

**Characterisation of protein-protein interactions of membrane
proteins from *Hordeum vulgare* and *Arabidopsis thaliana*
involved in plant immunity**

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I. Summary

Plants possess a sophisticated immune system, which is capable of defending a plant against the vast majority of microbes. For the recognition of different microbes, plants use receptor-like kinases (RLKs) and receptor-like proteins (RLPs), which are localised at the plasma membrane. These receptors can bind microbe-specific molecules on the extracellular side and can transmit the signal by phosphorylation reactions into a cellular signal. A defence signalling cascade is started to prevent the growth of the microbe. Not only RLKs are present in the plasma membrane, but also other proteins that play a crucial role in plant immunity. How these proteins interact with other proteins and the meaning of this interaction is only partially solved.

The overall aim of this thesis was the identification of new physical interaction partners of plasma membrane-localised proteins involved in plant immunity. The main approach that was used in this thesis was the split-ubiquitin yeast two-hybrid (Y2H) system. This yeast-based system was designed to identify protein-protein interactions at the plasma membrane, wherefore the classical Y2H system is not applicable. Two different split-ubiquitin Y2H systems were tested, and various cDNA library screens were performed.

A library composed of effector proteins of the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) was used to screen it against barley and wheat RLKs, which should have a proposed role in non-host resistance. Potential interaction partners with two of these RLKs could be identified and validated in yeast.

The split-ubiquitin Y2H system was further applied for the identification of new interaction partners of the barley Mlo protein. The biochemical function of this seven transmembrane-containing protein is to date unknown, but a knock-out of specific genes of MLO family leads to complete powdery mildew resistance. The interaction screen should help to find new indications of the biochemical function. Two new potential interacting proteins of barley could be identified, which have to be further validated in the future. The overexpression of one of the candidates, an O-methyltransferase, leads to enhanced resistance against *Bgh*. Two potential interacting CSEPs could be determined, although one candidate (CSEP0515) could not be validated in an *in planta* bimolecular fluorescence complementation (BiFC) assays. The other effector needs to be tested.

Finally, the yeast system was used for the identification of new interaction partners of the *Arabidopsis thaliana* chitin receptor CERK1. Yeast and *in planta* split-luciferase experiments validated that CERK1 interacts with calmodulin (CaM), a plasma membrane H⁺ ATPase (AHA2) and the protein PCC1 (PATHOGEN AND CIRCADIAN CONTROL 1). The function of the interaction of CaM and AHA2 needs further investigation in the future. PCC1 belongs to a family of biotic and abiotic stress response proteins in eukaryotes. It possesses a specific domain, which is called cysteine-rich transmembrane domain (CYSTM). *In silico* analysis and interaction data indicate that different CYSTM family members form homo- and heterodimers and that specific members show a similar expression pattern. For the CERK1-PCC1 interaction could be shown: 1. The potential interaction site of PCC1 was narrowed down; 2. It could be shown that also other family CYSTM members can interact with CERK1; 3. Indication of likely phosphorylation of the N-terminus of PCC1 could be revealed and 4. It could be displayed that also other RLKs involved in plant immunity, like LYK4, LYK5, BAK1, EFR and FLS2, can interact with PCC1. Two *Arabidopsis* mutant lines of PCC1 revealed an unfortunately inconclusive ROS burst phenotype. A knock-down mutant showed a reduced ROS response after chitin and elf18 trigger, whereas the knock-out mutant exhibited a wild type-like reaction.

II. Zusammenfassung

Pflanzen besitzen ein komplexes Immunsystem, welches fähig ist eine Pflanze gegen eine Vielzahl von Mikroben zu verteidigen. Um verschiedene Mikroben wahrzunehmen, haben Pflanzen Rezeptor-ähnliche Kinasen und Rezeptor-ähnliche Proteine entwickelt, welche in der Plasmamembran lokalisiert sind. Diese Rezeptoren binden mikrobenspezifische Moleküle an der extrazellulären Seite und können ein Signal durch Phosphorylierungsreaktionen in ein zelluläres Signal umwandeln. Eine Abwehrsignalkaskade wird gestartet, um das Wachstum der Mikrobe zu verhindern. Neben Rezeptor-ähnliche Kinasen befinden sich in der Plasmamembran auch andere Proteine, die in der Pflanzenabwehr eine wichtige Rolle spielen. Wie diese Proteine mit anderen Proteinen interagieren und welche Bedeutung die Interaktion spielt, ist nur teilweise geklärt.

Das übergreifende Ziel dieser Arbeit ist die Identifizierung von neuen physikalischen Interaktionspartnern von plasmamembran-lokalisierten Proteinen, denen eine Aufgabe bei der Pflanzenabwehr zukommt. Als Hauptmethode wurde das Split-Ubiquitin Hefe-Zwei-Hybrid-System genutzt. Dieses hefebasierte System wurde designt, um Protein-Protein-Interaktionen an der Plasmamembran zu identifizieren, wozu das klassische Hefe-Zwei-Hybrid-System nicht in der Lage ist. Zwei verschiedene Split-Ubiquitin Hefe-Zwei-Hybrid-Systeme wurden getestet und verschiedene cDNA-Bibliothek-Screens wurden durchgeführt.

Es wurde eine Bibliothek verwendet, die aus Effektorproteinen des Mehltaupilzes *Blumeria graminis* f. sp. *hordei* besteht. Diese Bibliothek wurde gegen Rezeptor-ähnliche Kinasen von Gerste und Weizen getestet, für welche eine Rolle in der Nicht-Wirts-Resistenz angenommen wird. Dabei konnten potenzielle Effektorproteine identifizieren werden, die mit zwei dieser Rezeptor-ähnlichen Kinasen interagieren, was im Hefesystem bestätigen werden konnte.

Zusätzlich wurde das Split-Ubiquitin Hefe-Zwei-Hybrid-System für die Identifizierung von neuen Interaktionspartnern vom Gersten Mlo-Protein verwendet. Die biochemische Funktion dieses Proteins mit sieben Transmembrandomänen ist bis heute unklar, aber ein Ausschalten von bestimmten Genen dieser Familie führt zur kompletten Mehltaresistenz. Ein Interaktionsscreen wurde herangezogen, um neue Anhaltspunkte auf die biochemische Funktion zu bekommen. Hierbei konnten zwei

potenzielle Interaktionspartner aus Gerste identifiziert werden, welche in der Zukunft noch weiter bestätigt werden müssen. Die Überexpressierung von einem Kandidaten – einer O-Methyltransferase – führt zur erhöhten Resistenz gegen Gerstenmehltau. Außerdem konnten zwei potenziell interagierende Effektorproteine bestimmt werden, wobei ein Kandidat (CSEP0515) nicht in BiFC Experimenten bestätigen konnte. Der zweite Effektor muss noch getestet werden.

Zuletzt wurde das Hefesystem verwendet, um neue Interaktionspartner des *Arabidopsis thaliana* Chitin Rezeptors CERK1 zu identifizieren. In Hefe und *in planta* Split-Luziferase Experimenten konnte gezeigt werden, dass CERK1 mit Calmodulin, einer Plasmamembran H^+ ATPase (AHA2) und dem Protein PCC1 interagiert. Die Funktion der Interaktion mit Calmodulin und AHA2 muss noch weiter untersucht werden. PCC1 gehört zu einer Proteinfamilie in Eukaryoten, die auf biotischen und abiotischen Stress reagiert. Es besitzt einen speziellen Bereich, welcher cystein-reicher-transmembran Bereich genannt wird. *In silico* Analysen und Interaktionsdaten weisen darauf hin, dass diese Familie Homo- und Heterodimere bildet und dass bestimmte Mitglieder ähnliche Expressionsmuster haben. Für die Interaktion mit PCC1 konnte folgendes gezeigt werden: 1. Die Interaktionsstelle in PCC1 konnte eingrenzt werden; 2. Es konnte gezeigt werden, dass auch andere Familienmitglieder mit CERK1 interagieren können; 3. Indizien für die Phosphorylierung des N-terminus von PCC1 konnten geliefert werden und 4. Es konnte gezeigt werden, dass auch andere Rezeptor-ähnliche Kinasen der Pflanzenabwehr, wie LYK4, LYK5, BAK1, EFR und FLS2 mit PCC1 interagieren können. Zwei *Arabidopsis thaliana* Mutantenlinien von PCC1 zeigten nicht aussagekräftige ROS Burst Phänotypen. Eine knock-down Mutante zeigte eine reduzierte ROS Antwort, wenn Chitin oder elf18 verwendet wurde, wobei die knock-out Mutante wildtyp-ähnliche Antwort zeigte.

III. General introduction:

Plants evolve sophisticated mechanisms to deal with biotic and abiotic stresses, by which they are constantly challenged in their environment. Biotic stresses are, for example, caused by microorganisms, like bacteria, oomycetes and fungi that attack and try to colonise plants. Plants employ different defence pathways to cope with these pathogens. The understanding of these immune mechanisms and the interplay between plant and pathogen has the potential to facilitate the improvement of pest resistance, for example, in crop plants.

PTI and ETI are important pillars of the plant innate immune system

The first barrier mounted by plants against pathogens is the cuticle and the cell wall. These physical barriers have to be overcome by pathogens to colonise the plant (Yeats & Rose, 2013). Some pathogens use natural openings, like stomata, to enter the plant, others enter through wounds on the surface and still others penetrate these barriers directly (Lo Presti et al., 2015, Xin et al., 2018). Closure of stomata or prevention of penetration requires detection of pathogens. For the recognition of pathogens, but also symbionts, plants use pattern recognition receptors (PRR) that can detect microbe-associated molecular patterns (MAMPs), including pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs). MAMPs and PAMPs refer to similar groups of molecules, but the term MAMPs further comprises molecules from non-pathogens, like symbionts. DAMPs are plant-derived substances, like the peptide 1 (Pep1) from *Arabidopsis* (Krol et al., 2010, Yamaguchi et al., 2006), which are generated during tissue damage. PRRs belong to the classes of receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Boller & Felix, 2009). Well-studied examples of plant RLKs are FLS2 (FLAGELLIN SENSITIVE 2)(Gómez-Gómez & Boller, 2000), EFR (ELONGATION FACTOR (EF)-TU RECEPTOR)(Zipfel et al., 2006) and CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1)(Miya et al., 2007). They consist of an extracellular ligand-binding domain, a transmembrane domain and, in case of RLKs, a kinase domain at the cytosolic side (Torii, 2000). Upon ligand binding at the extracellular site, the receptor undergoes conformational changes. This initiates cis and/or transphosphorylation of the receptor and co-receptors and elicits intracellular signal transmission, eventually mediating modulation of further proteins (e.g. via phosphorylation) and triggering of signalling cascades prompting a defence reaction. This mechanism is called MAMP-triggered immunity (MTI) or PAMP-triggered

immunity (PTI) (Chisholm et al., 2006, Jones & Dangl, 2006). The ligands of PRRs are often indispensable and highly conserved molecules for a specific group of pathogens, like flg22, a peptide epitope of the flagella of bacteria and chitin, a primary compound of the fungal cell wall (Newman et al., 2013).

One of the earliest detectable plant responses upon MAMP recognition is the influx of Ca^{2+} via yet unknown calcium channels. Ca^{2+} is an important second messenger, which, e.g. activates calcium-dependent protein kinases (CDPKs/CPKs), these phosphorylate protein targets and hence transmit the Ca^{2+} signal into a cellular response (Boudsocq et al., 2010). For example, Arabidopsis CPK5 phosphorylates the NADPH-dependent oxidase RBOHD (RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D) and regulates its activity (Dubiella et al., 2013). The activation of RBOHD leads to the generation of reactive oxygen species (ROS) by the conversion from O_2 to superoxide ($\text{O}_2^{\cdot-}$) and the further processing by superoxide dismutases (SOD) to H_2O_2 (Auh & Murphy, 1995, Suzuki et al., 2011). This ROS burst is initiated after the Ca^{2+} influx and binding to RBOHD and serves, among other tasks as a signalling mechanism (Miller et al., 2009, Mittler et al., 2011). Besides RBOHD, also apoplastic peroxidases contribute to the ROS production upon MAMP recognition (Daudi et al., 2012, O'Brien et al., 2012). Other targets of CDPKs are WRKY transcription factors (contain the amino acid sequence WRKYGQK)(Eulgem et al., 2000), like WRKY8, WRKY28 and WRKY48, which are activated after phosphorylation by CPK4, CPK5, CPK6 and CPK11 (Gao et al., 2013). Additional transmitters of Ca^{2+} signalling are the Ca^{2+} -binding calmodulin (CaM) proteins. These calcium sensors bind four Ca^{2+} ions and transduce the signal by modulating the activity of other proteins, like calmodulin-binding proteins (Du et al., 2011). CaMs bind, among others, to the CaM-binding transcription factor CBP60g (CALMODULIN BINDING PROTEIN 60 g) to activate the expression of ICS1 (isochorismate synthase) (Wan et al., 2012a, Wang et al., 2009, Zhang et al., 2010). ICS1 is a crucial enzyme in the synthesis of the phytohormone salicylic acid (SA) (Dempsey et al., 2011). Accumulation of SA after pathogen attack results in activation of different signalling processes and e.g. results in the expression of pathogenesis-related (PR) genes (Pieterse et al., 2012, Seyfferth & Tsuda, 2014, Vlot et al., 2009).

The MAPK (MITOGEN-ACTIVATED PROTEIN KINASE) cascade is another signalling pathway, which activated after the recognition of MAMPs. This cascade consists of an

MPK-kinase-kinase (MEKK), an MPK-kinase and an MPK, which mostly activate each other via trans-phosphorylation upon a stimulus (Meng & Zhang, 2013). Especially MPK3, MPK4 and MPK6 play an essential role during defence-related signal transduction as they phosphorylate specific protein targets, like transcription factors to regulate defence gene expression (WRKY29 and FRK1) and camalexin biosynthesis (Mao et al., 2011, Ren et al., 2008). Camalexin belongs to the class of phytoalexins, which are antimicrobial molecules that are produced after the recognition of a pathogen (Ahuja et al., 2012, Dixon, 2001). One example of deactivation of an MPK pathway is EDR1 (ENHANCED DISEASE RESISTENCE1), an MPKKK, which negatively regulates the MKK4/MKK5-MPK3/MPK6 kinase cascade (Zhao et al., 2014).

Pathogens evolved strategies to suppress MTI by the secretion of effector proteins, which interfere with plant defence proteins/pathways (Franceschetti et al., 2017, Toruno et al., 2016). Bacterial pathogens use, among others, their type III secretion system to deliver effectors into the host cell. They possess a relatively small set of effectors, which are very well studied for some pathogens so that for several effectors, their targets and functions are well known (Deslandes & Rivas, 2012). However, for the vast majority of predicted fungal/oomycete effectors, the exact function and the delivery mechanism into the host cell is unknown (Petre & Kamoun, 2014). The suppression of MTI leads to effector-triggered susceptibility (ETS). The exchange of small RNAs (sRNAs) is a mechanism that has only recently been described to contribute to the interplay of plant and pathogen. Pathogens and the host plant produce sRNAs to interfere with the host immune system or inhibit the pathogen. Both sides secrete these sRNAs to silence target genes, which is called cross-kingdom RNA interference (RNAi) (Hua et al., 2018, Weiberg et al., 2014). The *Arabidopsis/Botrytis cinerea* interaction is one example, in which both sides secrete sRNAs to interfere with genes either involved in plant defense or in fungal pathogenicity (Cai et al., 2018, Wang et al., 2017b).

In an arms-race dependent manner, plants counteract ETI *via* recognition of specific effectors by resistance proteins (R-proteins). These R-proteins often belong to the class of nucleotide-binding and leucine-rich repeat receptors (NLR). This family consist of a central nucleotide-binding domain (NB), a C-terminal leucine-rich repeat (LRR) domain and an N-terminal coiled-coil (CC) or Toll interleukin1 (TIR) domain (Jones et al., 2016, Maekawa et al., 2011). One example for this family is the *Mildew locus a*

(*Mla*) of barley (*Hordeum vulgare*), where one specific *Mla* protein encoded by the *Mla* locus can recognise a specific effector of the barley powdery mildew *Blumeria graminis* f. sp. *hordei* (*Bgh*). R-protein recognised effectors are called avirulence effectors or short AVR. Direct interaction between receptor and effector is suggested for this type of NLR (Saur et al., 2019). By contrast, other NLRs do not bind effectors directly but detect the interaction of an effector with the target protein, the modification of the target by the effector or the absence of the target after disruption by the effector. This indirect recognition is summarised in the guard-hypothesis (Dangl & Jones, 2001). The Arabidopsis NLR ZAR1 (HOPZ-ACTIVATED RESISTANCE 1) forms a complex with the kinase RKS1 (RESISTANCE-RELATED KINASE 1), which recognises the modification (uridylation) of the decoy kinase PBLs ((AvrPphB SUSCEPTIBLE PROTEIN 1) PBS1-LIKE PROTEIN 2) by the *Xanthomonas* effector AvrAC. The NLR forms a wheel-like structure, the resistosome, after the recognition (Wang et al., 2019a, Wang et al., 2019b). As upon recognition of many effectors by suitable R-proteins, resistosome formation initiates a hypersensitive response, whereby the attacked cell undergoes cell death to prevent the growth of the bacterium. Strikingly, some R-proteins employ integrated sensor or decoy domains, which mimic structures of effector targets to capture effectors and facilitate binding (Sarris et al., 2016, van der Hoorn & Kamoun, 2008). This part of the immune system is called effector-triggered immunity (ETI). The Zic-Zac model describes the constant evolution of (new) effectors to suppress immunity (ETS) and of suitable plant R-proteins counteracting these effectors (ETI) (Jones & Dangl, 2006) (Cui et al., 2015) (Figure 1).

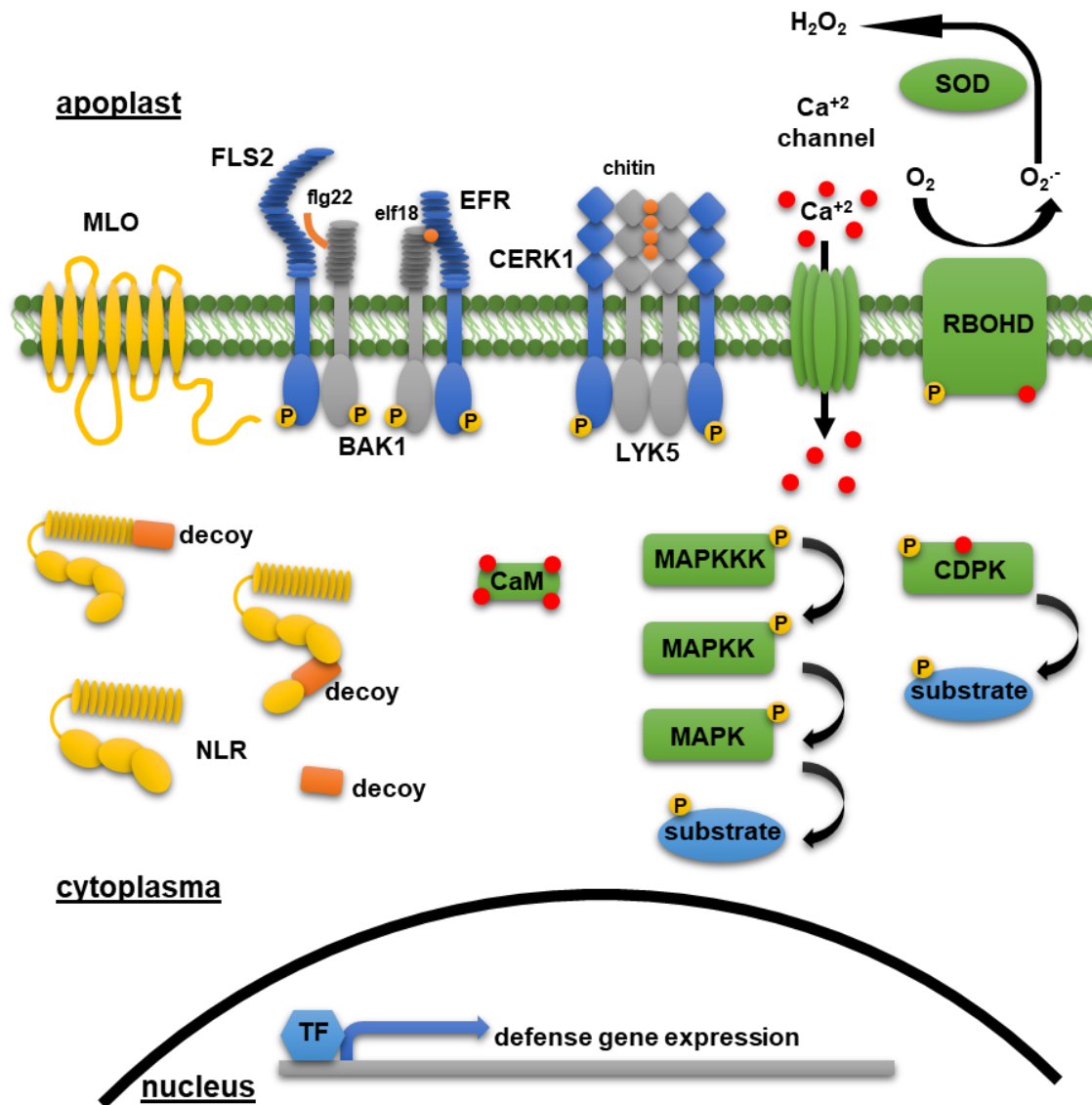


Figure 1: Schematic model of the plant immunity system. Pattern recognition receptors, which often belong to the class of receptor-like kinases bind specific microbial-associated molecular pattern. FLS2 and BAK1 cooperatively bind flg22. BAK1 further serves as a coreceptor for elf18 perception by EFR. CERK1 and LYK5 form a chitin-binding complex. RLK complexes undergo auto-phosphorylation and phosphorylate their target proteins. MAMP perception initiates Ca²⁺ influx that triggers further downstream signalling cascades. Ca²⁺-dependent protein kinases (CDPKs) and calmodulins (CaMs) bind Ca²⁺ to modulate the activity of other proteins and transmit the Ca²⁺ signal into a cellular response. MAMP perception-activated MAPK cascades further transmit the signal *via* phosphorylation of target proteins, e.g. transcription factors (TFs) that regulate defence gene expression. A ROS burst is generated by RBOHD activity. RBOHD generates superoxide (O₂⁻), which is converted to H₂O₂ by superoxide dismutases (SOD). These signalling pathways initiate MAMP-triggered immunity. Effector-triggered immunity is mediated by nucleotide-binding and leucine-rich repeat receptors (NLRs) that perceive the presence of pathogen effectors. Mimicry of potential effector targets can be employed by NLRs (*via* individual decoy proteins or integrated decoy domains) to bind effectors more efficiently. Effector recognition often initiates a hypersensitive response resulting in cell death. Besides proteins involved in mediating resistance, plants also possess proteins that contribute to susceptibility against pathogens. A critical protein for the powdery mildew interaction is the MILDEW LOCUS O (MLO). The biochemical function of this protein is unknown, but the loss-of-function mutation leads to complete pre-penetration resistance to powdery mildews.

The life cycle of *Blumeria graminis* f. sp. *hordei* reflects its biotrophic lifestyle

Powdery mildews are biotrophic epiphytically growing ascomycetes that can colonise a wide variety of plants, including important crop plants, like barley and wheat (Glawe, 2008). In case of the barley powdery mildew *Bgh*, a spore landing on the plant surface initially forms a primary germ tube (Figure 2). A secondary germ tube appears after ca. 4 h and forms an appressorium at its tip after ca. 8 h. When the appressorium is attached to the leaf surface, the penetration peg starts to grow into the plant epidermis cell at around 15 h. The penetration peg uses mechanical force rather than degradational enzymes to penetrate the cell wall (Pryce-Jones et al., 1999). Consequently, the fungus establishes its feeding structure, the haustorium, without disruption of the plant plasma membrane. A plant-derived extrahaustorial membrane surrounds the haustorium, and its formation is completed around 24 h after the first contact (Both & Spanu, 2004). It is suggested that the haustorium is necessary for the secretion of effectors into the host and for the uptake of nutrients from the host (O'Connell & Panstruga, 2006). The fungus starts to grow secondary hyphae on the plant surface after three days, which will penetrate adjacent epidermis cells and produce new haustoria. After five days, the fungus begins to produce new spores on the surface that will infect new parts of the plant or new plants when spread by wind. During the infection, *Bgh* secretes effector proteins, so-called candidates for secreted effector proteins (CSEPs) (Pedersen et al., 2012), to suppress the plant immunity and promote its growth (Aguilar et al., 2016, Ahmed et al., 2016, Pliego et al., 2013). The *Bgh* genome encodes a limited gene set of approximately 7100 genes, while most of the genome (around 74 %) consists of transposable elements (Frantzeskakis et al., 2018). This reduced gene number is considered to be a result of the biotrophic lifestyle of the fungus (Spanu et al., 2010). Strikingly, over 10 % of all *Bgh* genes encode for the around 700 predicted CSEPs, substantiating the pivotal importance of effectors for fungal survival.

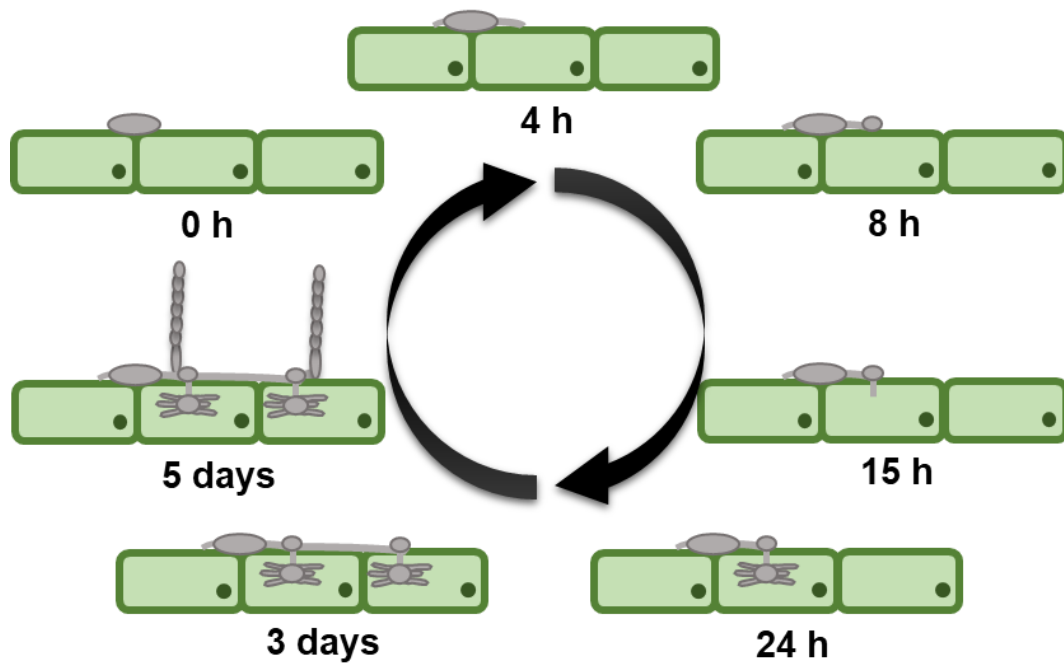


Figure 2: Schematic life cycle of *Blumeria graminis* f. sp. *hordei* (Bgh). When a spore of Bgh lands on the leaf surface, it forms a primary and subsequently a secondary germ tube after four hours. An appressorium develops at the end of the secondary germ tube and attaches to the leaf surface. The appressorium starts to penetrate the plant cell wall with mechanical force. After successful penetration, the fungus forms its feeding structure (haustorium) after 24 h. The haustorium is surrounded by the plant-derived extrahaustorial membrane. Bgh forms secondary hyphae on the leaf surface to penetrate surrounding cells and to establish new haustoria. After five days, the fungus produces new spores that can be distributed by the wind and infect new plants.

***mlo* mediates powdery mildew resistance**

Besides resistance proteins, plants encode proteins contributing to susceptibility against pathogens. *Vice versa*, loss of function of these proteins mediates resistance against pathogens. A durable type of resistance against powdery mildews is mediated by mutation or absence of the *mildew resistance locus o* (*Mlo*) gene. Loss of function of Mlo proteins confers pre-penetration resistance against all isolates of powdery mildew fungus (Büschges et al., 1997, Jørgensen, 1992). This type of resistance is widely used in the cultivation of barley and has been shown to be superior to *R*-gene mediated resistance regarding its stability under field conditions. Nevertheless, *mlo* mutation also induces pleiotropic effects, like early senescence (Consonni et al., 2006, Piffanelli et al., 2002). Mlos are seven-transmembrane domain-containing proteins first described in barley (Büschges et al., 1997, Jørgensen, 1992) and later in many other plant species (Acevedo-Garcia et al., 2014, Kusch et al., 2016). However, the biochemical functions of Mlo protein are largely unknown. Not all Mlo proteins are involved in mildew resistance since *Arabidopsis* encodes fifteen MLOs and only the

mlo2 mlo6 and *mlo12* mutations cooperatively contribute to resistance (Consonni et al., 2006). *AtMLO7* (NORTIA), for example, is involved in pollen tube perception, and its loss-of-function mutation leads to pollen tube overgrowth in the synergid, causing reduced fertility (Kessler et al., 2010).

Calmodulin (CaM) is one interaction partner of Mlo proteins. The binding-site for CaM is at the C-terminal domain of Mlos (Kim et al., 2002) and in barley, it was shown that this interaction is necessary for Mlo function (Bhat et al., 2005).

Protein-protein interactions are crucial for plant defence

Protein-protein interaction plays an important role within the cell for almost every kind of process. During plant defence, proteins interact to perceive MAMPs or to transmit a signal. PRRs physically interact with each other and/or co-receptors to bind MAMPs. FLS2 and BAK1, for example, interact to cooperatively bind flg22 (Chinchilla et al., 2007) (figure 1). Other examples are kinases, which must bind their substrate in order to mediate phosphorylation. One example is the interaction of MEKK2 with MPK4, whereby MEKK2 gets phosphorylated by MPK4 (Kong et al., 2012).

Researchers developed different types of methods to investigate protein-protein interaction *in vitro* or *in vivo*. The most commonly used assay is probably the classical yeast two-hybrid (Y2H) approach, where one protein of interest is fused to a DNA binding domain (BD) and the other protein to an activation domain (AD). Interaction of both proteins inside the nucleus initiates reporter gene expression and allows growth selection or detection of lacZ activity (Fields & Song, 1989). The split-ubiquitin Y2H system was developed for the detection of protein interactions of membrane proteins in yeast. It relies on the reconstitution of a split ubiquitin and its recognition by ubiquitin-specific proteases, which cleave off the reporter from the protein of interest (Johnsson & Varshavsky, 1994, Stagljar et al., 1998, Wittke et al., 1999). Fluorescence-based methods are, for example, the bimolecular fluorescence complementation (BiFC) (Ghosh et al., 2000) or Förster resonance energy transfer (FRET) (Heim & Tsien, 1996), which can be performed *in planta*. These assays further provide information about the subcellular localisation of the interaction. BiFC is based on the reconstitution of a split fluorophore, like yellow fluorescent protein (YFP), while FRET relies on the energy transfer from one fluorophore to another. Another light-based *in vivo* method is the split-luciferase assay, which uses the enzyme luciferase to produce a

luminescence signal after reassembly (Ozawa et al., 2001). *In vitro* methods are co-immunoprecipitation (CO-IP) (Kaboord & Perr, 2008) or microscale thermophoresis (MST) (Asmari et al., 2018, Wienken et al., 2010), which measure the binding of both partners via antibody detection or the molecular movement changes in a temperature gradient, respectively. Each of these methods was optimised and evolved differently over the past decades to be suitable for different application. Nevertheless, each assay possesses distinct advantages and drawbacks, which have to be considered during the decision for a particular assay (Xing et al., 2016). At least two different methods should be applied to avoid artefacts during protein-protein interaction detection and to validate an interaction. Preferably, three methods should be performed and at least one should be conducted in the natural system.

IV. Materials and Methods

1. Materials:

1.1 Plants:

1.1.1 *Hordeum vulgare*

The *H. vulgare* (barley) cultivar Margret, which was used in this study, was provided by I.G. Pflanzenzucht GmbH and the seeds were stored at 4 °C.

1.1.2 *Arabidopsis thaliana*

All used *A. thaliana* wt, and T-DNA insertion lines are listed in Table 1. All T-DNA insertion lines are based on Col-0. The seeds were stored at 4 °C

Table 1: List of *Arabidopsis thaliana* wild type and T-DNA insertion lines

line	reference/source
Col-0	J. Dangl ¹
<i>cerk1-2</i>	NASC ²
<i>fls2</i> (SAIL_691_C04)	S. Robatzek ³
821C03	GABI-KAT ⁵ / A. Slusarenko ⁴
528H09	GABI-KAT ⁵

¹University of North Carolina, Chapel Hill, NC, USA; ²Nottingham Arabidopsis Stock Centre, Nottingham (UK); ³(Sessions et al., 2002); ⁴RWTH Aachen University, Worringer Weg 1, 52074 Aachen, Germany; ⁵Bielefeld University, 33501 Bielefeld, Germany, (Kleinboelting et al., 2012)

1.1.3 *Nicotiana benthamiana*

N. benthamiana DOMIN was used for transient overexpression of bimolecular fluorescence complementation (BiFC) and split-luciferase constructs via *Agrobacterium*-mediated transformation.

1.2 Microorganisms:

1.2.1 Powdery mildew fungi

For the infection assay of barley, the barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) isolate K1 (Wiberg, 1974) was applied.

1.2.2 Bacteria

Agrobacterium tumefaciens was used for transient *N. benthamiana* transformation, and *Escherichia coli* was applied for general cloning. More information is provided in Table 2.

Table 2: List of *E. coli* and *A. tumefaciens* used in this study

strain	resistance	genotype	reference/source
<i>E. coli</i> TOP10	Str	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80/ <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> DB3.1	ccdB, Str	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80/ <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>ara</i> Δ139 Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i> <i>fhuA::IS2</i>	Invitrogen
<i>A. tumefaciens</i> AGL1	Amp/Carb, Rif	pTiBo542D T-DNA	(Lazo et al., 1991)
<i>A. tumefaciens</i> GV3101 pMP90RK	Rif, Gent, Kan		(Koncz & Schell, 1986)

1.2.3 Yeast strains:

Two different *Saccharomyces cerevisiae* strains (Table 3) were used for the two different split-ubiquitin yeast-two hybrid systems.

Table 3: List of *S. cerevisiae* strains used for split-ubiquitin Y2H assays

strain	split-ubiquitin system	genotype	reference/source
THY. AP4	Lex-based split-ubiquitin	MATa; <i>ade2</i> ; <i>his3</i> ; <i>leu2</i> ; <i>trp1</i> ; <i>ura3</i> ; <i>lexA::ADE2</i> ; <i>lexA::HIS3</i> ; <i>lexA::lacZ</i>	(Obrdlik et al., 2004)
JD53	Ura3-based split-ubiquitin	MAT α; <i>his3</i> -Δ200; <i>leu2</i> -3,112; <i>lys2</i> -801; <i>trp1</i> -Δ63; <i>ura3</i> -52	(Dohmen et al., 1995)

1.3 Media:

1.3.1 Luria-Bertani medium

The ingredients (Table 4) were mixed in ddH₂O and autoclaved for 20 min at 121 °C. 15 g of agar was added to produce a solid medium.

Table 4: Luria-Bertani medium (LB medium, 1 L)

component	amount
tryptone	10 g
yeast extract	5 g
NaCl	10 g
agar	15 g

1.3.2 Yeast media:

1.3.2.1 Yeast extract peptone dextrose medium (YPD)

Peptone and yeast extract were mixed in 900 ml ddH₂O, and the pH was set to 6.0. The medium was autoclaved at 121 °C for 20 min. Before usage, 100 ml filter-sterilised 10 x glucose stock (200 g/l) was added as a carbon source. 20 g of agar was added for solid medium (Table 5).

Table 5: Yeast extract peptone dextrose medium (YPD) (1 L)

component	amount
peptone	20 g
yeast extract	10 g
agar	20 g
before usage	
10 x glucose stock (200 g/L)	100 mL

1.3.2 Synthetic complete mixture dropout medium (SC-medium)

The following media were used for the LexA-based split-ubiquitin Y2H system (Table 6). The components in Table 6 were dissolved in 900 ml of ddH₂O. The pH was set to 6.0 – 6.3 with KOH. 20 g of agar was added if a solid medium was needed. Then the mixture was autoclaved for 20 min at 121 °C. Methionine was added as a sterile 0.1 M stock solution resulting in final concentrations of 75 µM, 150 µM, 300 µM and 600 µM when repression of the bait expression was required. Before usage, 100 mL filter-sterilised 10 x glucose stock (200 g/L) was added as a carbon source.

Table 6: Synthetic complete mixture (SC; Kaiser) dropout medium (1 L), LexA-based split-ubiquitin Y2H system

component	-L ¹ -M ²	-L -T ³ -M	-L -T -H ⁴ -A ⁵ -M
Yeast nitrogen base	6.9 g	6.9 g	6.9 g
Dropout mix -Ade, -Arg, -His, -Leu, -Met, -Thr, -Trp, -Ura	1.148 g	1.148 g	1.148 g
Arg	76 mg	76 mg	76 mg
Thr	76 mg	76 mg	76 mg
Ura	76 mg	76 mg	76 mg
Ade	18 mg	18 mg	
His	76 mg	76 mg	
Trp	76 mg		
Agar	20 g	20 g	20 g

¹L: leucine; ²M: methionine; ³T: tryptophan; ⁴H: histidine; ⁵A: adenine

The following media were used for the Ura3-based split-ubiquitin Y2H system (Table 7). The components in Table 7 were dissolved in 900 mL of ddH₂O. The pH was not adjusted. FOA selection breaks down above pH 6 and fully works at pH 4.5 and below (<https://www.usbio.net/protocols/5-FOA-protocol#ref>; accessed:19.6.19). 20 g of agar was added for solid medium. The mixture was autoclaved for 20 min at 121 °C. Methionine was added to repress the bait expression in concentrations of 75 µM, 150 µM, 300 µM and 600 µM (as described above). 5-FOA was added in concentrations of 0.7 g or 1 g per litre medium for the protein-protein interaction medium. Before usage, 100 ml filter-sterilised 10xglucose stock (200 g/L) was added as a carbon source.

Table 7: Synthetic complete mixture (SC; Kaiser) dropout medium (1 L), Ura3-based split-ubiquitin Y2H system

component	-H ¹ -M ²	-H -M -U ³	-H -T ⁴ -M	-H -T -M + 5-FOA ⁵
Yeast nitrogen base	6.9 g	6.9 g	6.9 g	6.9 g
Dropout mix -Ade, -Arg, -His, -Leu, -Met, -Thr, -Trp, -Ura	1.148 g	1.148 g	1.148 g	1.148 g
Arg	76 mg	76 mg	76 mg	76 mg
Thr	76 mg	76 mg	76 mg	76 mg
Ura	76 mg		76 mg	76 mg
Ade	18 mg	18 mg	18 mg	18 mg
Leu	380 mg	380 mg	380 mg	380 mg
Trp	76 mg	76 mg		
5-FOA				0.7-1 g in 10 ml DMSO
Agar	20 g	20 g	20 g	20 g

¹H: histidine; ²M: methionine; ³U: uracil; ⁴T: tryptophan; ⁵5-FOA: 5-Fluoroorotic acid

1.4 Antibiotics/Fungicides:

All antibiotics, except Chloramphenicol, were stored as a 1000 x fold stock solution at -20 °C and were used for the selection of transformed plasmids in *E. coli* or *A. tumefaciens*. Chloramphenicol was stored as a 5000 x fold stock solution at -20 °C. The fungicide Benzimidazole was stored as a 10 x stock solution at RT. All used solutions are listed in Table 8.

Table 8: List of antibiotics and fungicides

name	abbreviation	solvent	concentration	company
Antibiotics				
Ampicillin	Amp	H ₂ O	100 mg/mL	Duchefa
Kanamycin	Kan	H ₂ O	50 mg/mL	Carl Roth
Carbenicillin	Carb	H ₂ O	50 mg/mL	Duchefa
Gentamycin	Gent	H ₂ O	25 mg/mL	Carl Roth
Rifampicin	Rif	DMSO	100 mg/mL	Duchefa
Chloramphenicol	Cm	EtOH	100 mg/mL	Duchefa
Spectinomycin	Spec	H ₂ O	100 mg/mL	Duchefa
Fungicides				
Benzimidazole	Benz	H ₂ O	2 mg/mL	Sigma-Aldrich

1.5 Antibodies:

The following Table 9 contains the antibodies used for immunoblot analysis

Table 9: List of antibodies						
antibody	raised in	dilution	buffer	conjugate	company/source	
α -luciferase	goat	1:1000	5%BSA in TBST		Merck	
α -goat	mouse	1:1000	5% nonfat dry milk in TBST	IgG-HRP	Santa-Cruz Biotech	
α -LexA	rabbit	1:5000	5% nonfat dry milk in TBST		Prof. Dr. Karin Römisch	
α -rabbit	goat	1:1000	5% nonfat dry milk in TBST	IgG-HRP	Cell Signaling/NEB	

1.6 Primer:

Primers were used for PCR, sequencing and cloning and were ordered as a desalted powder by IDT (Leuven, Belgium). A 100 μ M stock solution was prepared with ddH₂O and diluted to 10 μ M as a working solution. All stocks and working solutions were stored at -20 °C. All primers are listed in Supplementary Table 1.

1.7 Vectors:

Plasmids were used in this study for Gateway cloning and gene expression in yeast and *in planta*. Table 10 summarises the essential properties of the vectors. Vector maps are provided in the Supplementary Figure 33-37.

Table 10: List of vectors for gateway cloning and gene expression in yeast and plants						
name	resistance/ selection marker	Used for	properties	reference/source		
DONR vector						
pDONR201	Kan	Gateway cloning	Gateway DONR vector	Invitrogen Karlsruhe (Germany)		
pDONR207	Gent	Gateway cloning	Gateway DONR vector	Invitrogen Karlsruhe (Germany)		
pENTR221	Kan	Gateway cloning	Gateway DONR vector	Invitrogen Karlsruhe (Germany)		
Destination vector						
pMetYC-Dest	Amp/Leu	LexA-based split- ubiquitin Y2H	methionine repressible promoter; low copy plasmid; C-terminal Cub-PLV	(Obrdlik et al., 2004)		
pNX32-Dest	Amp/Trp	prey expression vector	N-terminal NubG version, C-terminal HA- tag; high copy plasmid;	(Obrdlik et al., 2004)		

				ADH1 promoter/terminator	
pNX33-Dest	Amp/Trp	prey expression vector		N-terminal NubG version, C-terminal HA- tag; low copy plasmid; ADH1 promoter/terminator	(Obrdlik et al., 2004)
pNX _{wt} -Dest	Amp/Trp	prey expression vector		N-terminal Nubl version, C-terminal HA- tag; high copy plasmid; ADH1 promoter/terminator	(Obrdlik et al., 2004)
pMet-GWY-Cub- R-Ura3-Cyc1	Amp/His	URA-based split- ubiquitin Y2H		methionine repressible promoter; low copy plasmid; C-terminal Cub-R-Ura3	(Deslandes et al., 2003, Wittke et al., 1999)
pCup-Nul-GWY- Cyc1	Amp/Trp	prey expression vector		N-terminal Nubl version, low copy plasmid; copper promoter	(Deslandes et al., 2003, Wittke et al., 1999)
pCup-NuA-GWY- Cyc1	Amp/Trp	prey expression vector		N-terminal NubA version, low copy plasmid; copper promoter	(Deslandes et al., 2003, Wittke et al., 1999)
pCup-NuG-GWY- Cyc1	Amp/Trp	prey expression vector		N-terminal NubG version, low copy plasmid; copper promoter	(Deslandes et al., 2003, Wittke et al., 1999)
pESPYNE	Amp	BiFC		N-terminal the N- terminal part of YFP; p35S promoter	(Walter et al., 2004)
pUC-SPYCE	Amp	BiFC		C-terminal the C- terminal part of YFP, p35S promoter	(Walter et al., 2004)
pCAMBIA1300- C-LUC-GWY	Kan	split-luciferase		N-terminal the C- terminal part of a firefly luciferase; p35S promoter	(Chen et al., 2008)
pCAMBIA1300- GWY-N-LUC	Kan	split-luciferase		C-terminal the N- terminal part of a firefly luciferase; p35S promoter	(Chen et al., 2008)
pUbi-CFP-GWY- nos	Amp	plant expression vector		N-terminal tagged CFP; ubiquitin promoter	
pUbi-DsRED-nos	Amp	plant expression vector		Expression of DsRED as transformation control; ubiquitin promoter	
pUbi-GWY	Amp	plant expression vector		ubiquitin promoter	

1.8 Software and databases:

All used databases and software applications are listed in Table 11.

Table 11: List of software and databases

name	function/purpose	reference
Arabidopsis eFP browser	Arabidopsis gene expression database	(Winter et al., 2007)
ATTED-II ver.9.2 (2017.12.14)	co-expression network database	(Obayashi et al., 2018)
BioGRID3.5	interactome database	(Chatr-Aryamontri et al., 2017, Oughtred et al., 2019)
Draw custom plasmids map	construction of plasmid maps	(Bond & Naus, 2012)
e! EnsembleFungi	BLAST search <i>Bgh</i> genome	(Kersey et al., 2018)
e! EnsemblePlants	BLAST search plant genome	(Kersey et al., 2018)
ePlant	database: subcellular localisation, expression level, interaction, genomic information	(Fucile et al., 2011)
InterPro	search/prediction protein domains	(Jones et al., 2014b, Mitchell et al., 2019)
IUPred2A	prediction of disordered and redox-sensitive regions	(Mészáros et al., 2018)
M.I.N.D. (membrane-based Interactome Network Database	database of Arabidopsis interactome database	(Chen et al., 2012, Jones et al., 2014a, Lalonde et al., 2010)
NCBI	BLAST search	(Altschul et al., 1997)
NetPhos 3.1 server	prediction of phosphorylation sites	(Blom et al., 1999, Blom et al., 2004)
phobius	prediction of transmembrane domains	(Kall et al., 2004)
Phyre ²	protein structure prediction	(Kelley et al., 2015)
PLAZA v4.0	GO term enrichment analysis	(Van Bel et al., 2018)
ProtComp 9.0	prediction of subcellular localisation	Softberry, Inc., Mount Kisco, NY
PubMed	literature search	NCBI
REVIGO	Reduce and visualisation GO terms	(Supek et al., 2011)
SignalP-4.1 Server	prediction of signal peptides	(Nielsen, 2017)
STRING	interactome database	(Szklarczyk et al., 2015)
TOPCONS	prediction of transmembrane domains and signal peptides	(Tsirigos et al., 2015)
TMHMM Server v. 2.0	prediction of transmembrane domains	(Krogh et al., 2001)
UniProt	database protein information	(UniProt, 2019)
Web of science	literature search	Clarivate Analytics
name	function/purpose	company
BZII Viewer	Keyence microscope software	Keyence Corporation
Clone Manager 9	primer design, <i>in silico</i> cloning	Sci-Ed Software
EndNote X7.8 (build 11583) / X9.1 (build 12691)	reference manager	Thomas Reuters
GraphPad Prism 6	statistical analysis	GraphPad Software Inc.
ImageLab Version 4.1 build 16	agarose gel/ western blot detection	BioRad
MEGA6 (build:6140226)	alignment, Phylogenetic tree analysis	(Tamura et al., 2013)
Office 2016/365	processing of text, tables, figures	Windows Corp.

1.9 Equipment, chemicals and consumables:

In the following Tables (12 – 14) equipment, chemicals and consumables are listed.

Table 12: List of equipment used in this study

equipment	company, location
Camera Coolpix P600	Nikon, Düsseldorf (Germany)
Centrifuge 5424	Eppendorf, Hamburg (Germany)
Centrifuge 5424 R	Eppendorf, Hamburg (Germany)
Centrifuge 5717 R	Eppendorf, Hamburg (Germany)
Centrifuge 5810 R	Eppendorf, Hamburg (Germany)
Centrifuge Sorvall RC 5B Plus	Thermo Scientific, Schwerte (Germany)
ChemDoc MP	BioRad, Munich (Germany)
Clean bench BSB 4A	Gelaire Flow Laboratories; Sydney (Australia)
Combimag Ret 800	IKA, Staufen (Germany)
Digital Orbital shaker SHAKER DOS20m	ELMI, Riga (Latvia)
Gel Doc XR+ Imaging System	BioRad, Munich (Germany)
Gel dry frame	Roth, Karlsruhe (Germany)
Glass beads 4 mm	soilGEN, Aachen (Germany)
Heating block Thermomixer comfort	Eppendorf, Hamburg (Germany)
HERA therm Incubator	Thermo Scientific, Schwerte (Germany)
Plate vortex IKA Vibrax VXR	IKA, Staufen (Germany)
Incubator BM 800	Memmert, Schwabach (Germany)
Innova 42 (New Brunswick Scientific)	Eppendorf, Hamburg (Germany)
Keyence Biorevo BZ-9000	Keyence Corp., Japan (Osaka)
MicroPulser TM	BioRad, Munich (Germany)
Mini-Protean system	BioRad, Munich (Germany)
MLR-352-PE	Panasonic, Hamburg (Germany)
Multi pipette Handy step S	Brand, Wertheim (Germany)
Nanodrop 2000c	Thermo Scientific, Schwerte (Germany)
Neubauer improve counting chamber	Marienfeld, Lauda-Königshofen (Germany)
PCR machine 2720 Thermal Cycler	Applied Biosystems, Darmstadt (Germany)
PDS-1000/He Hepta	BioRad, Munich (Germany)
pH meter	Mettler Toledo, Gießen (Germany)
Pipette 0.2-2µl	Gilson, Den Haag (Netherlands)
Pipette Research Plus 0.5-5ml	Eppendorf, Hamburg (Germany)
Pipette Research Plus 100-1000µl	Eppendorf, Hamburg (Germany)
Pipette Research Plus 2-20µl	Eppendorf, Hamburg (Germany)
Pipette Research Plus 20-200µl	Eppendorf, Hamburg (Germany)
Power Pac 300	BioRad, Munich (Germany)
Power Pac Sonopuls GM70	Bandelin, Berlin (Germany)
Retsch MM 400	Retsch GmbH, Haan (Germany)
SDS PAGE System Mini-PROTEAN Tetra System	BioRad, Munich (Germany)
Sonicator HD70	Bandelin, Berlin (Germany)
Sonorex super RK 510	Bandelin, Berlin (Germany)
TSC-SP8 Confocal microscope	Leica Microsystems, Wetzlar (Germany)
UV Transilluminator	UVP Inc., Upland (USA)
Vortex-Genie 2	Scientific Industries, New York (USA)
Vacuum gas pump	VWR

Table 13: List of chemicals used in this study

chemicals	company, location
100% Ethanol	Roth, Karlsruhe (Germany)
100% Ethanol, denatured	Roth, Karlsruhe (Germany)
10xNEB Buffer	New England Biolabs;NEB, Ipswich (USA)
5xHF Buffer	Thermo Fisher Scientific, Waltham (USA)
Acetone	Roth, Karlsruhe (Germany)
Acetosyringone	Sigma-Aldrich, Dreisenhofen (Germany)
Agar-Agar	Roth, Karlsruhe (Germany)
Agarose (universal)	Bio-Budget Technology GmbH, Krefeld (Germany)
Ammonium acetate	Roth, Karlsruhe (Germany)
Ammonium persulfate (APS)	Roth, Karlsruhe (Germany)
Ampicillin sodium	Duchefa, Dreisenhofen (Germany)
Bromphenol blue R	Applied Biosystems, Darmstadt (Germany)
Calcium chloride	Roth, Karlsruhe (Germany)
Carrier DNA	Applichem, Darmstadt (Germany)
Carrier DNA (herring sperm)	Applied Biosystems, Darmstadt (Germany)
CHAPS	
chitin	Sigma-Aldrich, Dreisenhofen (Germany)
Chloroform	Applied Biosystems, Darmstadt (Germany)
Coomassie Brilliant Blue G250	Sigma-Aldrich, Dreisenhofen (Germany)
D-(+)-Glucose monohydrate	Applied Biosystems, Darmstadt (Germany)
D-(+)-Saccharose	Sigma-Aldrich, Dreisenhofen (Germany)
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe (Germany)
Dipotassium phosphate	Roth, Karlsruhe (Germany)
Disodium phosphate	Roche, Grenzach-Wyhlen (Germany)
Dithiothreitol (DTT)	Invitrogen, Karlsruhe (Germany)
D-Luciferin	Roth, Karlsruhe (Germany)
DNase I	Sigma-Aldrich, Dreisenhofen (Germany)
dNTPs	Genecraft, Köln (Germany)
dNTP mix (10mM)	Invitrogen, Karlsruhe (Germany)
elf18	EZBiolab Inc. (Carmel, USA)
Ethidium bromide	Roth, Karlsruhe (Germany)
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe (Germany)
flg22	CenticBiotec, Weimar (Germany)
Glacial acetic acid	Applichem, Darmstadt (Germany)
Glycerin	Roth, Karlsruhe (Germany)
Isopropanol	Roth, Karlsruhe (Germany)
LB-medium	Roth, Karlsruhe (Germany)
Lithium acetate	Applied Biosystems, Darmstadt (Germany)
Luminol	Sigma-Aldrich, Dreisenhofen (Germany)
Magnesium chloride	Roth, Karlsruhe (Germany)
Magnesium sulphate	Duchefa, Dreisenhofen (Germany)
MES	Roth, Karlsruhe (Germany)
Methanol	Sigma-Aldrich, Dreisenhofen (Germany)
Monopotassium phosphate	Roth, Karlsruhe (Germany)
Monosodium phosphate	Roth, Karlsruhe (Germany)
Oligo dT	Roth, Karlsruhe (Germany)
oNPG	Roth, Karlsruhe (Germany)
Perfluorodecalin	Sigma-Aldrich, Dreisenhofen (Germany)
Phenol	Roth, Karlsruhe (Germany)
Phenol:chloroform:isoamyl alcohol	Roth, Karlsruhe (Germany)
Poly(ethylene glycol) PEG 4000	Invitrogen, Karlsruhe (Germany)
PolyAcrylamide	Roche, Grenzach-Wyhlen (Germany)
Ponceau S	Applichem, Darmstadt (Germany)
Potassium acetate	Roth, Karlsruhe (Germany)
Potassium chloride	Sigma-Aldrich, Dreisenhofen (Germany)
Potassium ferricyanide	Sigma-Aldrich, Dreisenhofen (Germany)
Potassium ferrocyanide	Sigma-Aldrich, Dreisenhofen (Germany)

Protease inhibitor tablet	Roth, Karlsruhe (Germany)
RNase A, DNase free	Applichem, Darmstadt (Germany)
Sodium acetate	Roth, Karlsruhe (Germany)
Sodium carbonate	Roth, Karlsruhe (Germany)
Sodium chloride	Roth, Karlsruhe (Germany)
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe (Germany)
Sodium hydroxide (NaOH)	Roth, Karlsruhe (Germany)
Spermidine	Sigma-Aldrich, Dreisenhofen (Germany)
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe (Germany)
Thiourea	Roth, Karlsruhe (Germany)
Trichloroacetic acid	Roth, Karlsruhe (Germany)
TRIS	BioRad, Munich (Germany)
Triton-X 100	Roth, Karlsruhe (Germany)
Trypton/ Pepton from casein	Sigma-Aldrich, Dreisenhofen (Germany)
Urea	Applichem, Darmstadt (Germany)
XenoLight D-Luciferin-K+ salt	PerkinElmer Inc., Waltham (USA)
x-Gluc	Apollo Scientific, Stockport (UK)
Yeast extract	Sigma-Aldrich, Dreisenhofen (Germany)
Yeast Nitrogen Base without Amino Acids	Formedium LTD, Hunstanton (GB)
Yeast synthetic drop out medium supplements - His, -Ade, -Leu, -Met, -Trp, -Thr, -Arg, -Ura	Formedium LTD, Hunstanton (GB)
β-Mercaptoethanol	Applichem, Darmstadt (Germany)

Table 14: List of consumables

Consumables	company, location
1kb ladder plus	Invitrogen, Karlsruhe (Germany)
1.5 ml reaction tube	Sarstedt, Nürnberg (Germany)
2 ml reaction tube	Sarstedt, Nürnberg (Germany)
Cellophane paper	Roth, Karlsruhe (Germany)
BP Clonase	Thermo Scientific, Schwerte (Germany)
LR Clonase	Thermo Scientific, Schwerte (Germany)
96well culture plate, sterile	PerkinElmer Inc., Waltham (USA)
Inoculation loop 1 µl	Sarstedt, Nürnberg (Germany)
Glass beads 0.25 – 0.5 mm	Roth, Karlsruhe (Germany)
Glass beads 0.75 - 1 mm	Roth, Karlsruhe (Germany)
Macrocarriers (165-2335)	BioRad, Munich (Germany)
Metal beads 1.5 mm	Mühlmeier mahltechnik Bärnau (Germany)
Microcarriers, 1.0 µm gold	BioRad, Munich (Germany)
Monarch DNA purification Kit	New England Biolabs; NEB, Ipswich (USA)
Monarch gel extraction Kit	New England Biolabs; NEB, Ipswich (USA)
NucleoSpin Plasmid/Plasmid (NoLid)	Macherey-Nagel, Düren (Germany)
NucleoBond Xtra Midi	Macherey-Nagel, Düren (Germany)
Petri dishes, round/square	Sarstedt, Nürnberg (Germany)
Precision Plus Protein Standard Dual Color	BioRad, Munich (Germany)
Parafilm M	Pechiney Plastic Packaging, Chicago (USA)
Rupture discs 900 psi (165-2328)	BioRad, Munich (Germany)
Gold particle	BioRad, Munich (Germany)
Pipette tip 10 µl	StarLab, Hamburg, (Germany)
Pipette tip 1000 µl	StarLab, Hamburg, (Germany)
Pipette tip 200 µl	StarLab, Hamburg, (Germany)
Pipette tip 5 ml	StarLab, Hamburg, (Germany)
Pots 9x9	Pöppelmann GmbH & Co. KG, Lohne (Germany)
Cryogenic vials	StarLab, Hamburg, (Germany)
Syringe filter 0.22 µm	VWR, Darmstadt (Germany)
Falcon tube 50 ml	Sarstedt, Nürnberg (Germany)
Falcon tube 15 ml	Sarstedt, Nürnberg (Germany)
Filter units 0.22 µm	Sarstedt, Nürnberg (Germany)
Cuvettes	Sarstedt, Nürnberg (Germany)

SuperSignal West Pico	Thermo Scientific, Schwerte (Germany)
SuperSignal West Femto	Thermo Scientific, Schwerte (Germany)
Syringe 20 ml	B. Braun, Melsungen, (Germany)
Syringe 5 ml	B. Braun, Melsungen, (Germany)
Syringe 2 ml	B. Braun, Melsungen, (Germany)
Syringe 1 ml	B. Braun, Melsungen, (Germany)
PCR tubes	Sarstedt, Nürnbrecht (Germany)
ZymoResearch DNA concentrator Kit	ZymoResearch, Irvine (USA)
ZymoResearch Gel extraction Kit	ZymoResearch, Irvine (USA)

2. Methods:

2.1 Plant growth conditions

2.1.1 *A. thaliana* and *N. benthamiana* growths conditions

A. thaliana and *N. benthamiana* plants were cultivated in SoMi 513 soil (Hawita, Vechta, Germany) in 9 x 9 cm pots. The stratification of the seeds was conducted overnight at 4 °C in darkness. The plants were grown under short-day conditions (8 h light at 22 °C, 16 h darkness at 20 °C; 80-90% relative humidity; light intensity 100 $\mu\text{mol s}^{-1} \text{m}^{-2}$) for four to seven weeks. Plants were transferred to the long day chamber (16 h light at 23 °C, 8 h darkness at 20 °C; 60-65% relative humidity; light intensity 105-120 $\mu\text{mol s}^{-1} \text{m}^{-2}$) for propagation. After the onset of flowering, the plants were moved to the greenhouse, and aerial parts were enclosed in paper bags. The seeds were collected after the siliques were fully-dried.

2.1.2 Barley growth conditions

Barley was sown in 9x9 cm pots in SoMi 513 soil. Seeds were covered with a thin layer of soil. The plants were grown under long-day conditions (16 h light at 23 °C, 8 h darkness at 20 °C; 60-65% relative humidity; 105-120 $\mu\text{mol s}^{-1} \text{m}^{-2}$ light intensity). One week old plants were used for experiments. After infection with *Bgh* K1, the plants were kept in the *Bgh* K1 growth chamber under long-day conditions (12 h light at 20 °C, 12 h darkness at 19 °C; 60% relative humidity and 100 $\mu\text{mol s}^{-1} \text{m}^{-2}$ light intensity).

2.2 Cultivation of microorganisms

2.2.1 *E. coli* growth conditions

E. coli were grown overnight at 37 °C on LB agar or in liquid LB medium supplemented with appropriate antibiotics. Liquid cultures were agitated at 250 rpm. Glycerol stocks were prepared with 800 μL densely grown liquid culture and 200 μL 100% sterile glycerol. Stocks were stored at -80 °C.

2.2.2 *A. tumefaciens* growth conditions

Agrobacteria were cultivated for a minimum of 2 days at 28 °C on LB agar supplemented with appropriate antibiotics. Liquid LB medium cultures were inoculated with single colonies and grown for 24 h at 28 °C and 250 rpm. Glycerol stocks were prepared with 800 µL densely grown liquid culture and 200 µL 100% sterile glycerol. Stocks were stored at -80 °C.

2.2.3 Yeast growth conditions

Yeast strains were cultivated on solid medium (for compositions see paragraph 1.3.2) for a minimum of 2 days at 28-30 °C. For liquid cultures, the appropriate medium was inoculated with a single colony and incubated at least 16 h at 28–30 °C at 250 rpm. Glycerol stocks were prepared with 800 µL densely grown liquid culture and 200 µL 100% sterile glycerol. Stocks were stored at -80 °C.

2.2.4 Powdery mildew growth conditions

Bgh isolate K1 was cultivated on barley plants. Seven days old plants were infected in an infection tower *via* dispersing and subsequent 20 min settlement of fungal spores. Inoculated plants were kept in a growth chamber and were used for experiments or further propagation of the fungus.

2.3 Transient protein expression assays in *N. benthamiana*

2.3.1 *A. tumefaciens*-mediated transformation of *N. benthamiana*

(protocol of Klaus Harter (Tübingen), modified after David Baulcombe lab)

Agrobacteria glycerol stocks were re-grown on LB plates with appropriate antibiotics for 2-3 days at 28 °C. A single colony was used to inoculate 50 mL LB (with suitable antibiotics) supplemented with 20 mM acetosyringone and 10 mM MES (pH 5.7). The cultures were grown at 28 °C and 250 rpm for 24 h. The bacteria were pelleted by centrifugation at 3500 x g for 30 min at RT. The supernatant was discarded, and the pellet was resuspended in 5 mL Agro-infiltration buffer (10 mM MgCl₂; 10 mM MES (pH 5.7); 200 µM acetosyringone (freshly added before usage)). The OD₆₀₀ was checked by preparing 1:100 dilution in H₂O, and the bacteria solution was set to OD₆₀₀ 1 with Agro-infiltration buffer. For every infiltration event, at least 2.5 mL of each strain were prepared and incubated 2 – 4 hours at RT. Four to six week old *N. benthamiana* plants were covered with a hood, which was sprayed with water from the inside to

create a high humidity environment. Two strains were mixed 1:1 before the infiltration, resulting in a final OD₆₀₀ of 0.5 for each strain. Infiltration was performed with a needle-free syringe. The syringe was pressed to the lower side of the leaf, and a counter-pressure was manually applied to the upper side. Successful infiltration of the complete leaf area was controlled by observing a darkening of the leaves. Cotyledons were avoided. After 2-3 days the leaves were used to detect fluorescence or luminescence.

2.3.2 Transient protein expression in barley epidermal cells via particle bombardment

Biolistic transformation *via* gold particle bombardment was performed (with the PDS-1000/He Hepta™ (BioRad) system) for the transient expression of proteins in barley epidermal cells. Highly pure plasmid DNA with a concentration of 1 µg/µL was prepared with the NucleoBond Xtra Midi kit (Macherey-Nagel). Leaves of seven days old barley plants were excised and placed side by side on 1% agar with benzimidazole. The leaves were allowed to recover for 2-4 h.

Preparation of microcarriers

30 mg of 1 µm gold particles (microcarriers) were added to a 1.5 mL Eppendorf tube for ten bombardments. 1 mL of 70% EtOH was added and vigorously mixed by vortexing for 3-5 min on a platform vortex. Afterwards, the gold particles were allowed to settle for 15 min and subsequently centrifuged for 5 sec at RT. The EtOH was discarded, and the following wash step was repeated three times. 1 mL of sterile water was added and vortexed for 1 min. After a 1 min break, the microcarriers were centrifuged for 5 sec to pellet the gold, and the supernatant was discarded. Finally, 500 µL of sterile 50% glycerol was added to adjust the concentration to 60 mg/mL.

Binding of DNA to the microcarriers

The binding of the plasmid DNA to the microcarrier was performed 1 h before the bombardment. Therefore, the gold particles were vortexed for 5 min to resuspend the pellet. For each bombardment, 50 µL of the microcarrier suspension were removed during vortexing and transferred to a new Eppendorf tube. During further vortexing the following components were added in the respective order: 5 µL DNA (1 µg/µL), 50 µL 2.5 M CaCl₂ and 20 µL 0.1 M spermidine. Finally, the solution was mixed for three further min. Afterwards, the microcarriers were allowed to settle for 1 min and centrifuged for 2 sec. The supernatant was removed, and 140 µL of 70% EtOH were

added to the pellet. The gold microcarriers were vortexed at low speed for 2 sec, and the supernatant was removed after 2 sec of centrifugation. This washing step was repeated as described but with 100% EtOH. Eventually, the pellet was resuspended in 60 μ L 100% EtOH and stored on ice.

Particle bombardment

Seven macro carriers were placed inside the macro carrier holder and fixed with the insertion tool for each particle bombardment. 7 μ L of the microcarriers were evenly distributed over one macro carrier foil. The 7 μ L were removed while vortexing the tube to ensure proper mixing of the gold particle. After evaporation of the EtOH, the rupture disk (900 psi, BioRad) was soaked in 2-propanol and put in the top part of the hepta adapter, which was screwed inside the bombardment chamber. Subsequently, the bombardment apparatus was assembled according to the manufacturer's instructions. The target shelf with the samples was placed in the bombardment chamber. The bombardment was performed at a vacuum of 27 inches of mercury and a pressure of 1100 psi according to the manufacturer's instructions.

2.4 Histochemical staining:

2.4.1 GUS staining

Two days after inoculation of barley leaves with *Bgh* K1, the leaves were collected in a falcon tube and submerged with GUS staining solution (Table 15). Vacuum infiltration was conducted by applying three times a vacuum for 10 min. After incubation overnight at 37 °C, the GUS staining solution was discarded, and the leaves were destained with 80% EtOH for three days at 37 °C to remove the chlorophyll.

Table 15: Composition of GUS staining solution

component	concentration
NaH ₂ PO ₄	42.3 mM
Na ₂ HPO ₄	57.7 mM
EDTA	10 mM
MeOH	20% (v/v)
K ₃ Fe(CN) ₆	5 mM
K ₄ Fe(CN) ₆ × 3 H ₂ O	5 mM
X-Gluc	1 mg/mL
Triton X-100	0.1%

2.4.2 Coomassie staining of fungal structures

Chlorophyll destained leaves were soaked in Coomassie staining solution (45% (v/v) MeOH, 10% (v/v) acetic acid, 0.05% (w/v) Coomassie blue R-250) for 15-30 sec and subsequently washed two times with tap water to remove remaining staining solution. The samples were put onto a slide, and 50% glycerol was added before it was sealed with a coverslip. The samples were analysed by brightfield microscopy.

2.5 Microscopy:

2.5.1 Brightfield microscopy

Brightfield microscopy was used for the quantification of the penetration rate in *Bgh* infection assays.

2.5.2 Epifluorescence microscopy

The epifluorescence microscope Keyence BZ9000 was used for the detection of fluorescence signals in BiFC experiments. This microscope was equipped with filter cubes for the detection of the following fluorophores GFP (excitation 442.5-502.5 nm, emission 485-555 nm), YFP (excitation 476-524 nm, emission 515-569 nm), CFP (excitation 416-456 nm, emission 420-520 nm) and mCherry/DsRED (excitation 536-556 nm, emission 545-645 nm). The BZII-viewer software (Keyence) was utilised for taking pictures.

2.5.3 Confocal laser scanning microscopy

The Leica SP8 confocal laser scanning microscope was used to evaluate BiFC experiments in barley leaves. Barley leaves were prepared with perfluorodecalin for the microscope. The following excitation and emission wavelengths were used: mCherry/DsRED, excitation 561 nm (diode laser), emission 595-645 nm; YFP, excitation 514 nm (Argon laser), emission 525-570 nm.

2.6 DNA/RNA isolation and purification:

2.6.1 Quick isolation of genomic DNA from *A. thaliana*

Modified Edwards method (Edwards et al., 1991)

One to two *A. thaliana* leaves were collected in a 1.5 mL reaction tube, and 400 µL Edwards Buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS)

was added. The tissue was homogenised with a pestle and centrifuged for 5 min at full speed and RT. 300 µL of the supernatant was transferred into a new 1.5 ml reaction tube, and the same volume of isopropanol was added. The sample was gently mixed and centrifuged for 5 min at full speed (RT). The supernatant was removed, and the pellet was washed with 300-500 µL 70% ethanol. The pellet was dried at 55 °C, resuspended in 50-100 µL ddH₂O and stored at 4 °C.

2.6.2 Plasmid isolation from *E. coli*

For the isolation of plasmid DNA from *E. coli*, either the Macherey-Nagel NucleoSpin Plasmid Kit or an alkali lysis protocol was used. For the preparation of highly concentrated and pure plasmid DNA, the NucleoBond Xtra Midi kit from Macherey-Nagel was utilised.

Two ml of an overnight culture of *E. coli* were transferred in a 2 mL reaction tube and centrifuged at 7000 rpm for 1 min. The supernatant was discarded, and this step was repeated once. The pellet was resuspended in 250 µL Buffer I (Table 16) and afterwards, 250 µL Buffer II were added. The suspension was inverted three times and incubated for 1-2 min at room temperature. Then 250 µL Buffer III was added and mixed by inverting. The mixture was incubated on ice for 1-2 min and centrifuged for 8 min at 14000 rpm and 4 °C. 600 µL of the supernatant was transferred to a new 1.5 mL reaction tube and 600 µL cold isopropanol was added. The solution was mixed using a vortex, and a centrifugation step was performed for 15 min at 14000 rpm and 4 °C. The supernatant was discarded, and the pellet was washed with 500 µL 70% ethanol. The tube was mixed and centrifuged for 1 min at 14000 rpm. The supernatant was discarded again. The pellet was dried at 55 °C and resuspended in 50 µL TRIS pH 8 buffer or H₂O.

Table 16: Composition of plasmid isolation buffer I, II, III

component	concentration
Buffer I	
glucose	50 mM
EDTA	10 mM
Tris-HCl pH 8	25 mM
before usage	
10 mg/ml RNase stock solution	10 µL per 1 ml Buffer I
Buffer II	
SDS	1% (w/v)
NaOH	0.2 M
Buffer III	
potassium acetate pH 5.2	3 M

2.6.3 Plasmid isolation from yeast

The following protocol was modified based on (Robzyk & Kassir, 1992). A densely grown overnight yeast culture in selection medium was spun down (7000 rpm) for 1 min, and the supernatant was removed. Then 100 μ L of STET (8% sucrose, 50 mM Tris pH 8, 50 mM EDTA, 5% Triton X-100) were added, and the pellet was resuspended. After the addition of two scoops of glass beads (ca. 200 mg) and vortexing for 5 min, another 100 μ L STET was added. The sample was vortexed at maximum speed for 8 min at 4 °C. Afterwards, the samples were boiled for 3 min and cooled on ice. The supernatant was separated from the precipitate, transferred to a new 1.5 mL reaction tube and centrifuged at 14000 rpm for 10 min (4 °C). 100 μ L of the supernatant was transferred to a new tube and 50 μ L 7.5 M NH_4OAc was added. After incubation at -20 °C for at least 1 h (or overnight), the samples were centrifuged for 10 min at 14000 rpm and 4 °C to get rid of impurities. Again 100 μ L of the supernatant was collected, transferred to a new tube, and 200 μ L of ice-cold 100% EtOH was added. After centrifugation at 14000 rpm for 10 min, the supernatant was discarded, and the pellet was washed with 70% EtOH. After the washing step, the supernatant was completely removed, and the pellet was dried at 55 °C. The pellet was resuspended in 20 μ L ddH₂O; it was used as a template for an insert spanning PCR.

2.6.4 Sodium acetate precipitation

This method was used to purify PCR samples, clean DNA after a restriction digest or concentrate DNA. The volume of the DNA sample was set to 100 μ L with ddH₂O and 4 μ L of 4 M NaOAc together with 275 μ L 100% EtOH were added. This mixture was centrifuged at 15000 rpm for 30 min at 4 °C. Afterwards, the supernatant was removed, and the pellet was washed with 500 μ L 70% EtOH. In the end, the pellet was dried at 55 °C in a heat block and resuspended in 10-20 μ L of ddH₂O.

2.6.7 Purification of DNA

For the purification and concentration of DNA from a solution the ZymoResearch DNA Clean & Concentrator Kit or the Monarch PCR & DNA Cleanup Kit (NEB) was used. For the same purpose from a gel, the ZymoResearch Gel DNA Recovery Kit or the Monarch DNA Gel Extraction Kit (NEB) was applied.

2.6.5 RNA isolation

RNA was isolated with the NucleoSpin RNA Plus kit from Macherey-Nagel according to the manufacturer's protocol. The quality of the RNA was verified photometrically and by agarose gel electrophoresis.

2.6.6 cDNA synthesis

The RNA was treated with DNase I (Sigma-Aldrich, Dreisenhofen, Germany) according to the manufacturer's protocol and tested *via* PCR for remaining DNA. DNA contamination-free RNA was reversely transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol.

2.7 Cloning:

2.7.1 Gateway cloning

The Gateway Cloning System (Invitrogen, Karlsruhe, Germany) was used for cloning purpose. Both the BP and LR protocol from Invitrogen were modified as described in Table 17. Equal concentrations of donor and acceptor DNA were used (100 ng/ μ l-300 ng/ μ L). The reactions were incubated overnight at RT and subsequently transformed into heat-shock competent *E. coli* TOP10 cells.

Table 17: Composition of BP and LR reaction

component	BP	LR
TE buffer pH 7.5	1 μ L	1 μ L
enzyme mix	1 μ L BP clonase	1 μ L LR clonase
ddH ₂ O	1 μ L	1 μ L
donor	1 μ L PCR fragment	1 μ L pDONR clone with insert
acceptor	1 μ L pDONR201/pDONR207	1 μ L destination vector

Large destination vectors (>12 kb) were linearised for cloning to increase efficiency: these vectors were cut inside the Gateway-cassette *via* restriction digest and purified *via* sodium acetate precipitation.

The donor PCR product for the BP reaction was cleaned *via* sodium acetate precipitation or with a kit to get rid of primers prior cloning.

2.7.2 Restriction digest

Restriction digest was performed to confirm successful cloning according to the enzymes manufacturer's protocol. Restriction enzymes were obtained from New

England Biolabs (NEB, Ipswich, USA) or Fermentas (Thermo Fischer, Waltham, USA). Composition of an exemplary digest is listed in Table 18, and the incubation time was 2 to 4 h. In some cases, an overnight digest was performed to ensure a complete digest.

Table 18: Composition of restriction digest

component	volume
ddH ₂ O	add to 20 μ L
plasmid DNA	2 μ L
buffer	2 μ L
enzyme	0.5-1 μ L
total volume	20 μL

2.7.3 Sequencing

Sequencing of PCR products and plasmids was conducted by Eurofins Genomics (Ebersberg, Germany).

2.8 Polymerase Chain Reaction (PCR)

2.8.1 PCR for cloning

A proof-reading Phusion polymerase was used for cloning purposes. cDNA was used as a template for the amplification of genes of interest with specific primers with implemented Gateway-sites. The composition of the PCR mix and the PCR program can be found in Table 19. In some cases, DMSO (0.7 μ L) was added to the reaction to increase efficiency. The annealing temperature was chosen according to T_m of the primer and the elongation time depended on the length of the amplicons.

Table 19: Composition of Phusion PCR master-mix and PCR program

component	volume
primer F	1.25 μ L
primer R	1.25 μ L
10 mM dNTPs	0.5 μ L
ddH ₂ O	15.5 μ L
Phusion polymerase	0.5 μ L
template DNA	1 μ L
5xHF-Buffer	5 μ L
total volume	25 μL
temperature	time
98 °C	45 sec
98 °C	10 sec
T_m+3 °C	30 sec
72 °C	30 sec/1 kb; repeat from 98 °C 10 sec, 35 cycles
72 °C	10 min
16 °C	20 min

2.8.2 PCR for genotyping

Phusion or Taq polymerases were used according to Table 19 (Phusion) or Table 20 (Taq) for genotyping. Genomic DNA from *A. thaliana* was used as a template. DNA from Col-0 was taken as WT control, and as a negative control, H₂O was applied. Specific primers were chosen for amplification of WT DNA and the T-DNA insertion.

Table 20: Composition of Taq PCR master-mix and PCR program

component	volume
primer F	1 µL
primer R	1 µL
10 mM dNTPs	0.5 µL
ddH ₂ O	17.5 µL
Taq polymerase	0.5 µL
template DNA	2 µL
10xTaq buffer NEB	2.5 µL
total volume	25 µL
temperature	time
95 °C	5 min
95 °C	30 sec
T _m -8 °C	30 sec
72 °C	1 min/1 kb; repeat from 95 °C 30 sec, 35 cycles
72 °C	10 min
16 °C	20 min

2.8.3 RT-PCR

Phusion polymerase was used for RT-PCR with the composition listed in Table 19. Specific primers were chosen for the cDNA to generate amplicons with a preferred size of 300 bp to 500 bp and a max. size of 1 kb. Genomic DNA and water were used as negative controls (Table 21).

Table 21: PCR program RT-PCR

temperature	time
98 °C	45 sec
98 °C	10 sec
T _m +3 °C	30 sec
72 °C	30 sec/1 kb; repeat from 98 °C 10 sec, x cycles

2.8.4 Colony PCR

Colony PCR was performed for the verification of the presence of a transformed plasmid in *E. coli*, *A. tumefaciens* and *S. cerevisiae*. Programs and PCR mix composition for Phusion and Taq polymerase PCR are found in Table 19 and 20, respectively. As a template, a colony of the cells was picked with a sterile toothpick and resuspended in 20 µl sterile water in case of bacteria or in 20 µl 20 mM NaOH in case of yeast. Specific primers against plasmid sequences were used.

2.8.5 Agarose gel electrophoresis

Agarose gels were prepared with 1xTAE (Table 22) and 0.0025% of Ethidium Bromide (EtBr) as an intercalating dye for the separation of DNA fragments (1% or 2%). The gels were run in a chamber with running buffer (1xTAE with 0.0025% EtBr) at ca. 130 V (depending on the gel size) for 30-45 min. The bands were detected by UV illumination using the GelDoc™ XR+ (BioRad, Munich, Germany).

Table 22: Composition of 50xTAE buffer (2 L)

component	amount
ddH ₂ O	1.4 L
TRIS	484 g
EDTA	37.2 g
mix until EDTA is dissolved	
acetic acid	114.2 mL
mix, fill up to final volume and dilute to 1xTAE	

2.9 Transformation of Bacteria

2.9.1 Transformation of *E. coli*

Heat-shock competent *E. coli* Top10 cells were prepared according to Inoue et al. (1990) and stored at -80 °C. Plasmid DNA was added to 50 µL of thawed cells and incubated 30 min on ice. Afterwards, a heat shock at 42 °C for 90 sec was performed. The cells were cooled down on ice for 5 min and 1 mL LB medium was added to the cells. The cells were recovered for at least 1 hour at 37 °C and subsequently centrifuged at 6000 rpm for 1 min. Approximately 800 µL supernatant was discarded, and the pellet was resuspended in the remaining medium. 20 µL of the cell suspension were plated on LB agar with appropriate antibiotics, and the rest was plated on a second plate. The plates were incubated overnight at 37 °C.

2.9.2 Transformation of *A. tumefaciens*

Electro competent *A. tumefaciens* were stored at -80 °C and thawed on ice. 2 µL of plasmid DNA was added to the cells, and the mixture was transferred into a 1 cm electroporation cuvette. The cuvette was inserted into the MicroPulser Electroporation Apparatus (BioRad, Munich, Germany), and the cells were shocked with one pulse of 2.2 kV. Immediately, 1 mL of LB medium without antibiotics was added to the cells. The suspension was transferred into a 1.5 mL reaction tube and incubated for at least 2 h at 28 °C to allow recovery of the bacteria. Subsequently, the cells were plated on LB agar with appropriate antibiotics and incubated for 2 days at 28 °C.

2.10 Protein extraction, SDS-PAGE and Immunoblotting:

2.10.1 Total denatured protein extraction of leaf tissue

The plant tissue (12 leaf discs) was transferred to a 1.5 mL reaction tube with metal beads (1.5 mm diameter), frozen in liquid nitrogen and stored at -80 °C. The sample was ground for 1 min at a frequency of 25/s to a fine powder in a tissue grinder (Retsch, Haan, Germany). The powder was washed twice with 900 µL 100% ice-cold acetone and each time centrifuged in a pre-cooled centrifuge at 13000 rpm and 4 °C for 5 min. The pellet was resuspended in 900 µL ice-cold 10% (w/v) TCA in acetone by vortexing. The tube was inserted into an ultrasound ice-water bath for 10 min at 4 °C. After centrifugation (5 min, full speed, 4 °C) the pellet was washed once with 900 µL ice-cold 10% (w/v) TCA in acetone, once with 1 mL ice-cold 10% (w/v) TCA and then with 1 mL ice-cold 80% (v/v) acetone. Afterwards, the supernatant was discarded completely, and the pellet was resuspended at RT in 300 µL freshly prepared dense SDS buffer (100 mM Tris-HCl pH 8.0, 30% saccharose, 2% SDS, 5% β-mercaptoethanol). 300 µL of saturated Phenol/Tris-HCl pH8.0 (only the lower/bottom phase of the phenol was used) were added to the tube and then vigorously mixed on a vortex (until completely white). The sample was centrifuged at 13000 rpm for 20 min at RT. Two times 90 µL of the upper phase were carefully removed, separately transferred to clean 1.5 ml reaction tubes and stored on ice. One of these samples was subsequently used for SDS PAGE and Western blot while the second was used for the Bradford assay. In both samples, the total protein was precipitated for 60 min at -20 °C by addition and mixing of five volumes of ice-cold 100 mM ammonium acetate in methanol to the 90 µL solution. The protein was collected by centrifugation at full speed for 5 min at 4 °C. The pellet was washed once with 900 µL ice-cold 100 mM ammonium acetate in methanol and once with 900 µL ice-cold 80% (v/v) acetone. Finally, all acetone was removed with a pipette, and the samples were dried on ice with the lid open under the fume hood (10-15 min). The pellets were stored at -20 °C. 50 µL of Buffer A (7.7 M urea, 2 M thiourea, 300 mM NaCl, 0.25% CHAPS, 50 mM NaH₂PO₄ pH 8.0, 50 mM Tris pH 8.0) were added to prepare the sample for the Bradford assay. The pellet was resuspended for 1 h at RT and then shortly centrifuged before the Bradford assay. An SDS containing buffer was used to resuspend the second pellet for the SDS PAGE.

2.10.2 Quick protein extraction from yeast

5-50 ml of an overnight yeast culture were precipitated in a falcon tube by centrifugation at 3500 rpm for 3 min and then transferred to a 1.5 mL reaction tube. Afterwards, 50 μ L of acid-washed glass beads and 150 μ L Lyse & Load (LL-) buffer (Table 23) were added. This mixture was vortexed 1 min at highest settings and incubated for 10 min at 65 °C while shaking. Cell debris was separated by centrifugation at 7000 rpm for 1 min, and the supernatant was transferred to a new tube. 10-15 μ L of the supernatant was loaded on an SDS-PAGE.

Table 23: Composition of Lyse & Load (LL-) buffer

component	concentration
Tris-HCl pH 6.8	50 mM
SDS	4%
urea	8 M
glycerol	30%
DTT	0.1 M
bromophenol blue	0.005%
store at -20 °C	

2.10.3 Bradford assay

The Bradford assay was conducted for assessment of protein concentrations. The 5 x Bradford reagent concentrate (Rotiquant/Carl Roth) was diluted with five volumes of water. 5-20 μ L of the protein extract was mixed in a cuvette with 1 mL of the 1 x reagent. The protein concentration was measured photometrically. A standard curve in the used buffer system and with BSA was prepared to calculate protein concentrations.

2.10.4 SDS-PAGE (Laemmli)

The SDS-PAGE assay with the Laemmli buffer system (Laemmli, 1970) was used for the separation of proteins by their mass. For SDS-PAGE and Western blot the Mini-PROTEAN® Tetra Handcast Systems and the BioRad Mini Trans-Blot Cell were utilised according to the manufacturer's protocols. The separating SDS gel was prepared in the exact order of components given in Table 24. The stacking gel was made according to Table 25. After polymerisation, the gels were removed from the casting stand and inserted into the electrophoresis chamber. Two gels or one gel and one dummy were used for one electrophoresis plate holder, and the space between was filled with 1xSDS-running buffer (Table 26) to check for any leaking. The chamber was filled with 1xSDS-running buffer until the mark on the chamber. The samples were adjusted to the same protein concentration, mixed with the 2 x loading dye (Table 27)

and pipetted into the wells of the gel. Loading dye was added to empty gel pockets for a uniform run. Gels were run at 100 V for ca. 2 h until the loading dye front left the gel.

Table 24: Composition of the SDS-PAGE separating gel

component	7%	8%	10%	12%	15%	Recommended Acrylamide %	Protein size range
	15 mL	20 mL	5 mL	15 mL	10 mL		
H ₂ O	5.5 mL	9.2 mL	4 mL	3 mL	2.3 mL	8%	40-200 kDa
PolyAcrylamide 30%	3.5 mL	5.4 mL	5 mL	6 mL	5 mL	10%	21-100 kDa
1 M Tris pH 8.8	5.7 mL	5 mL	5.7 mL	5.7 mL	2.5 mL	12%	10-40 kDa
10% SDS	150 µL	200 µL	150 µL	150 µL	100 µL		
10% APS	150 µL	200 µL	150 µL	150 µL	100 µL		
TEMED	10 µL	12 µL	6 µL	6 µL	4 µL		

Table 25: Composition of the SDS-PAGE stacking gel

component	5 mL	10 mL	15 mL
H ₂ O	3.4 mL	6.8 mL	10.2 mL
PolyAcrylamide 30%	830 µL	1.66 mL	2.49 mL
1 M Tris pH 6.8	630 µL	1.26 mL	1.89 mL
10% SDS	50 µL	100 µL	150 µL
10% APS	50 µL	100 µL	150 µL
TEMED	5 µL	10 µL	15 µL

After the run, the chamber was disassembled, the stacking gel was discarded, and the separating gel was used for Coomassie staining or western blot.

Table 26: Composition of 10 x SDS running buffer (1 L)

component	amount
Tris Base	30.2 g
glycine	188 g
H ₂ O	600 mL
heat up to dissolve	
10% SDS	100mL
fill up to 1 L with H ₂ O	

Table 27: Composition of 2 x loading dye (40 mL)

component	amount
H ₂ O	5 mL
1 M Tris-HCl (pH 6.8)	5 mL
10% SDS	20 mL
glycerol	10 mL
bromophenol Blue	0.01 g
before use, mix	
2xLoading dye	800 µL
1 M DTT	200 µL

2.10.5 Coomassie staining

The gel was stained in Coomassie staining solution (Table 28) overnight on an orbital shaker or by heating in a microwave for a few seconds. The gel was treated with destain solution overnight to reduce background staining. The solution was changed multiple times until protein bands were visible. Coomassie gels were documented by taking a photo or drying the gel between cellophane sheets in a drying frame and subsequent scanning of the gel.

Table 28: Composition of Coomassie staining and destaining solution

component	amount
Coomassie staining solution (0.5 l)	
Coomassie	0.5 g
acetic acid (100%)	50 mL
methanol	250 mL
fill up with H ₂ O	
Coomassie destain solution (1 l)	
glycerol	30 mL
acetic acid (100%)	100 mL
methanol	400 mL
fill up with H ₂ O	

2.10.6 Western blot

Wet blot sandwiches and apparatuses were assembled according to the manufacturer's protocol for the transfer of proteins from gels to nitrocellulose membranes. Cold transfer buffer (Table 29) was used for blotting. The blot was run for 1 h at 100 V or 250 mA or overnight at 45 mA.

Table 29: Composition of 10X stock solutions of transfer buffer, PBS and TBS

component	amount
10xTransfer Buffer (1 L)	
Tris base	30.3 g
Glycine	144.1 g
fill up with H ₂ O	
before use, mix	
10xstock solution	100 mL
EtOH	200 mL
fill up to 1 L with H ₂ O	
10xPBS (pH 7.5, 1 L)	
Na ₂ HPO ₄	115 g
NaH ₂ PO ₄	29.6 g
NaCl	58.4 g
10xTBS (pH 7.6, 1 L)	
1 M Tris-HCl (pH 7.5)	200 mL
NaCl	90 g
for TBST and PBST	add Tween20 1:1000 dilution

2.10.7 Ponceau staining

After the Western Blot, the membrane was washed with water to remove residual SDS and stained for 10 min in Ponceau staining solution (0.5% Ponceau in 5% glacial acetic acid). After removal of the background with ddH₂O, the membrane was scanned and documented.

2.10.8 Blocking, probing and detection

The membrane was washed three times 5 min with 1XPBST or 1xTBST to remove the Ponceau staining for immunodetection of proteins. Blocking of non-specific binding sites was conducted with 5% nonfat milk powder in 1XPBST or 1xTBST for 1 h. Afterwards, the primary antibody was diluted to the recommendations of the manufacturer in the appropriate buffer and incubated overnight at 4 °C with the membrane on an orbital shaker. Residual primary antibody was removed by washing the membrane three times for 5 min with 1xTBST or 1XPBST before the second antibody was added to the membrane and incubated for 1 h at RT. After additional three 5 min washing steps, the immunodetection was performed by adding 500 µl of a 1:1 mixture of SuperSignal West Femto Chemi-luminescent substrate (Thermo Scientific) and SuperSignal West Pico Chemi-luminescent substrate (Thermo Scientific) and detecting the signals in the ChemDoc™ MP (Bio-Rad).

2.11 Measurement of plant responses to biotic triggers:

2.11.1 Chemiluminescence-based measurement of MAMP-induced ROS formation

Leaf discs from six to seven-week-old *A. thaliana* plants were prepared and transferred into a 96-well plate filled with 200 µL sterile ddH₂O per well. The leaf discs were incubated overnight on the bench. The next morning, the water was replaced by 50 µL sterile ddH₂O per well, and the leaf discs were allowed to rest for 30 min to avoid measuring a touch response. A 10 mg/mL chitin stock suspension was prepared freshly before usage: 5 mg chitin from shrimp shells (C9752-1G) was added to a 1.5 mL reaction tube with six metal beads. The powder was ground for 3 min at 25/s in a Retsch Mixer Mill, and ddH₂O was added to give a final concentration of 10 mg/mL. The suspension was constantly mixed until usage. A master mix of 2 µM flg22 (1 mM stock; -20 °C), 2 µM elf18 (1 mM stock, -20 °C) or 200 µg/mL chitin (freshly prepared;

see below) with 400 μ M Luminol (20 mM stock in DMSO; -20 °C), 20 μ g/mL horseradish peroxidase (1 mg/mL stock, -20 °C) in ddH₂O was prepared to trigger ROS formation. 50 μ L of the master mix was added to each well with the help of a multi pipettor. A timer was started at the first well, and the master mix was added in 2 s intervals to each well. Afterwards, the plate was inserted into the luminometer, and the measurement was started 1 min after finishing pipetting. Luminescence was recorded for 2 s/well in the same order as the master mix had been added. The luminescence from every well was repeatedly measured over a total time of 2700 sec in 120 sec intervals (23 repeats in total) in a Berthold Luminometer Centro XS³ 960:

2.11.2 Powdery mildew infection assay

Leaves of seven days old barley plants on 1% agar with benzimidazole were inoculated in an inoculation tower with *Bgh* K1 24 h after particle bombardment. Fungal spores were dispersed into the tower and let to settle for 20 min. Leaves were incubated for 48 h in a growth chamber (12 h light at 20 °C, 12 h darkness at 19 °C; 60% relative humidity and 100 μ mol s⁻¹ m⁻² light intensity), and afterwards sampled for evaluation of penetration rate via GUS staining, Coomassie staining and bright field microscopy as described above. Formation of secondary hyphae was considered as an indication for successful penetration.

2.12 Split-ubiquitin yeast 2-hybrid methods:

2.12.1 Split-ubiquitin procedure

A split-ubiquitin yeast 2-hybrid (Y2H) screen was conducted via the LexA-based or the Ura3-based split-ubiquitin Y2H system. Transformation of suitable yeast strains with the matching bait plasmids was performed as described in section 2.12.2. Presence of the bait protein was detected via western blot in case of the LexA-based system or via growth on SC-His-Met-Ura plates in case of the Ura3-based system. The bait fusion protein with the C-terminally-fused Ura3 enzyme complements lack of Ura3 in the employed yeast strain in the Ura3-based system. If the bait protein was properly expressed, the negative control was transformed into the yeast and tested for autoactivation of the system. When both tests were successfully passed, bait containing strain was used for cDNA library screening.

For the screening, the suitable yeast strain was transformed with 1-2 µg of the respective library, the negative and the positive control (if available) and plated entirely on the appropriate interaction selection agar. For the Ura3-based system agar with 5-FOA and for the LexA-based system agar lacking His and Ade was used. Transformation efficiency was calculated by plating 1:10 and 1:100 dilutions of the transformation reaction on agar selecting for presence of the plasmids.

Appearing colonies were re-streaked on new selection plates to reduce the risk of false positives. Plasmid DNA was extracted from re-growing colonies (see protocol 2.6.3) and used as a template for an insert spanning PCR (PCR with a primer located in the vector backbone) to amplify the inserted DNA. The PCR products were purified and sent for sequencing, and the results were analysed *via* BLAST search.

Interesting candidates (e.g. genes involved in the specific bait-related pathway) and multiple times appearing genes were checked for completeness of the insert and correct reading frame with the tag. Potential interaction partners were retested in yeast and/or *in planta*.

2.12.2 High-efficiency transformation of competent yeast cells

the following protocol was modified from (Daniel Gietz & Woods, 2002)

A 10 mL overnight culture was grown at 30 °C and 250 rpm in YPD or in the appropriate selection medium, in case the strain contained a plasmid. The next morning, a 50 mL culture was prepared in prewarmed YPD, and the OD₆₀₀ was set to 0.2 for conventional transformation and to OD₆₀₀ 0.4 for high-efficiency transformation. The culture was incubated at 30 °C and 250 rpm until the OD₆₀₀ reached 1 or 2, respectively. The cells were pelleted at 3500 rpm for 3 min in 50 ml falcon tubes, washed in 25 ml cold-sterile water and afterwards resuspended in 1 mL of 100 mM LiAc. The cell suspension was transferred to a 1.5 mL reaction tube and centrifuged at 13000 rpm for 10 sec. The supernatant was discarded and resuspended in 500 µL of 100 mM LiAc. For each transformation, 50 µL of the cell suspension was centrifuged at 1300 rpm for 15 sec. The supernatant was removed, and at least 1 µg of plasmid DNA was added. 300 µL of the transformation mix (Table 30) was added during vortexing the cells at low speed. Then the cells were incubated at 30 °C for 30 min and subsequently transferred to 42 °C for 60 min for a heat shock treatment. After centrifugation at 6000 rpm for 10 seconds, the supernatant was removed entirely, and the cells were carefully

resuspended in 500 µL sterile water by inverting the tube. 1:10 and 1:100 dilutions were plated on plasmid selection agar to estimate the transformation efficiency, and 100 µL was used for selection of positive transformants.

Table 30: Composition of the transformation mix (1 mL)

component	volume
50% PEG 3350	680 µL
1 M LiAc	100 µL
10 mg/mL carrier DNA ¹	14 µL
Sterile water	206 µL

¹ boil before use 5 min and chill on ice (reboil/use three times)

The efficiency was calculated with the following formula:

colony-forming units (CFU) x reaction volume / volume plated x µg DNA

2.12.3 Preparation and transformation of competent yeast cells

Yeast cells were grown overnight in 5-10 mL cultures in YPD medium (without plasmid) or SC medium minus the selection marker (carrying plasmid) at 30 °C and 250 rpm. The next day 50 mL cultures were prepared in prewarmed YPD. The OD₆₀₀ was set to 0.4, and the cells were incubated at 30 °C and 250 rpm. The cells were pelleted at an OD₆₀₀ of 2 by centrifugation at 3000 g for 5 min and washed in 0.5 volumes of sterile water. In a second wash step, 0.01 volumes of sterile water were used. Finally, the cells were resuspended in the same volume of filter sterile frozen competent cell (FCC) solution (5% v/v glycerol, 10% v/v DMSO). Samples of 50 µL were aliquoted and slowly cooled down to -80 °C in an isolated styrofoam box to prevent the formation of ice crystals.

For transformation, aliquots were thawed at 37 °C for 30 sec and centrifuged at 13.000 x g for 2 min. The FCC solution was removed, and plasmid DNA (300 ng - 1 µg) was added to the pellet. The following steps were performed as described above (2.12.2) for high-efficiency transformation.

2.12.4 Drop test assay

For the Drop test assay, 5 mL overnight cultures were grown in medium selecting for presence of bait and prey vectors. The next day, the cells were pelleted by centrifugation at 6000 rpm for 1 min in 2 mL reaction tubes. The cells were washed once with sterile water and resuspended in 500 µL sterile water. The OD₆₀₀ was measured in a 1:100 dilution and set to OD₆₀₀ 1 in a sterile 96 well plate. Afterwards,

10-fold dilution series until 0.001 were prepared with a multichannel pipette. Finally, 4 μ L of each dilution were dropped onto two different agars: once for selection for presence of bait and prey plasmids and documentation of even growth of all tested constructs, and second for selection of positive interaction.

2.12.5 LacZ reporter assay of the LexA-based split-ubiquitin Y2H system

At least 5 ml cultures were incubated at 28-30 °C and 250 rpm overnight in a shaking incubator in selection medium for both plasmids. Yeast cells were pelleted (centrifugation at 6000 rpm for 1 min) at an OD₆₀₀ of 0.8-1 and washed with 1 mL sterile 4 °C-cold Z-buffer (60 mM Na₂HPO₄ 2H₂O, 40 mM NaH₂PO₄ X H₂O, 10 mM KCl, 1 mM MgSO₄ 7H₂O; pH 7.0). The cells were resuspended in 650 μ L Z-buffer and disrupted by three freeze (liquid nitrogen) and thaw (37 °C for 1 min) cycles. 50 μ L 0.1% SDS and 50 μ L chloroform were added, and the samples were mixed by vortexing at maximum speed for 1 min. The cell debris and the lysate were separated by a centrifugation step for 10 min at 10000 x g at 4 °C. 600 μ L of the supernatant was transferred to a new tube and kept on ice. The protein concentration was measured via Bradford assay. The protein concentrations of all samples to be tested were adjusted to the concentration of the lowest concentrated sample. 800 μ L 37 °C-warm oNPG-solution (1 mg/mL *ortho*-Nitrophenyl- β -galactoside in Z-buffer) and 200 μ L of the yeast extract were mixed, incubated at 37 °C and yellowing of the solution was monitored over time. The reaction was stopped with 0.5 mL 1 M Na₂CO₃ before saturation of the colour change. The extinction was measured in a spectral photometer at 420 nm. In case the extinction exceeded an OD₄₂₀ of 1.0, the samples were diluted, and the measurement was repeated. The Specific enzyme activity (U/mg) was calculated with the following formula:

$$[\text{U/mg}] = (E_{420} \times V) / (\epsilon \times d \times v \times t \times P)$$

V = volume of reaction (1500 μ L)

ϵ = extinction coefficient of o-nitrophenol (4.5 mL/ μ mol cm)

d = thickness of the cuvette (1 cm)

v = volume of yeast extract (200 μ L)

t = incubation time [min]

P = protein concentration [mg/mL]

2.13 Library construction for the split-ubiquitin Y2H systems

2.13.1 *Bgh* effector library construction

Based on the 2010-2012 annotation of the *Bgh* genome (Pedersen et al., 2012, Spanu et al., 2010), around 534 effectors were purchased as pre-cloned constructs in pENTR221 gateway vectors from ThermoFisher (Waltham, USA). Ten randomly picked clones were sent for sequencing to confirm the identity. All clones were transformed in *E. coli*, plasmids were isolated, and glycerol stocks were prepared and stored at -80 °C. The plasmids were used as entry clones in Gateway LR reactions to shuttle the effectors into the yeast expression vector pNX32. The LR reactions and transformations were performed in 96-well plate format. From each LR reaction, one colony was grown as 5 mL overnight culture in LB medium supplemented with ampicillin. The plasmids were re-isolated by mini-prep and checked for successful recombination *via* PCR with insert-spanning primers specific for the vector backbone. The DNA concentration of all plasmids was measured and adjusted, and all vectors were mixed for one complete library.

The following formula was used to calculate the required number of yeast transformation colonies for complete coverage of the library (Clarke & Carbon, 1976):

$$P = 1 - (1 - f)^N$$

P = probability (0.95)

N = number of transformed colonies

f = ratio of the average insert size to total library size (619 bp/330462 bp = 1.873×10^{-3})

$$N = \ln(0.05) / \ln(1 - 1.873 \times 10^{-3}) = 1600$$

At least 1600 colonies are necessary to cover the entire library once.

2.13.2 Barley cDNA library construction

The library was constructed in cooperation with the lab of Pietro Spanu (Imperial College, London; UK). Both groups collected epidermal peels of barley at 0, 6, 12, 24, 48 and 96 hpi with *Bgh*. While the Spanu lab used the barley cultivar Golden Promise and the *Bgh* isolate DH14, the Panstruga lab used the barley cultivar Margret, and the *Bgh* isolate K1. For the epidermal peels, the epidermis was incised orthogonally to the leaf axis with a razor blade and peeled off. Epidermal peels were directly frozen in liquid nitrogen and stored at -80 °C. For each time point at least 3 g of material was collected and sent to Pietro Spanu's lab for RNA isolation. The isolated RNA was sent

to ThermoFisher Scientific for library construction (cDNA synthesis and cloning into pENTR vectors). The library inserts were transferred into a classical Y2H prey destination vector and into the pNX32 vector of the split-ubiquitin Y2H system. ThermoFisher Scientific specified the number of clones in the split-ubiquitin Y2H library with 3.2×10^6 CFU and indicated an average insert size of 1.7 kb. 1 μ l of the library was transformed into *E. coli* to confirm the quality, plasmids were re-isolated and sent for sequencing. In parallel, insert spanning PCRs were performed to estimate the average insert size. The average insert size of 1 kb was determined, and 2/10 of the obtained sequences were confirmed to be of *Bgh* origin.

2.14 *In planta* protein-protein interaction assays

2.14.1 Bimolecular Fluorescence Complementation (BiFC)

N. benthamiana leaves, or barley epidermal cells were transformed with the methods described in section 2.3 for BiFC experiments. Two plasmids were transformed in parallel: one carrying the bait protein fused to either the C-terminal or the N-terminal part of the fluorescent protein (FP; YFP or VENUS), and the second carrying the prey protein with the respective another half of the FP. Together with these plasmids, a transformation marker plasmid mediating the constitutive expression of mCherry or DsRed was transformed. After an incubation time of 2-3 days, the appearance of fluorescence in comparison to positive and negative control was examined *via* epifluorescence microscopy or confocal microscopy. Successfully transformed cells were identified with the help of the transformation marker. Multiple cells were examined to check for a difference in intensity of fluorescence or number of fluorescent cells compared to the negative control.

2.14.2 Split-luciferase assay (Split-LUC)

N. benthamiana leaves were transiently transformed with the method described in section 2.3.1. Plants of the same age and comparably old leaves were used for minimisation of the variability between experiments. Two leaves of the same plant were infiltrated with two *Agrobacteria* strains carrying both genes of interest with either the C-terminal or N-terminal part of the firefly luciferase. After 2-3 days, eight leaf discs (4 per leaf) were excised and placed into wells of a 96-well plate, which were filled with 200 μ L buffer (10 mM $MgCl_2$, 10 mM MES pH 5.7). Prior measurement, the buffer was

replaced by 100 μ L buffer supplemented with 1 mM Luciferin. Leaf discs were vacuum-infiltrated in an exicator for 5 min and subsequently placed in a luminometer. After a 5 min delay, the luminescence was measured repeatedly every 150 sec for 1 sec/well over a total time of 30 min. The raw data were examined and, if luminescence curves appeared normal, the relative light units (RLU) of the 5 min time point were used. The mean of 8 samples was calculated and used as an indicator of interaction strength. The values of all tested combinations were normalised against the negative control. After the measurement, samples for detection of fusion protein abundance *via* western blot were frozen in liquid nitrogen and stored at -80 °C.

Chapter 1: Comparison of two different split-ubiquitin yeast two-hybrid systems

Introduction:

The method of choice for the identification of protein-protein interaction is the classical Y2H system. This system is designed for soluble proteins and rely on the translocation of bait and prey protein, which are fused to a DNA-binding (BD) or an activation domain (AD), into the nucleus and further activation of reporter genes. The split-ubiquitin yeast two-hybrid (Y2H) system was designed to overcome the disadvantages of the classical Y2H system (Fields & Song, 1989), which is unsuitable for membrane-bound proteins since the interaction partners have to enter the nucleus. Truncation of membrane proteins enables their use in a classical system; however, this will change the 3D structure of the proteins, which will potentially impact interactions. The split-ubiquitin yeast two-hybrid system is based on the ability of ubiquitin, an abundant eukaryotic protein, which marks proteins for degradation, to reassemble if expressed as two independent peptides in yeast. The reassembled ubiquitin is recognised by ubiquitin-specific proteases, which mediate cleavage and release of a fused effector leading to reporter activation. For interaction screens, the C-terminal (Cub) and the N-terminal (Nub) regions of ubiquitin are fused to the bait and the prey candidate interactors, respectively (Johnsson & Varshavsky, 1994).

The LexA-based system uses an artificial transcription activator (PLV) as effector, which consists of ProteinA, LexA and VP16. The ProteinA domain contains two IgG-binding (immunoglobulin G) sites enabling immunoblot detection and prolonging the lifetime of the fusion protein (Xing et al., 2016). The LexA acts as a DNA-binding motif (Fogh et al., 1994), while the VP16 serves as the transactivation domain of the transcription activator (Hirai et al., 2010). The bait fusion protein is composed of the protein of interest C-terminally fused to Cub and PLV. The bait construct requires membrane localisation to hinder the fusion protein from entering the nucleus and from activation of reporter genes prior to the establishment of an interaction. If a physical interaction takes place, both fusion proteins associate, facilitating the reassembly of ubiquitin. Consequently, the PLV tag is cleaved off by ubiquitin-specific proteases (Stagljar et al., 1998), relocates into the nucleus and activates the reporter genes *His3*, *Ade2* and *lacZ*. The prey protein can either be membrane-bound or cytosolic (Figure 3). One advantage of this system is the presence of three different reporter genes,

which allows stringent dual auxotrophy selection mediated by *His3* and *Ade2* as well as quantification of the interaction *via lacZ*-based enzymatic assays (Obrdlik et al., 2004)

The bait-Cub construct is fused to the Ura3 protein in the Ura3-based system. Upon successful interaction, an arginine at the N-terminus of the Ura3 mediates its quick degradation *via* the N-end rule pathway after release from the bait-Cub-Ura3 fusion by ubiquitin-specific proteases (Bachmair et al., 1986, Tasaki et al., 2012). Rapid degradation of Ura3 leads to uracil auxotrophy and resistance to 5-FOA (5-Fluoroorotic acid) (Wittke et al., 1999). If present and stable, Ura3 converts 5-FOA to cytotoxic 5-Fu (Boeke et al., 1987), the thymidylate synthase inhibitor is blocking the synthesis of thymidine (Longley et al., 2003) and consequently mediating incorporation of uracil into the DNA (Seiple et al., 2006). The advantages of this system are its suitability for membrane-bound and cytosolic baits and the possibility to test for efficient expression of the bait construct by Ura3-mediated uracil auxotrophy (Figure 4).

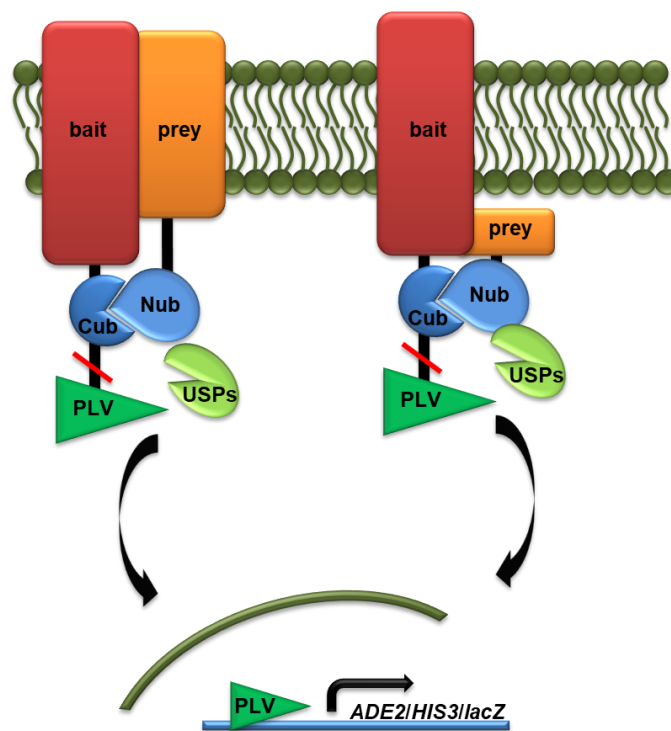


Figure 3: Model of the LexA-based split-ubiquitin yeast two-hybrid system. In the LexA-based split-ubiquitin Y2H system, the protein of interest (bait) is C-terminally fused to Cub (C-terminal part of ubiquitin) and the artificial transcription factor PLV. The potential interaction partner (prey) is fused N- or C-terminally to Nub (N-terminal part of ubiquitin). Both constructs are encoded by different plasmids and expressed in the *S. cerevisiae* strain THY.AP4. When both proteins interact, ubiquitin is reassembled, and the PLV is released by ubiquitin-specific proteases (USPs). PLV translocates into the nucleus, where it activates the reporter genes *ADE2*, *HIS3* and *lacZ*. The bait has to be membrane-anchored to avoid nuclear entry of PLV.

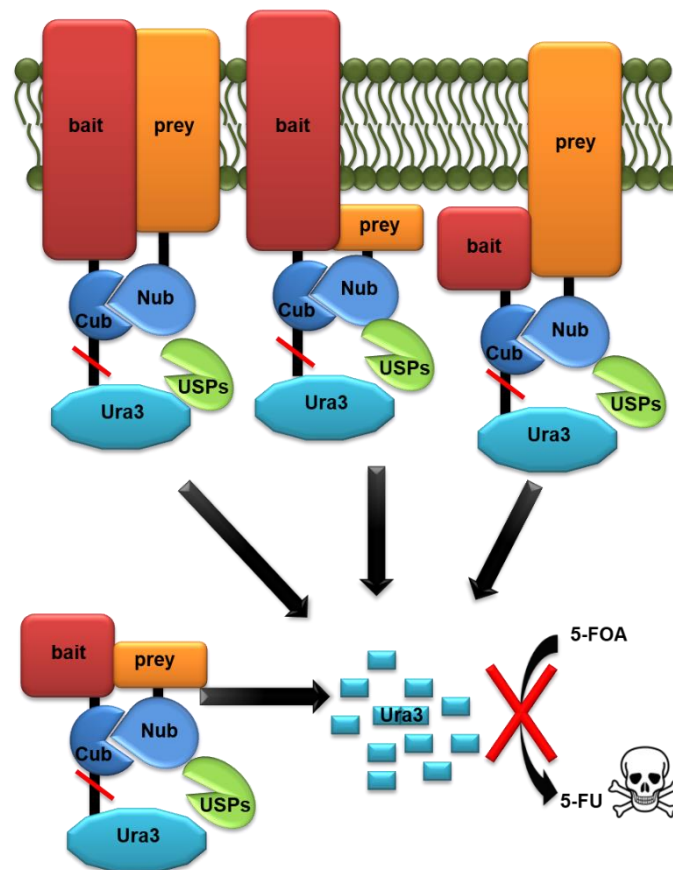


Figure 4: Model of the Ura3-based split-ubiquitin yeast two-hybrid system. In the Ura3-based split-ubiquitin Y2H system, the protein of interest (bait) is C-terminally fused to Cub (C-terminal part of ubiquitin) and an Ura3 protein with an N-terminal arginine. The potential interaction partner (prey) is fused N- or C-terminally to Nub (N-terminal part of ubiquitin). Both constructs are encoded by different plasmids and expressed in the *S. cerevisiae* strain JD53. When both proteins interact, ubiquitin is reassembled, and the Ura3 is released by ubiquitin-specific proteases (USPs). Due to the N-terminal arginine, the Ura3 is rapidly degraded. If degraded, Ura3 will not convert 5-FOA to toxic 5-FU. The bait protein can be membrane-anchored or cytosolic.

Three Nub versions are available with different affinities to Cub (Figure 5). Nubl is the wildtype version with the highest affinity and possesses isoleucine on position 13. NubA has an alanine on this position and a medium affinity, whereas NubG harbours a glycine and the lowest affinity. This modification was made to repress its tendency for self-assembly and consequently to reduce false positives. NubG cannot reassemble by itself; it needs the interaction of bait and prey to restore (Johnsson & Varshavsky, 1994), whereas Nubl is capable of forming a complete ubiquitin-protein without the interaction of bait and prey. I used different vector combinations in this study to test the suitability of the Nub variants for different baits. The vectors pNX_{wt} and pCup-Nubl-GWY-CYC1 encode Nubl, whereas pNX32, pNX33 and pCup-NubG-GWY-CYC1 carry NubG. Only pCup-NubA-GWY-CYC1 has NubA. The pNX vectors

possess the ADH1 promoter and terminator, whereas the pCup vectors harbour a copper-inducible promoter and CYC1 terminator. The pCup vectors and the pNX33 are low copy vectors (1-2 copies per yeast cell; (Clarke & Carbon, 1980, Tschumper & Carbon, 1983), while pNX_{wt} and pNX32 are high copy vectors (50-100 copies per yeast cell (Romanos et al., 1992)). These properties are critical for the selection of suitable vectors since they influence expression and sensitivity of the systems. All prey vectors are compatible with both split-ubiquitin systems (Grefen et al., 2007, Grefen et al., 2009, Stagljär et al., 1998, Wittke et al., 1999). Both bait vectors use the Met-repressible promoter Met25 (Obrdlik et al., 2004, Thomas et al., 1989) for fine-tuned expression of the bait fusion protein.

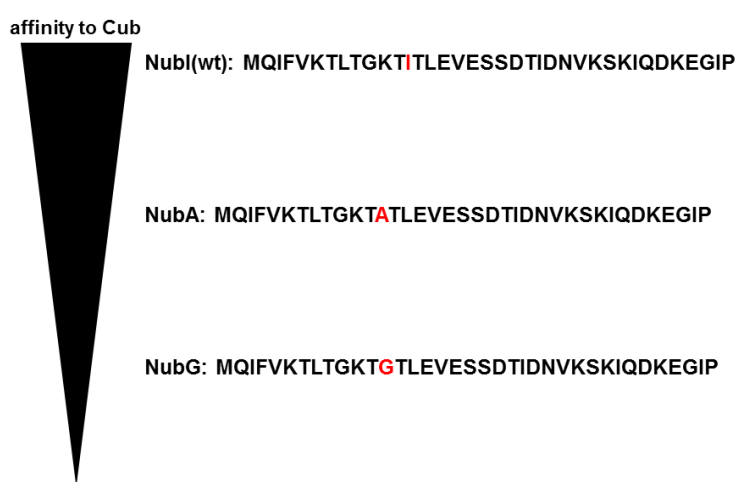


Figure 5: Scheme of the Nub variants. The three different N-terminal ubiquitin (Nub) versions Nubl, NuA and NubG have different affinities to the C-terminal part of ubiquitin (Cub). An amino acid exchange at position 13 of the wildtype Nubl with the highest affinity to Cub decreases the affinity gradually over a medium affinity (alanine; NubA) to a low affinity (glycine; NubG). Distinct Nub-Cub affinities are used to fine-tune the Y2H assays and consequently to suppress false-positive results.

Figure 6 shows the workflow of the screening. Pretests are performed to ensure a properly working system before the actual screen. Expression of the bait fusion protein is tested by western blot (LexA system) or auxotrophy selection (Ura3 system) after the transformation of the bait construct into the respective yeast strain. While conduction of the growth assay is faster and more convenient, the western blot is more informative since it provides evidence on the expression level and protein stability. The prey expression is not tested since each prey plasmid in the libraries contains different inserts, which are differently expressed or stable. The negative control should be tested when the bait is expressed. Either the empty vector backbone of the library can

be used, or the same vector with a protein that not interact with the bait. The empty vector can cause some background signal, like in many other assays, and that should be tested before the actual screen. When an interaction partner is known, then it could serve as a positive control. The transformation efficiency of the strain carrying the bait construct should be tested before the screen. Consequently, the efficiency of the transformation can be optimised without wasting the library. The library is transformed with a high-efficiency transformation protocol to ensure proper coverage. Depending on the intended coverage, the setup has to be modified. Positively selected colonies are selected again on selection agar to reduce the risk of false positives. Repeatedly growing colonies are used for plasmid extraction and either insert spanning PCR or plasmid retransformation in *E. coli*. Finally, the reisolated vectors or the PCR products are sent for sequencing, and the results are blasted against the reference genome. In case the obtained candidates are in frame with the tag, the number of hits of the same gene is evaluated. Confirmed candidates are further investigated *in silico* for their function and subcellular localisation. Promising candidates are retested in a targeted Y2H in yeast and/or in additional protein-protein interaction assays.

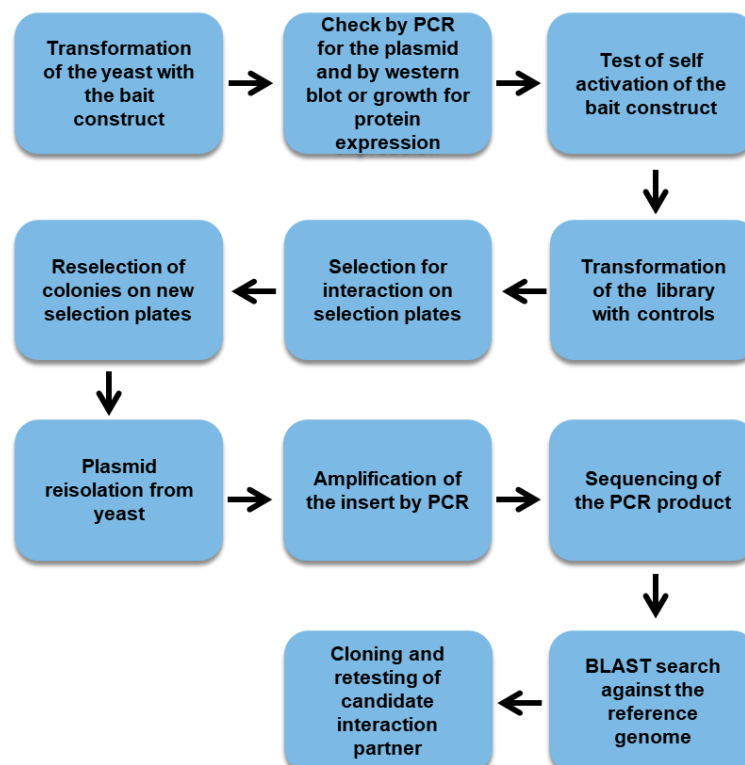


Figure 6: Schematic procedure of a split-ubiquitin Y2H screen. This scheme depicts the procedure of the performed H2Y screens. Transformation of the bait is followed by evaluation of the expression, the examination of the negative control and a possible autoactivation. The actual screen starts with the library transformation and is followed by a second selection of obtained colonies. After plasmid reisolation, PCR and sequencing, the sequencing results are blasted against the reference genome. Candidates are selected and revalidated.

The aim of this study

Previous high-throughput yeast screens investigated membrane interactome networks in *Arabidopsis* (Jones et al., 2014a, Lalonde et al., 2010). These mating/array-based approaches used a set of full-length cDNA clones as preys and did not capture all genes of *Arabidopsis*. However, this suggests that our knowledge on the interactome of membrane-bound proteins is still incomplete. Furthermore, Y2H screen data on membrane protein interaction networks of plants other than *Arabidopsis* is largely lacking. This thesis aims to identify new interaction partners of plant (membrane) proteins involved in plant immunity. In order to identify the most suitable assay for the investigation of membrane-bound protein interaction in different organisms, I compared two different split-ubiquitin Y2H systems with respect to reliability and efficiency.

Results:

Interaction of MLO2 and CaM as a case study to compare different split-ubiquitin Y2H systems

I made use of the well-characterised interaction of *Arabidopsis* MLO2 with CaM (Kim et al., 2002) to compare the LexA-based with the Ura3-based split-ubiquitin Y2H system. In this assay, barley *HvCaM3* was employed as prey since *Arabidopsis* CaMs share at least 97% amino acid identity with *HvCaM3*, and, in contrast to *Arabidopsis* CaMs, the interaction of *HvCaM3* with MLO2 has been validated (Kim et al., 2002). As CaM requires an accessible C-terminus for interaction with MLO2 (personal communication Ralph Panstruga), I employed prey vectors with N-terminally tagged Nub. MLO2 was labelled at its C-terminus as the N-terminus locates at the extracellular space, which would render the tag inaccessible for cytoplasmically located Nub. I further used six different prey vectors with distinct characteristics like different copy numbers, various promoters/terminators and various Nub versions to test which conditions were most suitable to detect the interaction reliably. The respective empty

prey vector (-GWY) was used as a negative control to investigate the effective suppression of background growth.

The LexA-based system resulted in the consistent growth of yeast on plasmid selection agar (-tryptophan -leucine) without methionine (Met; Figure 7 A), indicating lack of toxic effects and proper preparation of dilution series. Additional auxotrophy selection for positive interaction (-tryptophan -leucine -histidine -adenine -methionine) revealed no suppression of background growth of empty vector (eV) controls if Met was not added to the agar (Figure 7 B). Upon addition of increasing Met concentrations to the interaction selection medium, mediating transcriptional repression of the bait construct, the selective effect became more visible (Figure 7 C-F). While yeasts with eVs grew only at an OD₆₀₀ of 1 (of Nubl-GWY, NubA-GWY and NubG-GWY) or 10⁻¹ (NX32-GWY), the respective *HvCaM3* prey constructs resulted in the growth of all (Nubl-*HvCaM3*, NX32-*HvCaM3*) or most (NubA-*HvCaM3*, NubG-*HvCaM3*) dilutions (Figure 7 D, F). No impact of Met on growth was demonstrated for the NX_{wt} constructs (-eV or -*HvCaM3*); they grew similarly well up to a dilution of 10⁻³, independent on the presence of Met (Figure 7 A-F). In conclusion, a difference between eV and *HvCaM3* constructs could only be detected for NX32 or the pCup vectors (Nubl, NubA and NubG) and was merely noticeable at increasing Met concentrations, which indicates a high background growth in this system. Only the pCup vectors showed a clear growth phenotype, whereas the NX32 vector depicted a qualitative difference at higher Met concentrations. The same trend was observed for all three biological replicates.

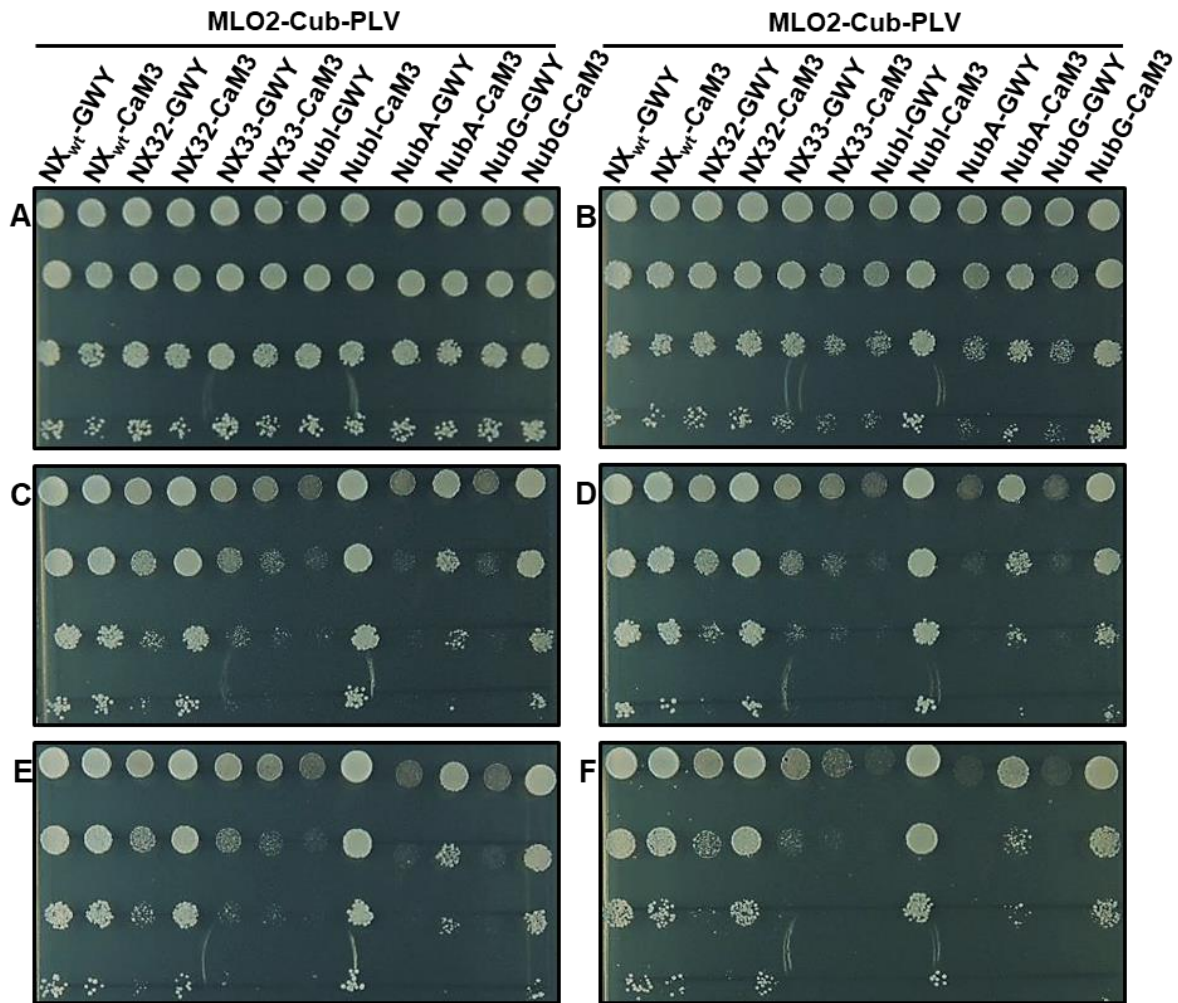


Figure 7: LexA-based split-ubiquitin assay for comparison of different prey vectors. The validated interaction of *AtMLO2* with *HvCaM3* was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). The empty vector was used as a background control (-GWY). 4 μ l of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD_{600} 1-10⁻³) on agar selecting for successful transformation (A) (SC -leucine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC -leucine -tryptophan -adenine -histidine). Different methionine (Met) concentrations were used as indicated to repress expression of the bait construct gradually. (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan - adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 75 μ M methionine, (D) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine, (E) SC -leucine -tryptophan -adenine -histidine + 300 μ M methionine, (F) SC -leucine -tryptophan -adenine -histidine + 600 μ M methionine. The experiment was repeated three times with similar results (Supplementary Figure 1+2).

In the Ura3-based system, all plotted yeast strains grew uniformly if selected for the presence of bait and prey vectors (-histidine -tryptophan; Figure 8 A). Upon addition of 1 g/l 5-FOA to select for positive interactions, only small quantitative differences in growth of yeasts carrying eV or *HvCaM3* constructs were visible (Figure 8 B). Yeasts with NX_{wt}-*HvCaM3*, NX33-*HvCaM3* and Nubl-*HvCaM3* grew slightly better compared

to the respective eV controls. Vice versa, the NubG-eV control unexpectedly facilitated growth in comparison with the NubG-*HvCaM3* construct. Supplementation of the interaction selection medium with increasing concentrations of Met did not result in altered growth but interfered with the selective effect of 5-FOA as all plotted yeasts grew equally well (Figure 8 C-D). Similar results were obtained in three experimental replicates (Supplementary Figure 3+4).

Importantly, 5-FOA is most effective at low pH medium (< pH 4,5). As the pH of the agar was initially set to six, only poor growth selection occurred upon addition of 5-FOA (Figure 8 B). When the pH was adjusted for later experiments and screening, the selection was fully functional.

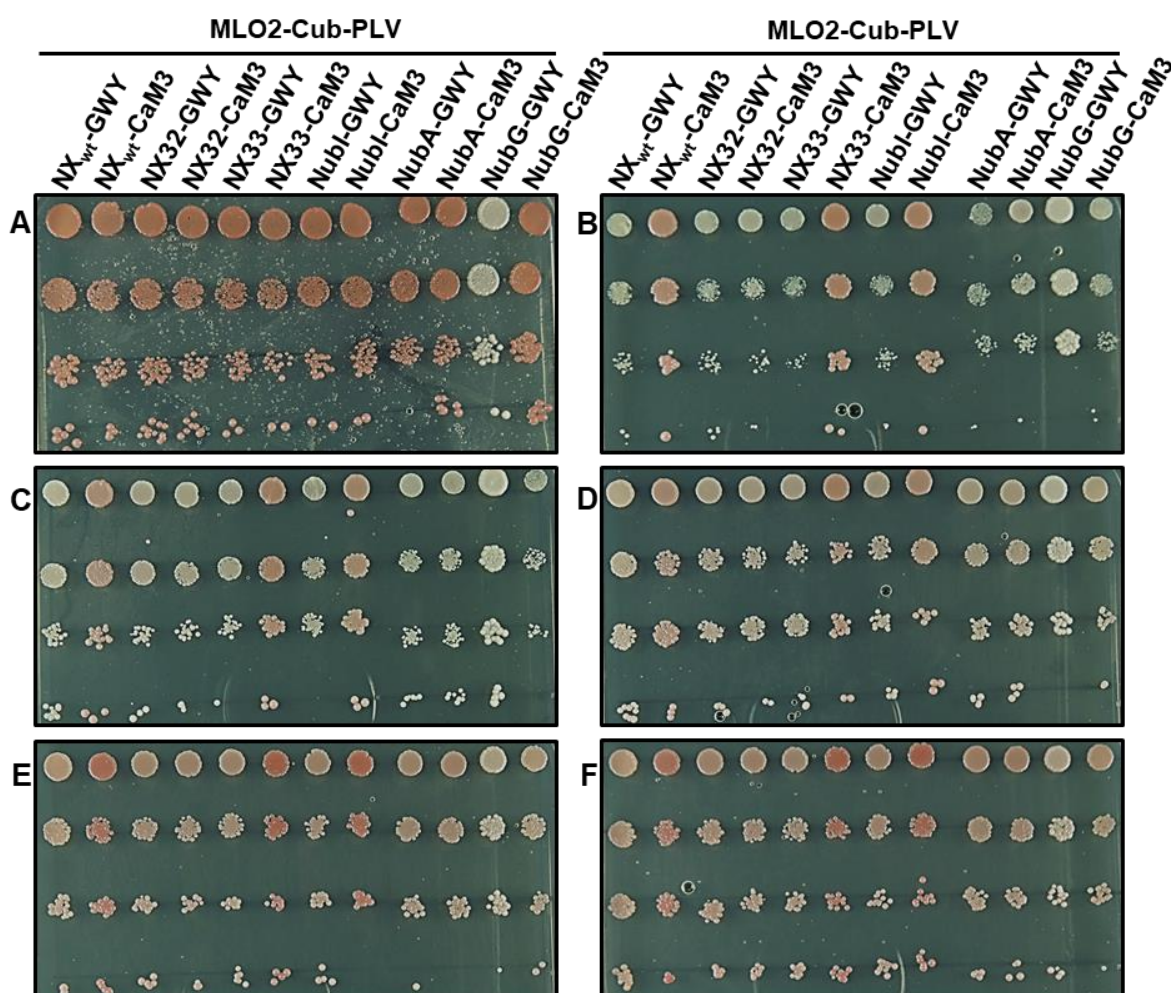


Figure 8: Ura3-based split-ubiquitin assay for comparison of different prey vectors. The validated interaction of AtMLO2 with *HvCaM3* was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). The empty vector was used as a background control (-GWY). 4 µl of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD₆₀₀ 1-10⁻³) on agar selecting for successful transformation (A) (SC -histidine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC

-histidine -tryptophan + 1 g/l 5-FOA). Different methionine (Met) concentrations were used as indicated to gradually repress expression of the bait construct. (A) SC -histidine -tryptophan -methionine, (B) SC -histidine -tryptophan - methionine + 1 g/l 5-FOA (C) SC -histidine -tryptophan + 75 μ M methionine + 1 g/l 5-FOA, (D) SC -histidine -tryptophan + 150 μ M methionine + 1 g/l 5-FOA, (E) SC -histidine -tryptophan + 300 μ M methionine + 1 g/l 5-FOA, (F) SC -histidine -tryptophan + 600 μ M methionine + 1 g/l 5-FOA. The experiment was repeated three times with similar results (Supplementary Figure 3+4).

Medium without Ura can be used as a counter-selection in the Ura3-based system. Presence of the bait fusion protein complements Ura3-deficient yeast strains and consequently enables growth. If bait and prey interact, the Ura3 is degraded, and the yeasts cannot resume growth on plates without Ura. As a result, only yeast cells lacking interaction will survive.

On agar with Ura, consistent growth of yeasts was observed for all tested constructs (Figure 9 A). Deprivation of Ura from the medium resulted in arrested growth of the prey constructs NX_{wt}- *HvCaM3*, NX33- *HvCaM3* and Nubl- *HvCaM3* as well as the negative control NubG-GWY. Notably, the same constructs had mediated improved growth on the 5-FOA and Ura3 containing agar (Figure 8 B), which confirms the evidence of interaction of the bait constructs. No growth of yeasts was visible on plates with an increased Met concentration (Figure 9 C-F).

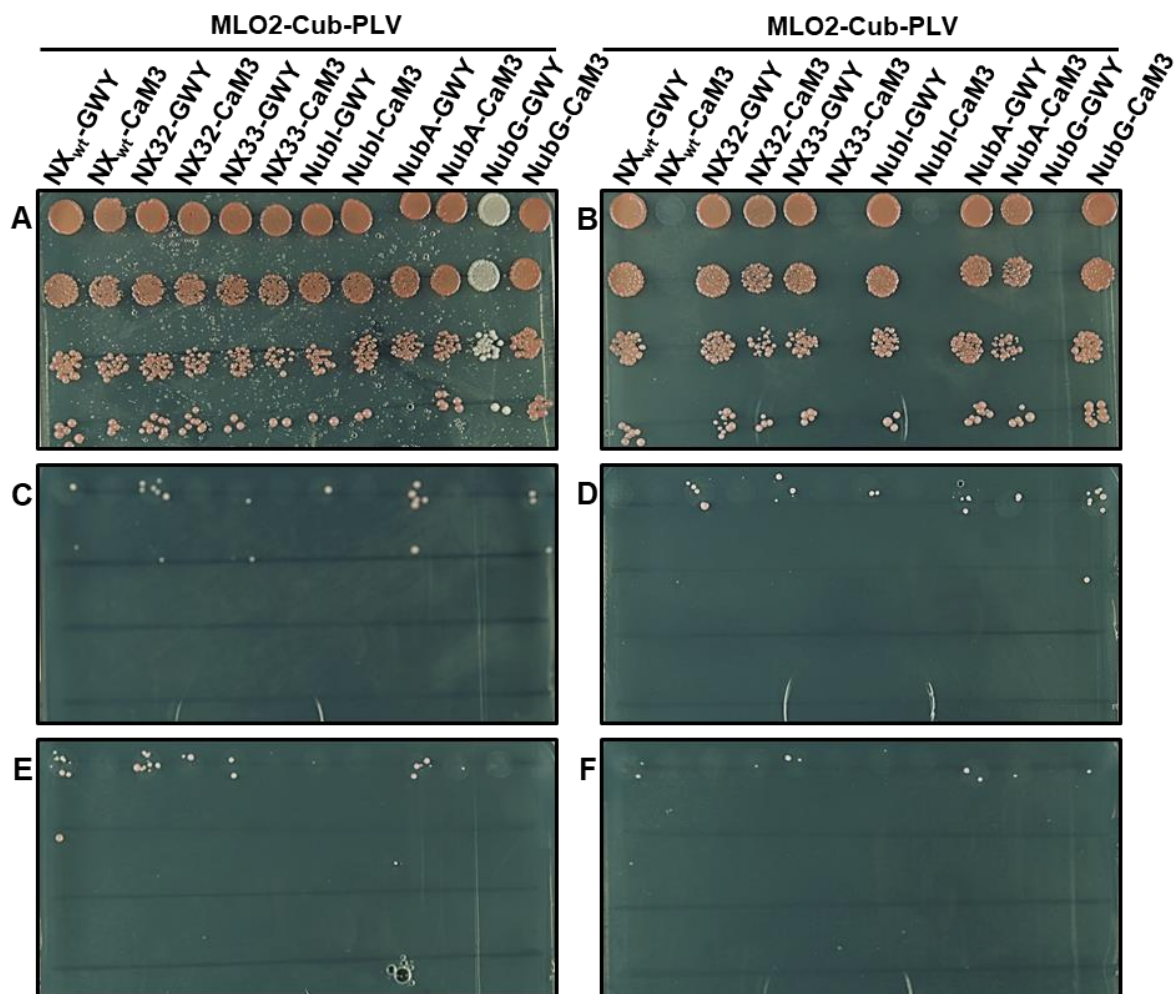


Figure 9: URA3-based split-ubiquitin assay with counter-selection for the comparison of different prey vectors. The validated interaction of *AtMLO2* with *HvCaM3* was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). The empty vector was used as a background control (-GWY). 4 μ l of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD₆₀₀ 1-10⁻³) on agar selecting for successful transformation (A) (SC -histidine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC -histidine -tryptophan -uracil). Different methionine (Met) concentrations were used as indicated to repress expression of the bait construct gradually. (A) SC - histidine - tryptophan -methionine, (B) SC -histidine -tryptophan -methionine -uracil (C) SC -histidine -tryptophan -uracil + 75 μ M methionine, (D) SC -histidine -tryptophan -uracil + 150 μ M methionine, (E) SC -histidine -tryptophan -uracil + 300 μ M methionine, (F) SC -histidine -tryptophan -uracil + 600 μ M methionine. The experiment was repeated three times with similar results (Supplementary Figure 4+5).

Performed LexA-based and Ura3-based split-ubiquitin Y2H screens

In total, 27 different bait proteins were used for screening against three distinct libraries with the LexA- and/or the Ura3-based Y2H systems (Figure 10). Forty-six different screens were conducted during this study, whereby varying degrees of colony number and verified interactions were achieved. Interpretation of the results of the different screens might serve as a measurement of the reliability of the two Y2H systems. Additionally, since CERK1 and FLS2 were screened in both systems, they can be used

to compare both systems. The LexA-based system is only capable of screening membrane proteins, whereas the Ura3-based system can also use cytosolic proteins. This is one reason why many proteins were only screened in the Ura3-based system since they are not membrane localised.

The conducted screens concentrated on plant membrane bait proteins involved in plant immunity from barley, wheat, rice and Arabidopsis as well as on two effector protein baits (AVR_{A1} and AVR_{A13}) from the barley powdery mildew *Blumeria graminis* f. sp. *hordei* (*Bgh*). Arabidopsis AtMLO2, AtMLO6, AtMLO12 (Devoto et al., 2003), AtFLS2 (Gómez-Gómez & Boller, 2000) and AtCERK1 (Miya et al., 2007) bait proteins were screened against an Arabidopsis/*G. orontii* cDNA library, which was prepared from powdery mildew-infected leaf material in the pCup-Nul-GWY-CYC1 prey vector (by Chiara Consonni) in order to identify new interaction partners. Barley and wheat bait proteins were screened against a *Bgh* CSEP library. These included thirteen integrated decoy domains from barley (Sarris et al., 2016) (provided by Dr. Panagiotis Sarris and Dr. Matthew Moscou), HvMlo (Büschges et al., 1997), HvMla10 (Seeholzer et al., 2010) and five RLKs from barley and wheat with proposed function in non-host resistance (Rajaraman et al., 2016), which were screened to find potential interacting CSEPs. The *Bgh* CSEP library was prepared with around 534 CSEPs annotated in the 2010 *Bgh* DH14 assembly (Pedersen et al., 2012, Spanu et al., 2010) in the NX32 prey vector. HvMlo, OsJAC1, as well as *Bgh* AVR_{A1} and AVR_{A13} (Lu et al., 2016) were screened against a barley/*Bgh* cDNA library to identify potential plant or fungal interaction partners and effector targets. This cDNA library was prepared in the NX32 prey vector from *Bgh*-inoculated barley epidermal peels to reduce the abundance of *RuBisCO* mRNA and to enrich fungal transcripts.

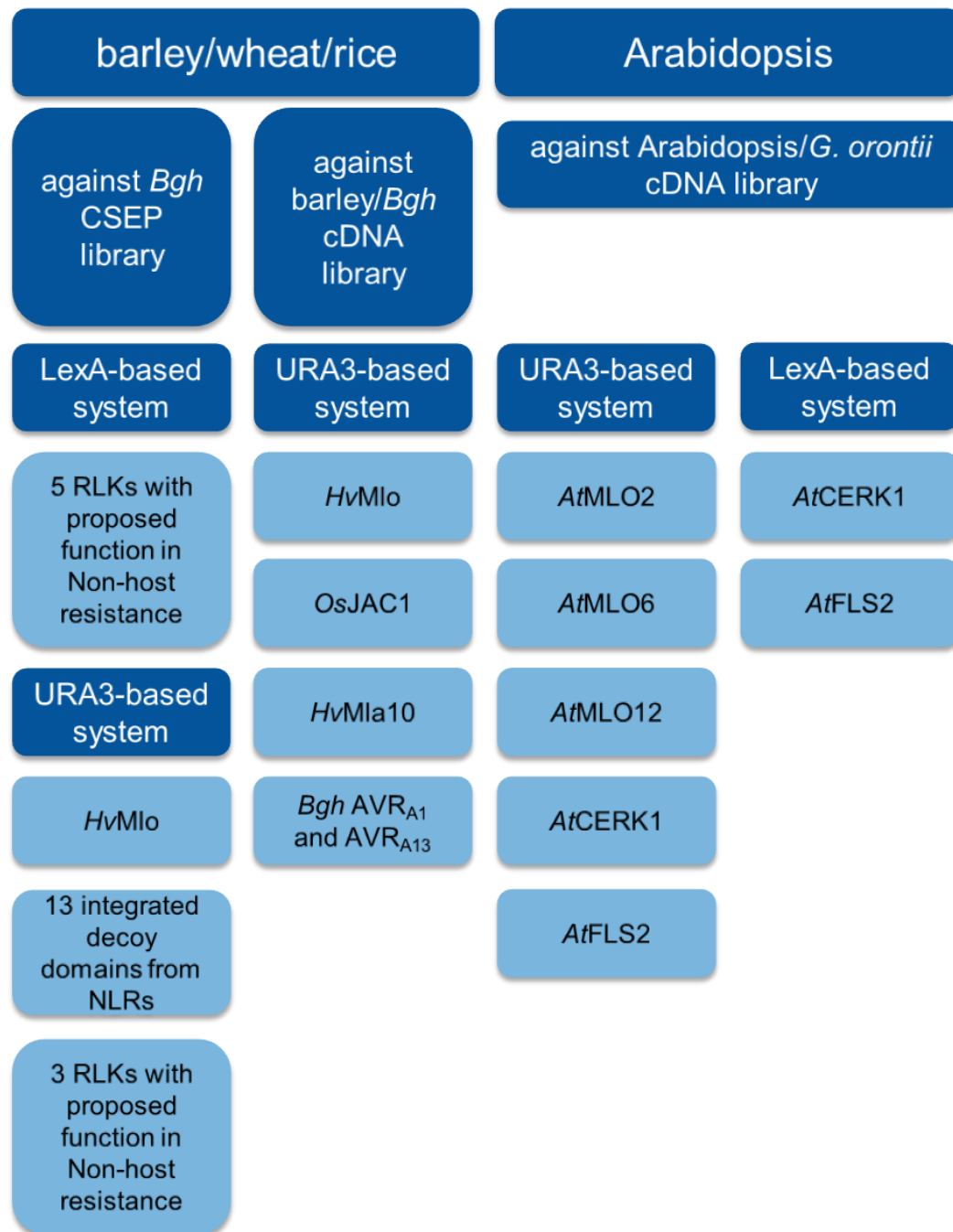


Figure 10: Schematic overview of conducted Y2H screens. Proteins from monocots (barley, wheat or rice), where either screened against a *Bgh* CSEP library and/or a barley/*Bgh* cDNA library. Arabidopsis proteins were screened against an Arabidopsis/*G. orontii* cDNA library. Avirulence effectors (AVRs) of *Bgh* were tested against a barley/*Bgh* cDNA library. The LexA-based split-ubiquitin Y2H system was used for *AtCERK1*, *AtFLS2*, *AtMLO2/6/12* and five receptor-like kinases from wheat and barley with proposed function in non-host resistance. The Ura3-based split-ubiquitin Y2H system was employed for screening of *AtMLOs* and *HvMlo*. Further *AtCERK1*, *AtFLS2*, 13 integrated decoy domains from barley, *HvMla10*, three of the *HvRLKs* and the two *Bgh*AVRs. *HvMlo* and the 13 decoy domains were tested twice against the *Bgh* CSEP library. The screen of *OsJAC1* (Jiang et al., 2006, Weidenbach et al., 2016) against a barley/*Bgh* cDNA library was performed in collaboration with Prof. Dr. Ulrich Schaffrath (RWTH Aachen).

LexA-based screens

Notably, many of the bait proteins (eight baits) showed no/low expression in yeast or auto-activity of the reporter system in the LexA-based vectors (Figure 11, Supplementary Figure 7, Table 31). The LexA-based screens with these baits resulted in either no colonies or were not performed, since a difference between the library and negative control was not expected (Table 31). These screens were discarded, and the baits were retested with the alternative Ura3-based system. For example, *HvLemk1* (formerly *HvRnr8*; (Rajaraman et al., 2016), and its wheat homologs *TaRnr8* p7 and p12 revealed no signal in the western blot of the LexA-based system (chapter 2; Figure 11) but were expressed (truncated and/or full-length version) in the Ura3-based system as indicated by the growth assay (Supplementary Figure 8). Interestingly, MLO proteins generally showed auto-activity in the LexA-based system (Table 31, Supplementary Figure 7), which is in accordance with the results obtained with *AtMLO2* and *HvCaM3* in the LexA-based system (Figure 7).

Consequently, the LexA-based screens resulted only for three baits in a reasonable number of colonies (Table 1). Two of these screens were performed against the CSEP library (*HvRlk7/HvRlk10*; see chapter 2 for more details) and one against the *Arabidopsis/G.orontii* cDNA library (*AtCERK1*). Mathematical evaluation (Clarke & Carbon, 1976) material and methods 2.13.1) suggested that at least 1500 successfully transformed colonies were required for full 1x coverage of the *Bgh* CSEP library, consisting of a defined number of clones. Accordingly, the recorded transformation efficiency of ca. 1×10^5 CFU/ μ g DNA represented a coverage of at least 10x for both baits (*HvRlk7/HvRlk10*). Indeed, certain CSEPs were identified multiple times (see chapter 2 for more details). By contrast, in the LexA-based *Arabidopsis/G.orontii* cDNA library screen with the *AtCERK1* bait, I captured only one candidate three times. However, this screen identified previously characterised interaction partners, like GPA1 (Aranda-Sicilia et al., 2015), CaM2 (Le et al., 2014) and ANN1 (Espinoza et al., 2017, Jones et al., 2014a), indicating its reliability (see chapter 4 for more details). Strikingly, all initial candidates from the LexA-based screens of *HvRlk7*, *HvRlk10* and *AtCERK1* could be further verified by targeted Y2H experiments and/or *in planta*.

Ura3-based screens

Many of the bait proteins obtained from collaborators were not membrane-associated (barley decoy domains, *Bgh* AVR_{A1}, *Bgh* AVR_{A13}, *HvMla10*, *OsJAC1*), disqualifying them for the LexA-based system. Hence these baits were screened with the Ura3-based system. Screening of *HvMlo* and the barley decoy domains against the *Bgh* CSEP library resulted in the identification of only a few potential interactors. Adjustment of the experimental parameters in a second screen revealed additional candidates only in case of the *HvMlo* bait.

As *HvLemk1* and its wheat homologs were not expressed in the LexA-based system, I truncated these proteins and expressed the kinase domain in the Ura3-based system. Since the split-ubiquitin system detects only cytoplasmic interactions and the cytosolic portion of *HvLemk1* and homologs is equivalent to the kinase domain, this truncation might most probably not circumvent detection of interacting candidates. The subsequent screen resulted in nine colonies, of which three represented the same CSEP. In parallel, also the full-length versions were used for the screen.

Screens, which revealed the most colonies with the Ura3-based system, were conducted with the *HvMlo*, *OsJAC1* and AVR_{A1} baits against the barley/*Bgh* cDNA library. All three screens showed a considerable variety of inserts (*HvMlo* screen chapter 3), which might indicate a high number of false positives.

The low number of colonies obtained in the Ura3-based system with many of the screened baits might suggest that for these baits, a less stringent selection is required. Evidence for this is given by the fact that, in contrast to the LexA-based screen, no colonies grew in the screen of the *AtCERK1* bait against the *Arabidopsis/G. orontii* cDNA library if the Ura3-based system was applied. The possibility to adapt the stringency of the Ura3-based system to the respective bait was subsequently tested by varying the 5-FOA concentration of the interaction selection agar. This was done for the screen with the decoy domains (reduction from 1 g/L to 0.7 g/L 5-FOA) and resulted in fewer colonies in the actual screen.

Table 31: Number of colonies obtained in split-ubiquitin Y2H screens indicative of possible interaction partners. Selection in Ura3-based screens was conducted with 1 g/L 5-FOA or 0.7 g/L 5-FOA. Grey background indicates screening combinations, which were not performed

bait	LexA-based		Ura3-based	
	CSEP library	Barley/ <i>Bgh</i> cDNA library	CSEP library	Barley/ <i>Bgh</i> cDNA library
Barley				
<i>HvRlk7</i>	47			
<i>HvRlk10</i>	20			
<i>HvRnr8/Lemk1</i>	no expression		9 (kinase domain)	
<i>HvMlo</i>	auto-activity	auto-activity	5/13	92
<i>HvMla10</i>			0	
Barley decoy domains				
Exo70			0/0	
HSF			1/0	
kinase			4/0	
kinase			9/0	
kinase			0/0	
PP2C			0/0	
TRX			0/0	
WRKY			0/0	
ZFBED			1/0	
ZFBED			1/0	
CG			0/0	
B3			3/0	
Wheat				
<i>TaRnr8 p7</i>	no expression		3 (kinase domain)	
<i>TaRnr8 p12</i>	no expression		0	
Rice				
OsJAC1				118
<i>Bgh</i>				
<i>BghAVR_{A1}</i>				50
<i>BghAVR_{A13}</i>				4
Arabidopsis		Arabidopsis/<i>G. orontii</i> cDNA library	Arabidopsis/<i>G. orontii</i> cDNA library	
<i>AtMLO2</i>		auto-activity		1
<i>AtMLO6</i>		auto-activity		5
<i>AtMLO12</i>		auto-activity		14
<i>AtFLS2</i>		no expression		2
<i>AtCERK1</i>		46		0

Discussion:**Plasmid properties influence split-ubiquitin Y2H results**

No differences in Y2H assays with the *AtMLO2* bait between eV and *HvCaM3* prey constructs were recognised in the LexA-based system if the expression of the bait was not repressed by addition of Met to the selection agar (Figure 7 B). This might indicate an unstable bait fusion protein resulting in entry of PLV into the nucleus and subsequent promotion of background growth in eV controls. This is supported by the auto-activity test without the prey plasmid. Yeast strains expressing MLOs show growth on SC -leucine -adenine -histidine -methionine plates, which indicates further an unstable protein (Supplementary Figure 7). In principle, PLV might be released after auto-assembly of Cub and Nub in eV constructs. However, at least the NubG variant alone is not capable of associating with Cub bait constructs but instead requires the interaction of the fused proteins (Johnsson & Varshavsky, 1994). Even when the Met concentration was increased, the background growth of some eV controls was not completely suppressed, rendering a screening approach with distinct vectors (pNX_{wt}, pNX32, pNX33) not promising. Nevertheless, quantitative differences in growth between several vectors were noticeable (Figure 7). The low copy pCup vectors worked fine with 75 µM Met and showed a clear growth difference between eV and *HvCaM3* constructs. Despite the different Nub version harboured by pCup vectors, no striking differences could be recorded in terms of suppression of background growth of eV controls. Interestingly, usage of the low copy pNX33 vector (promoter: ADH1) that differs from the pCup-NubG (promoter: CUP1) plasmid merely in the promoter region revealed only background growth that was indistinguishable in eV control and *HvCaM3* bait constructs. Hence, the suitability of the promoter is one critical factor that should be considered for selection of the vector. Employment of the high copy vectors pNX_{wt} and pNX32 resulted in a high background growth of eV controls. In the case of pNX_{wt}, even the addition of Met was not capable of reducing this effect. This vector features the high-affinity variant Nubl, expressed under control of the constitutive ADH1 promoter. Consequently, all of its properties favour a high expression of the prey and therefore probably facilitate unspecific auto-reassembly of Nubl with Cub, promoting false positives. By contrast, growth differences between eV and *HvCaM3* became visible at higher Met concentration with the pNX32 vector. As discussed above, the NubG variant encoded by pNX32 should not trigger false-positive results, high protein abundance, however, might still cause background growth. In addition to

the already mentioned properties, the linker between the protein of interest (*AtMLO2*) and the tag differs between pNX and pCup vectors. As previously shown, this might further influence the interaction by modulating protein stability and flexibility of the different domains (Klein et al., 2014, Prescott et al., 1999, van Rosmalen et al., 2017).

Repression of bait expression by the addition of Met resulted in the opposite effect in the Ura3-based system compared with the LexA-based assay (Figure 8 C-F). The effect of 5-FOA-mediated selection disappeared, since the repression of the bait fusion protein coincided with reduced conversion of 5-FOA to toxic 5-FU by Ura3 (Boeke et al., 1987). By contrast, if Ura was withheld from the selection agar, and the bait fusion protein was repressed by addition of Met, the growth of yeasts was arrested completely. Sparsely abundant Ura3 failed to mediate sufficient biosynthesis of Ura for cell survival (Figure 9).

Interestingly, application of the pNX_{wt} and pNX33 prey vectors succeeded to detect *AtMLO2* and *HvCaM3* interaction while usage of pNX32 did not (Figure 8 A, Figure 9 B). pNX33 is a low copy vector encoding low-affinity Nub (NubG), and NX_{wt} is a high copy vector plasmid mediating the expression of high-affinity Nub (NubI). The high copy vector pNX32, which encodes low-affinity Nub (NubG) combines the properties of pNX_{wt} and pNX33 but completely failed to mediate growth differences between eV control and *HvCaM3* prey construct.

The false-positive behaviour of the NubG-eV construct (Figure 8 A, Figure 9 B) might be caused by contamination with a different yeast strain because the NubG variant cannot auto reassemble with Cub unless the expression of the fusion proteins is too high. The only vector, which reliably detected positive interaction of *AtMLO2* and *HvCaM3* and repressed false-positive growth of eV controls in both systems (LexA and Ura3), was the pCup-NubI-GWY-CYC1 vector (Figure 7 C-F; Figure 8 A, Figure 9 B). This combination of a low copy vector encoding a high-affinity Nub variant revealed to be the most suitable option for Y2H-based investigation of *AtMLO2* interactions (Kim et al., 2002).

In conclusion, all vector properties (promoter, terminator, Nub version, copy number and protein linker) will influence whether an interaction can be detected in a Y2H screen and should be considered carefully. In conclusion, the tests performed with *AtMLO2* and *HvCaM3* visualised that even, if the same prey vectors are used, and both bait vectors employ the same promoter, there are still differences in the sensitivity

of both Y2H systems. Further, even the same bait/prey combination might result in distinct results, if different prey vectors are used. The applied vector combinations influence the outcome of a Y2H experiment or a screening approach and hence, should be tested in advance. Optimally, different vectors should be used to confirm the outcome of such an approach independently. However, the construction of a Y2H library is time-consuming and expensive, hampering such extensive experimental approaches. The comparison to a non-interacting protein as negative control can further help to improve the setup.

Advantages and disadvantages of the LexA- and the Ura3-based system

The LexA-based system was used successfully in three of the Y2H screens. I identified potential interaction partners that were further validated in targeted Y2H experiments and/or *in planta* with BiFC and/or split-luciferase assays (chapter 2 and 4). A drawback of the LexA system is the fact that only membrane-anchored proteins can be used as baits, as cytoplasmic fusion proteins might enter the nucleus and activate the reporter genes. This fact disqualified a large proportion of the bait proteins to be screened with this system. A newly available vector for this system introduces a membrane-anchor at the N-terminus of the bait fusion protein to enable usage of additional bait proteins (Mockli et al., 2007, Xing et al., 2016). However, the attachment of a second tag and aberrant targeting of a protein to the membrane might alter the 3D topology of the protein or block interaction domains. This would most likely influence protein-protein interactions. Despite this disadvantage, several significant benefits render the LexA system an interesting option for Y2H assays: the availability of antibodies binding to PLV enables visualization of the bait fusion protein abundance and stability; the possibility to select against two auxotrophy selection markers increases the stringency of the system compared to a single auxotrophy selection marker system, and the lacZ reporter allows quantification of the interaction strength *via* enzymatic assays. Initially, His auxotrophy was exclusively used for selection (Stagljar et al., 1998). The Ade marker was added to increase stringency (Grefen et al., 2007).

The auto-activity and low protein abundance observed for many of the tested baits (Figure 11, supplement Figure 7, Table 31) are not necessarily a problem of the LexA system, particularly. Auto-activity often is a result of a high expression level or instability of the fusion protein. In theory, all yeast strain should express the fusion construct similarly strong. Nevertheless, some strains produce proteins better as

others (Strucko et al., 2015) and exploiting these differences in the expression capacity could help to modulate the abundance of the bait fusion protein and hence to reduce auto-activity. The LexA-based system was further designed as a mating-based system, which results in the selection in a diploid yeast strain. I used only the haploid strain to select for interaction. This difference could also influence the expression.

Fusion to a tag can generally influence protein stability, and large fusion proteins can be challenging to express in a heterologous system (Booth et al., 2018, Saiz-Baggetto et al., 2017). Incubation at a lower temperature might help to increase the abundance of low expressed proteins. This approach was successfully used to test the interaction of MLAs with AVR_s in a classical Y2H system (Saur et al., 2019).

The pivotal advantage of the Ura3-based system is its suitability for cytosolic bait proteins. The option to perform an easy and fast test for the presence of the bait fusion protein featuring Ura3 via Ura auxotrophy selection turned out to be less beneficial and informative as expected. Positive growth might be mediated by low amounts of the bait fusion protein or by Ura3 that was severed from the bait. Hence, Ura auxotrophy selection does not indicate to which extent the protein is expressed or if the fusion protein is unstable. Consequently, a growth test is inferior to western blot detection in the LexA system. Truncated versions of the bait fusion protein, in which the N-terminal arginine of Ura3 is still masked, preventing its degradation, could enable yeast growth but would circumvent correct functioning. On the other hand, expression of the bait fusion protein might be too low to detect interactions, but still high enough to facilitate yeast growth on medium without Ura. These possibilities might explain the low outcome of some of the performed screens. A specific Ura3 antibody would solve these issues with the unknown bait expression level and protein stability.

In contrast to the LexA-mediated Y2H, the Ura3-based system is a single reporter system, which could be overcome by mutations in the Ura3 region that block FOA binding or conversion of the substrate to 5-FU. The great advantage of multiple reporters is reflected by commercially available Y2H kits. The ProQuest Y2H system (ThermoFisher Scientific) and the Matchmaker gold Y2H system (TaKaRa) use three reporter genes to select for interactions.

The best comparison of both systems allows the CERK1 screen. *AtCERK1* was screened in both systems and resulted in the LexA-based system in forty-six and in the Ura3-based system in zero colonies (see Table 31). In both cases, the same library

was used, and the results primarily show the sensitivity of the systems. Already a small amount of cleaved off PLV can allow the growth of the yeast in the LexA-based system and is therefore suitable for weak interactions. The Ura3-based system is maybe more suitable for strong interactions since the amount of available Ura3 proteins has to be reduced until a certain level to allow the growth of the yeast.

Negative results in the decoy screen

Integrated decoy domains of NLRs R-proteins are domains derived from protein targets of pathogen effectors that were integrated into plant immune receptors. The domains represent different structural motifs, which potentially are targeted by effector proteins of various plant pathogens. The plant uses these decoys to detect the effectors and initiate immune responses (Sarris et al., 2016, van der Hoorn & Kamoun, 2008). Similarly, Y2H screens were performed with decoy domains (see Table 31) in order to capture interacting CSEPs. In principle, these decoys should form complete independent structures, since they represent functional domains and therefore be stable without integration into the respective NLR. However, the Y2H baits were designed based on predictions from the genome and transcriptomic data, and additional attachment of a tag might alter their stability or three-dimensional structure. This might explain the generally low number of potential interaction partners found in all of the respective Ura3-based screens. The different decoy domains used as baits were selected to representatively cover a broad spectrum of different structural domains (for example kinases, WRKY and Exo70 domains). It was expected that this should increase the possibility to find potential interacting CSEPs of *Bgh*, but either no effectors bound to these domains or the screening conditions were too stringent to detect interactions. The previous tests with *AtMLO2/HvCaM3* revealed that not all vector combinations were suitable to detect a positive interaction. Indeed, the employed vector pNX32 worked poorly also in case of the *AtMLO2/HvCaM3* interaction partners, and usage of a more suitable vector might facilitate identification of interacting CSEPs. The pNX32 vector is the recommended choice in the LexA-based system (Grefen et al., 2009) since the pNX vectors previously belong to this system, and the pCUP vectors belong to the Ura3-based system. The prey vectors are compatible between both systems. The pNX32 vector was chosen for the CSEP library because I used the LexA-based system when the CSEP library was constructed. This vector was also used for the barley/*Bgh* cDNA library since no differences were visible

between the pNX32 and pCUP-Nul-GWY-CYC1 vector in a test with *HvMlo*. We decided on the pNX32 to reduce the chance of false-positive.

The minimal inhibitory 5-FOA concentration repressing the growth of the respective eV negative control was investigated to improve the screening conditions in the Ura3-based system. It turned out that, in the case of the decoy domain baits, 0.7 g/L was sufficient, to suppress background growth in targeted Y2H experiments (Dirnberger et al., 2008). Reduction of 5-FOA from the initially applied 1 g/L to 0.7 g/L; however, the overall low outcome of the screen was reduced to zero in all cases. In conclusion, the employed conditions of a Ura3-based Y2H screen proved unsuitable to capture CSEP interaction partners of integrated decoy domains. Alternatively, to changing the prey vector, application of a classical Y2H system might be more promising to identify interaction partners for these baits.

Advantages and disadvantages cDNA library vs CSEP library

The successful screens against the CSEP library used the LexA-based system and RLKs from barley as baits. I could validate four CSEPs, which interact with *HvRlk7* and *HvRlk10*. More information about these screens is accessible in chapter 2.

Only three screens of the barley/*Bgh* cDNA library revealed a promising number of potential interaction partners in the Ura3-based system (*HvMlo*, *OsJAC1*, *Bgh* AVR_{A1}). All three screens possessed a high potential background since the coverage of the same gene or gene family was low. Instead of aiming for a high library coverage, only a small amount of the library was transformed in order to perform a shotgun screening. This might explain that, despite the reasonable number of obtained colonies, I did not find multiple hits in the experiments. A successful cDNA library screen was performed with *AtCERK1* in the LexA-based system (chapter 4), where already known interaction partners were found, and new interaction partners were validated in further experiments (see chapter 4). A disadvantage of a cDNA library compared to the CSEP library is the occurrence of out of frame insertions and truncated protein versions. Naturally, a cDNA library is enriched in highly abundant mRNAs such as *RuBisCO* as well as in shorter transcripts since these are more efficiently transcribed from mRNA to cDNA. This is one reason why the barley cDNA library was produced from barley epidermal peels since the epidermis contains fewer chloroplasts (Barton et al., 2016). The CSEP library was made from synthesised gene models (without signal peptide) of

CSEPs from *Bgh*. The complexity of the CSEP library with its 534 effectors (Pedersen et al., 2012, Spanu et al., 2010) is also lower compared to the cDNA libraries.

Another problem of library screens is the fact that, upon library transformation, one yeast cell might perceive more than one plasmid, which impedes the identification of potential interaction partners (Scanlon et al., 2009, Scanlon et al., 2010). This process results in multiple bands in the insert spanning PCR, which indeed was recorded for the library screens. In such a case, both bands were extracted from the gel and sent for sequencing.

Conclusion:

I tested both bait vector systems (LexA- and Ura3-based) and six prey vectors in a pre-characterized interaction and in routine screening of a defined and two cDNA libraries. These tests revealed that in case of proper and stable expression of the bait protein, the LexA-based system has certain advantages over the Ura3-based system: the bait fusion protein can be detected by the western blotting allowing to evaluate expression level and stability. Availability of a specific Ura3 binding antibody would overcome the problem that currently the Ura3-based system relies on auxotrophy selection for verification of bait expression. Employment of two selectable markers in the LexA system, render the screening more stringent as Ura3-based selection, and the lacZ reporter enables quantification of the interaction additionally *via* enzymatic assay.

Unfortunately, only the tested pNX vector system allows investigation of the prey fusion protein abundance *via* western blot. This assay would be critical to test whether high prey expression correlates with more reliable detection of positive interaction or if also low abundant preys might result in robust selection and consequently would enable judgement of the sensitivity of the prey vector.

Taken together, despite the high occurrence of baits resulting in auto-activation, the screens performed with the LexA-based system revealed more reliable results. All potential interactions identified with the LexA-based system could be further validated in targeted Y2H experiments and/or *in planta* assays, which reflects reliability and stability of this system. The identified potential interaction partner from the Ura3-based screens are still under investigation. For these interactions, it remains to be seen if they can be validated. Availability of prey vectors with combinations of different critical properties and future development of novel bait vectors allows optimisation of the LexA-based system for each bait and will help to overcome problems with auto-activation and low bait expression.

Chapter 2: Interaction of RLKs involved in non-host resistance with *Bgh* effector proteins

Introduction:

Non-host resistance (NHR) is the most durable and broadly acting form of resistance that prevents the colonisation of plants by pathogens, which are not adapted to the host immune system. Non-host resistance is defined as that a whole plant species is resistant against all isolates of a particular pathogen.

NHR is a complex network of different mechanisms of plant immunity. For example, pattern recognition receptors (PPR) are involved in the perception of a microbe-associated molecular pattern (MAMPs) to trigger the MAMP-triggered immune (MTI) response. Pathogens that have not evolved mechanisms, like suitable effectors, to overcome/suppress this line of defence cannot colonise this plant species. Other components of the plant immune system, like effector-triggered immunity (ETI), secondary metabolites and cell wall composition, contribute to this kind of resistance (Lee et al., 2017). In contrast to ETI, which relies on the recognition of effectors by R-proteins and which is usually associated with a local programmed cell death response, NHR can be effective without local cell death. NHR relies on the lack of adaptation of a particular microorganism to a plant immune system.

Classic examples for proteins involved in non-host resistance are PEN1, PEN2 and PEN3 (PENETRATION1/2/3) from *Arabidopsis thaliana*. PEN1 is a syntaxin (Collins et al., 2003), which interacts with VAMP721/722, SNAP33, an ADP ribosylation factor (ARF) GTPase and the GTP exchange factor GNOM to facilitate secretion of exosomes for papilla formation (Nielsen & Thordal-Christensen, 2013). PEN2 is a myrosinase, which is involved in the hydrolysis of indole glucosinolates (Lipka et al., 2005) and PEN3 is an ABC transporter (Stein et al., 2006). PEN3 is an unspecific transporter and it is suggested that PEN3 is involved in the secretion of IBA (indole-3-butyric acid; an Auxin precursor) (Aryal et al., 2019), cadmium (Kim et al., 2007), camalexin (He et al., 2019) and 4-methoxyindol-3-yl-methanol (Matern et al., 2019). Knock-out mutants are more susceptible against the non-host pathogen *Bgh* that means the penetration rate of *Bgh* on *Arabidopsis* increases from 10% to 60% in the *pen1* mutant and to 40% in the *pen2* mutant. Both proteins work in different pathways

since the penetration rate in a double mutant increases to around 80% (Lipka et al., 2005).

Wheat and barley are important crop plants, and the specific interaction between wheat (*Triticum aestivum*) – *Blumeria graminis* f. sp. *tritici* (*Bgt*) and barley (*Hordeum vulgare*) – *Blumeria graminis* f. sp. *hordei* (*Bgh*) represent test systems to investigate NHR since *Bgh* is highly adapted to barley (host interaction) and cannot colonise wheat (non-host interaction) and *vice versa*. Which components are responsible for this phenomenon are so far unclear.

Many studies were conducted to investigate NHR in barley, and wheat (Delventhal et al., 2017, Douchkov et al., 2014, Zellerhoff et al., 2010) since NHR could be used as a durable resistance mechanism in plant breeding to produce more resistant plants. This effort lead to the identification of *HvRnr8* (REQUIRED FOR NON-HOST RESISTANCE 8) (synonym *HvLemk1*, an LRR-malectin domain-containing RLK), and it's wheat homologs. When the receptor-like kinase *Rnr8/Lemk1* is silenced in transgenic barley lines, the non-adapted wheat powdery mildew *Bgt* can colonise barley (Rajaraman et al., 2016).

During the infection of barley, *Bgh* secretes hundreds of candidates of secreted effector proteins (CSEPs) to suppress plant immunity and favour its own growth. The classical definition of an effector protein is a small protein with a signal peptide, which shows no homology outside the fungal family. There is evidence that this definition is somehow outdated since high-throughput genomics and functional analysis showed that some effectors are conserved in different families (Irieda et al., 2019). The CSEP library, which I use in this study, was made based on the *Bgh* DH14 genome assembly (v3) from 2010 with around 534 predicted effectors (Pedersen et al., 2012, Spanu et al., 2010). A new and improved assembly was published in 2018 that increases the number to around 700 CSEPs (Frantzeskakis et al., 2018). The function and plant targets of only a few CSEPs are known. For example, CSEP0064 (BEC1054) interacts *in planta* with PR10 and RNA (Pennington et al., 2016, Pennington et al., 2019). CSEP0064 was predicted to have an RNase-like structure but misses the essential amino acids for the catalytic activity. The resolved crystal structure, which is, by the way, the first crystal structure of a *Bgh* effector, confirmed the prediction. (Pennington et al., 2019). CSEP0064 belongs to a subgroup of *Bgh* effectors, so-called RALPHs

(RNase Like Proteins expressed in Haustoria), which are primarily abundant during infection and so might play a crucial role for pathogenesis. Other examples of effectors with known plant targets are CSEP0055, which interacts with PR17c (Zhang et al., 2012) and CSEP0105 and CSEP0162, which interact with HSP16.9 and HSP17.5 (HEAT SHOCK PROTEIN). For CSEP0105, it was shown that it can compromise the chaperone activity of the heat shock proteins (Ahmed et al., 2015).

It is described for *Pseudomonas syringae* effectors that these interact with RLKs to suppress immunity (Li et al., 2016b, Xiang et al., 2011, Zhou et al., 2014). *Bgh* CSEPs may also target barley RLKs towards suppressing MTI and defence signalling.

The aim of this study:

This study aimed to identify potential interaction partners of barley RLKs with a proposed role in non-host resistance and barley powdery mildew effector proteins (CSEPs). The LexA-based split-ubiquitin Y2H system and a *Bgh* CSEP library were used to perform this work.

Results:

I used the LexA-based split-ubiquitin Y2H system to screen the *Bgh* CSEP library (material & methods 2.13.1) against the receptor-like kinases (RLKs) *HvRlk7*, *HvRlk10*, *HvRnr8* and the wheat homologs *TaRnr8* p7 and *TaRnr8* p12, which show evidence for an involvement in non-host resistance in barley and wheat. They were identified by transient RNAi experiments in barley, which lead to an increased susceptibility to *Bgt* and transient expression of the barley gene in wheat, which lead to increased resistance to *Bgt* (Rajaraman et al., 2016) (unpublished data, group of Patrick Schweizer). The expression of these RLKs was tested *via* western blot analysis and only barley *HvRlk10* and *HvRlk7* revealed detectable expression in yeast. *Rnr8* and its wheat homologs showed no expression (Figure 11). The background was not detected using the empty vector pNX32 (NubG-GWY) vector (Figure 12). Thus, *Rlk7* and *Rlk10* were used for the screen in the LexA-based system.

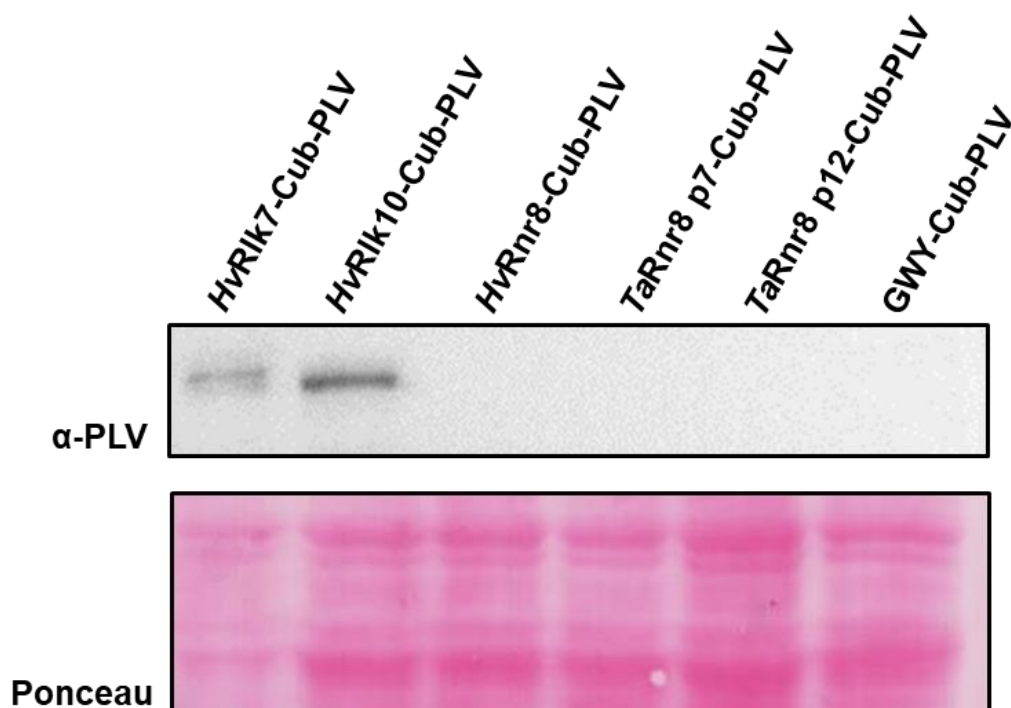


Figure 11: Barley and wheat RLK fusion protein expression in yeast. Cells expressing the different RLKs C-terminally fused to Cub (C-terminal part of ubiquitin), and PLV (artificial transcription activator ProteinA-LexA-VP16) were harvested, and the total protein was extracted. A specific antibody against PLV was used to detect bait protein expression. Expected size of the fusion proteins: *HvRlk7*: 157 kDa; *HvRlk10*: 123 kDa; *HvRnr8*: 165 kDa; *TaRnr8 p7*: 164 kDa; *TaRnr8 p12*: 163 kDa; PLV alone: 50 kDa. Ponceau staining was applied to visualise equal protein loading. The full-size blot is attached in the supplement (Supplementary Figure 11).

I performed multiple screens without methionine and later with 100 μ M Met to test and optimise the LexA-based split-ubiquitin Y2H. CSEP0045 was the most abundant effector identified in the screens (without methionine) with both bait RLKs, Rlk7 and Rlk10 (Table 32). The other CSEP identified with both RLKs was CSEP0310, which appeared in seven colonies (Rlk7) or in eight colonies (Rlk10) in the screens. Another effector often found (in 4 colonies with Rlk7) was CSEP0372, which is also called AVR_{A13} and interacts with Mla13 (Lu et al., 2016, Saur et al., 2019). Finally, CSEP0350 was found in a more stringent screen with an increased Met concentration of 100 μ M against Rlk7.

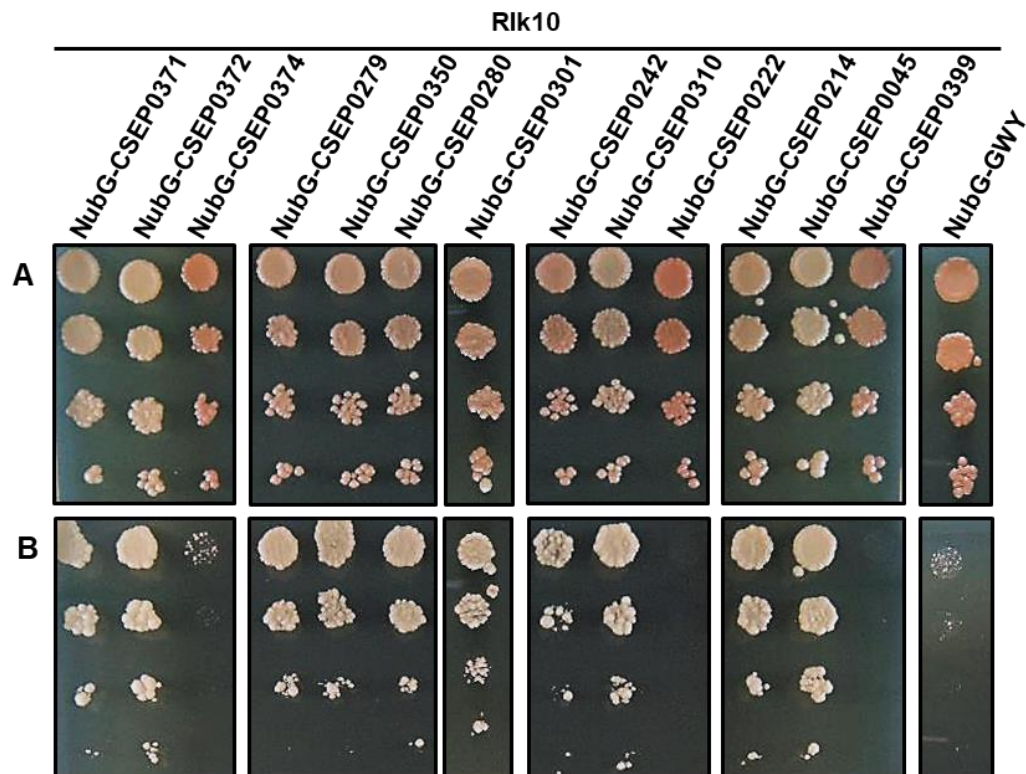
Table 32: List of potential interacting *Bgh* CSEPs with *HvRLK10* and *HvRLK7*.

Bait	CSEP	Number of colonies
Rlk10	CSEP0045	18
Rlk10	CSEP0310	7
Rlk10	CSEP0307	2
Rlk10	CSEP0329	2
Rlk10	CSEP0302	1
Rlk7	CSEP0310	8
Rlk7	CSEP0045	5
Rlk7	CSEP0372	4
Rlk7	CSEP0125	2
Rlk7	CSEP0279	2
Rlk7	CSEP0306	2
Rlk7	CSEP0350	2
Rlk7	CSEP0111	1
Rlk7	CSEP0110	1
Rlk7	CSEP0123	1
Rlk7	CSEP0134	1
Rlk7	CSEP0254	1
Rlk7	CSEP0264	1
Rlk7	CSEP0276	1
Rlk7	CSEP0301	1

This list summarises all potential interacting *Bgh* CSEPs of the *HvRlk7* and *HvRlk10* screen. More information about the CSEP library is in the materials & methods part 2.13.1. The tables show the bait, the potential interacting CSEP with CSEP number and the number of colonies in the screen that carried this effector.

Next, I performed targeted split-ubiquitin Y2H experiments between Rlk7/Rlk10 and CSEP0045, CSEP0310, CSEP0372, and the two closest homologs according to the genome annotation v3 (Spanu et al., 2010). The three closest homologs were used for CSEP0350. The homologs were used to test for the specificity of the interaction. The identified candidate effectors belong to different CSEP families according to the phylogenetic tree (Pedersen et al., 2012), and have no structural similarities, like cysteine residue number and position. I tested CSEP0045, CSEP0310, CSEP0350, and CSEP0372 with their homologs (CSEP0214, CSEP0399 and CSEP0242, CSEP0222 and CSEP0279, CSEP0280, CSEP0301 and CSEP0371, CSEP0374) against both Rlk7 and Rlk10 to validate the interaction and test for protein interaction specificity. All RLK and CSEP combinations show an even growth on plate A and C (Figure 12), which selects only against the bait and prey plasmids. All CSEPs that

interact with Rlk10 also interacted with Rlk7 (the amino acid identity of the kinase domain 26%) (Figure 12 B and D and Supplementary Figure 12). The next closest homolog of CSEP0372 and of CSEP0045, CSEP0371 and CSEP0214, respectively, also showed detectable yeast growth until OD₆₀₀ 0.001. The more distantly related CSEPs, CSEP0374 and CSEP0399, did not show any growth on selective medium (B and D). The closest homolog of CSEP0310 (CSEP0222) did not grow, but the one further related (CSEP0242) could grow. For CSEP0350 and the homologs (CSEP0279, CSEP0280 and CSEP0301) was no difference detectable, all combination could grow on selection medium. Therefore, the LexA-based split-ubiquitin Y2H system indicated that certain *Bgh* effector candidates, i.e. CSEP0045, CSEP0310, CSEP0372 and at least one homologue could interact with both barley receptor-like kinases Rlk7 and Rlk10.



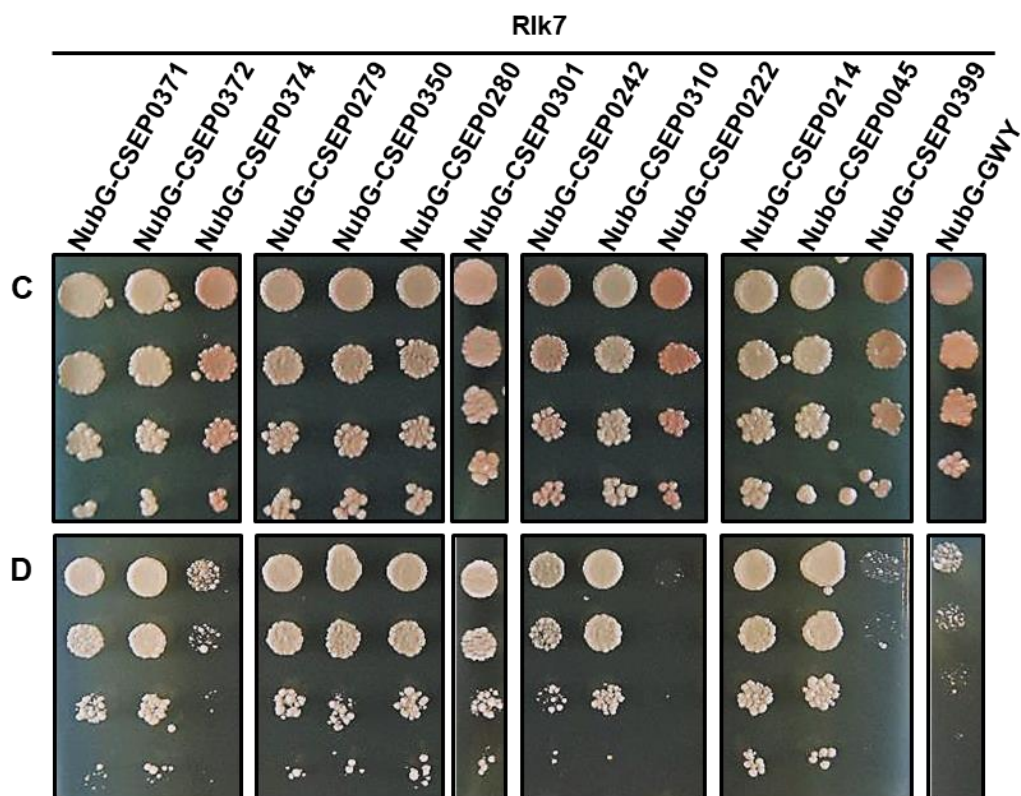


Figure 12: Validation of Rlk10 and Rlk7 interacting *Bgh* CSEPs in a split-ubiquitin Y2H assay. The LexA-based split-ubiquitin Y2H system was used to validate the identified potential interaction partners CSEP0045, CSEP00310, CSEP0350, and CSEP0372. Each potential interaction partner was dropped next to its closest homologs to test for specificity. The following combinations belong together: CSEP0372 with CSEP0371/CSEP0374, CSEP0350 with CSEP0279/CSEP0280/CSEP0301, CSEP0310 with CSEP0242/CSEP0222, and CSEP0045 with CSEP0214/CSEP0399. NubG-GWY was used as a negative control. NubG is the low-affinity version of the N-terminal ubiquitin part, which is tagged to the N-terminus of the effector. Panel A and C show the growth control, which selects for both plasmids on SC -leucine -tryptophan -methionine. Panel B and D depict the interaction plate on SC -leucine -tryptophan -adenine -histidine -methionine. 4 μ l overnight yeast culture is dropped in a 10-fold dilution series starting from OD₆₀₀ 1. Two more replicates were performed with the same result (Supplementary Figure 12).

Discussion:

Several publications reported the interaction of bacterial and fungal effectors with RLKs involved in plant immunity. A few examples are the conserved fungal (e.g. present in *Colletotrichum* spp. and *Magnaporthe oryzae*) effector NIS1 targets BAK1 and BIK1 (Irieda et al., 2019), the *Pseudomonas syringae* effectors HopF2, AvrPto and AvrPtoB target BAK1 (Zhou et al., 2014) and the effector AvrPto also targets SOBIR1 (Wu et al., 2017). All these effectors interfere in this way with plant immunity. These receptors represent an essential target for pathogens since these receptors are the first line of recognition in MTI. The selected RLKs from barley and wheat should have a function in the non-host resistance, which serves as an important defence mechanism in plants.

A hypothesis was that these RLKs could be targets of *Bgh* CSEPs, which can interfere with the receptor function, whereby *Bgt* CSEPs are unable to suppress the signalling.

I screened barley Rlk7 and Rlk10 against the *Bgh* CSEP library to identify potential interaction partners. Rlk7 and Rlk10 were expressed as full-length proteins in yeast, but the split-ubiquitin Y2H system is only capable of detecting interaction at the cytosolic side, which means only on the kinase region of the RLKs. Both RLKs show only a sequence similarity of 26% in this region, but a structural similarity could cause this result since different amino acid chains can fold into similar 3D structures. I identified CSEP0045, CSEP0310, CSEP0350 and CSEP0372 as potential interaction partners in a LexA-based split-ubiquitin yeast-two-hybrid assay. All of these CSEPs belong to different families but can interact with both RLKs, which are also not closely related. CSEP0372 belongs to family 34, CSEP0350 belongs to family 7 and the other two belong to no family. They show only a sequence similarity of 5%-10% between each other and have no putative structures in common. CSEP0372 shows a RNase-like structure and belongs therefore to the groups of RALPH effectors (Pedersen et al., 2012, Spanu, 2015). The NubG-tag of the pNX32 vector is not able to reassembly with Cub by itself, because of that, it needs interacting proteins. False positives can occur in this system when the prey unspecific binds to bait or the prey has ubiquitin or protease function. Unspecific binding can happen since some proteins tend to interact because of certain protein domains facilitate protein-protein interactions (Deng et al., 2002, Xia et al., 2008). Other examples are so-called sticky proteins which tend to interact with many proteins, because of misfolding, overexpression or a protein tag (Feller & Lewitzky, 2012). Proteins that are structurally related to ubiquitin and proteases could trigger the system without a real interaction, especially certain proteases could cleave off the PLV tag (Xing et al., 2016). The negative control (NubG-GWY, pNX32) could only rule out that the tag binding causes an activation of the reporter system. Further, this assay is performed in yeast, a heterologous system, which could lead to misfolding. Therefore, these candidates need to be validated *in planta* since specificity between the two RLKs and CSEPs could not be seen, but a list of over 500 CSEPs could be reduced to four potential interaction partners.

The *Bgh* effector CSEP0064 (BEC1054) interacts with many plant proteins in yeast. Pennington and colleagues identified glutathione-S-transferase (GST), Malate dehydrogenase (MDH), pathogenesis-related protein 5 (PR5) and elongation factor-1-

gamma (eRF1G) as potential plant targets in a co-immunoprecipitation pull-down assay and validated these candidate interaction partners in a classical yeast two-hybrid experiment (Pennington et al., 2016). However, CSEP0064 only interacted with PR10 in *in planta* BiFC assays, which was found as potential interaction partner in the pull-down assays but not validated by Y2H (Pennington et al., 2019). This case highlights that artificial systems such as pull-down assays and the heterologous yeast system can only identify potential targets, but the verification in a more natural plant system is necessary. Besides the physical interaction, the biological meaning of an interaction needs to be tested in the context of the barley/*Bgh* interaction.

In general, only a few *Bgh* CSEPs plant targets are known. For example, CSEP0105 and CSEP0162 interact with Hsp16.9 and Hsp17.5 (HEAT SHOCK PROTEIN) from barley in yeast and BiFC experiments. CSEP0105 is further capable of compromising HSP chaperone activity (Ahmed et al., 2015). CSEP0055 interacts with the pathogenesis-related protein PR17c in yeast and BiFC experiments, although the function of the effector is not known (Zhang et al., 2012). Further, (BEC3, CSEP0497) was found to interact with a barley thiopurine methyltransferase (TPMT) and (BEC4, not included in the CSEP list) appears to interact with a barley ubiquitin-conjugating enzyme (UBC) (Schmidt et al., 2014). This small number of interactions reflect the difficulty of finding meaningful interactions and their validation.

A general challenge when working with obligate biotrophic pathogens such as powdery mildews is that cultivation, overexpression and knock-out studies are not possible since these genomes are often not amenable to genetic manipulation. Therefore, sophisticated functional studies conducted for example with *Ustilago maydis* (Ma et al., 2018, Tanaka et al., 2014) or *Magnaporthe oryzae* (Ortiz et al., 2017) are not available. Host-induced gene silencing (HIGS) assays (Nowara et al., 2010) is one method to study effectors in powdery mildew and relies on over-expression of a silencing construct in the host plant and transfer of small RNAs into the pathogen. These studies can be very useful because a reduction in the penetration rate upon silencing of specific fungal CSEPs indicates a necessary function in virulence (Aguilar et al., 2016, Ahmed et al., 2016, Pliego et al., 2013). The RALPH effector BEC1054 (CSEP0064) was identified in such an assay (Pliego et al., 2013). This assay is, however, limited since it gives no information about the function of the effector or the potential plant target protein. Recent advances in next-generation sequencing technologies further

help in identifying potentially functional *Bgh* CSEPs and their interactors. Saur et al. identified avirulence effectors of *Bgh* and showed their direct physical interaction with the specific MLAs (R-protein). They identified the AVR_s over a transcriptome-wide association study (TWAS) instead of a classical protein-protein interaction screen. AVR_{A1} (CSEP0008) and AVR_{A13} (CSEP0372) were first identified (Lu et al., 2016), and later the cognate effectors of Mla7, Mla9, Mla10, and Mla22, i.e. AVR_{A7} (CSEP0059), AVR_{A9} (CSEP0174), AVR_{A10} (CSEP0141), and AVR_{A22} (CSEP0141), were found (Saur et al., 2019). Yet, while such methods aid in identifying relevant *Bgh* CSEPs as well as putative interaction partners, the mode of action of both CSEP/AVR as well as their interacting proteins remains unknown.

Another difficulty with fungal biotrophic pathogens such as powdery mildews and rust fungi is their large number of predicted effector proteins, which can range between hundreds (powdery mildews) to thousands (rusts) of effectors in one pathogen (Cantu et al., 2011, Duplessis et al., 2011, Frantzeskakis et al., 2018, Menardo et al., 2017, Muller et al., 2019, Nemri et al., 2014). These huge number of candidate effectors make the investigation of plant targets more challenging. Many studies used HIGS experiment results to narrow down potential important CSEPs for pathogenicity (Ahmed et al., 2015, Pliego et al., 2013). The tremendous amount of candidate effectors leads to the question if some effector can act redundantly as CSEP0105 and CSEP0162 can interact with Hsp16.7 and Hsp17.5 (Ahmed et al., 2015). The current annotation of the *Bgh* genome predicts around 700 CSEPs (Frantzeskakis et al., 2018). The CSEP library with 534 CSEP is based on the old assembly (Spanu et al., 2010). The assemblies differ in some new gene models for the additional CSEPs, and others were deleted from the set or got new gene models. All four candidate effectors from the screens are still present in the new assembly, and the gene models are unchanged.

The lack of structural information is another difficulty of this protein class because only the 3D structure of CSEP0064 was solved so far (Pennington et al., 2019) and for the vast majority of CSEPs, the function and functional domains are unknown. That makes the *in silico* analysis less informative. One possibility could also be that the identified candidates are not related on the amino acid level, but share a structural similarity, which could interact with conserved kinase domains of the RLKs. But without the structural information of other effectors, the prediction of the 3D structure results in low

confidence level models. Nevertheless, the low confidence prediction of CSEP0064 as RNase-like effector was confirmed by the crystal structure, which shows that this approach can lead to useful information (Pennington et al., 2019). RALPH effectors belong to a family of around 72 members and were identified over 3D structure prediction and by InterProScan (Pedersen et al., 2012). Another example, where the 3D structure leads to the identification of potential function, was AvrP from the rust fungus *Melampsora lini*. The structure revealed a zinc-finger-like domain and together with the localisation inside the nucleus, a transcriptional regulatory function is suggested (Zhang et al., 2018b).

In the end, the identified CSEPs that can interact with Rlk7 and Rlk10 should be validated *in planta* via split-luciferase, FRET and/or BiFC experiments to verify that they can interact. These effectors could be further investigated *via* HIGS or overexpression experiments in barley epidermal cells inoculated with *Bgh* and/or *Bgt*. If the RLKs have a function in non-host resistance, then the penetration rate of *Bgh* on barley may be reduced if the effectors targeting these RLKs are silenced in HIGS experiments, and the penetration rate could be increased in overexpression experiments with *Bgt* on barley. However, due to the fact that at least four CSEPs putatively target these RLKs, these effects may be masked due to their functional redundancy.

Conclusion:

I identified potential interaction partners of the barley RLKs, Rlk7 and Rlk10, using a *Bgh* CSEP library containing more than 500 CSEPs. CSEP0045, CSEP0350, CSEP0310 and CSEP0372 interacted with Rlk7 and Rlk10 in a LexA-based split-ubiquitin assay in yeast, though these interactions need to be validated *in planta* to verify the interactions. These interactions suggest that *Bgh* deploys effectors to target defence-related RLKs, potentially in order to suppress plant defence.

Chapter 3: A Y2H screen to identify novel interaction partners of barley Mlo

Introduction:

The MLO protein family is unique to plants (Kusch et al., 2016) and a loss-of-function mutation of distinct MLO proteins confers durable resistance against powdery mildews, e.g. of barley, Arabidopsis, pea and tomato (Bai et al., 2008, Büschges et al., 1997, Devoto et al., 2003, Humphry et al., 2011, Jørgensen, 1992). The number of MLO proteins per species varies between 10-20 genes per plant species, and not all of the family members contribute to powdery mildew susceptibility (Kusch et al., 2016). In Arabidopsis, for example, *Atmlo4* and *Atmlo11* mutants show a root curling phenotype (Acevedo-Garcia et al., 2014, Chen et al., 2009) and *Atmlo7* (*NORTIA*) results in a pollen overgrowth phenotype and hence reduced fertility (Kessler et al., 2010). Further, not all involved MLOs contribute to powdery mildew susceptibility to an equal degree: While mutation of *HvMlo* is sufficient to confer powdery mildew resistance in barley (Büschges et al., 1997, Jørgensen, 1992), in Arabidopsis, *mlo2*, *mlo6* and *mlo12* cooperatively mediate resistance, with *mlo2* having the strongest contribution. A triple mutant of *Atmlo2*, *Atmlo6* and *Atmlo12* is needed for complete powdery mildew resistance of Arabidopsis. An *Atmlo2* single mutant shows already reduced fungal entry and no conidia formation, whereas *Atmlo6* and *Atmlo12* single mutants depict wild type like penetration rates. This indicates a predominant role of *Atmlo2* in the powdery mildew interaction (Consonni et al., 2006). In addition to their role in powdery mildew resistance, mutation of the same MLOs induces pleiotropic effects like early leaf senescence and spontaneous callose deposition (Consonni et al., 2006, Piffanelli et al., 2002).

MLO proteins show a seven-transmembrane topology with an extracellular N-terminus and an intracellular C-terminus (Figure 13)(Devoto et al., 1999). The distal end of the C-terminus is intrinsically disordered, which is indicative of a non-fixed secondary (3D) structure (Kusch et al., 2016). This kind of region undergoes conformational changes after binding of ligands, including other proteins or after post-translational modification (Dyson, 2016, Uversky, 2015). This flexibility could be essential for the biochemical function of MLO. Markedly, the calmodulin (CaM) binding site is located near this region (Kim et al., 2002). CaMs are the only known interaction partners of MLOs and their binding site is necessary for the functionality of barley Mlo (Bhat et al., 2005).

Besides mutations of the CaM-binding domain that interfere with CaM binding, also other point mutations in the second and third cytoplasmic loop are essential for HvMlo function. Some of these mutations lead to complete or partial resistance of barley against powdery mildews (Reinstadler et al., 2010).

Different studies tried to identify new interaction partners of MLO proteins from barley and Arabidopsis in a cDNA library-based screen against certain MLOs (Consonni, 2005) or high-throughput mating-based screen of membrane proteins (Jones et al., 2014a, Lalonde et al., 2010). All of these yeast-based screens could not reliably confirm new interaction partners since they did not exceed the yeast test stage. Biochemical approaches in Arabidopsis based on protein crosslinking, purification and mass-spectrometry (XL-TAP-MS strategy) also did not reveal novel interaction partners so far (Leißing, 2018). In her thesis, Chiara Consonni (2005) screened *AtMLO1*, *AtMLO2*, *AtMLO3*, *AtMLO4*, *AtMLO5*, *AtMLO10* and *HvMlo* against an Arabidopsis cDNA library, which resulted in no confirmed clones for *AtMLO2* and *HvMlo* (Consonni, 2005). I screened *HvMlo* against a barley cDNA library (generated from *Bgh* infected epidermal peels sampled at different time points after inoculation) to increase the repertoire of potentially interacting proteins. The difference in this approach compared to the former screens by Consonni is the used library. On the one hand, Consonni screened *HvMlo* against an Arabidopsis cDNA library, and I use a barley cDNA library, and on the other hand, the epidermal peels should increase transcripts involved in the interaction between the plant and the fungus. This approach could increase the chance to capture potential interaction partners. The Arabidopsis library is made of whole leave samples infected with powdery mildew.

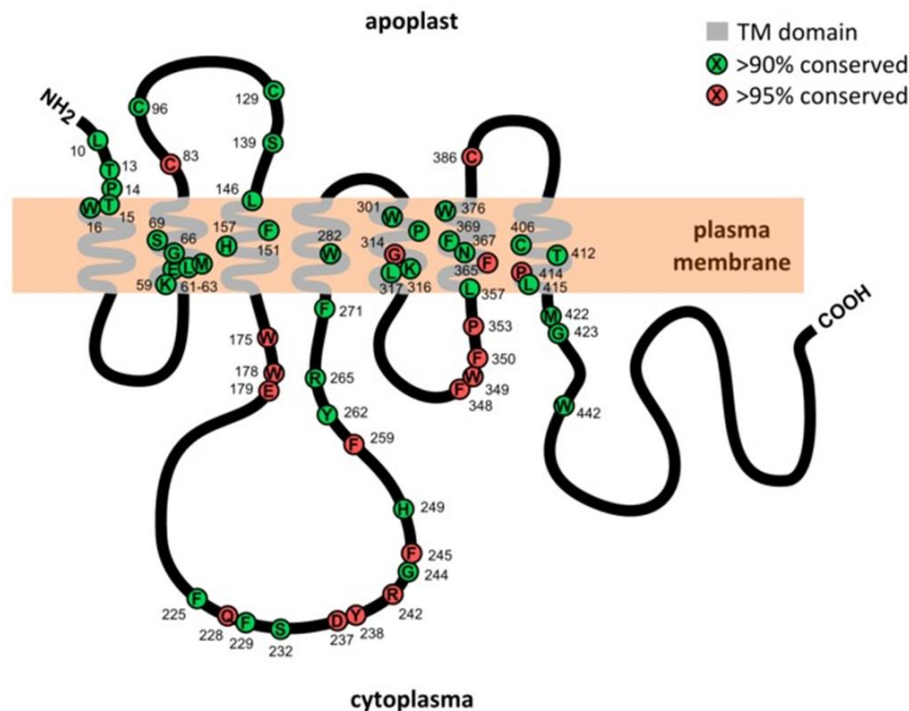


Figure 13: Predicted topology and conserved amino acids of MLO proteins in land plants. The figure depicts the predicted seven-transmembrane topologies of MLOs (Devoto et al., 1999). Conserved amino acids are indicated in green (> 90% conservation) and red (> 95% conservation). The figure is taken from (Kusch et al., 2016).

Aim of this study:

This study aimed to identify new interaction partners of the barley Mlo protein with the ultimate goal to collect information about pathways involving Mlo. As it is possible that Mlo interacts with plant proteins expressed explicitly during infection or even fungal proteins, the bait was screened against a barley cDNA library consisting of fungal and plant-derived transcripts (constructed from *Bgh*-infected plant material). The library most probably harbours additional effector proteins that were not included in the *Bgh* CSEP library, which was based on the 2010 genome assembly. This is advantageous since the presence of *HvMlo* is essential for the successful penetration of *Bgh*, rendering it conceivable that the protein is a possible target of effector proteins.

Results:

Y2H screening reveals potential interaction partners of *HvMlo*

Successful transformation of yeast with the bait construct (Figure 14 A), as well as expression and Ura3 functionality of the bait fusion protein *HvMlo*-Cub-R-URA3 (Figure 2 B), was confirmed *via* growth on SC selection medium lacking histidine

(auxotrophic marker in the vector backbone), methionine (derepression of bait promoter) and uracil (auxotrophic marker mediated by bait fusion protein). While both, the bait vector and the empty vector pMet-GWY-Cub-R-Ura3, grew equally well on selection medium without histidine and methionine (Figure 2 A), lack of uracil supported the growth of four *HvMlo* expressing strains until OD₆₀₀ 0.01 but not of the empty vector (Figure 2 B). Taken together, the fusion protein construct of *HvMLO*-Cub-R-Ura3 was expressed in yeast and could be used for the screening against the barley cDNA library.

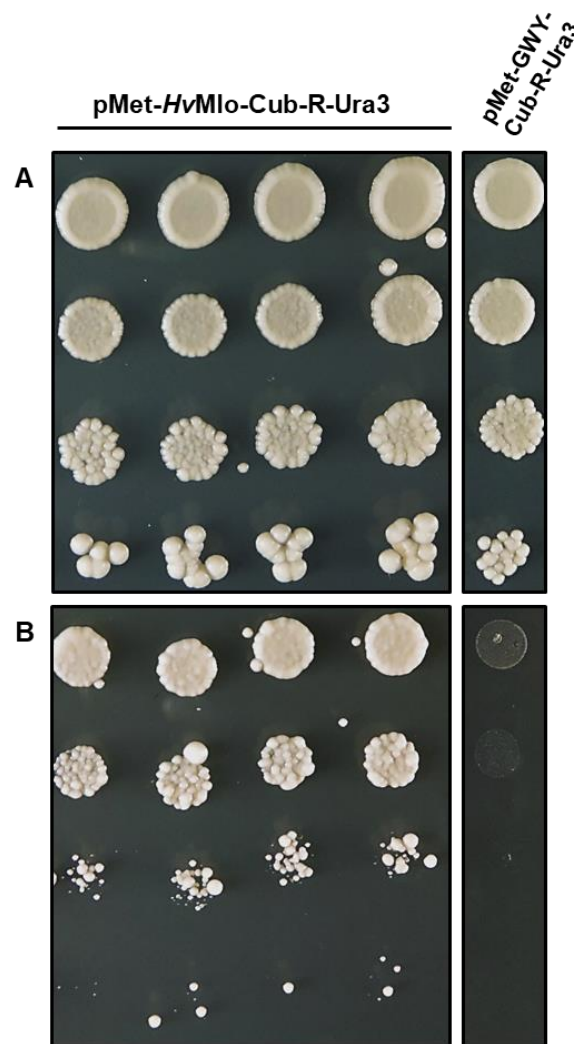


Figure 14: The *HvMlo*-Cub-R-Ura3 fusion protein is expressed and complements yeast Ura deficiency. Yeast JD53 was transformed with the *HvMlo*-Cub-R-Ura3 bait construct or the empty vector pMet-GWY-Cub-R-Ura3. Four independent transformants of the bait construct and one transformant of the empty vector were plotted in 10-fold dilution series. (A) Growth on SC -histidine (-methionine) confirms the presence of the vector (histidine auxotrophy). (B) Growth on SC -histidine -uracil (Ura3 complementation) -methionine (promoter derepression) confirms expression and Ura3 functionality of the bait construct but not of the empty vector.

Transformation of the prey library was performed with an efficiency of 1.58×10^5 CFU/ μ g DNA. This effort resulted in 234 colonies on 5-FOA containing plates (selection for positive interaction). Initially obtained colonies were reselected twice to reduce the occurrence of false positives. The confirmed 101 clones were used for plasmid extraction and PCR. Sequencing of PCR products revealed 73 hits in the barley genome and 12 in the *Bgh* genome (Table 33). For 18 clones either no PCR product could be generated or the resulting sequence had no match inside the genomes of *Bgh* and barley. Two clones (No. 69 and 80) matched to barley and *Bgh* genes: clone No. 69 encodes a barley Histidine triad (HIT) protein and No. 80 a 72 aa protein with no predicted domains in barley. Both clones additionally match with CSEP0421 of *Bgh*. In total, 7 of the 12 *Bgh* clones represented effector proteins. CSEP0515 was observed four times, which renders this effector an interesting candidate for further experiments.

HvMlo is a plasma membrane-resident protein (Devoto et al., 1999). Consequently, all of the identified 73 barley clones that were not predicted to localise to the plasma membrane or the cytosol (56 clones) were discarded for further inspection as these are unlikely interactors of HvMlo (Figure 15).

The remaining 17 barley clones (Figure 15) represented different families of proteins. Only domains or domain superfamilies could be predicted for some of the candidates, which made an assignment of a particular protein function more challenging. In total, the clones represented a diverse mix of proteins with no apparent pattern or preference for certain families. Families that could be identified in this subset included protein kinases (3 genes; clones 16, 26, 56), an EF-hand domain-containing protein (clone 185), an ABI family protein (clone 66), two transferases (clone 23, 40), two isomerases (clone 30, 154), two cytochrome proteins (clone 57, 151), two ribosomal proteins (clone 108, 208), an zinc-finger, AN1-type protein (clone 36), an inorganic pyrophosphatase (clone 48), an zinc/iron permease (clone 88), an arogenate dehydratase (clone 171) and a blue copper-binding protein (clone 180). Other protein families, which appeared multiple times in the total set, but not localise to the plasma membrane, cytosol or showed an ambiguous prediction of localisation were peptidases (3 genes; clones 33, 97, 120), NLRs (1 gene represented by the 2 clones 106 and 222), a bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain protein (1 gene represented by the 2 clones 7 and 202) (Table 33).

Table 33: Results of the Ura3-based split-ubiquitin Y2H screen of HvMlo against the barley-Bgh cDNA library

clone nr.	Gene ID	E-Value	%ID	description/function	localisation
4	HORVU4Hr1G064380	0.0	97	Purple acid phosphatase, a purple acid phosphatase, also a potential interaction partner MLO6	Extracellular (Secreted)
7	HORVU6Hr1G077890	0.0	100	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain (IPR016140)	Vacuole
8	HORVU7Hr1G008840	2.0e-147	96	SNAP25 homologous protein SNAP33	ambiguous
10	HORVU2Hr1G059620	0.0	99	MD-2-related lipid-recognition domain (IPR003172)	Extracellular (Secreted)
12	HORVU7Hr1G045710	1.8e-104	99	Dual specificity phosphatase (IPR024950)	Nuclear
16	HORVU2Hr1G117290	0.0	96	Serine/threonine-protein kinase, S-receptor-like serine/threonine-protein kinase, (kinase domain), Bulb-type lectin domain, PAN/Apple domain	Plasma membrane
17	HORVU6Hr1G014130	1.0e-169	100	undescibed protein, LOW PSII ACCUMULATION 2 (LPA2)	ambiguous
23	HORVU7Hr1G048970	0.0	99	O-methyltransferase, family 3 (IPR002935), also a potential interaction partner MLO6	Cytoplasmic/Nuclear
26	HORVU2Hr1G039070	8.0e-71	100	calcium-dependent protein kinase 20	Plasma membrane
30	HORVU3Hr1G003940	0.0	100	Mannose-6-phosphate isomerase (IPR016305)	Cytoplasmic
32	HORVU4Hr1G083840	0.0	98	Ribosomal protein L14P (IPR000218)	Nuclear
33	HORVU2Hr1G113460	6.7e-157	97	Peptidase C1A (IPR013128)	Extracellular (Secreted)
35	HORVU3Hr1G059830	0.0	96	ISC system FeS cluster assembly, IscU scaffold (IPR011339)	Mitochondrial
36	HORVU7Hr1G050270	0.0	99	Zinc finger, AN1-type (IPR000058)	Cytoplasmic
38	HORVU1Hr1G079410	0.0	99	Protein of unknown function DUF1639 (IPR012438)	ambiguous
39	HORVU5Hr1G050990	0.0	100	Ribulose biphosphate carboxylase small chain	Chloroplast
40	HORVU1Hr1G054100	0.0	99	Nucleotide-diphospho-sugar transferases (IPR029044)	Plasma membrane
41	HORVU7Hr1G077740	0.0	98	Ferredoxin	Chloroplast
42	HORVU6Hr1G085170	0.0	100	Photosystem II PsbR (IPR006814)	Chloroplast
43	HORVU2Hr1G125110	1.2e-132	78	Plant peroxidase (IPR000823)	Extracellular (Secreted)
47	HORVU2Hr1G028370	0.0	100	Defective-in-cullin neddylation protein (IPR014764)	ambiguous
48	HORVU3Hr1G116650	0.0	99	Inorganic pyrophosphatase (IPR008162)	Cytoplasmic
49	HORVU5Hr1G051010	0.0	100	Ribulose biphosphate carboxylase small chain	Chloroplast
52	HORVU7Hr1G094640	0.0	100	Tetratricopeptide-like helical domain superfamily (IPR011990), DYW domain (IPR032867), Pentatricopeptide repeat (IPR002885)	Mitochondrial
56	HORVU1Hr1G000660	0.0	96	Leucine-rich repeat RLK (Kinase domain)	Plasma membrane
57	HORVU3Hr1G025920	0.0	98	Cytochrome P450 (IPR001128)	Plasma membrane
61	HORVU1Hr1G043830	0.0	99	Aldolase-type TIM barrel (IPR013785), Glycoside hydrolase, family 27	Extracellular (Secreted)
62	HORVU2Hr1G057700	1.5e-97	100	Outer membrane protein/outer membrane enzyme PagP, beta-barrel (IPR011250), Photosystem II PsbO, manganese-stabilising (IPR002628)	Chloroplast
63	HORVU1Hr1G043910	8.7e-46	96	Uncharacterised protein family, basic secretory protein (IPR007541)	Extracellular (Secreted)
64	HORVU2Hr1G074730	0.0	100	no predicted domain	Nuclear
66	HORVU3Hr1G055780	0.0	98	Protein ABIL1	ambiguous
69	HORVU4Hr1G051830	0.0	100	Histidine triad (HIT) protein (IPR001310), nucleotide-binding proteins, [part of the sequence overlap with Bgh effector 421]	Mitochondrial

clone nr.	gene ID	e-value	%ID	description/function	localisation
70	HORVU0Hr1G027130	1.5e-85	100	Small GTPase superfamily, ARF/SAR type (IPR006689), Small GTP-binding protein domain (IPR005225)	Golgi
70	HORVU1Hr1G068790	1.5e-85	100	Small GTPase superfamily, ARF/SAR type (IPR006689), Small GTP-binding protein domain (IPR005225)	Golgi
71	HORVU0Hr1G003270	0.0	100	Oxygen-evolving enhancer protein 3 (IPR008797)	Chloroplast
72	HORVU5Hr1G109250	0.0	99	Chlorophyll A-B binding protein (IPR022796)	Chloroplast
75	HORVU5Hr1G111680	0.0	100	Zinc finger, RING/FYVE/PHD-type (IPR013083),	ambiguous
80	HORVU6Hr1G006840	0.0	100	protein 72aa, no domain annotation	ambiguous
88	HORVU2Hr1G112230	0.0	99	Zinc/iron permease (IPR003689)	Plasma membrane
90	HORVU1Hr1G088870	0.0	97	Chlorophyll A-B binding protein (IPR022796)	Chloroplast
93	HORVU1Hr1G006950	4.4e-147	100	Armadillo-type fold (IPR016024), Protein HGH1 N-terminal (IPR007205)	ambiguous
94	HORVU4Hr1G071050	0.0	99	Ribosomal protein S10 (IPR001848)	ambiguous
97	HORVU2Hr1G026340	1.9e-175	98	Peptidase C15, pyroglutamyl peptidase I (IPR000816)	Chloroplast
104	HORVU4Hr1G059860	0.0	95	HAD hydrolase, subfamily IA (IPR006439), HAD-like domain (IPR023214), Pyrimidine 5-nucleotidase	ambiguous
106	HORVU7Hr1G120540	0.0	100	Leucine-rich repeat domain, L domain-like, NB-ARC, P-loop containing nucleoside triphosphate hydrolase, Winged helix-turn-helix DNA-binding domain	ambiguous
108	HORVU4Hr1G001350	1.7e-95	99	SUZ domain (IPR024771), R3H domain (IPR001374), RNA/DNA-binding, Ribosomal protein	Cytoplasmic
115	HORVU5Hr1G088430	5.0e-18	85	Tetratricopeptide repeat (TPR)-like superfamily protein	ambiguous
116	HORVU1Hr1G035720	0.0	98	Ribulose biphosphate carboxylase small chain	Chloroplast
119	HORVU4Hr1G027660	0.0	96	Metallothionein, family 15, plant (IPR000347)	ambiguous
120	HORVU7Hr1G119930	0.0	100	Peptidase C1A	Endoplasmic reticulum
120	HORVU7Hr1G120060	0.0	100	Peptidase C1A	Endoplasmic reticulum
123	HORVU3Hr1G070950	7.0e-61	89	undescribed protein	Extracellular (Secreted)
126	HORVU1Hr1G045590	0.0	100	Glutamyl-tRNA reductase, conserved site (IPR018214), Quinate/shikimate 5-dehydrogenase/glutamyl-tRNA reductase (IPR006151), Tetrapyrrole biosynthesis, glutamyl-tRNA reductase (IPR000343)	Chloroplast
128	HORVU3Hr1G078680	8.6e-106	100	Cystatin domain, Proteinase inhibitor I25, cystatin, conserved site, They mainly inhibit peptidases belonging to peptidase families C1 (papain family)	Extracellular (Secreted)
130	HORVU5Hr1G119030	0.0	99	no predicted domain	Chloroplast
151	HORVU6Hr1G035970	0.0	99	Cytochrome b245, heavy chain, NADPH oxidase Respiratory burst	Plasma membrane
154	HORVU6Hr1G012570	1.7e-72	93	Cyclophilin-type peptidyl-prolyl cis-trans isomerase domain (IPR002130), potential interaction partner MLO3	Cytoplasmic
167	HORVU4Hr1G020030	0.0	99	ABA/WDS induced protein (IPR003496)	Nuclear
171	HORVU2Hr1G072210	0.0	100	Arogenate dehydratase: ACT domain, Bifunctional P-protein, chorismate mutase/prephenate dehydratase	Plasma membrane
175	HORVU2Hr1G017470	0.0	100	NAC domain (IPR003441)	Nuclear
176	HORVU2Hr1G111300	1.3e-138	99	Glutamine synthetase, catalytic domain (IPR008146)	Chloroplast
180	HORVU4Hr1G072480	0.0	100	Blue copper-binding protein, Cupredoxin (IPR008972), Phytocyanin domain (IPR003245)	Plasma membrane

clone nr.	gene ID	e-value	%ID	description/function	localisation
181	HORVU6Hr1G064620	2.5e-70	99	Dehydrin (IPR000167)	Extracellular (Secreted)
185	HORVU2Hr1G016740	4.1e-154	100	EF-hand domain (IPR002048), Serine hydrolase FSH (IPR005645), domain structure depending on the gene model, but both domains are not connected in one ORF	Plasma membrane
190	HORVU1Hr1G055440	5.9e-168	100	Nucleoside diphosphate kinase (IPR001564)	Chloroplast
191	HORVU5Hr1G033540	0.0	100	Ribulose biphosphate carboxylase small chain	Chloroplast
199	HORVU5Hr1G082990	0.0	100	PLC-like phosphodiesterase, TIM beta/alpha-barrel domain, Phosphatidylinositol-specific phospholipase C, X domain, Phospholipase C also interact with MLO2	ambiguous
202	HORVU6Hr1G077890	0.0	98	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain (IPR016140)	Vacuole
206	HORVU1Hr1G043910	8.3e-141	93	Uncharacterised protein family, basic secretory protein (IPR007541), believed to be part of the plant defence mechanism against pathogens	Extracellular (Secreted)
207	HORVU3Hr1G059210	1.4e-118	92	WRKY domain (IPR003657)	Nuclear
207	HORVU3Hr1G059220	1.4e-118	92	WRKY domain (IPR003657)	Nuclear
208	HORVU3Hr1G015190	1.78e-123	97	Ribosomal protein S5/S7 (IPR000235)	Cytoplasmic
216	HORVU0Hr1G007360	2.8e-50	91	Chlorophyllide a oxygenase, Rieske [2Fe-2S] iron-sulphur domain (IPR017941), Pheophorbide a oxygenase (IPR013626),	Chloroplast
222	HORVU7Hr1G120540	4.1e-36	95	Leucine-rich repeat domain, L domain-like, NB-ARC, P-loop containing nucleoside triphosphate hydrolase, Winged helix-turn-helix DNA-binding domain	ambiguous
230	HORVU3Hr1G056580	0.0	100	Ribosomal protein L31	ambiguous
Bgh					
46	CSEP515	2.1e-151	100	effector	
69	CSEP0421	3.0e-63	93	effector	
77	CSEP515	4.3e-76	99.3	effector	
80	CSEP0421	4.7e-66	82	effector	
82	BLGH_06411-mRNA-1	0.0	100	signal peptide (secreted), no known domains	
85	BLGH_03290-mRNA-1	0.0	99	no domains known, nuclear localisation predicted	
95	CSEP515	4.7e-15	85.7	effector	
139	BGHDH14_bgh05284	0.0	100	Eukaryotic translation initiation factor 3 subunit M (IPR027528)	
146	CSEP515	1.1e-132	96.4	effector	
173	BGHDH14_bgh05284	9.8e-129	100	Eukaryotic translation initiation factor 3 subunit M (eIF3m)	
186	BGHDH14_bgh05456-1	1.3e-149	100	2-oxoisovalerate dehydrogenase subunit alpha (IPR034616)	
234	CSEP262/CSEP485	4.4e-87	100	effector	

The identified proteases and peptidases were considered as false positives as they can cleave off the Ura3 tag and lead to 5-FOA resistance rendering the split-ubiquitin system sensitive against this protein class. Additionally, these proteins were predicted to localise to different compartments. By contrast, the identified kinases or kinase domains that included an S-receptor-like serine/threonine-protein kinase, CPK20 and

a leucine-rich repeat RLK were considered as interesting candidates. It is known that the C-terminus of Arabidopsis MLO2 can be phosphorylated (Leißing, 2018), which might indicate that similar modifications by kinases are possible for *HvMlo*. The CPK20 clone included only the EF-hand domain of this protein. The fact that a constitutively active form of CPK19/CDPK3 partially compromises *mlo*-mediated resistance (Freyemark et al., 2007) confirms the potential of CPKs as an interesting candidate interactor.

Similarly, the ABI family proteins might be connected to MLO functioning: the presence of *HvMlo* interferes with actin focussing towards the *Bgh* attack site during powdery mildew attack of barley (Miklis et al., 2007) and ABI family proteins are involved in the regulation of the actin cytoskeleton organisation.

Interestingly, several obtained clones were previously identified as potential interactors of Arabidopsis MLOs in high-throughput Y2H screens (Jones et al., 2014a, Lalonde et al., 2010): a purple acid phosphatase (clone 4) and an *O*-methyltransferase (clone 23) were identified as potential interactor of *AtMLO6*, while a cyclophilin-type peptidyl-prolyl cis-trans isomerase domain protein (clone 154) and a PLC-like phosphodiesterase (clone 199) were published as possible interaction partners of *AtMLO3* and *AtMLO2*, respectively.

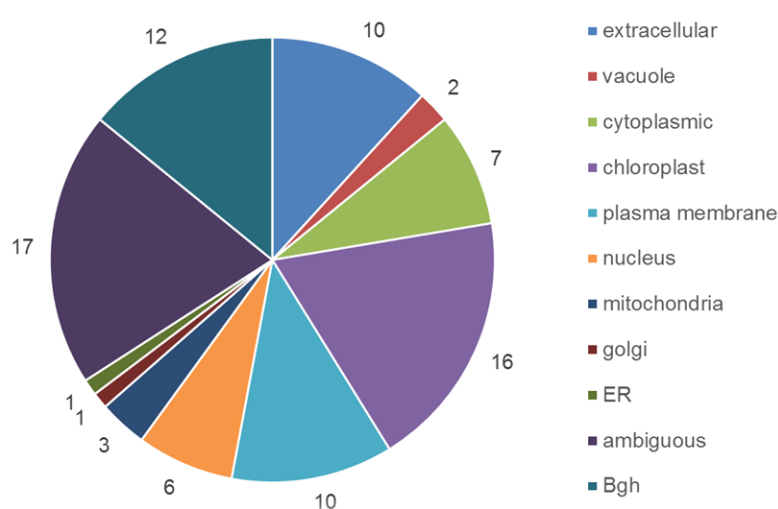


Figure 15: Predicted subcellular localisation of potential interaction partners of *HvMlo*. The subcellular localisation of potential interaction partners of *HvMlo* from the Ura3-based split-ubiquitin Y2H screen was predicted with ProtComp 9.0 (<http://www.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc>). The number of potential interaction partner is indicated. Predictions with similar scores for different compartments were summarised as ambiguous.

Finally, I decided to further investigate *Bgh* CSEP0515 as well as the barley homologs of the potential interactors of the powdery mildew susceptibility factors *AtMLO2* and *AtMLO6* (PLC-like phosphodiesterase (clone 199), purple acid phosphatase (clone 4), O-methyltransferase (clone 23)) and the ABI family protein (clone 66) as candidates for further investigation. Additionally, I included two clones that comprised complete open reading frames and a predicted cytosolic localisation (clone 36, a Zinc finger, AN1- type protein and clone 48, an inorganic pyrophosphatase). A test of all remaining 17 candidates was not possible, so the above-mentioned candidate genes were selected on the bases of former screens against MLOs or because the clones showed a complete open reading frame. All selected candidates were in frame with the tag. The kinases were not chosen since they were only present as truncated versions and the phosphorylation of the C-terminus of MLO2 was still under investigation (Leißing, 2018). They could serve as interesting candidates for future experiments, as well as the NLR protein. Taken together, I selected six genes from barley for further investigation in the split-luciferase assay in *N. benthamiana* and CSEP0515 for BiFC experiments in barley epidermal cells (Table 33).

Interaction of *HvMlo* and CSEP0515 could not be confirmed

Since CSEP0515 appeared four times in the cDNA library screen, it was chosen as the most promising effector candidate for *in planta* validation. *HvMlo*-cYFP and nYFP-CSEP0515, nYFP-*HvCaM3* (positive control) or nYFP-*HvGα* (negative control) was co-bombarded together with DsRED as a transformation marker (Figure 16). As expected, the positive control showed YFP fluorescence, while the negative control *HvGα* resulted in no fluorescence signal. The potential interaction of barley Mlo and CSEP0515 could not be confirmed in BiFC experiments in barley epidermal cells since no fluorescence could be detected in multiple cells of two replicates.

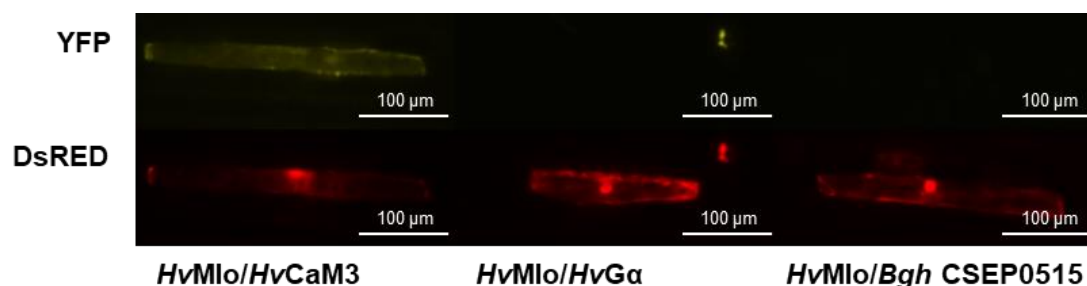


Figure 16: *HvMlo* and CSEP0515 do not interact in a barley BiFC assay. *HvMlo*-cYFP (pUCSPYCE-*HvMlo*) was co-bombarded with nYFP-*HvCaM3* (pESPYNE-*HvCaM3*; positive control), nYFP-*HvGα* (pESPYNE-*HvGα*; negative control) or nYFP-CSEP0515 (pESPYNE-CSEP0515) into 7-day-old barley (cv. Margret) leaf epidermal cells. DsRED was co-bombarded as a transformation control. Multiple transformed cells were examined in two independent bombardments. Upper panel: YFP fluorescence, lower panel: DsRED fluorescence. Scale bar indicates 100 µm.

HvMlo* interacts with a barely O-methyltransferase and an inorganic pyrophosphatase *in planta

A split-luciferase assay was performed in *N. benthamiana* to validate the interaction of *HvMlo* with the barley O-methyltransferase (clone 23; HORVU7Hr1G048970), the PLC-like phosphodiesterase (clone 199; HORVU5Hr1G082990), the purple acid phosphatase (clone 4; HORVU4Hr1G064380), the ABI family protein (clone 66; HORVU3Hr1G055780), the Zinc finger, AN1- type protein (clone 36; HORVU7Hr1G050270) and the inorganic pyrophosphatase (clone 48; HORVU3Hr1G116650) (Figure 17 A). Agrobacteria strains carrying *HvMlo*-nLUC were co-infiltrated with N-terminal cLUC fusions of one of the candidates. The luminescence was measured after two days and compared to co-infiltration of *HvMlo*-nLUC with cLUC-*HvCaM3* (positive control;(Kim et al., 2002)) or cLUC-*HvGα* (negative control). The absolute luminescence was normalised against co-infiltrated *HvMlo*-nLUC/cLUC-*Gα* (negative control), and at least four independent biological replicates were performed (Figure 17 A). The split-luciferase assay is compared to the BiFC assay faster, which allows the testing of different combination in a shorter time. But the BiFC assay can be performed in barley leaves which makes is the method of choice for smaller sets of proteins. HORVU4Hr1G064380, HORVU7Hr1G050270, HORVU3Hr1G055780 and HORVU5Hr1G082990 infiltration resulted in no or low luciferase activity. However, except for HORVU4Hr1G064380, none of these constructs revealed expression in the western blot, besides unspecific bands (Figure 17 B). Besides the positive control, only HORVU7Hr1G048970 and HORVU3Hr1G116650 showed a statistically significant difference compared to the negative control. Taken together, I could confirm the interaction of *HvMlo* with the barley O-methyltransferase (clone 23; HORVU7Hr1G048970) and the inorganic pyrophosphatase (clone 48; HORVU3Hr1G116650) *in planta* while further candidates could not be verified.

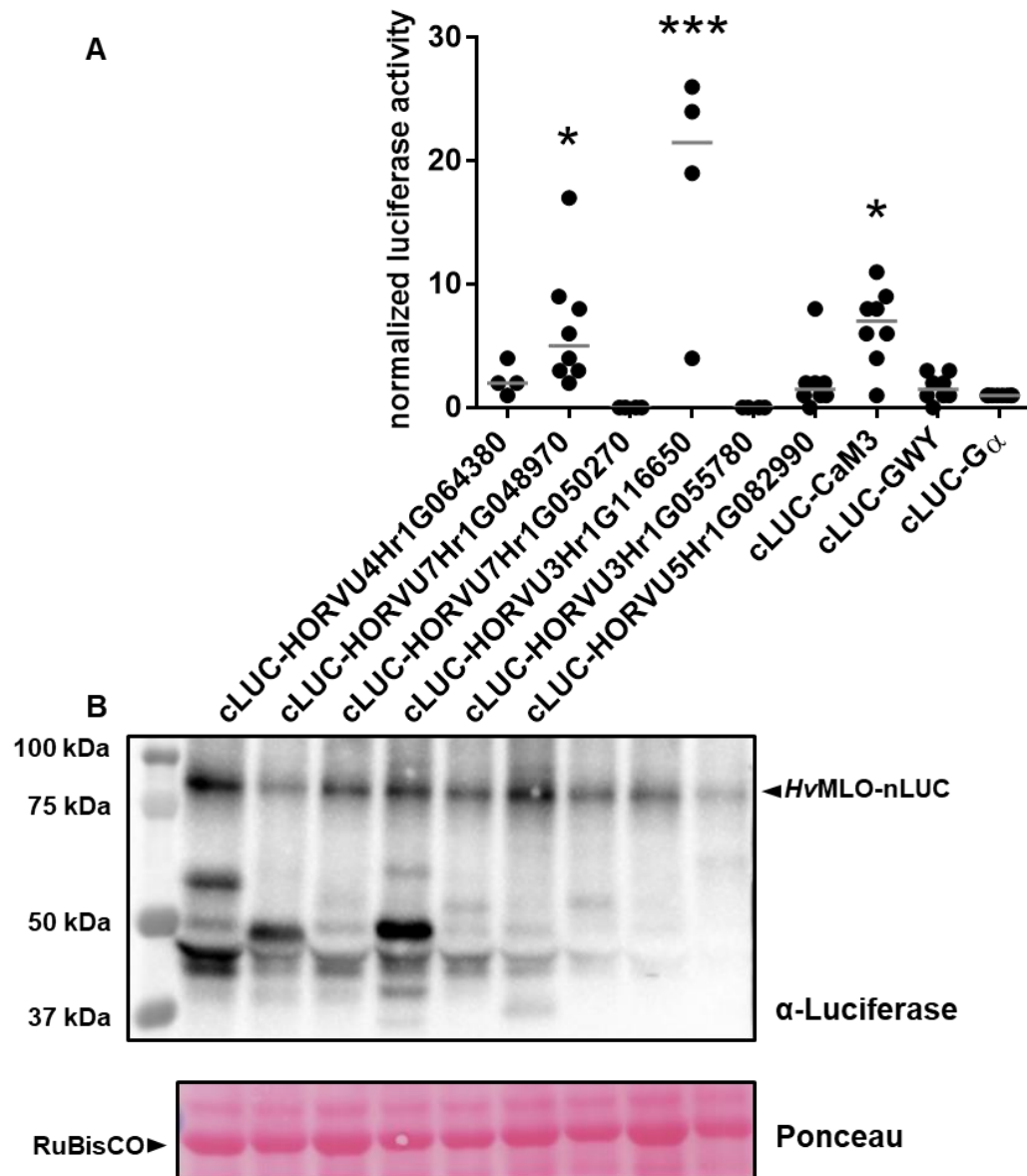


Figure 17: Split-luciferase validation of potential interaction partners of HvMlo. (A) The split-luciferase assay allows the quantification of *in planta* protein-protein interaction. Suspensions of *A. tumefaciens* strains carrying plasmids with the indicated fusion proteins and with HvMlo-nLUC were co-infiltrated in *N. benthamiana* leaves and incubated for two days. In total, luminescence from eight leaf discs (four discs from 2 individual leaves) of each combination was recorded. D-Luciferin was infiltrated as a substrate before the measurement. The mean values of the leaf discs after 5 min of measurement were normalised to the negative control HvGα. Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Asterisks indicate the following P-values: * = $P \leq 0.05$ and *** = $P \leq 0.001$. The horizontal bar marks the median. (B) The leaf discs were collected for total protein extraction and western blot analysis after measurement of luminescence. The polyclonal anti-luciferase antibody binds to the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was performed to visualise equal loading and membrane transfer of the proteins. The expected sizes of the split-luciferase fusion constructs are: HvMlo-nLUC: 107 kDa; cLUC-HORVU4Hr1G064380: 58 kDa; cLUC-HORVU7Hr1G048970: 47 kDa; cLUC-HORVU7Hr1G050270: 37 kDa; cLUC-HORVU3Hr1G116650: 46 kDa; cLUC-HORVU3Hr1G055780: 54 kDa; cLUC-HORVU5Hr1G082990: 57 kDa; cLUC-CaM3: 37 kDa; cLUC-GWY: 20 kDa; cLUC-Gα: 64 kDa. This experiment was repeated at least in four independent biological replicates. An image of an uncut western blot is included in the supplements.

The barley *O*-methyltransferase localises at the cytoplasm and interferes with *Bgh* penetration of barley leaf epidermal cells

Since HORVU7Hr1G048970 (*O*-methyltransferase) showed comparable luciferase activity to the positive control, a robust expression level and an *O*-methyltransferase was previously identified as a potential interactor of AtMLO6 (Jones et al., 2014a), I selected this gene for further characterisation of its subcellular localisation and a potential impact on the powdery mildew interaction.

In silico analysis of the subcellular occurrence of the *O*-methyltransferase suggested either a nuclear or cytoplasmic localisation of this interactor, depending on the prediction algorithm. As restriction of the protein to the nucleus would disqualify it as an interaction partner of *HvMlo*, I investigated its subcellular abundance in barley. The split-luciferase results were a first indication that this protein could not localise to the nucleus. The interaction can only take place when the *O*-methyltransferase is cytosolic or plasma membrane localised since *HvMlo* should localise at the plasma membrane. Expression of the N-terminal CFP-tagged *O*-methyltransferase (HORVU7Hr1G048970) in barley epidermal cells, together with DsRED as a transformation marker *via* bombardment, revealed a cytosolic localisation of the protein overlapping largely with cytoplasmic DsRED fluorescence (Figure 18). In contrast to CFP-HORVU7Hr1G048970 fluorescence, DsRED fluorescence could further be detected in the nucleus, suggesting that the *O*-methyltransferase is mainly abundant in the cytoplasm, where this protein could interact with Mlo.

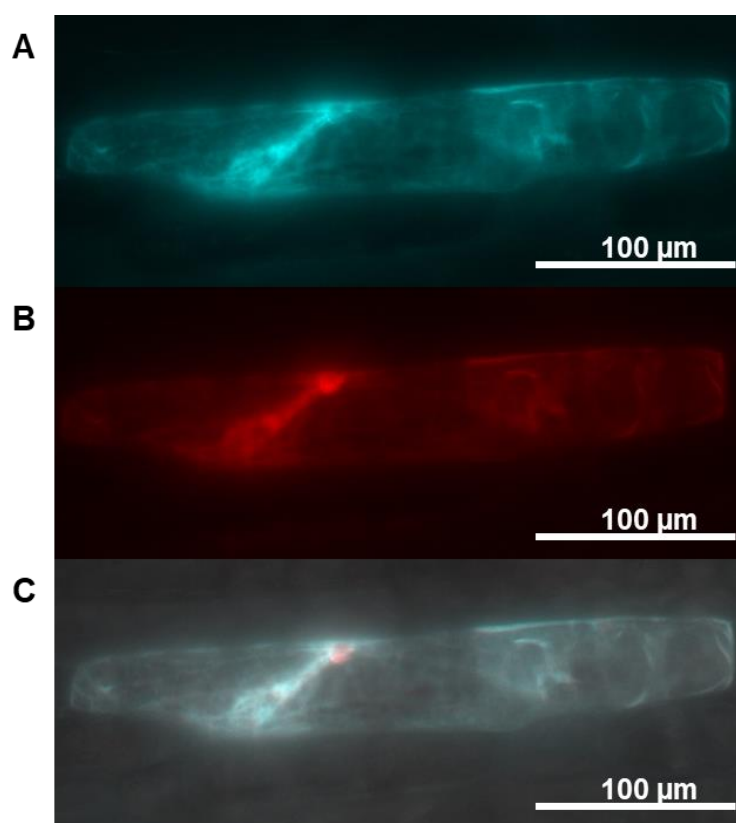


Figure 18: The *HvMlo* interacting *O*-methyltransferase HORVU7Hr1G048970 localises in the cytoplasm of barley leaf epidermal cells. The potential interaction partner HORVU7Hr1G048970 of *HvMlo* was fused N-terminally with CFP and transiently expressed together with DsRED in barley (cv. Margret) leaf epidermal cells via co-bombarded. A: CFP fluorescence, B: DsRED fluorescence, C: overlay. Scale bar indicates 100 µm.

As *HvMlo* is critical for infection of barley by *Bgh*, it is conceivable that an interacting protein might interfere with or facilitate this function of *HvMlo*. Consequently, a possible role of the *O*-methyltransferase (HORVU7Hr1G048970) in the barley/powdery mildew interaction was tested by overexpression in barley epidermal cells, inoculation of *Bgh* K1 and scoring the penetration rate of the fungus in GUS (control) and GUS + *O*-methyltransferase bombarded cells. The penetration rate of GUS expressing cells was set to 100%, and the penetration rate of *O*-methyltransferase overexpressing cells normalised to this control (Figure 19). Overexpression of the *O*-methyltransferase in barley cv. Margret resulted in ca. 50% reduction of the penetration rate compared to cells expressing GUS. This result was confirmed in three biological replicates; however, the difference between both constructs was not statistically significant. Still, reproducibility of the effect of *O*-methyltransferase overexpression suggests a negative impact of the protein on powdery mildew infection. Although the observed reduction of the penetration rate is no evidence for the interaction with *HvMlo*, an involvement of this gene in powdery mildew defence is likely possible.

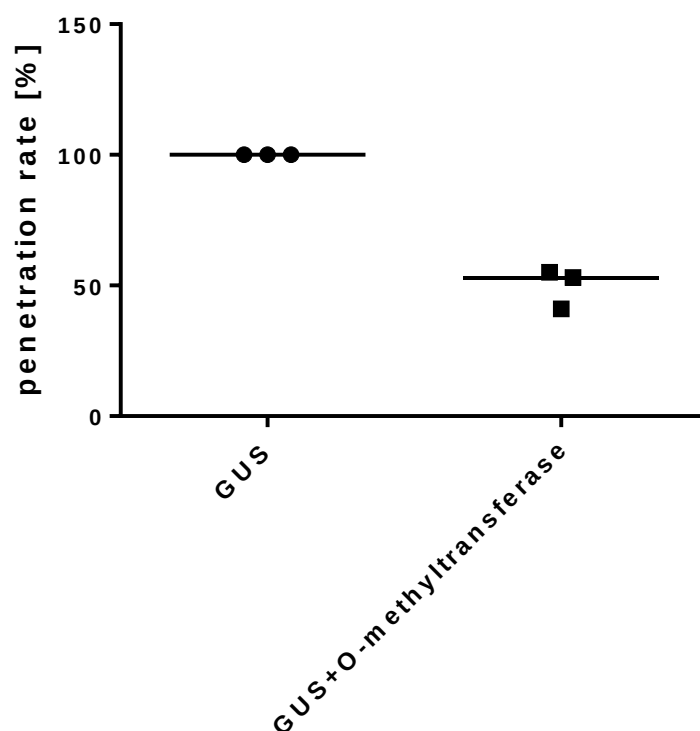


Figure 19: O-methyltransferase overexpression impacts the penetration rate of *Bgh* K1 on barley epidermal cells. GUS alone or in combination with the O-methyltransferase HORVU7Hr1G048970 was transiently expressed under control of the ubiquitin promoter via co-bombardment in barley leaf epidermal cells. Bombarded leaves were inoculated with *Bgh* K1, and at 1 dpi the entry rate in transformed cells was investigated after staining for GUS activity. The penetration rate of the GUS construct in each of three independent biological replicates was set to 100%, and the penetration rate of the O-methyltransferase was normalised to this control. The horizontal bars indicate the median. A Wilcoxon matched-pair signed-rank test did not reveal statistically significant differences.

Discussion:

The biochemical function of *HvMlo* is still unknown, and the identification of interacting proteins has the potential to provide novel insights helping to answer this question. The performed split-ubiquitin Y2H screen against the barley cDNA library resulted in an initially large set of potential interaction partners (Table 33), which could be due to the drawbacks of the used Ura3-based split-ubiquitin system (Wittke et al., 1999). This system uses only one reporter (Ura3), which renders it less stringent than the LexA-based system that employs two growth reporters (Obrdlik et al., 2004). In the case of *HvMlo*, the LexA-based system could not be used, because this bait fusion protein was unstable and resulted in an autoactivation of the reporter system (Supplementary Figure 7). Despite the high number of obtained clones, the number of repetitive hits of one gene was rather low (two genes were covered by two individual clones). As only

a small part of the cDNA library (2 µg DNA) was used, the transformation of a larger amount of the cDNA library could result in a more significant coverage. This small amount of the library was chosen as a test for the screening set up and already 2 µg of the cDNA library resulted in a considerable number of colonies, which had to be processed.

Besides *in silico* prediction of subcellular localisation, I compared my data with results of previous Y2H studies of other MLO proteins (Consonni, 2005, Jones et al., 2014a, Lalonde et al., 2010) to narrow down the list of candidate interactors. This revealed that four of the identified genes had been described as potential MLO-interacting proteins (proteins belonging to the same protein family), previously. Finally, additionally considering available functional data on HvMlo (Miklis et al., 2007) and coverage of genes by clones, I selected six genes for further investigation.

The cDNA library for this screen was prepared from *Bgh*-infected leaf material to capture also potential fungal as well as defence-activated plant interactors. Epidermal peeling was performed to concentrate transcripts involved in the defence to powdery mildew, which should increase the chance to find potential interaction partner. Indeed, CSEP0515 was found four times in the yeast screen and selected for further validation, but the interaction could not be confirmed *in planta* by BiFC experiments in barley epidermal cells (Figure 16). However, the expression of the effector could not be tested *via* western blot as biolistic transformation results in only a few cells expressing the constructs. Tagging with a complete YFP could show if this effector is correctly expressed in plant cells. Alternatively, usage of different linkers and tags in the BiFC system, or employment of other interaction assays (e.g. FRET or Co-IP) could circumvent possible steric problems interfering with YFP reassembly. A second *Bgh* effector captured by the initial screen, CSEP0421 was not tested *in planta* yet and needs to be validated in future.

The split-ubiquitin system is only capable of detecting interactions of proteins occurring in the cytosol, but the subcellular localisation of most *Bgh* CSEPs is unknown. They were expressed without signal peptide to ensure that they are expressed inside the cell. It is for a certain part of the CSEPs possibly the wrong localisation, which could lead to misfolding. The membrane-anchored ligand and receptor yeast two-hybrid (MALAR-Y2H) split-ubiquitin system (Li et al., 2016a) is capable of detecting

extracellular interactions. However, it involves attachment of tags on both sites of the prey protein, which could result in misfolding and loss of interaction. This system further requires prey sequences to lack a stop codon, which renders it incompatible with our CSEP library (chapter 2) and cDNA libraries. Another approach for high throughput detection of extracellular interactions could be the transfer of a split-reporter system, like the split-luciferase or split-horseradish peroxidase system to yeast (Martell et al., 2016, Ozawa et al., 2001, Schonberger et al., 1996) or adaptation of the split-invertase for this purpose (Schonberger et al., 1996). The split-horseradish peroxidase is used in the mammalian field (Martell et al., 2016) and specific luciferases can be expressed in the extracellular space (Bonaldi et al., 2017, Enjalbert et al., 2009). As an example, the *HvMlo* bait protein could be fused with nLUC at its extracellular N-terminus while the effector could be N-terminally tagged with a signal peptide-cLUC fusion mediating secretion of the prey. Cultivation of yeast in a buffered medium would provide suitable conditions for extracellular luciferase activity. Additionally, the substrate of the specific luciferase needs to be added, which is in case of the firefly luciferase D-Luciferin and ATP (Gould & Subramani, 1988). This reaction could be monitored in 96-well plates in a luminometer. This set up would not be suitable for a standard library screen but provide the possibility for an array-based approach in a mating based set up. Yet another approach to find new interaction partner would include transgenic barley lines, expressing *HvMlo* fused, to a biotin-ligase. The proximity-based labelling method relies on the biotinylation of proteins, which come into close contact with the bait protein. The biotinylated proteins can be purified and send to mass-spectrometry for the identification. The big advantages are that the prey carries no tag and the systems works in the host system (Branon et al., 2018, Khan et al., 2018, Trinkle-Mulcahy, 2019). Also, other systems tried to solve the issue of detecting extracellular interaction in different ways (Bushell et al., 2008, Urech et al., 2003, Younger et al., 2017). These assays could be an alternative approach to find interaction partner of *HvMlo*.

In addition to the *Bgh* effector, I selected six barley genes for further investigation *in planta*. Conducting a split-luciferase assay, I could confirm that HORVU7Hr1G048970 (O-methyltransferase) and HORVU3Hr1G116650 (inorganic pyrophosphatase) interact with *HvMlo* in *N. benthamiana* (Figure 17). The other four tested proteins were either not or only weakly expressed (HORVU5Hr1G082990, HORVU3Hr1G055780, HORVU7Hr1G050270) or did not interact with *HvMlo* (HORVU4Hr1G064380) (Figure

17 B). The inorganic pyrophosphatase was highly expressed *in planta* split-luciferase assay and facilitated high luciferase activity (Figure 18). Whether the latter result is an artefact of its high abundance or whether the protein is a true interactor of HvMlo needs to be further tested in additional protein-protein interaction assays. Similarly, the interaction of HvMlo with the O-methyltransferase should be confirmed with additional protein-protein interaction assays.

According to *in silico* analysis, both confirmed interactors potentially localised at the cytosol, which enables these candidates to interact with HvMlo. I could confirm the cytosolic localisation for the O-methyltransferase (Figure 18). A C-terminal tagged version should be additionally tested to verify this finding since the N-terminal tag could influence the subcellular localisation.

Plant O-methyltransferases (OMTs) transfer a methyl group to an oxygen atom of different substrates, like phenylpropanoids, flavonoids and alkaloids. Hence, they play important roles in the biosynthesis of lignin or in biotic and abiotic stress tolerance/resistance, since also phytoalexins belong to their products (Lam et al., 2007). Subclasses are caffeoyl-CoA 3-O-methyltransferases (CCoAOMT) and caffeic acid O-methyltransferase (COMT), which also use SAM (S-adenosyl-L-methionine) as a cofactor. Expression of the O-methyltransferase in the split-luciferase assay resulted in comparable luciferase activity as HvCaM3, which is a well-known interaction partner of HvMlo (Figure 17) (Kim et al., 2002). That is one reason why this candidate was chosen. Overexpression of the protein in barley epidermal cells further resulted in a powdery mildew phenotype, since the penetration rate was reduced by 50% (Figure 19). Nevertheless, this assay should be repeated to generate more robust data since the number of evaluated cells was low in some of the independent replicates. These overexpression experiments could be ideally complemented with RNAi experiments in the wild type or *mlo* background. The O-methyltransferase is predicted to belong to the group of caffeoyl-CoA 3-O-methyltransferase. Enzymes of this group transfer a methyl group from the cofactor SAM to the acceptor caffeoyl-CoA. O-Methyltransferases are involved in the phenylpropanoid pathway, which is necessary for the lignin biosynthesis in plants, but also for the production of SA, flavonoids and coumarins (Dixon et al., 2002, Xie et al., 2018). Many studies show the importance of O-methyltransferases in the production of monolignols (Do et al., 2007, Guo et al., 2001, Li et al., 2013, Maury et al., 1999, Tu et al., 2010, Wagner et al., 2011, Zhong et al., 2000). A BLAST search

of the amino acid sequence encoded by HORVU7Hr1G048970 in other species reveals the Tricin synthase 1 (ROMT-15) of rice (*Oryza sativa*) as one best match. ROMT-15 is necessary to produce the flavonoid Tricin and its derivatives in rice (Lee et al., 2008), which is incorporated in lignin of monocot plants (Lan et al., 2015, Li et al., 2016c). Future investigation of the enzymatic activity of heterologously expressed barley *O*-methyltransferase in an enzymatic assay could reveal which compounds are produced by the identified enzyme and whether it contributes to lignin biosynthesis. These assays could also reveal, to which group of *O*-methyltransferases, either caffeoyl-CoA 3-*O*-methyltransferases (CCoAOMT) or caffeic acid-*O*-Methyltransferase (COMT) this enzyme belongs. Both groups use different substrates and take part in different steps in a biosynthetic pathway of monolignols. For enzymes involved in the lignin biosynthetic pathway, an infection-related phenotype was reported in different plant species and for distinct pathogens (Liu et al., 2018b, Miedes et al., 2014). A maize caffeoyl-CoA *O*-methyltransferase confers resistance to southern leaf blight, gray leaf spot and northern leaf blight by an increase of the lignin precursor Coniferin and lignin and also other metabolites of the phenylpropanoid pathway. Yang and colleagues suggest that the increase of resistance is due to an alteration of the lignin, but they also found an increase in other metabolites, which could have signal molecule or antimicrobial function. They showed further that the expression of the caffeoyl-CoA *O*-methyltransferase in *N. benthamiana* suppresses cell death (Yang et al., 2017). Another study showed that the silencing of different enzymes of the lignin biosynthetic pathway resulted in an increased susceptibility against *Bgt* and *Bgh* on wheat (Bhuiyan et al., 2009). This finding is in accordance with the results of the overexpression of the identified *O*-methyltransferase in barley cells (Figure 19). But the overexpression of the *O*-methyltransferase could further influence other metabolites of the phenylpropanoid pathway, like SA or metabolites with antimicrobial activity since overexpression could lead to an imbalance in the distribution of precursors. For example, the SA level of Arabidopsis *mlo* mutants is increased (Gruner et al., 2018, Kuhn et al., 2017), which could be a result of misregulation. Metabolic comparison of a barley wild type cultivar with the respective *mlo* mutant showed differential accumulation of p-coumaroyl-hydroxyagmatine, a compound with antimicrobial activity, which is produced via p-Coumarate (von Röpenack et al., 1998). Interestingly, the downregulation of a methylenetetrahydrofolate reductase (MTHFR) and a caffeic acid *O*-methyltransferase (COMT) in switchgrass result in lesion-mimic cell death (Liu

et al., 2017). MTHFR provides the methyl units for the COMT. Spontaneous cell death is besides cell wall appositions (CWAs) without pathogen attack, a pleiotropic effect in the *mlo* mutant and appears in older leaves. Which changes on the metabolic level are responsible in case of this protein remains unclear. The increased expression of HORVU7Hr1G048970 during powdery mildew infection supports a participation of this enzyme in the defence to powdery mildews. The Expression Atlas (<https://www.ebi.ac.uk/gxa/home>; data obtained April 2019) shows a 2.4 – 2.5 log₂-fold change (*p*-values: 3.9×10^{-6} – 3.5×10^{-8}) between 20 – 32 h after powdery mildew infection, which corresponds with the time point of *Bgh* haustoria formation. During appressorium formation, the expression is only increased by 1.6 log₂-fold (*p*-value: 1×10^{-3}). Time points between 8 and 20 h would be interesting to test since the actual penetration happens in this time frame. Since *mlo* mutants exhibit a pre-penetration resistance, lignin could be involved in cell wall reinforcement preventing fungal entry. Overall, misregulation of the phenylpropanoid pathway by *O*-methyltransferases could partially explain *mlo* phenotypes like the early senescence and the pre-penetration resistance.

Although I successfully identified new potential interactors of *HvMlo*, it cannot be excluded that further proteins that are important for Mlo functioning do not interact directly with *HvMlo* but require other interaction or complex formation with CaM (or with proteins of *Bgh*). Such interactors could be captured in split-ubiquitin Y2H assay expressing *HvCaM3* on a third plasmid. However, this set up could possibly result in reduced transformation efficiency, since multiple vectors are a metabolic burden and reduce the life span of a cell (Falcon & Aris, 2003, Karim et al., 2013). Alternatively, the bait construct and CaM could be integrated into the genome of the yeast strain to circumvent low transformation rates and establish a screening set up for complex interactions. Other approaches are the proximity-based labelling method (Branon et al., 2018, Khan et al., 2018, Trinkle-Mulcahy, 2019) or the XL-TAP-MS strategy (Leißing, 2018), which could be used in barley to identify this kind of interactions. The advantage would be that only one Mlo is responsible for the powdery mildew phenotype and not three, like in *Arabidopsis*. The disadvantage is the transformation of barley, which is more time-consuming.

Conclusion

In summary, I could identify two new potential interaction partners of barley *HvMlo* (HORVU7Hr1G048970 and HORVU3Hr1G116650), whose interaction has to be further validated. One of the genes (HORVU3Hr1G116650) encodes an inorganic pyrophosphatase a class of enzymes that regulates intracellular levels of pyrophosphate, which is a by-product of many metabolic reactions (Kajander et al., 2013). The second gene (HORVU7Hr1G048970) codes for an *O*-methyltransferase with a potential role in the phenylpropanoid pathway and a positive effect on powdery mildew resistance. These findings provide novel insights into the functioning of *HvMlo* with the potential to contribute to the elucidation of its biochemical activity. A comparison with newly available data of a biochemical interaction screening approaches based on *in vivo* crosslinking of potential interaction partners of MLO2 (Leißing, 2018) and already available data might further identify potential interaction partner of MLOs.

Chapter 4: Identification and validation of potential interaction partner of Arabidopsis CERK1

Introduction:

Plants perceive conserved microbe-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs) on the cell surface to recognise phytopathogens and symbionts. These receptors are usually receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Newman et al., 2013, Zipfel & Oldroyd, 2017). Both consist of an extracellular domain and a single transmembrane domain, but only RLKs possess a kinase domain at the intracellular site. MAMPs are conserved molecules like flg22, an epitope of flagellin from bacteria, or the fungal cell wall component chitin, both of which are indispensable for the particular organism. RLKs bind MAMPs on the cell surface and transmit the signal intracellularly by a series of phosphorylation reactions as part of a signal transduction cascade, which ultimately leads to defence response.

Two of the most studied leucine-rich repeat (LRR) RLKs in Arabidopsis are FLS2 (FLAGELLIN SENSITIVE 2), which detects the flagellin epitope flg22 (Gómez-Gómez & Boller, 2000), and EFR (EF-TU RECEPTOR), which recognises the elongation factor TU (elf18) (Zipfel et al., 2006). However, RLKs do not act alone in the signal transduction. Instead, they associate with co-receptors or other RLKs to bind one MAMP (Monaghan & Zipfel, 2012, Newman et al., 2013, Zipfel & Oldroyd, 2017). One example of a co-receptor is BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1), which forms complexes with FLS2 and EFR (Kim et al., 2013).

The LysM (lysine motif) receptor CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) is involved in the perception of the main fungal cell wall component chitin (Liu et al., 2012b, Miya et al., 2007, Petutschnig et al., 2010, Shimizu et al., 2010, Wan et al., 2008) and the bacterial cell wall component peptidoglycan (Kouzai et al., 2014, Willmann et al., 2011) in Arabidopsis and rice (*Oryza sativa*). An Arabidopsis *cerk1* knock-out mutant cannot recognise chitin and trigger MTI (Miya et al., 2007). *AtCERK1* is also involved in the recognition of β -1,3-linked glycans (Mélida et al., 2018) and *OsCERK1* participates in recognition of lipopolysaccharide (LPS) (Desaki et al., 2018b). These different carbohydrate MAMPs elucidate the central role of CERK1 as an essential receptor. CERK1 does not act alone in recognition of these MAMPs but instead needs the LysM receptors LYK4 (LYSM-CONTAINING RECEPTOR-KINASE

4) and LYK5 (LYSM-CONTAINING RECEPTOR-KINASE 5) for the chitin perception in Arabidopsis (Cao et al., 2014, Wan et al., 2012b). The interaction of CERK1 with LYK5, but not with LYK4, was demonstrated. The chitin-binding affinity of LYK5 is higher than of CERK1, which suggests that LYK5 is the primary chitin receptor, but the kinase domain of LYK5 is not functional, and CERK1 is necessary as co-receptor to transmit the signal (Cao et al., 2014). For the perception of bacterial cell wall peptidoglycans, *AtCERK1*, *AtLYM1* and *AtLYM3* (LYSM DOMAIN GPI-ANCHORED PROTEIN 1 and 3) act together. LYM1 and LYM3 lack the cytoplasmic signalling domain, and CERK1 is also here necessary for the signal transduction. Physical interaction of these receptors was not yet shown (Willmann et al., 2011). *OsCERK1* forms a complex with the LysM-RLP CEBiP (CHITIN ELICITOR BINDING PROTEIN) (Shinya et al., 2012). CEBiP possesses no kinase domain and is GPI-anchored to the membrane, like LYM1 and LYM3. *OsCERK1* is responsible for the signal transduction, whereby CEBiP is necessary for the chitin-binding since *OsCERK1* has no chitin-binding activity (Gong et al., 2017, Kaku et al., 2006, Kouzai et al., 2014, Shimizu et al., 2010). The homologous of CEBiP, LYP4 and LYP6 (LYSIN MOTIF-CONTAINING PROTEIN 4/6) also show chitin-binding properties and form potentially a complex with *OsCERK1*. Both receptors are further involved in the peptidoglycan perception with CERK1 (Liu et al., 2012a). All these studies show the importance of CERK1 as co-receptor of different MAMP-triggered immunity pathways (Desaki et al., 2018a).

After chitin perception, the kinase domain of CERK1 is autophosphorylated and activated to phosphorylate target proteins. Three phosphorylation sites play an essential role (T479, T573 and Y428) in the activation of immune responses (Petutschnig et al., 2010, Suzuki et al., 2016). Y428 is phosphorylated before the chitin perception and is dephosphorylated by CERK1-interacting protein phosphatase 1 (CIPP1) after chitin trigger to downregulate chitin signalling (Liu et al., 2018a). Other interacting proteins are receptor-like cytoplasmatic kinases (RLCKs), like *OsRLCK185* and *AtPBL27*. *OsRLCK185* is phosphorylated by *OsCERK1* and directly phosphorylates MAPKKK18 and 24, which activate a MAPK cascade (Wang et al., 2017a, Yamada et al., 2017, Yamaguchi et al., 2013). In Arabidopsis, *AtPBL27* is phosphorylated by *AtCERK1* and phosphorylates MAPKKK5 to activate MAPK cascade (Shinya et al., 2014, Yamada et al., 2016). An *OsRacGEF* protein is also described as an interaction partner of *OsCERK1*. *OsRacGEF* is activated and activates

OsRac1 (a small GTPase) to regulate chitin signalling (Akamatsu et al., 2013). Publicly available data of a screen for *AtCERK1* interaction partners used the kinase domain as bait in a classical Y2H system and identified LIK1 (LYSM RLK1-INTERACTING KINASE 1) as interaction partner. This LRR-RLK is phosphorylated by CERK1 and regulates MAMP-triggered immunity since *lik1* mutants are more resistant to the hemibiotrophic pathogen *Pseudomonas syringae*, but more susceptible to the necrotrophic pathogen *Sclerotinia sclerotiorum* (Le et al., 2014). It was reported that *AtCERK1* interacts with the G α (GPA1) and G γ (AGG1/AGG2) subunits of a heterotrimeric G protein, but the function of this interaction in chitin perception is still unknown (Aranda-Sicilia et al., 2015). Studies showed that subunits of heterotrimeric G protein can be phosphorylated to modulate the activity (Chakravorty & Assmann, 2018). For example, the non-canonical G α subunit XLG2 interact with FLS2 and BIK1 (BOTRYTIS-INDUCED KINASE 1) in complex with AGB1 (G β) and AGG1/2 (G γ). After the activation of FLS2 by flg22, the heterotrimeric G protein dissociates from the complex and XLG2 is phosphorylated by BIK1. The phosphorylated XLG2 version interacts with RBOHD to induce ROS burst (Liang et al., 2016). Another interaction partner of *AtCERK1* is *AtANN1*, a NaCl-induced calcium channel, which results in an increased MAMP-induced immune response by elevated ROS level and stronger MAPK activation in *ann1* mutants (Espinoza et al., 2017, Jones et al., 2014a). Besides receptors, CERK1 interacts with different proteins (like heterotrimeric G proteins) to transmit the signal inside the cell and start signal transduction cascades. CERK1 is part of a complex interaction network to perceive and transmit a MAMP stimulus and start the cellular response of the cell.

CERK1 is an essential receptor in MAMP-triggered immunity, not only in Arabidopsis, and interacts with different kind of proteins through phosphorylation of the target protein to activate a signalling pathway. Various screening methods result in different sets of the potential interaction partner, what opens the question if all possible interaction partners of CERK1 are known. Most likely not, since other yeast-based screens used only the kinase domain of CERK1 (Le et al., 2014) or followed an array-based approach with membrane proteins (Jones et al., 2014a). These approaches have certain disadvantages, like either wrong localisation of the interacting couple or a limited set of the potential interaction partner. The LexA-based split-ubiquitin Y2H

system in combination with an Arabidopsis/*G. orontii* cDNA library solves both problems of the former screens.

The aim of this study:

The CERK1 interactome of Arabidopsis is possible still incomplete since different methods possess different drawbacks and will not cover all possible interactions. Therefore, this study aimed to use the LexA-based split-ubiquitin yeast two-hybrid system in combination with an Arabidopsis cDNA library produced from powdery mildew-infected material to identify new potential interaction partners of CERK1.

Results:

Identification of potential CERK1 interaction partners in a split-ubiquitin Y2H cDNA library screen

I conducted the protein-protein interaction screening using the LexA-based split-ubiquitin system (Grefen et al., 2007, Grefen et al., 2009). This system has the advantage of using the full-length protein and thus ensures a higher chance of adequate folding of the protein in its native location in the plasma membrane.

After cloning and transforming the bait constructs into the THY.AP4 yeast strain, I first tested the resulting strains for bait protein expression. The western blot showed clear expression of CERK1, BAK1 and LYK4 at around 120 kDa, which corresponds to the expected protein size of the fusion proteins (Figure 20). The second lower band (middle panel) probably represents degradation products with the PLV attached or artefacts from unspecific binding from the yeast background. Both scenarios are possible since this band is also present in the eV control (GWY-Cub-PLV). In the case of FLS2, EFR, and LYK5 no band was detected that corresponds to the full-length fusion constructs. I transformed the yeast (carrying the bait plasmid with CERK1) with the eV (pCup-Nubl-GWY-Cyc1), which is the vector backbone of the Arabidopsis cDNA library constructed by Chiara Consonni from *G. orontii*-infected rosette leaves of *Arabidopsis thaliana* to exclude background growth of the CERK1 expressing strain. No growth could be detected upon transformation with eV on SC -leucine -tryptophan -methionine -adenine -histidine medium, which serves as a selection medium in the LexA-based split-ubiquitin Y2H system.

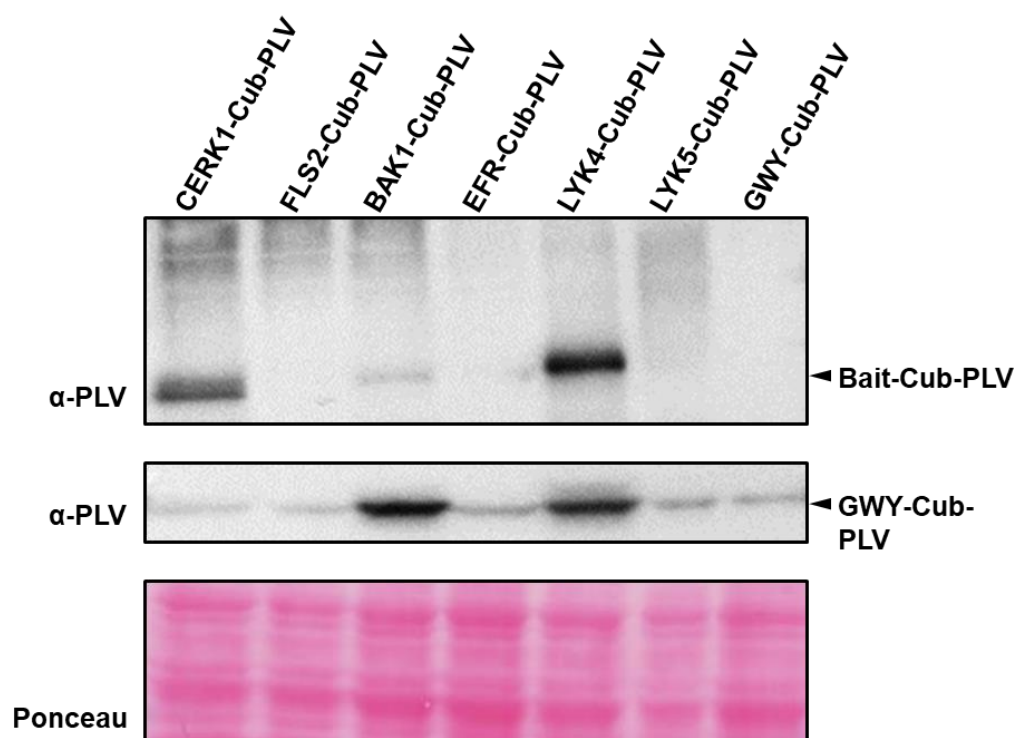


Figure 20: Western blot of Arabidopsis RLK expression in yeast. Cells expressing the different RLKs C-terminally fused to Cub (C-terminal part of ubiquitin) and PLV (artificial transcription activator ProteinA-LexA-VP16) were harvested, and the total protein was extracted. A specific antibody against the PLV was used to detect bait protein expression. The expected size of the fusion proteins: CERK1-Cub-PLV: 121 kDa; FLS2-Cub-PLV: 182 kDa; BAK1-Cub-PLV: 121 kDa; EFR-Cub-PLV: 176 kDa; LYK4-Cub-PLV: 120 kDa; LYK5-Cub-PLV: 126 kDa; GWY-Cub-PLV: 50 kDa. Ponceau staining was applied to visualise total protein loading. Full-size western blot is attached in the supplement (Supplementary Figure 11).

All colonies from the screen, which showed growth after cultivation on new selection plates and where the plasmid extraction and the insert spanning PCR was successful, are listed in Table 34. 140 colonies were streaked on new selection medium, of which 60 grew again, and the extraction and PCR were successful for 46 colonies. In three clones (clone 121/131/139), two bands were observed after PCR, which could be a result of multiple vector transformation (Scanlon et al., 2009, Scanlon et al., 2010). All sequenced products were searched for the best match in the Arabidopsis genome via BLASTN in the EnsemblPlants database (http://plants.ensembl.org/Arabidopsis_thaliana/Info/Index, accessed 29-Nov-2016) and further examined for their function, localisation and if the gene was in frame with the N-terminally fused Nub (N-terminal part of ubiquitin) tag to form a functional protein. Figure 21 summarises the putative localisation of the candidate interacting proteins on the base of the UniProt database annotation. Some of these localisation data were

compiled experimentally, and some were based on computational prediction. Only plasma membrane-localised and cytosolic proteins could interact with the kinase domain of CERK1. Genes involved in photosynthesis were excluded as potential interaction partners of CERK1 because they are separated in different compartments. Also, proteins that localise to other compartments like the ER or other organelles were excluded because the interaction is then unlikely to take place *in planta*. Thus, 11 out of 46 candidates were considered possible interaction partners of CERK1 based on the LexA-based split-ubiquitin system.

Table 34: List of potential interaction partners of CERK1 after the LexA-based split-ubiquitin cDNA library screen

clone number	gene ID	gene name	e-value BLASTN	%ID	description	localisation
1	At5g15600	SP1L4	0.0	100	Protein SPIRAL1-like 4	Cytoskeleton
6	At5g38430	RBCS-1B	0.0	100	Ribulose bisphosphate carboxylase small chain 1B	chloroplast
7	At5g38430	RBCS-1B	0.0	100	Ribulose bisphosphate carboxylase small chain 1B	chloroplast
8	At4g02770	PSAD1	0.0	100	Photosystem I reaction center subunit II-1	chloroplast
9	At5g17170	ENH1	3.4e-140	100	InterPro: PDZ domain/ Rubredoxin-type fold, ENH1, ENHANCER OF SOS3-1	chloroplast
13	At1g08380	PSAO	0.0	100		chloroplast
16	At3g22231	PCC1	2.0e-149	100	Cysteine-rich and transmembrane domain-containing protein	plasma membrane
17	At1g54390	ING2	1.9e-169	99.4	PHD finger protein ING2	nucleus
20	At5g38430	RBCS-1B	0.0	100	Ribulose bisphosphate carboxylase small chain 1B	chloroplast
21	At4g34870	CYP18-4	0.0	100	Peptidyl-prolyl cis-trans isomerase CYP18-4	cytosol
25	At1g32070	NSI	5.70e-144	100	Acetyltransferase	nucleus
34	At5g38430	RBCS-1B	0.0	100	Ribulose bisphosphate carboxylase small chain 1B	chloroplast
36	At5g38410	RBCS-3B	0.0	100	Ribulose bisphosphate carboxylase small chain 3B	chloroplast
39	At5g38430	RBCS-1B	0.0	100	Ribulose bisphosphate carboxylase small chain 1B	chloroplast
44	At2g36060	UEV1C	4.70e-170	100	Ubiquitin-conjugating enzyme E2 variant 1C	cytosol /nucleus
57	At2g18960	AHA1	1.2e-99	100	ATPase 1, plasma membrane-type	plasma membrane
61	At2g26460	SMU2	0.0	100	Suppressor of mec-8 and unc-52 protein homolog 2	nucleus
65	At2g31040	CGL160	7.20e-163	100	NCBI BLAST: ATP-Synthase subunit I	chloroplast
70	At4g39260	RBG8	0.0	100	Glycine-rich RNA-binding protein 8	cytosol /nucleus
71	At1g50430	DWF5	1.70e-123	100	7-dehydrocholesterol reductase	ER
77	At1g67090	RBCS-1A	0.0	100	Ribulose bisphosphate carboxylase small chain 1A	chloroplast
79	AT2g47070	SPL1	0.0	100	Squamosa promoter-binding-like protein 1, nucleus	nucleus
81	AT3g17930	DAC	7.40e-144	100	NCBI BLAST: DUF3007	chloroplast
85	At5g38410	RBCS-3B	0.0	100	Ribulose bisphosphate carboxylase small chain 3B	chloroplast
87	AT2g34460		0.0	100	NCBI BLAST: NADB_Rossmann superfamily	chloroplast
93	AT3g63410	VTE3	0.0	100	2-methyl-6-phytyl-1,4-hydroquinone methyltransferase	chloroplast
94	AT2g34460		0.0	100	NCBI BLAST: NADB_Rossmann superfamily	chloroplast

clone number	gene ID	gene name	e-value BLASTN	%ID	description	localisation
97	At4g28750	PSAE1	6.30e-44	100	Photosystem I reaction center subunit IV A	chloroplast
102	At1g67090	RBCS-1A	0.0	99.8	Ribulose biphosphate carboxylase small chain 1A	chloroplast
104	AT4g10480		0.0	100	Nascent polypeptide-associated complex subunit alpha-like protein 4	cytosol /nucleus
105	At1g06680	PSBP1	0.0	100	Oxygen-evolving enhancer protein 2-1	chloroplast
107	At5g38420	RBCS-2B	0.0	100	Ribulose biphosphate carboxylase small chain 2B	chloroplast
108	AT5g23575		9.0e-180	100	NCBI BLAST: CLPTM1 superfamily	ER
109	At5g38410	RBCS-3B	0.0	100	Ribulose biphosphate carboxylase small chain 3B	chloroplast
110	At1g79040	PSBR	3.0e-154	100	Photosystem II 10 kDa polypeptide	chloroplast
113	At3g43810	CaM7	7.0e-96	84.0	Calmodulin-7	cytosol
116	At5g38410	RBCS-3B	0.0	100	Ribulose biphosphate carboxylase small chain 3B	chloroplast
121.1	At3g43810	CaM7	6.0e-18	90.5	Calmodulin-7	cytosol
121.2	At2g26300	GPA1	1.5e-12	92.5	Guanine nucleotide-binding protein alpha-1 subunit	plasma membrane
124	AT1g52870		0.0	100	NCBI BLAST: Mpv17_PMP22 superfamily	peroxisome
131.1	At3g43810	CaM7	5.9e-18	90.5	Calmodulin-7	cytosol
131.2	At1g35720	ANN1	0.0	100	Annexin D1	cytosol
132	At1g67090	RBCS-1A	0.0	100	Ribulose biphosphate carboxylase small chain 1A,	chloroplast
139.1	At5g02500	MED37E	0.0	100	Probable mediator of RNA polymerase II transcription subunit 37e,	nucleus
139.2	At3g62030	ROC4	0.0	100	Peptidyl-prolyl cis-trans isomerase CYP20-3	chloroplast

The table includes the number of the clone, which regrew after the second round of selection and where the plasmid could successfully reextracted for sequencing. The gene ID and name is included for the best BLASTN match against the Arabidopsis genome. E-value and %ID were also noted down for evaluation. A short description and predicted localisation were necessary to reduce the list of the potential interaction partner.

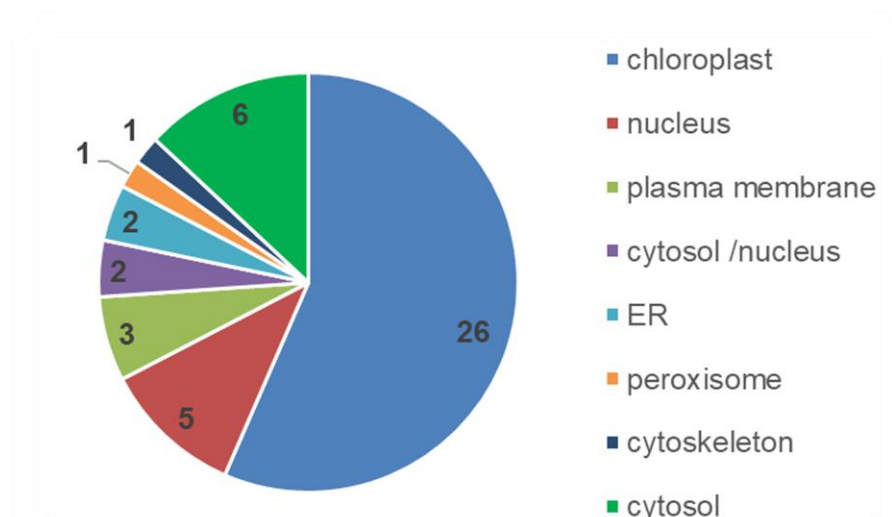


Figure 21: Pie chart summarising the predicted localisation of candidate CERK1 interaction partners. The pie chart summarises the localisation of potential interaction partner from the cDNA library screen based on the uniprot (<https://www.uniprot.org/>) annotation (experimental or predicted data).

The 11 candidate CERK1 interaction partners were At3g22231 (PCC1), At3g43810 (CaM7), At4g34870 (CYP18-4), At4g39260 (RBG8), At4g10480, At1g35720 (ANN1), At2g26300 (GPA1), At2g36060 (UEV1C), and At2g18960 (AHA1). Except for CaM7 (found three times), each of the candidates was present in the set only once, which could be explained by the small size of the set. This means that only one colony corresponds to this interaction partner. RBG8 and UEV1C are proteins that are predicted to localise to the cytosol and the nucleus. Furthermore, UEV1C may play a role in the DNA damage repair system (Wen et al., 2008) and RBG8 is involved in the regulation of alternative splicing (Fuhrmann et al., 2009, Schoning et al., 2008, Streitner et al., 2012). At4g34870 (CYP18-4) was considered interacting with CERK1 because of its peptidyl-prolyl cis-trans isomerase activity, which helps proteins to fold (Barbosa Dos Santos & Park, 2019). Therefore, this interaction may not have any meaning in the context of plant immunity. At4g10480 is a protein associated with ribosomes. Thus, due to their function, these proteins are unlikely to be functionally relevant interactors of CERK1. The candidates ANN1 and GPA1 were already published as interaction partners of CERK1, suggesting that the system is sensitive enough to detect true interactions. ANN1 (ANNEXIN D1) is a calcium- and lipid-binding protein, which has peroxidase activity (Gorecka et al., 2005) and was shown in BiFC and CoIP experiments to interact with CERK1 (Espinoza et al., 2017). The interaction of CERK1 with GPA1, a guanine nucleotide-binding protein alpha-1 subunit of a G protein that is involved in signal transduction (Assmann, 2002, Jones, 2002), was confirmed in BiFC experiments and in the same LexA-based split-ubiquitin Y2H system as I used in this study (Aranda-Sicilia et al., 2015). This circumstance made further investigation obsolete for these candidates.

Calmodulin (CaM) is a calcium sensor in all eukaryotes. CaM is capable of binding four Ca^{2+} ions, and after that undergoes a conformational change (Day et al., 2002, McCormack et al., 2005). In Arabidopsis, seven CaM genes are known, which encode for near-identical proteins (Day et al., 2002, McCormack & Braam, 2003). These CaMs can interact with other proteins and modulate their activity (Snedden & Fromm, 2001). As signal transducer, CaMs play an essential role in physiological processes, but also in abiotic and biotic stress responses (Aldon et al., 2018, Ranty et al., 2006, Yuan et al., 2017, Zeng et al., 2015, Zhang et al., 2014). It was published that CaM2 (Le et al., 2014) interacts with CERK1; here, I identified CaM7 in three colonies, which is also an

interaction partner of the ABC transporter PEN3 (Campe et al., 2016). Plasma membrane H⁺ ATPases (AHAs) are ten transmembrane domain proteins (Pedersen et al., 2007), which pump protons into the extracellular space under ATP consumption. As a consequence, an electrical and chemical gradient is built up along the membrane. This potential is used for the transport of nutrients into the cell and is therefore necessary for fundamental physiological processes like growth and development. AHA1 is, besides AHA2, one of the highest expressed AHAs in Arabidopsis (Haruta et al., 2010). AHAs are post-translationally regulated by phosphorylation of specific residues at the C-terminus to increase or decrease their activity (Haruta et al., 2015). It is also known that AHA1 and CNGC17 (CYCLIC NUCLEOTIDE-GATED ION CHANNEL) form a functional cation-translocating unit, which is activated by PSKR1/BAK1 (PHYTOSULFOKINE (PSK) LEUCINE-RICH REPEAT RECEPTOR KINASE 1) (Ladwig et al., 2015). Pathogen and circadian controlled 1 (PCC1) is an 81 aa long protein with a cysteine-rich transmembrane domain (CYSTM) that is located in the C-terminal region (Venancio & Aravind, 2010), and the protein family is involved in abiotic and biotic stress response. Overexpression and knock-down lines featured a more resistant or more susceptible phenotype compared to the wild type. A PCC1 overexpression line was more resistant against the oomycete *Hyaloperonospora parasitica* (Sauerbrunn & Schlaich, 2004). A second study by Mir and colleges showed that RNAi lines (knock-down lines) were more susceptible to *P. brassicae* and more resistant against *Botrytis cinerea* (Mir et al., 2013). Due to their promising functions, I selected CaM7, AHA1 and PCC1 for further investigation.

Validation of potential interaction partners of CERK1 in yeast and *in planta*

I identified 11 candidate interaction partners of CERK1 using the LexA-based split-ubiquitin system, but after further evaluation of the candidates, I decided to have a closer look at PCC1, CaM and AHA. However, since these putative interactors were identified from a cDNA library, most of the cDNAs do not include complete open reading frames, and hence the resulting proteins were truncated.

I performed targeted split-ubiquitin Y2H experiments to validate the new potential interaction partners of CERK1. Therefore, I cloned the full-length versions of the candidate proteins and included the colonies from the actual cDNA screen as controls. I was not able to clone AHA1, but its closest relative AHA2. They show an amino acid identity of 94% and act redundantly when one is knocked out (Haruta et al., 2010). For

the calmodulin interaction, I used the barley CaM3, since Arabidopsis CaM7 and barley CaM3 are 99% identical on the amino acid level and the clone *HvCaM3* was already available. The only difference is an amino acid substitution from S to A at position 11. That *HvCaM3* is capable of mimicking *AtCaMs* was reported in the MLO2/CaM interaction (Kim et al., 2002). PCC1 was cloned in both tag orientations (N- and C-terminal) because a transmembrane domain is located at the C-terminus and the interaction may be blocked when it is tagged at the carboxy-terminus. The experimental procedure is described in the material and methods under 2.12.2; 2.12.4; 2.12.5.

Figure 22 shows a 10-fold dilution series ranging from OD₆₀₀ 1 to OD₆₀₀ 0.001. Nubl-GWY (eV) was used as negative control and GPA1, the truncated ("trun.") and full-length version were plotted as positive controls. Plate A selects only for the bait and prey plasmid on an SC -leucine (Leu) -tryptophan (Trp) -methionine (Met) plate, and it is the growth control to ensure equal growth rates of all strains. These three amino acids are missing in the medium since leucine is the marker for the bait construct with CERK1, tryptophan is the marker for the prey vector with the potential interaction partners, and methionine can repress bait expression when added. All strains displayed equal growth on this medium (Figure 22). In plate B also adenine (Ade) and histidine (His) are missing; these two are the reporter markers for the respective synthesis genes only expressed if the interaction takes place. The bait vector has a Met-repressible promoter, and the addition of Met can be useful to remove background growth in the negative control or to test the strength of the interaction. When the bait expression is repressed, less protein is available in the cell to interact with the prey, and the chance of interaction is decreased. Strong interactions can compensate for that. Plate C and D contain 100 µM, and 300 µM Met, respectively, to estimate the strength of the interactions. The Nubl-GWY (eV) showed no growth on SC -Leu -Trp -Met -Ade -His plates, while both GPA1 versions were detectable in OD₆₀₀ 0.1 dilutions to a concentration of 300 µM of Met, although the truncated version from the cDNA library seemed to grow a bit better than the full-length version (Figure 22). Thus, the controls showed the expected outcome. The C-terminally tagged PCC1 variant showed no growth on SC -Leu -Trp -Met -Ade -His plates irrespective of the presence or absence of Met, while N-terminally tagged PCC1 was detectable until OD₆₀₀ 0.01. 300 µM of Met strongly reduced the growth of these strains, however, to detectable

colonies only at OD₆₀₀ 1. The CaM and AHA1/2 carrying strains were detectable until OD₆₀₀ 0.01 on SC -Leu -Trp -Met -Ade -His plates. The addition of Met did not affect the CaM strains, but almost entirely abolished growth of the AHA1/2 carrying strains.

The second reporter of this system is based on red-white selection as a result of Ade auxotrophy, which relies on the formation of a red-coloured intermediate of the adenine biosynthetic pathway. When no interaction takes place, the yeast accumulates this intermediate due to the absence of Ade2. The Nubl-GWY (eV) and PCC1-Nubl carrying strains showed the strongest red colour, Nubl-PCC1 trun., Nubl-PCC1, Nubl-GPA1, Nubl-AHA1 trun. and Nubl-AHA2 showed a light red colour and Nubl-GPA1 trun. and both Nubl-CaM strains did not show accumulation of red colour. This outcome was in accordance with the result of the growth phenotype. Taken together, GPA1 and CaM appeared to be the strongest interactors in the LexA-based split-ubiquitin assay, while PCC1 and AHA1/2 may be weaker interactors of Arabidopsis CERK1.

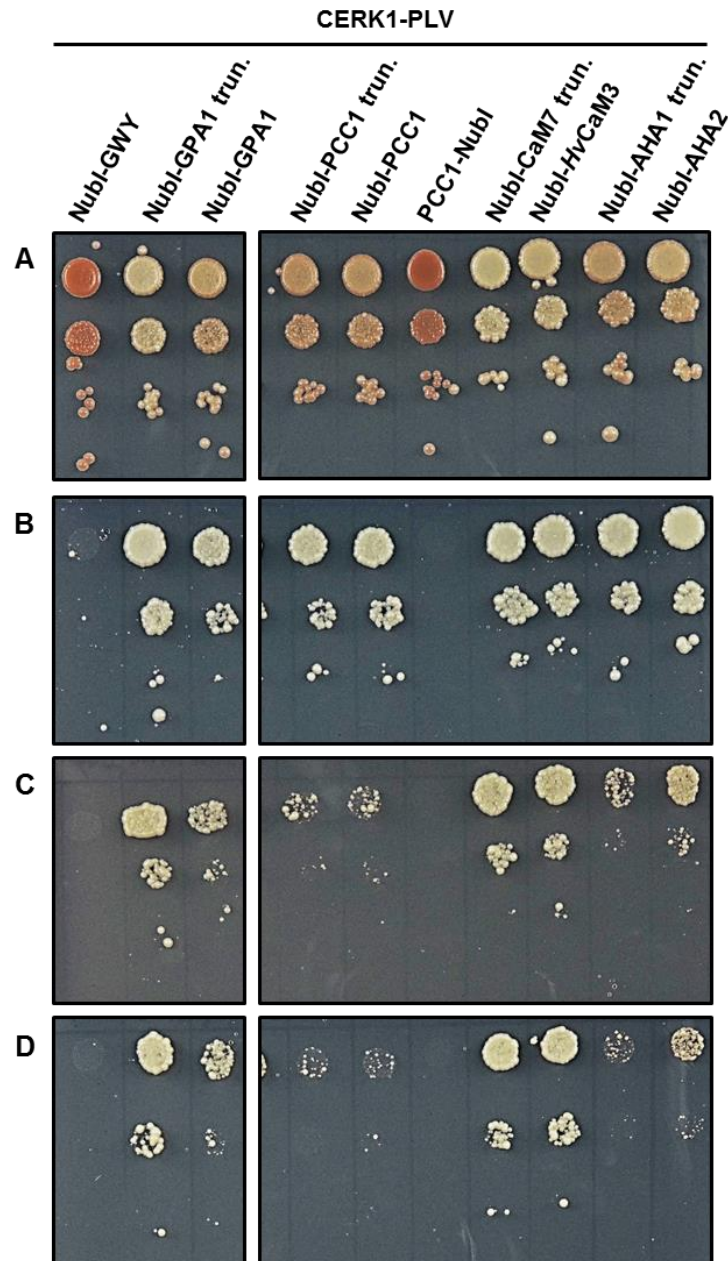


Figure 22: LexA-based split-ubiquitin assay for the validation of potential interaction partners of Arabidopsis CERK1. The colonies from the cDNA screen with truncated (trun.) protein versions were compared to the cloned full-length versions of the protein in the same vector backbone (pCup-Nubl-GWY-Cyc1). 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series ranging from OD₆₀₀ 1 on the top to OD₆₀₀ 0.001 at the bottom. The media were as follows: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 100 μ M methionine, (D) SC -leucine -tryptophan -adenine -histidine + 300 μ M methionine. This experiment was repeated three times, and the replicates are attached in the supplement (Supplementary Figure 12-14).

In addition, the LexA-based split-ubiquitin system has the *lacZ* gene as a third reporter under control of the LexA transcription factor. This reporter allows quantifying the interaction strength *via* enzymatic assays. I used the substrate oNPG (ortho-Nitrophenyl- β -galactoside) that is catalysed into the yellow product o-nitrophenol by the lacZ protein when incubated at 37 °C. The reaction was stopped with Na₂CO₃, and the OD was measured at 420 nm to calculate the specific enzyme activity (Figure 23). Only Nubl-GPA1 and Nubl-CaM from the cDNA library (trun.) were significantly different from the negative control Nubl-GWY (eV) according to a Kruskal-Wallis test followed by a Dunn`s multiple comparison test. However, this assay also showed a high variation, both between technical and between independent replicates. Indeed, all constructs showed somewhat detectable oPNG conversion to different degrees, which was not the case for Nubl-GWY (eV). The results of the LacZ assay are in accordance with the results of the growth reporter assay (Figure 22).

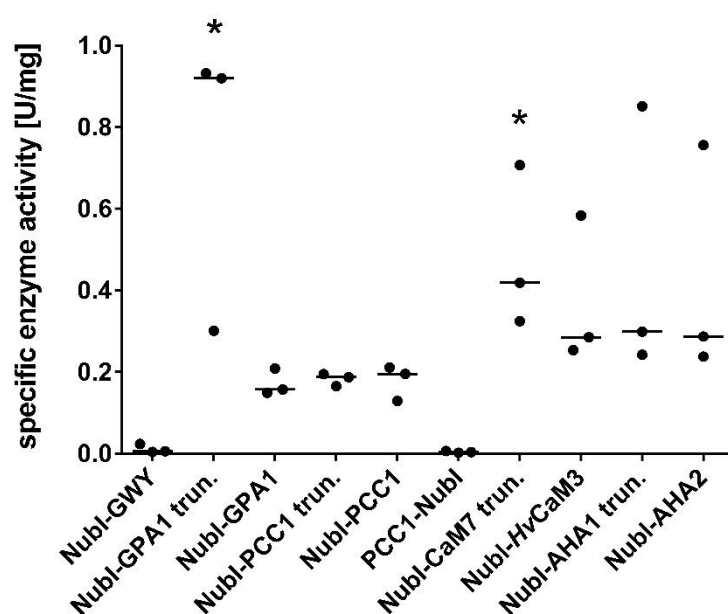


Figure 23: LacZ reporter assay for yeast lines expressing CERK1 and the potential interaction partners. The LacZ reporter assay was used for the quantification of the interaction between CERK1 and the potential interaction partner. Yeast cells expressing both fusion constructs were disrupted and the protein concentration of the supernatant was measured *via* the Bradford assay. The reaction buffer (Z-Buffer) was mixed with cell extract and the substrate oNPG. The reaction was incubated at 37 °C until the solution turned yellow, then the reaction was stopped by adding Na₂CO₃. The OD was measured, and the specific enzyme activity was calculated. The Kruskal-Wallis test followed by a Dunn`s post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (Nubl-GWY), and an asterisk indicates P≤0.05. The horizontal line indicates the median and “trun.” indicates the truncated protein versions from the cDNA library screen. The unit of specific enzyme activity is U/mg. This experiment was repeated in three independent replicates.

I performed split-luciferase assays by transient heterologous expression in *N. benthamiana* for further validation. Eight leaf discs per independent biological replicate were measured, and the mean of each tested combination was normalised to the mean of the negative control AGB1, a protein which does not interact with CERK1 (Aranda-Sicilia et al., 2015). The means of six independent biological replicates were used for the statistical analysis. I used a kinase-dead version of CERK1 since an active kinase lead to cell death in *N. benthamiana* plants (Pietraszewska-Bogiel et al., 2013). *HvCaM3*, *AHA2* and *PCC1* showed a statistically significant difference when compared to AGB1 (AGB1 control was set to 1) as a negative control. *PCC1* showed a highly significant difference with a p-value of ≤ 0.001 . Interestingly, *GPA1* depicts a low value in the range of the negative controls *cLUC-GWY* (eV) and *AGB1* (Figure 24 A). Figure 24 B depicts one of the western blots of the luciferase assays. Except for *AHA2*, it was possible to detect the CERK1 fusion protein and the different prey fusion proteins. However, the protein level of the controls was less than the protein levels for *HvCaM3* and *PCC1*, which could explain the low luciferase values. Nevertheless, all three candidates interact in two different protein-protein interaction assays, suggesting that *PCC1*, *AHA2*, and *HvCaM3* physically interact with *AtCERK1* in yeast and *in planta*. *PCC1* was chosen for a further investigation because this protein family is also known to be in general involved in abiotic and biotic stress responses, which classifies *PCC1* as an interesting interaction partner of CERK1.

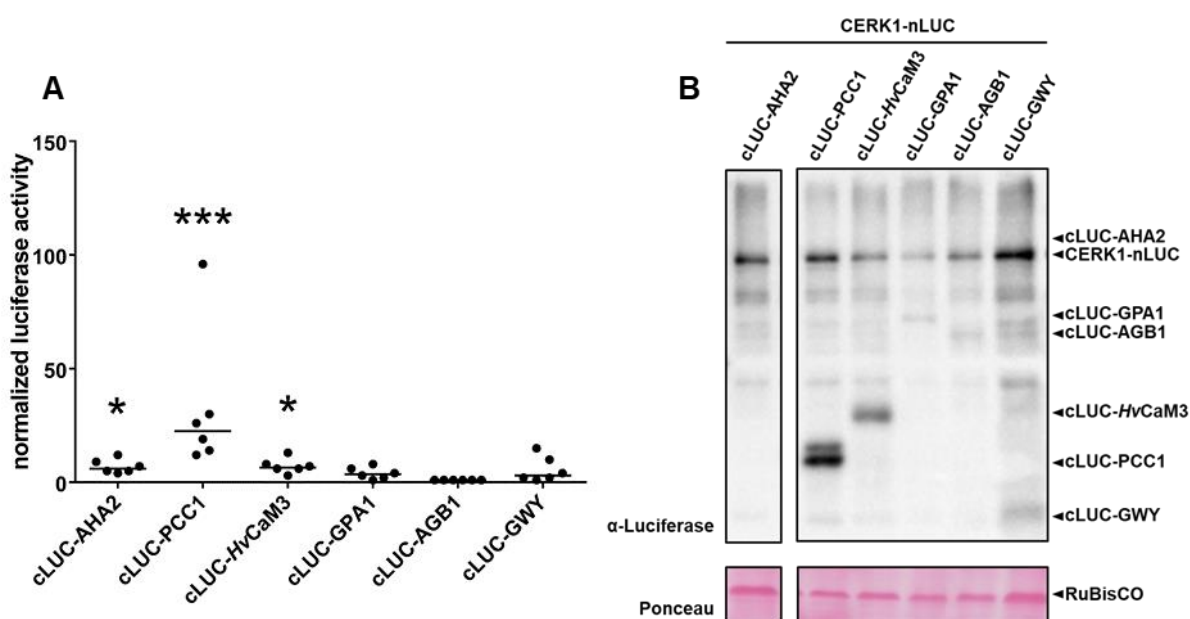


Figure 24: Split-luciferase assay of CERK1 and the potential interaction partners by heterologous expression in *N. benthamiana*. The split-luciferase assay allows the quantification of *in planta* protein-protein interaction. (A) *N. benthamiana* leaves were infiltrated with Agrobacteria carrying the fusion protein encoded on plasmids and incubated for two days. In total, eight leaf discs (four discs from 2 leaves each) of each combination were measured. D-Luciferin was infiltrated as a substrate before the measurement. The mean value of the leaf discs after 5 min of measurement was normalised with the negative control AGB1. (B) The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise the loading of the protein extract. The expected sizes with the fused split-luciferase parts are as followed: CERK1-nLUC: 116 kDa; cLUC-PCC1: 28 kDa; cLUC-AHA2: 124 kDa; cLUC-HvCaM3: 37 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa. Six independent biological replicates were measured. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1), which was set to 1. The asterisks indicate the following p-values: * = $P \leq 0.05$ and *** = $P \leq 0.001$. The horizontal bar marks the median. Full-size western blot is attached in the supplement (Supplementary Figure 15).

Protein-protein interaction studies with truncated PCC1 versions to find important regions

Next, I focused on PCC1 for detailed functional examination, since the corresponding gene is upregulated after *P. syringae* infection and shows increased and decreased resistance phenotypes when it is knocked down and overexpressed, respectively (Mir & Leon, 2014, Sauerbrunn & Schlaich, 2004). I constructed four different truncated versions of PCC1 to identify the functional moiety of the protein (Figure 25). For example, PCC1¹⁻⁵⁹ lacks the transmembrane domain in the C-terminal region, whereas PCC1⁵⁵⁻⁸¹ only consists of the transmembrane domain. All domains are based on a prediction or published data. Mir *et al.* showed that PCC1¹⁻⁵⁹ could not anchor to the plasma membrane (Mir & Leon, 2014). Both PCC1¹⁷⁻⁸¹ and PCC1³⁶⁻⁸¹ are lacking parts of the N-terminal region of the protein, where the phosphorylation sites are predicted to reside according to NetPhos 3.1 Server prediction (marked with circles) (Blom *et al.*, 1999, Blom *et al.*, 2004) and the iPTMnet proteomics database (marked with asterisk) (Nuhse *et al.*, 2004, Reiland *et al.*, 2009) (Figure 25).

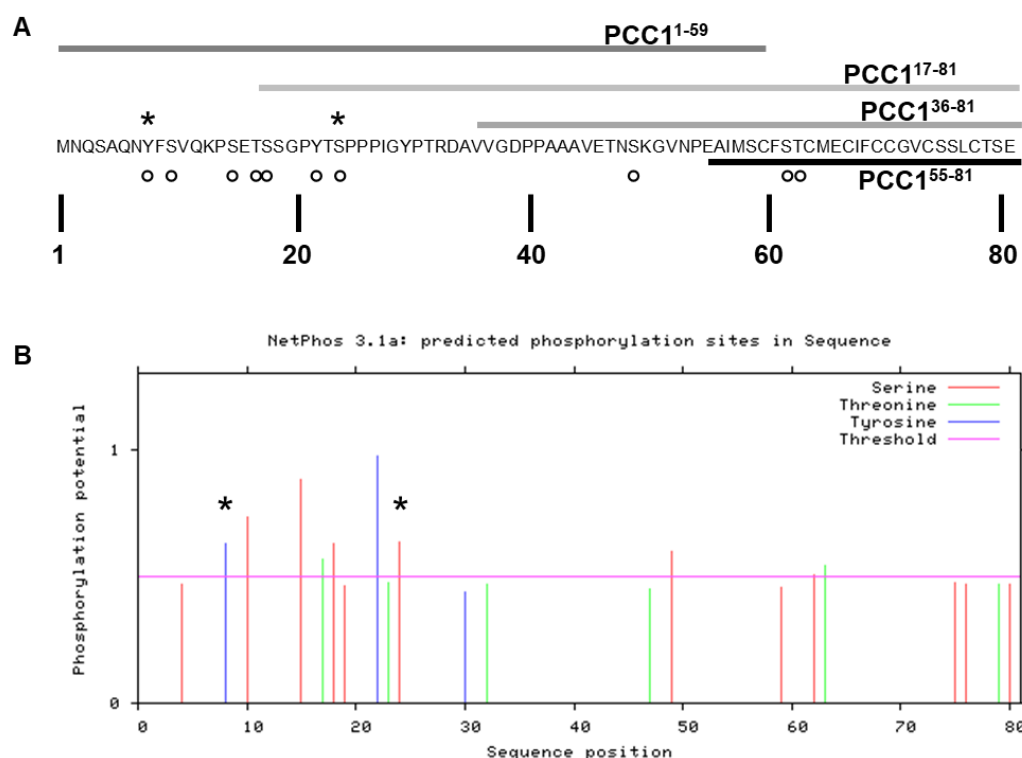


Figure 25: Model of the truncated versions and predicted phosphorylation sites of PCC1. (A) This scheme represents the truncated versions of PCC1 used in the split-ubiquitin and split-luciferase assays. Numbers indicate the amino acid position of the 81 amino acids long peptide. Phosphorylation sites identified in phosphoproteomics experiments are labelled with an asterisk and were extracted from the iPTMnet database (<https://research.bioinformatics.udel.edu/ipmnet/entry/Q94C26/>, accessed 11-Feb-2019) (Huang et al., 2018). The phosphorylation sites of PCC1 were predicted with the NetPhos 3.1 web tool (<http://www.cbs.dtu.dk/services/NetPhos/>, accessed 30-Apr-2019) (Blom et al., 1999, Blom et al., 2004) and are labelled with a circle. (B) The diagram of the phosphorylation potential of the amino acids, as predicted via NetPhos3.1. Most of the predicted sites with the highest potential lie in the N-terminal region.

I used the four truncated PCC1 versions for testing with the split-ubiquitin Y2H system using CERK1-Cub-PLV as bait. Plate A showed equal growth of all yeast strains (Figure 26). There was no visible growth for Nubl-GWY (eV) and AGB1 on selective SC medium (Plate B and C; selecting for interaction). GPA1 was detectable until OD₆₀₀ 0.01, both on SC -Leu -Trp -Med -Ade -His (plate B) and with 150 μ M Met (plate C). Both, the full-length PCC1 and the PCC1³⁶⁻⁸¹, versions displayed good colony growth until OD₆₀₀ 0.01 on selective medium, while PCC1¹⁷⁻⁸¹ and PCC1¹⁻⁵⁹ expressing yeast were only well detectable until OD₆₀₀ 0.1 (plate B). PCC1⁵⁵⁻⁸¹ did not grow on the interaction selection plate B. All PCC1 versions except PCC1⁵⁵⁻⁸¹ exhibited the same growth on selective medium with 150 μ M Met and were still visible at dilution 0.1 (plate C).

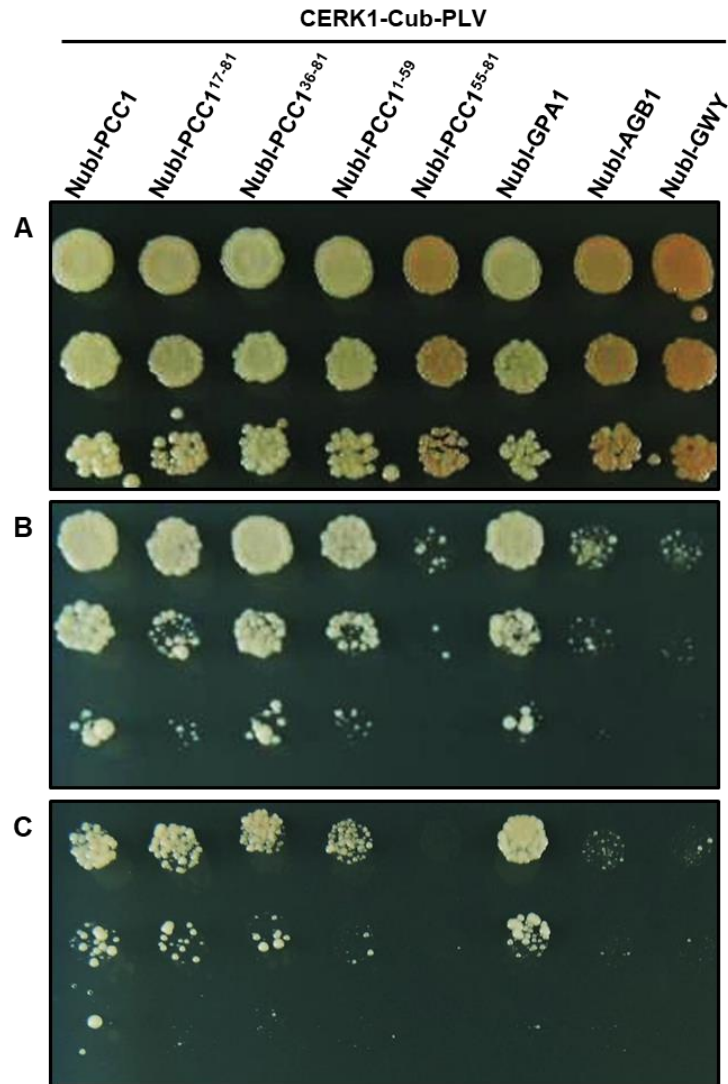


Figure 26: Comparison of truncated PCC1 versions against CERK1 in yeast. For the identification of the interacting domain of PCC1, full-length and truncated versions were tested against CERK1. GPA1 serves as a positive control as well as AGB1 and empty vector (Nubl-GWY) as negative controls. The numbers indicate which amino acids are present in the 81 amino acid long peptide. 4 μ l of overnight cultures of transformed yeast were drop-inoculated in a 10-fold dilution series starting at OD₆₀₀ 1 on the top until 0.01. (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine. This experiment was repeated in three independent biological replicates, and the replicates are attached in the supplement (Supplementary Figure 16).

Next, I used the *lacZ* reporter assay to quantify the interaction strength (Figure 27). Here, all PCC1 versions exhibited almost the same specific enzyme activity (median of ca. 0.07 U/mg), except for PCC1⁵⁵⁻⁸¹ (median of ca. 0.02 U/mg). However, only GPA1 and AGB1 were significantly different in terms of specific enzyme activity according to the Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test. Therefore, both yeast assays suggest that PCC1⁵⁵⁻⁸¹ does not interact with CERK1, while all other truncated PCC1 versions do. However, the LacZ assay failed

to show a statistical difference between the PCC1 versions, which was seen in the growth reporter assay.

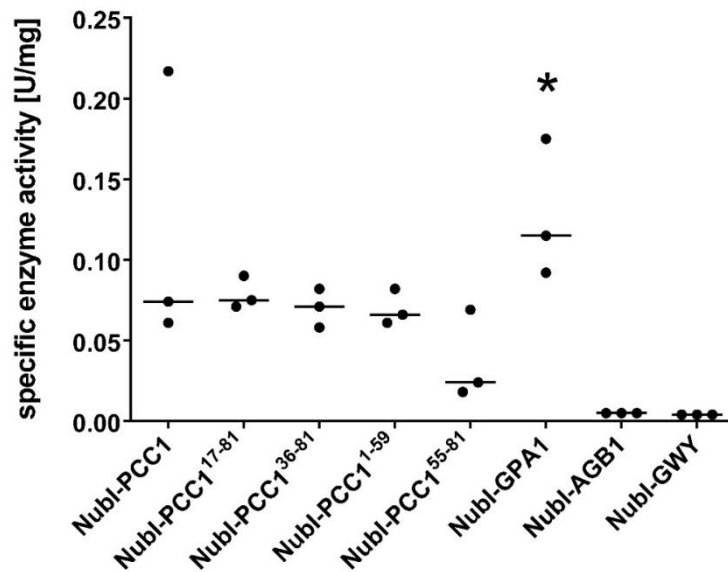


Figure 27: LacZ reporter assay to test the interaction between CERK1 and four truncated versions of PCC1. The LacZ reporter assay was used for the quantification of the interaction between CERK1 and the potential interaction partner. Yeast cells expressing both fusion constructs were harvested, and the cells were disrupted. The cell debris was separated by centrifugation, and the protein concentration was measured via the Bradford assay. The reaction buffer (Z-Buffer) was mixed with cell extract and the substrate oNPG, and the reaction was incubated at 37 °C. When the solution turned yellow, the reaction was stopped by adding Na₂CO₃. The OD was measured, and the specific enzyme activity was calculated. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1) and an asterisk indicates $P \leq 0.05$. The horizontal bar marks the median. The specific enzyme activity is given as U/mg. This experiment was performed in three independent biological replicates.

I also performed the split-luciferase assay for these combinations *in planta*. PCC1 full-length and PCC1³⁶⁻⁸¹ showed a median normalised luciferase activity of ca. 20, one order of magnitude higher than the cLUC-GWY (eV) and AGB1 controls at <5 ($P \leq 0.01$; Figure 28 A). PCC1¹⁷⁻⁸¹ showed similar activity of above 20 ($P \leq 0.001$). Both PCC1¹⁻⁵⁹ and PCC1⁵⁵⁻⁸¹ showed almost no activity with <5, suggesting that these peptides do not interact with CERK1 *in planta*. All samples exhibit almost the same protein amount except PCC1¹⁻⁵⁹, which showed a much lower protein amount compared to the others (Figure 28 B). Hence, the lack of interaction may be due to low expression of PCC1¹⁻⁵⁹ in *N. benthamiana*. The expression level of GPA1 and AGB1 lies between PCC1¹⁻⁵⁹ and the other PCC1 versions. The full-length PCC1 version shows a clear double band, and the PCC1¹⁷⁻⁸¹ version shows a less distinct double band. This may indicate phosphorylation of PCC1. Taken together, both the yeast split-ubiquitin assays and the

split-luciferase assay based on expression in *N. benthamiana* suggest that the truncated versions of PCC1, PCC1¹⁷⁻⁸¹, and PCC1³⁶⁻⁸¹ interact with CERK1 at similar levels as the full-length PCC1. PCC1¹⁻⁵⁹ may interact with CERK1 as well, but the data derived from expression in *N. benthamiana* is ambiguous due to the low expression of the construct. Thus, the 35 N-terminal amino acids of PCC1 are dispensable for the interaction with CERK1.

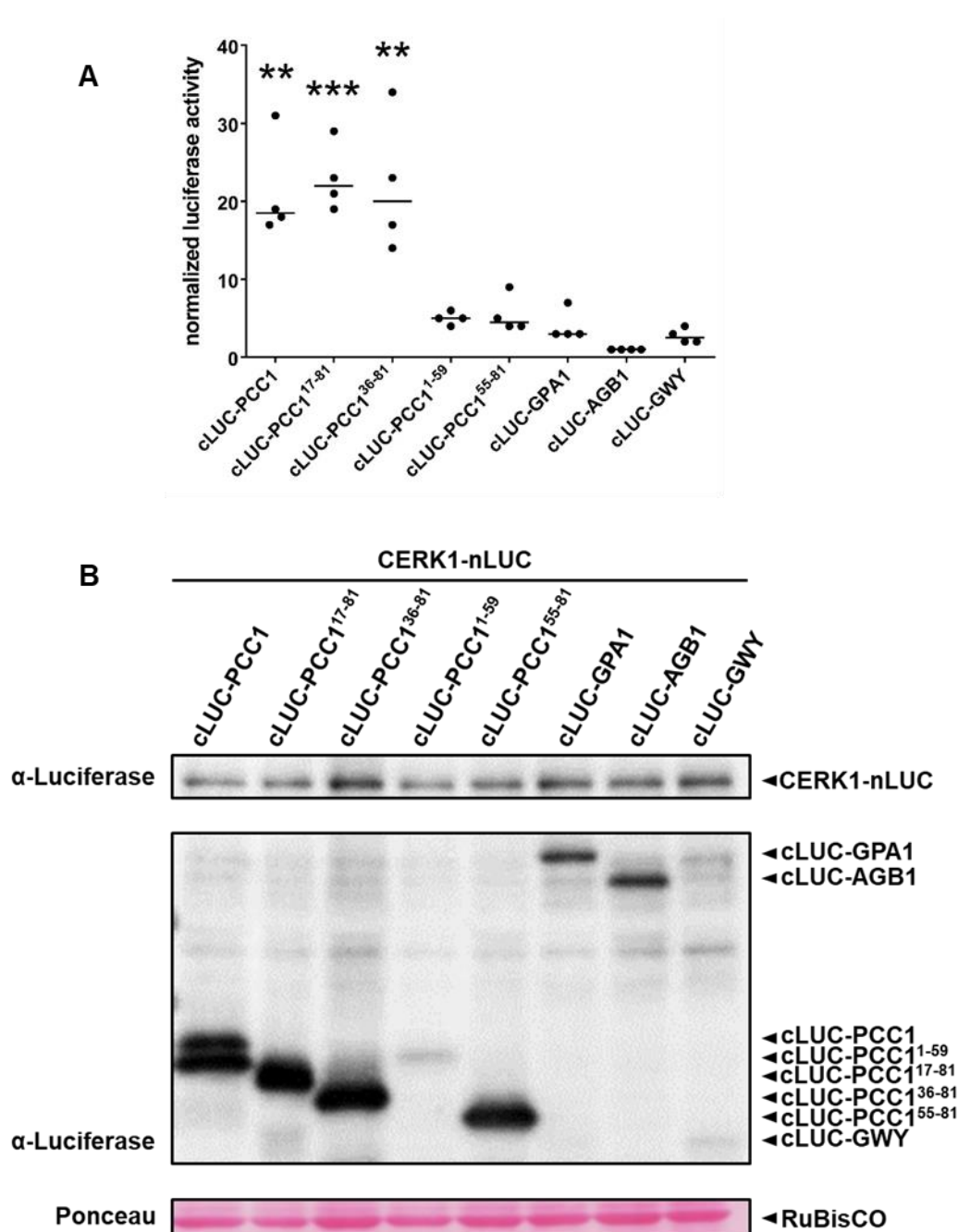


Figure 28: Split-luciferase assay to test the interaction between CERK1 and four truncated PCC1 versions. (A) The split-luciferase assay allows the quantification of *in planta* protein-protein interaction. Agrobacteria with the fusion protein encoded on plasmids were infiltrated in *N. benthamiana* and incubated for two days. In total, eight leaf discs (four discs from 2 leaves each) of each combination were measured. D-Luciferin was infiltrated as a substrate before the measurement. The mean value of

the leaf discs after 5 min of measurement was normalised with the negative control AGB1. (B) The total protein of 8 measured leaf discs was extracted, and a western blot was performed. The polyclonal anti-luciferase antibody is capable of detecting both the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes with the fused split-luciferase parts are as follows: CERK1-nLUC: 116 kDa; cLUC-PCC1: 28 kDa; cLUC-PCC1¹⁷⁻⁸¹: 26 kDa; cLUC-PCC1³⁶⁻⁸¹: 25 kDa; cLUC-PCC1¹⁻⁵⁹: 26 kDa; cLUC-PCC1⁵⁵⁻⁸¹: 23 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa. The numbers at the truncated versions indicate which amino acids are present from the 81 amino acid PCC1 peptide. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1), which was set to 1. The asterisks indicate the following p-values: ** = $P \leq 0.01$ and *** = $P \leq 0.001$. The horizontal bar indicates the median. This experiment was repeated in four independent biological replicates. Full-size western blot is attached in the supplement (Supplementary Figure 17).

Examination of predicted secondary structures of PCC1

I used the prediction tools Phyre² (Kelley et al., 2015, Kelley & Sternberg, 2009) and IUPred2A (Mészáros et al., 2018) to predict secondary structures and disordered regions of PCC1. Figure 29 A shows the prediction of Phyre², which revealed four predicted alpha-helices, whereby only the largest helix (54-79 aa) at the C-terminus has a confidence score of 9. The confidence score indicates the likelihood of the prediction. This alpha helix lies within the transmembrane region of PCC1. The other three alpha-helices have a confidence score between 8 and 5. Phyre² also predicts three disordered regions at the N-terminus (1-22 aa), in the middle (38-52 aa) and at the extreme C-terminus (78-81 aa). A region at the extreme C-terminus revealed a high confidence of ≥ 8 as well as an area at the extreme N-terminus (1-7 aa) and an area (17-21 aa). The other parts of these disordered regions show a confidence score between 8 and 5. I used IUPred2A to verify the Phyre² prediction, which revealed a disordered region between amino acid 20 and 50 (red graph) (Figure 29 B). This region revealed a score (probability) of above 0.5 and only partially overlap with the Phyre² prediction. IUPred2A also predicts areas of disordered binding regions (ANCHOR2; blue graph) (Figure 29 B), which indicates binding sites or sites of phosphorylation. ANCHOR2 predicted within the first 20 amino acids such a binding site with a score of around 0.6. IUPred2A is further capable of predicting redox-sensitive disordered regions (Figure 29 C), which will undergo conformational changes upon redox change. PCC1 shows almost in its whole protein structure this kind of region, which could indicate structural changes in response to redox changes. In summary, these results suggest that PCC1 is structurally disordered, except in the transmembrane region, which suggests that this protein undergoes structural changes upon stimulus like redox change or phosphorylation.

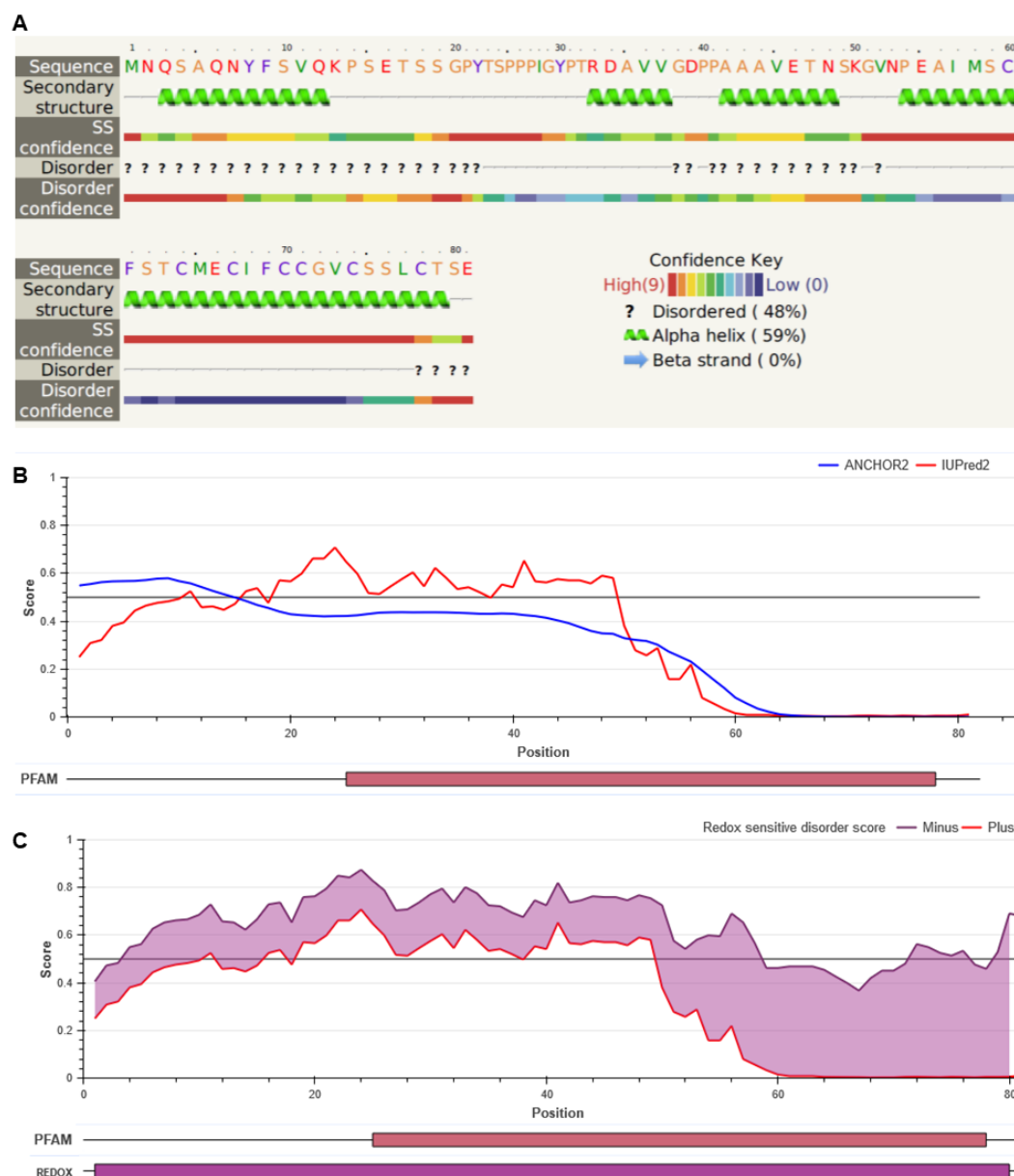


Figure 29: Secondary structure and disorder prediction of PCC1. (A) Secondary structure and disorder prediction of PCC1 by Phyre² (Kelley et al., 2015). Alpha helices are depicted in green and disordered region with a ?. The prediction confidence score is rainbow colour coded from red (9) high confidence to blue (0) low confidence. (B) Prediction of disordered regions (intrinsically unstructured) with IUPred2A (Mészáros et al., 2018). The red graph shows the prediction of disordered regions, which was calculated with IUPred2A algorithm. ANCHOR2 (blue graph) depicts the prediction of disordered binding site. (C) IUPred2A also incorporates a prediction of redox sensitive regions. (B/C) The graphs show a score for each amino acid. The threshold is 0.5 and corresponds to a probability. PFAM domain is depicted as a red bar and the region of redox sensitivity as a purple bar.

Phylogeny of the PCC1-containing CYSTM protein family

PCC1 is not the only cysteine-rich transmembrane protein in *Arabidopsis thaliana*. Sauerbrunn and Schlaich described five more family members (Sauerbrunn & Schlaich, 2004) and a paper from 2018 incorporates 13 members from Arabidopsis into this family (Xu et al., 2018). They were named after their main domain, CYSTM (cysteine-rich transmembrane module) (Venancio & Aravind, 2010), though PCC1 is not included in the phylogenetic tree in the study from Xu and colleagues (Xu et al., 2018). Since no tree, including all CYSTM family members, were available, I constructed a tree with all 14 members using the Maximum-Likelihood method, and 1000 bootstrap replicates (Figure 30 A). Both close relatives of PCC1, At3g22235 (CYSTM8) and At3g22240 (CYSTM9), cluster with PCC1 in the same chromosomal region (chromosome 3) and form a clade (I a) of the tree at 98% bootstrap support. These three members are separated on chromosome 3 by 7.6 kb and 7.8 kb, respectively (Figure 30 B). Also, At2g32190 (CYSTM4), At2g32200 (CYSTM5), and At2g32210 (CYSTM6) cluster in the same chromosomal region on chromosome 2 and form a common clade (I b) with At1g05340 (CYSTM1) (89% support). The CYSTM members of these two branches were described before (Sauerbrunn & Schlaich, 2004). The other clade, here named clade II, includes At1g56060 (CYSTM3), At2g33520 (CYSTM7), At3g49845 (CYSTM10), At4g33660 (CYSTM11), At5g04080 (CYSTM12), and At5g67600 (CYSTM13), which were described by Xu and colleagues (Xu et al., 2018). CYSTM2 groups in none of the three clades (Figure 30 A).

Next, I analysed the protein sequence alignments of PCC1 and the CYSTM family members. PCC1 and At3g22235 are identical from amino acid 1 to 55, while the C-terminal part is variable except for four cysteines, an isoleucine and a valine. In the region from amino acid 1-51, the close relative At3g22240 exhibits only eight amino acid substitutions compared to PCC1 and At3g22235. With respect to the C-terminus, At3g22235 and At3g22240 are more similar to each other, since there are only five amino acid substitutions (Figure 30 C). The overall amino acid identity with PCC1 is around 75% (At3g22235) and 60% (At3g22240). The identity of the other members (clade I) is much lower with about 31%-36%. However, all of these members share a few common motifs. The cysteine-rich region is only missing in the two alternative splice versions At2g32190.2 and At2g32210.2 (Figure 30 D, green box). Besides the cysteine-rich domain in the C-terminus (transmembrane domain), all members share

a proline/glutamine-rich domain (Figure 30 D, red box) in the middle of the protein. These two domains are typical for CYSTM proteins in all organisms. The conserved cysteine residues in the transmembrane domain are unique because usually only hydrophobic amino acids are conserved in transmembrane domains, suggesting a specific function of these cysteine residues (Venancio & Aravind, 2010).

I used further the TOPCONS webserver to predict transmembrane and signal peptides (Tsirigos et al., 2015). This online tool incorporates multiple predictions tools and uses the result of each tool to predict transmembrane domains and signal peptides. CYSTM proteins, which possess a transmembrane according to TOPCONS, are marked with « in Figure 30 A. Most of the CYSTM family members show a predicted transmembrane domain in the C-terminal region like it was demonstrated for PCC1 (Mir & Leon, 2014). For At3g22235, At2g32210 and At4g33660 was no transmembrane domain predicted, and all investigated proteins also show no signal peptide.

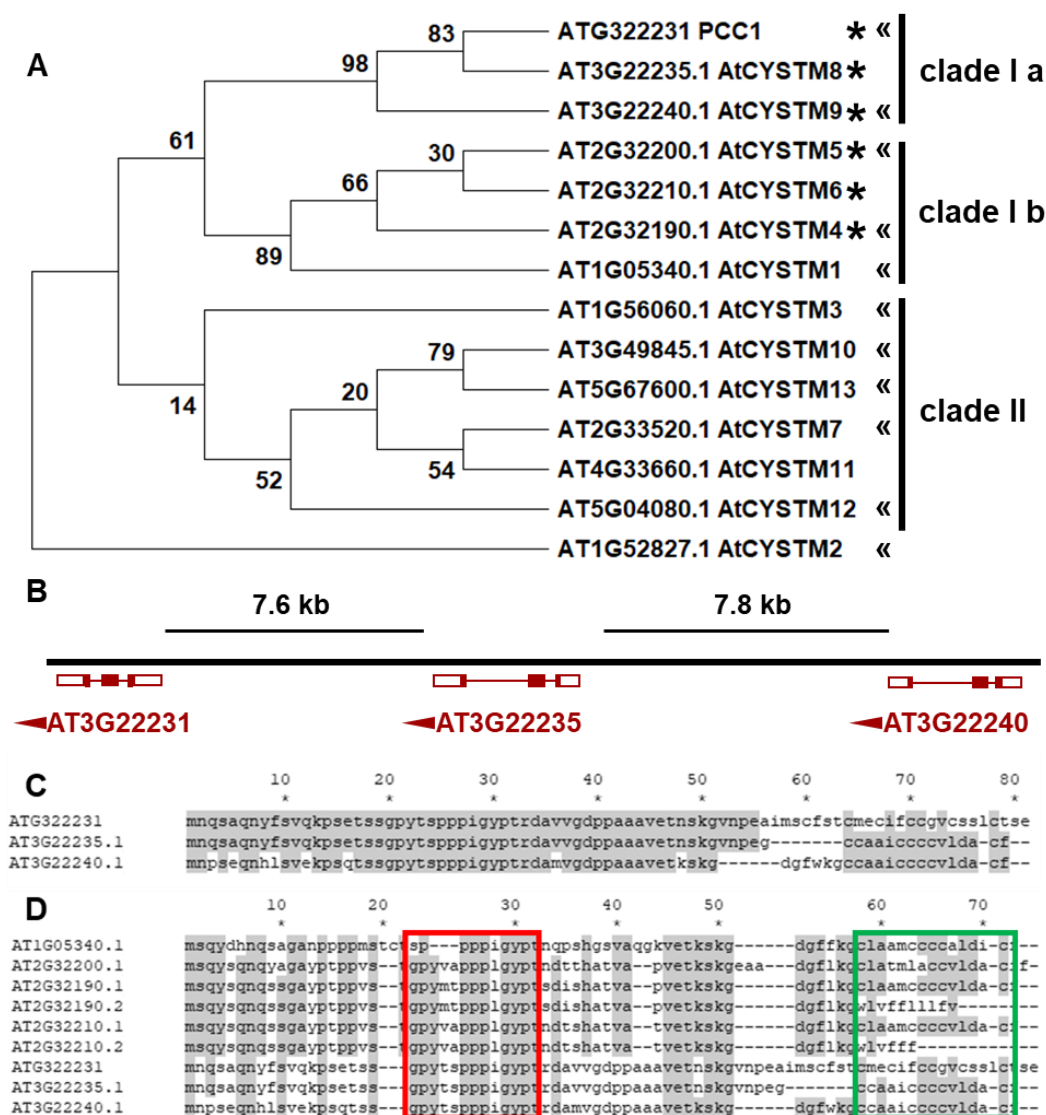


Figure 30: Protein alignments and Maximum-Likelihood phylogenetic tree of PCC1 and the CYSTM family. (A) The phylogenetic tree was calculated based on amino acid sequences by using the Maximum-Likelihood method and JTT matrix-based model with 1000 bootstraps in MEGAX (Jones et al., 1992, Kumar et al., 2018). PCC1 was incorporated into the tree of the CYSTM family of Arabidopsis identified in (Xu et al., 2018). The asterisks highlight the CYSTM family members investigated in this study. Transmembrane (TM) prediction was performed with TOPCONS (Tsirigos et al., 2015) and proteins with TM are marked with «. (B) This panel shows the genomic organisation of PCC1 and the close relatives At3g22235 and At3g22240 on chromosome 3. (C) Amino acid sequence alignment of PCC1, At3g22235 and At3g22240 was performed with CloneManager using the BLOSUM 64 scoring matrix. (D) Amino acid alignment of clade I a/b homologs of PCC1. The alignment was performed with CloneManager by using BLOSUM 62 scoring matrix. The red box labels the proline/glycine-rich region and the green box labels the cysteine-rich region. Matching amino acid residues are highlighted in grey. Amino acid alignment of all CYSTM members is attached in the supplements (Supplementary Figure 18).

Expression pattern of PCC1 and the CYSTM family

I used publicly available microarray data from the eFP browser (Winter et al., 2007) to determine if *PCC1* family members are expressed in response to pathogens or rather at developmental stages in Arabidopsis. The heatmap in Figure 31 depicts the relative expression values of *PCC1* and the *CYSTM* family in different tissues and developmental stages of *Arabidopsis thaliana*. For At3g22235, At2g32200 and At1g52827, no expression data were available on the eFP browser website. *PCC1* and At3g22240 were expressed in all leaf tissues at higher fold changes (>3), whereby the relative expression fold change of At3g22240 was around two-fold lower than those of *PCC1*. Besides these two, also At1g56060 showed approximately the same fold change in leaf tissue than At3g22240. The fold change is the ratio of the expression level of the gene of interest (e.g. *PCC1*) and a control signal, which is either the median of all expression values in a certain category (like development) or the mock treatment (biotic stress). At1g05340, At2g32190, At2g32210, (clade I b) and At2g33520, At5g67600 and At3g49845 (clade II) show large fold changes in different seed stages and dry seeds, except for At3g49845, which shows a decrease in fold change in dry seeds. A great fold change was detected in the roots for At3g49845. At5g04080, At1g05340, At2g32190, At2g32210, At5g67600 and At1g56060 (>3) depict a higher fold change in some flowering stages. The remaining family members show low fold change in all tested tissues and stages.

Next, I assessed the relative expression changes upon biotic stress (Figure 32). *PCC1* and At3g22240 show almost identical fold change patterns in the different biotic stress conditions. *PCC1* and At3g22240 were significantly induced upon *Phytophthora infestans* infection (after 24 h, >4.7) and infection with *Hyaloperonospora arabidopsidis* (*Hpa*) strain Emwa1 (2 dpi, >2.3). Conversely, 24 h after infection with a virulent *P. syringae* strain, both genes were downregulated compared to the control. Interestingly, the opposite, namely upregulation, was reported before (Sauerbrunn & Schlaich, 2004). When Arabidopsis leaves were challenged with *G. orontii*, *PCC1* showed a two-fold upregulation in infected cells isolated by microdissection. Here, no change was found for At3g22240. *G. orontii* infection resulted in down-regulation of *PCC1* in the *eds16* mutant. Unfortunately, no expression data are available for At3g22235, which would be interesting to compare the whole set of Arabidopsis clade I *CYSTM* members.

CYSTM family members from the other clades show a different fold change pattern upon biotic stress. For example, At2g32210, At2g322190 and At1g05340 (clade I b) are more than 12-fold upregulated after treatment with the fungal pathogen *Botrytis cinerea* (after 18 h). They are also strongly upregulated 24 h after *P. syringae* treatment (both virulent and avirulent strains) and 6 h after *Phytophthora infestans* treatment. At1g56060 shows overall the same pattern with less upregulation compared to At2g32210, At2g322190 and At1g05340. At1g56060 depicts a great fold change in microdissected *G. orontii* infected material. In case of the CYSTM members of clade II, At2g33520 shows very little deregulation due to pathogen stress, only upon *G. orontii* infection an 8-fold upregulated in the *eds16* mutant background and down-regulated upon *Peronospora parasitica* infection were found. At5g67600 is upregulated after treatment with *B. cinerea*, *P. syringae* (24 hpi) and *G. orontii*. The strongest upregulation of At5g04080 was found 2 h after *P. syringae* treatment (>3). By contrast, At4g33660/At3g49845 show mostly downregulation due to the different treatments, though At3g49845 depicts an 11-fold increase in expression 6 h after infection with an avirulent strain of *P. syringae* (>11) and a 15-fold upregulation due to NPP treatment (*Phytophthora*-associated elicitor, (Fellbrich et al., 2002)). In summary, the different CYSTM family members exhibit a variable expression pattern upon interaction with various pathogens, but many of the CYSTM genes are upregulated during infection of specific sets of pathogens at least at defined time points.

	AT3G22231	At3g22235	AT3G22240	AT2G32200	AT2G32210	AT2G32190	AT1G05340	AT1G56060	AT3G49845	AT5G67600	AT2G33520	AT4G33660	AT5G04080	AT1G52827
Tissue														
Dry seed	0	n/a	0	n/a	13	13	52	1	0.5	5.6	139	0.8	0.1	n/a
Imbibed seed, 24 h	0	n/a	0	n/a	1	1	0.7	0.3	0.6	2.4	3.6	0.4	0	n/a
1st Node	0.9	n/a	0.3	n/a	0.4	0.4	1.2	0.9	0.8	2.2	1.4	0.5	1	n/a
Flower Stage 12, Stamens	0.1	n/a	0.1	n/a	2	2	8.6	0.5	2.3	1.8	1.3	0.5	0.8	n/a
Cauline Leaf	3.8	n/a	2.3	n/a	0.4	0.4	1.2	0.9	0.8	1.2	0.9	1.1	1.6	n/a
Cotyledon	5.8	n/a	2.9	n/a	1.2	1.2	0.9	2.3	0.7	0.9	0.9	1.6	2.9	n/a
Root	0	n/a	0.5	n/a	1.6	1.6	4.1	1.2	51	1.4	0.8	0.2	4.2	n/a
Rosette After Transition to Flow.	8.3	n/a	3.7	n/a	0.2	0.2	0.3	1.2	1.2	0.9	0.7	1.2	0.9	n/a
Flower Stage 9	0.2	n/a	0.1	n/a	0.1	0.1	0.1	0.4	0.6	0.7	0.8	1	0.3	n/a
Flower Stage 10/11	0.3	n/a	0.2	n/a	0.2	0.2	0.2	0.9	0.7	0.8	0.6	1.4	0.5	n/a
Flower Stage 12	0.8	n/a	0.5	n/a	0.9	0.9	1.3	1.6	0.5	1.1	0.9	0.8	0.7	n/a
Flower Stage 15	1.8	n/a	1.4	n/a	2.2	2.2	4	2.9	0.8	1.7	0.9	0.8	2.1	n/a
Flower Stage 12, Carpels	0.1	n/a	0.6	n/a	0.3	0.3	0.3	0.7	0.5	0.6	0.4	0.8	0.3	n/a
Flower Stage 12, Petals	0.2	n/a	0	n/a	0.2	0.2	0.3	0.9	0.5	1.2	0.8	1.8	0.9	n/a
Flower Stage 12, Sepals	1.1	n/a	1	n/a	4.1	4.1	3.6	4.1	1.1	4	0.9	2.2	4.8	n/a
Flower Stage 15, Carpels	0.1	n/a	0.7	n/a	0.5	0.5	0.3	0.9	0.8	1	0.7	0.7	0.5	n/a

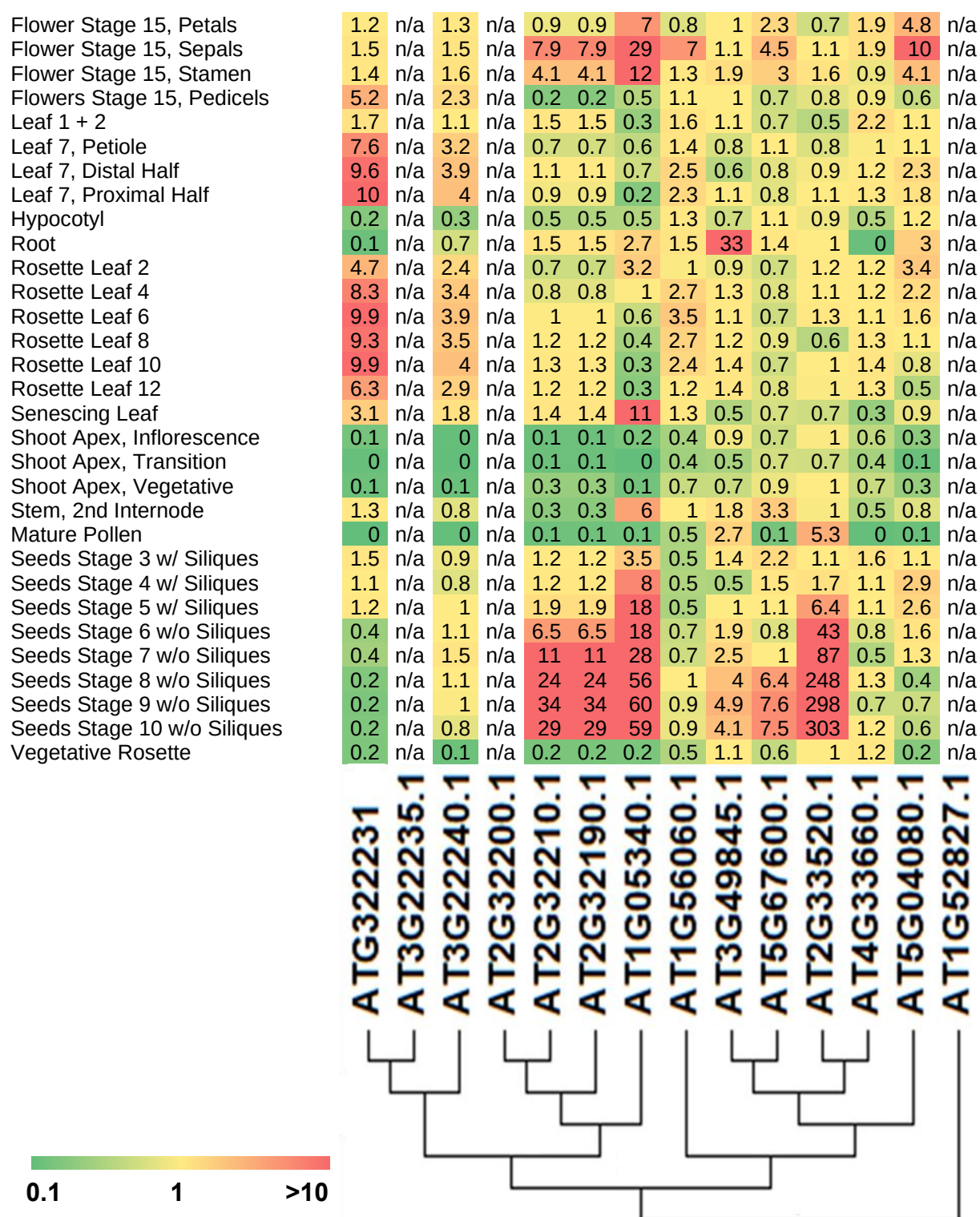


Figure 31: Heatmap based on relative expression values of *PCC1* and *CYSTM* family members in different Arabidopsis tissues during development. The relative expression data were extracted from the eFP Browser, and the fold change is depicted. For At3g22235, At2g32200, and At1g52827 no data were available (n/a). Green indicates (0.1) low, yellow (1) intermediate and red (>10) high fold change. The heatmap visualises the different expression patterns in different Arabidopsis tissues and different developmental stages.

Tissue	AT3G22231	At3g22235	AT3G22240	AT2G32200	AT2G32210	AT2g32190	AT1G05340	AT1G56060	AT3G49845	AT5G67600	AT2G33520	AT4G33660	AT5G04080	AT1G52827
Control B.c. at 18 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Treated B.c. at 18 h	1.1	n/a	1.0	n/a	12	12	16	3.6	2.4	1.9	1.0	0.6	0.8	n/a
Control B.c. at 48 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Treated B.c. at 48 h	1.0	n/a	1.0	n/a	13	13	22	5.1	1.3	3.2	1.5	0.4	0.7	n/a
Control Half P.s at 4 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Avirulent Half P.s at 4 h	0.9	n/a	0.9	n/a	2.8	2.8	2.3	2.8	0.5	1.2	0.9	0.9	1.0	n/a
Virulent Half P.s at 4 h	0.9	n/a	0.9	n/a	1.8	1.8	2.2	0.9	0.3	1.0	0.5	1.1	1.2	n/a
Control Half P.s at 8 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Avirulent Half P.s at 8 h	1.1	n/a	1.1	n/a	3.9	3.9	3.8	2.9	0.9	1.5	0.8	0.6	1.1	n/a
Virulent Half P.s at 8 h	1.0	n/a	1.0	n/a	1.4	1.4	1.4	1.3	0.6	1.1	0.4	0.8	1.0	n/a
Control Half P.s at 16 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Avirulent Half P.s at 16 h	1.0	n/a	0.9	n/a	2.2	2.2	5.1	3.4	1.2	1.8	1.7	0.6	0.9	n/a
Virulent Half P.s at 16 h	1.0	n/a	0.8	n/a	1.1	1.1	2.1	1.7	1.3	1.3	0.7	0.4	1.0	n/a
Control Half P.s at 24 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Avirulent Half P.s at 24 h	1.1	n/a	0.9	n/a	2.5	2.5	4.5	3.0	1.1	1.6	1.0	0.7	1.5	n/a
Virulent Half P.s at 24 h	1.2	n/a	1.0	n/a	2.2	2.2	3.3	3.3	0.5	1.2	0.6	0.5	1.3	n/a
Control Half P.s at 48 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Avirulent Half P.s at 48 h	1.0	n/a	1.0	n/a	1.3	1.3	1.8	2.3	1.3	0.9	1.1	0.9	1.1	n/a
Virulent Half P.s at 48 h	1.0	n/a	0.9	n/a	1.5	1.5	7.6	1.3	0.8	1.1	0.7	0.8	1.3	n/a
Control P.s. at 2 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Virulent P.s. at 2 h	0.6	n/a	0.7	n/a	1.5	1.5	1.5	1.6	2.5	1.4	1.1	0.8	3.3	n/a
Avirulent P.s. at 2 h	0.7	n/a	0.8	n/a	3.8	3.8	2.3	4.1	5.0	2.0	0.9	1.2	2.4	n/a
Deficient P.s. at 2 h	0.9	n/a	1.0	n/a	1.2	1.2	1.1	1.6	0.8	1.1	1.0	1.1	3.1	n/a
Nonhost P.s. at 2 h	0.8	n/a	1.0	n/a	1.8	1.8	1.8	3.0	4.0	1.5	0.8	0.8	3.5	n/a
Control P.s. at 6 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Virulent P.s. at 6 h	0.8	n/a	0.8	n/a	0.5	0.5	0.2	0.4	0.9	0.7	0.6	0.7	0.9	n/a
Avirulent P.s. at 6 h	1.1	n/a	1.1	n/a	2.8	2.8	3.3	2.3	11	1.5	0.8	0.7	1.5	n/a
Deficient P.s. at 6 h	0.9	n/a	1.0	n/a	0.4	0.4	0.3	0.4	0.7	0.8	0.6	0.7	0.6	n/a
Nonhost P.s. at 6 h	1.0	n/a	0.9	n/a	2.4	2.4	1.3	3.3	4.2	1.0	0.3	0.5	0.7	n/a
Control P.s. at 24 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Virulent P.s. at 24 h	0.2	n/a	0.4	n/a	10	10	84	1.1	0.5	4.7	1.0	0.5	2.2	n/a
Avirulent P.s. at 24 h	0.7	n/a	0.8	n/a	18	18	83	4.5	0.5	3.7	1.0	0.6	1.8	n/a
Deficient P.s. at 24 h	1.0	n/a	0.9	n/a	1.5	1.5	1.8	0.9	0.5	1.1	0.7	0.4	0.9	n/a
Nonhost P.s. at 24 h	0.9	n/a	0.8	n/a	6.0	6.0	8.9	1.8	0.5	2.3	0.7	0.4	0.9	n/a
H ₂ O at 1 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
FLG22 at 1 h	1.2	n/a	1.1	n/a	2.5	2.5	2.4	3.0	1.1	0.8	1.0	0.9	0.8	n/a
HrpZ at 1 h	0.8	n/a	0.8	n/a	3.7	3.7	2.4	3.4	1.3	0.8	0.9	1.0	0.9	n/a
Ca+Mg at 1 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
LPS at 1 h	0.9	n/a	0.8	n/a	1.1	1.1	1.1	1.3	1.2	0.9	0.9	0.7	0.8	n/a
GST at 1 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
NPP at 1 h	1.0	n/a	0.9	n/a	2.0	2.0	1.4	1.9	1.8	0.9	0.6	0.9	1.4	n/a
H ₂ O at 4 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
FLG22 at 4 h	0.8	n/a	0.9	n/a	2.4	2.4	2.7	1.3	0.6	1.0	0.2	0.4	0.9	n/a
HrpZ at 4 h	0.9	n/a	0.9	n/a	8.1	8.1	7.2	4.0	5.7	1.2	0.3	0.5	1.1	n/a
Ca+Mg at 4 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
LPS at 4 h	1.0	n/a	1.1	n/a	1.1	1.1	1.2	1.0	1.3	1.0	1.7	1.0	0.9	n/a
GST at 4 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
NPP at 4 h	0.9	n/a	1.0	n/a	2.2	2.2	1.8	1.8	14	1.2	0.7	0.7	1.0	n/a
Control P.i. at 6 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Treated P.i. at 6 h	1.2	n/a	1.1	n/a	13	13	14	9.7	6.9	1.2	0.9	0.5	1.3	n/a
Control P.i. at 12 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Treated P.i. at 12 h	0.9	n/a	1.1	n/a	6.3	6.3	6.4	2.0	1.0	1.5	1.0	0.7	0.9	n/a
Control P.i. at 24 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Treated P.i. at 24 h	5.8	n/a	4.7	n/a	0.7	0.7	3.6	0.8	1.0	1.1	1.5	0.7	0.9	n/a
Control E.o. at 6 h	1.0	n/a	1.0	n/a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	n/a
Treated E.o. at 6 h	0.8	n/a	0.8	n/a	0.9	0.9	0.8	1.3	0.9	0.9	0.8	1.1	0.8	n/a

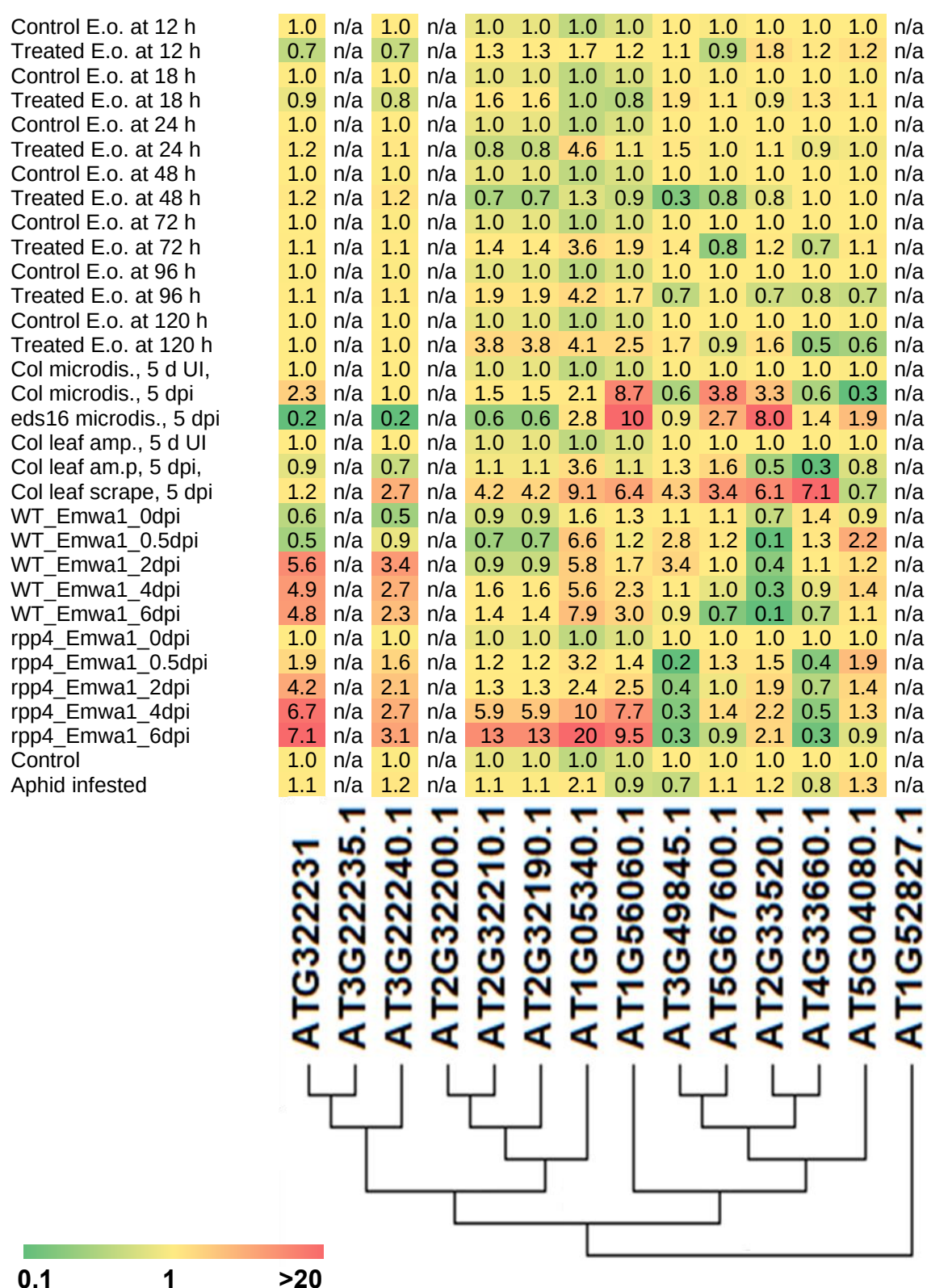


Figure 32: Heatmap of relative expression values of *PCC1* and *CYSTM* family members upon different biotic stresses. The relative expression data were extracted from the eFP Browser, and the fold change is depicted. For At3g22235, At2g32200, and At1g52827 no data were available (n/a). Scale bar indicates fold expression change; green decrease, yellow no change and red increase. The heatmap visualises the expression patterns after treating Arabidopsis with different biotic stresses. B.c.: *Botrytis cinerea*; P.s.: *Pseudomonas syringae*; P.i.: *Phytophthora infestans*; E.o.: *Erysiphe* (syn. *Golovinomyces orontii*). h: hours, dpi: days post-infection.

Co-expression analysis of PCC1 and the CYSTM family

In order to find a pathway in which PCC1 is involved, I investigated the top 300 co-expressed genes using ATTED-II (Obayashi et al., 2018), analysed these for GO term enrichment with PLAZA v4.0 (Van Bel et al., 2018) and filtered the resulting list with REVIGO (Supek et al., 2011) to reduce redundancy of GO terms. Figure 33 shows the GO term enrichment as $-\log_{10}$ (p-value) and only GO terms are displayed that showed a $-\log_{10}$ (p-value) of above 3 (corresponds to a p-value of 0.001). The GO term defence response was the most enriched one in this set with a $-\log_{10}$ (p-value) of 47. Also, other GO terms correlated with defence, immunity and response to SA are enriched with at least $-\log_{10}$ (p-value) of 23. GO terms corresponding to signalling and cell death showed a $-\log_{10}$ (p-value) between 10 and 20 (Figure 33 A). GO terms belonging to molecular function are depicted in Figure 33 B and show an increase in GO terms associated with kinase activity and binding of carbohydrates, ions, and ADP-binding, which indicate enrichment of protein kinases or receptor-like kinases in this data set. Regarding the category cellular components (Figure 33 C), the GO term correlated with cell periphery was enriched with the highest $-\log_{10}$ (p-value) of 18. Interestingly, the top 300 co-expressed genes include numerous defence genes like *NPR4*, *PR5*, *PR1*, *PR2*, *EDS1*, *SID2*, *MPK3*, *MKK1* and *MPK11*.

I then extracted all top 300 co-expressed genes of all CYSTM members from ATTED-II and searched for overlapping gene sets. No co-expression data could be extracted for At3g22235, At2g32200, At2g32210, At2g32190 and At1g52827. This analysis revealed an overlap of 67 genes for PCC1 and At3g22240 and 41 genes for PCC1 and At1g56060 (Figure 34). Clade-specific co-regulation was not found since overlap of co-expressed genes can also be seen between clade I and clade II members, while members of the same clade do not show any overlap in their co-expression networks in some cases. PCC1, At3g22240, At5g67600, At5g67600 and At1g56060 showed 10% overlap (>30 common co-expressed genes) with at least two other CYSTM family members. The highest numbers of co-regulated genes were found between PCC1 and At3g22240 (67) and between the clade II members At5g04080 and At5g67600 (68).

Next, I investigated the co-expressed genes of At3g22240 (clade I a) and of At1g05340 (clade I b) in more detail. Both genes were the only members of clade I of these were co-expression data available, and these represent a close homolog (At3g22240) and a further related relative (At1g05340) of PCC1. I performed a GO term enrichment

analysis for the category biological processes to search for an overlap with PCC1. At3g22240 shows a co-expressed gene overlap of 67 genes, whereas At1g05340 has no overlap with PCC1. The GO term enrichment for At3g22240 looks similar to PCC1 with the highest $-\log_{10}$ (p-value) of 23 found for defence response. In total, the $-\log_{10}$ (p-values) are lower than for PCC1. The top 8 enriched GO terms of At3g22240 are also present in the list of the top PCC1 enriched terms (Figure 35 A). The co-expressed genes for At3g22240 include, for example, *PR4*, *PR3*, *PR1*, *PAD4*, *PAD3*, *MLO2*, *MLO6*, *SID1* and, interestingly, *CERK1*. At1g05340 shows no significant overlap with PCC1 and At3g22240 since these GO terms are more correlated with responses to abiotic stress and compounds. Overlapping GO terms are response to external stimulus, response to other organism and response to biotic stimulus that each show enrichment of less than a $-\log_{10}$ (p-value) of 5 (Figure 35 B). A complete list of the co-expressed gene IDs is attached as Supplementary Table 3.



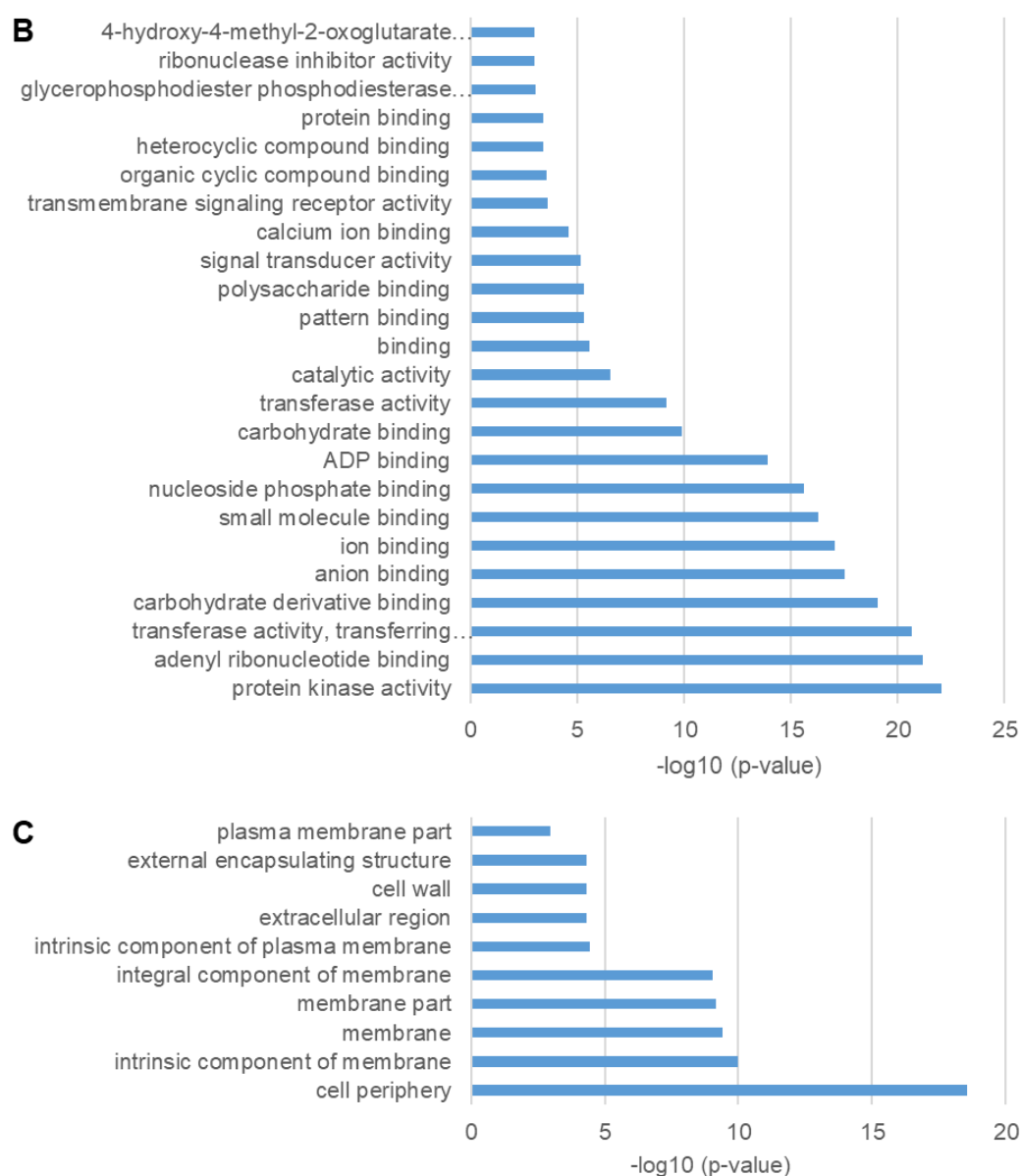


Figure 33: GO term enrichment analysis of the top300 co-expressed *PCC1* genes. The top 300 co-expressed genes of *PCC1* were extracted from ATTED-II (Obayashi et al., 2018), the GO term enrichment analysis was performed with PLAZA v4.0 (Van Bel et al., 2018) and the resulting GO term list was filtered with REVIGO (Supek et al., 2011) to reduce the number of redundant GO terms. (A) The $-\log_{10}(\text{p-value})$ of GO terms belonging to biological processes, (B) molecular function and (C) cellular component are shown. The cut-off was set at $-\log_{10}(\text{p-value}) = 3$ (equals a p-value of 0.001).

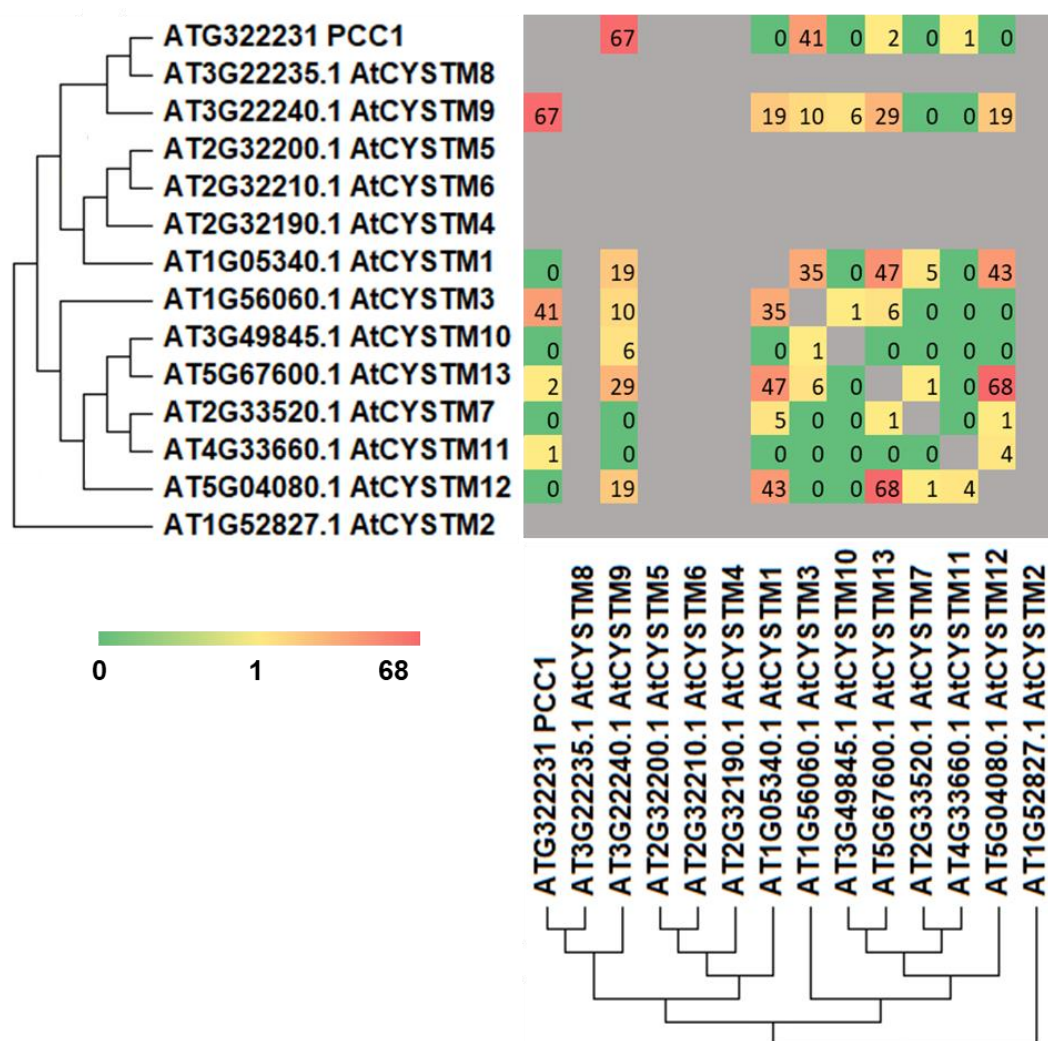
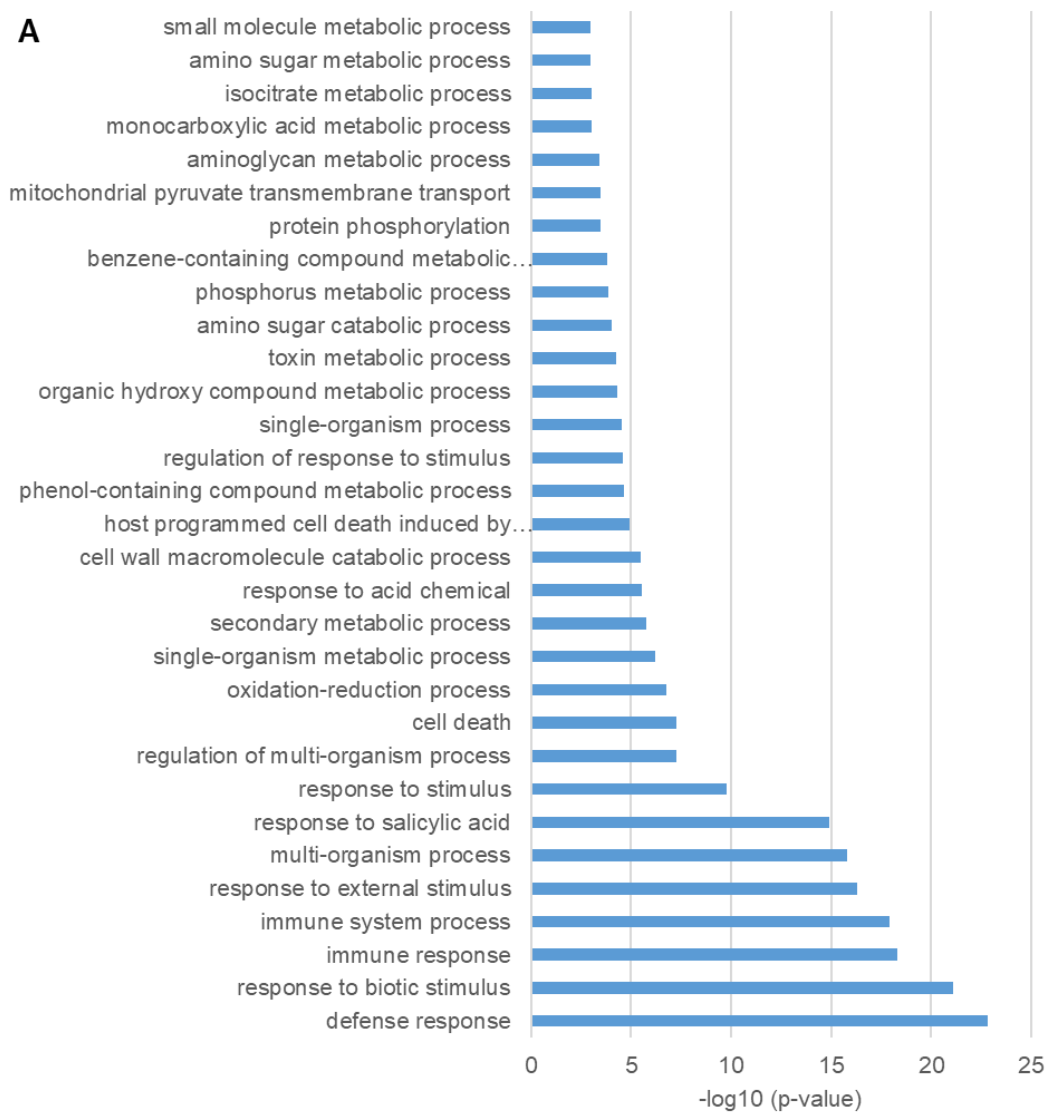


Figure 34: Co-expressed genes between *PCC1* and CYSTM family members. The figure depicts the number of co-expressed genes between *PCC1* and the CYSTM family members. The top300 co-expressed genes of each family members were extracted from Atted-II (<http://atted.jp/>) (data accessed 03-Apr-2019) and compared between the different members. For some CYSTM members, no co-expression data could be extracted, which is indicated by the grey boxes. The phylogenetic tree was generated as described in figure 30.



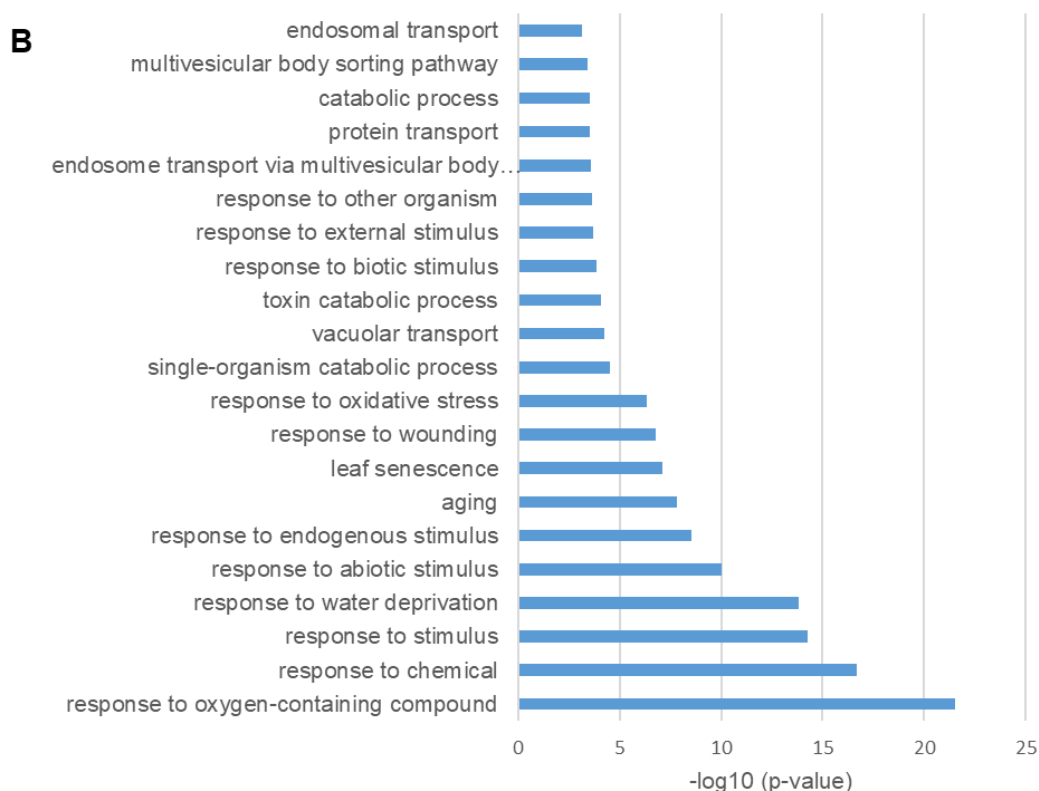


Figure 35: GO term enrichment analysis of the top300 co-expressed *At3g22240* and *At1g05340* genes. The top 300 co-expressed genes of PCC1 were extracted from ATTED-II (Obayashi et al., 2018) and the GO term enrichment analysis was performed with PLAZA v4.0 (Van Bel et al., 2018) and the resulted GO term list was filtered with REVIGO (Supek et al., 2011) to reduce redundant GO terms. The $-\log_{10}(\text{p-value})$ of GO terms belonging to biological processes are shown. (A) Depicts GO terms from the closely related clade I a homologue *At3g22240* and (B) from the further related clade I b homologue *At1g05340*. The cut-off was set at $-\log_{10}(\text{p-value}) = 3$ (equals a p-value of 0.001).

I also performed a GO term analysis for overlapping genes between PCC1 and *At3g22240* (Figure 36 A and B). Like the separated GO term analysis of both (PCC1 and *At3g22240*) suggested, also the common overlapping co-expressed genes are involved in defence, immunity, response to biotic stimuli and SA (Figure 36 A). The GO terms associated with molecular function suggest enrichment in genes involved in protein phosphorylation (Figure 36 B).

Taken together, the co-expression analysis revealed an enrichment of co-expressed genes with function in plant defence, immunity, and SA metabolism in the datasets of PCC1 and *At3g22240* (clade I a). The analysis further showed that overlap in co-expressed genes does not only occur within members of the same CYSTEM clade but also between different clades.

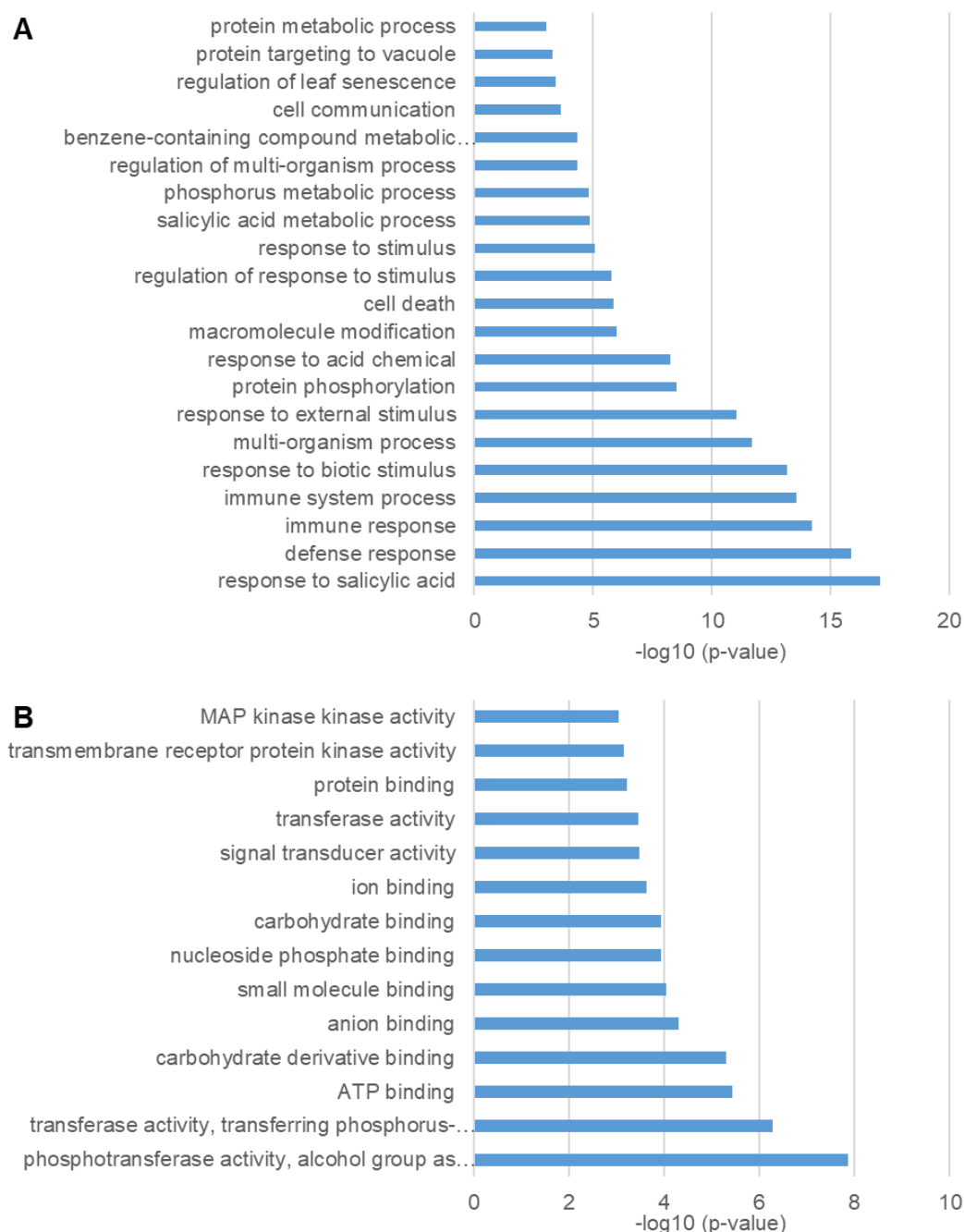


Figure 36: GO term enrichment analysis of overlapping co-expressed genes between *PCC1* and *At3g22240*. The top 300 co-expressed genes of *PCC1* were extracted from ATTED-II (Obayashi et al., 2018) and the GO term enrichment analysis was performed with PLAZA v4.0 (Van Bel et al., 2018) and the resulted GO term list was filtered with REVIGO (Supek et al., 2011) to reduce redundant GO terms. The overlapping co-expressed genes (67 genes) of *PCC1* and *At3g2240* were used for the GO term analysis. (A) The $-\log_{10}(\text{p-value})$ of GO terms belonging to biological processes and (B) molecular function are depicted. The cut-off was set at 3 $-\log_{10}(\text{p-value})$

Protein interaction studies of CERK1 with PCC1-related CYSTM family members

I cloned the clade I members of the Arabidopsis CYSTM family, except At1g05340 which I could not amplify from cDNA. I was able to clone two splice versions for At2g322190 (CYSTM6) and At2g32190 (CYSTM4). The second splice versions of both genes are shorter and partially lack the cysteine-rich transmembrane domain (Figure 30 B). First, I used the LexA-based split-ubiquitin assay to test if these PCC1-related proteins interact with Arabidopsis CERK1. Plate A selected only against the bait and prey plasmids and verified equal growth of all strains (Figure 37). GPA1 was a positive control, and AGB1 and Nubl-GWY (eV) were included as negative controls. Except for At3g22235, At2g32190.2 and the negative controls, all yeast strains were detectable until OD₆₀₀ 0.01 on the selective medium SC -Leu -Trp -Met -Ade -His (plate B). However, on the medium including 150 µM Met, only the GPA1 interaction strain grew until OD₆₀₀ 0.01, while the CYSTM members only showed growth at OD₆₀₀ 1.

Further, the *lacZ* reporter assay showed the same trend in three independent biological replicates (Figure 38), although none of the combinations displayed a statistical difference from AGB1. Most CYSTM combinations with CERK1 exhibited around 0.1 U/mg median specific enzyme activity in this assay, except for At3g22235 and At2g32190.2 with ca. 0.02 U/mg. The negative controls showed no enzyme activity. Some of the samples displayed a relatively high variation in the data set.

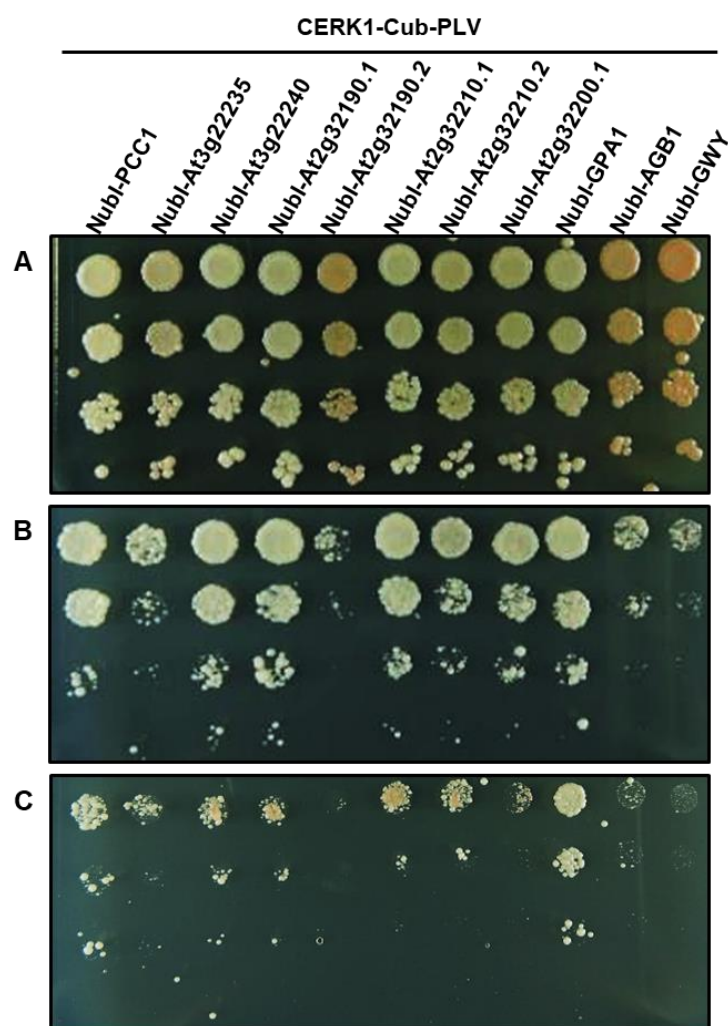


Figure 37: Comparison of PCC1 family members against CERK1 in a LexA-based split-ubiquitin Y2H. For the validation of the specific interaction of PCC1 with CERK1, also other PCC1 family members were tested. GPA1 serves as a positive control, and AGB1 and empty vector (Nubl-GWY) as negative controls. 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series starting at OD₆₀₀ 1 on the top and until OD₆₀₀ 0.001. Selection media were as follows: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine. This experiment was repeated in three independent biological replicates, and the replicates are shown in Supplementary Figure 19.

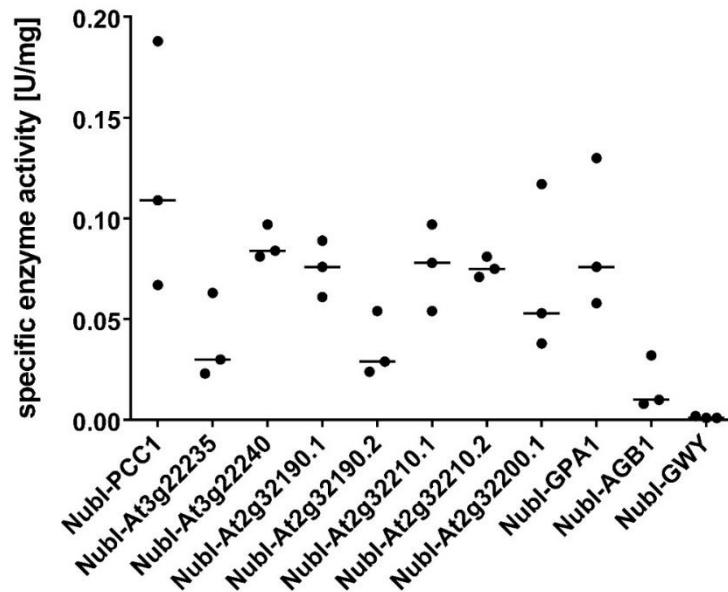


Figure 38: LacZ reporter assay of CERK1 and the PCC1 family members. The LacZ reporter assay was used for the quantification of the interaction between CERK1 and PCC1 family members. Yeast cells expressing both fusion constructs were harvested, and the cells were disrupted. The cell debris was separated by centrifugation, and the protein concentration was measured via the Bradford assay. The reaction buffer (Z-Buffer) was mixed with cell extract and the substrate oNPG and the reaction was incubated at 37 °C. When the solution turned yellow, the reaction was stopped by adding Na₂CO₃. The OD was measured, and the specific enzyme activity was calculated. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1) and an asterisk indicates $P \leq 0.05$. The horizontal bar marks the median. The specific enzyme activity is given as U/mg. This experiment was repeated in three independent biological replicates.

Next, I performed the *in planta* split-luciferase assay for PCC1 and its CYSTM relatives in *N. benthamiana*. Here, the luciferase activity for the PCC1-CERK1 interaction was significantly different ($P \leq 0.001$) compared to the negative control AGB1 (Figure 39 A). In this assay, At3g22235 and At3g22240 (clade 1 a) also showed significantly increased median luciferase activity of almost 20 ($P \leq 0.01$), which was at similar levels as PCC1. In yeast, At3g22235 grew a bit less than At3g22240, but the median luciferase activity of these two interactions was almost identical *in planta*. The interaction of the clade I b CYSTMs (At2g32190 and At2g32210) with CERK1 showed a significant difference to AGB1 as well. Both median values were around ten, thus lower than for PCC1, At3g22235, and At3g22240. For the second splice variants of these CYSTMs, I obtained values in the range of the cLUC-GWY (eV) and AGB1, suggesting no interaction with CERK1. However, both of the splice variants also displayed lower protein levels than any other construct (Figure 39 B). Altogether, both yeast split-ubiquitin and *in planta* split-luciferase assays indicate that except

At2g32200 and the tested splice variants, most PCC1-like clade 1 CYSTM proteins interact with Arabidopsis CERK1 to different extents.

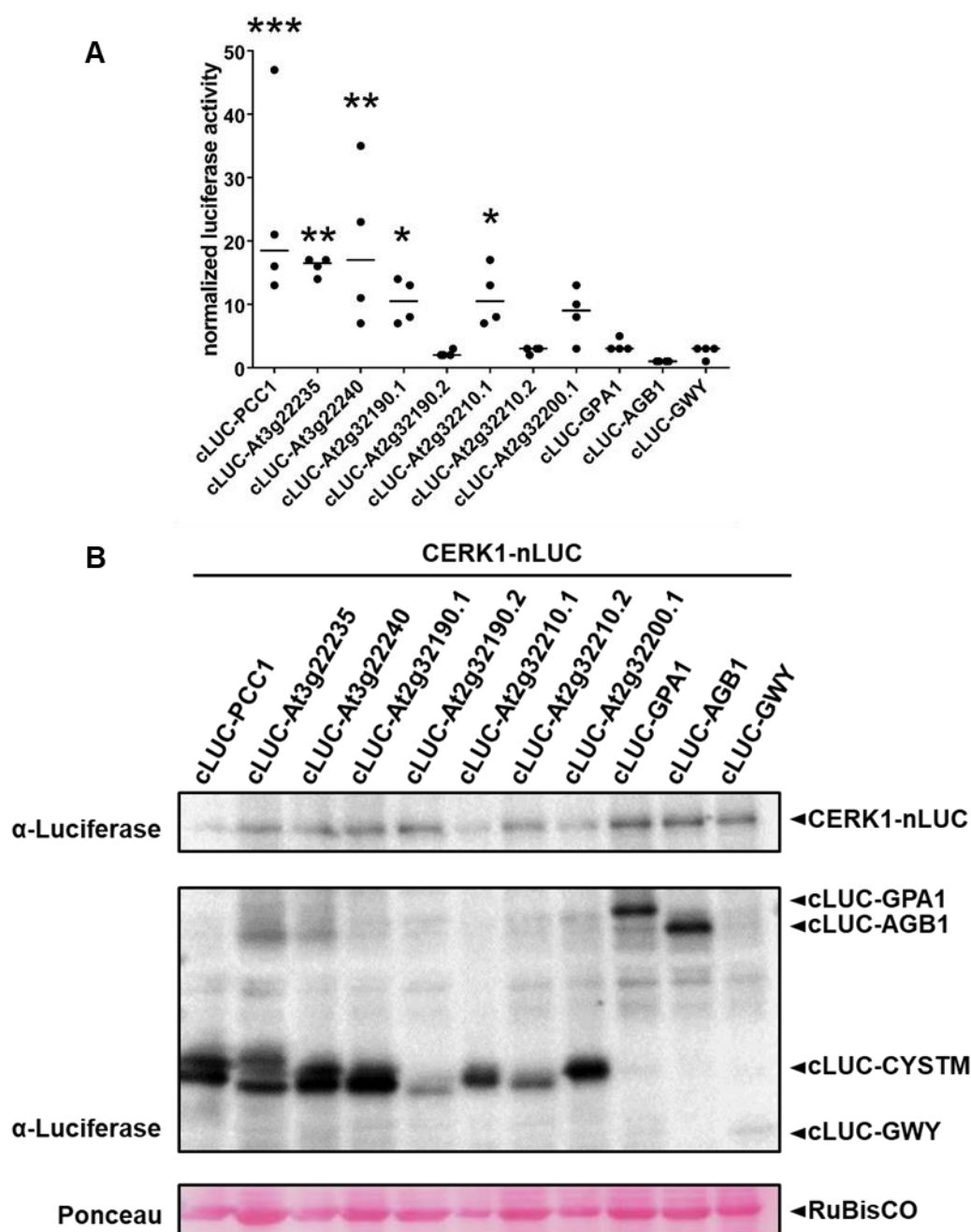


Figure 39: Split-luciferase assay of CERK1 and PCC1 family members. (A) The split-luciferase assay allows the quantification of *in planta* protein-protein interaction. Agrobacteria with the fusion protein encoded on plasmids were infiltrated in *N. benthamiana* and incubated for two days. In total, eight leaf discs (four discs from 2 leaves each) of each combination were measured. D-Luciferin was infiltrated as a substrate before the measurement. The mean value of the leaf discs after 5 min of measurement was normalised with the negative control AGB1. (B) The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase protein fusions are as follows: CERK1-nLUC: 116 kDa; cLUC-PCC1 and the other family members around 28 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1), which was set to 1. The asterisks indicate the

following p-values: * = $P \leq 0.05$, ** = $P \leq 0.01$, and *** = $P \leq 0.001$. The horizontal bar indicates the median. This experiment was repeated in four independent biological replicates. Full-size western blot is attached in the supplement (Supplementary Figure 20).

Do RLKs involved in innate immunity interact with PCC1 in yeast and *in planta*?

Since I found reproducible interaction of CERK1 with PCC1 full length and specific truncated versions, I was wondering if other RLKs also involved in plant immunity may interact with PCC1 as well. I included the closest paralogs of PCC1 from clade I a At3g22235 and At3g22240. I studied the interaction of these CYSTM proteins with known receptors involved in chitin perception, LYK4 and LYK5 (Cao et al., 2014, Wan et al., 2012b). I further broadened the set by including RLKs of bacterial MAMPs, the flagellin receptor FLS2, the Ef-Tu receptor EFR, and the co-receptor BAK1 (Kim et al., 2013). AGB1 and eV served as negative controls against all these RLKs, but GPA1 is only known to interact with CERK1, BAK1 and BIR1 (Aranda-Sicilia et al., 2015).

The LysM receptor-like kinases LYK4 and LYK5 are involved in chitin perception and activation of downstream signalling. Unfortunately, the LYK5 fusion protein was not detected in the transformed yeast; LYK4 showed a clear band in the western blot (Figure 20). LYK5 only showed weak interaction with At3g22240 in yeast (Figure 40 B), which was abolished in Met-containing medium. In the case of LYK4, a strong band was detected in the western blot, but there were no differences between all strains on the selection medium (plate B). The addition of 150 μ M Met visualised variations: The negative controls could only grow at OD₆₀₀ 1, while At3g22235 was well detectable at OD₆₀₀ 0.1, and GPA1, PCC1, and At3g22240 grew up to OD₆₀₀ 0.01.

The *in planta* split-luciferase assay was conducted to validate the yeast data. For LYK4, the interaction with At3g22240 was found to be statistically significant (Figure 41 A). The other two candidates showed slightly less luciferase activity with a possible outlier of PCC1. In contrast to the yeast system, no background by the negative control could be seen. The luciferase activity of LYK5 with these proteins was in the same range as for LYK4 (Figure 41 B). Here, only PCC1 was statistically significantly different from AGB1. GPA1, AGB1 and the cLUC-GWY (eV) showed the same low luciferase activity. The western blot depicts an overall equal expression of LYK4 and slight variations for LYK5 *in planta* (Figure 41 C). Also, the prey proteins were all detectable, although the levels seem to differ between the samples. Therefore, PCC1,

At3g22235 and At3g22240 seem to interact with the Arabidopsis receptors LYK4 and LYK5 in yeast and *in planta*.

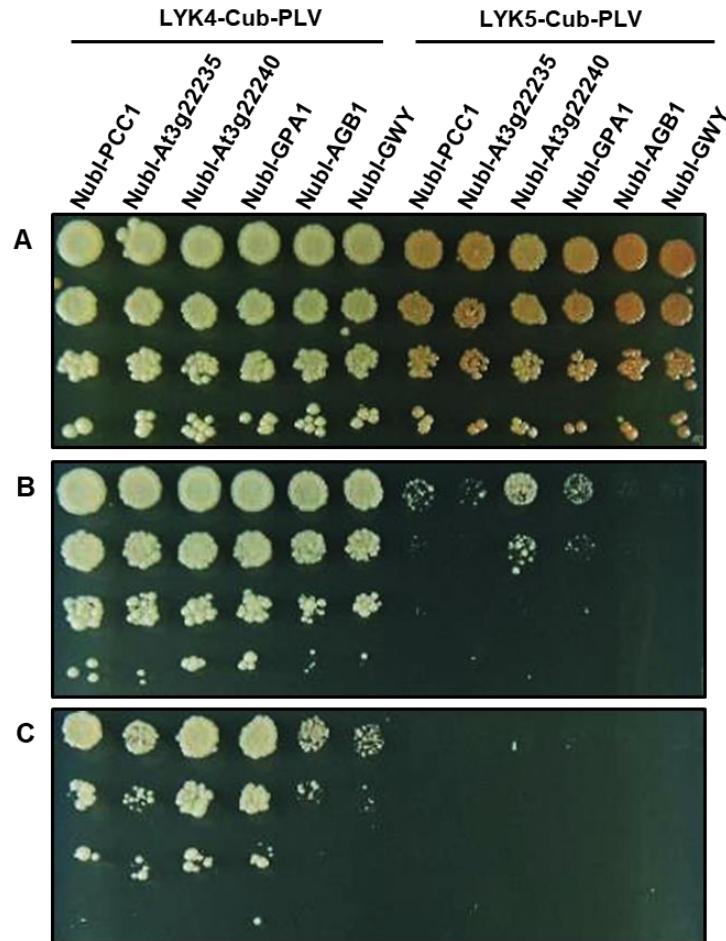


Figure 40: Testing the interaction of PCC1 against LYK4 and LYK5 in a LexA-based split-ubiquitin Y2H. To test the interaction of PCC1 with other RLKs, PCC1 and both close family members At3g22235 and At3g22240 were tested against LYK4 and LYK5. GPA1 serves as a positive control as well as AGB1 and empty vector (Nubl-GWY) as negative controls. 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series starting at OD₆₀₀ 1 on the top and until OD₆₀₀ 0.001 at the bottom. The different plates are supplemented with increasing concentration of methionine to repress the bait protein expression: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine. This experiment was repeated in three independent biological replicates, and the replicates are attached in the supplement (Supplementary Figure 21).

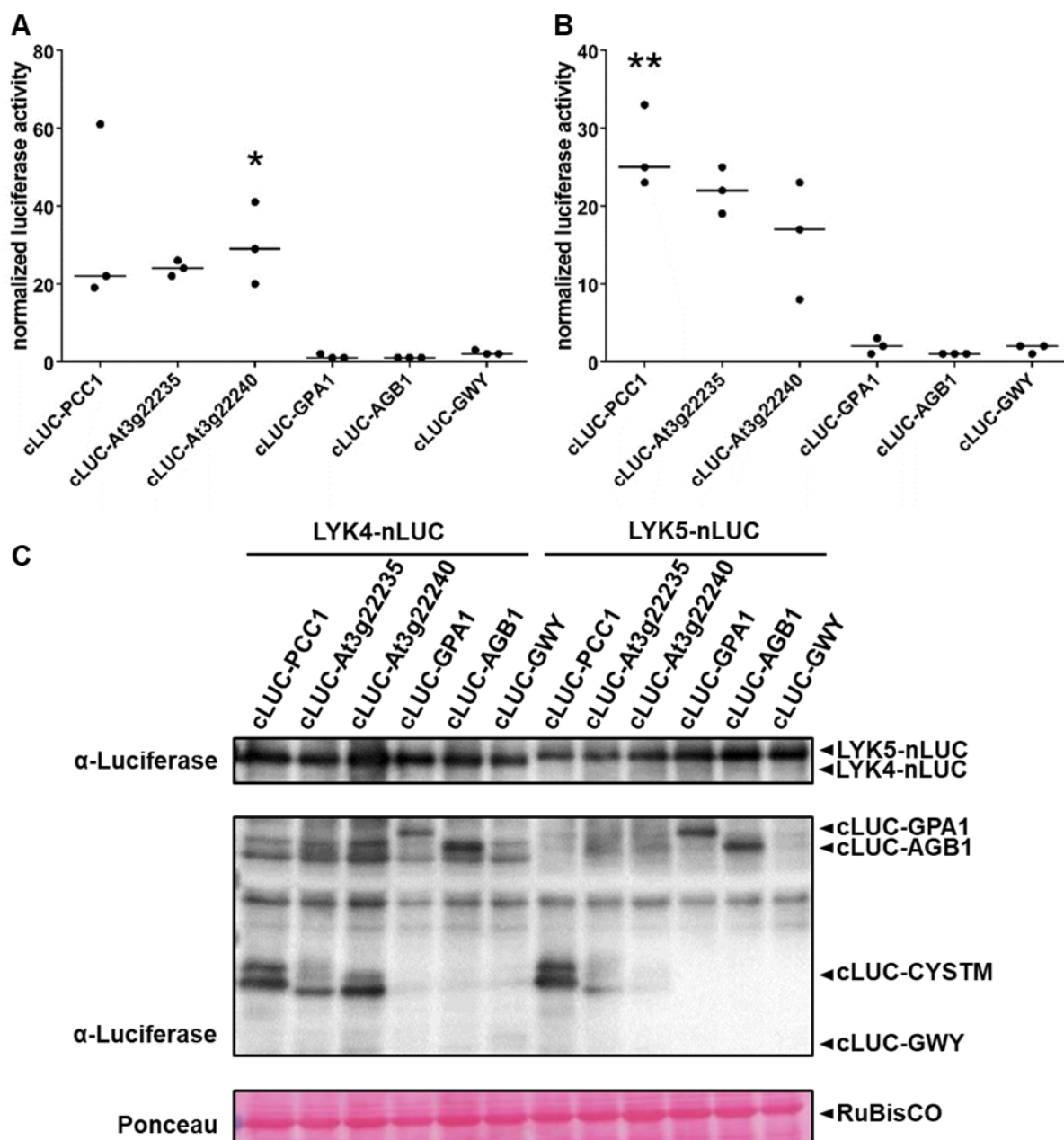


Figure 41: Split-luciferase assay of LYK4 and LYK5 against PCC1. (A/B) The split-luciferase assay allows the quantification of *in planta* protein-protein interaction. Agrobacteria with the fusion protein encoded on plasmids were infiltrated in *N. benthamiana* and incubated for two days. In total, eight leaf discs (four discs from 2 leaves each) of each combination were measured. D-Luciferin was infiltrated as a substrate before the measurement. The mean value of the leaf discs after 5 min of measurement was normalised with the negative control AGB1. (A) depicts the split-luciferase assay of LYK4 and (B) the assay with LYK5. (C) The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes with the fused split-luciferase parts are like followed: LYK4-nLUC: 116 kDa; LYK5-nLUC: 122 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235: 27 kDa; cLUC-At3g22240: 27 kDa; cLUC-GPA1: 64 kDa, cLUC-AGB1: 61 kDa and cLUC-GWY: 20 kDa. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1), which was set to 1. The asterisks indicate the following p-values: * = $P \leq 0.05$ and ** = $P \leq 0.01$. The horizontal bar marks the median. This experiment was repeated in three independent biological replicates. Full-size western blot is attached in the supplement (Supplementary Figure 22).

Another famous and maybe even more studied receptor-like kinase is FLS2, the Arabidopsis flagellin receptor. Even though no bait expression could be initially detected of the fusion protein FLS2-Cub-PLV by a western blot (Figure 20), I performed the split-ubiquitin experiment. The yeast system could detect a weak interaction for PCC1, At3g22240 and GPA1 with FLS2 on selective medium, possibly weak due to low bait expression (Figure 42 A, 42 B). Two other replicates revealed the same trend (Supplementary Figure 23). In one replicate also At3g22235 grew almost the same way as PCC1 and At3g22240. When the methionine was added to the selective medium, the interaction was not detectable in all cases (data not shown).

The other two RLKs in the set were BAK1 and EFR. For EFR, no bait expression and for BAK1, only a weak bait expression was detected in yeast (Figure 20). Accordingly, EFR yeast strains showed no growth on the interaction medium (Figure 42; plate D), possibly due to the low bait expression in yeast. For BAK1, yeast growth was detected on selection medium, although the addition of Met inhibited any yeast growth with BAK1 baits. Here, At3g22235 followed the overall trend in all split-ubiquitin experiments and exhibited less growth (to OD₆₀₀ 0.1) compared to PCC1, At3g22240, and the positive control GPA1 (detected at OD₆₀₀ 0.01). The negative controls AGB1 and Nubl-GWY (eV) did not exhibit colonisation on selection medium.

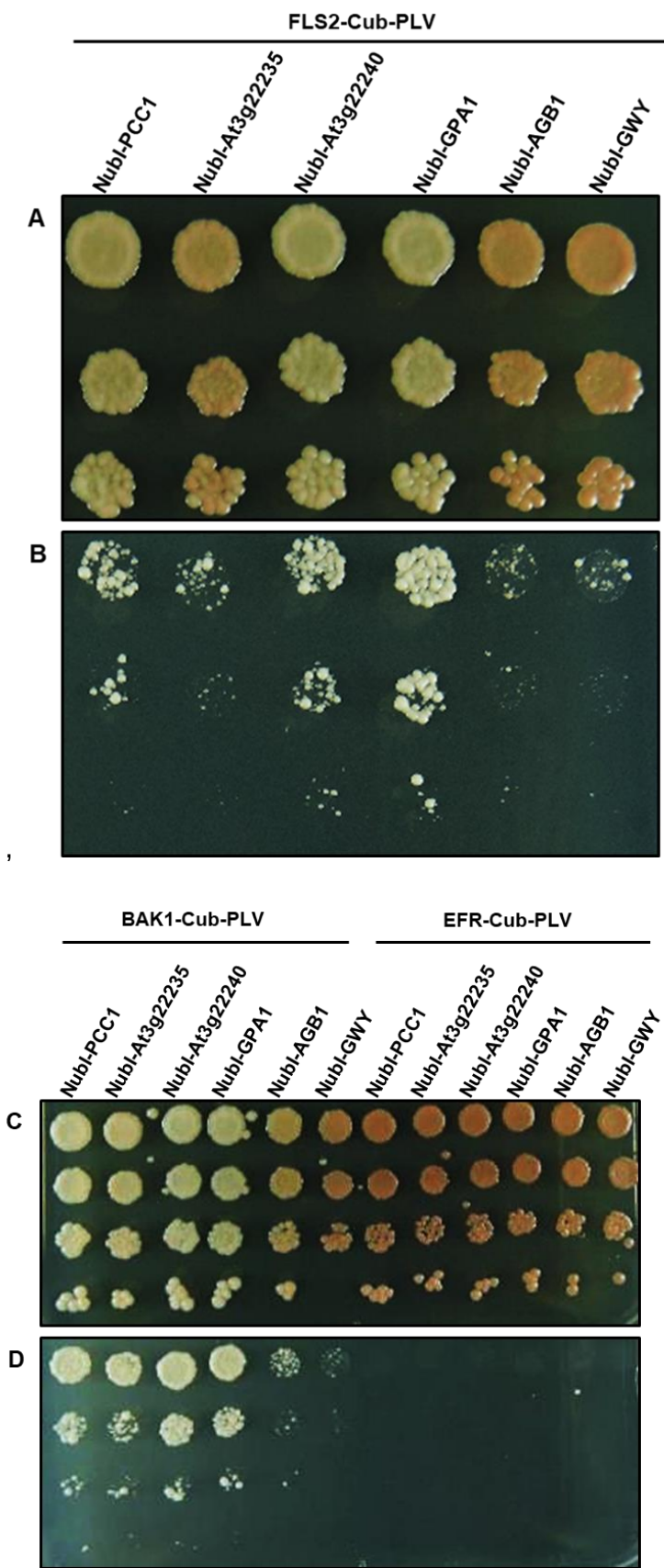


Figure 42: Testing interaction of PCC1 against FLS2, BAK1 and EFR in a LexA-based split-ubiquitin Y2H. To test the interaction of PCC1 with other RLKs, PCC1 and both close family members At3g22235 and At3g22240 were tested against FLS2, BAK1 and EFR. GPA1 serves as a positive control as well as AGB1 and empty vector (Nubl-GWY) as negative controls. 4 µl of overnight cultures of transformed yeast were plotted in a 10-fold dilution series starting at OD₆₀₀ 1 on the top and until OD₆₀₀ 0.01 at the bottom. The different plates are supplemented with increasing concentration of methionine to repress the bait protein expression: (A/C) SC -leucine -tryptophan -methionine, (B/D) SC -leucine -tryptophan -adenine -histidine -methionine. This experiment was repeated in three independent biological replicates, and the replicates are attached in the supplement (Supplementary Figure 23+24).

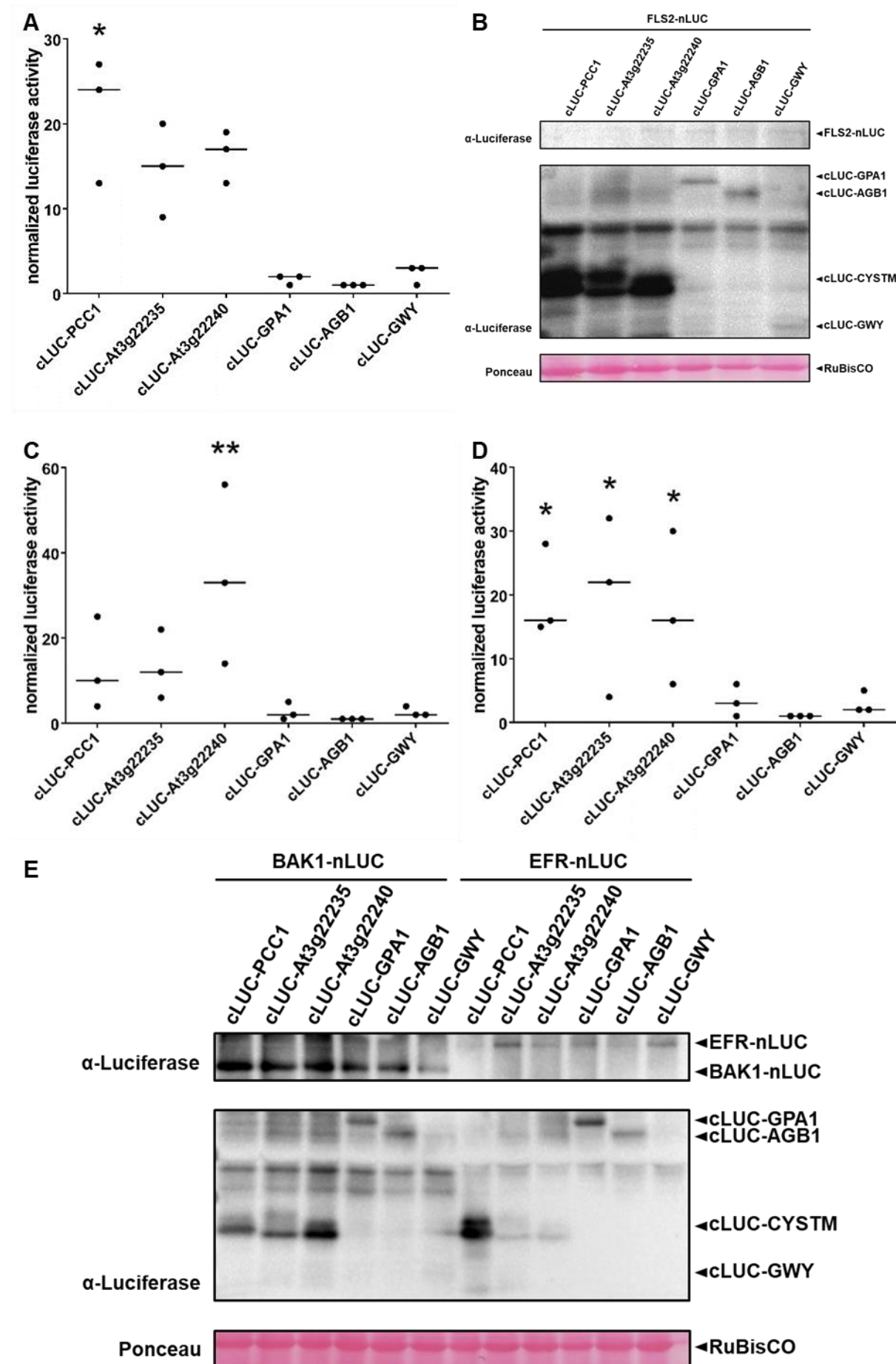


Figure 43: Split-luciferase assay of FLS2, BAK1 and EFR against PCC1. (A/C/D) The split-luciferase assay allows the quantification of *in planta* protein-protein interaction. Agrobacteria with the fusion protein encoded on plasmids were infiltrated in *N. benthamiana* and incubated for two days. In total, eight leaf discs (four discs from 2 leaves each) of each combination were measured. D-Luciferin was infiltrated as a substrate before the measurement. The mean value of the leaf discs after 5 min of measurement was normalised with the negative control AGB1. (A) depicts the split-luciferase assay of FLS2, (C) the assay with BAK1 and (D) the assay with EFR. (B/E) The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase protein fusions are as follows: FLS2-nLUC: 177 kDa; BAK1-nLUC: 117 kDa; EFR-nLUC: 162 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235 27 kDa, cLUC-At3g22240 27 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1), which was set to 1. The asterisks indicate the following p-values: * = $P \leq 0.05$ and ** = $P \leq 0.01$. The horizontal bar marks the median. This experiment was repeated in three independent biological replicates. Full-size western blot is attached in the supplement (Supplementary Figure 25+26).

Next, I performed the split luciferase activity assays in *N. benthamiana* to test the interactions between the three RLKs and PCC1, At3g22235, and At3g22240. In case of the FLS2 receptor, PCC1 showed a median normalised activity of around 25 ($P < 0.05$) and both At3g22235 and At3g22240 had a median activity of ca. 15 (Figure 43 A). In this assay, GPA1 did not show luciferase activity, which is in contrast to the result from the yeast system. *In planta*, all family members were expressed equally according to the western blot (Figure 43 B). In this assay, only a very faint band of the bait protein could be detected. The split-luciferase assay for EFR showed a statistically significant difference for all three proteins compared to the negative control AGB1, and they displayed a median luciferase activity of around 20 (Figure 43 D). The western blot confirmed that all constructs were expressed (Figure 43 E). In the case of BAK1, a lower luciferase activity of around 10 for PCC1 and At3g22235 was found (Figure 43 C). The value for At3g22240 was three-fold higher and was statistically significantly different compared to AGB1 ($P < 0.01$). However, the western blot indicated that the expression level of At3g22240 was higher compared to the other prey protein constructs (Figure 43 E).

Taken together, PCC1 and At3g22240 appear to interact both with FLS2 and the co-receptor BAK1, while the interaction of At3g22235 with these receptors was less clear in the yeast-based assays. These proteins may interact with EFR as well according to *in planta* assays, but the Y2H assays remained inconclusive due to the lack of expression of EFR.

Heterodimerization of PCC1 with its two closest family members *in planta*

In 2014, Mir and Leon showed the homodimerization capability of PCC1 in a classical Y2H (lacking the C-terminal transmembrane domain), pull-down by CoIP, and BiFC experiments (Mir & Leon, 2014). Moreover, Xu *et al.* demonstrated that other CYSTM members could form heterodimers in split-ubiquitin Y2H and split-luciferase assays, but PCC1 was not included in this data set (Xu *et al.*, 2018). Since I did not want to mask the C-terminal transmembrane domain with the Cub-PLV tag, which would be fused C-terminally in the bait vector, I could not use the split-ubiquitin system to study this question. However, the split-luciferase system for expression in *N. benthamiana* was suitable to address this question, because, for this system, all four possible combinations of tag orientation were available. Hence, I generated N-terminally tagged nLUC-PCC1, -At3g22235 and -At3g22240 constructs. GPA1, AGB1 and cLUC-GWY (eV) were used as negative controls. The published interaction of PCC1 with itself was used as a positive control.

The median of PCC1 and At3g22235 was with 15 – 20 normalised luciferase activity in the same range as in the other experiments and suggested interaction of these proteins with PCC1 ($P < 0.05$). At3g22240 exhibited somewhat lower activity with between 10-15 normalised activity (Figure 44 A). The control constructs displayed consistently low activity below 5. PCC1 and both closely related proteins were strongly expressed as baits *in planta* (Figure 44 B).

In the complementary approach, using At3g22235 as bait, only PCC1 showed a statistical difference to AGB1 with a luciferase activity of around 10 (Figure 44 C). At3g22235 and At3g22240 had median activities at similar levels but were not significant. When At3g22240 was used as bait, PCC1, At3g22235, and At3g22240 showed increased luciferase activity (median ~15) compared to the negative control AGB1, though the difference was statistically significant for the former two proteins only at $P < 0.05$ (Figure 44 D). All constructs were expressed, though the bait protein was generally more strongly expressed compared to the prey versions (Figure 44 E). Altogether, these experiments suggest that PCC1, At3g22235, and At3g22240 likely form homo- and heterodimers *in planta*.

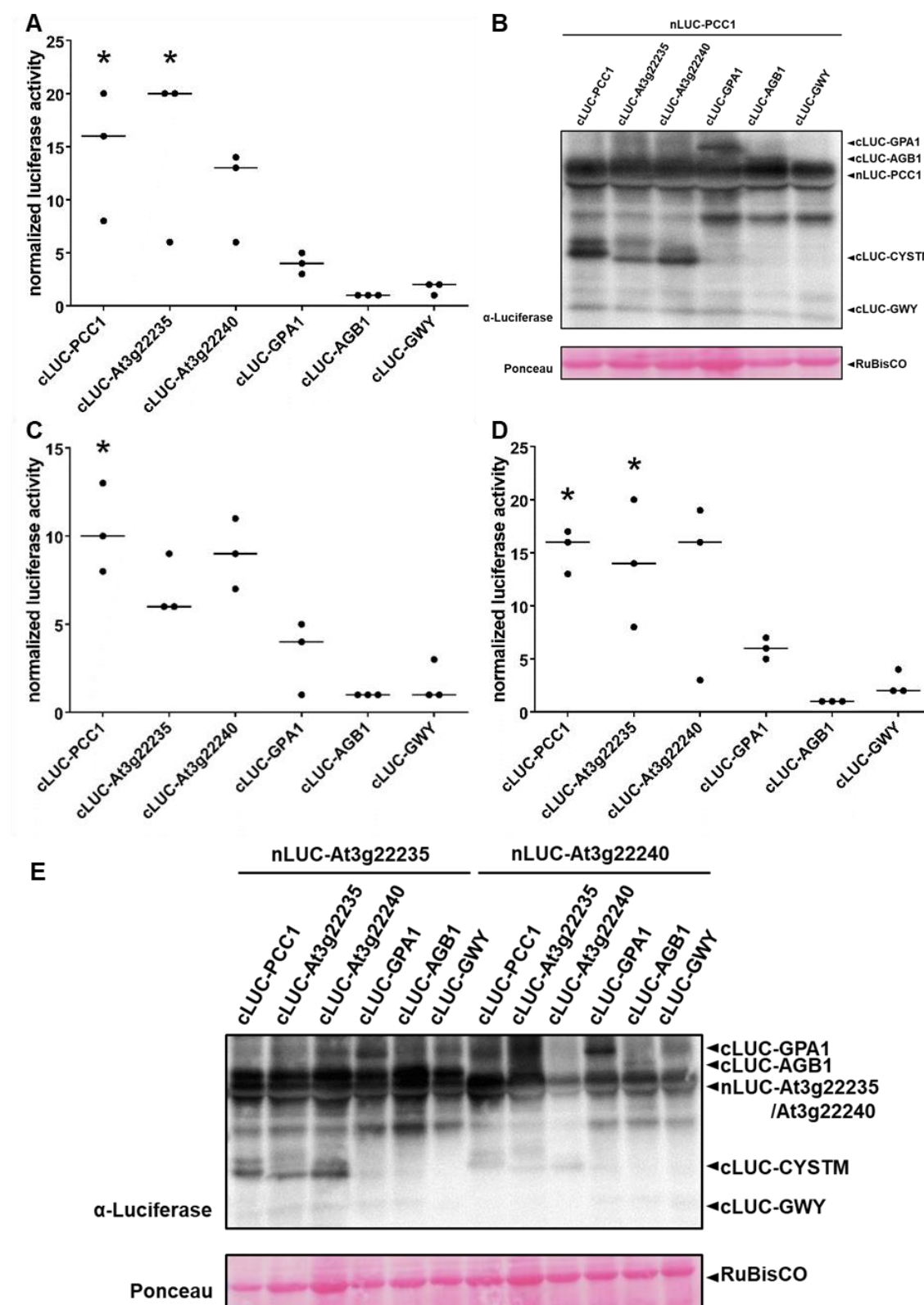


Figure 44: Split-luciferase assay to test for homo- and heterodimerization of PCC1 with itself and its closest homologs. (A/C/D) The split-luciferase assay allows the quantification of *in planta* protein-protein interaction. Agrobacteria with the fusion protein encoded on plasmids were infiltrated in *N. benthamiana* and incubated for two days. In total, eight leaf discs (four discs from 2 leaves each) of each combination were measured. D-Luciferin was infiltrated as a substrate before the measurement. The mean value of the leaf discs after 5 min of measurement was normalised with the negative control AGB1. (A) depicts the split-luciferase assay of PCC1, (C) the assay with At3g22235 and (D) the assay with At3g22240. The leaf discs were collected for total protein extraction and western blot analysis. The

polyclonal anti-luciferase antibody is capable of detecting both the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase fusion constructs are: nLUC-PCC1: 56 kDa; nLUC-At3g22235: 55 kDa; nLUC-At3g22240: 55 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235: 27 kDa; cLUC-At3g22240: 27 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa. The mean values were normalised with the negative control AGB1. The Kruskal-Wallis test followed by a Dunn's post-hoc multiple comparison test was used to test for statistically significant differences. Each group was compared to the negative control (AGB1), which was set to 1. The asterisks indicate the following p-values: *= $P \leq 0.05$. The horizontal bar marks the median. This experiment was repeated in three independent biological replicates. Full-size western blot is attached in the supplement (Supplementary Figure 27+28).

Investigation of the biological relevance of the PCC1 interaction

Since the protein interaction studies suggest that PCC1 and its closest paralogs associate with PRRs, i.e. CERK1, LYK4, LYK5, FLS2, EFR, and BAK1, the clade I CYSTM proteins may be involved in MAMP-triggered immunity in Arabidopsis. For PCC1 knock-down RNAi lines and a PCC1 overexpression line, different pathogen phenotypes were described. For example, the PCC1 overexpression line is more resistant to the oomycete *Hyaloperonospora parasitica* (Sauerbrunn & Schlaich, 2004). The RNAi lines are more resistant against *Botrytis cinerea* and more susceptible to *Phytophthora brassicae* (Mir et al., 2013). An early response to the recognition of elicitors such as chitin or the flagellin epitope flg22 is the accumulation of reactive oxygen species (ROS burst). Therefore, I acquired two GABI-Kat T-DNA insertion lines, one of which (GABI_821C03) carries a T-DNA insertion in the promoter region, and the other (GABI_528H09) has a T-DNA insertion in the second exon (Figure 45). For 821C03, a second T-DNA insertion was reported on the GABI-Kat homepage. The presence of all T-DNA insertions was tested by PCR genotyping (Alonso et al., 2003) and only the T-DNAs in the gene PCC1 were identified (Supplementary Figure 29). I determined the transcript accumulation of PCC1 by RT-PCR (Supplementary Figure 30) and found the highest transcript accumulation for the wild type Col-0. The line 821C03 line, which will be called PCC1^{KD} (KD: knock-down), displayed weaker PCC1 expression than in Col-0, while no transcript was detected for the line 528H09. It was also not possible to amplify the full-length PCC1 ORF from cDNA obtained from the line 528H09, indicating that this line is a true knock-out of PCC1, called PCC1^{KO} in the following (Supplementary Figure 31). The neighbouring closely related genes of PCC1 had no T-DNA insertions (Supplementary Figure 32).



Figure 45: Schematic gene model of *PCC1*. In the schematic gene model of *PCC1*, the T-DNA insertion sites are indicated by the triangles. All T-DNA mutants are Gabi-Kat lines. 821C03 lies in the promoter region; it knocks down the expression. 528H09 lies within the gene and disrupts the expression, and a full-length transcript is no longer produced.

Then, I performed ROS burst assays using the elicitors chitin, flg22, and elf18 (Figure 46). In case of chitin trigger, leaf discs from Col-0 wild type plants showed a peak of ROS accumulation between 10 and 20 min after the addition of chitin. The *cerk1-2* mutant is insensitive to chitin because CERK1 is missing (Miya et al., 2007) and was used as a negative control. Col-0 and *PCC1*^{KO} exhibited the same ROS response curve (peak at more than 15,000 RLU). The mutant *PCC1*^{KD} showed a reduced ROS burst response of ca. 5,000 RLU at the peak. The ROS formation is around one-third of that of the wild type (Figure 46 A).

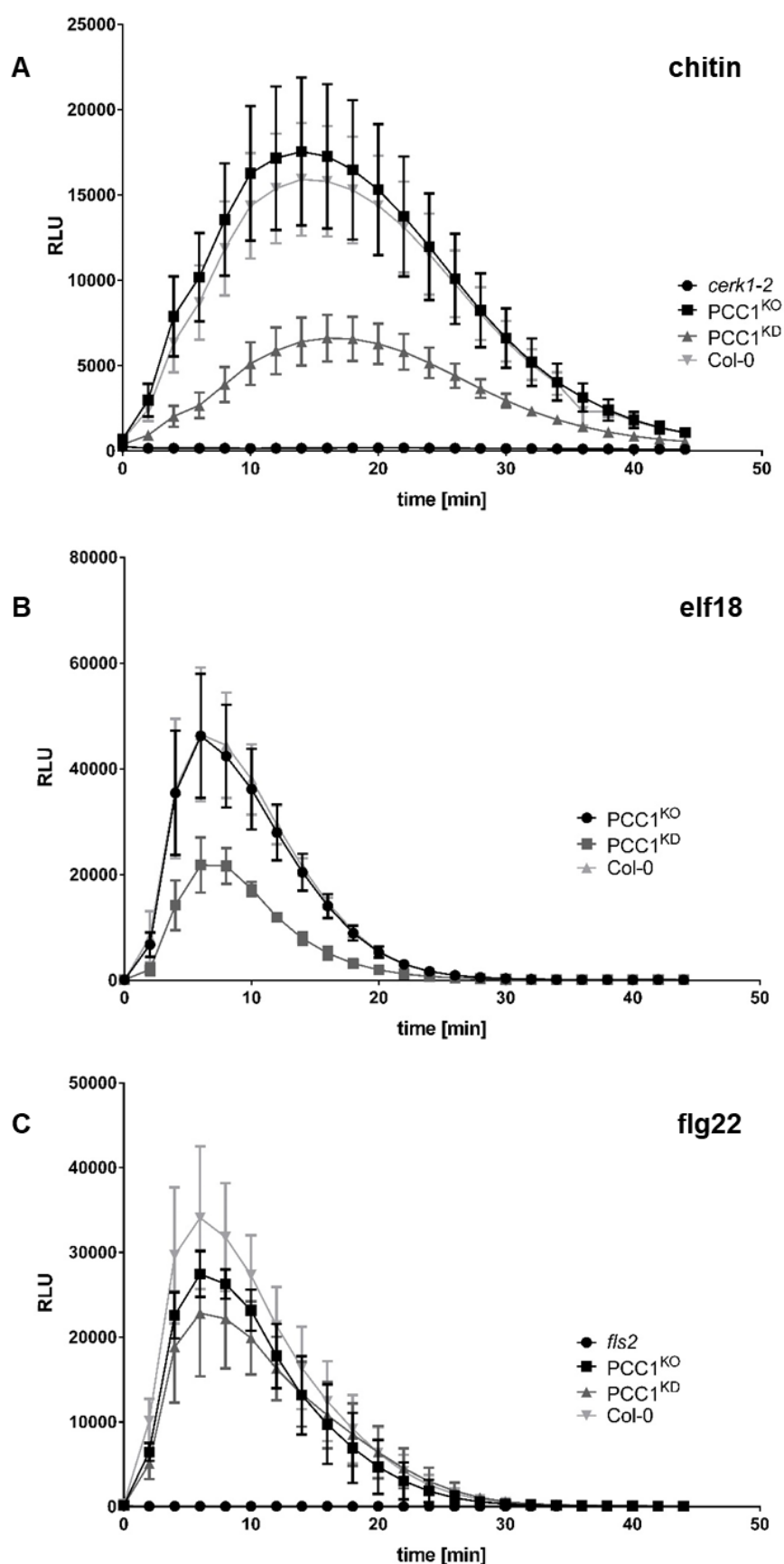


Figure 46: Luminescence-based measurement of ROS burst formation after chitin, elf18 and flg22 trigger in Arabidopsis leaf discs. Twelve leaf discs were prepared from 6-7week old Arabidopsis plants one day before the analysis and stored in 96-well plates with 200 μ l sterile water. The next day

the water was replaced with 50 µl sterile water. After 30 min of rest to ensure no measurement of other responses, 50 µl of the mix of (A) 200 µg/ml chitin, (B) 2 µM elf18, (C) 2 µM flg22 and 400 µM Luminol, 20 µg/ml peroxidase was added in 2-sec intervals to each well. The plate was measured with a luminometer for 45 min with a cycle time of 2 min and measurement of 2 sec per well. The experiment was repeated in four independent biological replicates. Error bars indicate standard error. The response was measured in relative light units (RLU) over time (min). The GABI-KAT lines are depicted as followed: PCC1^{KD} = GABI_821C03 and PCC1^{KO} = GABI_528H09.

I then triggered both mutants, the wild type Col-0 and an *fls2* mutant with flg22. The mutant *fls2* lacks the FLS2 receptor and is insensitive to flg22 (Gómez-Gómez & Boller, 2000). The peak of the ROS burst appeared at around 6-8 min (Figure 46 C). The Col-0 wild type and the two PCC1 T-DNA insertion lines exhibited similar ROS accumulation, with peaks between 20,000 and 30,000 RLU, suggesting no difference between the genotypes. I also used elf18, perceived by the receptor EFR (Zipfel et al., 2006), to test the ROS response (Figure 46 B). Similar to flg22 trigger, the peak was observed around 6-8 min after the trigger. I observed a similar reaction, as seen in the chitin-challenged samples. Col-0 and PCC1^{KO} showed a near-identical response with a peak at almost 50,000 RLU; the line PCC1^{KD} showed a reduced response with a peak at 20,000 RLU. In conclusion, PCC1^{KO} exhibited the same ROS accumulation pattern as the wild type, while the knock-down line PCC1^{KD} showed a reduced ROS peak upon chitin and elf18 trigger. Hence, it is unlikely that PCC1 knock-out plants are affected in MAMP-triggered ROS burst elicitation.

Discussion:

Until now, mostly other kinases or receptor-like proteins were identified as interaction partners of CERK1 either to bind MAMPs cooperatively or to transmit the phosphorylation signal to start a signalling cascade. Examples of cooperative binding are OsCEBiP and AtLYK5, which show a higher chitin-binding affinity than CERK1 but possess no kinase or a not functional kinase domain. CERK1 forms a complex with these receptors to transmit the binding of chitin by phosphorylation reactions into a cellular response (Cao et al., 2014, Gong et al., 2017, Kaku et al., 2006, Shimizu et al., 2010, Shinya et al., 2012). Another example is PBL27, a receptor-like cytoplasmic kinase, which is activated through phosphorylation by CERK1 and then activates the MAPK signalling pathway (Shinya et al., 2014, Yamada et al., 2016). CIPP1 is a phosphatase involved in the downregulation of chitin-triggered signalling by dephosphorylation of the kinase domain of CERK1 (Liu et al., 2018a). Other described interaction partners are involved in different signalling pathway like GPA1 (G α subunit), AGG1/AGG2 (G γ subunit) (Aranda-Sicilia et al., 2015) and ANN1 (Espinoza et al., 2017). ANN1, a NaCl-induced calcium channel, is not phosphorylated by CERK1, but an *ann1* mutant depicts a reduced calcium influx after chitin-trigger and an elevation in ROS formation and stronger MAPK activation (Espinoza et al., 2017). Heterotrimeric G protein subunits can interact with different RLKs and are targets of these receptors. Heterotrimeric G proteins are signalling components, which can activate other protein. For example, XLG2 (non-canonical G α subunit) is phosphorylated by BIK1 and activates RBOHD (Liang et al., 2016). The exact function of the interactions of GPA1, AGG1/AGG2 and ANN1 is still unclear.

This study aimed to identify new interaction partners of CERK1 by the use of a LexA-based split-ubiquitin Y2H system. The screen resulted in a list of potential candidates, and I selected PCC1, CaM7 and AHA1/2 for a further investigation, because other studies reported for these proteins/protein families an involvement in defence (Frei dit Frey et al., 2012, Kim et al., 2002, Liu et al., 2009, Mir et al., 2013, Sauerbrunn & Schlaich, 2004). Most of the other potential candidates were filtered out, because of either their predicted localisation to different compartments than CERK1, which would make interactions impossible to occur *in planta* or a function not connected to plant immunity. I could also identify the known interaction partners GPA1 and ANN1 in the screen.

I could show that *HvCaM3* (substituted *AtCaMs*), *PCC1* and *AHA2* can interact with CERK1 in split-ubiquitin Y2H assays and *in planta* split-luciferase experiments (Figure 22 – 24). Calmodulin is a well-known sensor in calcium signalling in plants that binds Ca^{+2} , undergoes conformational changes and modulates the activity of other proteins (Aldon et al., 2018, Cheval et al., 2013, Day et al., 2002, McCormack & Braam, 2003, Snedden & Fromm, 2001). As signal transducer, CaMs play an essential role in physiological processes, but also in abiotic and biotic stress responses (Aldon et al., 2018, Ranty et al., 2006, Yuan et al., 2017, Zeng et al., 2015, Zhang et al., 2014). The importance of CaMs as interaction partners can be seen in the interactions with *PEN3*, an ABC transporter that is involved in nonhost resistance. *PEN3* interacts with *CaM7*, and a *cam7* mutant shows compromised immunity against the non-host pathogens *Phakopsora pachyrhizi* and *Bgh* (Campe et al., 2016). Another example is *HvCaM3*, which can bind to the C-terminus of *HvMLO* and is needed for its function (Bhat et al., 2005, Kim et al., 2002). Another calmodulin, namely *CaM2*, was earlier reported to interact with CERK1 in a classical Y2H screen, but this interaction was not further validated (Le et al., 2014). These reports suggest that the interaction could be meaningful in the context of plant immunity, but additional investigation is needed to solve the function of this interaction. Questions that could be explored in future are for example if CaM is a phosphorylation target of CERK1 since CaMs can be phosphorylated, which modulate its binding activity to other proteins (Benaim & Villalobo, 2002, Sacks et al., 1996) or if CaM regulates the activity of CERK1. It was reported that the phytosulfokine receptor *PSKR1* (PHYTOSULFOKINE RECEPTOR-LIKE KINASE1), which is involved in the regulation of growth responses, can bind CaMs at the kinase domain. A mutation in the CaM-binding site results in a kinase-inactive phenotype, which proves the potential significance of this interaction (Hartmann et al., 2014, Zhang et al., 2018a). The *BRI1* kinase (BRASSINOSTEROID-INSENSITIVE 1) is also capable of binding CaM *in vitro*. Calmodulin binding reduces the auto and transphosphorylation activity of *BRI* (Oh et al., 2012). Whether CaM is a target or a regulator of CERK1 remains unknown. Another question will be if there are differences in the specificity of the various near-identical CaMs? However, since I could substitute *AtCaM7* with *HvCaM3* in the assays with the same results, the investigation of the binding specificity of the so similar CaMs (at least 97% identical on amino acid level) could be challenging. Especially the overexpression of the proteins in these assays could force them to interact, limiting the specificity of these assays to a level

that would make differences, if they exist, undetectable. No binding specificity was reported in the PSKR1/CaM interaction; all tested CaMs could interact with the receptor (Hartmann et al., 2014). Whether this also happens under normal expression level, needs to be investigated. When CERK1 possesses a CaM-binding site, then a mutated version of this binding motif could be transformed into the *cerk1* mutant background, and this complemented line could be tested for normal MAMP-triggered immunity responses like ROS formation, calcium influx and MAPK activation. Altered responses would suggest an involvement of CaM-binding in the regulation of CERK1. To answer the question, if CaM is a phosphorylation target of CERK1, *in vitro* kinase assays with heterologously expressed CERK1 kinase and CaMs could be tested.

AHAs are ten transmembrane domain-containing proteins (Pedersen et al., 2007), which pump protons into the extracellular space under ATP consumption. This built up an electrical and chemical gradient. The potential is used for transport processes over the membrane, and therefore AHAs are important for essential physiological processes like growth and development. The lethality of an AHA1/2 double mutant underlines the importance of these proteins (Haruta et al., 2010). A following up question would be if AHA1 can also interact with CERK1, and what the function of this interaction would be? I was not able to clone this candidate, which were found in the screen, but the close homologue AHA2. It is known for AHA2 that a regulatory element in the C-terminus can be phosphorylated. Specific phosphorylation sites can increase the activity, whereas others decrease the activity (Haruta et al., 2015). A fragment, containing 315 amino acids of the C-terminus of AHA1 was identified in the screen, and this fragment contains the regulatory phosphorylation sites. A possible role for AHA1/2 could be the regulation of the alkalinisation of the extracellular space, which is one response to the detection of plant pathogens (Elmore & Coaker, 2011). For example, the RALPH (RAPID ALKALINIZATION FACTOR) receptor FERONIA (Fer) phosphorylates Ser899 of AHA2, which results in inactivation and the alkalinization of the extracellular space (Haruta et al., 2014). Experiments with a constitutively active version of AHA1 showed that the ROS burst is reduced after the challenge with flg22 (Keinath et al., 2010). AHA1 and AHA2 undergo changes in their phosphorylation status after the recognition of flg22, which is another indication of a role in MTI (Benschop et al., 2007, Nuhse et al., 2007). AHA1 and AHA2 are also able to interact with CNGC17 (CYCLIC NUCLEOTIDE-GATED CHANNEL) and form a complex with BAK1 and PSKR1 (PHYTOSULFOKINE (PSK) LEUCINE-RICH REPEAT RECEPTOR

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KINASE 1) (Ladwig et al., 2015). Since AHAs are regulated through phosphorylation and show a response after flg22-trigger, it is also possible that CERK1 could regulate AHAs. AHA1 and AHA2 can act redundantly and a double mutant is lethal (Haruta et al., 2010), which make knock-out mutants not a promising choice to explore this question. The constitutively active version of AHA1 could be interesting to test after chitin-trigger, if this mutant shows differences in the ROS formation. The C-terminus of AHA1/2 (with the phosphorylation sites) and the CERK1 kinase domain could be tested in *in vitro* kinase assay, if CERK1 can in principle phosphorylate AHAs or if these interaction takes place, because both proteins are membrane localized. The reported results of other studies support the possible interaction of CERK1 with AHA1/2, whereby CERK1 could regulate AHAs activity.

Taken together, I could validate new interaction partners of CERK1, namely AHA2, PCC1 and CaM in yeast and *in planta*, although it needs to be tested, if also AHA1 and other Arabidopsis CaMs can interact with CERK1. I will discuss the interaction of CERK1/PCC1 in the following section in more detail. The biological function of these interactions remain unknown but opens the field for further research. The power of the chosen split-ubiquitin Y2H system in combination with the used cDNA library was further shown since around 10% of the selected colonies harboured already known or potential new interaction partner of CERK1. Two independent methods are considered to be enough to assume an interaction. In the best case, the interaction can also be observed in a third independent system like BiFC, Co-IP or MST (Jerabek-Willemsen et al., 2014, Lee, 2007, Xing et al., 2016). This should be done for these interactions in the future to support the findings even more.

Interaction of PCC1 and CYSTM members with RLKs involved in plant immunity

I identified and validated the interaction of CERK1/PCC1 and continued with PCC1 for further investigation since pathogen-related phenotypes were reported (Mir et al., 2013, Sauerbrunn & Schlaich, 2004). PCC1 was discovered since it displayed an up-regulation after treatment of Arabidopsis plants with virulent and avirulent *P. syringae* strains. Northern blot and mutant line analysis also showed a circadian clock-dependent regulation of PCC1 with the highest expression at the end of the day, as well as SA dependency of this expression (Sauerbrunn & Schlaich, 2004). Overexpression and knock-down lines featured a more resistant or more susceptible phenotype compared to the wild type. A PCC1 overexpression line was more resistant

against the oomycete *Hyaloperonospora parasitica* (Sauerbrunn & Schlaich, 2004). A second study by Mir and colleges showed that RNAi lines (knock-down lines) were more susceptible to *Phytophthora brassicae* and more resistant against *Botrytis cinerea* (Mir et al., 2013). PCC1 belongs to a protein family in eukaryotes that is associated with biotic and abiotic stress response or tolerance. For example, CDT1 (CADMIUM TOLERANCE 1) confers tolerance to cadmium in *Digitaria ciliaris* and *Oryza sativa* (Kuramata et al., 2009) and the genes *YDL012C* and *YDR210W* from *Saccharomyces cerevisiae* and *Uvi15* from *Schizosaccharomyces pombe* are involved in the resistance to various substances (Andreeva et al., 2017, Lee et al., 1995, Smith et al., 1999). Another example is CYSTM3 in Arabidopsis, which is a negative regulator of salt tolerance (Xu et al., 2019). This group of proteins possesses a specific domain, a cysteine-rich transmembrane domain (CYSTM) that is located in the C-terminal region (Venancio & Aravind, 2010). The role in biotic and abiotic stress response in eukaryotes qualifies the PCC1 family as interesting candidates for further analysis.

I tested other CYSTM family members for the exploration of the interaction specificity of CERK1 with PCC1, namely At2g32190, At2g32200, At2g32210 (clade I b), At3g22235 and At3g22240 (clade I a) (Figure 30). Thus, all tested CYSTMs seem to interact with CERK1 to different extents. According to the *in planta* data, PCC1 and the two closest homologs, At3g22240 (CYSTM9) and At3g22235 (CYSTM8), interact the strongest with CERK1. All CYSTM members of clade I share the same motif (VET[N/K]SKG) (Figure 30) that is possibly required for the interaction of PCC1 with CERK1 (Figure 28). Experiments with truncated PCC1 versions (Figure 26-28) support this finding, since the first 35 amino acids of PCC1 are dispensable for the interaction and the C-terminus (transmembrane domain; 55-81 aa) alone is not capable of interacting with CERK1. The VET[N/K]SKG motif lies within the region that can interact with CERK1 and is located before the transmembrane area. The stronger interaction of clade I a CYSTMs may be due to the amino acids directly in front of this motif (VET[N/K]SKG), where clade I a CYSTMs harbour GDPPAAA, while clade I b have SHATVA[X] and CYSTMs of clade II do not have the motif VET[N/K]SKG. These different residues in front of the motif may affect the structure and properties of this region, affecting the stability or strength of the interaction (Planas-Iglesias et al., 2013). The interaction strength of clade I b members is around the half of clade I a members, which could imply that only a part of the necessary interaction domain is present in clade I b. Prediction tools for secondary structures and disordered region revealed that

possibly the major part of PCC1 (except the transmembrane domain) is a disordered region, which indicates that this protein undergoes structural changes after a stimulus. Disordered regions are often responsible for the binding of molecules or other proteins (Dyson, 2016, Uversky, 2015) and could be necessary for the interaction with RLKs. Taken together, the data suggest that a region within amino acid 36-59 is responsible for the interaction with CERK1. The test of clade II members could clarify if this motif is necessary for the interaction because these members lack this motif. Afterwards, the exchange of this region with the motif of clade I b or clade II members in PCC1 could be tested to abolish the interaction.

I also tested LYK5, LYK4, FLS2, EFR and BAK1 against PCC1, At3g22235 and At3g22240 as a *vice versa* experiment of specificity. All three CYSTMs could interact to the same extent in *in planta* experiments with the RLKs. The only exception was present in the interaction with BAK1, where At3g22240 showed a stronger interaction. Some split-luciferase experiments show a high variation to draw a robust conclusion. More replicates are necessary to solve this problem. All these RLKs are involved in plant immunity. The next step would be to test RLKs, which have no connection to plant immunity if they also show interaction with PCC1. For example, the brassinosteroid receptor BRI (BRASSINOSTEROID INSENSITIVE 1) (Li & Chory, 1997) or the phytosulfokine (PSK) receptor 1 (PSKR1) (Wang et al., 2015), which are involved in plant growth regulation, could be tested for interaction. It would be crucial to identify common motifs in the kinase domains to pin down the interaction site of CERK1. These sites could be truncated and tested in yeast and split-luciferase assays.

Mir and colleagues (Mir & Leon, 2014) and Xu *et al.* (Xu et al., 2018) showed that the transmembrane domain or the C-terminus with the conserved cysteine residues are important for the dimer formation (homo and heterodimers). I tested the homo- and heterodimerisation of PCC1, At3g22235 and At3g22240 and all combinations indicate an interaction, even though not always statistically significant in all orientations. These experiments have to be further validated in other protein-protein interaction methods, like BiFC or Co-IP, to confirm these interactions because this experiment is only a hint in the direction of dimerisation. I did not use the split-ubiquitin system, like in Xu *et al.* (Xu et al., 2018), because the bait vector requires a C-terminal tagging, which could block the plasma membrane localisation of PCC1 and could lead to false positives or negatives. It would also be interesting to test if the CYSTM family members form higher

forms of complexes than dimers and if PCC1 forms heterodimers with other CYSTM members. A further question will be if the dimerization is necessary for the interaction with RLKs.

The conserved cysteine residues inside the transmembrane domain are uncommon because usually, only hydrophobic amino acids are conserved in this kind of region (Von Heijne, 2007). Already Venancio and Aravind suggested an enzymatic or ligand-binding function of these conserved cysteines (Venancio & Aravind, 2010). It is also not clear if all CYSTM members possess a transmembrane domain (Figure 30), but the conserved cysteines are always present in the same region at the C-terminus. Depending on the algorithm, no transmembrane domain is predicted for some CYSTM family members, like At3g22235. This could suggest that a plasma membrane localisation for some CYSTM members is not necessary for their function. Cysteines are known for forming disulfide bonds, either intramolecular or intermolecular and that the formation of these bonds is dependent on the redox state (Nagahara, 2011). Together with the predicted redox sensitivity of the protein (Figure 29), this evidence suggests in combination with the overall disordered structure of PCC1 a structural change after redox alteration. Venancio and Aravind proposed a potential alteration of the redox potential of the membrane by CYSTM members, which could chelate metal ions since At1g52827 (CYSTM2) is involved in cadmium resistance (Kuramata et al., 2009, Venancio & Aravind, 2010). Another possibility would be that PCC1 forms in a more oxidising environment, like after a ROS burst in the apoplast, intra or intermolecular disulphide bonds to form or disrupt dimers. No enzymatic or signalling activity is reported so far for CYSTM members, activation or repression upon redox stimulus classify this family as a redox responsive protein/sensor (Nagahara, 2011, Spoel & van Ooijen, 2014). An approach to test this hypothesis could be the exchange of the conserved cysteines and the complementation of a mutant line, which show a phenotype and check for alteration in this phenotype. It was reported that the cysteines do not affect the interaction of other CYSTM members in split-ubiquitin and split-luciferase assays. Xu and colleagues tested CYSTM3 (At1g56060), exchanged the cysteine residues systematically to serines and could not see a difference in interaction (Xu et al., 2019). To test, if disulfide bonds are formed and if they are either intramolecular or intermolecular, PCC1 could be expressed *in planta* with a protein tag. The cell lysate can be treated with and without reducing agents and separated on a SDS-PAGE/Western Blot. When intermolecular bonds are formed, then the band

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should shift to a higher mass, and by intramolecular bonds, the band should shift to a lower mass, since the molecule is smaller and can run faster through the gel.

Fluorophore-tagged versions would also be helpful to investigate if localisation changes appear after a stimulus and to test heterodimer formation with FRET. The advantage of FRET is the live cell imaging with no stabilisation of interaction compared to BiFC. A subcellular localisation change could be possible since CYSTM3 showed a cytoplasmatic localisation in *N. benthamiana* and localisation in the mitochondria in Arabidopsis protoplast. This could be due to the different expression system or due to the different conditions, which could trigger a different localisation (Xu et al., 2018, Xu et al., 2019). Taken together, the conserved cysteine residues possess probable a critical function, which could be connected to redox changes. These cysteines have further no influence on the dimer formation of CYSTM members. The split-luciferase assays also suggest a dimerization of PCC1 and its close homologues.

Additionally, to the interaction with RLKs and the formation of at least dimers, I observed double bands on western blots with PCC1, which would indicate post-translational modification of PCC1 (Figure 28). Phosphorylation is the most common modification (Khoury et al., 2011) and PCC1 possesses predicted and known phosphorylation sites in the N-terminal region (Figure 25). When PCC1 was truncated in the N-terminus, the double band disappeared. As additional support shows the IUPred2A prediction of the disordered binding site (Figure 29, blue graph) that the extreme N-terminus (1-12 aa) harbours such a binding site, what is an additional hint for phosphorylation sites (Mészáros et al., 2018). Phosphorylation sites would classify PCC1 as a potential target of RLKs. Phosphorylation of proteins can alter the conformation, which is again a connection to the disordered regions in PCC1. This conformational change can activate or deactivate protein function. One example of activation is PBL27, which is phosphorylated by CERK1 and then activates a MAPK cascade (Shinya et al., 2014, Yamada et al., 2016). If the potential conformational changes of a redox stimulus effect the possible phosphorylation or *vice versa* need to be evaluated. Another option would be that both changes are independent of each other and have different functions. Nevertheless, the N-terminal part is dispensable for the interaction with CERK1 but could have regulatory functions. PCC1 could be expressed in *N. benthamiana* as tagged version, and the protein extract could be treated with and without phosphatases to validate the phosphorylation. When the

double band disappears, then it is an indication of phosphorylation. For further investigation, *in vitro* kinase experiments with mutated PCC1 versions could validate, which amino acids are phosphorylated and if there is any specificity of the kinase domains of different RLKs or kinases in general because the double band in the split-luciferase assays can also be seen when no RLK is co-expressed. This could indicate unspecific phosphorylation since PCC1 is transiently overexpressed in *N. benthamiana* and the total protein is extracted. At this point, also other kinases could phosphorylate PCC1. I also used a kinase death version of CERK1 because an active kinase would lead to cell death in *N. benthamiana* (Pietraszewska-Bogiel et al., 2013). Further, LYK5 possesses a not functional kinase (Cao et al., 2014), which indicate that both RLKs are not responsible for the phosphorylation that is observed in these assays. In the end, complemented mutant lines with different mutations in the N-terminus that block, or mimic phosphorylation would be interesting if they influence PCC1 activity, like the reported pathogen phenotypes (Mir et al., 2013, Sauerbrunn & Schlaich, 2004).

Complex expression network of the CYSTM family

I extracted the expression data of different tissues and under biotic stresses from the eFP browser database to investigate the overall expression patterns of this family compared with *PCC1*. Unfortunately, for *At3g22235*, *At2g32200* and *At1g52827*, no data were available. Especially for *At3g22235*, the closest homolog of *PCC1*, expression data would be interesting (Figure 31+32). I also extracted the top 300 co-expressed genes from Atted-II for all available CYSTM members and performed a GO-term enrichment analysis for *PCC1*, *At3g22240* and *At1g05340*. The co-expression data were obtained for nine of the fourteen members; unfortunately, for the closest homolog *At3g22235* of *PCC1*, co-expression data were not available (Figure 33-36). Tissue-wise *PCC1* and *At3g22240* are ubiquitously expressed, except in pollen, shoot apex, root and some seed stages, but the greatest fold change is in leaf tissue. By contrast, other members have their expression fold change maxima in some seed stages or the root. A common observation is that individual members show great relative fold changes in the same specific tissues, like clade I b members in seed stages, which could support a possible heterodimer formation of these proteins and a potential overlapping function. This observation is further visible in the reaction after different biotic stresses that certain family members behave the same way. The physical heterodimer formation of different CYSTM members was shown by Xu *et al.* (Xu et al., 2018). Another observation is the downregulation of mostly all members of

the CYSTM family in the shoot apex material, which suggests that the CYSTM family would negatively interfere in this developmental process. Some of the CYSTM members also show an overlap in co-expressed genes, which is another indication that they could act in the same pathways or have an overlapping function, but for 1/3 of the genes are no co-expression data available to draw a more definite conclusion. For example, *PCC1* and *At3g22240* are co-expressed and share 67/300 co-expressed genes, and the GO-term analysis (Figure 34+36) revealed an enrichment in defence-related and SA responsive genes, like it was seen in the *PCC1* GO-term enrichment. A SA dependency of *PCC1* expression was already demonstrated in a *NahG* Arabidopsis mutant (Delaney et al., 1994), which expresses a salicylate hydroxylase to degrade SA *in planta*. This mutant could not accumulate *PCC1* transcript (Sauerbrunn & Schlaich, 2004). SA is also closely linked to systemic acquired resistance (SAR) (Gruner et al., 2013) and in general with defence (Dempsey & Klessig, 2017). Under the co-expressed genes are defence involved genes like *EDS1*, *NPR4*, *PR1*, *PR2*, *PR5* (Antoniw et al., 1980, Falk et al., 1999, Fu et al., 2012, Van Loon, 1982). When the GO-terms of biological function were evaluated in the set of *PCC1* and *PCC1/At3g22240* co-expressed genes, then GO-terms were enriched, which show a connection to kinases and receptor function. This fact is also another hint in the direction of interaction with RLKs since RLKs and *PCC1* are co-regulated. Additionally, *CERK1* is also under the top 300 co-expressed genes of *At3g22240*, which further support the relevance of the interactions. The overlap of co-expressed genes is not only within a clade visible, because *PCC1* and *At1g56060*, a clade II member, show an overlap of forty one genes and *At3g22240* has further overlapping genes with *At5g04080* (19 genes), *At5g67600* (29 genes), *At1g56060* (10 genes) and *At1g05340* (19 genes). These results fit as well in the assumption of heterodimer formation or functional overlap since *At1g56060* is also expressed in leaf tissue, but to lesser extent. The assumption that all members of one clade show an overlap in co-expressed genes is not the case, because *PCC1* and *At1g05340* revealed no overlap and the GO-terms of *At1g05340* depict rather an involvement in abiotic stress response.

Interestingly, when I looked at the expression data under biotic stress, then no upregulation with *P. syringae* can be seen for *PCC1* (Figure 32) as it was described by Sauerbrunn and Schlaich (Sauerbrunn & Schlaich, 2004) with their northern blot analysis. They also report a transcript increase in mock-treated samples, which may

account for these conflicting results, because the mock-treated sample is used to calculate the relative expression. For the eFP browser data, it was also not indicated when the samples were taken, since the expression of *PCC1* increases over the day until it reaches highest expression levels at the end of the day (Sauerbrunn & Schlaich, 2004). This makes the comparison between the different experiments difficult. *PCC1* and *At3g22240* show high basal relative expression in leaves, which could mask any changes. A clear upregulation is visible in both genes after oomycete infection (after 24 h). Other CYSTM members also show an upregulation after treatment with different pathogens. They are generally expressed in much lower levels so that the upregulation appears more dramatically. Surprisingly almost every CYSTM member shows differential expression for specific treatments. *CYSTM3* (*At1g56060*) was reported as a negative regulator of salt stress tolerance since overexpression lines prevent Na⁺ efflux and inhibit the activities of a range of ROS scavenging enzymes (Xu et al., 2019), but this member revealed also upregulation after *Botrytis cinerea*, *Pseudomonas syringae* and *Phytophthora infestans* treatment. For this protein family an involvement in different kind of stress responses, biotic and abiotic stresses is reported (Andreeva et al., 2017, Kuramata et al., 2009, Lee et al., 1995, Smith et al., 1999) and the expression pattern after biotic stress further supports the assumption of heterodimerisation and functional overlap, since specific CYSTM members show an upregulation after the same stimuli. This assumption is further supported by the promoter regions of the CYSTM family investigated by Xu et al. (Xu et al., 2018), which showed different response elements for various stress stimuli. In summary, the results suggest that certain family members, like *PCC1* and *At3g22240*, act in the same pathway because they show an overlap in co-regulated genes and they are expressed in the same tissue or under the same stress. The data suggest further an involvement of different CYSTM in biotic stress response.

First, it could be interesting to test if *PCC1* and *At3g22240* mutants show a SAR phenotype since both are co-regulated with SAR genes. *At3g22240* mutants have not been tested for any pathogen phenotype, which could also be informative. The generation of double mutants could be difficult due to the close genomic localisation of *PCC1* and *At3g22240*. A CRISPR/Cas9 approach could be used to generate double or higher orders of mutant lines (Wu et al., 2018). Overall it would be interesting to examine for all CYSTM mutants, which show a differential regulation upon pathogen

treatment, if they are altered in the ROS response or in a pathogen phenotype in general, like the PCC1^{KD} mutant (Figure 46).

PCC1 involved in ROS response?

The ROS burst is one of the earliest measurable responses after the recognition of a MAMP and initiated after the Ca²⁺ influx. ROS serves, among other tasks as a signalling mechanism in plants (Miller et al., 2009, Mittler et al., 2011). Some studies in yeast and rice suggest that the CYSTM family has a metal ion-binding function at the C-terminus and therefore chelate metal ions, which could also influence the redox state in the extracellular space (Andreeva et al., 2017, Kuramata et al., 2009, Lee et al., 1995, Smith et al., 1999). The binding activity is not yet shown. Cysteine residues are not only necessary to bind metal ions but also to form disulfide bonds, which can react to/sense redox changes (Nagahara, 2011) or form protein structural relevant bonds. Another hint in the direction of ROS effects is the knock-out mutant of the CYSTM member At1g56060 (CYSTM3), which shows under salt stress conditions a reduced ROS production and an increase in enzymes that scavenge ROS molecules (Xu et al., 2019). I performed luminescence-based ROS measurements with two GABI-Kat mutants to test if PCC1 is involved in ROS response after pathogen recognition (Figure 46). No difference could be observed with the two mutant alleles and flg22 as a trigger (Figure 46), but with chitin and elf18, respectively, the ROS response was reduced in the PCC1^{KD}, but not in the PCC1^{KO} line. According to GABI-Kat, the line PCC1^{KD} that I obtained from the lab of Prof. Slusarenko (RWTH Aachen) should have a second T-DNA insertion in the promoter region of *At5g13920* (Zinc-finger containing protein). However, the second mutation is not present according to the genotyping (Supplementary Figure 32), which means it was either back-crossed or the T-DNA was sorted out during propagation and screening for homozygous lines. 821C03 (PCC1^{KD}) is a knock-down line because the T-DNA is inserted in the promoter region, and RT-PCR confirmed reduced transcript abundance (Figure 45 and Supplementary Figure 30). 528H09 (PCC1^{KO}) is a complete knock-out due to the insertion in the coding-region of PCC1 (Figure 45 and Supplementary Figure 31). An alternative explanation of this phenotype could be compensation of the total knock-out by a close relative of PCC1, which does not occur in the knock-down line, where the reduced transcript level may be sufficient to induce a phenotype (El-Brolosy & Stainier, 2017, Wilkinson, 2019). RT- or qRT-PCRs of the close homologs in these lines would be necessary to verify their altered expression to test this hypothesis. The results and other reports suggest

involvement of PCC1 in ROS formation, regulation or signalling, but a final conclusion could not be drawn since both GABI-Kat lines show different effects.

Are CYSTMs stress redox responsive proteins?

I constructed a hypothetical model of CYSTM function to explain the reported phenotypes and results (Figure 47). This gene family is not only present in plants but instead reported in a wide range of eukaryotes, like yeast and humans (Venancio & Aravind, 2010). Additionally, this family is involved in stress response/tolerance, either from abiotic or biotic stimuli (Andreeva et al., 2017, Kuramata et al., 2009, Lee et al., 1995, Sauerbrunn & Schlaich, 2004, Smith et al., 1999, Xu et al., 2018, Xu et al., 2019). These proteins possess a similar cysteine-rich motif in a transmembrane region at the C-terminus, which implies a conserved function. This assumption is further supported by the fact that transmembrane regions usually show conservation only of hydrophobic residues (Von Heijne, 2007). Additionally, cysteine residues are known for the formation of disulfide bonds, which react to redox changes (Nagahara, 2011) or they are involved in chelating metal ions. The appearance of this family in many eukaryotes also suggest a function in a conserved pathway. The results from Xu *et al.* with *At1g56060* (CYSTM3) (Xu et al., 2019), which showed an decrease in H₂O₂ and O₂^{·-} in the knock-out mutant and an increase in overexpression lines, and the reduction in the ROS burst in the PCC1^{KD} mutant in this study further point in the direction of ROS signalling/regulation. The *cystm3-2* mutant showed increased enzyme activity in ROS scavenging enzymes (catalase (CAT) and superoxide dismutase (SOD)) and ROS scavenging molecules (glutathione). The overexpression lines showed a decrease in these components. ROS is participating in many stress responses as signalling molecule (Baxter et al., 2013, Farnese et al., 2016, Torres et al., 2006) and could be one intersection to explain all different mutant phenotypes for PCC1, like the pathogen phenotypes, altered reaction of light stimuli and gibberellin and abscisic acid (Mir et al., 2013, Mir & Leon, 2014). Until now, a ROS receptor is also unknown, but it is discussed, if RLKs with a cysteine-rich extracellular domains could be responsible (Noctor & Foyer, 2016, Podgórska et al., 2017, Shapiguzov et al., 2012).

It is further known from Arabidopsis that the family members can form homo- and heterodimers, which could help to regulate the signals differently since the various phosphorylation sites at the N-terminus may have a regulatory function. Additionally, different members localise to different subcellular compartments, where they could

perceive ROS from different locations, and they show different expression patterns. Besides the tested RLKs, PCC1 also interacts with CSN5A and CSN5B (CSN: COP9 SIGNALOSOME) (Mir & Leon, 2014). They are subunits of the COP9 signalosome, which is a conserved multi subunits protein complex that has a central role in regulating the ubiquitin/26S proteasome pathway (Nezames & Deng, 2012). CSN5B interacts further with VTC1, an enzyme responsible for the ascorbic acid biosynthesis, which serves as an antioxidant. The interaction leads to the degradation of VTC1 and reduced ascorbic acid concentrations (Wang et al., 2013). Mutants of *vtc1* exhibit expression of PR genes and increased basal resistance to *Pseudomonas syringae* (Pavet et al., 2005). Xu et al. also reported changes in the ROS scavenging system. Other studies argue for the involvement in the ubiquitination pathway through the interaction with the CSN5 subunits in general, which could either bind CSN5 to block the complex formation or the induction of the complex formation at the plasma membrane to target plasma membrane proteins (Mir et al., 2013, Mir & Leon, 2014).

The circadian clock plays a vital role in the transcriptional regulation of developmental and defence-related processes. Through this regulation, phytohormones (like SA) and ROS productions show an oscillating rhythmic (Lai et al., 2012, Spoel & van Ooijen, 2014). PCC1 also shows a circadian clock regulation, which could indicate that the increase in expression over the day is essential for the function. The RT-PCR also revealed a higher transcript level than the actin control that indicates further the importance of this protein. Upon stress stimuli, the plants react with changes in the redox state and hormone changes, which result in a cellular response. These regulatory mechanisms can be influenced by the circadian clock (Spoel & van Ooijen, 2014).

Nevertheless, this is only a model, which need to be further validated by mutant analysis and complementation studies with modified protein versions to test the influence of different protein regions.

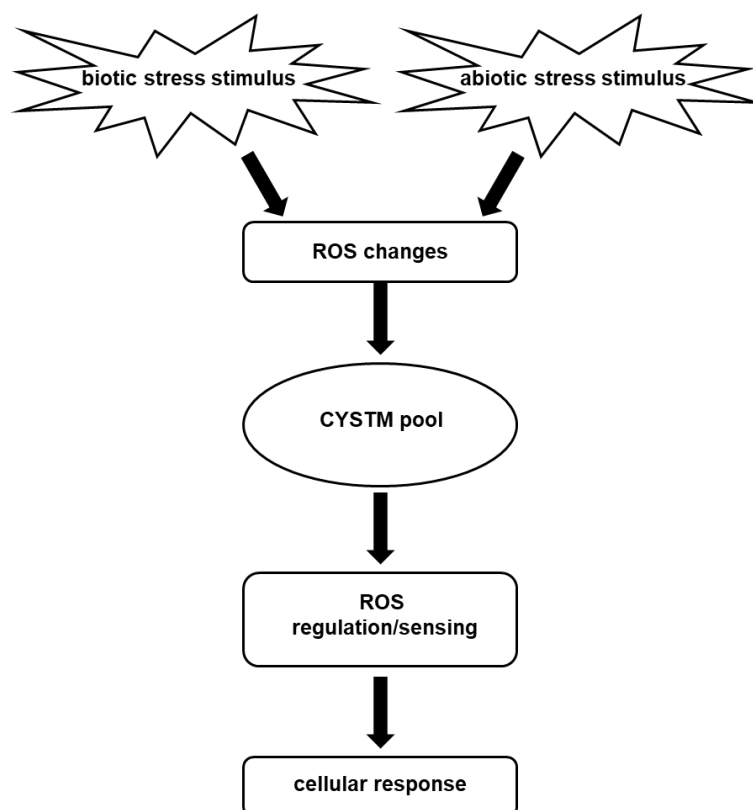


Figure 47: Model of CYSTM function. CYSTMs are stress involved (biotic and abiotic) proteins, which localised to different subcellular compartments and show distinct tissue-wise expression and a complex expression pattern under biotic stress. CYSTM possess a conserved cysteine-rich module in the C-terminus, which could be involved in ROS/REDOX sensing. A model a function as homo- or heterodimer to response to different ROS signals. After ROS sensing, they either regulate ROS accumulation, and this regulates indirectly downstream cellular responses, or they regulate cellular responses.

Conclusion:

To summarise this chapter, I could identify and validate CaM, PCC1 and AHA2 as interaction partners of CERK1 in yeast and *in planta*, whereby the biological function is still unclear. I further narrowed down the potential interaction site in PCC1 to a region between amino acid 35 and 55. This region includes a conserved motif and has a predicted intrinsically disordered region, which can be important for protein-protein interactions. The results indicate further that PCC1 and its close homologs At3g22235 (CYSTM8) and At3g22240 (CYSTM9) could be phosphorylated, which could have a regulatory function. I could show that all tested CYSTM family members could interact with CERK1, but some in the split-luciferase assay to a lesser extent, that could be explained by the differences in the conserved motif region. Clade I a members can interact with different RLKs, but more experiments would be necessary to show any specificity, and it would be interesting to test RLKs, which are not involved in plant immunity. I could experimentally observe that PCC1 and both close homologs can form

homodimers and heterodimers in the split-luciferase system. The ROS experiments were unfortunately inconclusive with respect to their outcome because both Gabi-Kat lines show a different behaviour, since the PCC1^{KO} showed no differences compared to wildtype, whereas the PCC1^{KD} mutant revealed a reduced ROS response with chitin and elf18. The lines should be tested for the expression of different PCC1 homologs to exclude compensation of the full knock-out. Finally, I could present *in silico* data that also other CYSTMs show a differential expression after pathogen challenge and that *PCC1* and *At3g22240* have an overlap in a GO term enrichment analysis in genes involved in plant immunity and SA. I could verify the interaction of PCC1, but the biological function, especially the involvement in which kind of defence pathway PCC1 is involved, will be an exciting challenge for the future. The data suggest involvement in ROS regulation/sensing, but more experiments are necessary to validate and explore this hypothesis.

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VII. Abbreviation list

abbreviation	full term
%	percentage
°C	degree Celsius
μ	micro
ε	extinction coefficient
α	anti
5-FOA	5-fluoroorotic acid
5-FU	5-fluorouracil
5xHF Buffer	5 x High-Fidelity Buffer
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
AD	activation domain
Ade	adenine
Amp	ampicillin
AMT	Agrobacterium-mediated transformation
APS	ammonium persulfate
Arg	arginine
<i>At</i>	<i>Arabidopsis thaliana</i>
AVR	avirulence protein
B.c.	<i>Botrytis cinerea</i>
BD	binding domain
BEC	Blumeria effector candidate
Benz	benzimidazole
<i>Bgh</i>	<i>Blumeria graminis</i> f. sp. <i>hordei</i>
<i>Bgt</i>	<i>Blumeria graminis</i> f. sp. <i>tritici</i>
BiFC	Bimolecular fluorescence complementation
bp	base pairs
BSA	bovine serum albumin
CaCl ₂	calcium chloride
CaM	calmodulin
Carb	carbenicillin
CCoAOMT	caffeoyl-CoA 3-O-methyltransferases
cDNA	complementary deoxyribonucleic acid
CDPK/CPK	Calcium dependent protein kinase
CFP	cyan fluorescent protein
CFU	Colony-forming units
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
cLUC	C-terminal part of luciferase
Cm	chloramphenicol
Co-IP	co-immunoprecipitation
Col-0	Columbia-0
COMT	caffeic acid O-methyltransferase
CSEP	candidate for secreted effector protein
Cub	C-terminal part of ubiquitin
cv	cultivar
cYFP	C-terminal part of YFP
CYSTM	cysteine-rich and transmembrane domain
d	thickness of the cuvette
DAMP	damage associated molecular pattern
dd	double distilled

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
dpi	days post inoculation
DTT	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
E.o.	<i>Erysiphe</i> (syn. <i>Golovinomyces</i>) <i>orontii</i>
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
ETI	effector triggered immunity
EtOH	ethanol
ETS	effector triggered susceptible
abbreviation	full term
eV	empty vector
FRET	Förster resonance energy transfer
fwd/ f	forward
g	gram
g	gravitational constant
<i>G. orontii</i>	<i>Golovinomyces orontii</i>
Gent	gentamycin
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hour
HIGS	Host-induced gene silencing
His	histidine
hpi	hours post inoculation
HRP	horseradish peroxidase
HSP	heat shock protein
<i>Hv</i>	<i>Hordeum vulgare</i>
IgG	immunoglobulin G
Kan	kanamycin
kb	kilobase
KCl	potassium chloride
KD	knock-down
kDa	kilo Dalton
KO	knock-out
l	litre
LB	Luria-Bertani
Leu	leucine
LiAc	lithium acetate
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LUC	luciferase
LysM	lysin motif
m	milli
m	meter
M	molar
MALAR	membrane-anchored ligand and receptor
MAMP	microbe associated molecular pattern
MAPK	Mitogen-activated protein kinase
MES	2-(N-morpholino) ethanesulfonic acid
Met	methionine
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
min	minute
mRNA	messenger RNA
MS	mass-spectrometry
MST	microscale thermophoresis
MTI	MAMP triggered immunity
<i>N. benthamiana</i>	<i>Nicotiana benthamiana</i>
Na ₂ HPO ₄	disodium hydrogen phosphate
NaCl	sodium chloride
NaH ₂ PO ₄	sodium dihydrogen phosphate
NaOAc	sodium acetate

VII. Abbreviation list

NaOH	sodium hydroxide
NB	nucleotide-binding
NEB	New England biolabs
NLR	nucleotide-binding and leucine-rich repeat receptor
nLUC	N-terminal part of luciferase
Nub	N-terminal part of ubiquitin
nYFP	N-terminal part of YFP
OD	optical density
OMT	O-methyltransferases
oNPG	<i>ortho</i> -Nitrophenyl- β -galactoside
Os	<i>Oryza sativa</i>
P	Protein concentration
abbreviation	full term
P.i.	<i>Phytophthora infestans</i>
P.s.	<i>Pseudomonas syringae</i>
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBST	phosphate buffered saline + tween20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PLV	ProteinA-LexA-VP16
PPR	pattern recognition receptors
PR	pathogenesis-related
PTI	PAMP triggered immunity
R	arginine
RALPH	RNase like proteins expressed in haustoria
rev/ r	reverse
Rif	rifampicin
RLCK	receptor-like cytoplasmatic kinases
RLK	receptor-like kinase
RLP	receptor-like protein
RLU	relative light units
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rotations per minute
R-protein	Resistance protein
RT	room temperature
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SA	salicylic acid
SAM	S-adenosyl-L-methionine
SC	synthetic complete
SDS	sodium dodecyl sulfate
Sec/s	second
Spec	spectinomycin
sRNA	small ribonucleic acid
Str	streptomycin
t	time
<i>Ta</i>	<i>Triticum aestivum</i>
TAE	tris base, acetic acid and EDTA
TAP	Tandem Affinity Purification
TBST	tris-buffered saline + tween20
TCA	trichloroacetic acid
T-DNA	transfer DNA
TE	TRIS-EDTA
TEMED	tetramethylethylenediamine
Thr	threonine
TIR	Toll interleukin 1
T_m	primer melting temperature
TRIS	tris(hydroxymethyl)aminomethane
Trp	tryptophan
trun	truncated
U	units/enzyme activity
Ura	uracil

USP	Ubiquitin specific proteases
V	volt
v	volume
wt	wild type
Y2H	yeast two-hybrid
YFP	yellow fluorescent protein
YPD	yeast peptone dextrose

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X. Acknowledgements

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The following students contributed to this work with their thesis: Steven Dreischhoff (Chapter 1: Comparison of the two split-ubiquitin Y2H systems and screening of the *HvRnr8* and wheat homologs in the Ura3-based system) (Dreischhoff, 2016), Jan Engels (Chapter 4: Validation of calmodulin, AHA2 and PCC1 as CERK1 interactors in yeast) (Engels, 2017) and Florian Kümmel (Chapter 1: Performing the AVR and the second decoy domain screen in the Ura3-based system) (Kümmel, 2018).

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XI. Curriculum vitae

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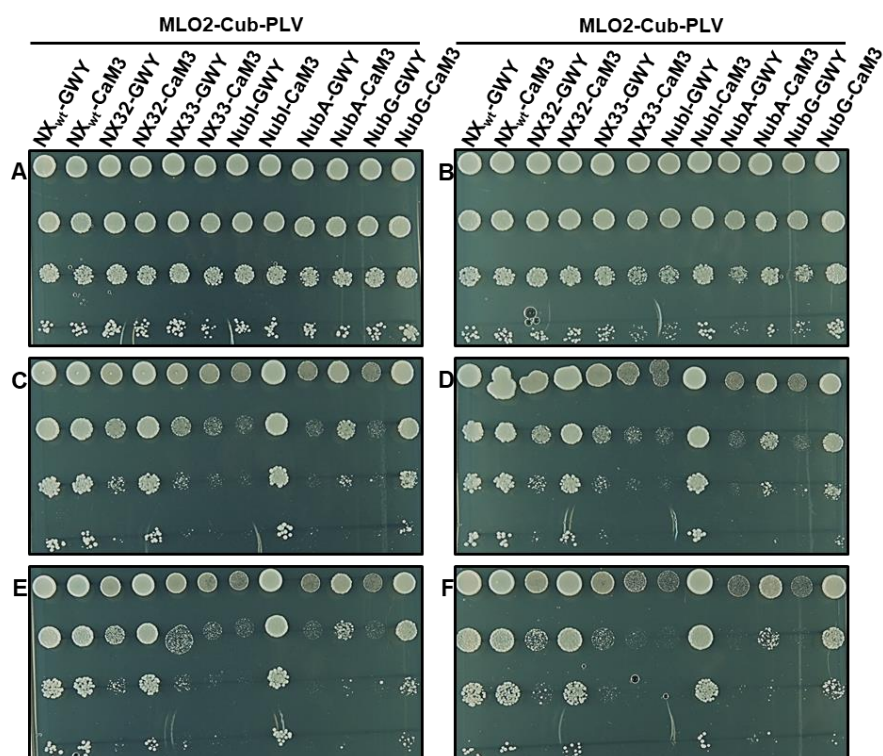
Publications

Sabelleck B, Panstruga R. 2018. Novel jack-in-the-box effector of barley powdery mildew pathogen? *Journal of Experimental Botany* 69, 3511-3514

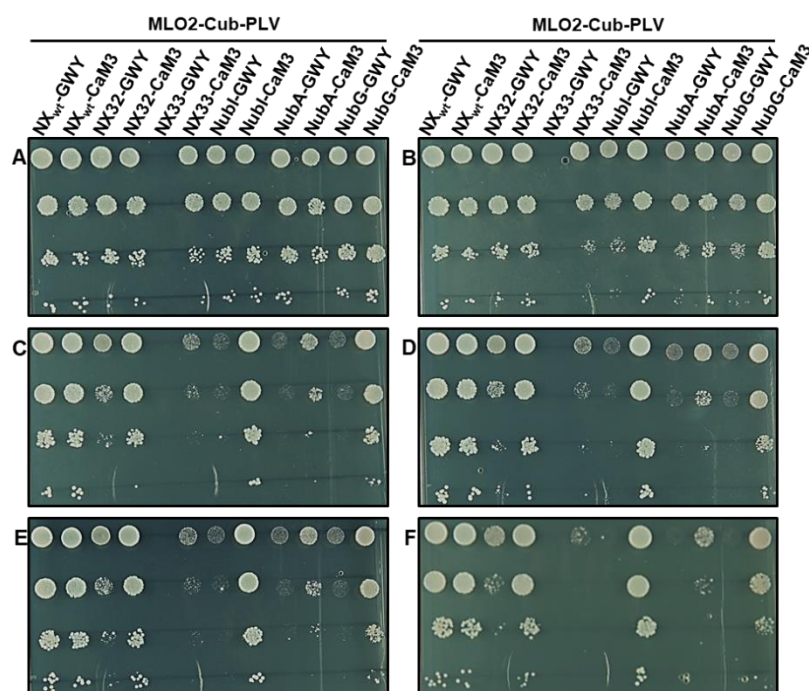
Saur I, Bauer S, Kracher B, Lu X, Franzeskakis L, Müller C, **Sabelleck B**, Kümmel F, Panstruga R, Maekawa T, Schulze-Lefert P. 2019. Multiple pairs of allelic MLA immune receptor-powdery mildew AVR_A effectors argue for a direct recognition mechanism. *eLife* 8:e44471

Barsoum M*, **Sabelleck B***, Spanu P, Panstruga R. 2019. Rumble in the effector jungle: Candidate effector proteins in interactions of plants with powdery mildew and rust fungi. (accepted in *Critical Reviews in Plant Science*) (*shared first author)

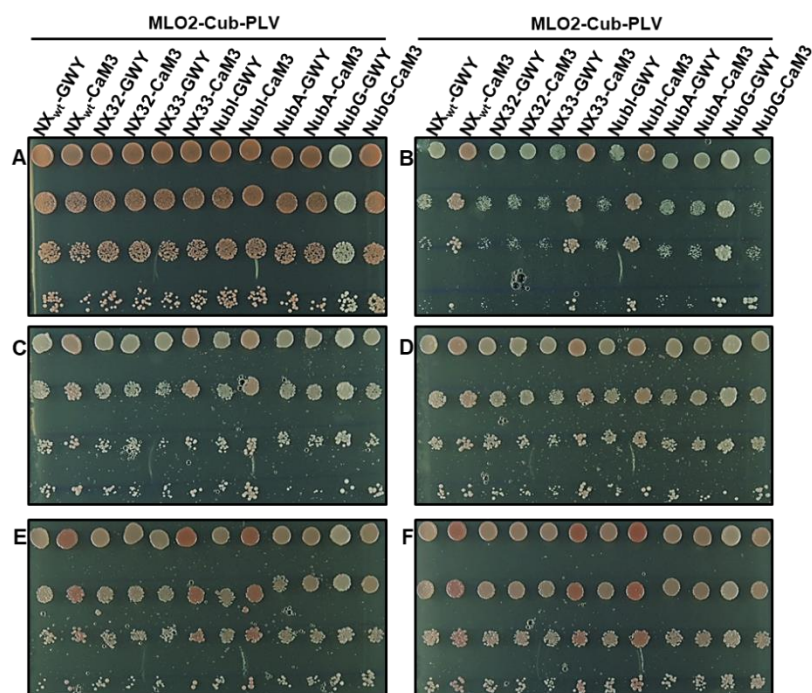
XII. Supplementary Material



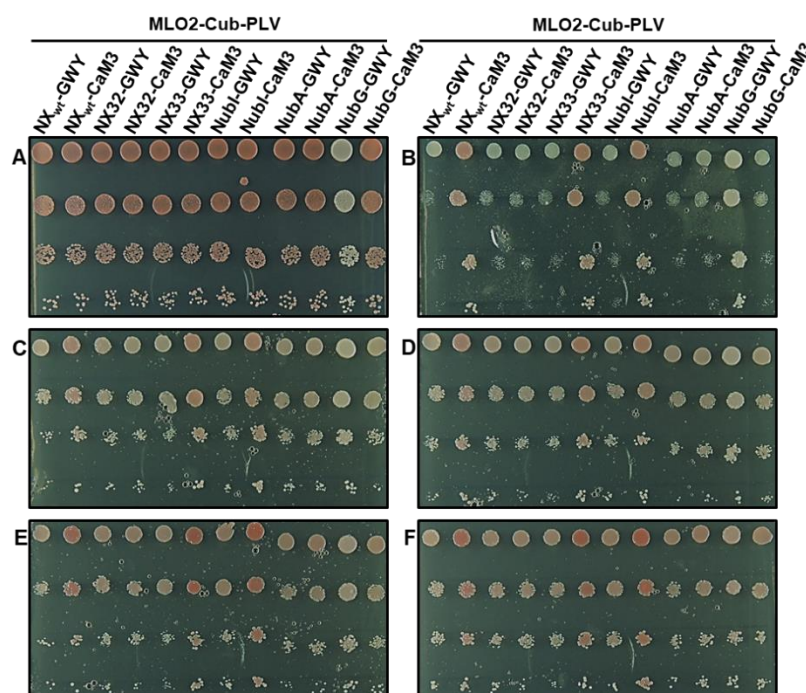
Supplementary Figure 1: LexA-based split-ubiquitin assay for the comparison of different prey vectors; replicate 2. The validated interaction of *AtMLO2* with *HvCaM3* was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). 4 μ l of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD₆₀₀ 1-10⁻³) on agar selecting for successful transformation (A) (SC -leucine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC -leucine -tryptophan -adenine -histidine). Different methionine (Met) concentrations were used as indicated to gradually repress expression of the bait construct. (A) SC -leucine -tryptophan - methionine, (B) SC -leucine -tryptophan -adenine -histidine - methionine, (C) SC -Leu -tryptophan -adenine -histidine + 75 μ M methionine, (D) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine, (E) SC -leucine -tryptophan -adenine -histidine + 300 μ M methionine, (F) SC -leucine -tryptophan -adenine -histidine + 600 μ M methionine. The experiment was repeated three times with similar results; replicate 2.



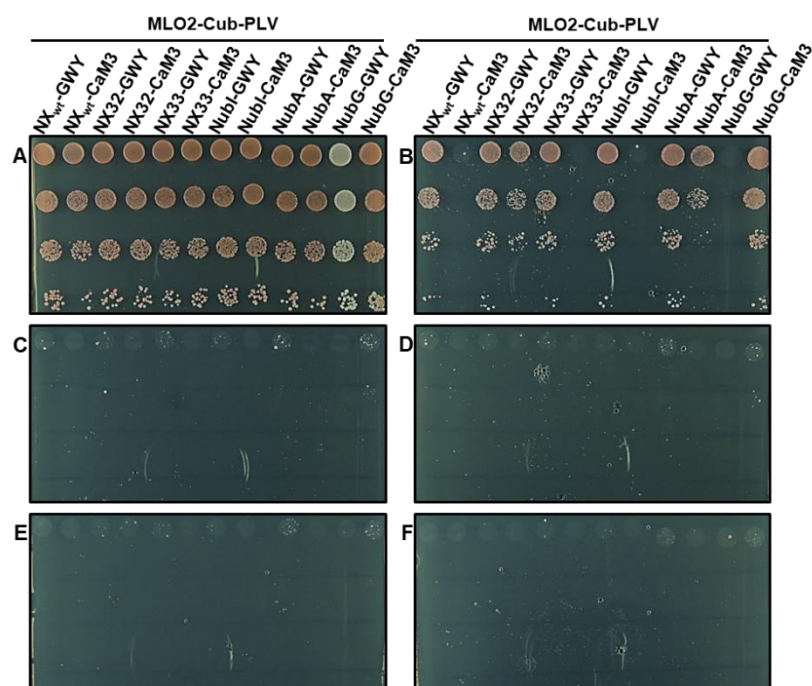
Supplementary Figure 2: LexA-based split-ubiquitin assay for the comparison of different prey vectors; replicate 3. The validated interaction of *AtMLO2* with *HvCaM3* was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). 4 μ l of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD₆₀₀ 1-10⁻³) on agar selecting for successful transformation (A) (SC -leucine -tryptophan -Met) or for positive interaction (B, C, D, E, F) (SC -leucine -tryptophan -adenine -histidine). Different methionine (Met) concentrations were used as indicated to gradually repress expression of the bait construct. (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 75 μ M methionine, (D) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine, (E) SC -leucine -tryptophan -adenine -histidine + 300 μ M methionine, (F) SC -leucine -tryptophan -adenine -histidine + 600 μ M methionine. The experiment was repeated three times with similar results; replicate 3.



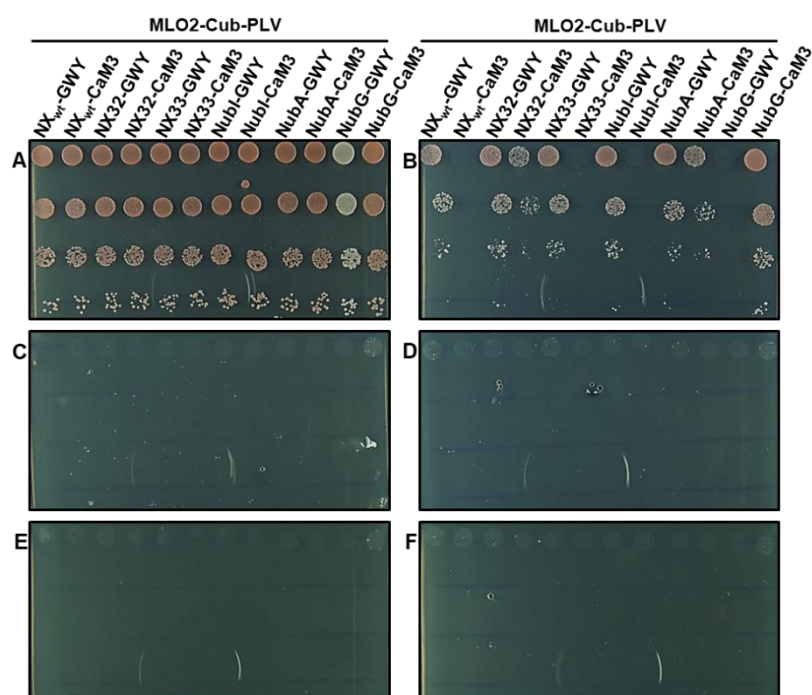
Supplementary Figure 3: Ura3-based split-ubiquitin assay for the comparison of different prey vectors; replicate 2. The validated interaction of AtMLO2 with HvCaM3 was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). 4 µl of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD₆₀₀ 1-10⁻³) on agar selecting for successful transformation (A) (SC -histidine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC -histidine -tryptophan + 1 g/l 5-FOA). Different methionine (Met) concentrations were used as indicated to gradually repress expression of the bait construct. (A) SC -histidine -tryptophan -methionine, (B) SC -histidine -tryptophan -methionine + 1 g/l 5-FOA (C) SC -histidine -tryptophan + 75 µM methionine + 1 g/l 5-FOA, (D) SC -histidine -tryptophan + 150 µM methionine + 1 g/l 5-FOA, (E) SC -histidine -tryptophan + 300 µM methionine + 1 g/l 5-FOA, (F) SC -histidine -tryptophan + 600 µM methionine + 1 g/l 5-FOA. The experiment was repeated three times with similar results; replicate 2.



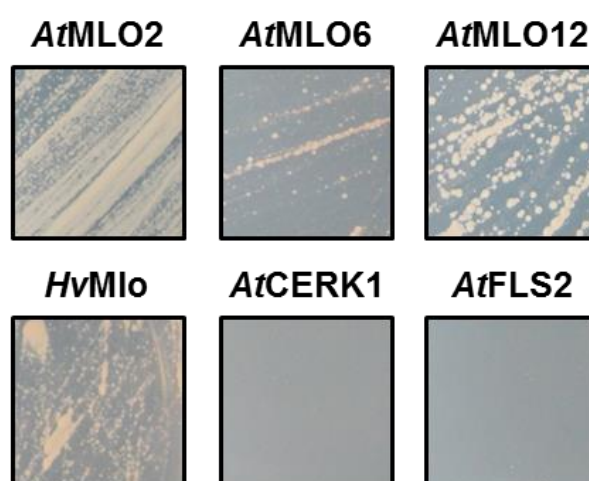
Supplementary Figure 4: Ura3-based split-ubiquitin assay for the comparison of different prey vectors; replicate 3. The validated interaction of AtMLO2 with HvCaM3 was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). 4 µl of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD₆₀₀ 1-10⁻³) on agar selecting for successful transformation (A) (SC -histidine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC -histidine -tryptophan + 1 g/l 5-FOA). Different methionine (Met) concentrations were used as indicated to gradually repress expression of the bait construct. (A) SC -histidine -tryptophan -methionine, (B) SC -histidine -tryptophan -methionine + 1 g/l 5-FOA (C) SC -histidine -tryptophan + 75 µM methionine + 1 g/l 5-FOA, (D) SC -histidine -tryptophan + 150 µM methionine + 1 g/l 5-FOA, (E) SC -histidine -tryptophan + 300 µM methionine + 1 g/l 5-FOA, (F) SC -histidine -tryptophan + 600 µM methionine + 1 g/l 5-FOA. The experiment was repeated three times with similar results; replicate 3.



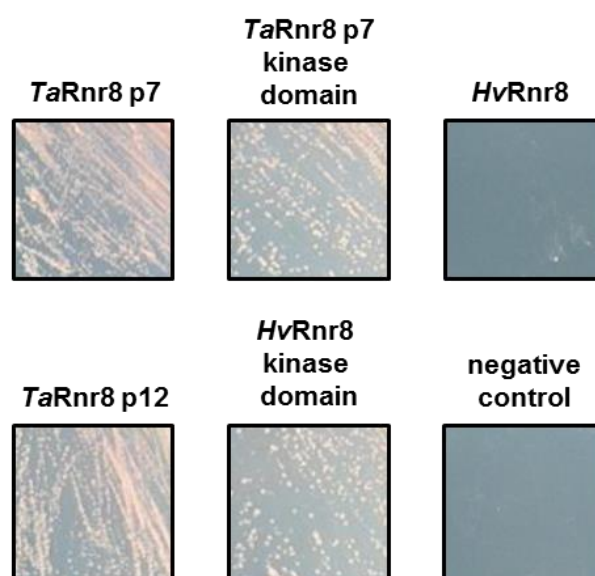
Supplementary Figure 5: Ura3-based split-ubiquitin assay with counter-selection for the comparison of different prey vectors; replicate 2. The validated interaction of AtMLO2 with *HvCaM3* was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). 4 μ l of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD_{600} 1-10⁻³) on agar selecting for successful transformation (A) (SC -histidine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC -histidine -tryptophan -uracil). Different methionine (Met) concentrations were used as indicated to gradually repress expression of the bait construct. (A) SC -histidine -tryptophan -methionine, (B) SC -histidine -tryptophan -methionine -uracil (C) SC -histidine -tryptophan -uracil + 75 μ M methionine, (D) SC -histidine -tryptophan -uracil + 150 μ M methionine, (E) SC -histidine -tryptophan -uracil + 300 μ M methionine, (F) SC -histidine -tryptophan -uracil + 600 μ M methionine. The experiment was repeated three times with similar results; replicate 2.



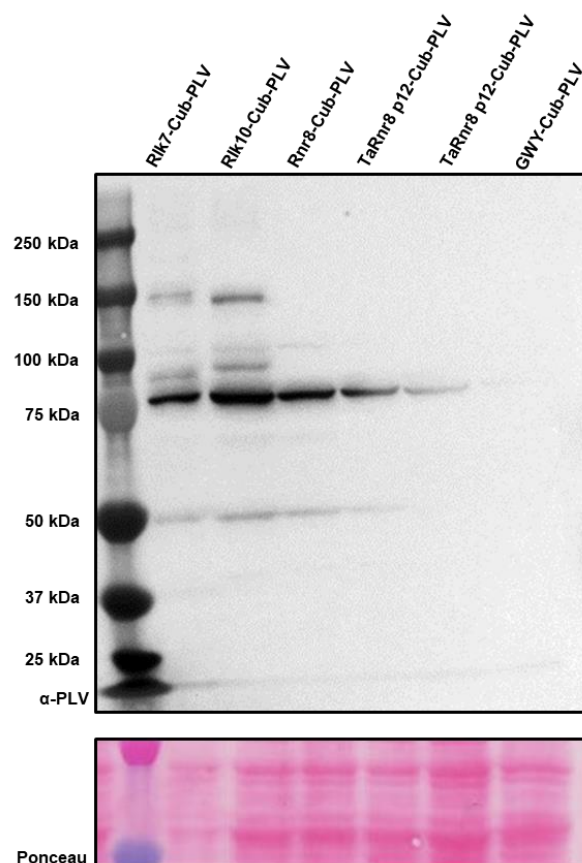
Supplementary Figure 6: Ura3-based split-ubiquitin assay with counter-selection for the comparison of different prey vectors; replicate3. The validated interaction of AtMLO2 with HvCaM3 was used in this assay to compare prey pCup vectors with different Nub variants (Nubl, NubA, NubG) and high/low copy vectors (NX_{wt}, NX32, NX33). 4 µl of overnight cultures of transformed yeasts (strain THY.AP4) were plotted in 10-fold dilution series (OD₆₀₀ 1-10⁻³) on agar selecting for successful transformation (A) (SC -histidine -tryptophan -methionine) or for positive interaction (B, C, D, E, F) (SC -histidine -tryptophan -uracil). Different methionine (Met) concentrations were used as indicated to gradually repress expression of the bait construct. (A) SC -histidine -tryptophan -methionine, (B) SC -histidine -tryptophan -methionine -uracil (C) SC -histidine -tryptophan -uracil + 75 µM methionine, (D) SC -histidine -tryptophan -uracil + 150 µM methionine, (E) SC -histidine -tryptophan -uracil + 300 µM methionine, (F) SC -histidine -tryptophan -uracil + 600 µM methionine. The experiment was repeated three times with similar results; replicate 3.



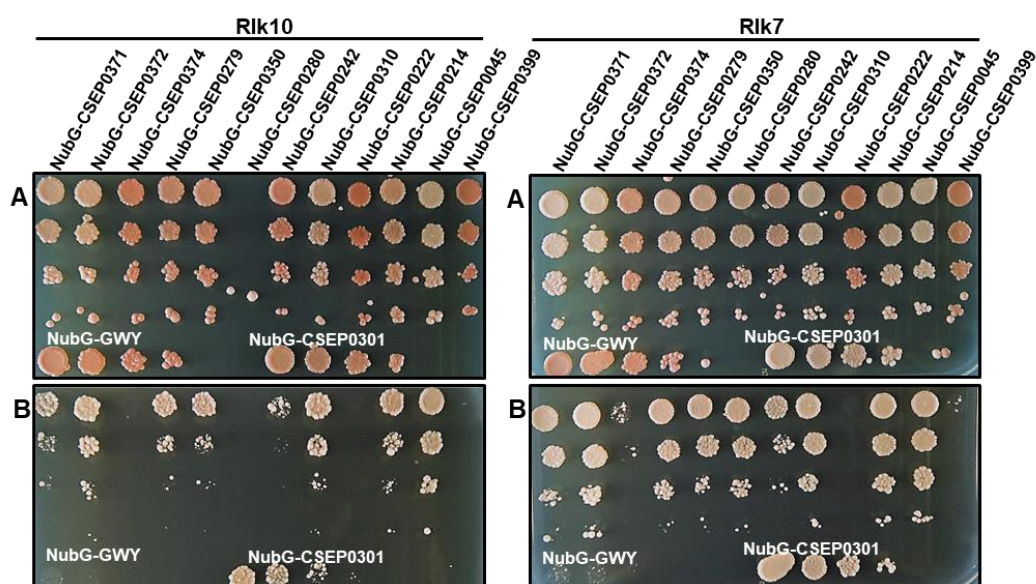
Supplementary Figure 7: Test of bait auto-activation in the LexA-based system. The THY.AP4 yeast strain was transformed with the pMetYC-GWY-Cub-PLV vector, expressing the indicated bait proteins as fusion proteins with Cub-PLV. The strains were streaked on SC -leucine -adenine -histidine -methionine and growth indicates auto-activation of the bait construct.

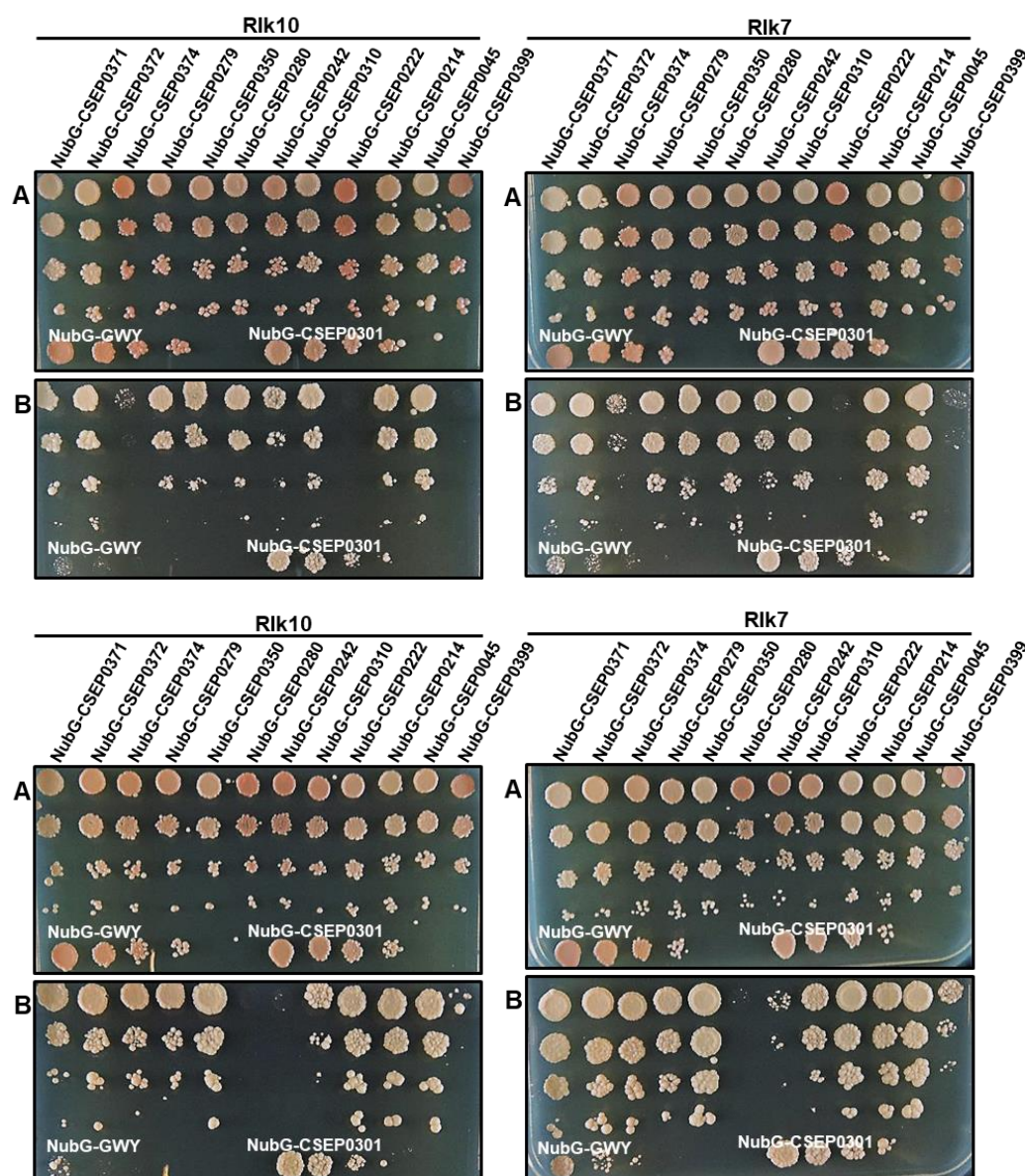


Supplementary Figure 8: Test of bait protein expression of HvRnr8 and its homologs in the Ura3-based system. Transformed yeast, expressing the indicated protein of interest as a fusion protein with Cub-Ura3, were streaked on SC -histidine -uracil -methionine. Growth indicates expression of the bait fusion protein.

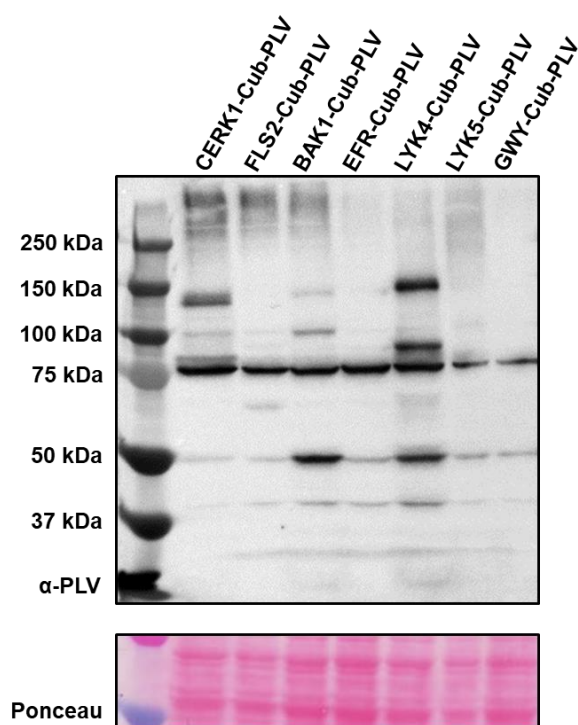


Supplementary Figure 9: Barley and wheat RLK fusion protein expression in yeast. Cells expressing the different RLKs C-terminally fused to Cub (C-terminal part of ubiquitin), and PLV (artificial transcription activator ProteinA-LexA-VP16) were harvested and the total protein was extracted. A specific antibody against PLV was used to detect bait protein expression. Expected size of the fusion proteins: *HvRik7*: 157 kDa; *HvRik10*: 123 kDa; *HvRnr8*: 165 kDa; *TaRnr8* p7: 164 kDa; *TaRnr8* p12: 163 kDa; PLV alone: 50 kDa. Ponceau staining was applied to visualise equal protein loading.

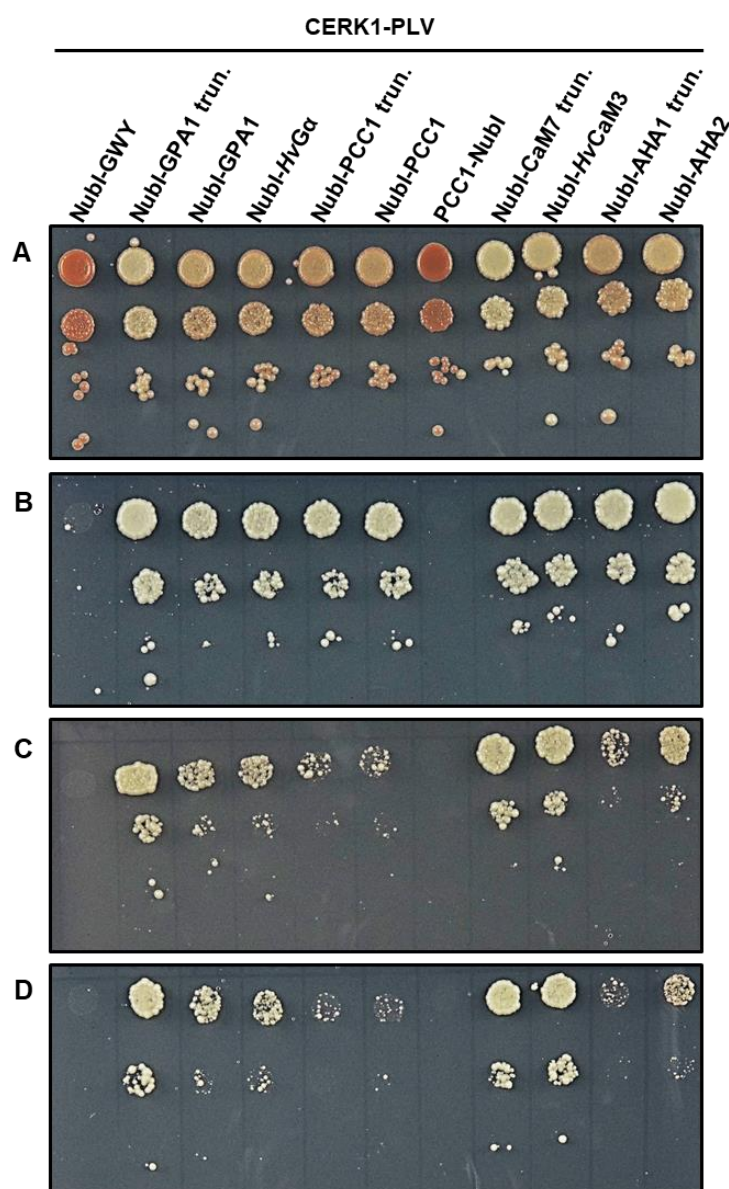




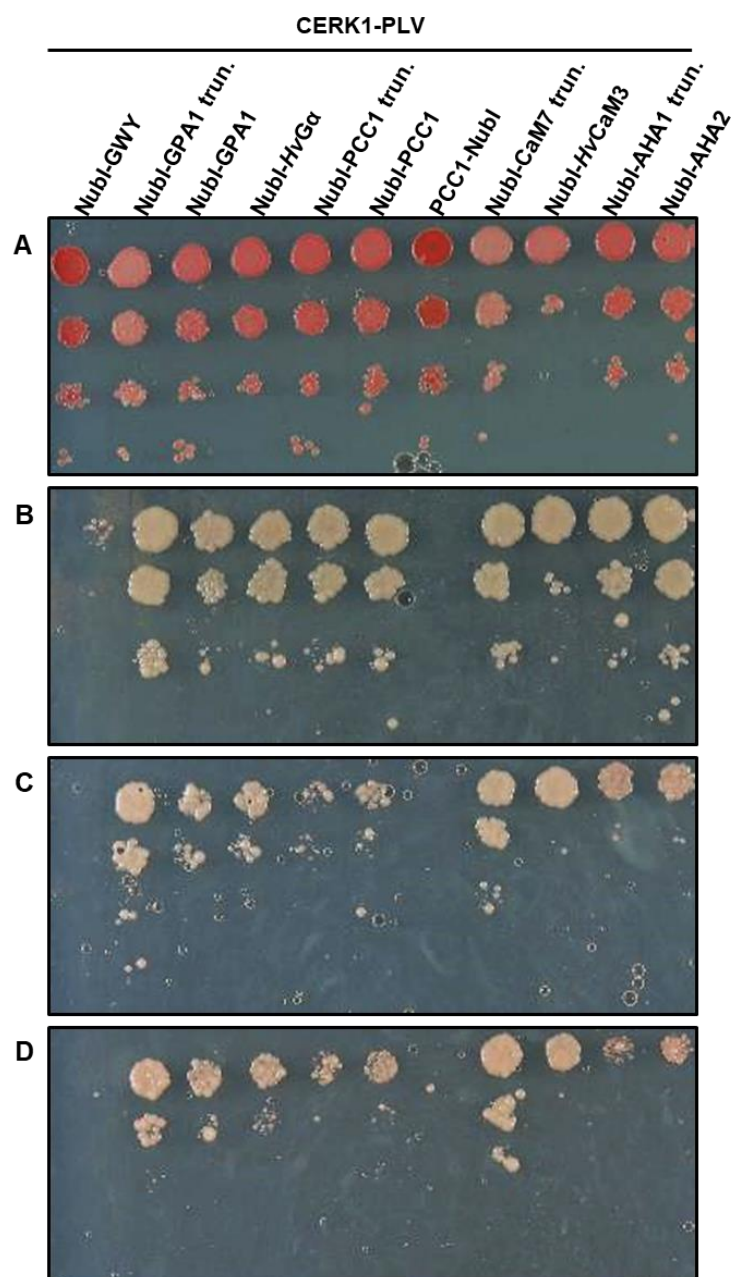
Supplementary Figure 10: Validation of Rik10 and Rik7 interacting *Bgh* CSEPs in a split-ubiquitin Y2H assay. The LexA-based split-ubiquitin Y2H system was used to validate the identified potential interaction partners CSEP0045, CSEP0310, CSEP0350, and CSEP0372. Each potential interaction partner was dropped next to its closest homologs to test for specificity. The following combinations belong together: CSEP0372 with CSEP0371/CSEP0374, CSEP0350 with CSEP0279/CSEP0280/CSEP0301, CSEP0310 with CSEP0242/CSEP0222, and CSEP0045 with CSEP0214/CSEP0399. NubG-GWY was used as a negative control. NubG is the low-affinity version of the N-terminal ubiquitin part, which is tagged to the N-terminus of the effector. Panel A shows the growth control, which selects for both plasmids on SC -leucine -tryptophan -methionine. Panel B depicts the interaction plate on SC -leucine -tryptophan -adenine -histidine -methionine. 4 μ l overnight yeast culture is dropped in a 10-fold dilution series starting from OD₆₀₀ 1. All three replicates are present here.



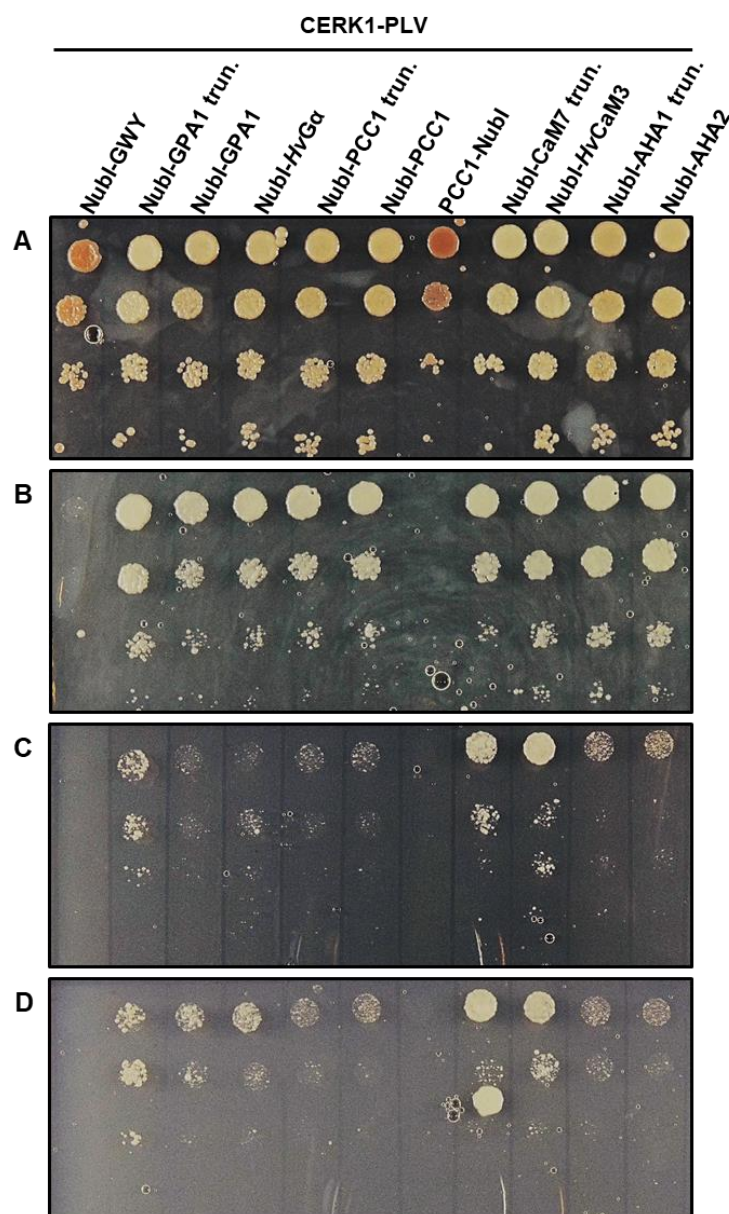
Supplementary Figure 11: Western blot of RLK expression in yeast. Cells expressing the different RLKs C-terminally fused to Cub (C-terminal part of ubiquitin) and PLV (artificial transcription activator ProteinA-LexA-VP16) were harvested and the total protein was extracted. A specific antibody against the PLV was used to detect bait protein expression. The expected size of the fusion proteins: CERK1-Cub-PLV: 121 kDa; FLS2-Cub-PLV: 182 kDa; BAK1-Cub-PLV: 121 kDa; EFR-Cub-PLV: 176 kDa; LYK4-Cub-PLV: 120 kDa; LYK5-Cub-PLV: 126 kDa; GWY-Cub-PLV: 50 kDa. Ponceau staining was applied to visualise total protein loading.



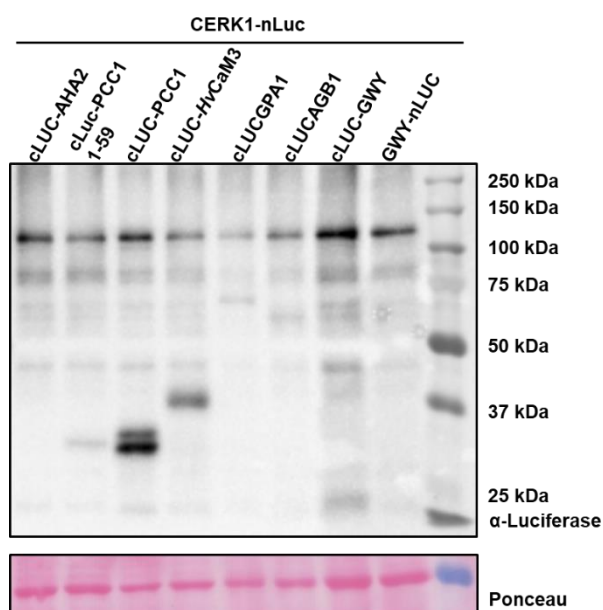
Supplementary Figure 12: LexA-based split-ubiquitin assay for the validation of potential interaction partner of CERK1; replicate 1. The colonies from the cDNA screen with truncated (trun.) protein versions were compared to the cloned full-length versions of the protein in the same vector backbone (pCup-Nubl-GWY-Cyc1). 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series ranging from OD₆₀₀ 1 on the top to OD₆₀₀ 0.001 at the bottom. The media were as follows: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 100 μ M methionine, (D) SC -leucine -tryptophan -adenine -histidine + 300 μ M methionine. This experiment was repeated three times and the replicates are attached in the supplement; replicate 1.



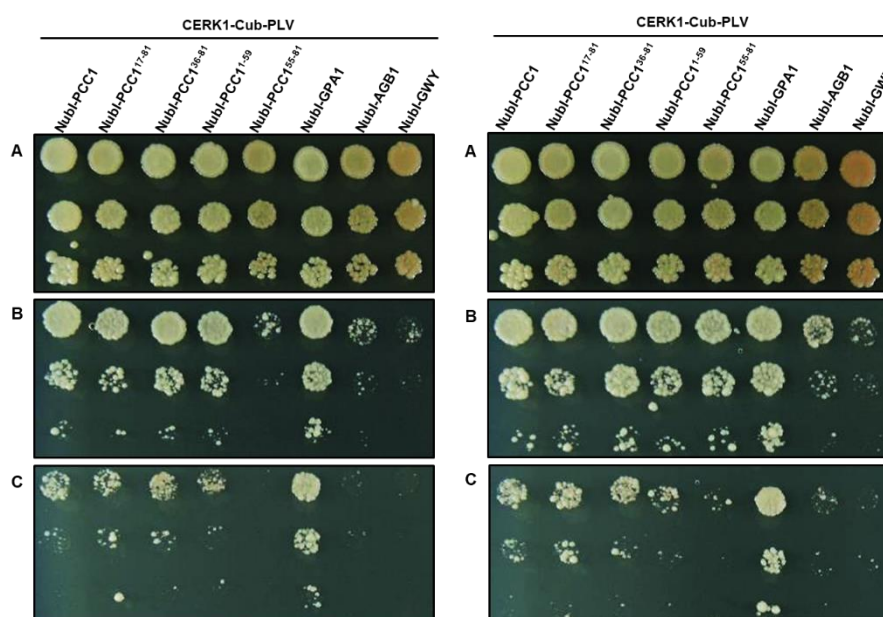
Supplementary Figure 13: LexA-based split-ubiquitin assay for the validation of potential interaction partner of CERK1; replicate 2. The colonies from the cDNA screen with truncated (trun.) protein versions were compared to the cloned full-length versions of the protein in the same vector backbone (pCup-Nubi-GWY-Cyc1). 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series ranging from OD₆₀₀ 1 on the top to OD₆₀₀ 0.001 at the bottom. The media were as follows: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 100 μ M methionine, (D) SC -leucine -tryptophan -adenine -histidine + 300 μ M methionine. This experiment was repeated three times and the replicates are attached in the supplement; replicate 2.



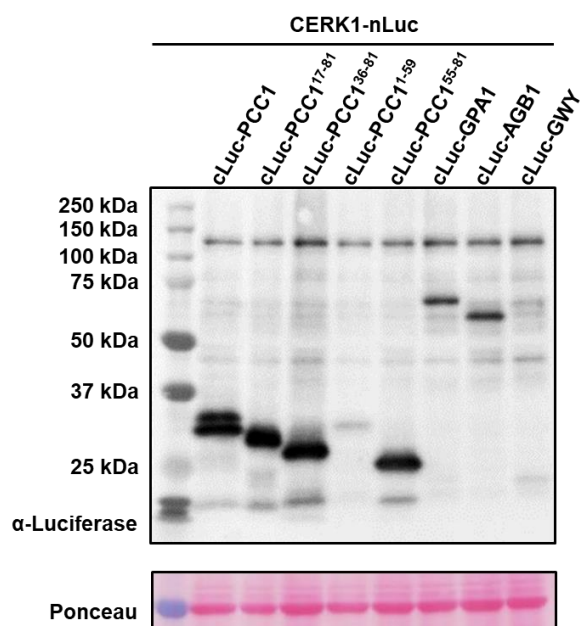
Supplementary Figure 14: LexA-based split-ubiquitin assay for the validation of potential interaction partner of CERK1; replicate 3. The colonies from the cDNA screen with truncated (trun.) protein versions were compared to the cloned full-length versions of the protein in the same vector backbone (pCup-Nubl-GWY-Cyc1). 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series ranging from OD₆₀₀ 1 on the top to OD₆₀₀ 0.001 at the bottom. The media were as follows: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 100 μ M methionine, (D) SC -leucine -tryptophan -adenine -histidine + 300 μ M methionine. This experiment was repeated three times and the replicates are attached in the supplement; replicate 3.



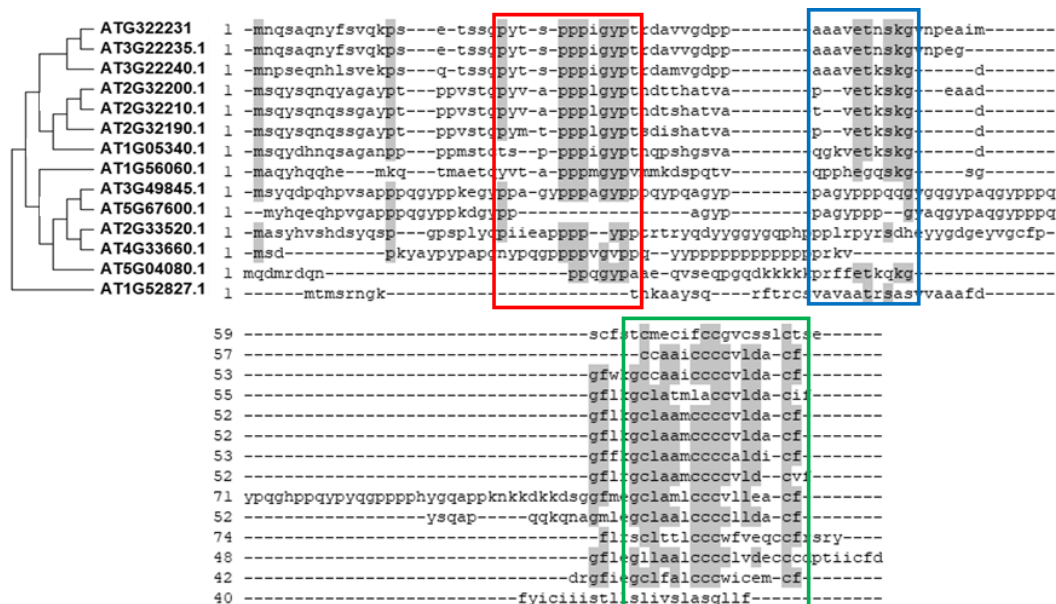
Supplementary Figure 15: Validation of expression of CERK1 and the potential interaction partners by heterologous expression in *N. benthamiana*. The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise the loading of the protein extract. The expected sizes with the fused split-luciferase parts are as followed: CERK1-nLUC: 116 kDa; cLUC-PCC1: 28 kDa; cLUC-AHA2: 124 kDa; cLUC-HvCaM3: 37 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa.



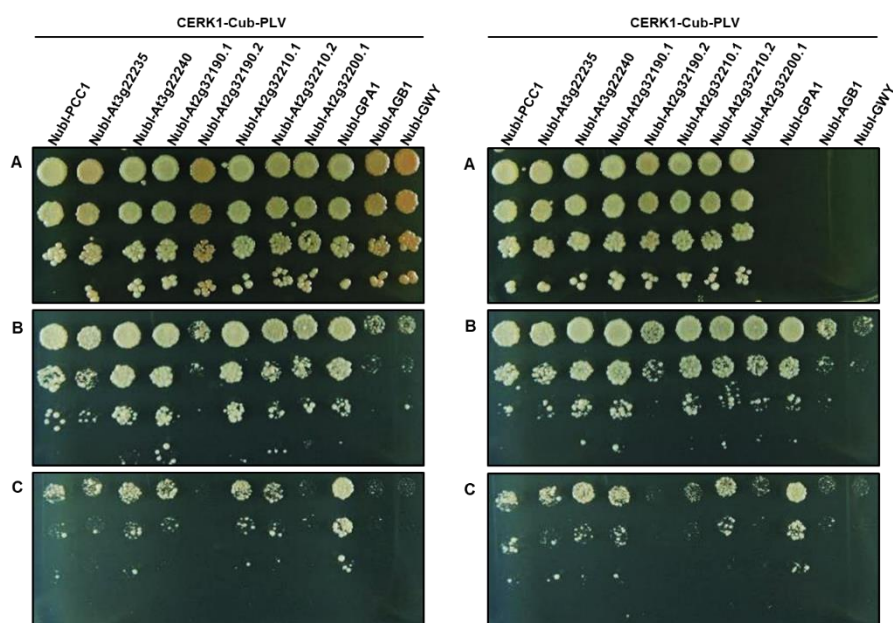
Supplementary Figure 16: Comparison of truncated PCC1 versions against CERK1 in yeast. For the identification of the interacting domain of PCC1, full-length and truncated versions were tested against CERK1. GPA1 serves as a positive control as well as AGB1 and empty vector (Nub1-GWY) as negative controls. The numbers indicate which amino acids are present in the 81 amino acid long peptide. 4 μ l of overnight cultures of transformed yeast were drop-inoculated in a 10-fold dilution series starting at OD₆₀₀ 1 on the top until 0.01. (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine. This experiment was repeated in three independent biological replicates; replicate 2 (left) and 3 (right).



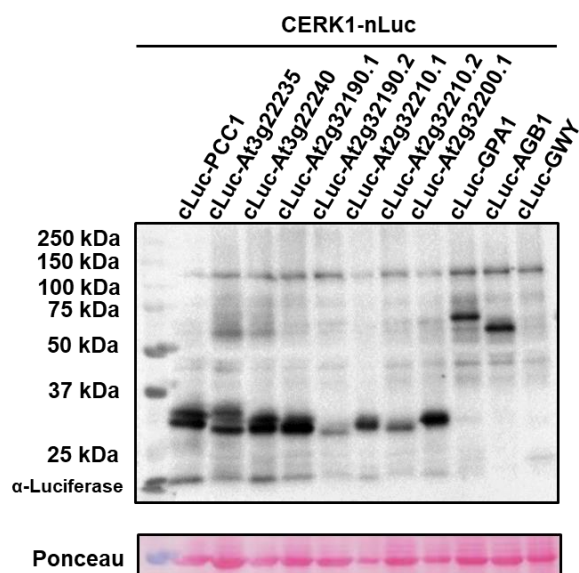
Supplementary Figure 17: Validation of expression of CERK1 and four truncated PCC1 versions in the split-luciferase assay in *N. benthamiana*. The total protein of 8 measured leaf discs was extracted and a western blot was performed. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes with the fused split-luciferase parts are as follows: CERK1-nLUC: 116 kDa; cLUC-PCC1: 28 kDa; cLUC-PCC1¹⁷⁻⁸¹: 26 kDa; cLUC-PCC1³⁶⁻⁸¹: 25 kDa; cLUC-PCC1¹⁻⁵⁹: 26 kDa; cLUC-PCC1⁵⁵⁻⁸¹: 23 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa. The numbers at the truncated versions indicate which amino acids are present from the 81 amino acid PCC1 peptide.



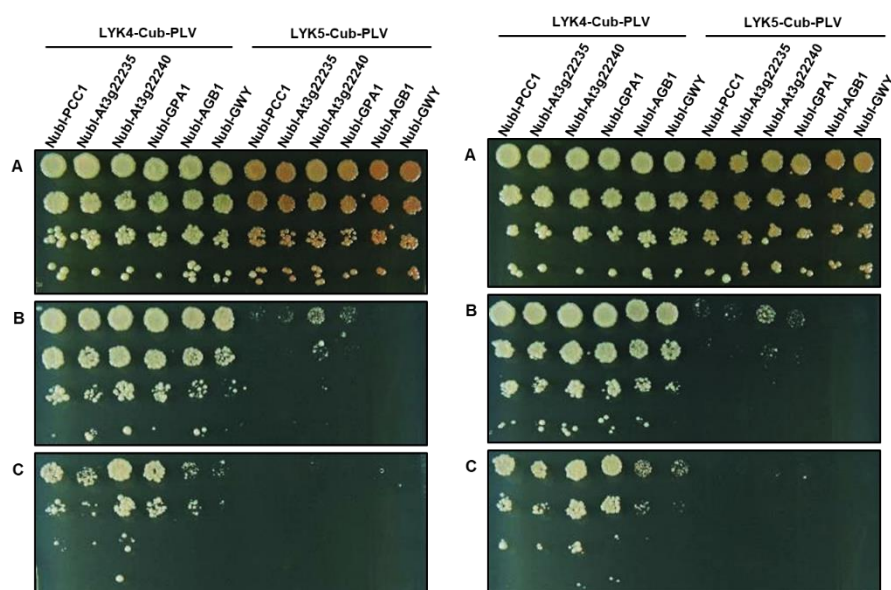
Supplementary Figure 18: Protein alignments of PCC1 and the CYSTM family. Amino acid alignment of all CYSTM family members. The alignment was performed with CloneManager by using BLOSUM 62 scoring matrix. The red box labels the proline/glycine-rich region, the blue box a conserved region of clade I members, which is potentially necessary for the interaction with RLKs and the green box labels the cysteine-rich region. Matching amino acid residues are highlighted in grey.



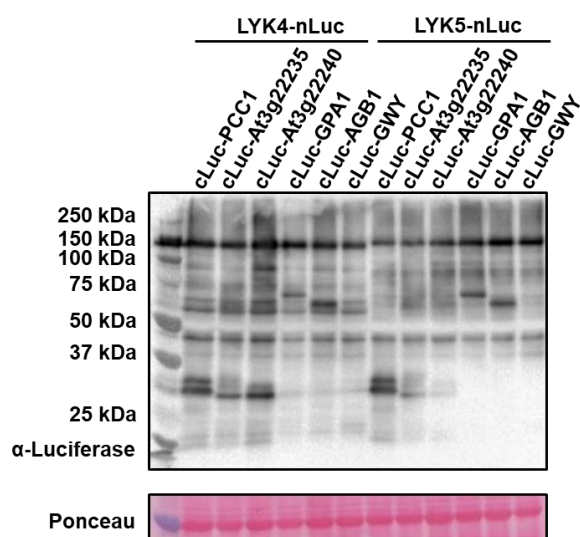
Supplementary Figure 19: Comparison of PCC1 family members against CERK1 in a LexA-based split-ubiquitin Y2H. For the validation of the specific interaction of PCC1 with CERK1, also other PCC1 family members were tested. GPA1 serves as a positive control, and AGB1 and empty vector (Nubl-GWY) as negative controls. 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series starting at OD₆₀₀ 1 on the top and until OD₆₀₀ 0.001. Selection media were as follows: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine. This experiment was repeated in three independent biological replicates; replicate 2 (left) and 3 (right).



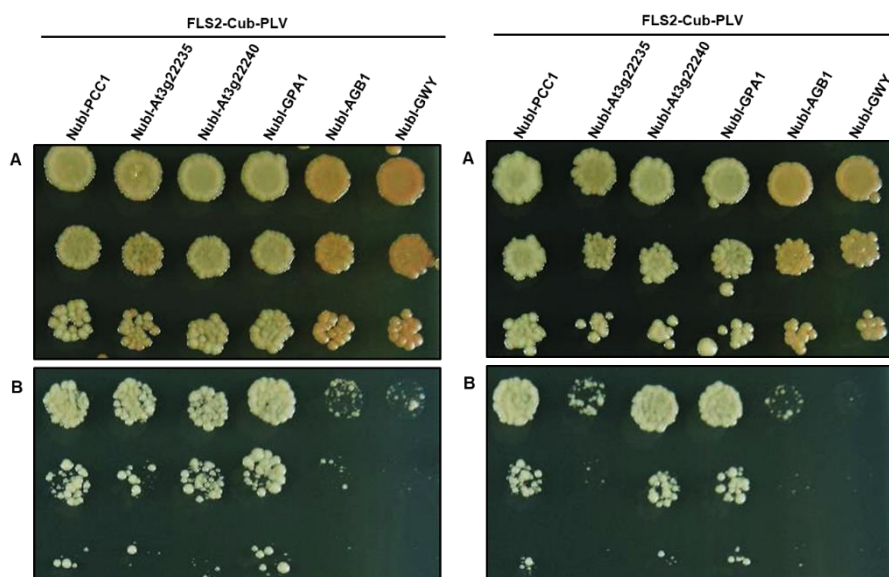
Supplementary Figure 20: Validation of expression of CERK1 and PCC1 family members in the split-luciferase assay in *N. benthamiana*. The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase protein fusions are as follows: CERK1-nLUC: 116 kDa; cLUC-PCC1 and the other family members around 28 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa.



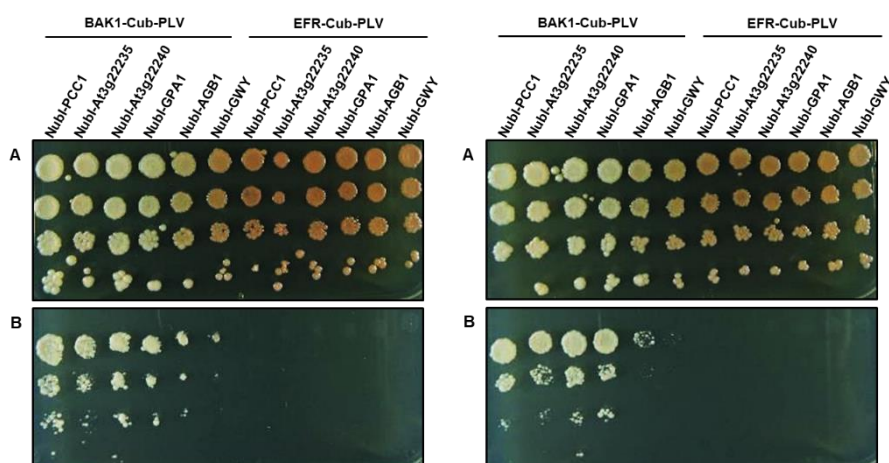
Supplementary Figure 21: Testing interaction of PCC1 against LYK4 and LYK5 in a LexA-based split-ubiquitin Y2H. To test the interaction of PCC1 with other RLKs, PCC1 and both close family members At3g22235 and At3g22240 were tested against LYK4 and LYK5. GPA1 serves as a positive control as well as AGB1 and empty vector (Nubl-GWY) as negative controls. 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series starting at OD₆₀₀ 1 on the top and until OD₆₀₀ 0.001 at the bottom. The different plates are supplemented with increasing concentration of methionine to repress the bait protein expression: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine, (C) SC -leucine -tryptophan -adenine -histidine + 150 μ M methionine. This experiment was repeated in three independent biological replicates; replicate 2 (left) and 3 (right).



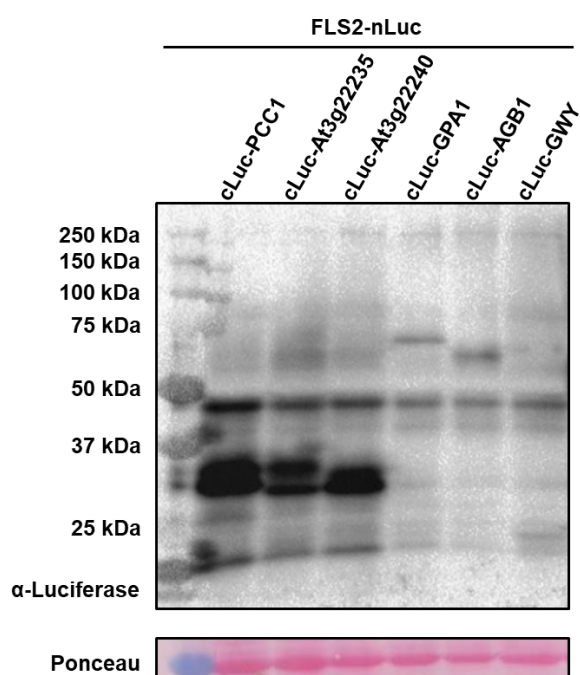
Supplementary Figure 22: Validation of expression of LYK4/LYK5 and PCC1 in the split-luciferase assay in *N. benthamiana*. The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes with the fused split-luciferase parts are like followed: LYK4-nLUC: 116 kDa; LYK5-nLUC: 122 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235: 27 kDa; cLUC-At3g22240: 27 kDa; cLUC-GPA1: 64 kDa, cLUC-AGB1: 61 kDa and cLUC-GWY: 20 kDa.



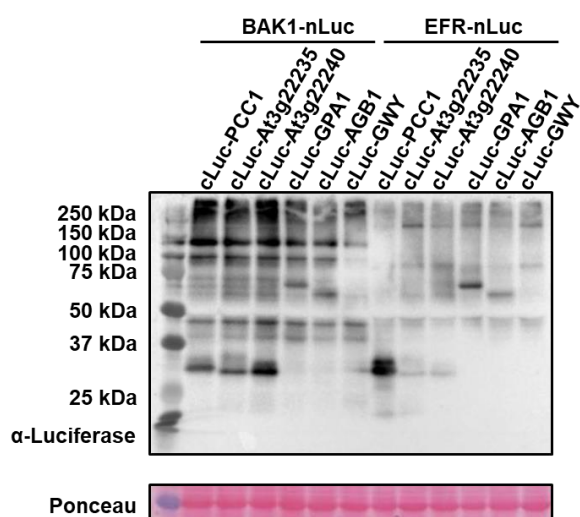
Supplementary Figure 23: Testing interaction of PCC1 against FLS2 in a LexA-based split-ubiquitin Y2H. To test the interaction of PCC1 with other RLKs, PCC1 and both close family members At3g22235 and At3g22240 were tested against FLS2. GPA1 serves as a positive control as well as AGB1 and empty vector (Nubi-GWY) as negative controls. 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series starting at OD₆₀₀ 1 on the top and until OD₆₀₀ 0.01 at the bottom. The different plates are supplemented with increasing concentration of methionine to repress the bait protein expression: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine. This experiment was repeated in three independent biological replicates; replicate 2 (left) and 3 (right).



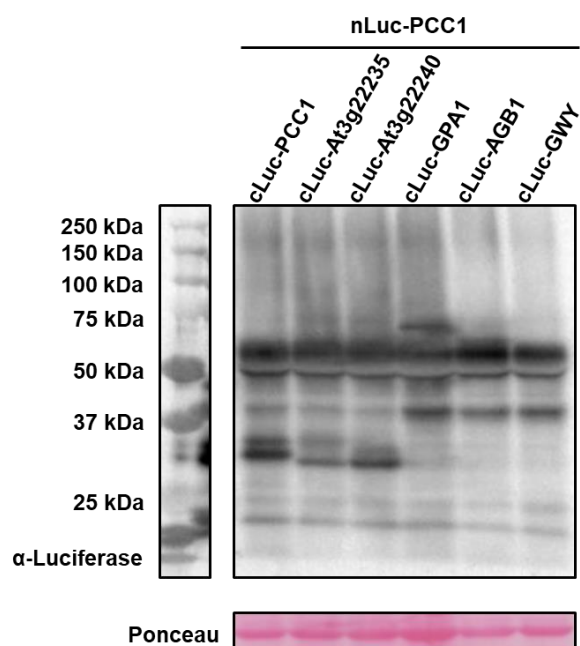
Supplementary Figure 24: Testing interaction of PCC1 against BAK1 and EFR in a LexA-based split-ubiquitin Y2H. To test the interaction of PCC1 with other RLKs, PCC1 and both close family members At3g22235 and At3g22240 were tested against BAK1 and EFR. GPA1 serves as a positive control as well as AGB1 and empty vector (Nubi-GWY) as negative controls. 4 μ l of overnight cultures of transformed yeast were plotted in a 10-fold dilution series starting at OD₆₀₀ 1 on the top and until OD₆₀₀ 0.01 at the bottom. The different plates are supplemented with increasing concentration of methionine to repress the bait protein expression: (A) SC -leucine -tryptophan -methionine, (B) SC -leucine -tryptophan -adenine -histidine -methionine. This experiment was repeated in three independent biological replicates; replicate 2 (left) and 3 (right).



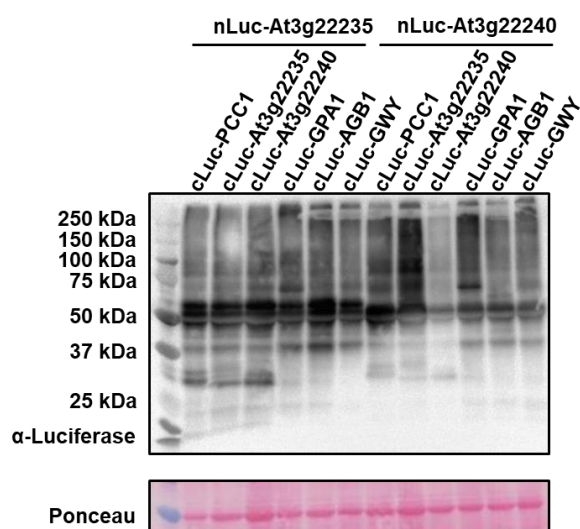
Supplementary Figure 25: Validation of expression of FLS2 and PCC1 in the split-luciferase assay in *N. benthamiana*. The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase protein fusions are as follows: FLS2-nLUC: 177 kDa; 162 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235 27 kDa, cLUC-At3g22240 27 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa.



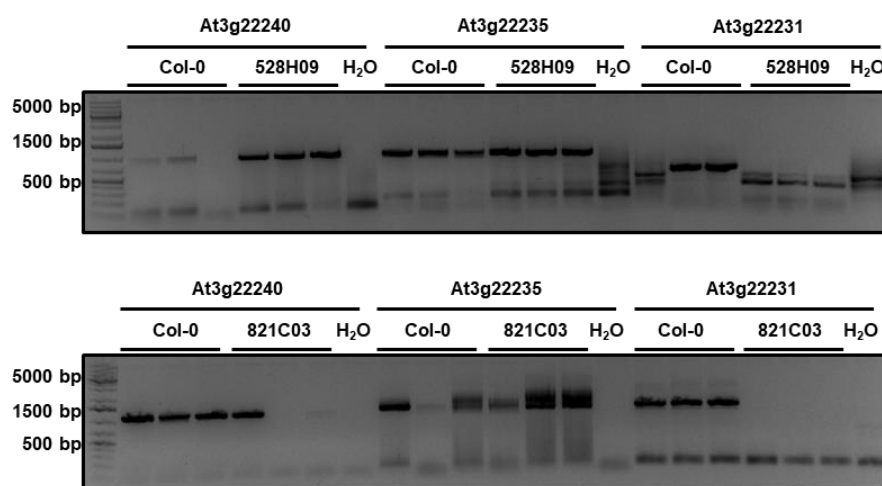
Supplementary Figure 26: Validation of expression of BAK1/EFR and PCC1 in the split-luciferase assay in *N. benthamiana*. The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase protein fusions are as follows: BAK1-nLUC: 117 kDa; EFR-nLUC: 162 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235 27 kDa, cLUC-At3g22240 27 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa.



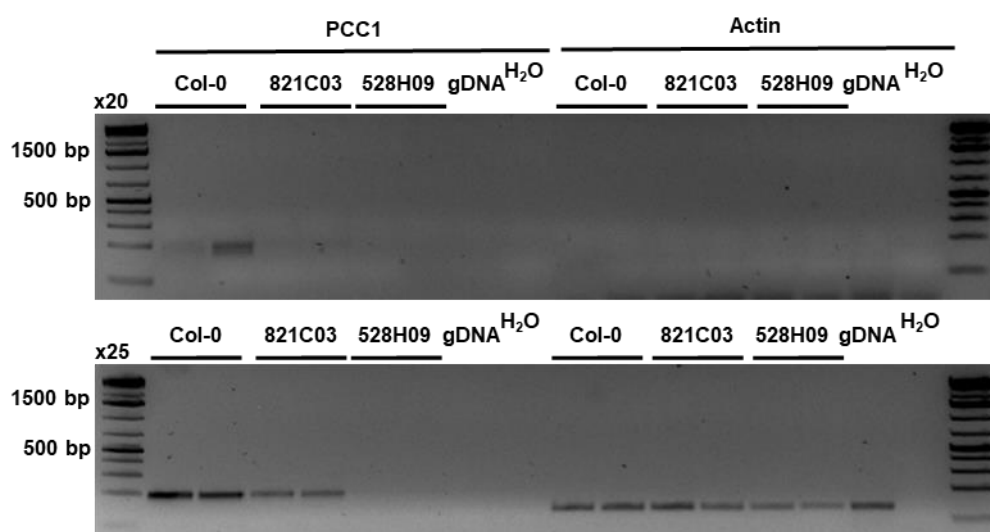
Supplementary Figure 27: Validation of expression of PCC1 and PCC1 in the split-luciferase assay in *N. benthamiana*. The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase fusion constructs are: nLUC-PCC1: 56 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235: 27 kDa; cLUC-At3g22240: 27 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa.



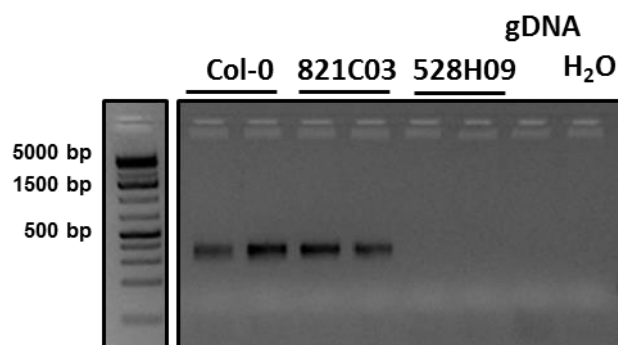
Supplementary Figure 28: Validation of expression of At3G22235/At3G22240 and PCC1 in the split-luciferase assay in *N. benthamiana*. The leaf discs were collected for total protein extraction and western blot analysis. The polyclonal anti-luciferase antibody is capable of detecting both, the C-terminal and N-terminal part of the split-luciferase. Ponceau staining was used to visualise equal loading of the protein extract. The expected sizes of the split-luciferase fusion constructs are: nLUC-At3g22235: 55 kDa; nLUC-At3g22240: 55 kDa; cLUC-PCC1: 28 kDa; cLUC-At3g22235: 27 kDa; cLUC-At3g22240: 27 kDa; cLUC-GPA1: 64 kDa; cLUC-AGB1: 61 kDa; cLUC-GWY: 20 kDa



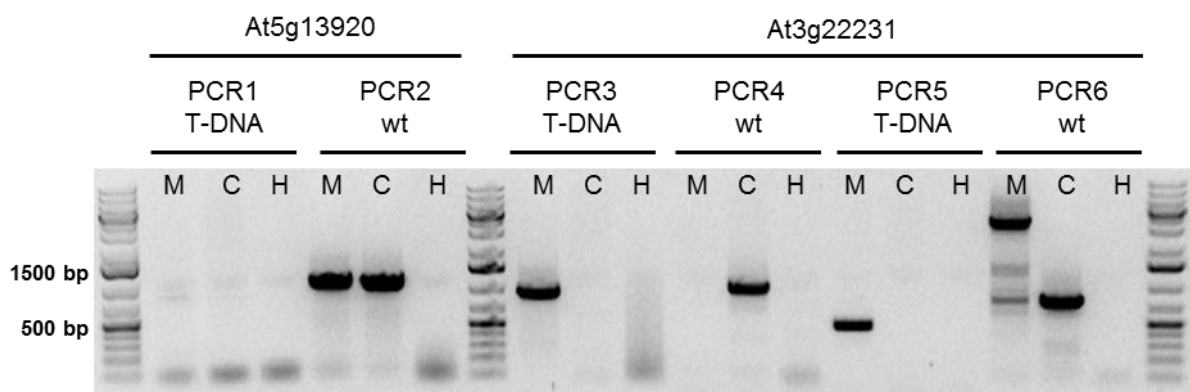
Supplementary Figure 29: Testing Gabi-Kat lines 821C03 and 528H09 for the correct T-DNA insertion. Specific primers were designed to amplify the region of the T-DNA insertion for each mutant line and for each of the three adjacent genes. For the line 528H09, the following band sizes were expected: At3g22231: 545 bp; At3g22235: 980 bp and At3g22240: 950 bp. For the line 821C03, the following band sizes were expected: At3g22231: 1385 bp; At3g22235: 1340 bp and At3g22240: 1160 bp. Three independent PCR reaction per wildtype and mutant were set up to ensure that no band formation belongs to the T-DNA and not to PCR failure. Upper panel wildtype and 528H09 are compared, and bottom panel wildtype and 821C03 are compared. Only for At3g22231, no band could be detected in both mutants.



Supplementary Figure 30: RT-PCR of PCC1. The transcript expression of both mutant lines were compared to the wildtype Col-0. Genomic DNA and water were used as a negative control to ensure no gDNA contamination, which would result in a band shift or unspecific bands. The expected size was around 200 bp for the PCC1 specific fragment. The RT-PCR was run in a 2% agarose gel. Specific primers for actin were used as a control for intact cDNA and comparison of transcript level. Upper gel shows the RT-PCR after 20 cycles and bottom gel after 25 cycles. The first PCC1 band could be detected after 20 cycles in the wildtype, where no actin band was visible. After 25 cycles also the knock-down 821C03 line showed a band. Also for actin bands were then visible. Only 528H09 depicted no bands until 25 cycles.



Supplementary Figure 31: Amplifying the full-length PCC1 from cDNA. Specific primers were used to amplify the full-length PCC1 in wildtype cDNA and cDNA from both mutant lines. Only for 528H09 it was not possible to amplify the gene, since the T-DNA lies within the coding region.



Supplementary Figure 32: Verification of the second T-DNA insertion in the Gabi-Kat 821C03 line. A second T-DNA insertion is annotated in the promoter region of At5g1392 according to the Gabi-Kat website. Specific primer were used to test this via PCR. PCR1 (0.8 kb) was against the T-DNA insertion in At5g1392 and PCR2 (1.2 kb) was the test for the wt allele. PCR3 (1 kb) and PCR5 (0.5 kb) were against the T-DNA in the promoter region of At3g22231 and PCR4 (1 kb) and PCR6 (0.8 kb) for the wt allele. In PCR6 the whole T-DNA was amplified. The abbreviations stand for M: mutant, C: Col-0 wild type control and H: water control.

Supplementary Table 1: Primer sequences In this table, all primers are listed, which were used in this study. The primer name and the sequence was noted down.

name	sequence
BS7_Seq_bait_f	CCAATTGAACACGCTCGATG
BS8_Met25_fwd	GTACCGGATGCAAGGGTTCG
BS9_Cub_rev	TACCACCTCTTAGCCTTAGC
BS10SeqProteinAr	CGCCTTCAGATGTTCTTCAG
BS11_Seq_VP16_r	CCTTCCGTGTTTCAGTTAGC
BS12_TermADH1_r	ACCTGACCTACAGGAAAGAG
BS13_PromADH1_f	GCCTTCCTTCCAGTTACTTG
BS14TaRNR8p12_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGGGCGCGGAGGACGC
BS15TaRNR8p12_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGAGTCGCAAAATGTCTCAAGCTACC
BS16TaRNR8p7_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGGGCGCGGAGGATGC
BS17TaRNR8p7_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGAGTCGCAAAATGTCTCAAGCTACC
BS18HvRNR8_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGAGTCGCAAAATGTCTCAAGCTACC
BS19HvRLK7_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGGGACGGGAAGAGGTAC
BS20HvRLK7_r	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTGCGTTGGCGAGCAAC
BS21SeqRNR8_f	GCATCTGGATCTGAGCTTTAATG
BS22SeqRNR8_2_f	CAATTCTATTGGGCAGGAAGAGGG
BS23HvRLK7_f	AAGCTGCCCAAGCTCAGGAC
BS24RLK7_2_f	GACCAGTTTGAGCGCAGTTC
BS25SeqRNR8_3_f	TGCCGGCTGCTCTTTCCTTG
BS27HvRLK10_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCGGCCACCACCATCCTG
BS28HvRLK10_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCTTGAGTTGTGACAGACATG
BS29Seq_HvRLK10_f	GGCGTCATGGACATGGAGAG
BS89 RLK3 f GW	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGGCGATGCCCACTGCCCTTAAGG
BS90 RLK3 r GW	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTCATCATCCTCCTCCGAGATC
BS91 SeqRLK7 f3	CCTTACACTCCCTCGATCTC
BS92 CYC1 r	GCGTGAATGTAAGCGTGAC
BS109 Seq CSEP pENTR	GATGCCTGGCAGTTCCTTAC
BS110 Seq RLK3 f	TTCGGATTCCATGGGATGAC
BS111 Seq pAG415GPD-GFP	CCCACGCATGTATCTATCTC
BS126 KanMX r geno.	CCTGAGCGAGACGAAATACG
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BS129 TaRNR8p12 kinase f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCTTTCTCCTCTTTCGCTCACAT
BS130 HvRNR8 kinase f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATTGGCTCATTTACCTTGAG
BS131 RLK3 kinase f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCAACAAGTGCTTGGCCCGAGGAG
BS132 Seq prey Nul f	CTTCCGATACCATCGACAAC
BS133 Seq prey r	TTTCGGTTAGAGCGGATGTG

BS134 Bgt CSEP45 f	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTCGTTGCAACAAAAGATGCACTGC
BS135 Bgt CSEP45 r	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAAACCACTTGAACCACGATAAG
BS136 GWY cassette Nul r	AGCCGGATCCTCTAGATTAC
BS137 Gwy cassette NX r	GAAAGCTGGCGCATGATGAC
BS138 TaRNR8 p7 GWY kinase f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATTGGCGAAGGTGGTTTTG
BS139 TaRNR8 p12 GWY kinase f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATTGGCTCATTTACCTTG
BS146 seq HA r	TGAGCAGCGTAATCTGGAAC
BS147 seq DsRed r	CTTGGTCACCTGCAGCTTGG
BS148 p12 kinase mutagen f	CCTTGAGACAAATCAAATCAGCAACGAGAACTTTG
BS149 p12 kinase mutagen r	CAAAGTTTCTCGTTGCTGATTTGATTTGTCTCAAGG
BS150 Mla10 GWY f	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGGATATTGTCACCGGTGCCATTTCC
BS151 Mla10 GWY r	GGGGACCACTTTGTACAAGAAAGCTGGGTGCATTAAATCGTCATCTTGAGCACCTTCT G
BS152 Mla27-1 GWY f	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGGATATTTTCACCGGTGCCATTTCC
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BS158 Seq Mla f 3	GTTGGCTGTCAGGTGGTTCC
BS159 Seq Mla f 4	GGATGTCGTCGACAAGTTCC
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BS180 GPA1 f	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCTTACTCTGCAGTAG
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BS183 CERK1 f	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGCTAAAGATTTCTCTAATC
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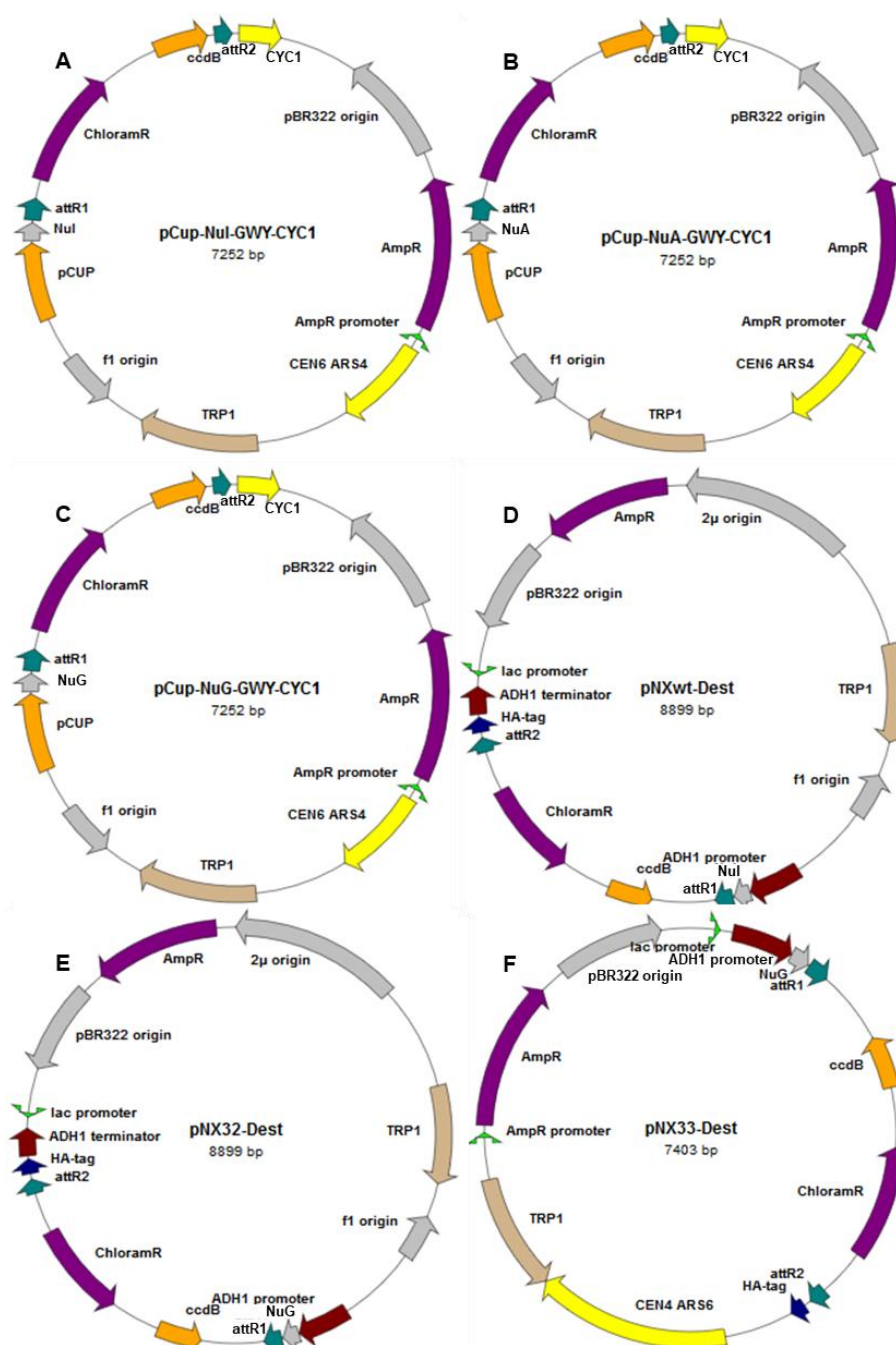
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BS243 N_Luc f	ACGCTGGGCGTTAATCAAAG
BS244 C_Luc r	CGGTCAACGATGAAGAAGTG
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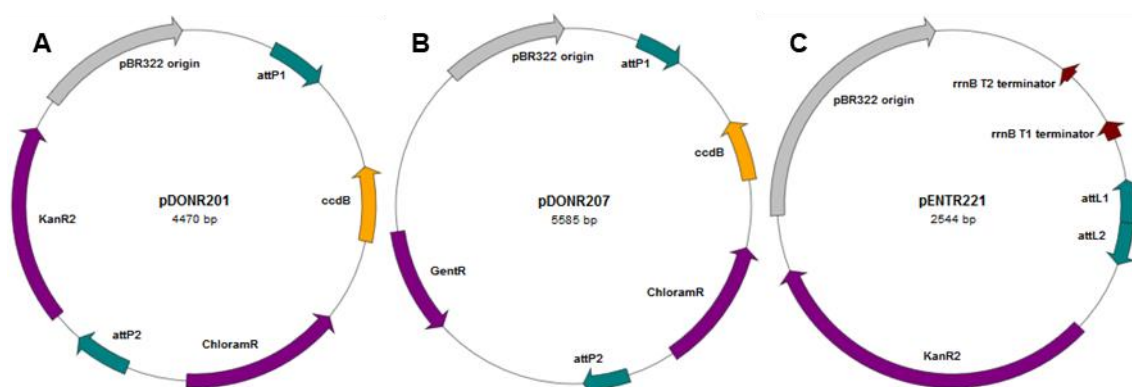
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BS325 mut. Primer PCC1 S24A r	GATAACCAATTGGTGGCGGAGCTGTGTACGGCCCTGATGAAG
BS326 mut. Primer PCC1 Y8F r	GAAGGTTTTTGCACGAAAAGAAATTTTGC GCGGATTGATTC
BS327 mut. Primer PCC1 Y8F f	GAATCAATCCGCGCAAAATTTCTTTTCCGTGCAAAAACCTTC
BS328 PCC1 GWY delta 1-120 f	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCGGTGGTGGGTGATCCTCC
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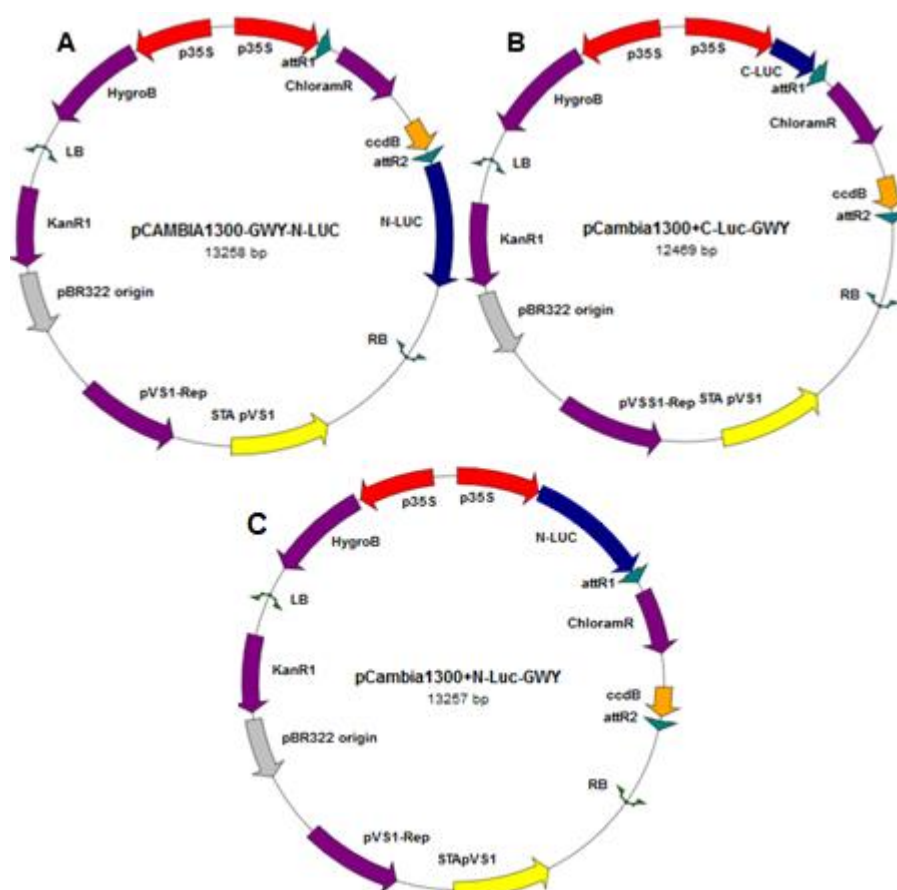
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BS378 PCC1 genotyp f	TGTACGTCATGTACGTGA
BS379 o8474	ATAATAACGCTGCGGACATCTACATTTT
BS381 At3g22240 gDNA r	TACTGACTGATGGTTCAATCATCG
BS382 At3g22240 gDNA f	TCTAGCGACTAGCGTACTTAATTG
BS383 At3g22235 gDNA r	CATCCCACCTAATTAATTCCCTTTG
BS384 At3g22235 gDNA f	CGACGTCATGTACGTGTACGTCATGTACGTGTAC
BS385 At3g22231 gDNA r	GGTCAGAAACAAGATGTAATTAAG
BS386 At3g22231 gDNA f	CGCTTTCCTCTGCCCTTGTG
BS395 528H09 geno. f	CATAGAGTTTAGAGCAATACCC
BS398 At5g13920 r geno.	TGTTACATTAGGTACGCCTCGTTT
BS399 At5g13920 f geno.	CTATGAGCTTTAACTTGCGAACG



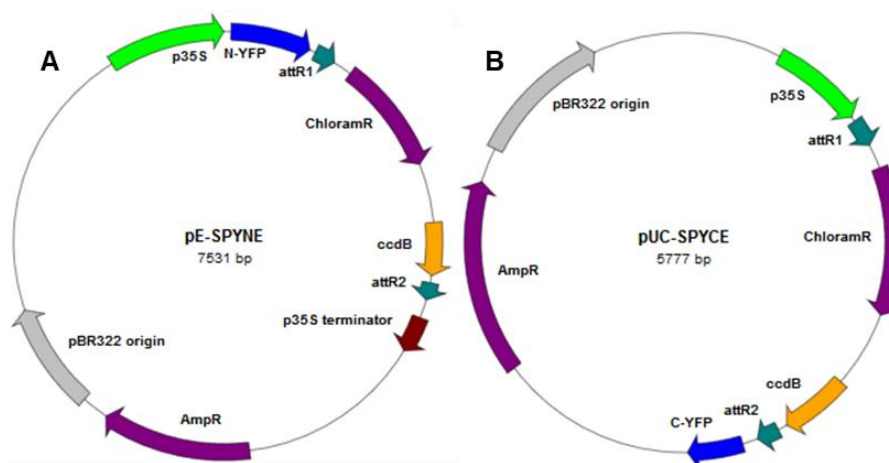
Supplementary Figure 33: Split-ubiquitin prey vector maps. All vectors are Gateway compatible vectors and use the *Trp1* (beige) as a selection marker in yeast, *Amp* resistance (purple) as a resistance marker in *E.coli* and have the Gateway cassette with the chloramphenicol resistance (purple) and the *ccdB* gene (orange). Gateway sites are marked in teal. All have the *f1* and *pBR322* origin of replication (silver). A-C use the *CEN4 ARS6* as the origin of replication in yeast and are low copy vectors. A-C have the copper-inducible promoter (*pCUP*, orange) and the *CYC1* terminator (yellow). A: N-terminal *NuI* (N-terminal part of ubiquitin, wildtype, highest affinity to Cub, silver), B: N-terminal *NuA* (N-terminal part of ubiquitin, middle affinity to Cub, silver), C: N-terminal *NuG* (N-terminal part of ubiquitin, lowest affinity to Cub, silver). D-F use the *ADH1* promoter and terminator and have a triple HA-tag after the Gateway cassette at the C-terminus. D-E have the *2μ* origin of replication in yeast and are high copy vectors (silver). F has the *CEN4 ARS6* origin of replication and is a low copy vector (yellow). D: N-terminal *NuI* (N-terminal part of ubiquitin, wildtype, highest affinity to Cub, silver), E-F: N-terminal *NuG* (N-terminal part of ubiquitin, lowest affinity to Cub, silver).



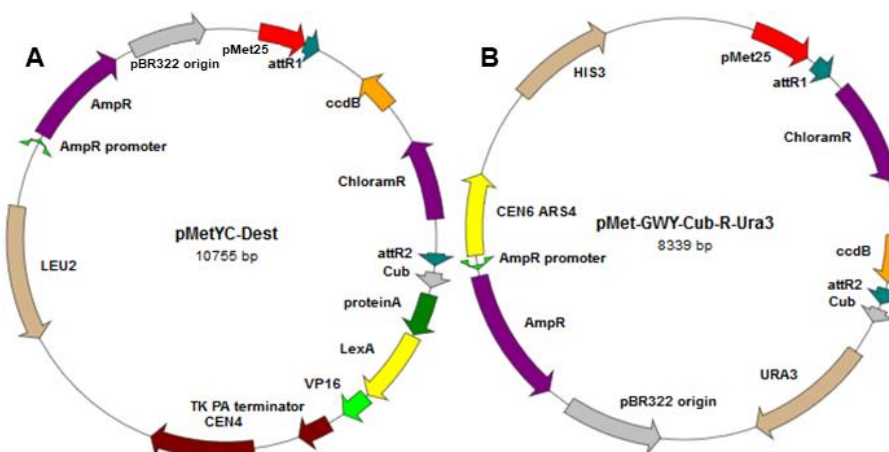
Supplementary Figure 34: ENTRY vector maps. ENTRY vectors for Gateway BP reactions. All have the pBR322 origin of replication (silver). A-B has the Gateway cassette with chloramphenicol resistance (purple) and the *ccdB* gene (orange). Gateway sites are marked in teal. A and C uses the kanamycin resistance marker (purple) and B the gentamycin as resistance marker (purple).



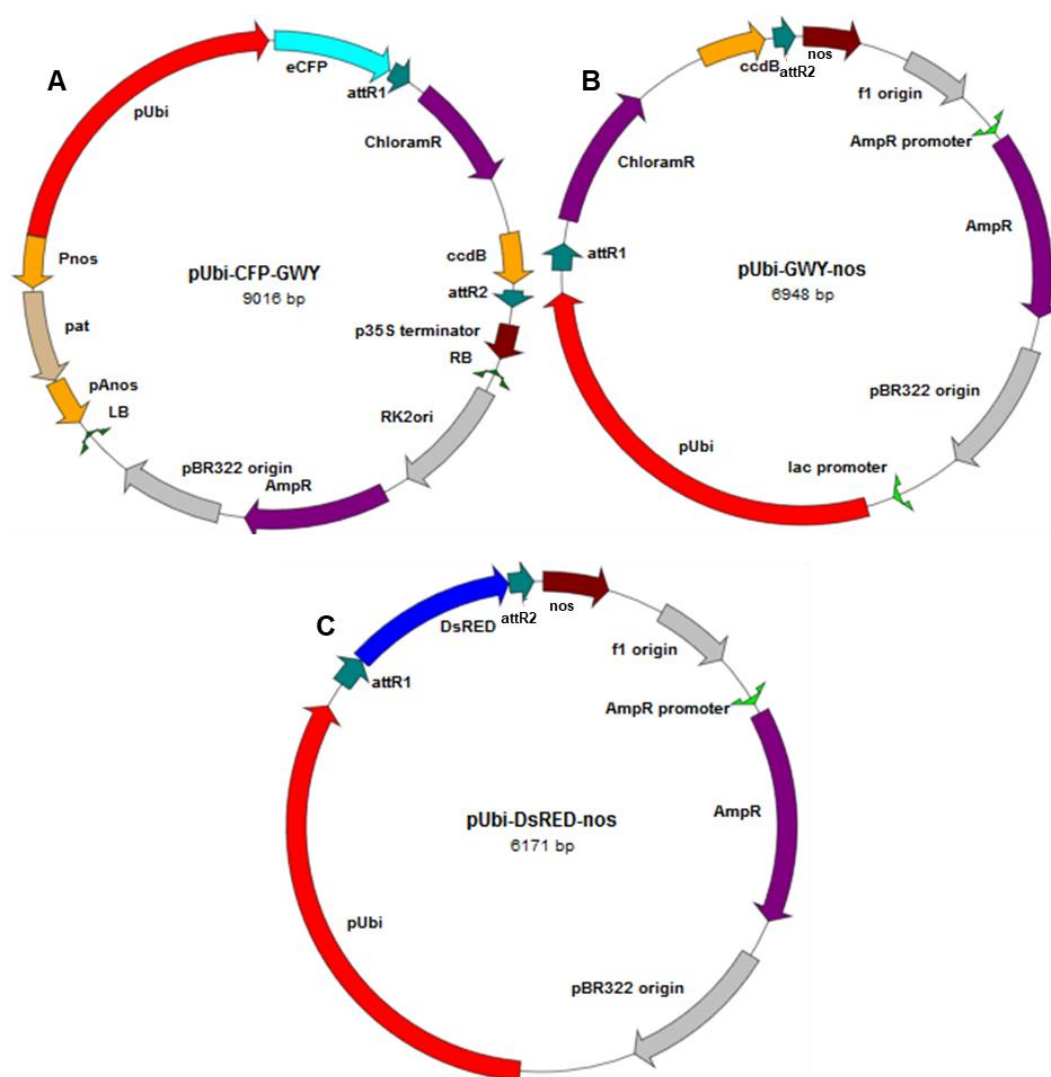
Supplementary Figure 35: Split-luciferase vector maps. These vectors were used for the split-luciferase assays. Both have a kanamycin resistance (purple), the pBR322 origin of replication (silver), pV51-Rep (purple), STA pV51 (yellow). Between LB and RB (teal), both use a hygromycin resistance (purple) marker under the control of a 35S promoter (red) and have a Gateway cassette with chloramphenicol resistance (purple) and *ccdB* gene (orange) between the gateway sites teal. The expression cassette is under control under the 35S promoter (red). A has the N-LUC (N-terminal part of the luciferase) at the C-terminus and B has the C-LUC (C-terminal part of the luciferase) at the N-terminus.



Supplementary Figure 36: BiFC vector maps. These vectors were used for the BiFC experiments. Both have an ampicillin resistance (purple) and an pBR322 ori (grey). Between LB and RB (teal), both have a Gateway cassette with chloramphenicol resistance (purple) and ccdB gene (orange) between the gateway sites (teal). The expression cassette is under control under the 35S promoter (green). A has the N-terminal part of the fluorophore YFP at the N-terminus (blue), whereas B has the C-terminal region of the fluorophore YFP at the C-terminus (blue).



Supplementary Figure 37: Split-ubiquitin bait vector maps. All vectors are Gateway compatible vectors and use the Amp resistance (purple) as a resistance marker in *E.coli* and have the Gateway cassette with the chloramphenicol resistance (purple) and the ccdB gene (orange). Gateway sites are marked in teal. All have the pBR322 origin of replication (silver). Both vectors have the Met25 promoter (red) as methionine repressible promoter. Both vectors have the Leu2 selection marker (beige) in yeast, whereas B uses His3 as a marker (beige). Both vectors are low copy vectors with the CEN4 ARS6 (A: red, B: yellow). After the expression cassette, A has in the following order at the C-terminus: Cub (C-terminal part of ubiquitin, silver), proteinA (green), LexA (yellow) and VP16 (bright green). ProteinA, LexA and VP16 from the artificial transcription factor PLV, which activates the reporter gene after an interaction. B has in the following order at the C-terminus: Cub (C-terminal part of ubiquitin, silver) and the Ura3 gene (beige) with a substitution in the first position, which leads to an exchange from Met to Arg.



Supplementary Figure 38: Plant expression vector. These vectors were used for fluorophore/non tagged expression of genes of interest *in planta* (barley). These vectors have an ampicillin resistance (purple) and an pBR322 ori (grey). (A) Between LB and RB (teal), the vector has a Gateway cassette with chloramphenicol resistance (purple) and ccdB gene (orange) between the gateway sites (teal). (B/C) have no LB and RB sites, but a Gateway cassette with chloramphenicol resistance (purple) and ccdB gene (orange) between the gateway sites (teal). The expression cassette is under control of the pUbi (ubiquitin) promoter (red) and p35S terminator (dark red)(A) or nos terminator (dark red) (B/C). (A)The vector has the fluorophore eCFP at the N-terminus (blue) and (C) has DsRED (blue) instead of the Gateway cassette. The pat (herbicide resistance) is under the control of the nos promoter and terminator (orange).

Supplementary Table 2: Co-expressed genes of PCC1, At3g22240 and At1g05340

Extracted top 300 co-expressed genes of PCC1, At3g22240 and At1g05340 from ATTED-II. Depicted are the gene ID and the alias.

PCC1		At3g22240		At1g05340	
co-expressed	alias	co-expressed	alias	co-expressed	alias
genes		genes		genes	
At5g03350	Legume lectin	At3g22231	PCC1	At1g05340	
At2g14560	LURP1	At1g19960		At3g49780	PSK4
At3g22240		At3g27890	NQR	At4g15610	UPF0497
At4g14400	ACD6	At2g14560	LURP1	At2g31945	
At1g19960		At2g40750	WRKY54	At3g06420	G8H
At2g41090	EF-hand	At5g55170	SUMO3	At4g22820	zinc finger
At2g25510		At3g28940	AIG2-like	At1g63720	
At1g31580	ECS1	At1g30900	VSR6	At3g48020	
At1g21250	WAK1	At3g20510	Transmembranes 14C	At1g68620	alpha/beta- Hydrolases
At5g54610	BDA1	At3g48080	alpha/beta- Hydrolases	At1g22985	CRF7
At2g40750	WRKY54	At3g22600	inhibitor	At1g45145	TRX5
At3g04210	TIR-NBS	At4g18930	phosphodiesterase	At1g67920	
At5g19250	GPI-anchored	At4g14400	ACD6	At2g22860	PSK2
At5g59670	kinase	At5g37740	Calcium-dependent lipid-binding	At5g13330	Rap2.6L
At2g41180	SIB2	At3g13610	2OG	At4g27410	RD26
At1g21270	WAK2	At3g51330	protease	At3g19580	ZF2
At1g66970	SVL2	At3g60450	mutase	At4g23450	AIRP1
At1g49750	LRR	At5g24530	DMR6	At1g24600	
At5g10760	protease	At5g52810	Rossmann-fold	At3g28210	SAP12
At5g55450	inhibitor	At1g18980	RmlC-like cupins	At1g71950	inhibitor
At3g57240	BG3	At4g20110	VSR7	At5g40690	
At1g75040	PR5	At5g44480	DUR	At1g10140	UCP031279
At3g56400	WRKY70	At1g13750	Purple phosphatases	At5g13080	WRKY75
At1g35710	Protein kinase with LRR domain	At3g43800	GSTU27	At5g13200	GRAM domain
At5g60900	RLK1	At5g03350	Legume lectin	At5g57910	
At1g52290	PERK15	At3g09490	Tetratricopeptide repeat (TPR)-like	At3g04010	hydrolase
At2g23200	kinase	At5g54610	BDA1	At2g34500	CYP710A1
At2g32160	transferase	At1g13830	Carbohydrate- binding X8 domain	At3g15500	NAC3
At2g14610	PR1	At4g18430	RABA1e	At3g20340	
At4g11900	kinase	At5g50760	SAUR-like auxin- responsive	At2g22470	AGP2
At4g26070	NMAPKK	At3g23600	alpha/beta- Hydrolases	At1g58340	ZRZ

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At1g03400	2OG	At5g39950	TRXH2	At2g37750	
At1g76960		At2g34940	VSR5	At2g28400	DUF584
At1g02450	NIMIN1	At3g09010	kinase	At1g13340	Regulator of Vps4 activity in the MVB pathway
At1g69730	kinase	At5g11670	NADP-ME2	At1g02310	MAN1
At1g17610	TIR-NBS	At3g62780	Calcium-dependent lipid-binding	At5g10695	
At3g45860	CRK4	At1g35260	MLP165	At1g69790	kinase
At4g02420	kinase	At1g47480	alpha/beta-Hydrolases	At5g18270	ANAC087
At4g21380	RK3	At2g44010		At5g04250	Cysteineases
At1g13470	DUF1262	At5g60950	COBL5	At3g62260	Protein phosphatase 2C
At3g57260	PR2	At3g22850	Aluminium induced with YGL and LRDR motifs	At1g80160	GLYI7
At1g11330	kinase	At2g25510		At1g08920	ESL1
At1g13750	Purple phosphatases	acid At5g46230	DUF538	At3g50970	XERO2
At1g68585		At1g74210	GDPD5	At4g12040	SAP7
At5g24210	alpha/beta-Hydrolases	At3g56400	WRKY70	At5g04760	Duplicated homeodomain-like
At1g78620	Protein of unknown function DUF92	At5g18780	F-box	At3g04070	NAC047
At5g36930	TIR-NBS-LRR	At4g37010	CEN2	At1g43160	related to AP2 6
At3g56710	SIB1	At4g01920	Cysteine/Histidine-rich C1 domain	At4g39670	GLTP
At3g59010	PME61	At4g25900	Galactose mutarotase-like	At2g45210	SAUR36
At5g64800	CLE21	At3g28950	AIG2-like	At3g04060	NAC046
At1g55910	ZIP11	At2g30870	GSTF10	At1g76070	
At2g37710	RLK	At5g19240	GPI-anchored	At1g71697	CK1
At2g40600	appr-1-p processing enzyme	At3g01290	HIR2	At3g50260	DEAR1
At3g25020	RLP42	At3g11340	UGT76B1	At4g03320	Tic20-IV
At2g04450	NUDX6	At1g23830		At4g33940	RING/U-box
At1g74440	DUF962	At5g42250	dehydrogenase	At3g60690	SAUR-like auxin-responsive
At2g27920	SCPL51	At1g09560	PDGLP1	At4g19200	proline-rich
At4g23130	RLK6	At5g48290	Heavy metal transport/detoxification	At4g21980	G8A
At1g16260	kinase	At3g56240	CCH	At4g25390	kinase

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At3g60420	mutase	At3g63080	MEE42	At5g17650	glycine/proline-rich
At5g52810	Rossmann-fold	At2g38860	YLS5	At2g35980	YLS9
At1g17600	TIR-NBS-LRR	At1g67940	NAP3	At1g02400	GA2OX6
At3g28540	hydrolase	At1g17745	PGDH	At5g05410	DREB2A
At2g18660	PNP-A	At1g21310	RSH	At1g08315	ARM repeat
At1g74300	alpha/beta-Hydrolases	At4g14390	Ankyrin repeat	At2g30250	WRKY25
At5g56260	transferase	At2g17720	P4H5	At3g51890	Clathrin
At1g31550	hydrolase	At2g20630	PIA1	At5g59820	ZAT12
At5g53370	PMEPCRF	At1g03850	GRXS13	At4g19640	RABF2B
At2g32680	RLP23	At4g00700	transferase	At1g01720	ATAF1
At4g29810	MKK2	At2g47130	SDR3	At1g21000	transcription
At2g33530	scpl46	At4g05590		At4g30390	
At3g28930	AIG2	At1g11310	PMR2	At3g29575	AFP3
At3g05650	RLP32	At3g12500	PR3	At2g02390	GSTZ1
At1g05630	5PTASE13	At1g11330	kinase	At1g19020	
At3g25010	RLP41	At2g25000	WRKY60	At5g04340	ZAT6
At2g24160		At2g41380	transferase	At2g28110	IRX7
At1g69720	HO3	At5g56610	Phosphotyrosine phosphatases	At5g45130	RHA1
At1g73805	SARD1	At4g05200	CRK25	At3g22260	Cysteineases
At5g44580		At4g26070	NMAPKK	At1g73030	CHMP1A
At2g24860	DnaJ/Hsp40 cysteine-rich domain	At2g27920	SCPL51	At3g60140	SRG2
At3g08870	kinase	At5g45000	TIR-NBS-LRR	At1g13990	
At5g19240	GPI-anchored	At3g21710		At4g20860	Berberine
At5g38980		At5g17330	GAD1	At5g18130	
At4g25940	ENTH/ANTH/VHS	At1g02360	Chitinase	At5g08240	
At2g26400	ARD3	At1g17080	L18ae	At5g63905	
At5g22570	WRKY38	At3g26440	DUF707	At4g01870	tolB
At4g13810	RLP47	At4g33360	FLDH	At1g53580	GLY3
At4g03450	Ankyrin repeat	At2g19130	kinase	At1g01480	ACC2
At2g14580	PRB1	At5g59670	kinase	At1g65510	
At3g26230	CYP71B24	At1g12140	FMO GS-OX5	At2g46270	GBF3
At4g23240	CRK16	At5g40690		At2g30360	SNRK3.22
At4g13510	AMT1;1	At1g77920	TGA7	At1g77450	NAC032
At2g24390	AIG2-like	At4g20780	CML42	At2g38340	DREB19
At3g14620	CYP72A8	At1g23140	Calcium-dependent lipid-binding	At5g62150	LysM
At2g17040	NAC036	At1g63460	GPX8	At5g54940	SUI1
At4g23150	CRK7	At5g47730	Sec14p-like phosphatidylinositol transfer	At1g63840	RING/U-box
At1g16500		At5g45380	DUR3	At2g46500	UBDK GAMMA 4
At2g26440	inhibitor	At3g22620	inhibitor	At1g22990	HIPP22
At2g26020	PDF1.2b	At1g76960		At5g20910	AIP2

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At5g10380	RING1		At5g08240		At3g01970	WRKY45
At2g28085	SAUR-like responsive	auxin-	At1g12200	FMO	At1g30700	Berberine
At2g24850	TAT3		At3g09270	GSTU8	At5g42050	DCD domain
At1g33960	AIG1		At1g21250	WAK1	At1g61820	BGLU46
At5g11250	TIR-NBS-LRR		At3g24503	REF1	At1g29970	RPL18AA
At3g23010	RLP36		At5g64370	PYD3	At4g37010	CEN2
At5g44420	PDF1.2A		At5g25940	early nodulin	At2g23810	TET8
At3g43800	GSTU27		At4g14610		At2g47270	UPB1
At4g08470	MEKK3		At3g56950	SIP2;1	At2g16720	MYB7
At4g23610	LEA		At5g37480		At3g17000	UBC32
At4g23220	CRK14		At2g37710	RLK	At4g05100	MYB74
At3g28890	RLP43		At3g57630	exostosin	At3g46930	kinase
At1g64360			At1g57560	MYB50	At5g17850	Sodium/calcium exchanger
At1g59620	CW9		At1g70690	PDLP5	At1g73260	KTI1
At5g52740	Copper transport		At5g04930	ALA1	At5g59220	SAG113
At5g61520	Major facilitator		At1g68300	hydrolase	At3g10640	SNF7
At2g15042	LRR		At5g22570	WRKY38	At1g48000	MYB112
At5g44820	transferase		At4g01480	PPa5	At1g52890	NAC019
At3g45290	MLO3		At4g23470	PLAC8	At1g30640	kinase
At4g23320	CRK24		At3g04720	PR4	At1g15430	DUF1644
At2g37540	Rossmann-fold		At3g53780	RBL4	At4g33420	Peroxidase
At4g04220	RLP46		At3g02770	transferase	At3g61980	Kazal-type
At3g48080	alpha/beta- Hydrolases		At2g41090	EF-hand	At3g22910	hydrolase
At5g52760	Copper transport		At1g05000	PFA-DSP1	At5g13190	GILP
At3g50480	HR4		At2g40600	appr-1-p processing enzyme	At5g04080	
At4g03080	BSL1		At3g17420	GPK1	At4g35860	GB2
At5g39030	kinase		At2g30840	2OG	At2g29460	GSTU4
At3g04480	nuclease		At4g30670	Putative membrane lipoprotein	At3g22600	inhibitor
At1g64710	dehydrogenase		At5g44820	transferase	At5g38710	reductase
At1g29720	kinase		At3g01420	PADOX-1	At3g57090	FIS1A
At5g52540	DUF819		At1g02450	NIMIN1	At4g28085	
At4g23180	RLK4		At2g44360		At2g35730	Heavy metal transport/detoxific ation
At4g00955			At1g66880	kinase	At1g01470	LSR3
At4g26090	RPS2		At3g49780	PSK4	At5g11680	
At1g08450	PSL1		At4g15093	LigB	At5g16830	SYP21
At5g05090	Homeodomain-like		At4g01700	Chitinase	At1g70590	F-box
At3g45140	LOX2		At1g67330	DUF579	At5g47120	BI1
At4g11000	Ankyrin repeat		At2g31090		At5g51070	SAG15
At5g17880	CSA1		At4g39030	SID1	At4g26750	EXT-like

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At5g61540	hydrolase	At5g44920	TIR	At3g48520	CYP94B3
At4g04500	CRK37	At5g11970	DUF3511	At1g76180	ERD14
At1g67865		At1g61560	MLO6	At1g23440	I-like
At1g66980	SNC4	At5g49760	kinase	At5g27520	PNC2
At4g23260	CRK18	At1g13950	ELF5A-1	At3g08720	S6K2
At4g19660	NPR4	At3g09810	IDH-VI	At1g28200	FIP1
At1g33840	DUF567	At4g13660	PRR2	At5g62480	GSTU9
At5g24530	DMR6	At2g40110	Yippee putative zinc-binding	At1g70140	FH8
At4g11890	ARCK1	At5g60900	RLK1	At3g55890	Yippee putative zinc-binding zinc finger
At3g19550		At1g65690	LEA	At5g62460	
At4g28490	RLK5	At4g10500	2OG	At1g62840	DUF1442
At3g62780	Calcium-dependent lipid-binding	At1g34750	Protein phosphatase 2C	At1g15010	
At3g50950	ZAR1	At5g38900	Thioredoxin	At3g47550	zinc finger
At1g52770	NPH3	At1g65820	transferase	At3g43230	zinc finger
At1g23840		At5g12880	proline-rich	At5g15190	
At4g16890	SNC1	At1g74440	DUF962	At2g47190	MYB2
At5g49760	kinase	At2g36330	UPF0497	At5g39610	ORE1
At3g47480	EF-hand	At5g53320	kinase	At2g33700	PP2CG1
At4g14365	XBAT34	At4g13510	AMT1;1	At2g30550	alpha/beta-Hydrolases
At5g25050	Major facilitator	At3g11840	PUB24	At4g31860	Protein phosphatase 2C NC
At4g24970	ATPase	At1g51830	kinase	At5g06370	
At2g29120	glutamate receptor 2.7	At2g45220	inhibitor	At3g49210	WSD1-like
At2g36430	DUF247	At1g74300	alpha/beta-Hydrolases	At3g10985	WI12
At1g22650	A/N-InvD	At3g29250	SDR4	At2g23450	kinase
At4g23200	CRK12	At5g54840	SGP1	At1g65690	LEA
At2g23680	WCOR413	At3g25610	hydrolase	At3g21520	DMP1
At2g17120	LYP1	At5g58375	transferase	At1g42990	BZIP60
At2g05520	GRP3	At5g19930	Protein of unknown function DUF92	At1g21010	
At5g44870	TTR1	At5g07460	PMSR2	At2g39050	EULS3
At1g67470	kinase	At1g45145	TRX5	At5g13820	TBP1
At3g57700	kinase	At4g08770	Prx37	At4g21560	VPS28-1
At4g34480	hydrolase	At4g34050	CCoAOMT1	At1g07870	kinase
At3g45260	zinc finger	At4g02860	PhzC/PhzF	At1g61810	BGLU45
At2g47880	Glutaredoxin	At3g13650	dirigent-like	At5g66070	RING/U-box
At3g26320	CYP71B36	At5g43910	kinase	At3g51130	
At5g52750	Heavy metal transport/detoxification	At3g13910	DUF3511	At1g78310	VQ motif
At4g16860	RPP4	At3g12700	NANA	At5g54840	SGP1
At4g23310	CRK23	At4g19660	NPR4	At4g39890	RABH1c

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At2g15080	RLP19	At2g37580	RING/U-box	At4g37370	CYP81D8
At5g44570		At4g17215	extensin	At2g31990	Exostosin
At1g11310	PMR2	At2g23680	WCOR413	At3g10500	NTL4
At1g67860		At5g17880	CSA1	At5g06760	LEA4-5
At5g60800	Heavy metal transport/detoxification	At1g16670	kinase	At1g62300	WRKY6
At2g36970	transferase	At5g25890	IAR2	At5g05600	2OG
At3g28940	AIG2-like	At3g52400	SYP122	At5g01380	Homeodomain-like
At2g31880	SOBIR1	At4g17030	EXPR	At5g46080	kinase
At1g78410	VQ motif	At2g48030	I-like	At2g18690	
At5g13320	WIN3	At5g63600	FLS5	At2g17500	Auxin efflux carrier
At3g48640		At5g03460		At3g01650	RGLG1
At4g14390	Ankyrin repeat	At1g02900	RALFL1	At3g63050	
At4g33050	IQM1	At3g19660		At5g37540	protease
At4g38550	lipase	At4g11850	PLDGAMMA1	At5g57900	SKIP1
At1g55450	transferase	At5g45500	RNI-like	At5g58730	Mik
At1g35230	AGP5	At1g48210	kinase	At1g35190	2OG
At1g56520	TIR-NBS-LRR	At1g22180	Sec14p-like phosphatidylinositol transfer	At2g37110	PLAC8
At1g64400	LACS3	At3g09020	transferase	At5g39520	DUF1997
At2g31230	ERF15	At2g02390	GSTZ1	At1g04960	DUF1664
At3g26600	ARO4	At2g23200	kinase	At1g54100	ALDH7B4
At1g21240	WAK3	At2g17120	LYP1	At1g75440	UBC16
At3g57460	catalytics;metal ion binding	At2g17130	IDH2	At1g76590	transcription
At5g22380	NAC090	At2g22480	PFK5	At2g32800	AP4.3A
At1g68600	transporter	At1g62790	inhibitor	At2g31350	GLX2-5
At5g07580	DNA-binding	At3g26830	PAD3	At1g23040	glycoprotein
At4g00330	CRCK2	At4g22530	transferase	At4g05050	UBQ11
At5g44070	PCS1	At5g10760	protease	At4g26060	L18ae
At5g40060	NBS-LRR	At4g00955		At5g56150	UBC30
At4g36500		At4g26060	L18ae	At2g06530	SNF7
At1g29240	DUF688	At3g45860	CRK4	At2g32150	HAD
At1g56150	SAUR-like auxin-responsive	At1g51070	bHLH115	At2g41380	transferase
At3g02770	transferase	At3g21230	4CL5	At2g38250	Homeodomain-like
At3g46530	RPP13	At4g29810	MKK2	At2g27310	F-box
At3g11090	LBD21	At4g19880	transferase	At1g08050	Zinc finger
At3g27890	NQR	At2g24040	Low temperature and salt responsive	At5g65300	
At1g23130	Polyketide cyclase/dehydrase and lipid transport	At3g03000	EF hand calcium-binding	At5g02020	SIS
At3g17700	CNGC20	At3g21240	4CL2	At4g34230	CAD5
At2g40270	kinase	At2g19570	DESZ	At3g18560	

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At1g50170	SIRB	At1g67800	Copine	At3g52800	zinc finger
At3g09010	kinase	At1g17430	alpha/beta-Hydrolases	At5g67340	ARM repeat
At3g48650		At4g21380	RK3	At1g13195	RING/U-box
At5g39020	kinase	At1g35720	OXY5	At4g29950	Ypt/Rab-GAP domain of gyp1p inhibitor
At1g74710	SID2	At3g25930	hydrolase	At2g45220	
At5g55170	SUMO3	At5g05960	inhibitor	At1g52690	LEA7
At1g55120	FRUCT5	At3g28930	AIG2	At3g14990	DJ1A
At3g05660	RLP33	At1g60680	oxidoreductase	At5g13810	Glutaredoxin
At3g55130	WBC19	At5g63590	FLS3	At2g36220	
At1g56190	kinase	At1g22410	synthetase	At3g59700	LECRK1
At4g17570	GATA26	At3g21630	LYSM RLK1	At1g17080	L18ae
At3g48090	EDS1	At5g63990	monophosphatase	At5g25450	Cytochrome bd ubiquinol oxidase
At1g01560	MPK11	At5g62630	HIPL2	At5g55970	RING/U-box
At1g66880	kinase	At1g08050	Zinc finger	At3g14060	
At1g65490		At5g19590	DUF538	At2g47770	TSPO
At5g08380	AGAL1	At3g07600	Heavy metal transport/detoxification	At4g24400	SnRK3.13
At5g23240	N-terminal	At5g45510	LRR	At5g64260	EXL2
At1g56510	WRR4	At4g38080	glycoprotein	At3g05200	ATL6
At5g09470	DIC3	At3g09960	phosphoesterase	At1g09070	SRC2
At1g53230	TCP3	At1g73805	SARD1	At3g14280	
At3g62370	heme binding	At1g35710	Protein kinase with LRR domain	At2g17840	ERD7
At3g44350	NAC061	At1g03210	PhzC/PhzF	At5g63790	NAC102
At2g25000	WRKY60	At3g52430	PAD4	At1g75810	
At1g63880	TIR-NBS-LRR	At5g62150	LysM	At1g07500	
At4g38560	lipase	At4g11290	Peroxidase	At3g13430	RING/U-box
At3g01080	WRKY58	At5g64890	PROPEP2	At5g11090	serine-rich
At3g62410	CP12-2	At1g51860	kinase	At3g12510	MADS-box
At5g58390	Peroxidase	At1g51890	kinase	At4g02380	SAG21
At5g04930	ALA1	At3g11280	Duplicated homeodomain-like	At3g07370	CHIP
At1g69550	TIR-NBS-LRR	At3g26470	Powdery mildew resistance	At4g34180	Cyclase
At1g31690	oxidase	At2g44790	UCC2	At3g02480	LEA
At2g03980	hydrolase	At1g07090	LSH6	At5g38895	RING/U-box
At4g27300	kinase	At3g21770	Peroxidase	At1g74020	SS2
At5g13720	UPF0114	At3g25600	EF-hand	At3g63310	BIL4
At5g54720	Ankyrin repeat	At3g50280	transferase	At4g18170	WRKY28
At5g11060	KNAT4	At3g44100	MD-2-related lipid recognition	At1g08940	mutase
At4g08480	SUMM1	At3g47540	Chitinase	At2g38410	ENTH/VHS/GAT

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At1g11300	protein serine/threonine kinases;protein kinases;ATP binding;sugar binding;kinases;carb ohydrate binding	At4g36430	Peroxidase	At3g03310	LCAT3	
At3g16530	Legume lectin	At1g01340	CNGC10	At4g04800	MSRB3	
At4g25110	MCP1c	At2g25450	2OG	At3g06490	MYB108	
At1g79110	BRG2	At5g61250	GUS1	At5g11650	alpha/beta- Hydrolases	
At1g07650	kinase	At5g19250	GPI-anchored	At5g61640	PMSR1	
At2g24600	Ankyrin repeat	At2g02360	PP2-B10	At2g37970	SOUL-1	
At1g65500		At2g16790	hydrolase	At4g04620	G8B	
At3g45640	MPK3	At4g23710	VHA-G2	At1g74360	kinase	
At1g51805	kinase	At3g13950		At5g67600	WIH1	
At1g74210	GDPD5	At3g53150	UGT73D1	At5g48180	NSP5	
At5g36220	CYP91A1	At2g23540	hydrolase	At2g01540	Calcium- dependent binding	lipid-
At1g72610	GLP1	At3g23200	UPF0497	At4g16760	ACX1	
At1g13110	CYP71B7	At5g20090	UPF0041	At3g50980	XERO1	
At2g03530	UPS2	At2g15390	FUT4	At1g21450	SCL1	
At2g34940	VSR5	At1g15120	Ubiquinol- cytochrome reductase hinge	At3g14560		
At2g46600	EF-hand	At2g26400	ARD3	At2g40000	HSPRO2	
At3g44450		At2g41180	SIB2	At5g26340	STP13	
At3g44630	TIR-NBS-LRR	At2g21100	dirigent-like	At1g17020	SRG1	
At5g40170	RLP54	At2g31880	SOBIR1	At1g74590	GSTU10	
At5g52320	CYP96A4	At1g64280	SAI1	At2g21620	RD2	
At5g58570		At1g07000	EXO70B2	At1g64610	Transducin/WD40 repeat-like	
At5g41410	BEL1	At3g02240	RGF7	At3g55470	Calcium- dependent binding	lipid-
At4g17695	KAN3	At4g11530	CRK34	At3g17110		
At5g22580	Domain	At5g54500	FQR1	At5g11970	DUF3511	
At4g00970	CRK41	At1g70810	Calcium-dependent lipid-binding	At5g27420	CNI1	
At4g38460	SSU	At1g31580	ECS1	At3g52400	SYN122	
At1g11350	SD1-13	At3g47480	EF-hand	At1g32350	AOX1D	
At5g63410	kinase	At1g64405		At5g58350	ZIK2	
At5g07770	FH2	At4g30190	PMA2	At5g63970	RGLG3	
At4g10500	2OG	At2g04430	NUDT5	At3g13910	DUF3511	
At4g18250	kinase	At1g34420	kinase	At3g10320	transferase	
At1g30900	VSR6	At3g48920	MYB45	At5g09440	EXL4	
At1g53350	CC-NBS-LRR	At2g01650	PUX2	At4g26080	ABI1	

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At5g55990	CBL2	At3g09220	LAC7	At3g01420	PADOX-1
At3g47250	DUF247	At2g47200		At5g58070	TIL
At1g28600	hydrolase	At2g39420	alpha/beta-Hydrolases	At2g15480	UGT73B5
At3g28480	oxygenase	At3g59660	GRAM	At5g42380	CML39
At1g48210	kinase	At3g01720		At1g27300	
At3g60540	Preprotein translocase Sec	At5g42830	transferase	At4g24990	ATGP4
At1g72920	TIR	At2g14610	PR1	At1g21550	EF-hand
At5g11910	alpha/beta-Hydrolases	At5g59530	2OG	At5g61820	
At4g35180	LHT7	At4g01070	UGT72B1	At5g20230	SAG14
At4g16950	RPP5	At3g62580	LEA	At5g06320	NHL3
At2g44210	DUF239	At4g26970	ACO2	At3g17410	kinase
At3g29400	EXO70E1	At1g09090	RBOHB	At1g48320	DHNAT1
At1g28230	PUP1	At5g25540	CID6	At3g17710	F-box
At4g29050	kinase	At1g66240	ATX1	At5g59490	HAD
At2g42690	alpha/beta-Hydrolases	At3g05920	Heavy metal transport/detoxification	At2g21820	
At3g07520	glutamate receptor 1.4	At4g26910	transferase	At5g62020	HSFB2A
At3g57260		At3g57260		At2g37150	RING/U-box
