# Generation of highly productive polyclonal and monoclonal tobacco suspension lines from a heterogeneous transgenic BY-2 culture through flow cytometric sorting

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vorgelegt von

Diplom-Biologin
**Janina Kirchhoff**
aus Dortmund

Berichter: Universitätsprofessor Dr. rer. nat. Rainer Fischer
Universitätsprofessor Dr. rer. nat. Dirk Prüfer

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# 1 Introduction

## 1.1 Plant cells as production systems for recombinant proteins

The ever increasing demand for complex pharmaceutical or industrial proteins that require regulatory and safety standards in combination with considerable advances in plant research over the past decade has emerged plant cell cultures as a suitable alternative production platform for recombinant proteins (Xu *et al.* 2011). Today, suspended plant cells offer a number of advantages for the expression of complex recombinant proteins over traditional microbial or mammalian production systems because of their intrinsic safety and economic bioprocessing of biologically active mammalian proteins.

### 1.1.1 Advantages of plant cells

Intact plants and plant tissues are widely documented as an attractive alternative to the established production technologies that use bacterial systems, yeasts and cultured animal cells, for several reasons. Factors that favour plant systems are the convenient production of active, complex recombinant proteins, the reduced risk of product contamination by human pathogens or bacterial endotoxins and the potential for economic large-scale production of agricultural biomass (Sharp and Doran 2001). Indeed, intact plants only need inexpensive inputs such as sunlight, water and nutrients and they require low harvest and processing technology for the production of the respective protein (Rigano and Walmsley 2005). Since sophisticated transformation techniques and a broad variety of signal sequences and regulatory elements already exist, genetic modifications for optimal transgene expression are simple and straightforward. Although a wide range of plant hosts have been developed, tobacco is a favoured model system for the production of recombinant proteins because it is amenable to genetic engineering and produces a high biomass. Given that tobacco is a non-food, non-feed plant minimizes the risk of plant-made recombinant proteins entering the food chain. Even though many low-nicotine varieties of tobacco circumvent the disadvantage of toxic alkaloids, the tobacco platform has still some drawbacks, i.e. the long creation time for transgenic plant lines and the limited biomass storage. In particular, proteins accumulated in leaves tend to be unstable and affected by proteolytic degradation, which requires the immediate processing of harvested genetic plant material (Conley *et al.* 2010). In this regard, targeting of therapeutic proteins to storage organs such as maize seeds (Stöger *et al.* 2000) or potato tubers (De Wilde *et al.* 2002) enhanced both protein yields and stability. However, the poor biosafety and uncertain containment of these forage crops should not be neglected.

Among various plant expression systems being explored, suspension-cultured plant cells are a promising alternative for the production of recombinant proteins. Being totally devoid of problems associated with the seasons, pesticides, soil nutrition or gene flow into the environment, plant suspension cultures have intrinsic advantages over greenhouse or

field-grown intact plants (Plasson *et al.* 2009). Compared to the growth of whole transgenic plants the time needed for recombinant protein production is considerably shorter and using bioreactor cultivation, transgenic plant suspension cultures have superior benefits over all established expression systems in terms of flexibility, scalability, and production costs (Boehm 2007). Since bioreactors facilitate a contained system, plant cell-based production systems can be easily cultivated at certified and controllable conditions. This provides advantages for regulatory GMP (good manufacturing practice) requirements for the production of recombinant proteins that can be obtained more easily using reactor grown cells (Doran 2000). Moreover, plant cell cultures enable greater opportunities for the manipulation of recombinant proteins levels, as culture conditions can be altered much more easily in bioreactors than in the field or greenhouse. For instance, supplementing culture medium with nitrogen significantly increased recombinant antibody yields (Holland *et al.* 2010) and several examples demonstrated that protein production in plant cell cultures can be enhanced by the modification of diverse bioprocessing parameters (Georgiev *et al.* 2009). The ability to fuse the protein of interest to a signal sequence that permits product secretion into culture medium is also advantageous for suspended plant cells, as cell disruption and product clarification can be circumvented, which contributes to a simplified downstream processing and thus reduction of the overall production cost (Xu *et al.* 2011).

Plant cell suspension cultures feature many merits of whole-plant systems with those of microbial and mammalian production systems. Like bacteria, cultured plant cells can be inexpensively cultivated in simple defined nutrient media and their growth kinetics in suspension culture is similar to microbial cell types (James and Lee 2006). However, plant cells are preferred over prokaryotic hosts, such as *Escherichia coli*, because these microbial systems lack the accurate post-translational modification of complex eukaryotic proteins. Moreover, the formation of insoluble inclusion bodies and the elimination of endotoxins produced during bacterial cultivation adds to the cost of recombinant protein production (Daniell *et al.* 2001, Basaran and Rodriguez-Cerezo 2008). As higher eukaryotes, one of the major benefits of plant cells is the ability to perform post-translational protein maturation including intron splicing, correct protein folding and glycosylation essential for protein activity and suitable kinetic properties (Gomord and Faye 2004). Therefore, plant cells are able to synthesize polypeptides and complex proteins that cannot be made in microbial, yeast or insect cell systems (Ma *et al.* 2003). Plant cells are also capable of recognizing and cleaving protein signal peptides, a trait that facilitates protein-targeting strategies such as secretion or localization within organells. Although yeast and insect cells are in general amenable for genetic manipulation and possess the capability for complex post-translational modifications through a eukaryotic protein synthesis pathway, both have a restricted ability for authentic human protein glycosylation. Using yeast expression systems like *Saccharomyces cerevisiae* or

*Pichia pastoris*, recombinant proteins often have altered biological properties compared to their native counterparts. In fact, yeast cells tend to hyperglycosylate proteins by adding too many mannoses to the glycans (Fischer *et al.* 1999, Chung *et al.* 2010), whereas proteins obtained from insect cell cultures revealed a limited number of complex glycan structures (Becker-Pauly and Stöcker 2011). However, engineered transgenic insect cell lines expressing mammalian glycosyltransferases are reported that produce human-like sialylated N-glycoproteins (Aumiller *et al.* 2003, Tomiya *et al.* 2004). Apart from only minor variations the plant cell glycosylation machinery is competitive to conventional mammalian cell cultures systems like CHO (Chinese hamster ovary) or HEK (Human embryonic kidney) cells that are commonly used to produce complex multimeric protein therapeutics. Especially, targeting of the recombinant proteins to the plant secretory pathway ensures *N*-glycosylation and other post-translational modifications of the protein to take place. Compared to their mammalian counterparts, slightly altered glycan structures in plants might influence the activity and stability of recombinant proteins or could cause potentially allergic responses against plant-specific glycans (Doran 2000). The latter still hampers the acceptance of plant-derived proteins and can be avoided by simple strategies that prevent plant specific glycan pattern, i.e. the humanization of plant-derived proteins by the introduction of a mammalian biosynthetic pathway comprising the expression of human glycosylation related enzymes like sialyltransferase (Castilho *et al.* 2010) or the retention of the recombinant protein to the endoplasmatic reticulum (ER) (Ko and Koprowski 2005). In particular, the mammalian-like N-glycosylation pattern of plant produced biotherapeutic antibodies is necessary to induce antibody-dependent cellular cytotoxicity (ADCC) (Ko *et al.* 2008). As the susceptible mammalian cell cultures are more expensive, difficult to cultivate and may harbor human pathogens, cultured plant cells have additional advantage over its mammalian counterpart.

Even though suspension cultures have been established from a wide range of plant hosts, tobacco cells such as NT-1 or Bright Yellow 2 (BY-2) represent frequently used systems for the production of recombinant proteins (Nagata *et al.* 1992). In comparison to alternative plant cell cultures established from rice (Huang *et al.* 2001), carrot (Aviezera *et al.* 2009) or soybean (Smith *et al.* 2002) both tobacco cultivars are favoured because of their rapid growth, inexpensive nutritional requirements and well-established transformation methods. Furthermore, NT-1 as well as BY-2 can be easily cultured without the need of light. Especially for the development of large-scale processes, BY-2 cells can be cultivated under controlled conditions in bioreactors up to the cubic meter scale without any difficulties (Eibl and Eibl 2008).

### 1.1.2 Plant-made pharmaceuticals from plant suspension cultures

Plant-based systems have been successfully established as a promising bioproduction platform of numerous recombinant proteins that are either therapeutically valuable or of industrial

interest for diagnostic and technical purposes (Thomas *et al.* 2011). For already a few decades most applications of plant suspension cells aimed primarily at the production of high value secondary metabolites of industrial importance, which are particularly used as agro-chemicals, food additives or pharmaceuticals. These include the commercial production of natural colours such as anthocyanins, various flavonoids and metabolites of therapeutic value e.g. the anti-inflammatory red pigment shikonin, the anti-tumor agent taxol or the antimalarial drug artemisinin (Rao and Ravishankar 2002, Weathers *et al.* 2010). With substantial progress in understanding the plant molecular biology, plant suspension cultures offer many benefits for recombinant human or animal proteins and are now used to produce a variety of pharmaceutical proteins. Although the recombinant proteins yield in transgenic plant suspension cultures are usually lower compared to mammalian and eukaryotic host systems, today many biopharmaceuticals of mammalian origin have been synthesized in suspension cultured plant cells including antibodies, vaccines and biopharmaceuticals (Xu *et al.* 2011). Thus far, a frequently reported host species for recombinant protein production in suspension cultured cells is tobacco, but several other cultures such as rice, carrot, soybean or tomato have also been reviewed (Xu *et al.* 2011). Apart from blood products like the α1-antitrypsin (Terashima *et al.*, 1999) or the thrombomodulin derivative solulin (Schinkel *et al.* 2005) and human growth hormones (Kim *et al*., 2008), the recombinant production of immunotherapeutic cytokines has been reported in multiple plant culture species and bioactive human interleukins such as hIL-2, hIL-4 or hIL-18 were of particular significance (Sharma *et al.* 2006, Shin *et al.* 2010). Recombinant enzymes with therapeutic or diagnostic applications e.g. ß-glucoronidase (Kurata *et al.* 1998), invertase (Des Molles *et al.* 1999) or transglutaminase (Sorrentino *et al.* 2005) are only some representatives of a variety of plant cell culture derived proteins that also include recombinant antibodies and antibody fragments (Sharp and Doran 2001, James and Lee 2006). Since expression of the first full-size immunoglobulines in plant suspension cultures more than 10 years ago (Fischer *et al.* 1999), suspended tobacco cells have been successfully used for the production of numerous therapeutic antibodies and antibody derivates covering functional Fab fragments, scFvs, diabodies and antibody-fusion proteins (Huang *et al.* (2009) and references therein). Especially, the human antibody against Hepatitis B virus surface antigen (Yano *et al.*, 2004), the TMV-specific antibody rAb24 (Fischer *et al.* 1999) or anti-HIV antibodies like the 2F5 (Sack *et al.* 2007) and the most recently published 2G12 (Holland *et al.* 2010) have been effectively produced in tobacco suspension cultures yielding up to 8 mg/l culture. In the recent years, the plant suspension culture platform has also gained attention for the production of vaccines, and multiple candidate antigens including several viral and bacterial pathogen antigens have been expressed in different plant suspension culture systems (Daniell *et al.* 2001). Prominent examples for plant cell produced vaccines target enterotoxigenic *E. coli* infections or viral

infections. Also, the production of antigens against Hepatitis B virus, Avian influenza or the infectious bursal desease (IBD) has attracted particular interest (Rybicki 2010, Xu *et al.* 2011).

Although the use of transgenic plant cultures for recombinant protein production has been comprehensively reviewed in several publications (Doran 2000, Hellwig *et al.* 2004, De Muynck *et al.* 2010) and commercial systems for plant cell cultures were established there has been little progress towards a wide application. Compared to conventionally used microbial and mammalian expression systems, the number of recombinant proteins produced at large-scale is still relatively low. This is mainly caused by several challenging drawbacks of plant cell cultures such as the low yields of recombinant products, their genetic instability and the lack of well-accepted technologies for production at commercial scale (Georgiev *et al.* 2009). In comparison to other heterologous production systems recombinant proteins from plant cell cultures, tend to accumulate at low levels ranging 1-8 mg/l suspension culture and only rarely, exeptional high protein yields were reported, for example, 200 mg/l α1-antitrypsin in rice cultures (Huang *et al.* 2001). To boost recombinant protein expression and to reach economic profitability, much effort should be put in consequent strain and genotype selection, media component optimization or genetic modification.

Although some obstacles still exist, plant-derived pharmaceuticals of significant relevance were already tested in clinical trials. In 2006, the hemagglutinin-neuraminidase (HN) protein of Newcastle Disease Virus, which was produced by Dow AgroSciences (Indianapolis) in transgenic tobacco suspension cells, was approved for commercial release and registered as a recombinant animal vaccine product (Evans 2006). Three years later the Food and Drug Administration (FDA) granted Phase III clinical trials for the recombinant glucocerebrosidase enzyme (prGCD), an orphan drug produced by Protalix in genetically modified carrot suspension cells for use in the treatment of Gaucher`s disease (Protalix 2008).

## 1.2 Optimization of plant cell culture expression platforms

Plant suspension cells are particularly well-suited and effectively employed host systems for the production of various foreign proteins. However, it has been challenging to achieve adequate yields of homogeneous, high-quality recombinant pharmaceutical proteins because the low yields present a bottleneck for their use as an economic feasible production platform. In the context of heterologous protein production, it has been repeatedly reported that transformation of plant suspension cultures frequently results in transgenic cultures that represent a mixture of epigenetically different cells and that show highly heterogeneous and inconsistent expression levels of the target protein. Apart from the heterogeneity originating from the primary regenerated plant cell tissue several other factors can lead to inconsistent protein production such as different loci of transgene insertion or different numbers of transgene

copies that also demonstrates serious problems in terms of production rates (Müller *et al.* 1996, Nocarova and Fischer 2009). In addition, proteolysis of the recombinant proteins by intracellular or secreted host proteases may as well prevent to reach satisfactory accumulation levels (Schiermeyer *et al.* 2005). Further, periodic subculture processes can also introduce variability and contribute to gradual loss of desirable characteristics e.g. decreased recombinant protein productivity over time, a frequently reported obstacle in the development of plant cell culture production systems (Qu *et al.* 2005, James and Lee 2006, Kolewe *et al.* 2008). Despite the apparent advantages of plant cell cultures solutions to these drawbacks need to be found, if plant-produced transgenic proteins are supposed to be utilized as commercial production platforms for therapeutic proteins. For a profitable protein production at high yields and quality, the development of plant suspension cultures producing sufficient levels of recombinant protein is therefore a continuous challenge (Huang *et al.* 2009). Since most plant products of commercial interest are proteins that require both high biomass and high product yields, complex approaches were applied to achieve consistent high culture productivity (Weathers *et al.* 2010).

Several strategies for the productivity improvement of plant cell-based processes have been published so far. In particular, progress was achieved by plant cell fermentation and specific cultivation vessels for plant cells have been developed. Sophisticated bioreactor engineering resulted in various reusable and single-use bioreactors suitable for optimal production of recombinant proteins from plant suspension cultures (Raven *et al.* 2011). Further, modifications on culturing conditions such as agitation speed or aeration rate, represent only some bioprocess modification strategies (Hellwig *et al.* 2004, Huang and McDonald 2009) and a number of plant species-specific advanced bioreactor culture strategies including fed-batch, two-stage, perfusion, semi-continuous and continuous cultivation have been developed to improve the overall productivity of the plant cell culture platform (recently reviewed by Xu *et al.* (2011) and references therein). In addition, pH adjustments, medium additives and medium exchange have been used to reduce or eliminate the degradation of secreted recombinant proteins by proteases (Huang *et al.* 2009). Along with these strategies the co-expression of protease inhibitors as stabilizing agents for the protection of recombinant proteins has been stated to improve recombinant protein levels in transgenic plant cells (Komarnytsky *et al.* 2006, Kim *et al.* 2008, Goulet *et al.* 2010). Since plant cell culture medium can be easily manipulated by changing the sugar compositions, the concentrations of plant growth hormones, or the amounts of phosphate and nitrogen, medium optimization strategies were also successfully applied to boost the yield of biomass, natural plant products and the biosynthesis of recombinant proteins (Georgiev *et al.* 2009, Holland *et al.* 2010). For example, Ketchum *et al.* (1999) optimized the biomass production of *Taxus* suspension cultures by comprehensive medium modifications and reached 5.3-fold increased growth

rates. Similarly, a statistical optimization of carbon and nitrogen source optimization, which was studied in suspended *Azadirachta* cultures, was favorable for both biomass and azadirachtin accumulation, a widely used biopesticide (Prakash and Srivastava 2005). Finally, exhaustive media analysis and alteration improved the production of the full-size human anti-HIV antibody 2G12 in transgenic tobacco BY-2 suspension cultures by 150-fold (Holland *et al.* 2010). Additional approaches to achieve high levels of recombinant proteins include molecular technologies that boost transgene copy number, transcription or translation, and that increase protein stability by targeting to specific subcellular compartments or through protein fusions (Streatfield 2007, Sharma and Sharma 2009).

Since only a small number of cells among the initial transformants can produce high yields of recombinant protein (Carroll and Al-Rubeai 2004), a promising strategy is to select elite single cells and use them to initiate high-productivity plant suspension cultures. Though not extensively investigated yet, it is frequently observed for various eukaryotic host systems that high recombinant protein production or increased secondary metabolite levels have a negative impact on the culture fitness and growth performance. In particular, highly productive cells often show slower growth and might be outcompeted by low producer cells in mixed cultures (Ikeda *et al.* 1981, James and Lee 2006, Jiang *et al.* 2006). Therefore, an ideal scenario would be to separate the highly productive cells shortly after the transformation from the low-productive ones. Common procedures include the isolation of individualized transgenic calli by plating thin layers of the transgenic plant cell suspensions onto agar plates (Nocarova and Fischer 2009) or the regeneration of callus tissue using high antibiotic pressure (Akashi *et al.* 2002). Further, Schweiger *et al.* (1987) published a microculture system by a computer-controlled set-up for the efficient separation, transfer and culture of individual plant cells. The fluorescence activated cell sorting (FACS) technology is an appropriate mean to separate single cells because it allows the simultaneous identification and isolation of high producing cells from a transgenic population, whenever specific staining or fluorescence can be used to identifiy desired cells (Naill and Roberts 2005). Compared to manual selection procedures the application of flow sorting is less labour- and time-intensive and most importantly an effective screening of thousands of cells becomes feasible.

### 1.3 Flow cytometric sorting and its application for plant cells

Flow cytometry is a powerful technology in biological research applied for the determination of multiple physical and chemical parameters of biological cells or for the identification and selection of single cells regardless of their origin. As individual cells are simultaneously analyzed with respect to multiple optical properties by passing through a laser beam at a very high rate, flow cytometry is a unique tool to analyze the characteristics of cell populations on a single-cell basis rather than on a population average (Redenbaugh *et al.* 1982). Even relationships between cell properties can be determined by examining diverse para-

meters for each cell, thus providing better understanding of factors responsible for particular cell culture characteristics (Yanpaisan *et al.* 1999). Fluorescence activated cell sorting (FACS, Figure 1) is a flow cytometric technique that combines the rapid and multiparametric evaluation of large cell numbers in real-time with the physical separation of desired single cells, e.g. the efficient selection of fluorescence tagged cells from a heterogeneous population (Galbraith *et al.* 1999).

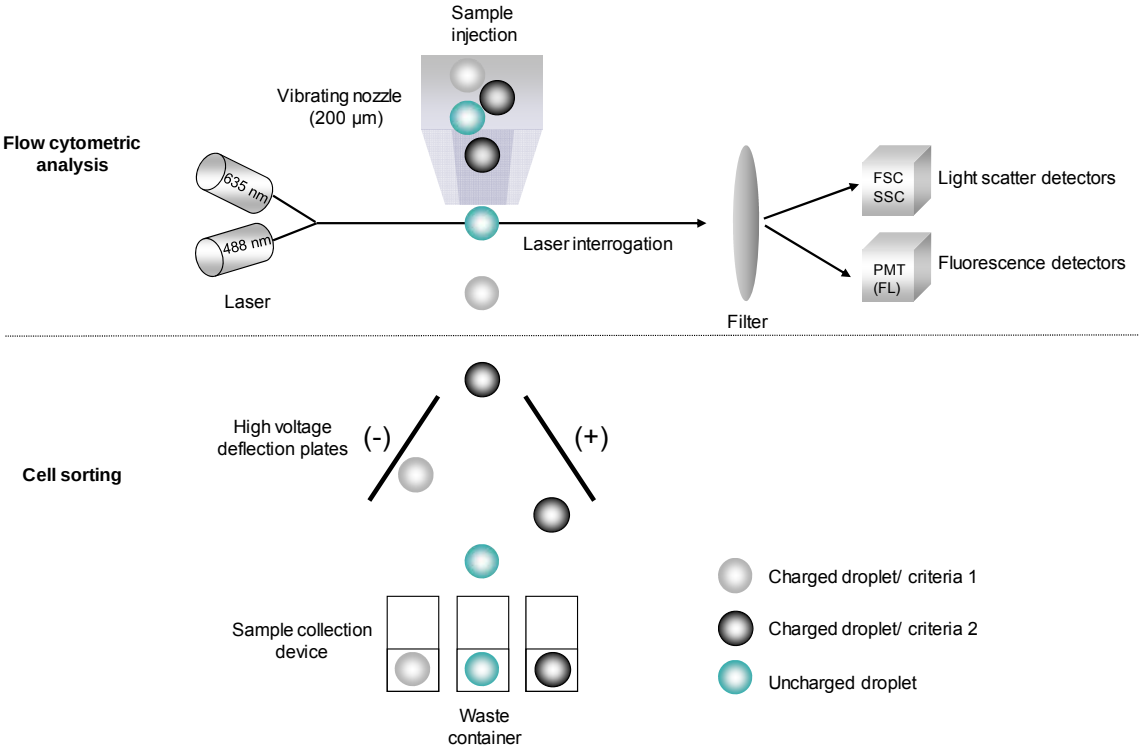



**Figure 1: Schematic overview of flow cytometric analysis and cell sorting**.
Suspended cells are hydrodynamically focused within a sheath stream that is intercepted by focused laser beams. The scattered light and fluorescence signals are collected by forward scatter channel (FSC), side scatter channel (SSC) or fluorescence channel (FL) photomultiplier tubes (PMT). User-defined parameters provide the basis to define and select individual cells of interest that are separated from the fluid stream as cell-containing drops, generated by slight vibration of the flow tip causing the fluid stream to break into regular droplets. The droplets are deflected in an electrical field and deposited in sample collection devices.

In principle, suspended cells are first pressurized into a directed fluid stream that becomes hydrodynamically focused within a sheath stream and then passed through focused laser beams that intercept the sample stream and illuminate the single cells. The scattered light signals are collected by multiple detectors and optical filters with the forward scattered light being proportional to cell size and perpendicularly scattered light correlating to cell granularity (Ibrahim and van den Engh 2007). Additionally, incident laser light might also be emitted by fluorochroms from the flowing cells. The light scatters and fluorescence data allow unlimited combinations of user-defined parameters that provide the basis to define and select individual cells of interest (see Figure 1). These can be separated from the fluid stream as cell-containing drops, which are generated by slight vibration of the flow tip causing the fluid

stream to break into regular droplets. The cell-containing droplets are then deflected in an electrical field and can be collected as viable and homogeneous fractions at high speed and purity (Ibrahim and van den Engh 2007).

Flow cytometry technology facilitates the determination of a wide range of cell variables covering genetic and molecular information, cell functions or morphologic characteristics, for instance, whereas cell sorting in particular enables the direct selection of cells with desirable properties (Yanpaisan *et al.* 1998). Flow cytometric applications are restricted to cell suspension solutions because individual cells have to pass the fluid stream (Ibrahim and van den Engh 2007). Most flow cytometric techniques were initially developed and are now routinely used for mammalian cells, but flow cytometry and cell sorting has also been adapted to various microbial cells focussing on the identification or discrimination of physiological cell properties, the assessment of cell viability and the enrichment of high-producing strains of biotechnological interest (reviewed by Davey and Kell, 1996, Müller and Nebe-von-Caron, 2010). Differing from microbial systems and mammalian cell cultures, plant suspended cells are larger and often tend to clump together, two features that challenge the application of flow cytometry (Yanpaisan *et al.* 1998, Galbraith *et al.* 2011).

Therefore, considerable efforts in the 80´s and early 90´s have been made to develop suitable conditions for rapid and gentle flow cytometric analysis and sorting of plant cells resulting in the adaption of flow cytometry to the plant field. In particular, comprehensive investigations focused on the optimization of instrument settings such as flow tip orifice (Jett and Alexander 1985), fluid stream pressure and droplet formation conditions for sorting of large cells (Harkins and Galbraith 1987), that allow high-frequency recovery of viable cells (Galbraith *et al.* 1984). These studies led to sophisticated cytometric approaches for plant microspores (Schulze and Pauls 1998) and pollen (Moon *et al.* 2011). However, flow sorting of other plant cells requires single protoplasts that can be obtained from the usually aggregated plants and cell cultures by enzymatic removal of cell walls. The production of small protoplasts i.e. miniprotoplasts (Glimelius *et al.* 1986) and the impact of different medium osmolarities on protoplast size and stability have already been evaluated to advance the flow sorting of plant protoplasts (Harkins and Galbraith 1984).

The successful recovery of vital plant protoplasts after flow sorting was first reported by Harkins and Galbraith (1984) for tobacco leaf protoplasts. Since then, the number of publications on flow cytometric methods for plant cells has increased steadily and current applications cover the analyses of various cellular characteristics and cell functions (Bergounioux *et al.* 1992) using flow sorting to pre-selected material for subsequent biochemical and genomic analyses (Birnbaum *et al.* 2005). Examples include e.g. the analyses of DNA and RNA contents used for cell cytokinesis or transgene expression studies. In this context, the measurement of DNA contents in the nuclei of *Solanum* suspension cell culture reported by

Yanpaisan *et al.* (1998) demonstrated an effective flow cytometric approach to indirectly monitor growth and cell cycle properties. In addition, the measurements of secondary metabolite levels and studies on plant cell chloroplasts or particular organells have been reported (reviewed in Doležel *et al.*, 2007 and Yanpaisan *et al.*, 1998).

Over the last thirty years, fluorescence activated cell sorting of plant protoplasts has been used as an alternative strategy to effectively separate desired plant cells mainly focusing their culture and plant re-growth (Harkins and Galbraith 1984). For instance, FACS has already been used to select and enrich cell pools from different plant species, e.g. the identification of carrot cell culture populations according to morphogenic and thus light scatter properties (Guzzo *et al.* 2002) or the separation of *Arabidopsis* or tobacco protoplast subpopulations based on single or dual fluorescence (Bargmann and Birnbaum 2009, Galbraith *et al.* 2011). Several earlier studies focused on the identification and isolation of plant heterokaryons from a mixed population and their regeneration to intact hybrid plants. Following protoplast fusions either endogeneous or introduced fluorescence signals utilized the discrimination between sub-populations i.e. parental and heterokaryont (Redenbaugh *et al.* 1982, Afonso *et al.* 1985, Alexander *et al.* 1985, Waara *et al.* 1998).

Finally, the work carried out for flow cytometry of plant cells was contemplated by Yanpaisan *et al*. (1999) to be deployed during bioprocessing of plant cell cultures. Multiparametric analyses of the biochemical state of cells in bioreactors such as size, DNA content or cell cycle activity could be elucidated for a better control of product synthesis. The benefit to immediately select for desired cell characteristics within process applications remains an important advantage associated with flow cytometry and cell sorting for plant cell systems.

### 1.4 Objective and experimental design of this work

Tobacco suspension cultures are considered as an attractive production system for recombinant biopharmaceutical proteins in plant research and for upscaled production processes because of their high propagation and beneficial growth conditions. However, phenomena such as culture heterogeneity, low recombinant protein yields or the decrease of recombinant product levels over time are frequently observed within transgenic plant suspension cultures and apparently hamper their economic profitability.

To counteract these problems we used the heterogeneous tobacco BY-2 suspension culture BY-2 MTED#18 that co-expresses genes of the human full-size antibody M12 and DsRed as a fluorescent marker protein physically linked on one T-DNA and applied FACS technology aiming at the development of a novel strategy for the selection of highly-productive plant suspension lines. The following objectives were set to achieve this goal (1) optimization of tobacco BY-2 protoplast preparation and regeneration, (2) flow sorting of BY-2 protoplasts in pool and single cell mode, (3) analysis of polyclonal and monoclonal lines to demonstrate increased protein yields and superior culture stability and (4) the establish-

ment of an accelerated transformation and culture regeneration strategy. To maximize the amounts of vital protoplasts various regeneration medium compositions and cultivation conditions should be tested for optimal survival and efficient proliferation at low protoplast densities. Within this work, two flow sorting strategies should be applied to generate homogeneous plant suspension cultures with improved recombinant protein productivity and culture stability. In a first approach strong DsRed fluorescent cells were deposited as pools of 20-cells because an efficient regeneration procedure for single cells still needed to be developed. Thereafter, flow sorting and a feeder cell-based regeneration process of highly fluorescent single BY-2 cells should be performed to create superior producing monoclonal tobacco lines. In this context, a potential contamination with feeder cells should be assessed by both resistance tests and flow cytometric analyses in an experiment using transgenic tGFP expressing feeder cultures. Several FACS rounds should be conducted with the primary sorted transgenic polyclonal and also with monoclonal cultures to further increase recombinant protein production and stability. The performance of the generated polyclonal and monoclonal suspension cultures in comparison to the parental BY-2 MTED#18 culture should be examined in terms of growth performances, recombinant M12 and DsRed protein production, long-term productivity and genetic stability. Considering the potential large-scale M12 antibody production an improved monoclonal cell line was then used to examine its cultivation in a stirred tank bioreactor. In addition, it should be investigated whether cultivation in nitrogen optimized medium further improved recombinant M12 antibody amounts.

Based on the developed single cell sort and regeneration procedure FACS should be applied to establish an accelerated approach for an efficient separation of highly productive transgenic cells at an early stage after transformation. Transgenic suspension cultures derived from this approach were examined by ELISA or immunoblot for recombinant protein levels and by flow cytometry for analysis of fluorescence. A schematic representation of the experimental design is presented in Figure 2.

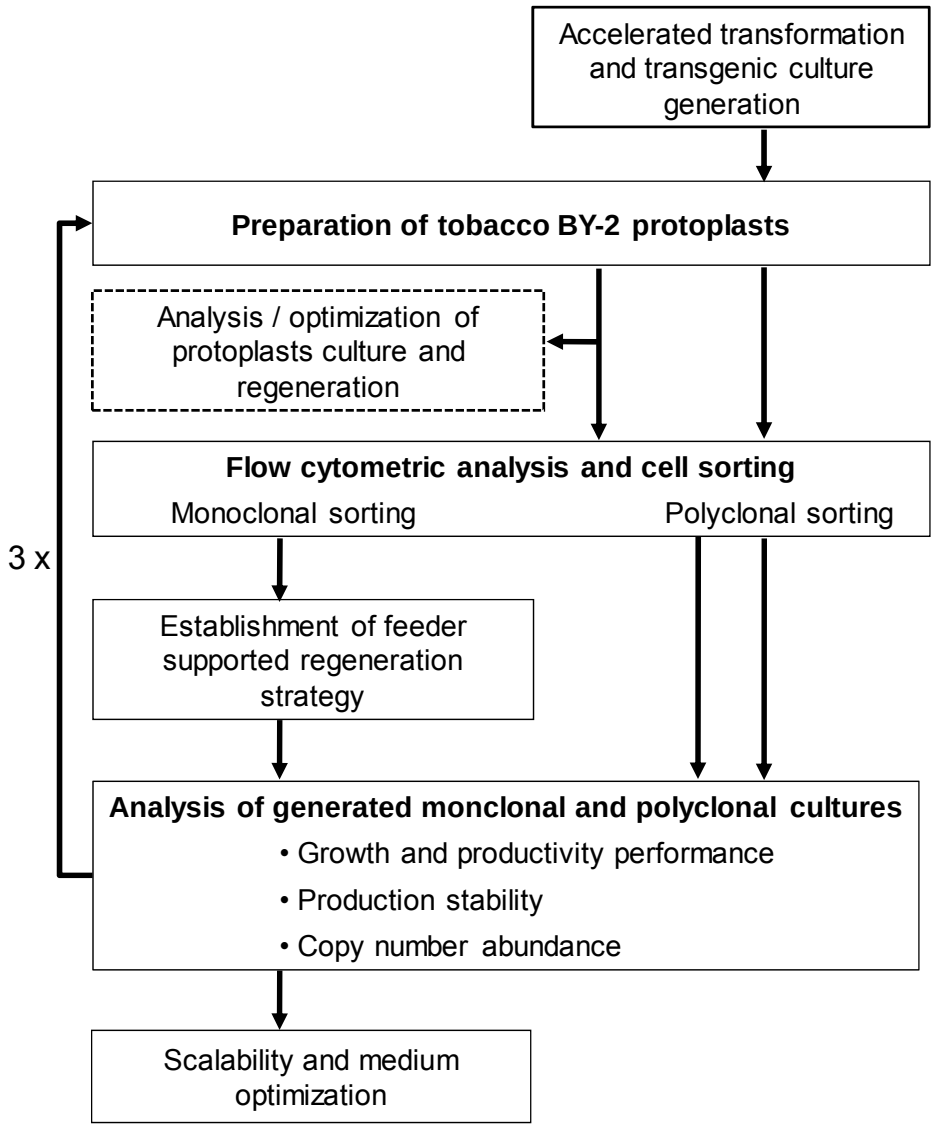



**Figure 2: Schematic representation of the experimental design.**

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Chemicals and consumables

All used chemicals for media, buffers and solutions as well as enzyme solutions were provided by the following companies:

Dianova (Hamburg), Duchefa (Haarlem, Niederlande), Evrogen (Moscow, Russia), Fermentas (St. Leon Roth), Jackson ImmunoResearch Laboratories Inc. (West Grove, USA), New England Biolabs (Frankfurt), Roth (Karlsruhe), Sigma-Aldrich (München).

Consumables were purchased from:

ABgene (Surrey, England), BioRad (München), Braun AG (Melsungen), Becton Dickinson (Heidelberg), Calbiochem (San Diego, USA), Chemicon, (Hampshire, England), Eppendorf AG (Hamburg), Greiner Bio-One (Solingen), Invitrogen GmbH (Karlsruhe), Macherey-Nagel (Düren), Millipore (Eschborn), Pall Life Science (Dreieich), Roth (Karlsruhe), Sarstedt (Nümbrecht), Satorius (Göttingen), Spectrum Laboratories (Breda), Thermo Scientific (Bonn), Whatman Schleicher und Schuell (Dassel).

### 2.1.2 Equipment and accessories

The used equipment is listed below.

| Equipment | Manufacturer |
|---|---|
| AIDA Image Software | Raytest GmbH, Straubenhardt |
| Avanti J26-XPI Centrifuge + Rotor 25.50 | Beckman Coulter GmbH, Krefeld |
| Biomek FX Liquid Handling Robotic System | Beckman Coulter GmbH |
| Bioreactor (3 liter scale, autoclavable) | Applikon Biotechnology, Schiedam |
| Bioconsoles, ADI1030, ADI1035 (wet unit) | Applikon Biotechnology |
| Centrifuge 5415R | Eppendorf, Wesseling-Berzdorf |
| ELX 405 Microplate Washer | BioTek Instruments GmbH, Bad Friedrichshall |
| Flow Cytometer FACSCalibur | Becton Dickinson, Heidelberg |
| Flow Cytometric Cell Sorter FACSVantage SE | Becton Dickinson |
| Freezer (- 80°C) | Kendro Laboratory Products GmbH, Hanau |
| Gelelectrophoresis equipment, Power supply | BioRad |
| Grinding Bead Mill MM300 | Retsch, Haan |
| Kühner Shaker ISF1-X | Kühner AG, Birsfelden, Switzerland |
| Laser Scanning Confocal Microscope (Leica) | Leitz, Wetzlar |

| Equipment | Manufacturer |
|---|---|
| Leica KL 1500 LCD lamp with excitation filter (BP: 545/30 nm) and foil no.182 light red | Leitz |
| Laminar Flow Hera Safe HS12 | Kendro |
| Innova Incubator Shaker 4430 | New Brunswick Scientific GmbH, Nürtingen |
| Invers Fluorescence Microscope (Leica) | Leitz |
| Multifuge 3S-R and 3S; Swinging-bucket rotor | Heraeus, Hanau; Heraeus Sorvall Langenselbold |
| Magnetic Stirrer Safety control | IKA RCT basic, Staufen |
| Novex Mini-Cell | Invitrogen Life Technologies, Darmstadt |
| Ocelot rocker | Boekel Scientific, Pennsylvania, USA |
| Olympus IX71 inverse fluorescence microscope | Olympus Microscopy Research Center, Hamburg |
| Osmomat 030 | Gonotec, Berlin |
| pH electrode PB-11 | Satorius AG, Göttingen |
| Scanjet 4890 Scanner | HP GmbH, Böblingen |
| Scales TE612, TE313, TE6101 | Satorius AG |
| SDS 7700 Real Time PCR Detection System | Applied Biosystems, Darmstadt |
| Synergy HT Microplate Reader | BioTek Instruments GmbH |
| Thermomixer comfort (1.5 ml) | Eppendorf, Wesseling-Berzdorf |
| Ultrasonic Control Unit Sonoplus HD 2070 MS 72, Sonotrode 492 MS 72 | Bandelin electronic, Berlin |
| Vortex Genie 2 | Scientific Industries Inc., New York, USA |
| WinMDI (Windows Multiple Document Interface for Flow Cytometry) | The Scripps Research Institute, Florida |

### 2.1.3 Reaction kits

For extraction of genomic DNA from plant material (2.4.1) the Chemagen Plant DNA Extraction Kit (Chemagen, Baesweiler) was used. The Platinum® SYBR®Green qPCR SuperMix-UDG with ROX Kit for quantitative real-time PCR (2.4.2) was purchased from Invitrogen (Darmstadt). Unless otherwise stated reaction kits and corresponding buffers were deployed according to manufacturer´s instructions.

### 2.1.4 Antibodies and substrates

The antibodies listed in Table 1 were applied in immunological methods such as enzyme linked immuno sorbent assay (2.3.4) and immunoblot analysis (2.3.3).

**Table 1**: **Antibodies**.

| Antibody | Abbreviation | Specificity | Property | Manufacturer |
|---|---|---|---|---|
| Goat-anti human IgG Fc | GAH IgG Fcγ | Fc-part of IgG | polyclonal | Dianova |
| Goat-anti human IgG-Fc AP | GAH IgG Fcγ$^{AP}$ | Fc-part of IgG | polyclonal, alkaline phosphatase (AP) conjugated | Dianova |
| Goat-anti human IgG λ LC AP | GAH IgG λ LC$^{AP}$ | λ-light chain | polyclonal, alkaline phosphatase (AP) conjugated | Dianova |
| Rabbit-anti TurboGFP | antiGFP | denaturated turboGFP | polyclonal | Evrogen |
| Goat anti Rabbit IgG H+L AP | GAR H+L$^{AP}$ | IgG heavy and light chain | Polyclonal, alkaline phosphatase (AP) conjugated | Jackson Immuno Research |
| M12 antibody | M12 | Mucin-1 | Tobacco produced human full-size $IgG_1$ | J. Neumann/ Dr. N. Raven, IME, Aachen |

### 2.1.5 Bacterial strains

The following two agrobacteria strains were used for stable transformation of tobacco BY-2 suspension cultures (2.2.4):

*Agrobacterium tumefaciens* strain GV3101::pMP90RK (Koncz and Schell 1986): The resident disarmed Ti helper plasmid pMP90RK encodes antibiotic resistance against gentamicin and kanamycin, respectively. Moreover, this plasmid contains a RK2 replicase as well as virulence (*vir*) and transfer (*tra*) genes essential for the horizontal transfer of T-DNA. This *Agrobacterium* strain is compatible with pTRA plant expression vectors (Dr. Thomas Rademacher, Fraunhofer IME, Aachen).

*Agrobacterium tumefaciens* strain LBA4404 (Hellens *et al.* 2000): The cells contain the disarmed Ti plasmid pAL4404, which carries the *vir* and *ori* region of the Ti plasmid. The resident plasmid encodes also the *aadA* gene that confers antibiotic resistance to streptomycin. These agrobacteria served as host cells for the plant expression vector pDAB9695 (2.1.7).

### 2.1.6 Plant suspension cultures

The suspension cell line *Nicotiana tabacum* L. cultivar Bright Yellow 2 (Nagata *et al.* 1992) established and cultured at the Fraunhofer IME ($BY\text{-}2_{IME}$) or at Dow AgroSciences ($BY\text{-}2_{DAS}$),

respectively, was used for transformation purposes (2.2.4) or within flow cytometric applications (2.2.7). In addition, the following transgenic BY-2 lines were used:

BY-2MTED#18: The transgenic tobacco suspension culture was generated by *Agrobacterium*-mediated stable transformation (2.2.4) of BY-$2_{IME}$ cells with the pTRAkc:MTED plasmid (2.1.7) as described by An (1985) followed by kanamycin-based selection and subsequent separation and screening of transformed callus tissue (Kirchhoff 2007). The transgenic MTED#18 cell culture that co-expresses the red fluorescent marker protein DsRed as well as the tumor-specific human antibody M12 (Raven *et al.* 2011) was identified to accumulate the maximum M12 antibody yield of 14.34 ± 4 µg/g fresh weight. The established suspension culture showed two subpopulations with regard to DsRed production in flow cytometric analysis (2.2.7.1) and was therefore used as starting material for subsequent selective flow cytometric sorting experiments (2.2.7, 2.2.8).

BY-2KDEL-GFP: The transgenic tobacco suspension cell culture expressing the ER-retrieved turboGFP was generated (Dr. Flora Schuster, Fraunhofer IME) by *Agrobacterium*-mediated stable transformation (2.2.4) of BY-$2_{DAS}$ cells with the pDAB9695 plasmid (2.1.7). Following initial selection on imazethapyr the strongest GFP fluorescent callus was selected as a candidate for flow sorting experiments (2.2.7, 2.2.8).

### 2.1.7 Plasmids

For the generation of the transgenic tobacco BY-2 suspension cultures (2.2.4) the following plant expression vectors have been used:

pTRAkc:MTED (Kirchhoff 2007): This plant expression vector is a derivate of the pTRA plasmid (Sack *et al.* 2007). The T-DNA contains three expression cassettes each under control of the doubled enhanced *Cauliflower mosaic virus* 35S promoter (Kay *et al.* 1987) for expression of the heavy and light chain genes of the M12 antibody and the red fluorescent protein DsRed (Jach *et al.* 2001), respectively. Expression cassettes of all three genes were cloned consecutively onto the same T-DNA to achieve co-expression (Figure 3). In addition, a *nos* expression cassette including the *nptII* gene serves as resistance marker for selection on kanamycin (Bevan *et al.* 1992). The M12 antibody had been isolated from a human naïve Fab library as a binder to the breast adenocarcinoma cell line MCF-7 (Wong *et al.* 2001). It had been converted to a fully human $IgG_1$ antibody by fusing the variable regions of the scFvM12 to the constant domains of human $IgG_1$ and the human lambda light chain, respectively. The coding sequence of the antibody heavy chain gene is fused to a SEKDEL retention signal (Munro and Pelham 1987) to ensure intracellular accumulation of the M12 antibody in the endoplasmic reticulum (ER). In contrast, DsRed is targeted to the proplastids of BY-2 cells by a GBSS transit peptide (Vanderleij *et al.* 1991).

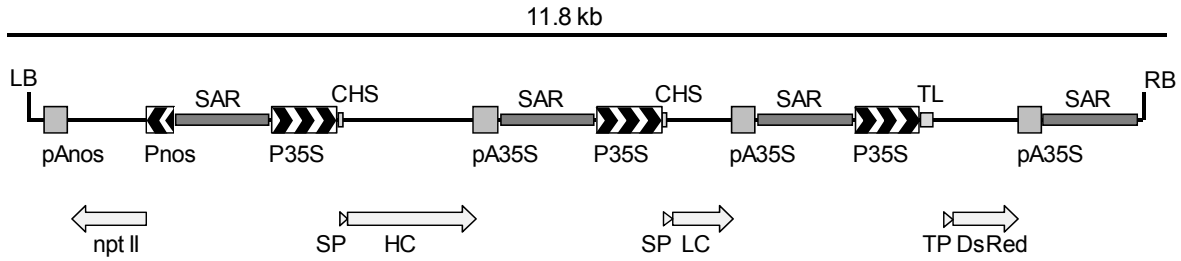



**Figure 3: Scheme of T-DNA of the pTRAkc-MTED plant expression vector.**
LB and RB: left and right border of the T-DNA; Pnos and pAnos: promoter and terminator of the nopaline synthase gene; *nptII*: coding sequence of the neomycine transferase gene; SAR: scaffold attachment region of *Nicotana tabacum*; P35S and pA35S: promoter with duplicated enhancer and terminator of the *Cauliflower mosaic virus* (CaMV) 35S gene; CHS: 5'-UTR of the chalcone synthase from *Petroselium crispum*; SP: SEKDEL signal peptide for ER retention; HC and LC: coding sequence of the heavy and light chain of the M12 antibody; TL: 5'-UTR of the *Tobacco etch virus* (TEV); TP: GBSS transit peptide for chloroplast targeting; DsRed: coding sequence for the red fluorescent protein from *Discosoma* spec.

pDAB9695 (Dr. Jean L. Roberts, Dow AgroSciences): The plant expression vector was constructed and kindly provided by Dow AgroSciences. For bacterial selection the binary plasmid encodes a streptomycin/spectinomycin resistance gene (*aad*, SpecR) (Hollingshead and Vapnek 1985). The T-DNA carries the imazethapyr selectable marker gene (acetohydroxyacid synthase, AHAS) for transgenic plant cell selection and the *turbo-gfp* gene (tGFP, Evrogen) that was fused to a SEKDEL signal peptide for ER retention. Expression of the *turbo-gfp* was driven by a melon actin promoter (*Cm*Act (Clendennen *et al.* 2000)).

### 2.1.8 Oligonucleotides

For the PCR amplification during real time quantitative PCR (2.4.2) several oligonucleotides were designed to amplify target DNA fragments. The primers were synthesized according to common primer design standards for qPCR and are listed in Table 2.

**Table 2: Oligonucleotides**.

| Target Gene | Primer | Sequence (5`→3`) | PCR product (bp) |
|---|---|---|---|
| M12 HC[1] | qPCR M12_HC_for | CTGAGACTCTCCTGTGCAGCCTCTG | 167 |
| | qPCR M12_HC_rev | GTCTCTGGAGATGGTGAATCGGC | 167 |
| DsRed | qPCR DsRED_forwa | CCTGAAGGATTTAAATGGGAAAGGG | 167 |
| | qPCR DsRED_rever | TCACGAGGATACAAACGCTCAGTG | 167 |
| PAL[2] | qPCR/Nt_PAL_for | TACTATGACTTGATGTTGTGTGGTGACTGA | 164 |
| | qPCR/Nt_PAL_rev | GAGCGGTCTAAATTCCGACCCTTATTTC | 164 |

[1] heavy chain of the M12 antibody; [2] phenylalanine ammonia-lyase gene

### 2.1.9 Antibiotics and herbicides

All antibiotics and herbicides were solubilised using ultrapure water, and stored at – 20°C as sterile filtrated (0.22 µm) aliquots. Unless otherwise stated all antibiotics were used at concentrations indicated in Table 3.

**Table 3: Antibiotics and herbicides**.

| Selective agent | Abbreviation | Stock solution | Working solution |
|---|---|---|---|
| Carbenicillin | Carb | 100 mg/ml | 25 µg/ml |
| Cefotaxime | Cef | 200 mg/ml | 200 µg/ml |
| Imazethapyr | Ima | 400 mg/ml | 400 µg/ml |
| Kanamycin | Kan | 100 mg/ml | 50 µg/ml |
| Rifampicin | Rif | 100 mg/ml | 25 µg/ml |
| Streptomycin | Strep | 30 mg/ml | 30 µg/ml |
| Spectinomycin | Spec | 100 mg/ml | 100 µg/ml |

### 2.1.10 Solutions, media and buffers

Standard solutions, buffers and media stock solutions were prepared using de-ionized water according to established procedures (Sambrook *et al.* 2001) unless otherwise stated. Media were sterilized by autoclaving (25 min, 121°C, 2 bar) or filter sterilization through a 0.22 µm filter. All heat-sensitive components, such as antibiotics, were prepared as stock solutions, filter-sterilized (0.22 µm) and added to the medium/buffer after cooling below 50°C.

## 2.2 Methods

### 2.2.1 Cultivation of *Agrobacterium tumefaciens*

The cultivation of agrobacteria was performed as liquid cultures in baffled Erlenmeyer flasks at 28°C with an orbital agitation of 160 rpm for 24 to 48 h. The cultures were routinely initiated from a glycerol stock. Recombinant A. *tumefaciens* strain GV3101 cultures were grown in liquid $YEB^{CarbKanRif}$ medium whereas $YEB^{StrepSpecRif}$ medium was needed for recombinant *A. tumefaciens* strain LBA4404 (2.1.5). Long-term maintenance of both recombinant cultures was achieved by preparation of glycerol stock cultures. Here, 10 ml of an overnight agrobacterium culture was centrifuged at 3.000 x g for approximately 10 min at RT to pellet the agrobacteria. After discarding the supernatant, the cell pellet was resuspended in 1-2 ml YEB medium and an equal volume of 40% (v/v) glycerol. *A. tumefaciens* glycerol stocks were stored as 200 µl aliquots at -80°C.

**YEB medium** (pH 7.4)

| | |
|---|---|
| Nutrient broth | 0.5% (w/v) |
| Yeast extract | 0.1% (w/v) |
| Peptone | 0.5% (w/v) |
| Sucrose | 0.5% (w/v) |
| $MgSO_4$ | 2 mM |

- Optional addition of 1.5% (w/v) agar for solid medium

### 2.2.2 Growth and induction of *A. tumefaciens* for plant cell transformation

Transgenic *A. tumefaciens* cultures were initiated from glycerol stocks and cultivated in 10 ml sterile YEB medium comprising strain-specific antibiotics. For optimal culture aeration disposable Cultiflask50 bioreactors (Sartorius) were used and positioned on an orbital shaker at 160 rpm and 27°C. Agrobacterium cultures were grown for 15 -20 h. Optical density of the cultures was photometrically measured and adjusted to an $OD_{600nm}$ of 1 using YEB medium if necessary. These cultures were subsequently employed for stable transformation of BY-2 suspension cells (2.2.4).

### 2.2.3 Cultivation of plant suspension cultures

The tobacco suspension culture *Nicotiana tabacum* L. cv. Bright Yellow 2 (BY-2) (Nagata *et al.* 2004) was cultured in darkness under sterile conditions as 50 ml aliquots in 100 ml glass Erlenmeyer flasks at 26°C, with a constant orbital agitation of 180 rpm. The cultivation medium MS (Murashige and Skoog 1962) comprised basal MSMO salts (pH 5.8) dissolved in deionized water supplemented with 3% (w/v) sucrose, 0.2 g/l potassium dihydrogen phosphate, 0.1 g/l myo-inositol, 1 mg/l thiamine and 1 mg/l 2.4-dichlorophenoxyacetic acid. For the nitrogen-enriched cultivation medium (MSN) the standard MS medium was supplemented with 100 mM potassium nitrate (Holland *et al.* 2010). Wild type suspension cells were subcultured at seven-day cycle by transfer of 2% (v/v) cell culture into fresh medium whereas transgenic cultures were inoculated with 5% (v/v) cell culture. Stock cultures were maintained as callus tissue on solid MS medium (including 0.8% (w/v) agar), comprising culture-specific selective agents (2.1.9). Callus plates were cultivated in darkness at 26°C and subcultured monthly. BY-2 suspension cultures were established through resuspension of approximately 1-2 cm callus tissue in liquid MS medium.

#### 2.2.3.1 Determination of growth parameters

Growth characteristics and antibody accumulation of transgenic BY-2 shake flask suspension cultures were investigated by time course experiments. In general, 500 ml Erlenmeyer flasks containing 200 ml culture volume were inoculated using 5% (v/v) suspension cells that had been cultivated for seven days under standard conditions (2.2.3) since the last passaging. Three biological replicates were run for each experimental setup and cultivations were incu-

bated under standard cultivation conditions (2.2.3) for a period of 10 days. Sampling was performed on day 0, 3, 4, 5, 6, 7, and 10 post inoculation („days past inoculation“, dpi). Two milliliters of the culture was required for the determination of M12 antibody accumulation (2.3.1, 2.3.4) and a 10 ml sample served for measuring several growth parameters. Through centrifugation (3000 x g/10 min/RT) of the 10 ml sample in a 15 ml tube (Greiner) the cells were sedimented and the “packed cell volume” (PCV) measured allowing a percentaged calculation of cell culture growth during cultivation. Fresh cell weight (FW) was gravimetrically measured by vacuum filtration of the 10 ml sample and weighing the biomass in a pre-weighed scale pan. Dry weight (DW) was estimated after incubation of the retained cells over night at 37°C. Both, fresh weight and dry weight were calculated as grams per liter (g/l). Besides these parameters the development of pH and osmolality of the medium during cultivation was monitored using the pH electrode PB-11 and the osmomat 030.

#### 2.2.3.2 Fermentation

The up-scaled cultivation of BY-2 suspension cultures was performed in a 3 liter stirred glass tank bioreactor (Applikon) with a single marine impeller containing a working volume of 2 liter autoclaved MS medium supplemented with 0.01% (v/v) anti-foaming agent (Pluronic L61). The starting suspension cultures were cultivated as described in 2.2.3 and optimized by maintenance in successive short cycles (5 day subcultivations) to generate 100 ml starting cultures in a logarithmical growth phase used to inoculate the bioreactor at a density of 3-5%. The bioreactor was maintained at 26°C, 200 rpm and a fixed nominal value of 20% dissolved oxygen saturation. During the cultivation the dissolved oxygen was regulated by controlled aeration when necessary (0.1 vvm; 200 ml/min). The oxygen uptake as well as the culture pH were monitored online by the Applikon ADI 1030 control unit and recorded by the BioXpert 3.0 software (Applikon). Culture samples from cultivation day 0, 3, 4, 5, 6 and 7 were collected to determine several off-line parameters such as PCV, fresh weight, dry weight and M12 antibody levels according to procedures described in 2.2.3.1, 2.3.1 and 2.3.4.

### 2.2.4 Stable transformation of tobacco BY-2 suspension cells

The creation of stable transformed BY-2 suspension cultures was achieved by an *Agrobacterium*-mediated method. Transgenic *Agrobacterium* strains were inoculated one day before transformation and adjusted to $OD_{600nm}$ of 1 as described in 2.2.2. Actively growing BY-2 wild type cells (day 3 of culture cycle; 2.2.3) were supplemented with 200 µM acetosyringone and 3 ml were subsequently transferred into a petri dish for co-cultivation with 150 µl agrobacteria ($OD_{600nm}$= 1) at RT under dark conditions. After three days of co-cultivation at room temperature the BY-2 cells were resuspended in 1 ml of MS medium supplemented with 200 mg/l cefotaxime and an appropriate selective agent. About 500 µl to

1000 µl of the co-cultivated cells were plated onto selective MS agar plates and cultivated for at least four weeks in darkness at 26°C. Transgenic callus tissue was transferred to fresh selection plates and suspension cultures were initiated from callus material of approximately 1-2 cm in diameter. Established transgenic suspension cultures were analyzed by ELISA (2.3.4), immunoblot (2.3.3) and flow cytometry (2.2.7.1) for accumulation of their respective recombinant protein.

### 2.2.5 Preparation of protoplasts from BY-2 suspension cultures

BY-2 protoplasts were isolated from wild type or transgenic suspension cells using a modified procedure based on the protocol described by Schinkel *et al.* (2008). Actively growing cell cultures in the logarithmic growth phase, i.e. day three after initial 2% (v/v) subculture, were harvested by centrifugation at 850 x g for 5 min in sterile conical plastic centrifuge tubes. The supernatant was removed and cells were incubated in 10 ml PNT solution (Schnorf *et al.* 1991) containing 1% (w/v) cellulose Onozuka R-10 (Serva, Heidelberg) and 0.3% (w/v) macerozyme R-10 (Serva). Digestion was carried out overnight for approximately 15 h at 26°C with optional gentle agitation to liberate individual protoplasts. Protoplasts were sieved through a 100 µm nylon mesh and subsequently floated to the surface during centrifugation (104 x g for 8 min at RT). Pellet and medium interface were removed and the protoplasts were washed twice with PNT solution and once with W5 solution (Menczel *et al.* 1981).

| **PNT solution** (pH 6) | |
|---|---|
| Kao Michayluk salts | 3.6 g/l |
| Sucrose | 136.9 g/l |
| NAA | 0.5 mg/l |
| BAP | 1 mg/l |

| **W5 solution** (pH 5.6) | |
|---|---|
| NaCl | 154 mM |
| $CaCl_2$ | 12 mM |
| KCl | 5 mM |
| Glucose | 5 mM |

Sedimented protoplasts were resuspended in a modified rich culture medium based on the published 8p medium composition (Kao and Michayluk 1975) and cultured for three days at 26°C to allow the formation of a primary cell wall. The modified regeneration medium was termed 8p2c (Table 4). The pre-regenerated protoplasts were sieved again through a 100 µm mesh and transferred into a sterile tube for flow cytometric sorting (2.2.7.2).

**Table 4**: **Composition of the 8p2c medium (pH 5.6)**

- Kao und Michayluk basal salt mixture (Duchefa)

- Kao und Michayluk vitamine solution (Sigma)

| | | | | | |
|---|---|---|---|---|---|
| 20 | µg/l | p-aminobenzoic acid | 1 | mg/l | nicotine amide |
| 2 | mg/l | L-ascorbic acid | 1 | mg/l | pyridoxine HCL |
| 10 | µg/l | biotin | 200 | µg/l | riboflavin |
| 1 | mg/l | D-calcium panthothenate | 1 | mg/l | thiamineHCL |
| 1 | mg/l | cholinechloride | 10 | µg/l | vitamin A |
| 400 | µg/l | folic acid | 20 | µg/l | vitaminB12 |
| 100 | mg/l | myo-inositol | 10 | µg/l | vitamin D |

- Organic acids (pH 5.5 with $NH_4OH$)

| | | |
|---|---|---|
| 20 | mg/l | sodium pyruvate |
| 40 | mg/l | malic acid |
| 40 | mg/l | citric acid |
| 40 | mg/l | fumaric acid |

- Sugar and sugar alcohols

| | | | | | |
|---|---|---|---|---|---|
| 0.25 | g/l | sucrose | 0.25 | g/l | cellobiose |
| 0.25 | g/l | mannose | 0.25 | g/l | ribose |
| 68.4 | g/l | glucose | 0.25 | g/l | sorbitol |
| 0.25 | g/l | rhamnose | 0.25 | g/l | xylose |
| 0.25 | g/l | fructose | 0.25 | g/l | mannitol |

- Hormones

| | | |
|---|---|---|
| 0.2 | mg/l | 2,4-D |
| 0.5 | mg/l | zeatin |
| 1.0 | mg/l | NAA |

- 2% (v/v) coconut water
- 500 mg/l casamino acid

### 2.2.6 Fluorescence staining of BY-2 protoplasts

Protoplasts from BY-2 suspension cultures were stained using fluorescent dyes to determine protoplast viability (2.2.6.1) and the formation of primary cell walls (2.2.6.2).

#### 2.2.6.1 Viability staining

The viability of freshly prepared protoplasts was determined by staining the protoplasts with fluorescein diacetate (FDA, Sigma Aldrich) 1 h after preparation (Yokoyama *et al.* 1997). A 2 ml aliquot of protoplast solution was incubated with 5 µg of FDA solution and gently inverted for 30 min at RT in the dark. The percentage of FDA stained viable protoplasts was determined by flow cytometric analysis (2.2.7) using a FACSCalibur Flow Cytometer (BD Bioscience) and calculated by the corresponding WinMDI Software (The Scripps Research Institute, Florida). Unstained protoplasts were used as a control to adjust the flow cytometer settings.

#### 2.2.6.2 Cell wall staining

Cellulose staining with Calcofluor White (CW), a fluorescent brightener, was used to detect the primary cell wall regeneration of protoplasts (Kwok *et al.* 2007). CW (Sigma) was solubilised in 250 mM $Na_2HPO_4$ as a stock solution of 1 mg/ml and stored in the dark. For cell staining, an aliquot of 400 µl protoplast solution was inverted gently for 10 min with 16 µl CW solution. Stained protoplasts were washed twice with 8p2c medium and resuspended in 300 µl of the same medium. The CW fluorescence was determined using a Leica fluorescence microscope (Leitz) equipped with a DAPI filter (450/40 nm). The formation of a primary cell wall was monitored by counting the number of stained protoplasts after 0, 1, 6, 24 and 48 h.

### 2.2.7 Flow cytometric analysis and cell sorting

Flow cytometric instrumentation is designed to examine optical properties of suspensions of single spherical particles (i.e. cells and protoplasts) as they are constrained to pass rapidly in a fluid stream through focused tuneable laser illumination. The individual particles scatter light or reemit fluorescence signals that are subsequently detected using wavelength-specific filters and sensitive photomultiplier tubes (PMT). Intensity values are recorded and flow cytometric data are presented as population distributions plotted graphically as dot plots or histograms. Flow cytometry has particular advantages in combination with cell sorting. The fluorescence-activated cell sorting (FACS) technology enables a simultaneously rapid identification and separation of cells from a heterogeneous population based on computerized sorting decisions (i.e. sort regions) that were selected on population data collected previously. FACS relies on the principle that fluid streams in air are unstable and decay into droplets by an electromechanical drive. The sample stream emerges through a periodic oscillating flow orifice of a precise diameter (typically 40–200 µm) and is charged by application of an electric voltage. The detached droplet containing a cell or protoplast of interest is collected following electrostatic deflection by passage through a fixed high-voltage field. Thereby, high rates of sorting can be achieved decreasing labour and time associated with standard manual selection methods.

#### 2.2.7.1 Flow cytometric analysis

For the flow cytometric determination of fluorescent protoplasts the FACSCalibur Instrument (BD Bioscience) was used. The flow cytometer was operated using a 488 nm laser for excitation. As light scatters i.e. forward scatter channel (FSC) and side scatter channel (SSC) determined protoplast size and granularity, different fluorescence channels measure the emission of fluorescence. Beside a 530/30 nm filter (FL 1 channel) for the detection of FDA stained protoplasts (2.2.6.1) or tGFP fluorescence, the 585/42 nm filter (FL-2 channel) was used for DsRed analysis. The amplification of light and fluorescence scatter multiplier

tube voltages was adjusted based on BY-2 wild type protoplasts. The signal distribution of the wild type population was used to define a threshold, which excluded all background signals caused by autofluorescence. According to this threshold the percentage of fluorescent protoplasts within the transgenic cultures were calculated using the WinMDI Software (The Scripps Research Institute).

#### 2.2.7.2 Fluorescence activated cell sorting (FACS)

The FACS Vantage SE (BD Bioscience) was used for sorting of transgenic tobacco protoplasts suspensions in sterile phosphate buffered saline (pH 7.4) as sheath fluid. Prior to sorting sample tubes were cleared of residual contaminants by passage of sterile sheath fluid. The cytometric sort settings were aligned with commercial standard calibration particles (BD Bioscience). The flow sorter was operated using a 488 nm argon ion laser with a laser power output of 175 mW for excitation. The total population was displayed in the light scatter channels (FSC/SSC) according to size and granularity and the emission of the fluorescent proteins were measured using a 575/26 nm filter for DsRed (PE-channel) and 530/30 nm for GFP (FITC-channel). The threshold value for event detection was set at 15 on forward scatter channel. Photomultiplier tube voltages were adjusted to approximately 290 V for SSC, 340 V for FSC and 300 V for the DsRed and the GFP channel, respectively. The light scatter signals were collected in linear amplification mode and fluorescence signals were collected by logarithmic amplification. Based on signals collected for FSC, SSC and fluorescence electronic sort windows were set that only the strongest 2% fluorescent and viable protoplasts were sorted. The signal data were processed by the FACSDiva software (BD Bioscience). Sorting was performed through a 200 µm flow tip (Galbraith and Lucretti 1992) with a system sheath pressure of 6 psi, a drop drive frequency of approximately 7.5 kHz and an amplitude of approximately 20 V. Tobacco protoplasts were deposited into 96 well microtiter plates (Greiner) containing 50 µl of regeneration medium (2.2.5, Table 4) supplemented with 200 mg/l cefotaxime. Flow cytometric selections were performed by sorting protoplasts either in a 20-protoplast (pool) mode (3.3.1) or in single cell mode (3.3.4).

### 2.2.8 Regeneration of FACS selected protoplasts

For successful protoplast proliferation and callus formation after flow cytometric sorting two different strategies were applied: FACS selected protoplasts pools were immediately cultured in regeneration medium (2.2.8.1), whereas a feeder cell based approach was used to regenerate single sorted transgenic protoplasts (2.2.8.2).

#### 2.2.8.1 Regeneration of sorted protoplasts at low density

The regeneration of low protoplast densities in general and of 20 protoplast pools in particular was realized as follows: Prior to flow sorting of high fluorescent protoplasts the micro-

titer plate wells (Greiner) were prefilled with 50 µl of sterile 8p2c protoplast regeneration medium (2.2.5). The sorted transgenic protoplasts were monitored by inverse fluorescence microscopy at different time points (directly after sorting and 2-3 weeks later) to track protoplast proliferation and microcolony formation, which occurred 14–20 days after sorting of the transgenic protoplasts. Cultivation of sorted protoplasts in microtiter plate wells took place at 26°C in darkness. The plates were closed with a sterile lid and sealed with Parafilm strips to avoid evaporation and maintain sterile conditions. Transgenic microcolonies were directly transferred by a wide pipette tip to solidified MS medium (2.2.3), containing an appropriate selective agent (2.1.9) and cultivated further at 26°C in the dark. Grown callus tissue was used to establish suspension cultures according to the procedure described in 2.2.3.

#### 2.2.8.2 Regeneration of single sorted protoplasts (monoclonal cultures)

The feeder cell approach enabled the regeneration of single flow sorted transgenic protoplasts. Single sorted protoplasts (2.2.7.2; single cell mode) were cultured in microtiter plates pre-filled with 50 µl of regeneration medium (2.2.5) containing wild type feeder protoplasts at densities of 2 x $10^3$/ml. Plates were closed with a sterile lid and sealed with parafilm strips to avoid evaporation and retain sterility. The efficiency of single cell sorting was monitored by inverse fluorescence microscopy (Leitz, Wetzlar) and cultivation was performed at 26°C in the dark for approximately 2 weeks. The formation of microcallus tissue was confirmed approximately 14 days post sorting by inverse fluorescence microscopy (Leitz) and microcallus tissue was transferred to solid MS medium supplemented with an appropiate selective agent using a wide pipette tip. After a second transfer to fresh agar plates callus tissue was used to establish suspension and stock cultures as described in 2.2.3.

### 2.2.9 Nomenclature of FACS generated suspension cultures

To optimize recombinant protein accumulation levels of the heterogeneously producing MTED#18 cell culture (2.1.6) flow cytometric sorting was applied to separate high producing protoplasts (2.2.7.2). Repeating this procedure resulted in several rounds of polyclonal sorted cultures and monoclonal cell lines regenerated from three consecutive flow-sorting processes (Table 5). The best producing polyclonal or monoclonal suspension culture from one FACS round served as a starting culture for subsequent flow sorting using equal sort settings for each FACS round and protoplast deposition mode.

**Table 5: Nomenclature of FACS generated tobacco BY-2 suspension cultures.**
Several cell lines and cultures were established after flow cytometric sorting of high DsRed fluorescent protoplasts of the heterogeneous BY-2 suspension culture MTED#18. Protoplasts were deposited as single protoplasts or in pools of 20 protoplasts respectively.

| Cell cultures/ cell lines | Abbreviation | Flow cytometric sort mode |
|---|---|---|
| **First round** | | |
| MTED#18 monoclonal line 6 | msR1C6 | Single cell |
| MTED#18 monoclonal line 21 | msR1C21 | Single cell |
| MTED#18 monoclonal line 24 | msR1C24 | Single cell |
| MTED#18 polyclonal culture 11 | psR1C11 | 20 cells pool |
| **Second round** | | |
| MTED#18 monoclonal line 6.5 | msR2C6.5 | Single cell |
| MTED#18 monoclonal line 21.1 | msR2C21.1 | Single cell |
| MTED#18 monoclonal line 24.3 | msR2C24.3 | Single cell |
| MTED#18 polyclonal culture 11.5 | psR2C11.5 | 20 cells pool |
| **Third round** | | |
| MTED#18 monoclonal line 6.5.8 | msR3C6.5.8 | Single cell |
| MTED#18 monoclonal line 21.1.36 | msR3C21.1.36 | Single cell |
| MTED#18 monoclonal line 24.3.29 | msR3C24.3.29 | Single cell |
| MTED#18 polyclonal culture 11.5.8 | psR3C11.5.8 | 20 cells pool |

ms: monoclonal sorted; ps: polyclonal sorted

## 2.3 Proteinchemical and immunological methods

### 2.3.1 Extraction of total soluble proteins from BY-2 suspension cultures

Total soluble proteins were extracted from samples of transgenic BY-2 suspension cultures for the analysis of recombinant protein accumulation levels (2.3.2, 2.3.3, 2.3.4). Transgenic suspension cells were harvested by centrifugation (13.000 x g, 20 min, 4°C) and subsequently resuspended in three volumes extraction buffer. In case of time course experiments, samples were stored at -20°C and collectively thawed for total protein extraction. Homogenisation and extraction was achieved by sonication for 1 min, 9 x 10% cycles and 40% power (Sonoplus, Bandelin). Total protein extracts were separated from cell debries by an additional centrifugation step. Cell extracts were transferred to 1.5 ml reaction tubes and stored at 4°C until testing.

**Extraction buffer** (pH 6)

| | |
|---|---|
| 2-Mercaptoethanol | 5 mM |
| EDTA | 5 mM |
| PBS (pH 6) | 50 ml |

**PBS buffer** (10x, pH 7.4)

| | |
|---|---|
| NaCl | 1.37 M |
| KCl | 27 mM |
| $Na_2HPO_4$ x $12H_2O$ | 81 mM |
| $KH_2PO_4$ | 15 mM |

### 2.3.2 Discontinuous SDS polyacrylamide gelelectrophoresis (SDS-PAGE)

The electrophoretic separation of proteins according to their specific molecular weight was performed by a standard discontinuous polyacrylamid gelelektrophoresis (PAGE) (Laemmli 1970). Protein samples (2.3.1) were mixed in a one to five ratio with reducing loading buffer. Due to the addition of the anionic detergent sodium dodecyl sulfate (SDS) all proteins were charged negatively. Boiling (99°C, 10 min) of the samples in the presence of the reducing agent 2-mercaptoethanol causes protein denaturation and reduction of protein disulfide linkages. The pre-treated protein samples were separated under reducing conditions in precast 4-12% (w/v) polyacrylamide gels (Thermo Fisher Scientific, Bonn). Gels were run in a Tris-HEPES-SDS running buffer at a constant voltage of 150 V for 45 min and subsequently processed for immunological analysis (2.3.3).

| **Reducing loading buffer** (5x) | |
|---|---|
| Tris-HCl (pH 6.8) | 1 M |
| SDS | 4% (w/v) |
| Bromphenol blue | 0.05% (w/v) |
| 2-Mercaptoethanol | 10% (v/v) |
| Glycerol | 30% (v/v) |

| **SDS PAGE Running buffer** (pH 8) | |
|---|---|
| Tris base | 100 mM |
| HEPES | 100 mM |
| SDS | 3.5 mM |

### 2.3.3 Immunoblot analysis

The integrity of the target proteins was examined by immunoblot analysis (Towbin *et al.* 1979) that was conducted after electrophoretic separation of the protein samples by SDS-PAGE (2.3.2). Proteins were electrotransferred onto a nitrocellulose membrane using transfer buffer and a constant voltage of 100 V for 60 min. Afterwards, blocking of non-specific binding sites on the nitrocellulose membrane was achieved by incubation with 5% (w/v) BSA in phosphate saline buffer for 30 min. The immunoblot membrane was rinsed with PBS containing 0.05% (v/v) Tween 20 and probed with antibodies (2.1.4) specific to the respective target protein (2.1.4). For M12 antibody determination the membrane was probed for 1 h with both primary AP-conjugated antibodies GAH $FC^{AP}$ (0.3 µg/ml) and GAH IgG $LC\lambda^{AP}$ (0.06 µg/ml) for heavy and light chain detection. The blotted tGFP protein was probed with the antiGFP primary antibody (50 ng/ml) for 1 h followed by the secondary antibody GAR $H+L^{AP}$ (0.12 µg/ml). After incubation at room temperature for 1 h and extensive washing, immunocomplexes were visualized with a NBT/BCIP solution (1:100) diluted in AP buffer. Determination of the tGFP concentrations were evaluated in comparison to a commercial tGFP standard (Evrogen) using the automatic imaging data analysis (AIDA).

| **Transfer buffer** | |
|---|---|
| Tris-HCl | 192 M |
| Glycin | 25 mM |
| Methanol | 20% (v/v) |

| **Alkaline phosphatase (AP) buffer** (pH 9.6) | |
|---|---|
| Tris-HCl | 100 mM |
| NaCl | 100 mM |
| $MgCl_2$ | 5 mM |

### 2.3.4 Enzyme linked immuno sorbent assay (ELISA)

The amount of M12 antibody in protein samples (2.3.1) was biochemically quantified by enzyme linked immuno sorbent assay (ELISA). M12 antibody accumulation levels were determined using the following immunological detection setup: High binding microtiter plates were coated with 70 ng/well GAH IgG Fc fragment specific serum (2.1.4) in sodium bicarbonate buffer at pH 9.6 and incubated overnight at 4°C. After washing the plates three-times with PBS containing 0.05% (v/v) Tween 20 (PBS-T) blocking was performed by addition of 200 µl/well of phosphate saline buffer containing 1% (w/v) BSA and incubation at room temperature for 1 h. The blocking buffer was removed and after washing 100 µl of two-fold serial diluted protein extracts (2.3.1) were added to the ELISA plate and incubated at room temperature for 2 h. Dilutions of protein A affinity chromatography purified M12 anti-body from tobacco plants served as a reference (14) for the determination of the M12 antibody concentration in the samples. The microtiter plates were washed with PBST and 4 ng/well of AP-labeled GAHλ LC specific serum (2.1.4) diluted in PBS were added. After incubation at room temperature for 1 h and washing, 100 ng/well of pNPP substrate in SP buffer were applied and detection was photometrically performed at 405 nm using a Multiscan spectrometer (BioTeck Instruments).

| **Coating buffer** (pH 9.6) | | **Substrate buffer** (SP, pH 9.8) | |
|---|---|---|---|
| Sodium carbonate | 15 mM | Diethanolamine | 0.1 M |
| Sodium hydrogen carbonate | 35 mM | $MgCl_2$ (x $6H_2O$) | 1 mM |

## 2.4 Recombinant DNA methods

### 2.4.1 Extraction of genomic DNA from plant material

Genomic DNA for quantitative real time PCR (2.4.2) was isolated from transgenic BY-2 suspension cultures. After vacuum filtration 200 mg cells (fresh weight) were transferred into a deep well plate (96 well) containing metal beads and 400 µl lysis buffer supplemented with 100 mg/ml RNase A (Chemagen, 2.1.3). Subsequently, the well plate was placed into a grinding bead mill (Retsch, Haan) and reciprocal shaking for 3 min at full speed disrupted the cells. Homogenized samples were centrifuged at 13.000 x g for 20 min at RT to pellet cell debries. Genomic DNA was isolated from cell extracts using the plant DNA extraction kit (Chemagen, 2.1.3) according to the manufactor`s instructions. The simultaneous extraction of genomic DNA from multiple samples was performed by an automated liquid handling workstation (Beckman Coulter GmbH, Krefeld).

### 2.4.2 Quantitative real time PCR

The detection and quantification of gene amplification and copy numbers was realized by the rapid and sensitive real-time Polymerase Chain Reaction technique (real- time qPCR) in

which the amplification of the PCR product is continuously monitored by fluorescence. Real-time qPCR was performed using an SDS 7700 real time PCR detection system (Applied Biosystems) in the presence of SYBR-Green while PCRs were carried out in 96 well PCR reaction plates (ABgene) at a 25 µl scale. Genomic DNA of each sample (2.4.1) was combined with a master mixture, supplemented with gene specific primers for three different target genes (Table 6), SYBR-Green premix (Invitrogen) and deionised water. The mixtures were distributed to three replicates and two non-template controls (NTC) for each sample. Sample amplification was performed using the following parameters: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec 95°C and 45 sec at 60°C. During the amplification process the accumulation of PCR products was detected by the simultaneous accumulation of the SYBR-green fluorescence. The system software provided the cycle number at which the fluorescence signal crossed a defined threshold (Cycle threshold, Ct) for each reaction. Based on this Ct values a quantitative calculation of the gene copy numbers was performed for each sample according to the common 2 -ΔΔCt method (Livak and Schmittgen 2001).

**Table 6: Target gene specific primer setup for real-time qPCR.**

| Heavy chain of M12 antibody | | DsRed | | PAL | |
|---|---|---|---|---|---|
| qPCR M12_HC_for | 500 nM | qPCR DsRED_forwa | 400 nM | qPCR/Nt_PAL_for | 500 nM |
| qPCR M12_HC_rev | 200 nM | qPCR DsRED_rever | 200 nM | qPCR/Nt_PAL_rev | 400 nM |

# 3 Results

Within this thesis flow cytometric sorting was applied to optimize an existing transgenic tobacco BY-2 suspension culture producing the visual marker protein DsRed and the human full-size antibody M12. Since the M12 and DsRed genes are located on the same T-DNA a co-selection strategy aimed at the simulateous identification of high M12 antibody producing cells by sorting strong fluorescent cells (3.3). Beside the evaluation of optimal preparation and cultivation conditions for low protoplast densities (3.1), a simple feeder cell-based strategy was developed to regenerate single cells and to establish monoclonal suspension cultures (3.1.5, 3.3.4). The FACS created polyclonal and monoclonal cultures were analysed with respect to growth performances, recombinant protein accumulation and production as well as genetic stability (3.3, 3.4). For selected monoclonal high-producing lines also culture scalability and medium optimization were investigated (3.5). Further, flow cytometric analysis and sorting was coupled to a transformation procedure (3.6) to establish an efficient screening and selection method for highly productive transgenic cells at an early stage after transformation in order to accelerate the generation of high-producing monoclonal cell lines.

## 3.1 Preparation of tobacco BY-2 protoplasts and generation of suspension cultures

For flow cytometric applications (2.2.7) using plant cells, single protoplasts represent an essential substitute for intact plant cells that were obtained by enzymatic digestion from the usually aggregated plant suspension cultures. Based on existing procedures, an efficient and gentle protocol was established that provided sufficient amounts of intact and vital tobacco BY-2 protoplasts for subsequent flow cytometric screening approaches. In particular, digestion conditions and regeneration medium compositions were optimized (3.1.1, 3.1.2). A robust and efficient method to regenerate transgenic cultures from separated BY-2 protoplasts was developed specifically for low protoplast densities prior to flow cytometric sorting experiments (3.1.4, 3.1.5).

### 3.1.1 Preparation of protoplasts from BY-2 suspension cultures

Protoplasts offer an excellent source material for analysis and optimization of plant suspension cultures via flow cytometry (2.2.7), but this method requires the isolation of a large number of vital protoplast. Based on existing preparation methods, the procedure was optimized for tobacco BY-2 suspension cultured cells with respect to enzyme concentration, digestion time and washing condition. In a first step, different concentrations of the cell wall specific enzymes cellulase and macerozyme were investigated in combination for efficacy in releasing large populations of vital protoplasts. Effective cellulase concentrations were tested for 1% (w/v), 2.5% (w/v) and 5% (w/v) enzyme resulting in an optimal cell wall digestion and formation of protoplasts using 1% (w/v) cellulase. In addition, effective protoplast liberation

was achieved by 0.3% (w/v) macerozyme identified through concentration tests using 0.075% (w/v), 0.15% (w/v) and 0.3% (w/v) of the enzyme. For all tested enzyme concentrations and combinations, cell digestion was performed in PNT solution (2.2.5) overnight (approx. 15 h) and the combination of 1% (w/v) cellulase and 0.3% (w/v) macerozyme resulted in almost 100% single protoplasts. All other assayed enzyme concentrations resulted merely in spherical shaped cell chains instead of individual protoplasts (data not shown).

Additional experiments were performed to identify the minimum time needed to liberate BY-2 protoplasts from suspension cells using the optimal cellulase and macerozyme combination. Therefore, the digestion of BY-2 suspension cells in a 1% (w/v) cellulase and 0.3% (w/v) macerozyme PNT solution was monitored microscopically after 1.5, 3, 5, 6 and 15 h, respectively. A short incubation time of 1.5 h to 5 h was not sufficient for protoplast liberation as the majority of cells still contain cell walls and existed as cell clusters and cell chains. By extending the incubation period initial release of spherical shaped protoplasts were observed after 6 h of digestion whereas overnight incubation of 15 h yielded exclusively liberated protoplasts (almost 100%, data not shown). As cellulase and macerozyme were known to have inhibitory effects on protoplast viability and cell wall regeneration (3.1.3) (Schilde-Rentschler 1977), it was evaluated whether an additional washing step (2.2.5) had a positive effect on protoplast proliferation. Compared to a single washing step routinely conducted in protoplast preparation protocols, two PNT washing steps turned out to be favorable over one washing step and resulted in slightly accelerated protoplast proliferation (3.1.2, Table 7). In summary, the following procedure turned out to be favorable for the preparation of large populations of vital protoplasts from BY-2 suspension cells: protoplasts were prepared from suspension cultures harvested in the logarithmic growth phase by overnight incubation at 27°C in the presence of 1% (w/v) cellulase and 0.3% (w/v) macerozyme using two washing steps with PNT solution after the isolation procedure (2.2.5). Examination of a typical preparation of freshly purified protoplasts determined an average yield of $6.2 \times 10^5 \pm 1 \times 10^5$ intact BY-2 protoplasts per milliliter assessed on distinct optical criteria including spherical shape, cytoplasmic strands and a defined and centered nucleus that is attached to the peripheral cytoplasm. Additionally, flow cytometric analysis (2.2.7.1) of vital stained protoplasts (2.2.6.1) confirmed high viability of the protoplasts since an average of 73.3% showed strong FDA fluorescence and were determined as viable approximately 1 h after the preparation of BY-2 protoplasts.

### 3.1.2 Optimization of the protoplast regeneration medium and conditions

Optimal conditions for robust and reliable BY-2 protoplast proliferation and cell wall regeneration will have a major impact on the generation of suspension cultures after flow cytometric sorting (2.2.7.2). Although some media were reported to be beneficial to culture tobacco derived protoplasts (i.e. mesophyll protoplasts) the medium compositions and cultivation con-

ditions were modified with respect to optimal regeneration of BY-2 protoplasts at low-densities. As a basic medium the protoplasts were regenerated in a rich culture medium containing diverse ingredients based on the published 8p medium composition (Kao and Michayluk 1975) (2.2.5). When cultured in this 8p regeneration medium sustainable protoplast proliferation was observed between 4-6 days after preparation. However, the published 8p medium failed to support protoplast regeneration when less than 5000 protoplasts were cultivated per ml. With respect to protoplast proliferation, several modifications of the basic 8p recipe were tested “one factor at a time” by the addition of cellobiose, the alteration of the sugar or various casein concentrations, the addition of the hormone phytosulfokine alpha and the variation of the pH value. The effects of medium variations and medium additives on the protoplast proliferation were assayed on lower protoplast densities of approx. $2 \times 10^2$ protoplast per ml. Proliferation of protoplast cultured in modified 8p medium compositions were monitored by microscopy and empirically assessed in comparison to the protoplast proliferation rate when cultured in the basic 8p medium (Table 7).

**Table 7: Influence of different medium components (A) and parameters (B) on protoplast proliferation.**
The tobacco BY-2 protoplasts were cultured in a nutrient-rich 8p medium (Kao and Michayluk 1975). Several listed modifications from the basic recipe (**A**) were tested as “one factor at a time” analysis using approx. $2 \times 10^2$ protoplasts per ml with the intention to optimize protoplast proliferation and cell culture regeneration. All components were added as sterile filtered solutions. The optimized 8p medium resulting from testings perfomed in (A) was used to evaluate optimal protoplast cultivation parameters (**B**). Proliferation of protoplasts was monitored by microscopy evaluated on day 3 and day 10, respectively. Reported effects resulted from day 10 observations of three replicate experiments and displayed unaltered (-) or faster and earlier (+) protoplast proliferation compared to the basic 8p medium.

| A | Medium component | Final concentration | Effect on protoplast proliferation |
|---|---|---|---|
| | **Sugars** | | |
| | Fructose | 0.5 g/l | - |
| | Ribose | 0.5 g/l | - |
| | Xylose | 0.5 g/l | - |
| | Mannose | 0.5 g/l | - |
| | Rhamnose | 0.5 g/l | - |
| | Cellobiose | 17.3 g/l | - |
| | | 34.4 g/l | - |
| | **Hormone** | | |
| | PSK-alpha | 0.1 µM | - |
| | | 1 µM | - |
| | | 1.5 µM | - |
| | | 2 µM | - |
| | **Casein hydrolysate** | | |
| | Casamino acid | 0.12 g/l | - |
| | | 0.25 g/l | - |
| | | 0.5 g/l | **+** |
| | **Gelling additives** | | |
| | Agarose | 0.2% | - |
| | Alginate | 0.2% | **+** |
| | Carrageenan | 0.2% | **+** |
| | Methyl cellulose | 0.2% | - |

| B | Parameter | Modification | Effect on protoplast proliferation |
|---|---|---|---|
| | Temperature | 22°C<br>27°C | -<br>**+** |
| | PNT wash step | once<br>twice | -<br>**+** |
| | Medium pH | 4.5 – 7.5<br>(0.5 steps) | **+**<br>(pH 5.5 – pH 6.5) |
| | Medium osmolality [mosmol/kg] | 0.2 -0.8<br>(0.2 steps) | **+**<br>(0.5 – 0.6 mosmol/kg) |

As increased sugar concentrations of five tested sugars and the addition of cellobiose had either no or in case of cellobiose only a moderate effect on the proliferation of BY-2 protoplasts rates, the basic 8p sugar concentrations were retained. In contrast, the alteration of the casein concentration to 500 mg/l significantly enhanced protoplast proliferation showing “four cell stages” instead of single protoplast divisions like in the basic 8p medium at the same time, but no specific impact was observed for lower casein concentrations (Table 7A). Finally, medium supplementation with the phytohormone PSK-α had no stimulating effect on protoplast proliferation. As most of the tested medium modifications had no enhancing influence on protoplast proliferation, the increasing casein levels turned out to be favourable for protoplast proliferation and protoplast proliferation was observed on day earlier. The casein modified regeneration medium for BY-2 protoplasts was termed 8p2c (2.2.5).

Furthermore, different cultivation parameters that are relevant for protoplast survival and proliferation such as temperature, medium pH and osmolality were investigated using the optimized 8p2c medium in order to identify sustainable regeneration conditions. When regeneration was performed at 27°C instead of 22°C, protoplast proliferation took place 24 h earlier. Maintainence of the protoplasts in 8p2c regeneration medium at 27°C resulted in microcalli formation within 10 days whereas the majority of protoplasts cultured at room temperature (22°C) died within the same time period. Microscopic observation of protoplasts cultivated in 8p2c media with pH values ranging between pH 4.5 to pH 7.5 revealed vital protoplasts that reached “four cell stage” after seven days when pH values range between pH 5.5 to pH 6.5. For lower pH values the inhibition of protoplast proliferation and death of protoplasts were observed (Table 7B). Therefore, the pH value of the 8p2c medium was always adjusted to pH 6.0. Testing of different medium osmolalities (Table 7B) identified 0.5 to 0.6 mosmol/kg to be essential for protoplast vitality and proliferation, whereas lower or higher osmolalities caused protoplast death.

Apart from the liquid cultivation of BY-2 protoplasts alternative procedures were also tested to regenerate low protoplast densities. Since the cultivation of protoplast (<10 protoplasts) in small droplets (approx. 5 µl – 10 µl medium) was inapplicable and resulted in either evaporation or protoplast death, the embedding of low protoplast densities in semi-solidified

8p2c medium was tested. In this context, the suitability of different gelling agents like alginate, carageenan, agarose and methyl cellulose was compared (Table 7A). For the application of solid medium the addition of carageenan was favourable over all other tested gelling agents as regeneration of callus tissue from initial protoplasts was possible and, unlike alginate, the medium remained solid over the total observation time of two to four weeks. Despite the benefit of fixed and physically separated protoplasts in semi-solid medium, the simple cultivation in liquid 8p2c medium was finally superior for BY-2 protoplast cultivation because of the easy handling and its feasibility with regard to flow cytometric sorting procedures (2.2.8).

To identify the minimal seeding density, i.e. densities at which BY-2 protoplasts can be successfully regenerated to suspension cultures, limiting dilution experiments were performed. Applying the 8p2c regeneration medium adjusted to pH 6.0 and about 0.6 mosmol/kg in combination with a cultivation at 27°C protoplasts revealed a minimal protoplasts density of 200-400 protoplasts per ml (corresponding to 10-20 protoplasts per well in 50 µl) (data not shown). Protoplast at these low densities underwent early mitotic divisions within two to three days and formed microcallus tissue within 10-14 days.

### 3.1.3 Monitoring of cell wall synthesis of regenerating BY-2 protoplasts

Being a prerequisite for cell division and cell regeneration, the re-formation of cell walls was monitored to assess the time point when BY-2 protoplasts were robust enough to survive the flow sorting procedure (2.2.7.2), but still are round-shaped and do not form aggregates. Since cellulose synthesis is a good indicator for plant cell wall formation, the protoplasts were stained by the cellulose-binding Calcofluor white (CW, 2.2.6.2). As documented in Figure 4, CW stained protoplasts were monitored at different timepoints after an optimized protoplast preparation procedure described in 2.2.5 and cultivation in 8p2c regeneration medium (3.1.2). The absence of cell walls shortly after protoplast preparation was proven for the majority of cultured protoplasts, as expected. In fact, within the first 4 h post preparation only a minor portion of 9.5% to 12.7% BY-2 protoplasts rebuild stainable primary cell walls (Figure 4). The robustness of the protoplasts to survive the following flow sorting process (2.2.7.2) was improved by further incubation in 8p2c regeneration medium for up to 48 h during which cell wall reassembly was observed. In fact, about 30% protoplasts commenced regeneration of detectable cell walls within 6 h and Calcofluor White staining confirmed the formation of a primary cell wall after 24 h for 90% of the viable protoplasts. Beside cell wall regeneration this experiment confirmed also protoplast viability by distinctly stained cell-cell septum proving protoplast divisions observed after 24 h – 48 h (Figure 4, 48 h). The formation of a primary cell wall does not alter the spherical shape of the protoplasts and protected them from shear forces that occur during flow sorting (2.2.7.2). The preliminary sorting of protoplast with pre-regenerated cell walls (after 48 h) revealed vital protoplasts that commenced

proliferation whereas flow sorting at an earlier time point after preparation resulted in protoplast death (data not shown). In conclusion, results of this experiment prompted to pre-regenerate isolated BY-2 protoplasts for at least 48 h ahead of flow cytometric sorting in order to obtain robust, but still spherical protoplast suspensions that resisted the flow cytometric sorting process.

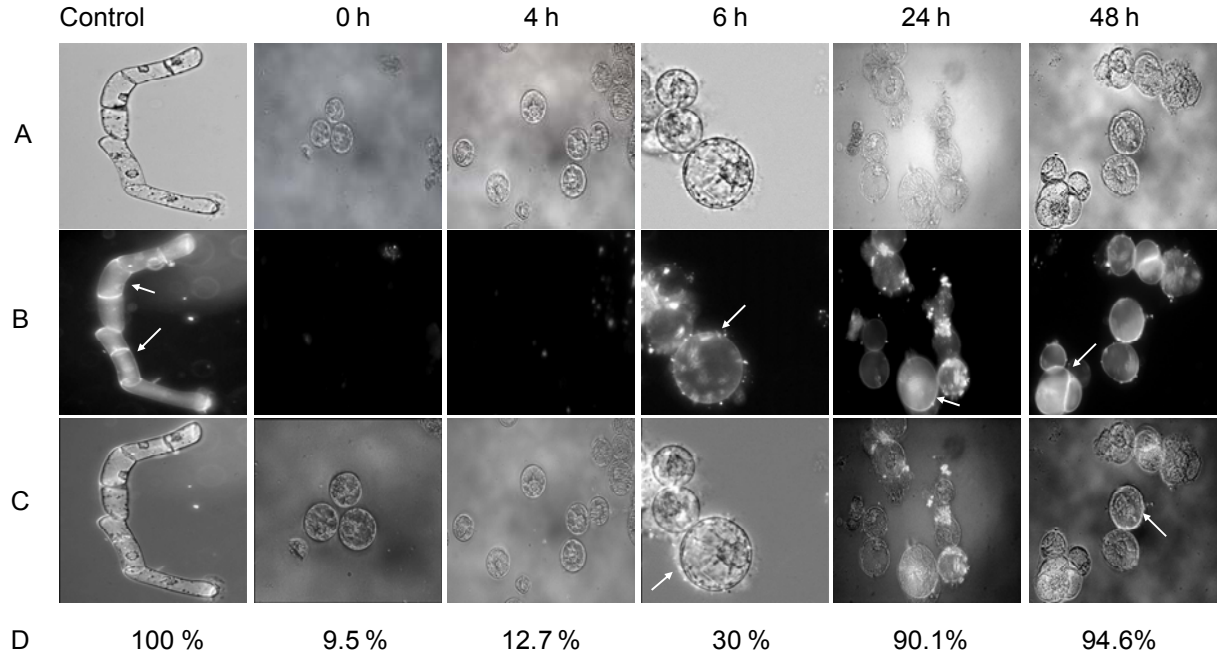



**Figure 4: Cell wall synthesis of cultured protoplasts.**
Protoplasts were prepared from BY-2 suspension cells (control, 3.1.1) and cultured in 8p2c medium (3.1.2). The regeneration of cell walls was monitored at different time points (0–48 h) by Calcofluor White staining and fluorescence microscopy (2.2.6.2). Fluorescence images were taken using a DAPI filter (BP 470/40 nm) with a 500 msec exposure time whereas transmission light images were taken with an exposure time of 300 msec. For all images a 100-fold magnification was used except for the 6 h time point (200-fold magnification). **A:** Transmission light image of cultured protoplasts regenerating cell walls. **B:** Fluorescence light image of cultured protoplasts regenerating cell walls. **C:** Overlay of transmission light and fluorescence light image of cultured protoplasts regenerating cell walls. **D:** Percentage of protoplasts regenerating a stainable cell wall determined for 120 counted protoplasts for each time point The white arrows indicate Calcofluor White stained cell walls.

### 3.1.4 Regeneration of flow cytometrically sorted protoplasts

For the practical application of flow cytometric sorting of plants cells two requirements had to be fulfilled, first the cell survival during the sorting procedure and second the regeneration of the deposited plant cells. Introducing a 48-72 h long pre-regeneration step between protoplast preparation and the flow sorting ensured the robustness of the BY-2 protoplasts as cell wall reassembly was initiated (3.1.3). These protoplasts with partially regenerated cell walls were hereinafter referred to as cells. Using the sort conditions for tobacco cells described in 2.2.7.2, facilitated the gentle sorting of the BY-2 cells in a viable state and at sterile conditions. As mentioned in 3.1.2, also flow sorted BY-2 cells failed to regenerate when cells were separated below a seeding density of $2 \times 10^2$ cells per ml. The optimal seeding density for polyclonal cell sorting was $4 \times 10^2$ protoplasts per ml corresponding to 20 cells in 50 µl culture

medium per 96 multiwell plate well (data not shown). When cell sorting was performed in polyclonal pools of 20 cells each and according to the electronic sort settings shown in Figure 7, an efficient deposition of viable BY-2 cell pools was achieved for up to 93% of the 96 multiwell plate wells. Moreover, about 35% of these flow sorted pools easily regenerated to polyclonal microcallus tissue within 15 weeks using the 8p2c cultivation medium (data not shown). In contrast, the direct cell sorting on semi-solid 8p2c medium, which was in addition investigated to gain microcallus tissue, resulted in cell death rendering the recovery of viable sorted BY-2 cells impossible. For the following flow cytometric experiments the separation of BY-2 cells was performed using liquid 8p2c regeneration medium.

Furthermore this study aimed at the flow cytometric separation of single tobacco BY-2 cells to achieve monoclonal cell lines. When BY-2 cells were sorted in the single cell mode, a deposition efficiency of 35% single cells was achieved using 96 multiwell plates. The remaining wells either lacked flow sorted cells or contained multiple deposited cells. Similar to earlier findings on the regeneration of low cell densities (3.1.2), no re-cultivation was observed here when single cells were deposited by FACS.

In summary, flow sorted polyclonal cultures originating from 20 pool sorted cells were successfully regenerated, but the proliferation of single sorted cells was hampered by the necessity of at least $2 \times 10^2$ cells per ml. Considering this, the application of feeder cells, simulating a small population, was required to develop a regeneration strategy for single sorted cells (3.1.5).

### 3.1.5 Establishment of a feeder cell-based strategy for monoclonal cultures

To enable the regeneration of single cells after flow cytometric sorting, a feeder-based strategy was established to support proliferation of single sorted transgenic BY-2 cells and to ensure the generation of monoclonal cultures. According to published procedures using feeder cultures of the same species than the sorted cells (Davey *et al.* 2005), approx. 100 BY-2 wild type cells in 50 µl per 96 microtiter plate well were provided as feeder suspensions in this work. In a first approach, it was tested whether the feeder and the flow sorted cells can be physically separated using nylon gauze inserts (data not shown), but this setup was discarded because the gauze inserts did not allow any microscopic monitoring of the single cells. As a consequence, within this thesis the direct co-cultivation of single transgenic cells with wild type feeder cells was investigated (Figure 5). The seeding of roughly 100 BY-2 wild type cells in 50 µl per 96-well plate well was sufficient for the survival of single sorted cells, their proliferation and promotion of monoclonal microcallus formation (Figure 5, 2-4). In fact, reliable mitotic division of single FACS selected cells was obtained after 48 h of co-cultivation and approximately half of the single flow sorted cells, i.e. 15% of initially 35% single deposited cells (3.1.4), proliferated and formed microcolonies within 2 weeks. These microcolonies were transferred onto selection plates, e.g. kanamycin containing plates, to promote the

exclusive growth of the transgenic cells and generate monoclonal callus tissue (Figure 5, 4). Occasionally, some of the transferred transgenic microcolonies failed to grow on selection plates and rarely cell tissue remained stuck to the pipette, which also contributed to a loss of microcolonies. As a consequence of gentle resuspension, required for the microcolony transfer to selection plates, multiple transgenic calli instead of a single callus were obtained. Even though the microcolony was fragmented, it was still monoclonal and originated from the same sorted single cell (Figure 5, 3+4). Finally, the monoclonal suspension cultures established from separated transgenic callus tissue were examined by molecular and flow cytometric analyses (3.3.4).

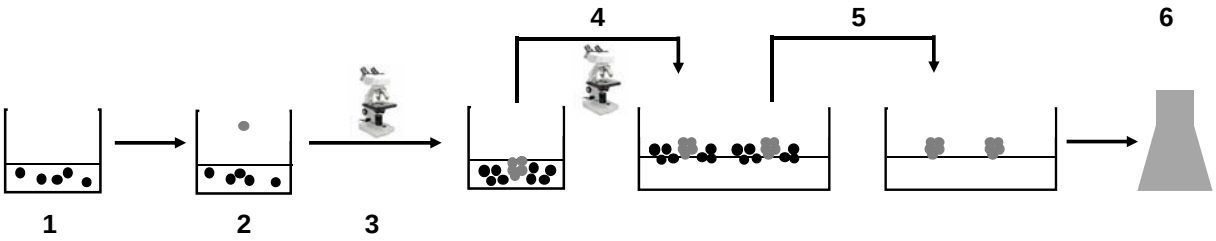


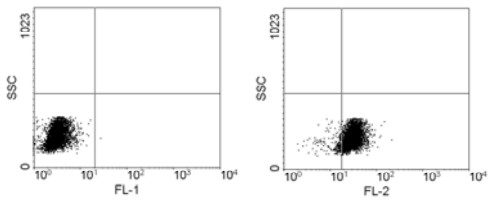

**Figure 5: Schematic overview illustrating the deposition of single, highly fluorescent transgenic protoplasts and the regeneration of monoclonal cell lines.**
1: Transfer of wild type feeder cells (black) into regeneration devices (e.g. 96 microtiter plate well). 2: Flow cytometric sorting and deposition of highly fluorescent single cells (grey) in regeneration devices prefilled with feeder cells. 3: Verification of single cell deposition and proliferation by inverse fluorescence microscopy. 4: Transfer of regenerated transgenic microcalli to selective solid cultivation medium for removal of wild type feeder cells. 5: Transfer of grown transgenic calli to fresh selective solid cultivation medium. 6: Establishment of monoclonal transgenic suspension cultures by transfer of callus tissue to liquid cultivation medium.

## 3.2 Proof of feeder cell removal during the regeneration of sorted protoplasts

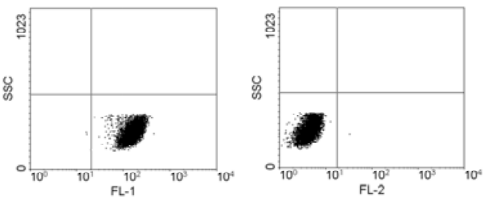
For the reliable regeneration of single FACS selected protoplasts to monoclonal cultures the presence of feeder protoplasts turned out to be absolutely required since a minimum seeding density of protoplasts was essential for protoplast proliferation (3.1.4, 3.1.5). Since the feeder protoplasts were temporarily co-cultivated with sorted transgenic protoplasts it was mandatory to exclude the survival of feeder protoplasts during the regeneration of monoclonal cultures (2.2.8.2). Therefore, the potential contamination of single sorted transgenic BY-2 cultures with feeder protoplasts was investigated. In particular, kanamycin resistant BY-2 cells producing DsRed (MTED#18, 2.1.6) were flow sorted as single cells (2.2.7.2) into imazethapyr resistant BY-2 feeder cells producing the green fluorescent protein tGFP (2.1.6) and *vice versa* meaning that single imazethapyr resistant tGFP expressing cells were deposited into MTED#18 feeder cells. After regeneration of the sorted cells (3.1.4), 40 monoclonal calli from both experiments were analyzed towards their respective resistance. Therefore, 20 monoclonal tGFP and 20 DsRed fluorescent calli, were transferred onto selection plates containing either imazethapyr or kanamycin and cultured for 14 days. Visual inspection confirmed that the 20 tGFP producing calli grew exclusively on medium containing imazethapyr

and the 20 tested MTED#18 calli grew exclusively on medium containing kanamycin (Figure 6C).

A




B




C

| Single sorted protoplasts | Feeder protoplast culture | Resistance of regenerated cell lines | | Proportion of fluorescent cells in regenerated cell lines | |
|---|---|---|---|---|---|
| | | Imazethapyr | Kanamycin | GFP | DsRed |
| BY-2 (GFP; Imazethapyr) | MTED#18 (DsRed; Kanamycin) | 20/20 | 0/20 | 94.2% 5.5% | 0 % |
| MTED#18 (DsRed; Kanamycin) | BY-2 (GFP; Imazethapyr) | 0/20 | 20/20 | 0 % | 93.7% 3.7% |

**Figure 6: Verification of feeder protoplast elimination in regenerated monoclonal cultures.** Representative flow cytometric dot plots and resistance characteristics of transgenic monoclonal BY-2 cultures regenerated with presence of approx. 100 fluorescent feeder protoplasts in 50 µl per well (3.1.5). **A:** Kanamycin-resistant BY-2 culture producing DsRed (MTED#18) that was regenerated by co-cultivation with imazethapyr-resistant tGFP producing feeder cells. **B:** Imazethapyr-resistant tGFP producing culture that was regenerated by co-cultivation with MTED#18 feeder cells. For fluorescence signal analysis (2.2.7.1) a total of 10.000 protoplasts were recorded (**A**, **B**). Cell debris were excluded from the analysis by gating the viable cell population in a forward/sideward scatter dot plot. The viable population was analyzed in the tGFP channel (FL-1) and DsRed channel (FL-2). For statistical calculation of fluorescent events a threshold (quadrant) was set based on wild type protoplasts. The FL-2 signals were compensated (- 30% of the FL1) due to overlapping fluorescence. **C:** Characteristics of monoclonal calli and suspension cultures obtained by flow cytometric sorting and regeneration of transgenic single cells. Resistance test data were obtained from 20 FACS generated monoclonal calli (n = 20) for each approach. The flow cytometric values represent means ± SD of four independent suspension cultures (n= 4) established from the 20 FACS generated calli for each sort approach.

In addition, four regenerated monoclonal suspension cultures of each type were analyzed by flow cytometry (2.2.7.1) to assess the presence of contaminating feeder cells. Protoplasts of the regenerated tGFP and MTED#18 suspension cultures were analyzed with respect to potential either DsRed or tGFP fluorescence introduced by the respectively regenerated feeder cells. The four monoclonal BY-2 MTED#18 cell lines cultured in the presence of kanamycin selection contained 93.7 ± 3.7% DsRed fluorescent cells (Figure 6C) that were entirely detected in the FL-2 channel and no green fluorescence representing feeder cells was recorded in the FL-1 channel (Figure 6A). Likewise, the four monoclonal BY-2 tGFP cell lines cultured in the presence of imazethapyr contained 94.2 ± 5.5% tGFP fluorescent cells (Figure 6C) exclusively recorded in the FL-1 channel and no DsRed fluorescence representing feeder cells was detected in the FL-2 channel (Figure 6B). The findings from the resistance assays and flow cytometric analysis clearly demonstrated that the selection strategy completely suppresses feeder cell growth during the regeneration of single sorted cells.

### 3.3 FACS-based optimization of existing transgenic plant cell lines

The applicability of fluorescence activated cell sorting was investigated to separate defined tobacco cells from a population of heterogeneous cells to enhance transgenic BY-2 suspension culture productivity. For the intended experiment, the heterogeneous MTED#18 suspension culture (2.1.6), producing 14.3 ± 4 µg per gram fresh weight of the recombinant M12 full-size antibody (corresponding to 10 mg/l culture volume), was chosen. The flow cytometric analysis (2.2.7.1) revealed two subpopulations of different fluorescence intensities, with only 24% of the viable population representing the high DsRed fluorescence (Figure 7, right). Because the DsRed and M12 antibody genes are linked on the same T-DNA, DsRed fluorescence was used as a indicator of M12 levels assuming that the selection for high-level DsRed fluorescence would identify clones producing as well high levels of the co-expressed recombinant antibody. Therefore, pools of 20 cells (3.3.1) and single cells (3.3.4) were separated by flow cytometric sorting to establish highly productive polyclonal and monoclonal cultures. For both strategies, the sort criteria were adjusted based on signals collected for cell size, cell granularity and fluorescence of the MTED#18 culture that only strong DsRed fluorescent and viable cells were sorted (Figure 7).

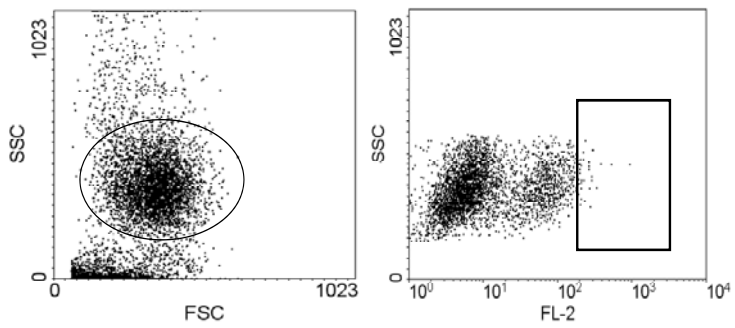



**Figure 7: Settings for the separation of strongly DsRed fluorescent protoplasts by flow cytometric sorting.**
Of the gated viable population (indicated with an ellipse) 2-5% cells, showing the strongest fluorescence signals in the DsRed detection channel (FL-2), were single cell or pool sorted into multi-well plates (indicated with a square).

#### 3.3.1 Enrichment of highly productive cells through sorting of protoplast pools

Screening and separation of intensive DsRed fluorescent cells of the heterogeneously producing model culture MTED#18 (3.3) was conducted by three consecutive rounds of polyclonal flow sorting to simultaneously increase the number of highly DsRed fluorescent and M12 antibody producing cells. More precisely, pools of 20 cells were flow sorted (2.2.7.2) from a portion of 2-5% strong DsRed fluorescent cells of the MTED#18 culture (3.3, Figure 7). Comparable to previously reported data (3.1.4), flow sorting of 20 cell pools resulted in a deposition efficiency of 90% achieved for each of the three sorting rounds and approx. 34% of the sorted pools regenerated to suspension cultures here. The polyclonal cultures of each FACS round were evaluated regarding their recombinant M12 antibody yield and DsRed fluorescent cell portion. For each round the best producing culture, documented in Figure 8, served as a starting culture for the following FACS round.

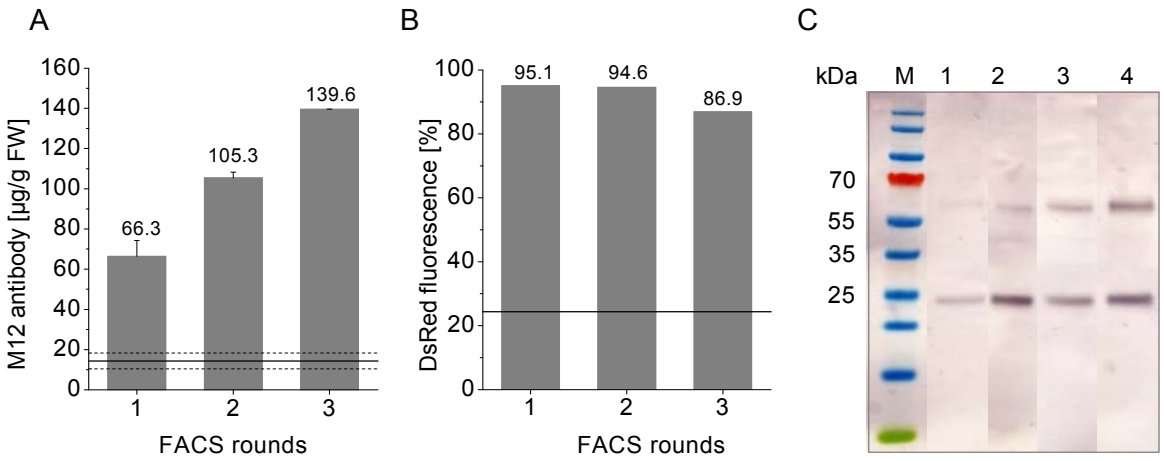



**Figure 8: Flow sorting mediated generation of an improved cell culture expressing DsRed and M12 antibody.**
Selection of highly productive polyclonal cultures was performed for the BY-2 MTED#18 cell line by flow cytometric separation (2.2.7.2). For each of the three successive FACS rounds 20 cell pools were separated from 2-5% viable cells showing the strongest DsRed signals in the fluorescence channel and seeded into multiwell plates. Regenerated cell lines of each FACS round were analyzed regarding recombinant M12 antibody and DsRed protein; the best producing culture of each round was used for subsequent FACS. **A**: Production of the M12 antibody in FACS-derived polyclonal lines. For each round the antibody yields were determined by ELISA (2.3.4). Error bars represent standard deviations of three technical replicates. The antibody level of the parental MTED#18 culture is displayed as solid black line with standard deviation (dash lines) of three biological replicates. 1: MTED#18 psR1C11, 2: MTED#18 psR1C11.5, 3: MTED#18 psR3C11.5.8. **B**: Determination of the DsRed marker protein in FACS-derived polyclonal lines by flow cytometric analysis (2.2.7.1). Data represent the percentage of viable fluorescent cells based on 10.000 analysed events. The DsRed fluorescent population of the parental MTED#18 line is displayed as a solid black line. 1: MTED#18 psR1C11, 2: MTED#18 psR1C11.5, 3: MTED#18 psR3C11.5.8. **C**: Immunoblot detection (2.3.3) of the M12 antibody. 20 µl of cell extract were separated on a 4-12% (w/v) SDS-PAGE gel and electro-blotted to nitrocellulose membrane (2.3.2). Antibody detection was performed by GAHλLC$^{AP}$ and GAHFc$^{AP}$ (2.1.4) incubation following NBT/BCIP substrate visualization. M: Page Ruler Protein Marker Broad Range (Fermenters), 1: parental MTED#18 culture; 2: MTED#18 psR1C11, 3: MTED#18 psR1C11.5, 4: MTED#18 psR3C11.5.8.

Compared to the parental MTED#18 culture, the evaluation of the first round of flow cytometric pool sorting resulted in six polyclonal suspension cultures that displayed up to 3.9-fold enriched portions of DsRed fluorescent cells and up to 4.7-fold increased levels of the M12 antibody, as presented for the best identified culture MTED#18psR1C11 (Figure 8A+B). Although this first round culture consisted of 95% DsRed fluorescent cells and co-expressed the M12 antibody at accumulation levels of 66 ± 7.8 µg/g fresh weight (corresponding to 35 mg/l culture volume) the MTED#18psR1C11 culture was further improved by subsequent flow cytometric sorting. From the second FACS selection according to DsRed intensity three polyclonal cultures were generated that showed a consistent distribution of strong DsRed fluorescent cells ranging between 90-95%. Despite no obvious differences in the DsRed producing cell population, the accumulation levels of the co-expressed M12 antibody were significantly enhanced up to 7.5-fold when compared to the parental MTED#18 culture. The MTED#18psR2C11.5 culture, procducing the recombinant M12 at yields of 105 ± 2.9 µg/g fresh weight (corresponding to 61 mg/l culture volume) and showing a portion of 94% strong DsRed fluorescent cells, was identified as the top producer of the

three tested second round polyclonal cultures (Figure 8A+B) and used for a following FACS round. The succeeding third round of cell sorting led to the generation of six polyclonal cultures were the M12 antibody levels, in relation to the parental MTED#18 culture, were once more increased to finally 9.7-fold. This was determined for the polyclonal MTED#18psR3C11.5.8 culture producing 139 ± 0.1 µg/g fresh weight (corresponding to 88 mg/l culture volume). However, a slightly decreased percentage of 87% DsRed fluorescent cells were detected for this culture by flow cytometric analysis (2.2.7.1).

Additionally, immunoblot analysis confirmed the integrity of the human M12 full-size antibody for the parental MTED#18 culture and the respective best polyclonal MTED#18 culture of each sorting round by successful detection of the 55 kDa heavy and 25 kDa light chain (Figure 8C). It was demonstrated that signal intensities for both antibody chains increased along with the subsequent rounds of FACS selection, even though this was more prominent for the heavy chain. In detail, protein signals detected for MTED#18psR2C11.5 showed greater intensities compared to the protein signals of MTED#18psR1C11, whereas the most intensive protein signals were detected for the MTED#18psR3C11.5.8 culture.

Confirming the calculated percentages of the DsRed fluorescent cell populations (Figure 8B), flow cytometric analysis (2.2.7.1) verified a considerable enrichment of the red fluorescent population in the MTED#18psR1C11 culture achieved by the first FACS round. This proportion of DsRed fluorescent cells remained consistent within the polyclonal cultures generated by the following two FACS rounds (Figure 9).

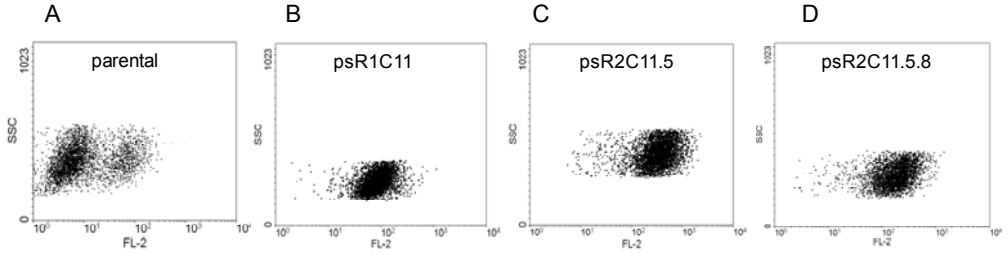



**Figure 9: Comparison of fluorescent marker distribution in the parental and FACS improved cultures.**
Improved cell cultures created within three rounds of successive polyclonal flow sorting (20 cells per pool) and the parental MTED#18 culture were analyzed by flow cytometry (2.2.7.1). The distribution of DsRed fluorescence signals was recorded for 10.000 events. Dot plots displayed the DsRed distribution of the viable population in a single parameter dot plot as events versus DsRed channel (FL-2). Names within the plots represent the respective cell culture. **A**: parental MTED#18 culture, **B**: first round culture MTED#18psR1C11, **C**: second round culture MTED#18psR2C11.5, **D**: third round culture MTED#18psR3C11.5.8.

Contrary to the heterogenic parental MTED#18 culture (Figure 9A), the three polyclonal sorted cultures (Figure 9B, C, D) displayed distinct populations expressing the recombinant DsRed protein at similarly high fluorescence intensity levels. The weak fluorescent population of the parental culture was successfully removed except for some events detected at

aberrant fluorescence intensities, possibly caused by variations in culture fitness and age, protoplast preparation or specific gating.

This experiment demonstrated that flow sorting of high fluorescent protoplast pools led to a simultaneous increase in culture productivity and homogeneity of the initially heterogeneous MTED#18 culture that can be further enhanced by successive rounds of sorting. Ultimately, the average time frame calculated for a single flow sorting round starting from an already established heterogenic suspension culture and implementing the cell regeneration process (2.2.8) was only 2.5 months.

### 3.3.2 Growth characteristics of polyclonal sorted cultures

The growth characteristics with regard to biomass accumulation and recombinant M12 antibody productivity were reviewed for the three rounds of polyclonal sorted cultures (3.3.1), in comparison to the parental MTED#18 culture, by time course experiments using routine shake flask conditions (2.2.3.1). Typical growth performances observed within three independent experiments were ascertained for a seven day cultivation and exemplarily presented for the fresh cell weight at 3 to 7 days after inoculation of the cultures (dpi) in fresh medium (Figure 10D-F). The M12 antibody accumulation levels in the cell extracts (2.3.1) were determined by sandwich ELISA (2.3.4) for the same sampling days (Figure 10A-C).

Growth performance of the parental MTED#18 culture was characterized by an initial lag-phase of approximately two days ahead of the exponential growth (data not shown), finally entering the stationary phase at 5 dpi at which a maximum fresh weight value of 766 g/l, corresponding to 77% PCV, was reached. After inoculation the medium pH dropped below pH 5 within the first 3 dpi and stayed constant with only minor changes between pH 5.5 and pH 6.0 during further cultivation. The medium osmolality declined coinciding with the exponential growth phase between 3 dpi and 5 dpi from 200 osmol/kg to 20 osmol/kg indicating the consumption of medium components (i.e. sugars, micro- and macroelements, data not shown). The parental MTED#18 culture typically accumulated maximum M12 antibody levels of 20 ± 10 µg/g fresh weight on day 4 of cultivation (Figure 10A-C).

Although, the lag phase period of all three polyclonal MTED#18 cultures was consistent with the one of the parental MTED#18 culture (data not shown), an appreciable extended logarithmically growth phase by one day was observed for the pool sorted cultures. As a consequence, the stationary phase was obtained at 6 dpi, reaching maximum biomass of 600 g/l fresh weight for MTED#18psR1C11 (corresponding to 65% PCV, Figure 10D) and 675 g/l for MTED#18psR3C11.5.8 (corresponding to 60% PCV, Figure 10F). Among the three polyclonal suspension cultures, no obvious differences of the assayed growth parameters were observed: as medium pH persisted similar values between pH 5.0 and pH 6.0 during logarithmically and stationary growth, the medium osmolality sharply declined from 200 osmol/kg on day three to 30 osmol/kg on day six (data not shown). Again this effect directly coincided with

the logarithmically growth phase as a consequence of nutrient consumption. However, after 7 dpi the achieved cell pellet weights and relating PCV were found to be approximately 22% less compared to the parental MTED#18 culture.

Consistently, for the polyclonal MTED#18 cultures, quantification of the recombinant M12 antibody typically yielded the highest accumulation levels at the end of cultivation. In this timecourse experiment, the M12 concentrations were determined in a range of 76 ± 0.6 µg/g fresh weight for MTED#18psR1C11 at 6 dpi (corresponding to 38 mg/l, Figure 10A), 111 ± 8 µg/g fresh weight for MTED#18psR2C11.5 (corresponding to 66 mg/l, Figure 10B) and 174 ± 13 µg/g fresh weight (corresponding to 117 mg/l) for MTED#18psR3C11.5.8 at 7 dpi (Figure 10C), respectively.

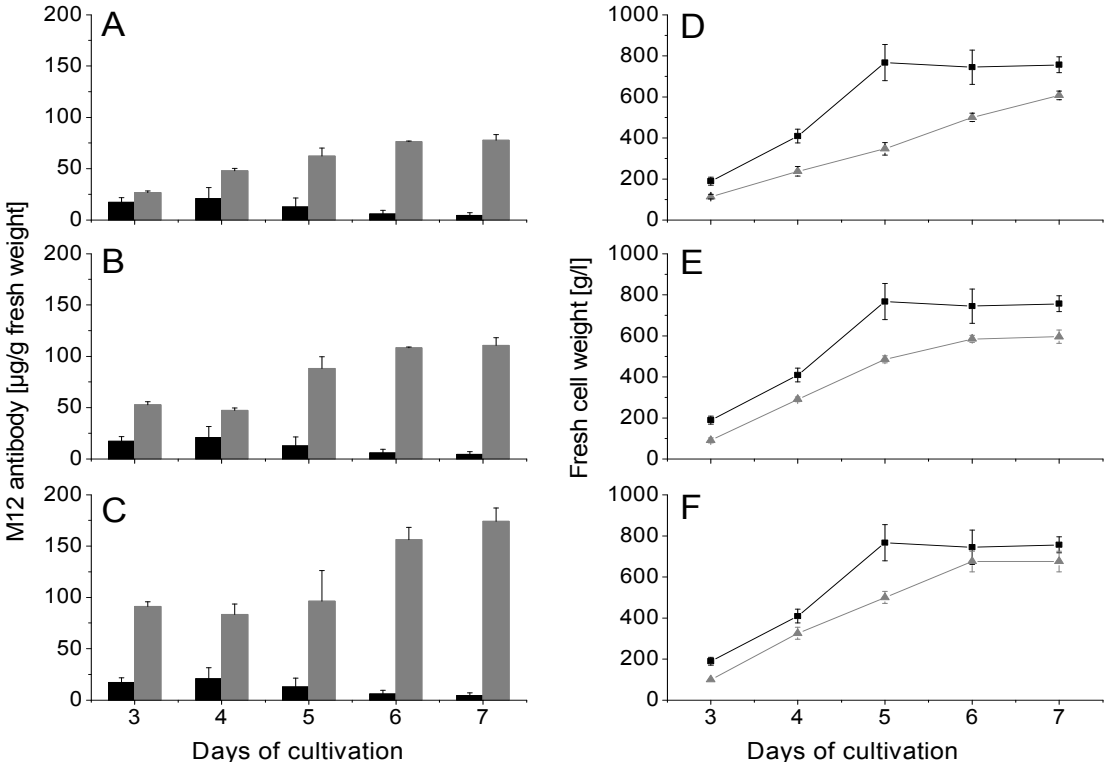



**Figure 10: Growth and production characteristics of improved MTED cultures.**
The FACS improved suspension cultures (three subsequent polyclonal rounds, 3.3.1) and the parental MTED#18 suspension culture (2.1.6) were cultivated in parallel as 200 ml cultures in 500 ml Erlenmeyer flasks using routine cultivation conditions (2.2.3). Sampling was performed daily on day 3 to 7 after inoculation. Growth and M12 antibody data were obtained from three independent cultivations of each culture: MTED#18 parental culture (black/ ■), MTED#18 culture 11 (first-third round; grey/▲). The M12 antibody accumulation levels were determined by ELISA (2.3.4) and displayed as columns. The antibody yield during standard cultivation was analysed from first round culture MTED#18psR1C11 (**A**), second round culture MTED#18psR2C11.5 (**B**) and third round culture MTED#18psR3C11.5.8 (**C**). Values of the measured fresh cell weight (2.2.3.1) were plotted as lines and symbol. The error bars represent the standard deviation of three biological replicates (n=3). Biomass production during standard cultivation was determined for first round culture MTED#18psR1C11 (**D**), second round culture MTED#18psR2C11.5 (**E**) and third round culture MTED#18psR3C11.5.8 (**F**). The error bars represent the standard deviation of three biological replicates (n=3).

### 3.3.3 Recombinant protein production stability in FACS improved suspension cultures

Continuous maintenance of the polyclonal suspension cultures in routine culture (2.2.3) provoked the investigation whether FACS improved recombinant protein levels remained stable. Since the polyclonal MTED#18psR3C11.5.8 culture was only recently established at this time and a re-establishment of the parental MTED#18 culture from callus tissue was required, the analysis of DsRed and M12 production with these cultures was not feasible and only investigated for the polyclonal cultures MTED#18psR1C11 and MTED#18psR2C11.5 (3.3.1). Over a period of one year the percentages of DsRed fluorescent cells were assessed by flow cytometry (2.2.7.1), whereas M12 antibody yields were quantified by ELISA (2.3.4) using extracts from 5 dpi harvested cells (2.3.1). The results obtained for both 12 months old polyclonal cultures were compared to values generated directly after culture establishment (0 months) and are displayed in Figure 11.

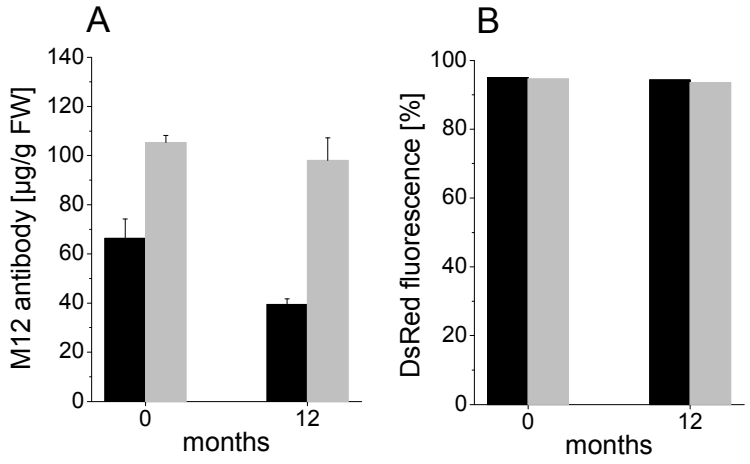



**Figure 11: Recombinant protein accumulation in FACS improved suspension cultures.**
The polyclonal cultures MTED#18psR1C11 (black) and MTED#18psR211.5 (grey) were maintained over 12 months in routine culture (26°C, 180 rpm) with a passaging cycle of 7 days (2.2.3). Sampling and analysis of both recombinant proteins was performed after one year on day 5 of routine subcultivation (2.2.3). **A**: Production of the M12 antibody in FACS improved cell lines. The M12 antibody accumulation levels were determined by ELISA (2.3.4). The error bars represent the standard deviation of three technical replicates. **B**: Determination of the DsRed fluorescent marker protein distribution by flow cytometric analysis (2.2.7.1). Data represent the percentage of viable fluorescent protoplasts based on 10.000 analyzed events.

As demonstrated by flow cytometric analysis, the viable DsRed fluorescent population remained consistent for the polyclonal cultures MTED#18psR1C11 and MTED#18psR2C11.5, showing about 94% highly fluorescent cells (Figure 11B) over a time period of 12 months. Likewise, consistent high M12 antibody yields of 98.1 ± 9 µg/g fresh weight were quantified for the second round MTED#18psR2C11.5 culture compared to 105.5 ± 2.9 µg/g fresh weight produced by this culture prior to routine maintenance (Figure 11A). Since this apparent decrease in antibody accumulation values resided within the range of standard deviation, the MTED#18psR2C11.5 culture appeared to be stable regarding the

production of both recombinant proteins. In contrast, a decreased M12 antibody production of 39.5 ± 2.1 µg/g fresh weight from previously 66.3 ± 7.8 µg/g fresh weight was observed for the first round MTED#18psR1C11 culture after one year. This observation provided evidence that polyclonal cultures were still composed of a heterogeneous mixture of cells, which were affected by variations associated with the repeated subculture process (Figure 11A).

### 3.3.4 Generation of monoclonal cell lines from a heterogeneous transgenic suspension culture

As a further aim, high recombinant protein producing monoclonal suspension cultures were generated from the heterogeneous transgenic MTED#18 culture. Therefore, separation of high producing cells was conducted by flow cytometric sorting of single intensive DsRed fluorescent cells. Again, a fraction of 2-5% strong red fluorescent cells of the parental culture (3.3, Figure 7) was flow sorted, but separation of cells was performed in single cell mode (2.2.7.2). To ensure the creation of monoclonal cultures from single deposited transgenic cells the feeder based regeneration strategy reported in 3.1.5 was applied. Basically, the identified best producing monoclonal cultures served as a new monoclonal starting culture for following, in total three, flow sorting rounds (Figure 12). In this experiment, successful seeding of single transgenic cells was achieved in 20% on average using single cell mode deflection in each of the three flow sorting rounds. Like reported previously (3.1.4), the remaining wells either lacked flow sorted cells or contained multiple deposited cells. About 15% of the single deposited cells reproducibly underwent proliferation and were successfully regenerated to suspension cultures.

For the first flow sorting round, about 24, from initially 55 regenerated monoclonal suspension cultures were pre-selected to identify the best producer based on criteria like growth and DsRed fluorescence intensity, which was optically monitored by a fluorescence lamp. M12 antibody accumulation levels were then quantified by ELISA (2.3.4) in cell extracts harvested on day five of subculture (2.3.1) identifying four monoclonal suspension cultures producing less M12 levels, compared to the parental MTED#18 culture (< 14.3 ± 4 µg/g fresh weight, 3.3), two monoclonal cultures exhibiting similar accumulation levels (data not shown) and eighteen monoclonal cultures showing significantly higher M12 production rates ranging from 43.7 ± 5.4 µg/g to 182.9 ± 7.8 µg/g fresh weight (data not shown). The best first round monoclonal suspension cultures MTED#18msR1C6, MTED#18msR1C21 and MTED#18msR1C24 (Figure 12), displayed 7.5 to 13-fold increased levels of the recombinant M12 antibody ranging from 105 ± 13 µg/g fresh weight (MTED#18msR1C6) to maximum values of 182.9 ± 7.8 µg/g fresh weight, corresponding to 64 mg/l when normalized to culture volume, as presented for the MTED#18msR1C24 culture (Figure 12A). Besides this, all three monoclonal cultures consisted of 90-96% DsRed

fluorescent cells, which is to a fourfold improvement compared to the parental DsRed distribution (Figure 12B).

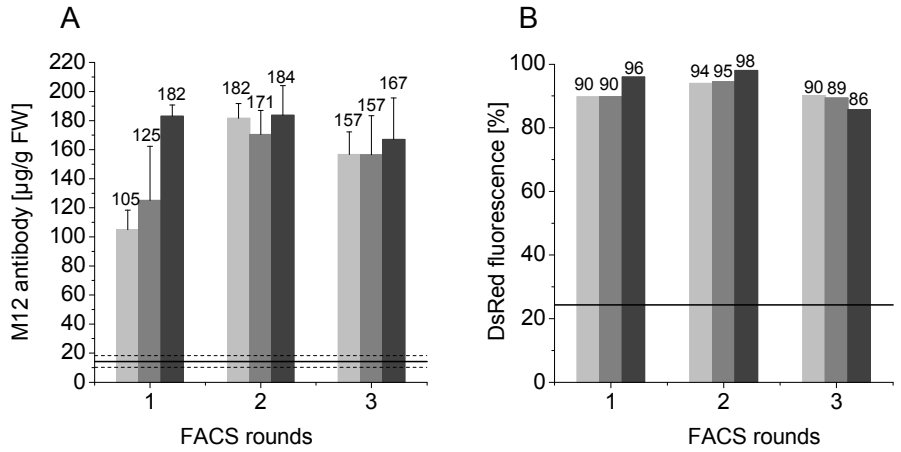



**Figure 12: Flow sorting mediated generation of monoclonal cell cultures from MTED#18.**
The generation of monoclonal cultures was performed for the BY-2 MTED#18 cell line by flow sorting in single cell mode (2.2.7.2). In each of three successive FACS rounds single cells were sorted from 2-5% cells showing the strongest DsRed intensity and deposited into multiwell plates pre-filled with feeder cells and regeneration medium. Generated monoclonal cultures of each FACS round (MTED#18msR1C6, MTED#18msR2C6.5 and MTED#18msR3C6.5.8 (light grey), MTED#18msR1C21, MTED#18msR2C21.1 and MTED#18msR3C21.1.36 (grey), MTED#18msR1C24, MTED#18msR2C24.3 and MTED#18msR3C24.3.29 (dark grey)) were analyzed for both recombinant proteins and best producing cultures of each round were used for subsequent FACS. **A**: Production of the M12 antibody in monoclonal cultures. The antibody levels were determined by ELISA (2.3.4) after each FACS round. Error bars represent standard deviations of three technical replicates. The antibody level of the parental MTED#18 cell line is displayed as solid black line with standard deviation (dash lines) of three biological replicates. **B**: Determination of the DsRed fluorescent marker protein accumulation in monoclonal cultures by flow cytometric analysis (2.2.7.1). Data represent the percentage of viable fluorescent protoplasts based on 10.000 analysed events. The DsRed fluorescent population of the parental MTED#18 line is displayed as a solid black line.

A second round of flow sorting on the three most productive first round monoclonal cultures was performed to investigate the consistency of high-level recombinant protein production. This flow sorting round led to the establishment of 30 suspension cultures (ten each from the three first-round lines) of which the most intense DsRed fluorescent cultures were again pre-selected. The best performing second round lines were MTED#18msR2C6.5, MTED#18msR2C21.1 and MTED#18 msR2C24.3. The proportion of cells showing DsRed fluorescence remained stable in these lines (94-98%). Likewise, the high-level M12 antibody accumulation was also maintained within the MTED#18msR2C24.3 culture producing 184 ± 20 µg/g fresh weight (consistent with the parental MTED#18msR1C24 line at 182.9 ± 7.8 µg/g fresh weight). The other two lines showed even higher expression levels than their first round counterpart cultures (Figure 12A), with MTED#18msR2C6.5 showing 30% improvement (182 ± 10.1 µg/g fresh weight) and MTED#18msR2C21.1 producing the recombinant antibody at 70% higher levels (171 ± 16.3 µg/g fresh weight).

A third round of flow sorting did not improve these antibody yields any further, but led to a 8-14% decline in the M12 culture productivity when compared to the best second round

monoclonal lines. In addition, flow cytometric analysis (2.2.7.1) demonstrated that the portion of highly DsRed fluorescent cells still remained consistent levels of 90% in the best third round monoclonal cultures MTED#18msR3C6.5.8, MTED#18msR3C21.1.36 and MTED#18msR3C24.3.29 (Figure 12B). These findings clearly demonstrated that two rounds of flow sorting were sufficient to reach maximum cell line productivity and any further FACS rounds were negligible.

For the three best monoclonal MTED#18 cultures of each FACS round (nine in total) the expression and integrity of the human M12 full-size antibody was successfully confirmed by immunoblot detection (2.3.3) of the 55 kDa heavy chain and 25 kDa light chain (Figure 13). Contrary to the parental MTED#18 culture (Figure 13A-C), rising signal intensities for both antibody proteins in general and for the heavy chain in particular were observed in the protein extracts of the first and second round monoclonal lines (Figure 13A-C). For the third FACS round monoclonal cultures the protein signal intensities remained constant (Figure 13B+C), except for the MTED#18msR3C6.5.8 culture showing even stronger protein signal besides the most likely heavy chain degradation at approximately 45 kDa (Figure 13A). Overall, these immunoblot results confirmed the recombinant M12 antibody levels previously quantified by ELISA (Figure 12A).

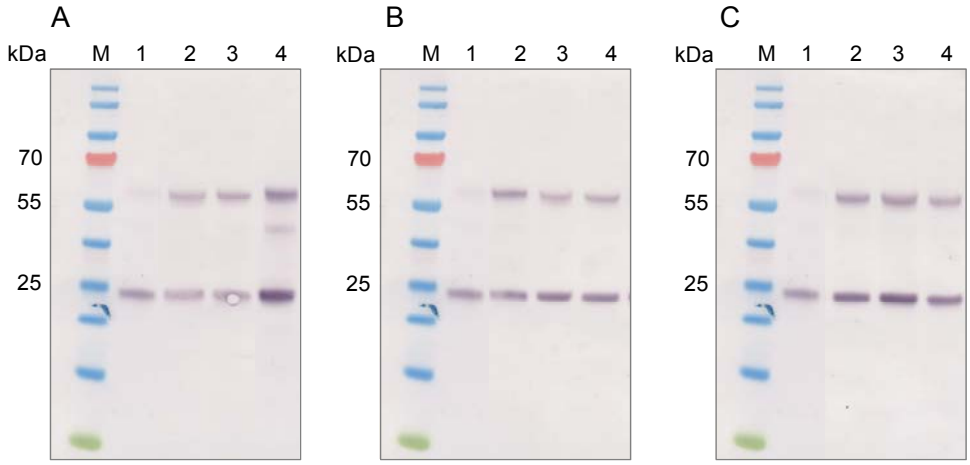



**Figure 13: Analysis of recombinant antibody integrity in monoclonal cell cultures derived from MTED#18.**
For immunoblot detection (2.3.3) of the recombinant antibody M12 20 µl of suspension cell extracts (2.3.1) were separated on a 4-12% (w/v) SDS-PAGE gel (2.3.2) and electroblotted onto nitrocellulose membrane. Antibody detection was performed by GAHλLC$^{AP}$ and GAHFc$^{AP}$ (2.1.4) incubation following NBT/BCIP substrate visualization. **A**: Detection of M12 antibody in three sorting rounds of MTED#18 monoclonal culture 6. M: Page Ruler Protein Marker Broad Range (Fermenters), 1: parental MTED#18 culture, 2: MTED#18msR1C6, 3: MTED#18msR2C6.5, 4: MTED#18msR3C6.5.8. **B**: Detection of M12 antibody in three sorting rounds of MTED#18 monoclonal culture number 21. M: Page Ruler Protein Marker Broad Range (Fermenters), 1: parental MTED#18 culture, 2: MTED#18msR1C21, 3: MTED#18msR2C21.1, 4: MTED#18msR3C21.1.36. **C**: Detection of M12 antibody in three sorting rounds of MTED#18 monoclonal culture number 24. M: Page Ruler Protein Marker Broad Range (Fermentas), 1: parental MTED#18 culture, 2: MTED#18msR1C24, 3: MTED#18msR2C24.3, 4: MTED#18msR3C24.3.29.

Moreover, the distribution and intensity of the DsRed fluorescent population was analyzed by flow cytometry (2.2.7.1) for the nine best producing monoclonal cultures (three of each flow sorting round) (Figure 14). As mentioned in 3.3.1, the parental MTED#18 culture expressed two subpopulations of different DsRed fluorescence intensities, while the high fluorescent population represents only 24% of the whole culture (Figure 14A). However, flow sorting of high fluorescent single protoplast resulted in a significant enrichment of the DsRed fluorescent population recorded as a defined population for all first round monoclonal cultures (Figure 14B), indicating that the majority of evaluated protoplasts expressed recombinant DsRed at similar and high intensity levels. The subsequent second (Figure 14C) and third round (Figure 14D) of monoclonal cultures exhibited permanent homogeneity of the considerable high fluorescent population. Apart from some events detected at less fluorescence intensities, possibly caused by variations in culture fitness, protoplast preparation or specific gating, the low fluorescent population was successfully removed. This observation was especially true for the second round monoclonal cultures (Figure 14C), but also for culture MTED#18msR3C6.5.8 and MTED#18msR3C24.3.29, respectively (Figure 14D). Overall, this kind of analysis verified the calculated percentages of DsRed fluorescent cells reported earlier (Figure 12B) and approved all tested monoclonal MTED#18 cultures (first, second and third round) to be consistently homogeneous for DsRed fluorescence, and probably also for M12 antibody production.

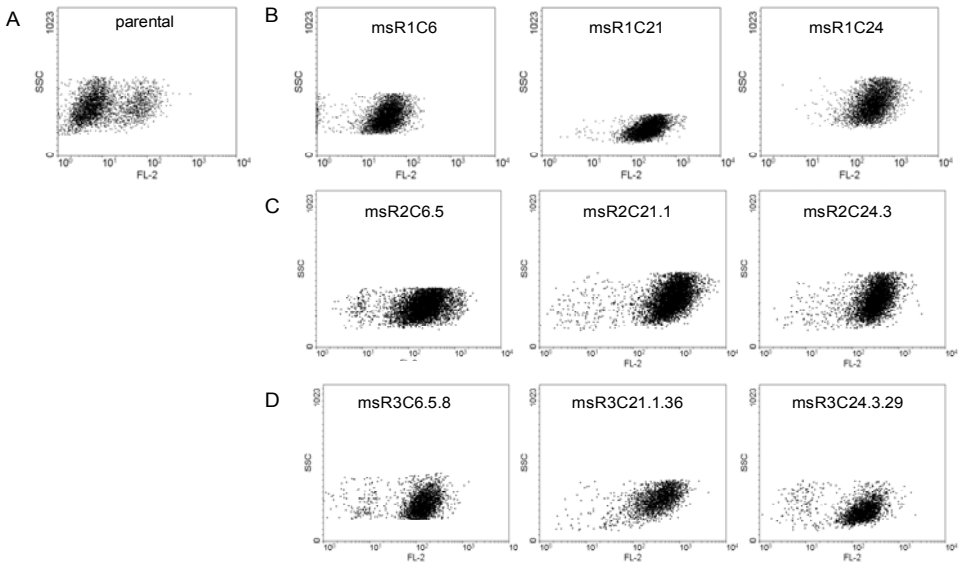



**Figure 14: Comparison of fluorescent DsRed distribution in FACS generated monoclonal cultures.**
Monoclonal cell lines generated within three rounds of successive flow cytometric single cell sorting (2.2.7.2) as well as the parental MTED#18 culture were analyzed by flow cytometry (2.2.7.1). The DsRed fluorescence signal distribution was recorded for 10.000 protoplasts each. Dot plots displayed DsRed distribution of the viable population in a single parameter dot plot as events versus DsRed channel (FL-2). Dot plots were labled with the respective cell culture name. **A**: parental MTED#18 culture, **B**: First round cultures MTED#18msR1C6, MTED#18 msR1C21 and MTED#18 msR1C24, **C**: Second round cultures MTED#18msR2C6.5, MTED#18msR2C21.1 and MTED#18msR2C24.3 **D**: Third round cultures MTED#18msR3C6.5.8, MTED#18msR3C21.1.36 and MTED#18msR3C24.3.29.

### 3.3.5 Growth performance of monoclonal cell cultures

The growth and recombinant M12 antibody production was monitored for the three best monoclonal MTED#18 cultures of each FACS round (nine in total) (3.3.4) by time course experiments using routine cultivation conditions (2.2.3.1) and assessed in comparison to the parental MTED#18 culture data. Typical growth characteristics observed within biological triplicates were assayed for a seven day cultivation period and exemplarily depicted for the fresh cell weight at three to seven days after culture inoculation in fresh medium (Figure 15D-F). The M12 antibody accumulation yields in cell extracts (2.3.1) were quantified by sandwich ELISA (2.3.4) for the same sampling days (Figure 15A-C) to evaluate the productivity of the single sorted monoclonal cultures.

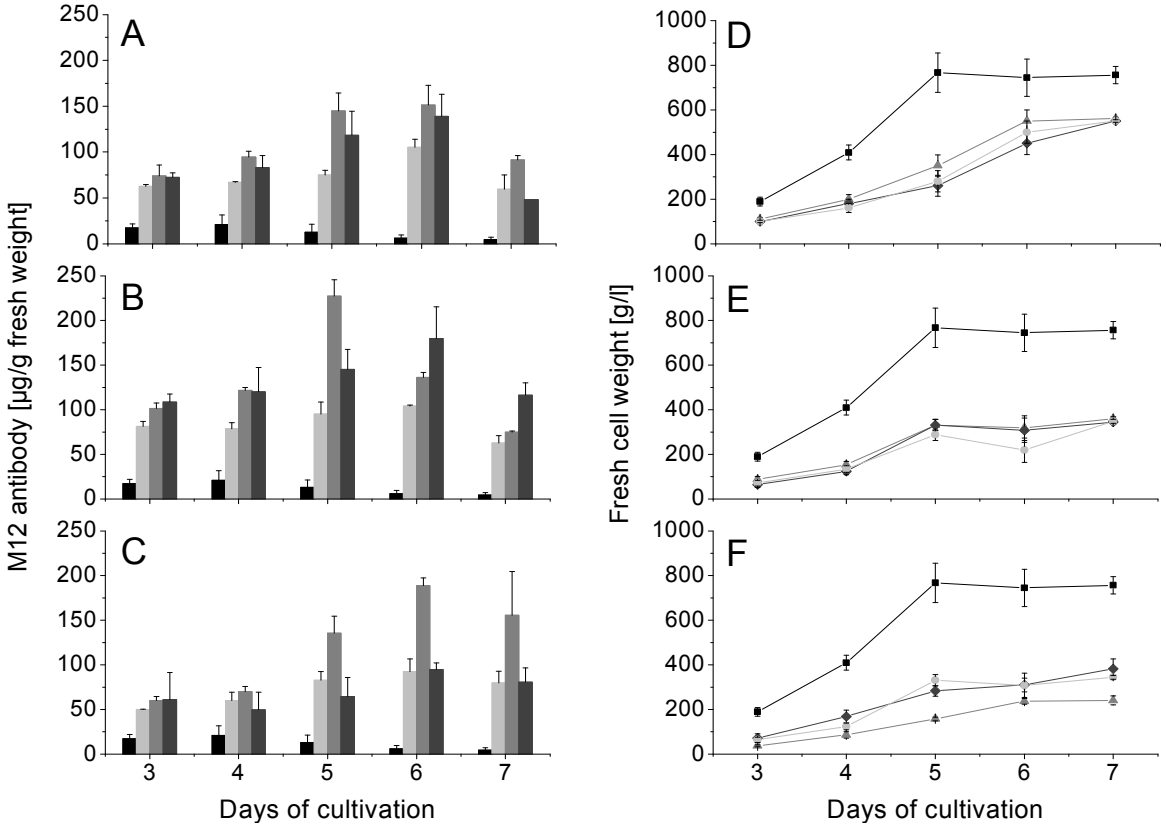



**Figure 15: Growth and production characteristics of monoclonal cell lines obtained by flow sorting.**
The monoclonal suspension lines (three monoclonal lines of three FACS rounds) and the parental MTED#18 suspension culture were cultivated in parallel as 200 ml cultures in 500 ml Erlenmeyer flasks using routine cultivation conditions (2.2.3). Sampling was conducted on day 3, 4, 5, 6 and 7 after subculture. Fresh weight and M12 antibody data were obtained from three independent cultivations of the following cultures: MTED#18 parental culture (black/ ■), MTED#18 monoclonal culture 6 (first-third round; light grey/●), MTED#18 monoclonal culture 21 (first-third round, grey/▲) and MTED#18 monoclonal culture 24 (first-third round, dark grey/♦). During standard cultivation the accumulation of M12 antibody was determined for first round monoclonal cultures (**A**), second round monoclonal cultures (**B**) and third round monoclonal cultures (**C**). All M12 antibody accumulation levels were determined by ELISA (2.3.4) and displayed as columns. The biomass production during standard cultivation was analysed for first round monoclonal cultures (**D**), second round monoclonal cultures (**E**) and third round monoclonal cultures (**F**). Percentages of the measured fresh cell weight were plotted as lines + symbol. The error bars represent standard deviations of three biological replicates (n=3).

As reported in 3.3.2, during a cultivation time of seven days the parental MTED#18 culture showed a characteristic development of biomass and nutrient consumption typically observed for transgenic BY-2 suspension cultures reaching maximum values of the intracellular M12 antibody at 4 dpi. On the contrary, the monoclonal MTED#18 cultures, displayed growth characteristics that clearly differed from the parental MTED#18 culture, even though they originated from different subsequent FACS rounds. The lag phase growth of the monoclonal lines lasted for approximately two days (data not shown) and entered an obviously extended logarithmically growth phase that remained until day six (Figure 15D-F), except for the second round lines that reached the stationary growth phase already at 5 dpi (Figure 15D,F). During cultivation, maximum biomass production for the three first round monoclonal cultures (Figure 15D) were obtained on day six with an average value of 500 ± 50 g/l fresh weight corresponding to 55% PCV. Much lower fresh weight values were obtained for the second round monoclonal cultures, although reaching 60% PCV, an average fresh weight of 315 ± 25 g/l was accumulated (Figure 15E). For the third round monoclonal cultures MTED#18msR3C21.1.36 and MTED#18msR3C24.3.29 final values of up to 381 ± 44 g/l fresh weight (corresponding to approx. 60% PCV) were determined at 7 dpi, whereas the MTED#18msR3C6.5.8 culture reached its maximum biomass already at 5 dpi (Figure 15F). However, at the end of cultivation cell pellet weights and relating PCV were found to be 26% to 57% less compared to the parental MTED#18 culture.

Considering growth, a steadily declining medium osmolality from 200 osmol/kg to 50-30 osmol/kg was commonly observed coincided with the logarithmically growth phase of all assayed monoclonal lines. Similar to the parental MTED#18 culture, the medium pH initially declined below pH 5, persisted constant values around pH 5.5 and raised up to pH 6 when cells entered the stationary growth phase (data not shown).

In terms of recombinant protein production, quantification of the recombinant M12 antibody usually yielded the highest accumulation levels at 6 dpi. For the first round monoclonal lines M12 values of 151 ± 21 µg/g fresh weight for MTED#18msR1C21 (Figure 15A) were observed, whereas the second round lines accumulated the antibody at up to 180 ± 35 µg/g fresh weight (MTED#18msR2C24.3) on day six. Maximum levels of 226 ± 18 µg/g fresh weight, quantified for MTED#18msR2C21.1 (Figure 15B), were detected after 5 dpi. Ultimately, for the three third round monoclonal cell lines the highest M12 antibody accumulation level, was 188 ± 8.5 µg/g fresh weight observed at 6 dpi for the cell line MTED#18msR3C21.1.36 (Figure 15C). As stated earlier (3.3.4) these data indicate that two rounds of flow sorting were sufficient to reach maximum cell line productivity and any further FACS rounds were not necessary.

### 3.3.6 Stability of recombinant protein Production in monoclonal suspension cultures

The standard maintenance of plant suspension cultures by routine subculturing (2.2.3) provoked the investigation whether the improved recombinant protein levels remained sustainable in the established monoclonal lines. Since a third FACS round was not yet established, the production of DsRed and M12 antibody was evaluated for each of the three first and second round cultures (3.3.1), respectively. During a cultivation period of 12 months both recombinant proteins were quantified in intervals of four month. The amount of M12 antibody, quantified from cell extracts of 5 dpi samples (2.3.1) by ELISA (2.3.4) and the portion of DsRed fluorescent cells calculated from flow cytometric analyses (2.2.7.1) are compiled in Figure 16.

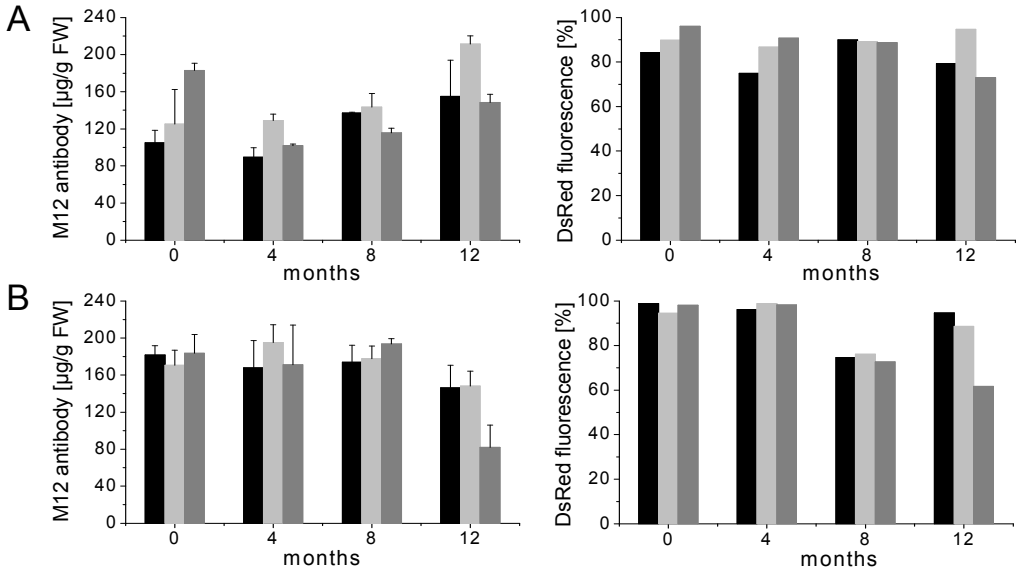



**Figure 16: Stability of recombinant protein accumulations in monoclonal suspension cultures.** The three monoclonal cultures of the first and second round, respectively, were maintained over 12 months in routine culture (26°C, 180 rpm, 2.2.3) with a passaging cycle of 7 days. Sampling and analysis of the recombinant proteins was conducted every fourth month on day 5 of routine subcultivation. The M12 antibody accumulation levels were determined by ELISA (2.3.4). Error bars represent the standard deviation of three technical replicates. Determination of the DsRed fluorescent marker protein was performed by flow cytometric analysis (2.2.7.1). Data represent the percentage of fluorescent protoplasts based on 10.000 analyzed events of the viable population. **A**: Analysis of first round monoclonal suspension cultures. MTED#18 msR1C6 (black), MTED#18 msR1C21 (light grey), MTED#18 msR1C24 (grey). **B**: Analysis of second round monoclonal suspension cultures. MTED#18 msR2C6.5 (black), MTED#18 msR2C21.1 (light grey), MTED#18 msR2C24.3 (grey).

Over a period of one year, consistent populations showing around 80 - 90% high DsRed fluorescent cells were recorded by flow cytometry for the three first round monoclonal cultures, indicating stable and homogeneous production of the recombinant visual marker protein (Figure 16A, right). However, the portion of DsRed fluorescent cells, determined after 12 months within the MTED#18msR1C24 culture, declined by 18%, possibly due to some degree of variability during periodic subculture. Contrarily, after one year, the recombinant M12 antibody was still produced at high levels of 148 ± 9 µg/g fresh weight for MTED#18msR1C24 up to 211 ± 9 µg/g fresh weight for MTED#18msR1C21, albeit the fact

that fluctuating accumulation levels were observed during periodic quantification (Figure 16A, left). In contrast to M12 antibody levels quantified for month 0, unexpected higher accumulation levels were observed, particularly for the monoclonal MTED#18msR1C21 line, after 12 months maintenance.

Stability analysis of the second round monoclonal cultures confirmed constant and high antibody yields with marginal value variation for the duration of eight months subculture continuation and the recombinant M12 antibody is produced at levels of 174 ± 18 µg/g fresh weight (MTED#18msR2C6.5) or up to 194 ± 6 µg/g fresh weight (MTED#18msR2C24.3). In comparison to the first round monoclonal cultures, all three second round monoclonal cultures appeared much more stable for the first eight months of investigation. Quite unexpectedly, decreased antibody values of 146 ± 24 µg/g fresh weight and 148 ± 16 µg/g fresh weight were determined for both cultures MTED#18msR2C6.5 and MTED#18msR2C21.1, respectively, after 12 months. The third monoclonal culture MTED#18msR2C24.3 produced even less than 50% of the previously quantified M12 antibody (82 ± 23 µg/g fresh weight, Figure 16B, left). The same trend was also observed for the DsRed fluorescent population of this monoclonal culture: the portion of red fluorescent cells decreased by approximately 30% between month 4 and month 12 of the observation period comprising a population of 62% DsRed fluorescent cells at the last timepoint (Figure 16B, right). Even though temporarily decreased percentages were detected for 8 months, both monoclonal cultures MTED#18msR2C6.5 and MTED#18msR2C21.1 maintained a stable population of about 91% high fluorescent cells throughout the cultivation period.

Overall, after more than 50 passages (one year) it was proven that the majority of selected first and second round monoclonal cultures stably maintained high levels of both recombinant proteins.

### 3.4 Determination of transgene copy numbers in FACS generated cultures

The remarkably enhanced yields of both recombinant DsRed protein and M12 antibody in polyclonal (3.3.1) and monoclonal cultures (3.3.4) prompted to investigate whether flow sorting preferentially selects for multiple transgene copy clones. In the following experiment, the abundance of transgene copy numbers in genomic DNA samples (2.4.1) of several FACS-enriched cultures was determined by a quantitative real-time PCR (RT-qPCR, 2.4.2). Since the PAL intron sequence is single copy and assumed to remain constant (proportional to the extracted and applied genomic DNA) this gene was used as an internal calibrator to normalize the amplification reactions. Thus, any variation of the respective transgene (DsRed and M12 heavy chain (HC)) to the PAL indicated differences in copy numbers. The RT-qPCR results were expressed as ratios of the gene-specific signal to the internal control signal (ΔCt) presenting a corrected relative value for the gene-specific product in each sample. The

subsequent evaluation by the comparative $2^{-\Delta\Delta Ct}$ method, documented in Table 8, indicated the change of copy numbers relative to the parental reference culture MTED#18.

**Table 8: Relative quantification of transgene copy numbers by real-time PCR.**
Genomic DNA (2.4.1) extracted from polyclonal (3.3.1) and monoclonal suspension cultures (3.3.4) was analyzed by real-time quantitative PCR (2.4.2) to detect differences in copy numbers compared to the parental MTED#18 culture. PCR product accumulation was detected by SYBR-Green fluorescence and provided as cycle numbers (Ct) crossing a defined threshold level. Ct values represent a mean of two PCRs. For sample normalization Ct values were expressed as ratios of the gene-specific signal (DsRed transgene (CtDsRed) and M12 antibody heavy chain transgene (CtM12HC)) to the internal PAL gene control signal (Ctcontrol) yielding corrected relative values (ΔCt) for the gene-specific product in each sample. The relative amount of target copies was calculated by using the comparative 2-ΔΔCt method (ΔΔCt = ΔCtsample - ΔCtreference) that divided the ΔCt for any sample by the ΔCt of the reference (parental culture).

| | Threshold Cycle numbers (Ct) | | | Relative amount transgene copies | |
|---|---|---|---|---|---|
| **Cell line** | $Ct_{control}$ [1] | $Ct_{DsRed}$ [2] | $Ct_{M12\ HC}$ [3] | $2^{-\Delta\Delta CtDsRed}$ | $2^{-\Delta\Delta CtM12HC}$ |
| **Parental MTED#18** | 24.5 | 22.5 | 22.3 | 1.0 | 1.0 |
| **Monoclonals** | | | | | |
| first round | | | | | |
| monoclone 6 | 23.3 | 21.1 | 20.6 | 0.9 | 0.7 |
| monoclone 21 | 24.4 | 22.4 | 22.9 | 1.0 | 1.5 |
| monoclone 24 | 21.5 | 20.4 | 20.2 | 1.9 | 1.9 |
| second round | | | | | |
| monoclone 6.5 | 23.4 | 21.8 | 21.5 | 1.3 | 1.2 |
| monoclone 21.1 | 22.2 | 20.0 | 20.3 | 0.9 | 1.2 |
| monoclone 24.3 | 22.2 | 21.1 | 21.4 | 1.9 | 2.3 |
| third round | | | | | |
| monoclone 6.5.8 | 25.5 | 22.8 | 23.5 | 0.6 | 1.2 |
| monoclone 21.1.36 | 23.7 | 20.8 | 22.3 | 0.5 | 1.7 |
| monoclone 24.3.29 | 23.0 | 21.2 | 23.1 | 1.2 | 4.9 |
| **Polyclonals** | | | | | |
| first round | | | | | |
| clone 11 | 20.2 | 24.5 | 19.3 | 1.1 | 1.4 |
| second round | | | | | |
| clone 11.5 | 20.4 | 19.0 | 19.8 | 1.5 | 3.0 |
| third round | | | | | |
| clone 11.5.8 | 20.2 | 19.0 | 18.8 | 1.6 | 2.1 |

[1] Endogenous single copy gene = phenylalanine ammonia lyase gene (PAL)
[2] Gene of the recombinant red fluorescent marker protein (DsRed)
[3] Gene of the recombinant M12 antibody heavy chain (M12 HC)

Equal amplification efficiencies of all three genes was ensured as the slopes observed from test PCRs with gene-specific primers on serial diluted plasmid DNA, were close to -3.3 required for an optimal PCR efficiency (data not shown). When both transgenes were amplified from genomic DNA of assayed cultures, in general, no obvious variations (± 0.3 Ct)

were detected between the duplicate reactions for the mean Ct values, indicating reproducible amplification for parallel reactions. Normalization of the uncorrected Ct values for the DsRed and M12HC genes generated mean ΔCt values that did not exceed a standard deviation higher than ± 0.5 ΔCt. Subsequent appliance of the comparative $2^{-\Delta\Delta Ct}$ method accomplished the calculation of relative changes in copy numbers and monoclonal as well as polyclonal cultures showed only marginal differences of the ΔΔCt value of the DsRed gene (Table 8). Therefore, results of the relative DsRed copy quantification was assessed as unaltered with respect to the parental MTED#18 culture. Similarly, calculation of M12 heavy chain ΔΔCt values again revealed only minor variations for the majority of assayed monoclonal cultures indicating consistent copy numbers of this transgene in each sample concluding unaffected copy numbers among the "sets" of monoclonal cultures or compared to the parental MTED#18 culture. In contrast to these results, altered copy numbers were observed for the monoclonal MTED#18msR1C24 culture as ΔΔCt values increased through three consecutive FACS rounds: for the MTED#18msR1C24 a ΔΔCt value of 1.9 was calculated, the MTED#18msR2C24.3 displayed a ΔΔCt value of 2.3 and finally a ΔΔCt value of 4.9 calculated for MTED#18msR3C24.3.29 (Table 8). Since copy numbers of the co-located DsRed gene remained constant among these cultures, the increase of M12HC gene copy numbers was unexpected. Likewise, for the tested polyclonal cultures, the M12HC ΔΔCt values varied between the three culture samples whereas the relative amount of DsRed copies showed consistent ratios (Table 8). Based on these preliminary RT-qPCR results there was neither evidence for an enrichment of multiple copy clones during FACS selection nor a clear consistence of transgene numbers among the sorted lines of subsequent FACS rounds.

## 3.5 Up-scaling and optimization of recombinant protein production of monoclonal cultures

The scale-up from shake flasks to bioreactors is an essential step towards biotechnological production of desired recombinant proteins. Thus, the growth and production performance of the monoclonal MTED#18msR2C21.1 line (3.3.4) was analyzed in a stirred tank bioreactor at a two liter scale (3.5.1) and compared to shake flask cultivation. Furthermore, it was evaluated whether the cultivation in nitrogen supplemented MSN medium (Holland *et al.* 2010) could boost the yield of recombinant proteins in the monoclonal cultures (3.3.4).

### 3.5.1 Fermentation and recombinant protein production in a monoclonal cell line

An up-scaled cultivation of the high producing monoclonal MTED#18msR2C21.1 line (3.3.4) proposed to verify the high M12 antibody yields observed for shake flask conditions. Therefore, batch fermentation in a 3 l stirred tank bioreactor with a 2 l working volume was compared to standard 1 l shake flask cultivation inoculated with a 200 ml culture volume. To provide an optimized starting inoculum of cells in the logarithmically growth phase, the mono-

clonal MTED#18msR2C21.1 line was propagated by short cycle subcultivations (three subcultivations on day 5, 2.2.3) and used to inoculate the stirred bioreactor and shake flasks with 3% (v/v) suspension culture, respectively. During cultivation the production of recombinant M12 antibody and the accumulation of biomass were monitored and documented in Figure 17.

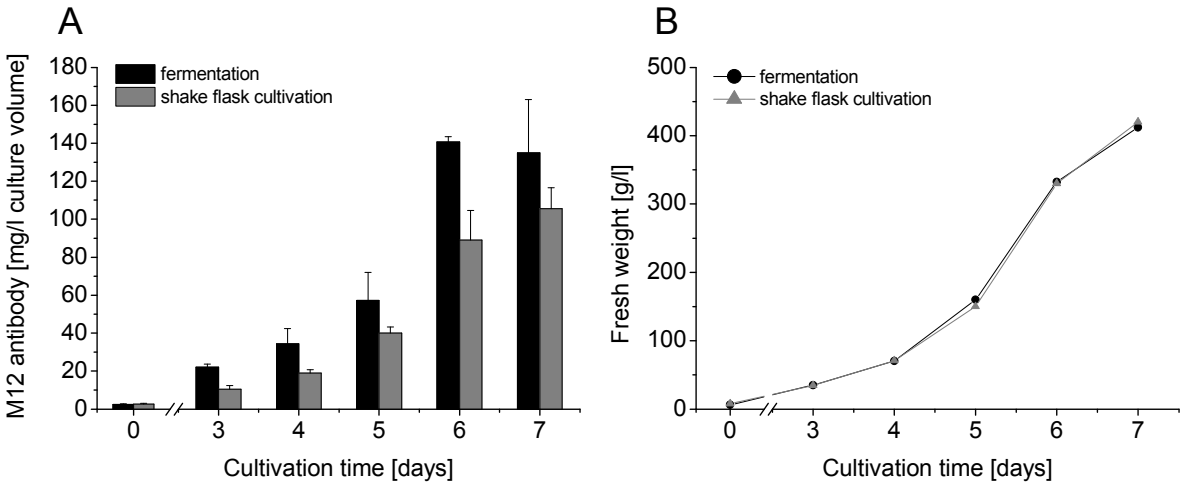



**Figure 17: Growth and M12 antibody accumulation of the MTED#18msR2C21.1 cell line during fermentation and shake flask cultivation.**
The transgenic monoclonal suspension culture MTED#18 msR2C21.1 (3.3.4) was cultivated at 2 l scale in a a 3 l stirred tank bioractor (black/●) (2.2.3.2). In parallel the monoclonal culture was cultivated in 1 l shake flasks at 200 ml scale (grey/▲) using routine cultivation conditions (2.2.3). An optimized 5 day old culture (three subcultivations on day 5) was used as inoculum of both cultivation vessels with a density of 3% (v/v). Sampling was conducted on day 0, 3, 4, 5, 6 and 7 after inoculation. The M12 antibody accumulation levels were determined by ELISA (2.3.4). The error bars represent the standard deviation of three technical replicates. The fresh cell weight was determined after centrifugation of 10 ml cell culture sample (2.2.3.1).

The up-scaled cultivation of the monoclonal MTED#18msR2C21.1 suspension line in a stirred bioreactor was successful and led to improved M12 antibody production. During the entire cultivation period of 7 days logarithmical cell growth was observed for the 200 ml shake-flask culture and for cells grown in the stirred bioreactor tank. Hence, in both cultivation devices neither an extended lag phase growth nor the entrance into the stationary growth phase were observed and the biomass accumulated to comparable values for each sampling point (Figure 17B). In fact, no obvious differences were determined for fresh and dry cell weight: at controlled fermentation conditions the culture yielded maximum levels of 411.8 g/l fresh weight and 15.9 g/l dry weight on day seven. Similar biomass levels of 420 g/l fresh and 15.5 g/l dry weight were achieved by cultivation in shake flasks.

The volumetric productivity of recombinant M12 antibody (mg per liter culture volume), was significantly higher throughout the cultivation process under bioreactor fermentation conditions compared to the shake flask control setup. Even though the M12 antibody accumulation per gram fresh weight substantially declined from day three (628 ± 46 µg/g fresh weight) to day seven (356 ± 37 µg/g fresh weight) of stirred tank cultivation, the volumetric

antibody level continuously increased during the entire cultivation period and reached maximum values of 141 ± 3 mg/l culture volume at 6 dpi, a 50% increase over the shake flask cultivation (Figure 17A). For the parallel cultivation of the monoclonal culture in shake flasks a maximum volumetric yield of 106 ± 11 mg/l culture volume was determined on day seven of cultivation (Figure 17A) whereas consistent M12 antibody levels of about 282 ± 31 µg/g were quantified for each sampling day when antibody yields were normalized to fresh cell weight (data not shown).

Finally, for the monoclonal line MTED#18msR2C21.1, it was confirmed that growth performances and recombinant antibody production observed for shake flask cultivations were comparable to those for cultivation in small bioreactors with controlled fermentation conditions.

### 3.5.2 Cultivation of monoclonal cell lines in nitrate-enriched medium

The supplementation of the standard plant cell cultivation medium (2.2.3) with an additional nitrogen source improved the intra- and extracellular accumulation of total and recombinant proteins produced by transgenic BY-2 suspension cultures (Holland *et al.* 2010). The impact of increased nitrogen levels on intracellular recombinant M12 antibody production in monoclonal MTED#18 lines (3.3.4) was investigated. For this purpose, monoclonal MTED#18 lines of the first and second round (3.3.4, i.e. six monoclonal lines in total) were adapted to MSN medium containing a total concentration of 100 mM $KNO_3$ (2.2.3) and subcultured four times using standard conditions (2.2.3). The M12 antibody levels of suspensions grown in MS and in MSN medium, respectively, were quantified by ELISA (2.3.4) in cell extracts at 5 dpi (2.3.1) and visualized in Figure 18.

The adaptation of already high recombinant protein producing monoclonal lines in nitrate supplemented MS medium (MSN) led to significantly enhanced M12 antibody concentrations, a tendency that was observed for all six assayed monoclonal suspension lines. The degree of improvement ranged between 18% determined for MTED#18msR1C21 to nearly 100% M12 antibody yields as presented for MTED#18msR1C24 or MTED#18msR2C6.5 (Figure 18). The antibody levels of the remaining monoclonal cultures resided between this range of improvement (i.e. 18% to 100%), but the degree of improved M12 yields varied broadly, i.e. the MSN-induced productivity increases of initially less productive cultures were clearly more pronounced than for highly productive ones. More precisely, monoclonal lines producing the recombinant antibody at already high levels in MS medium like MTED#18msR1C21 or MTED#18msR2C21.1 showed less improvement upon cultivation in MSN medium compared to monoclonal lines producing initially lower levels of antibody such as MTED#18msR1C6 and MTED#18msR1C24. However, this experiment demonstrated the potential of further boosting the M12 antibody production of already high producing monoclonal lines by means elevated nitrate concentrations.

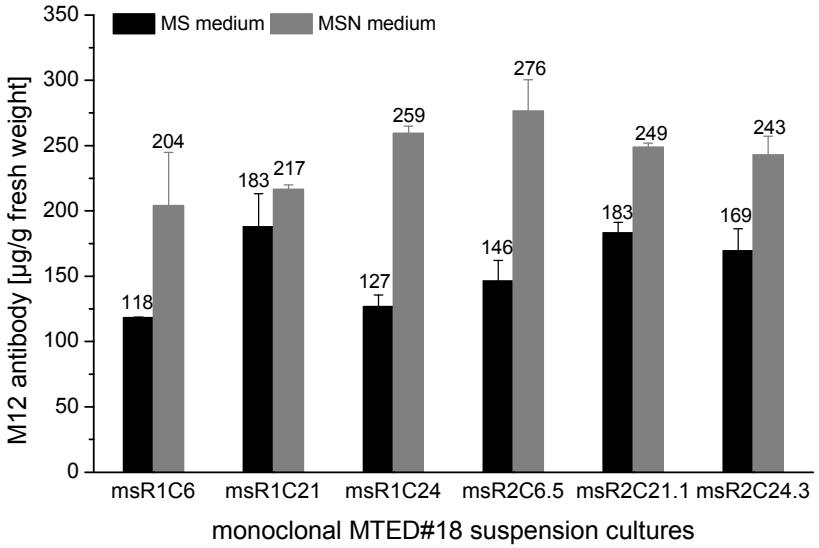



**Figure 18: Accumulation of M12 antibody in monoclonal cultures cultivated in standard and optimized medium.**

The three first (MTED#18msR1C6, MTED#18msR1C21, MTED#18msR1C24) and second (MTED#18msR2C6.5, MTED#18msR2C21.1, MTED#18msR2C24.3) round monoclonal cultures were cultivated under standard conditions (2.2.3) in MS medium (black) and MSN medium (grey), respectively. Sampling was performed after four routine subcultivations on day 5 of the cultivation cycle. Accumulation levels of the recombinant M12 antibody were determined by ELISA (2.3.4) from crude cell extracts (2.3.1). The error bars represent the standard deviation of three biological replicates.

## 3.6 Development of a transformation procedure for rapid flow cytometric analysis and sorting of elite transgenic events

The establishment of transgenic BY-2 cell cultures that produce a recombinant protein at stable and high levels usually implements time-consuming and tedious screening processes and it is often hampered through the heterogeneity of the cell populations present in the transgenic suspension cultures. As a consequence, an efficient flow cytometric pre-screening of transformed cells at an early stage after transformation procedure for rapid identification of rare high producing and probably stable cells is desirable. For the development of such an effective selection strategy, our attempt included an accelerated generation of transgenic material by immediate establishment of suspension cultures after *Agrobacterium*-mediated transformation, which served as starting material for flow cytometric selection of productive BY-2 cells shortly after transformation (3.6.1). To compare the impact of early FACS screening efforts on recombinant protein production, the FACS-derived cultures were compared with cultures established by the conventional transformation setup including the plating of cells on selective medium and the screening of callus tissue (3.6.2).

### 3.6.1 Generation of high producing suspension cultures from liquid regenerated transgenic events (accelerated transformation strategy)

Transgenic tobacco suspension cultures are typically established after analysis of recombinant protein levels from callus tissue, a process that usually takes several weeks

(Figure 22, 3.6.3). As an alternative approach, the direct regeneration of transgenic BY-2 cells from *Agrobacterium*-mediated transformation events (2.2.4) in liquid cultivation medium is proposed to shorten the timeline needed to gain a transgenic starting culture suitable for the selection of high producers by flow cytometry (2.2.7.2). For this purpose, BY-2 suspension cells were temporarily co-cultivated with engineered agrobacteria, delivering constructs containing fluorescence marker proteins, and expanded to a suspension culture by liquid MS medium cultivation in the presence of appropriate selective agents (2.1.9). Beside the MTED construct (pTRAkc:MTED, 2.1.7), that encodes genes for DsRed and the ER-targeted M12 antibody, an expression construct encoding for an ER-targeted tGFP was tested for its suitability as an alternative visual marker (pDAB9695, 2.1.7). The repression of transgenic agrobacterium growth was effectively circumvented by the addition of antibiotics and transformed suspension cultures usually exceeded a 50% PCV - which is a criterion for suitable cell culture growth - within eight to fifteen days after inoculation. This culture was further used to inoculate a starting culture (2%, v/v) for flow cytometric analysis and flow sorting (2.2.7, Figure 19).

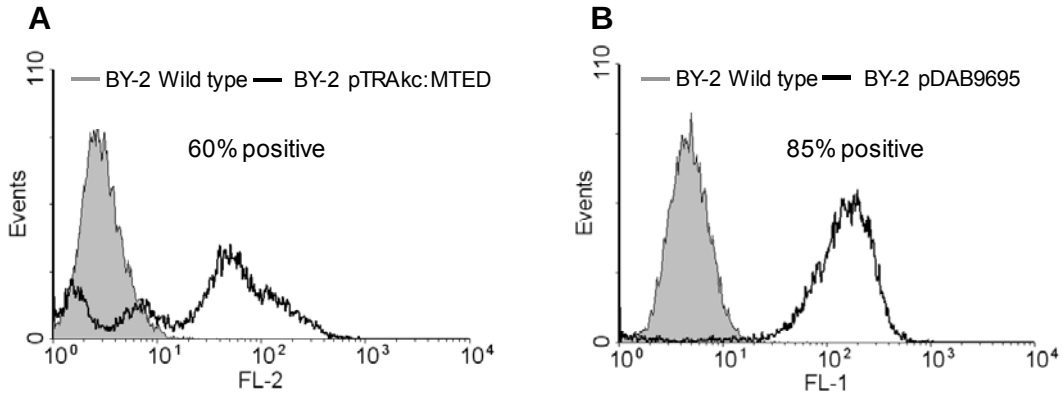



**Figure 19: Flow cytometric histograms illustrating the distribution of fluorescent marker proteins following accelerated transformation.**
Flow cytometric analysis (2.2.7.1) of transgenic BY-2 suspension cultures established by immediate inoculation of suspension cultures after transformation (accelerated transformation procedure, 3.6.1). For each transformed culture 10.000 protoplasts were recorded and plotted, in comparison to untransformed wild type protoplasts (grey curves), as histograms on logarithmic scale. **A:** Analysis of pTRAkc:MTED transformed suspension cultures. The fluorescence of the DsRed marker protein was detected by the FL-2 channel. **B:** Analysis of pDAB9695 transformed suspension cultures. The fluorescence of the visual tGFP marker protein was recorded by the FL-1 channel. The numbers in the graphs display the percentage of fluorescent cells above wild type background level.

Flow cytometric analysis for the determination of transformation efficiency proved successful expression of the respective fluorescence protein in transformed BY-2 cells showing significant shifts in the fluorescence channels. When transformation was conducted with agrobacteria harbouring pTRAkc:MTED, around 60% of the recorded protoplasts produced DsRed at fluorescence signal intensities that were above the background levels of untransformed wild type protoplasts (Figure 19A). Even higher transformation efficiencies were achieved after transformation of the pDAB9695 construct, which resulted in 85% tGFP produ-

cing protoplasts (Figure 19B). In summary, both transgenic suspension cultures expressed the recombinant protein at heterogeneous levels detected by a broad distribution of the fluorescence signals in the respective fluorescence channel instead of relative distinct peaks.

The discrimination of wild type and tGFP fluorescent protoplasts was easily manageable using flow cytometry, however, reliable microscopic investigations were strongly hampered by the weak signal intensity of the tGFP marker protein on the one hand and considerable autofluorescence of the feeder protoplasts on the other. This observation, together with the fact that polyclonal sorting was quite satisfactory for this proof of concept study, prompted to sort approximately 2-5% of highly fluorescent protoplasts with 20 protoplasts per well (polyclonal) in 96 well plates for both transformation approaches. Subsequent microscopic analysis of sort efficiencies revealed intact tGFP fluorescent protoplasts in 80% of the plate wells and 82% of the wells contained intact and DsRed fluorescent protoplasts. Ultimately, transgenic callus tissue and suspension cultures producing either DsRed or tGFP, respectively, were established from 10-12% of the obtained microcolonies and directly subjected to recombinant protein analyses (3.6.2).

In the following parts of this thesis the suspension cultures created by this approach are termed “accelerated cultures” or “FACS-derived cultures” and the reported procedure for the rapid generation of transgenic cultures is named “accelerated transformation” or “FACS strategy”.

### 3.6.2 Comparative analysis of conventional and accelerated transformation procedures for transgenic cell line generation

To examine the impact of immediate flow sorting shortly after transformation (3.6.1) on recombinant protein levels, the productivity of suspension cultures derived by the FACS strategy, were quantified and compared to transgenic cultures coevally established by the conventional transformation approach (2.2.4). Suspension cultures derived from the latter procedure were established from transgenic callus tissue showing the highest fluorescence intensity measured macroscopically. Upon stable BY-2 transformation with the pTRAkc:MTED and pDAB9695 vectors (3.6.1), six suspension cultures per construct and respective screening strategy (conventional *versus* FACS) were exemplary investigated. At first, the cultures were screened for recombinant DsRed or tGFP production by flow cytometric analysis (2.2.7.1) in the attempt to compare the distributions of the respective fluorescent protein. The results of these investigations are compiled in Figure 20.

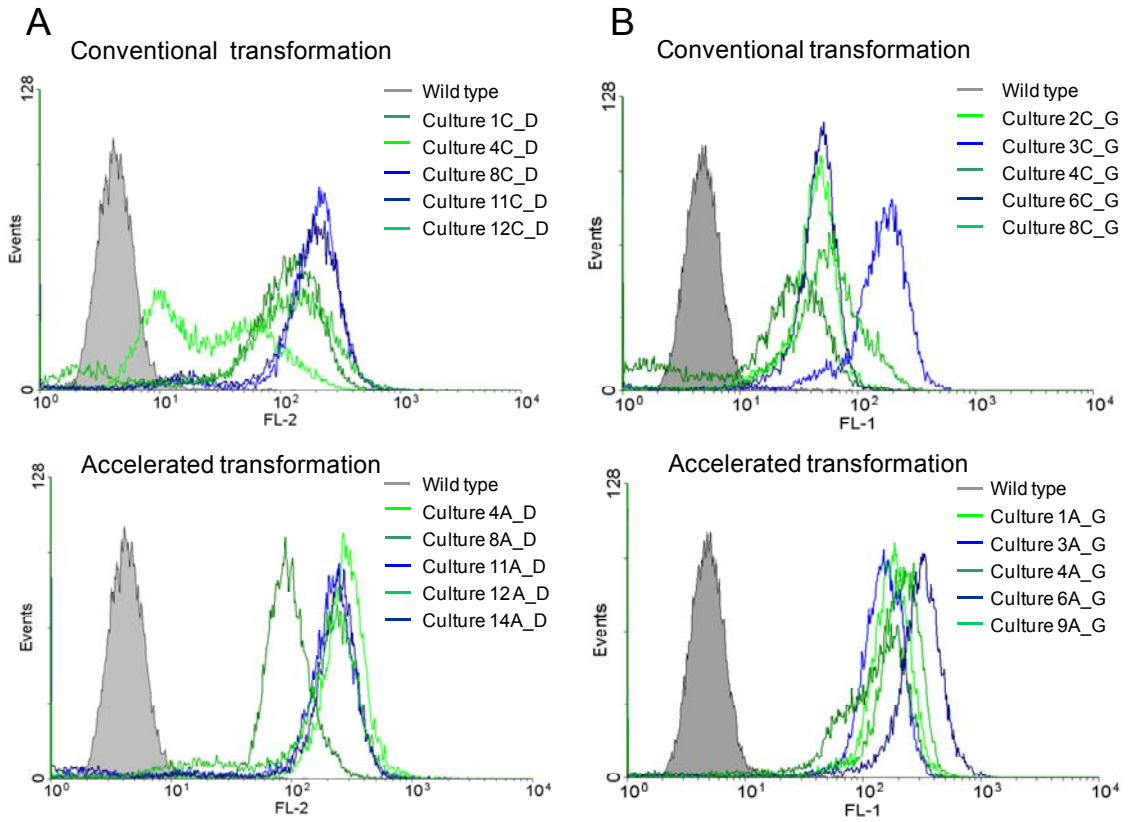



**Figure 20: Flow cytometric histograms displaying the distribution of the fluorescent marker protein expression in the generated transgenic BY-2 suspension cultures.**
Representative flow cytometric analyses (2.2.7.1) of transgenic BY-2 suspension cultures established by conventional and accelerated transformation procedures are presented. For each culture, 10.000 protoplasts were analyzed and plotted as histograms. **A:** Flow cytometric analysis of suspension cultures transformed with pTRAkc:MTED. The fluorescence intensities of the DsRed marker protein (D) are detected by the FL-2 channel on a logarithmic scale. **B:** Flow cytometric analysis of suspension cultures transformed with pDAB9695. The fluorescence of the tGFP marker protein (G) in the FL-1 channel are given on a logarithmic scale. The upper histograms show the fluorescence distribution of six representative transgenic cultures that were obtained by the conventional procedure (C) compared to untransformed wild type protoplasts (grey curves). The lower diagram displays the fluorescence signal distribution of six representative suspension cultures that were obtained using the accelerated procedure (A) compared to wild type protoplasts (grey curves).

Comparative fluorescence analyses of the six DsRed producing suspension cultures originating from the accelerated FACS-based approach displayed homogeneously distributed cells of similar and strong red fluorescence intensities. Apart from culture 8A_D, which showed lower DsRed intensity, narrow fluorescence signal peaks located on the far right on the FL-2 fluorescence channel axis were determined (Figure 20A, lower histogram). In contrast, the six suspension cultures established by the conventional transformation strategy exhibited partially heterogeneous signal distributions of DsRed fluorescence intensities, which are recorded by broad histogram curves e.g. culture 4C_D (Figure 20A, upper histogram). However, when implementing all fluorescence signals above wild type background levels the calculation of the fluorescent cell portions resulted in similar percentages of 76-78% for suspension cultures of both transformation approaches (data not shown).

As expected, similar results were achieved for the examined tGFP suspension cultures. Whereas the green fluorescent protein was homogeneously produced at high fluorescence intensity among cultures derived from the FACS approach (Figure 20B, lower histogram), partially broad and diversified histogram curves indicated heterogeneously distributed tGFP expression of various intensities within most suspension cultures established by the conventional strategy (Figure 20B, upper histogram). Likewise, no obvious differences in tGFP fluorescent cell portions between accelerated and conventional generated cultures were observed, in fact 80-88% of the assayed cultures displayed the visual green marker protein (data not shown).

Furthermore, recombinant protein accumulation levels from 5 dpi cell extracts (2.3.1) were analyzed for each transformation approach: recombinant tGFP levels were determined densiometrically based on an immunoblot (2.3.3). Due to the lack of a suitable quantification method for the reporter protein DsRed (i.e. immunological or spectrometrical), we quantified the co-produced M12 antibody by ELISA (2.3.4) assuming that the levels of M12 correlate with the DsRed accumulation. The determined accumulation levels of the respective recombinant proteins from both generation approaches are presented in Figure 21.

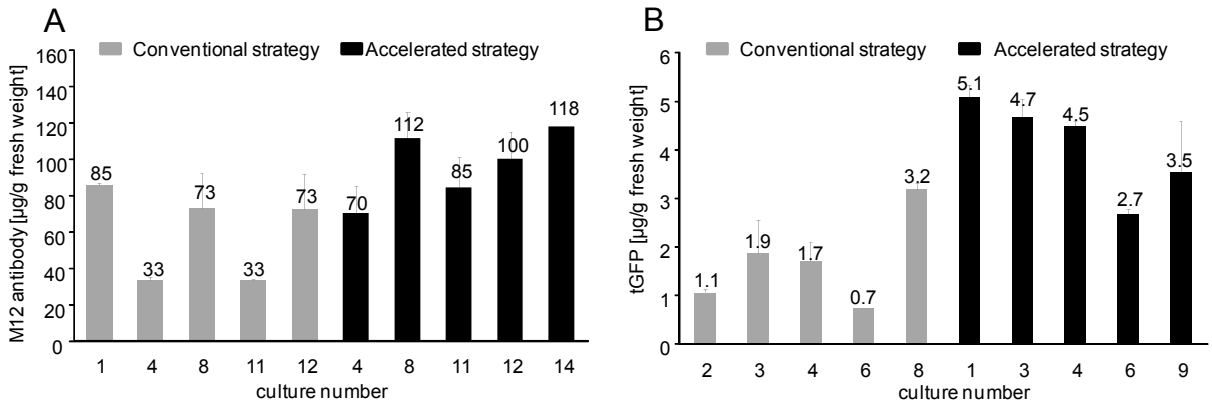



**Figure 21: Quantification of recombinant protein production in transgenic BY-2 suspension cultures.**
The productions of the recombinant proteins in BY-2 suspension cultures established by conventional transformation (grey) were compared to those of suspension cultures that were generated by the accelerated transformation strategy (black). **A:** Quantitative analysis of M12 antibody produced by BY-2 suspension cultures transformed with pTRAkc:MTED. The M12 antibody accumulation levels were determined by ELISA (2.3.4). **B:** Quantitative analysis of tGFP produced by BY-2 suspension cultures transformed within pDAB9695. The amount of expressed tGFP was quantified based on a commercial tGFP standard by image analysis (AIDA) of an immunoblot (2.3.3). Values represent the mean of two biological replicates (n=2).

Immunological analysis of the M12 antibody accumulation in 5 dpi cell extracts of suspension cultures transformed with pTRAkc:MTED revealed higher accumulation levels of the recombinant antibody for three out of the six assayed cultures derived from the FACS-based approach compared to conventionally generated transgenic cultures. In fact, considering the small number of tested cultures, the M12 antibody was produced in a range of 33 ± 1.7 to

85 ± 1.5 µg/g fresh weight in conventional suspension cultures, but accumulation levels determined for some of the FACS-derived cultures somewhat exceeded these levels by reaching 118 ± 20 µg/g fresh weight (Figure 21A). Interestingly, even after conventional transformation, the observed antibody levels were much higher compared to the M12 amount quantified for the parental MTED#18 culture (14.34 ± 4 µg/g fresh weight).

Comparative quantification of suspension cultures transformed with tGFP demonstrated even more strikingly that cultures obtained from the accelerated transformation and FACS-based strategy produced on the average higher recombinant protein amounts than the conventional cultures. In particular, a maximum level of 3.2 ± 0.1 µg/g fresh weight tGFP was quantified for a single conventional culture whereas already three of the FACS-derived tGFP cultures produced the green fluorescent protein at higher levels up to 5.1 ± 0.2 µg/g fresh weight (Figure 21B).

As a conclusion, the results obtained from this experiment confirmed that the FACS-based approach was superior to the conventional culture generation in several ways: (1) the direct cultivation of transformed BY-2 cells in suspension allowed the faster establishment of transgenic starting material, (2) flow cytometric clone selection enabled the screening of a significantly higher number of transformed cells and (3) increased the number of highly productive and homogeneously producing transgenic suspension cultures.

### 3.6.3 Timelines for FACS-based generation of high recombinant protein producing cell lines

For the generation of transgenic BY-2 suspension cultures with high recombinant protein levels, two FACS-based approaches were employed within this thesis: On the one hand the generation of high producing polyclonal or monoclonal cultures from an already existing heterogeneously expressing starting culture (conventional strategy, 3.3) and on the other hand the FACS-based separation of elite transgenic cells shortly after transformation (accelerated strategy, 3.6). To rate the temporal advantages of the novel accelerated strategy for the generation of transgenic BY-2 culture the timelines of both FACS procedures were compiled in Figure 22.

The applied conventional strategy to generate highly productive BY-2 cultures, such as MTED#18 psR1C11 (3.3.1) or MTED#18 msR1C24 (3.3.4), was calculated to take at least 27 weeks. This time-span primarily included the previous establishment and screening of transgenic callus tissue, whose generation required one-time 12 weeks. Thereafter, the FACS-based selection and regeneration of cells as well as the biochemical analyses took approximately 15 weeks. This workflow was performed in triple selection rounds during this thesis (3.3). The applied accelerated transformation approach (3.6.1) provided a transformed suspension culture within 2-3 weeks instead of 6-8 weeks omitting the establishment and screening of callus tissue. In contrast to the conventional method, the subsequent flow sor-

ting allowed a screening of thousands of transgenic cells shortly after transformation, facilitated the early elimination of poor recombinant protein producing cells and amplified the generation of highly productive cultures at the same time. The time period needed for the regeneration of flow sorted cells and the analysis of suspension cultures was similar to the conventional strategy and required 15 weeks, respectively. In conclusion, the combination of an accelerated transformation procedure and subsequent FACS selection shortened the time to establish high producing transgenic BY-2 lines from 6.7 months required for the conventional approach to 4.5 months.

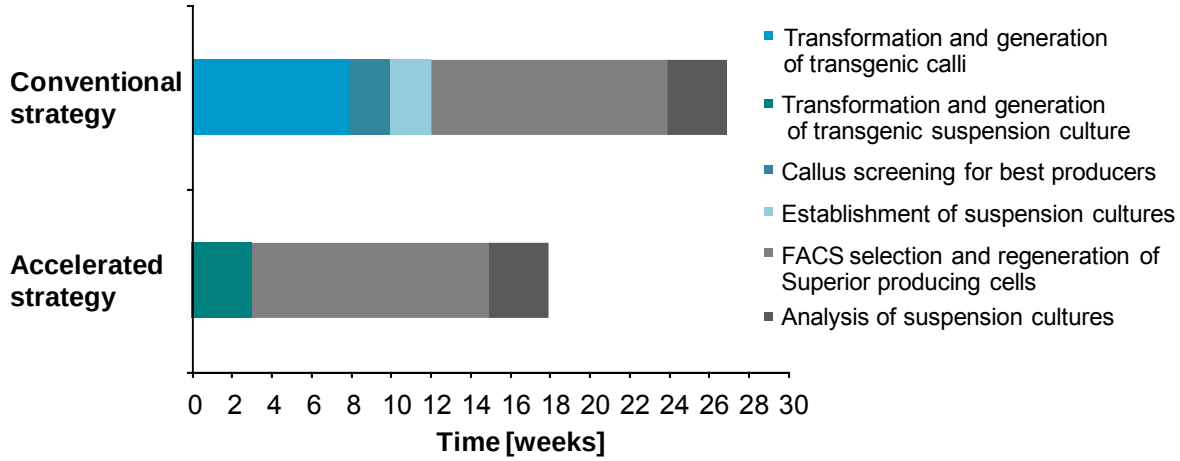



**Figure 22: Timelines for FACS-based generation of high producing suspension cultures**.
Two strategies were applied to generate high recombinant protein producing suspension cultures. The generation and screening of transgenic callus tissue after *Agrobacterium*-mediated transformation of tobacco BY-2 cells and the establishment of transgenic suspension cultures from calli constituted the first steps of the conventional strategy (upper bar) followed by flow cytometric sorting and regeneration of desired single protoplasts. Within the accelerated strategy (lower bar) tobacco suspension cells were transformed and subsequently used for the direct establishment of a transgenic suspension culture that was further used for flow cytometric separation of elite single transformation events. Once polyclonal or monoclonal suspension cultures were regenerated they were subjected to recombinant protein analyses with both strategies.

# 4 Discussion

Within the scope of this PhD thesis two major objectives were pursued: first, the generation of highly productive polyclonal and monoclonal cell lines from a heterogeneous tobacco BY-2 culture (3.3) and second, the rapid establishment of high-producing transgenic BY-2 cultures shortly after a transformation event (3.6).

The initial part of this thesis focused on the optimization of protoplast preparation and pre-culture conditions that resulted in the generation of viable and robust protoplasts suitable for flow sorting (3.1). This was an important step for the establishment of robust and reproducible flow cytometry and subsequent cell regeneration procedures. For the generation of polyclonal cultures the modification of cultivation conditions reduced the required seeding density to only 2 x $10^2$ flow sorted cells per ml. Further, the establishment of monoclonal lines from single sorted cells was realized by a feeder cell-supported procedure (3.1.5). These adjustments resulted in the generation of elite suspension cultures with up to 13-fold improved M12 antibody productivity. In addition, medium optimization and bioreactor cultivation of the new monoclonal cultures were applied (3.5) that boosted the recombinant M12 antibody levels by an additional 50% -100%.

The second part of this thesis focused on the FACS-supported generation of transgenic polyclonal cell lines at an early stage after a transformation (3.6). This strategy resulted in a faster creation of transgenic cells, less manual work and drastically increased screening throughput using flow cytometry. Comparative immunological and flow cytometric analyses demonstrated the capability of this strategy to isolate highly productive transgenic tobacco cultures with a time saving of 2.5 months.

## 4.1 Preparation of tobacco BY-2 protoplasts for flow cytometric sorting and its regeneration

Flow cytometric applications request the availability of single cell suspensions, but BY-2 suspension cells grow in chains and tend to form clusters. Therefore, the sorting of BY-2 protoplasts instead of intact plant cells is mandatory (Galbraith *et al.* 2011) and their efficient preparation in large amounts is an essential prerequisite. Although protocols for the isolation of vital protoplast populations from various plant species and tissues are available, the specific preparation procedure still needs to be adjusted empirically for the respective protoplast source (Power *et al.* 2004).

Within this thesis the optimal amounts of the cell wall degrading enzymes, cellulase and macerozyme, the composition and pH of the regeneration medium and the ideal regeneration temperature were investigated. In the attempt to isolate reproducible high amounts of viable protoplasts from tobacco BY-2 suspension cultures, the protoplasts were released and isolated from cell aggregates according to an improved protocol reported by Schinkel *et al*.

(2008). When testing different concentrations of cell wall degrading enzymes it was demonstrated that the combination of 1% (w/v) cellulase and 0.3% (w/v) macerozyme in a slightly hypertonic osmoticum turned out to be most suitable setup for the preparation of protoplasts from BY-2 suspension cells (3.1.1). This treatment provided efficient removal of the cell walls without imposing excessive stress on the plant cells confirming a result that had been previously reported by Uchimiya and Murashige (1974) for the protoplast preparation from tobacco BY-2 suspension cultures using the same enzyme concentrations. In this work, the isolation procedure was based on the sucrose flotation method and provided reproducible amounts of about 1 x $10^6$ protoplasts per gram fresh weight with 80% viability. These data are comparable to results that had been reported in earlier studies for the preparation of tobacco mesophyll protoplasts in which up to 8 x $10^5$ protoplasts per gram fresh weight and vitalities of 80-98% were achieved (Harkins and Galbraith 1984, Rakosy-Tican and Menczel 1998).

The next challenge was the cultivation and regeneration of the isolated protoplasts at the lowest possible seeding densities aiming at the regeneration of single protoplasts. Thus far, minimal protoplast seeding densities are reported from 5 x $10^2$ to 1 x $10^6$ protoplasts per ml whereas the regeneration at higher densities is limited due to the depletion of nutrients and growth factors (Power *et al.* 2004). When using the nutrient-rich 8p medium formulation, which had been published by Kao and Michayluk (1975) as ideal for the regeneration of *Vicia* protoplasts at low population densities, the regeneration of BY-2 protoplasts at seeding densities exceeding 5 x $10^3$ protoplasts per ml was achieved. However, for the regeneration of BY-2 protoplasts at seeding densities lower than 5 x $10^3$ protoplasts per ml, the medium had to be modified. Therefore, the impact of various published supplements, i.e. the addition of cellobiose (Schilde-Rentschler 1977), the phytohormone phytosulfokine alpha (Matsubayashi and Sakagami 1996) or altered sugar (Godo *et al.* 1996) and casamino acid (Raveh and Galun 1975, Schilde-Rentschler 1977) was tested at different concentrations (3.1.2). While most supplements did not substantially improved the BY-2 protoplast regeneration, the addition of the doubled amount of casamino acids resulted in faster proliferation with initial protoplast divisions taking place approximately after 24 h – 48 h, which is 24 h earlier than with the basic 8p medium. It is assumed that the addition of casamino acids compensates the loss of organic constituents important for cell proliferation, which occurs as a consequence of cell wall removal (Meyer and Abel 1975). The modified BY-2 protoplast regeneration medium suitable for the regeneration of BY-2 protoplasts at low cell densities was termed 8p2c medium. This medium was used to determine the best pH value for protoplast regeneration. When testing different pH values of the 8p2c medium ranging from pH 4.5 to pH 7.5 observations from Meyer and Abel (1975a) were confirmed, namely that a medium pH value of 6.0 is the most suitable one for protoplast regeneration. The influence of the

cultivation temperature on protoplast proliferation was also taken into consideration and maintenance of the isolated protoplasts in regeneration medium at 27°C was preferable to cultivation at room temperature (22°C) because protoplast proliferation started faster at the elevated temperature. This beneficial effect of increased temperature on protoplast proliferation has also been described in earlier studies on tobacco protoplasts by Meyer and Abel (1975a) and Shepard and Uyemoto (1976). As a possible explanation Shepard and Uyemoto (1976) suggested that protoplasts cultured at lower temperature enlarge prior to the initiation of division, whereas relatively little cell enlargement preceded first division at higher cultivation temperatures. After the determination of the most suitable protoplast regeneration medium and the optimal incubation temperature sustained mitotic division was achieved already at a seeding density of $2 \times 10^2$ BY-2 protoplasts per ml, which is considerably lower compared to other published data (i.e. $5 \times 10^2$ to $1 \times 10^6$) on minimal seeding densities for plant protoplasts (Power *et al.* 2004).

For the practical application of flow sorting of plant protoplasts two challenging requirements had to be fulfilled, i.e. the cell survival during the sorting procedure and the regeneration of single deposited plant protoplasts (Lührs and Lorz 1988). The first requirement was achieved by introducing a 48-72 h long pre-regeneration step between protoplast preparation and the flow sorting that allowed the formation of a primary cell wall without changing the spherical shape of the cells (3.1.3). The formation of primary cell walls is obviously a prerequisite for cell division in tobacco protoplasts (Meyer and Abel 1975) and it is assumed that it also protects the cells from flow cytometric shear forces. Furthermore, the three-day pre-regeneration step of the protoplast minimised the occurence of spontaneous protoplast fusions, which represents a potential contamination risk of flow sorted cultures (Chen *et al.* 2004).

Using the appropriate medium and cultivation conditions, isolated BY-2 protoplasts subsequently commenced cell wall regeneration comparable to what has been reported for tobacco mesophyll or carrot protoplasts (Nagata and Takebe 1970, Guzzo *et al.* 2002). The development of cell wall formation was monitored by specific staining of cellulose with Calcofluor White and it was observed that after 48 h about 95% of the cells had built a primary cell wall (3.1.3). The pre-regeneration step and the application of flow sort settings for large particle as recommended by Jett and Alexander (1985), which include the use of phosphate buffered saline as sheath fluid and a 200 µm flow tip orifice operated at low sheath pressures, finally resulted in the efficient and gentle deposition of viable BY-2 cells after flow sorting. In contrast, the immediate sorting of freshly prepared protoplasts apparently affected their vitality making the recovery of adequate amounts of viable sorted BY-2 protoplasts impossible (3.1.4). In earlier studies of Harkins and Galbraith (1987) the flow sorting of $1\text{-}5 \times 10^4$ tobacco mesophyll protoplast pools was reported to achieve about 50%

sorting efficiencies and 25% of the initial protoplasts recovered in a viable state. In comparison, within this work sorting efficiencies exceeded these reported data (3.1.4); we reproducibly obtained up to 93% efficiencies when protoplasts were sorted in 20-cell pools. Similar to Harkins and Galbraith (1987) half of the sorted protoplasts recovered in a viable state. For the single cell sort approach reduced sorting efficiencies of about 35% were reached and again 50% of those cells recovered. As to our best knowledge the sorting and deposition of single protoplast has not been published yet, no reference exists for a comparison or an assessment of our results.

Depending on the respective sort approach, two different procedures had to be applied for the regeneration of pool and single flow sorted cells. As mentioned above, BY-2 protoplasts failed to regenerate when cultivated below a seeding density of $2 \times 10^2$ protoplasts per ml. The lowest seeding density for the polyclonal sorting approach was $4 \times 10^2$ protoplasts per ml and they easily regenerated to suspension cultures within 15 weeks using the 8p2c cultivation medium (3.1.4). However, for the regeneration of monoclonal cells, a more efficient regeneration strategy had to be established. Thus far, research has either focused on the recovery of single cells from unsorted protoplasts (Kyozuka *et al.* 1987, Eigel and Koop 1989) or on the regeneration of flow-sorted protoplast pools (Afonso *et al.* 1985, Birnbaum *et al.* 2005), but never on the regeneration of single cells after flow sorting.

A potential strategy for single cell recovery from protoplasts is the use of semi-solidified medium for the embedding of protoplasts and in this context the applicability of the two gelling agents alginate and carageenan was compared within the scope of this thesis. Sustained protoplast divisions were obtained with both gelling agents (3.1.2), but the alginate containing medium gradually liquefied caused by the presence of citric acid in the 8p2c regeneration medium. In contrast, the carageenan containing solid medium remained polymerized (Ichi *et al.* 1986, Dovzhenko and Koop 2003) and is consequently the preferable gelling agent for the embedding of BY-2 protoplasts to achieve regeneration. Various suitable semi-solid cultivation systems have been described for culturing plant protoplasts at low seeding densities (Davey and Kell 1996), however, the regeneration of truly single protoplast using this technique has not been reported until now and it also failed when tested within this PhD work.

An alternative strategy for the regeneration of single protoplasts employs feeder cultures that stimulate the proliferation of plant protoplasts by the release of growth factors during division (Davey *et al.* 2005). Manifold setups using feeder cultures for the regeneration of protoplasts have been reviewed by Davey *et al*. (2005) and employ either the embedment of the feeder cells, or the use of complex layer structures to efficiently separate the feeder cells from the regenerating protoplasts. In a first approach, it was tested if the feeder and the sorted cells can be physically separated using nylon gauze inserts, but this setup was discar-

ded because the nylon gauze inserts did not allow any microscopic monitoring of the single protoplasts. As a consequence, within this thesis the direct co-cultivation of single transgenic protoplasts with wild type feeder cells was investigated (3.1.5). The subsequent selection on antibiotics (Eigel and Koop 1989) practiced feasible to facilitate single protoplast regeneration without physical separation of feeder and sorted cells. In addition, this simplified procedure consists of only a few working steps, which makes it not only convenient but also highly efficient because of minimal exposure of the protoplasts to shear forces and other mechanical stress. Using the direct co-cultivation strategy, approximately 50% of the viable, single sorted cells regenerated and reached microcallus stage within two weeks. Taking into consideration that for the single cell sort approach sorting efficiencies of about 35% were obtained, this means that almost 17% of all single sorted and deposited cells regenerated.

To demonstrate the complete exclusion of feeder cells an experiment was performed in which kanamycin-sensitive tGFP producing BY-2 protoplasts were used as feeder cells and kanamycin-resistant DsRed producing BY-2 protoplasts represented the flow sorted single protoplasts. The inverted experimental setup was also tested meaning that this time the imazethapyr-sensitive, kanamycin-resistant DsRed producing BY-2 protoplasts served as feeder cells and the imazethapyr-resistant, kanamycin-sensitive tGFP producing BY-2 protoplasts were flow sorted. After regeneration and establishment of new suspension cultures resistance tests in which twenty cultures were tested for resistance towards kanamycin and imazethapyr proved the absence of any feeder cell in the newly generated monoclonal BY-2 lines (3.2). Corresponding flow cytometric analyses of four cultures for each setup also confirmed that no feeder cells had survived the antibiotic or herbicide selection procedure because no fluorescence originating from the respective feeder cells was detectable.

In conclusion, the optimization of protoplast preparation and regeneration resulted in sustained BY-2 protoplasts proliferation and the reproducible establishment of polyclonal cultures using 2 x $10^2$ protoplasts per ml. Moreover, the development of a feeder cell-based regeneration strategy enabled the generation of monoclonal cell lines from a single sorted cell and the complete exclusion of feeder cells was clearly demonstrated. Finally, sort efficiencies of 35% for single and 95% for pool sorted cells were achieved and in both cases 50% of the sorted cells regenerated to callus stage.

## 4.2 Application of flow cytometric sorting for the establishment of highly productive BY-2 suspension cultures

Flow cytometric analysis and sorting has been repeatedly deployed for the selection of desired fluorescent plant protoplasts from mixed populations (Waara *et al.* 1998, Bargmann and Birnbaum 2009, Galbraith *et al.* 2011). In this work using a co-selection strategy, DsRed fluorescence based flow sorting of the heterogeneous transgenic MTED#18 culture that produces the fluorescent protein DsRed and the full-size IgG antibody M12 was applied to

establish polyclonal and monoclonal suspension cultures with improved recombinant protein productivity and culture stability (3.3). As the M12 and DsRed expression cassettes are physically linked on one T-DNA, flow sorting of strong fluorescent protoplasts was performed assuming this would also identify clones producing high levels of the antibody. The repetitive co-selection of the two recombinant proteins in three subsequent rounds of flow cytometric sorting proved to be an excellent strategy that resulted in the generation of polyclonal cultures producing up to 9.7-fold higher yields of the recombinant M12 antibody (3.3.1) and the selection of monoclonal cultures showing up to 13-fold increased productivity (3.3.4). Differing from the heterogeneous parental MTED#18 culture, which showed two distinct populations varying in fluorescence intensity, the novel cultures consisted of homogeneous and strong fluorescent populations with about 96% highly DsRed fluorescent cells. The factors of improvement achieved during three repetitive flow sorting rounds of all generated polyclonal and monoclonal lines are listed at the end of this chapter in Table 9.

For the polyclonal as well as for the monoclonal cultures the major improvement in M12 antibody productivity and DsRed accumulation was obtained after the first round of flow sorting. With respect to the antibody amounts the observed 4.7-fold enhanced productivity of the polyclonal culture was lower compared to the M12 levels accumulated in the selected monoclonal lines, which showed up to 13-fold improved productivity. On the contrary, the population of strong DsRed fluorescent cells was equally increased in the most productive polyclonal and monoclonal cultures to a portion of 96% homogeneous fluorescent cells. While for the majority of established cultures an apparent correlation between DsRed fluorescence signal intensity and M12 productivity was obvious, in a few cases the accumulation of the two recombinant proteins diverged. The observation that the fluorescence intensity does not necessarily correlate with target protein accumulation has already been described for the selection of polyclonal cultures (James and Lee 2006) and may reflect a reduced but still existing culture heterogeneity. Based on the findings of the first FACS round, two more sorting rounds were performed aiming at further increase of productivity and stability of the polyclonal culture MTED#18psR1C11 and the three established monoclonal cultures MTED#18msR1C6, MTED#18msR1C21 and MTED#18msR1C24. Thus far, repetitive flow sorting has only been described for mammalian production systems and is recommended to further enrich or maintain the portion of stable, high producing cells in polyclonal cultures (Brezinsky *et al.* 2003, Carroll and Al-Rubeai 2004, Moretti *et al.* 2010). In 1999, Yanpaisan *et al*. put forward the necessity to periodically select highly productive plant cells in order to avoid a reduction in productivity over time, but until now such strategies are rarely followed and the eventual loss of culture productivity during successive rounds is still a drawback of genetically modified plant suspension cells (James and Lee 2006).

After a second sorting round of the polyclonal culture MTED#18psR1C11 M12 antibody productivity was further 2.6-fold enhanced resulting in a final improvement factor of 7.3 for MTED#18psR2C11.5 when compared to the parental culture (3.3.1). Highest accumulation levels of about 105 ± 2.9 µg/g fresh weight were achieved corresponding to a volumetric productivity of 88 mg/l. Just recently, Ye *et al.* (2010) described similar increases in productivity for the CHO system after repetitive rounds of flow cytometric pool sorting. Additional flow sorting rounds of monoclonal cell lines led to improved M12 antibody yields for two out of three lines (3.3.4). In fact, the second FACS round led to a productivity increase for the monoclonal lines MTED#18msR2C6.5 and MTED#18msR2C21.1 derived from lower producing ancestors MTED#18msR1C6 and MTED#18msR1C21. Given that both lines originated from an already monoclonal line the further increase in culture productivity was unexpected and was probably caused by reversion of the sorted culture to a heterogeneous state or by the weak correlation of the flow sorting marker DsRed and the M12 antibody, an aspect that has already been addressed. On the contrary, flow sorting of the highest producing MTED#18msR1C24 line did not result in the identification of novel superior monoclonal cultures, but the M12 production levels remained constant. These findings might indicate that the flow cytometric based optimization of recombinant protein accumulation has reached its limit or that the best producing lines were already identified. In summary, after the second flow sorting round all monoclonal cultures showed M12 accumulation levels up to 182.9 µg/g fresh weight in total, which reflects an improvement of 13-fold when compared to the parental MTED#18 culture.

In contrast to the improved M12 productivities observed for polyclonal and monoclonal BY-2 cultures from the second flow sorting round, the amount, intensity and homogeneity of DsRed fluorescence did not further rise, but remained at constant levels of 95% compared to the first FACS round (3.3.1, 3.3.4). The diverging trend of fluorescence signals and M12 accumulation levels, which had already been sporadically spotted for the monoclonal cultures of the first sorting round, was even more pronounced for the cultures of the second sorting round. These findings might be due to the variable expression of the two genes or due to limitations of the flow cytometric detection procedure. The variable expression of genetically linked transgenes in plant cells, as commonly observed in transformation experiments, has already been described in several earlier studies by Peach and Velten (1991), Akashi *et al*. (2002) or Melander *et al*. (2006). They reported that two genes positioned on the same T-DNA region directed by the same promoter did not express coordinately and suggested that this reflects interclonal and intraclonal variabilities. Regarding potential limitations of the flow cytometric detection procedure it should be taken into account that generated DsRed fluorescence data in percentages are relative values compared to a non-fluorescent wild type reference material. The actual concentration of the DsRed protein is not

quantified and thus the accuracy of this method might be too low to resolve minor differences in DsRed protein amounts. As mentioned earlier (3.6.2), the spectrometic quantification of fluorescent DsRed protein amounts was not practicable during this work due to a lack of DsRed reference material. However, we consider the variable expression of the two genes more probable because initial experiments indicate a high degree of convergence between the two DsRed detection methods spectrometry and flow cytometry (data not shown).

Within the third round of flow sorting the productivity of the polyclonal culture MTED#18psR2C11 was once more increased resulting in a final improvement factor of 9.7-fold for MTED#18psR3C11.5.8 compared to the parental culture (3.3.1). We also found that monoclonal cell line productivity became limited and even declined by 8-14% (3.3.4) whereas the portion of homogeneous DsRed fluorescent cells remained constant in the polyclonal and monoclonal lines, respectively. These results indicate that after two rounds of single cell flow sorting a productivity plateau of the cultures was reached and further selection rounds resulted in a decline, as it has been reported by Ye *et al.* (2010) for stable transfected CHO cell cultures. In this context, Ye and co-workers assumed that the sorted cells were unstable in nature.

Based on these findings, a comparison of the two sorting strategies leads to the following conclusions: (1) the cell lines generated from polyclonal or monoclonal sorting showed similar high productivities for DsRed and M12 antibody, (2) the application of the single-cell sorting approach was more straightforward because it required only two selection rounds for the identification of highest producing lines and (3) since the regeneration of single BY-2 cells was difficult and laborious the polyclonal sorting strategy might be preferable for other plant suspension cells as the establishment of sophisticated regeneration protocols is not necessary.

The comparison of the maximum recombinant M12 anibody amounts obtained after flow sorting, which reached 182.9 ± 7.8 µg/g fresh weight for the monoclonal lines (corresponding to 64 mg/l culture volume), with published data clearly emphasizes that we achieved the highest values so far reported for an IgG antibody in tobacco suspension cultures. Until now, documented yields for intracellular recombinant antibodies range from 0.5 mg/l for the anti-rabies virus antibody (Girard *et al*., 2006), to 1.8 mg/l for the human anti-HIV antibody 2F5 (Sack *et al*., 2007) or 2.3 µg/ml for the immunoglobulin GI-like murine antibody 14D9 (Lopez *et al*., 2010). Higher IgG levels were only published when the recombinant protein was secreted to the culture medium. Thus, antibody production levels of 5.5 mg/l for the secreted M12 antibody (Raven *et al.* 2011), 8 mg/l for the secreted anti-HIV antibody 2G12 (Holland *et al*., 2010) and 15 mg/l of a human antibody against HepatitisB Virus (Yano *et al*., 2004), were already obtained for tobacco suspension cultures. Even higher yields of full-size recombinant IgGs in plant suspension cultures such as 25 mg/l for tobacco culture were only realized

when culture conditions and the purification protocols were optimized (Fischer *et al.* 2003), as discussed later in chapter 4.4. In fact, the M12 antibody productivity of the polyclonal and monoclonal BY-2 cell lines were in the same range as yields commonly observed for flow sorted mammalian HEK and CHO cell cultures that usually produce recombinant proteins at 20-160 mg per liter culture broth non-optimized in batch cultures (Sleiman *et al.* 2008, Ye *et al.* 2010) highlighting the competitiveness of plant production systems towards mammalian counterparts.

Cell line development is one strategy to optimize the productivity of plant expression systems. Other strategies deploy media optimization, bioreactor design or bioprocess modification to enhance the amounts of recombinant proteins in plant suspension cultures yielding 4- to 20-fold increases (Georgiev *et al.* 2009, Huang *et al.* 2009, Holland *et al.* 2010). The flow cytometric sorting of transgenic BY-2 cultures proved an alternative and promising strategy to boost recombinant protein levels with increased and homogeneous product formation that are comparable to those resulting from either of the above mentioned optimization techniques.

**Table 9: FACS-derived cell lines for high yield recombinant protein production.**
Shown are the respective improvement factors obtained by three repetitive flow sorting rounds of one polyclonal and three monoclonal cell lines. The improve factors were determined for M12 antibody productivity and DsRed fluorescent cell portions. Both were calculated based on the parental MTED#18 culture that produced 14.4 µg/g fresh weight M12 antibody and comprised 24% DsRed fluorescent cells. The total improvement factor represents the sum.

| | Improvement factors | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DsRed population | | | | M12 antibody productivity | | | |
| FACS round | **1** | **2** | **3** | **total** | **1** | **2** | **3** | **total** |
| Polyclonal culture* | 4.0 | 0 | 0 | 4.0 | 4.6 | 2.7 | 2.4 | 9.7 |
| Monoclonal cultures[1] | 4.0 | 0 | 0 | 4.0 | 7.2 | 5.8 | 0 | 13.0 |
| Monoclonal cultures[2] | 4.0 | 0 | 0 | 4.0 | 8.6 | 3.4 | 0 | 12.0 |
| Monoclonal cultures[3] | 4.0 | 0 | 0 | 4.0 | 13.0 | 0 | 0 | 13.0 |

FACS = Fluorescence activated cell sorting; *= MTED#18 psR1C11, MTED#18 psR2C11.5 and MTED#18 psR3C11.5.8; 1 = MTED#18msR1C6, MTED#18msR1C21 and MTED#18msR1C24; 2 = MTED#18msR2C6.5, MTED#18msR2C21.1 and MTED#18msR2C24.3; 3= MTED#18msR3C6.5.8, MTED#18msR3C21.1.36 and MTED#18msR3C24.3.29

## 4.3 Characterization of FACS-derived polyclonal and monoclonal cultures

In chapter 4.2 the efficiency of flow cytometric sorting for the generation of highly productive polyclonal and monoclonal tobacco BY-2 suspension cultures was discussed. Within the scope of this thesis, further features of the parental and flow sorted cell cultures were also compared, namely the growth performance, the abundance of transgene copy number and the stability of cell culture productivity.

In this work, all flow sorted cultures with improved M12 productivity revealed characteristic differences with respect to growth and productivity when compared to the parental cell culture MTED#18. The growth of the polyclonal and monoclonal cultures was clearly slower with a delay of approximately 30 h (3.3.2, 3.3.5). As a consequence, average biomass accumulation was reduced by 45% for the flow sorted cultures and highest cell masses were reached approximately 6.5 days after routine subculture. Similarly, the maximal M12 antibody yields were most often attained 6 days after subculture, which was approximately two days delayed compared to the parental culture. However, when considering the impact of culture growth on the volumetric M12 productivity, we still observe up to 10-fold increased antibody levels for the flow sorted cultures.

Although it has not been studied in detail, we consider it rather unlikely that the flow sorting process itself led to changes in the growth characteristics of the cells because a negative effect of the flow sorting process on either deposited or regenerated cells has not been reported by others so far (Harkins and Galbraith 1984, Naill and Roberts 2005, Galbraith *et al.* 2011). Instead, we propose that reduced culture growth is due to the strongly enhanced culture M12 productivity and might reflect the increased metabolic burden to produce the elevated levels of recombinant proteins. Actually, there are several reports describing slower growth of highly productive transgenic hybridoma (Kromenaker and Srienc 1994) and CHO cells (Jiang *et al.* 2006). Likewise, Ikeda, *et al.* (1981) observed halved growth rates for tobacco BY-2 suspension cultures producing high levels of the metabolite ubiquinone-10 compared to low producers.

It is known that the levels of transgene expression and the recombinant protein productivity is influenced by the locus of the genomically integrated T-DNA and by the number of transgene integration events into the plant cell genome (Mason *et al.* 2002). In this work, it was assayed whether DsRed-based sorting might preferentially select cells with multiple transgene integration events that account for the elevated recombinant protein yields observed for the polyclonal and monoclonal MTED#18 cultures (3.3). In addition, it was investigated whether the transgene copy numbers remain consistent in cultures from subsequent flow sorting rounds. Information on the gene copy numbers of the M12 heavy chain and of DsRed in the parental and in various flow sorted cultures were compiled using the RT-qPCR method. RT-qPCR in combination with the $2^{-\Delta\Delta Ct}$ quantification method is a common method for the determination of relative transgene copy numbers with high accuracy enabling the reliable distinction between one and two transgene copy events (Callaway *et al.* 2002, Bubner and Baldwin 2004). Moreover, this method is highly recommended for the screening of great numbers of independent samples (Ingham *et al.* 2001, Shepherd *et al.* 2008). To assure the sound quantification of relative transgene numbers, RT-qPCR specific

PCR conditions were chosen that led to similar amplification efficiencies and low standard deviation of the amplicon Ct values (Livak and Schmittgen 2001).

To investigate whether flow sorting preferentially selects for multiple copy clones, the relative copy numbers of the parental MTED#18 culture was compared with those of the first FACS selection round. Respective data revealed consistent relative copy numbers for all lines except MTED#18msR1C24 for which both transgenes doubled (3.4). Thus, there is no clear evidence for an enrichment of multiple copy clones during FACS selection.

When investigating the consistency of transgene numbers among the sorted lines of subsequent FACS rounds, variations became obvious and no common pattern was apparent (3.4). The relative M12 and DsRed transgene numbers of the MTED#18msR1C6 remained constant in the lines of the second and third FACS round. Unlike, in case of the MTED#18msR1C21 the $2^{-\Delta\Delta Ct}$ values halved for the DsRed gene of the line isolated from the third FACS round (MTED#18msR3C21.1.36). Further, discrepancies of relative gene copies were even more pronounced for lines originating from MTED#18msR1C24. As mentioned above, duplication was found for both transgenes in MTED#18msR1C24 compared to the parental line, which then remained consistent with the following FACS selected line (MTED#18msR2C24.3). The $2^{-\Delta\Delta Ct}$ values of the expression cassettes for best monoclonal line originating from the third FACS round (MTED#18msR3C24.3.29) diverged. More precisely, the values for the DsRed expression cassette almost halved and the values for the M12HC expression cassette doubled indicating uncoupled transgene propagation.

The extent of transgene changes was unexpected considering the supposingly genetic uniformity of the monoclonal cultures. However, instability of transgene integration in general is a known phenomenon (Tax and Vernon 2001) and might be a possible explanation of the discrepancies in relative DsRed and M12 heavy chain copy numbers observed within this work. Similarily to our results, Melander *et al.* (2006) found discrepancies in transgene copy numbers in different generations of oilseed rape plants transformed with a double-gene construct and identified the loss of one of the inserted transgenes.

In addition, the explicit fluctuations of transgene copy numbers might also originate from the fuzzy assignment of $2^{-\Delta\Delta Ct}$ values to copy numbers, i.e. values that are not a multiple or a quotient of the reference value; a drawback that complicates the sound interpretation of the data. In fact, Bubner *et al.* (2004) found that two-fold differences of transgene copy numbers in plants were the resolution limit of this quantification method. Also Subr *et al.* (2006) report difficulties in the estimation of relative potato virus A gene copy number changes produced by the T1 generation of tobacco plants. Bubner and co-workers (2004) also suggest to use the RT-qPCR rather as a complementary tool than as a replacement of more suitable methods such as Southern blot analysis. Unfortunately, the determination of absolute transgene copy numbers by Southern blot analysis was beyond the scope of this thesis.

Beside the genetic characterization of the flow sorted polyclonal and monoclonal cell lines, their recombinant protein productivity was monitored over a time span of 12 months to investigate the production stability of the FACS generated cultures. Even though some references describe the stable, long-term recombinant protein production in plant cultures, e.g. the production of carrot invertase in tobacco BY-2 suspensions (Des Molles *et al.* 1999) or the $IgG_1$ antibody production by tobacco hairy root cultures (Wongsamuth and Doran 1997), culture instabilities and gradual decreases of the recombinant protein yields over time are an obstacle commonly observed but rarely described in the development of plant cell culture production systems (Kolewe *et al.* 2008). Thus far, the loss of desirable cell characteristics like high recombinant protein production is frequently observed for periodically subcultured plant cells with gene mutations, recombination events or inherent variabilities supposingly causing these changes (James and Lee 2006, Kolewe *et al.* 2008). For example, Qu *et al.* (2005) describe decreased anthocyanin accumulation in *Vitis vivifera* suspensions after a cultivation period of eight months. Moreover, both Gao *et al.* (1991) and Lambe *et al.* (1995) claimed significant reduction in the yield of recombinant beta-glucoronidase (GUS) for transgenic plant cell cultures when maintained by subculturing.

In this work, a reduction in M12 protein productivity was observed for some lines and it was most pronounced in the first round polyclonal culture (MTED#18psR1C11) that showed up to 50% productivity loss (3.3.3) after one year. Contrary, both the succeeding polyclonal culture (MTED#18psR2C11.5) and all except one of the monoclonal cell lines remained stable M12 producers. Considering these results and the fact that the polyclonal MTED#18psR1C11 culture still consists of a mixture of cells, it is possible that the highly productive cells were outcompeted over time by faster growing low producers dominating the culture. It has already been described for tobacco suspension cells (Lee *et al.* 1991, James and Lee 2006) that long-term maintenance of transgenic suspension cultures leads to the divergence of cultures resulting in the loss of productivity. Such alterations of cell populations are generally favoured by the presence of genetic or epigenetic culture variabilities, which are highly abundant in heterogeneous cultures (Henderson and Jacobsen 2007, Kolewe *et al.* 2008). In contrast to the reduction in M12 productivity, the percentage and intensity of the DsRed fluorescent marker protein remained constant in the polyclonal MTED#18psR1C11 culture during long-term cultivation. This observation does not support our initial assumption that highly productive cells are prone to be overgrown by low producing ones as a consequence of increased metabolic burden as stated earlier. Instead, a more likely explanation for this striking counter-development of the two recombinant protein yields is the inactivation of the M12 expression cassette by mechanisms of gene silencing (Stam *et al.* 1997) probably comparable to findings of Fu *et al.* (2000). They reported the individual silencing of single transgenes initially present on one T-DNA in transgenic rice lines. In addition, the long-term

maintenance of plant cells in the presence of phytohormones is cited to cause mutations that negatively effect cell culture performance (Zhang and John 2005, Kolewe *et al.* 2008) and might have provoked the decrease in recombinant protein productivity.

A 50% reduction in M12 yields was also determined for the monoclonal line MTED#18msR2C24.3 after 12 months coinciding with a 30% decrease in the portion of DsRed fluorescent cells (3.3.6). Apart from this exception, recombinant protein productivity remained stable for all other assayed monoclonal cultures over the whole period of investigation with MTED#18msR2C21.1 being the most stable one. Minor fluctuations in the M12 antibody production might be attributed to the lack of cell cycle synchronization, variations in cell fitness or differences in the periodic subculture process by which all cultures were maintained (Yanpaisan *et al.* 1999, James and Lee 2006). Similar to our data, Ketchum *et al*. (1999) also found monthly changes in the yield of paclitaxel that was accumulated in *Taxus* suspension cultures.

Conclusively, it was clearly shown in this thesis that multiple rounds of polyclonal sorting or the direct generation of monoclonal cell lines are promising strategies to stabilize culture productivities and thus confirm the suggestion of Yanpaisan *et al.* (1999) to apply several rounds and periodic repetition of flow cytometric selection to keep cell lines highly productive.

### 4.4 Application of monoclonal BY-2 cultures for the production of M12 antibody

As mentioned in the previous chapter the monoclonal culture MTED#18msR2C21.1, which has gone through two FACS selection rounds, had shown consistently high levels and lowest fluctuations in M12 productivity (171 ± 16.3 µg/g fresh weight M12). Therefore, this cell line was selected for subsequent cultivation studies in which (1) shake flask cultivation was compared with controlled bioreactor cultivation and (2) standard BY-2 medium was compared with nitrogen-enriched medium to further boost M12 antibody production.

There are several reports reviewing the opportunities to optimize recombinant protein production and product accumulation focusing on bioreactor design and bioprocess development (Huang and McDonald 2009, Desai *et al.* 2010). Aiming at a further increase in M12 yields, we compared the cultivation of MTED#18msR2C21.1 cells in shake flask and in a 3 l stirred tank bioreactor (2 l working volume). For both cultivation approaches similar growth data were obtained and maximum fresh weights of 420 g/l were measured (3.5.1). These values are in the same range as those reported in earlier studies for batch fermentations of BY-2 cells (Schmale 2007, Holland *et al.* 2010). Suehara *et al.* (1996) obtained up to 610 g/l fresh weight of tobacco suspension cells when applying a fed-batch fermentation strategy indicating the potential of bioprocess optimization. Regarding the volumetric M12 antibody production, stirred tank bioreactor cultivation was superior to shake flask cultivation reaching 1.6-fold higher yields of up to 140 mg/l M12 antibody during the end of cultivation (corresponding to 356 ± 37 µg/g fresh weight, 3.5.1). This yield not only exceed the initial

productivity of the monoclonal cell line determined from routine maintenance cultivation 2.1-fold, but are also 55% higher than any previously published values for an ER-retarded full-size antibody produced in BY-2 cells so far (Holland; 2006).

Several authors propose inadequate oxygen transfer (Eibl and Eibl 2008, Huang and McDonald 2009, Huang *et al.* 2009) and restricted mixing efficiencies (Nisi *et al.* 2010) are factors that limit the suitability of shake flasks for plant cell-based production systems. Therefore, the constant oxygen supply under controlled fermentation condition might have led to the enhanced M12 antibody production. Similar observations were also made by Lee and Kim (2004), who reached up to 9.4-fold higher recombinant hGM-CSF protein yields for stirred tank bioreactor cultivated transgenic tobacco cultures compared to shake flasks. They also claim improved aeration rates plus optimal agitation of the culture broth in the bioreactor vessels being causative for the increase in product formation.

In conclusion, the up-scaled cultivation of the MTED#18msR2C21.1 suspension line in a 3 l bioreactor was successful and even more the modification of cultivation conditions boosts the recombinant M12 antibody production.

Apart from bioreactor and bioprocess modification, plant cell cultivation and recombinant protein production can also be optimized by changing the culture medium composition (Georgiev *et al.* 2009, Xu *et al.* 2011). The most common plant cell culture media are MS (Murashige and Skoog 1962), Gamborg B5 (Gamborg *et al.* 1968) and LS (Linsmayer and Skoog 1965), which all provide sufficient nutrients necessary for cell growth. Further manipulation of these media compositions has resulted in a broad spectrum of different media for plant cell cultivation (Gamborg *et al.* 1976, Leifert *et al.* 1995) that enable efficient tissue/cell propagation. However, during early medium development, the exploitation of plant cell culture for the production of secondary metabolites or recombinant proteins were neglected, thus, research on the optimization of media composition is still ongoing. The modification of nutritive factors is frequently deployed to customize the culture medium and to identify the most suitable medium composition improving cell growth, culture productivity or product quality, e.g. by addition of salts or sugars (Vazquez-Flota *et al.* 1994, Kawana and Sasamoto 2008, Xu *et al.* 2011). The methodic alteration of macro- and micronutrients, carbon sources and phytohormones (Zhong 2001) can be accomplished by extensive statistical experiments (Bensaddek *et al.* 2001) and factorial designs (Nas *et al.* 2005). Beside the variation of nutrients and phytohormones, the addition of particular agents like bovine serum albumin (James *et al.* 2000), polyethylene glycol, gelatin and polyvinylpyrrolidone (LaCount *et al.* 1997, Lee *et al.* 2002, Kwon *et al.* 2003) often improves recombinant protein quality and recovery. Just recently, the comprehensive determination of various nutrient levels in spent BY-2 medium resulted in the targeted supplementation of depleted medium compounds and doubled amounts of recombinant tGFP protein (Ullisch *et al.* 2011) In case of recombinant

IgG production in tobacco cell cultures, the level of rAB24 was increased three-fold by amino acid supplementation of the suspended cells (Fischer *et al.* 1999). In 2010, Holland *et al*. achieved up to 20-fold improved levels of the secreted human full-size antibody 2G12 produced in BY-2 cultures after increasing the nitrate concentration in the medium.

These promising results prompted us to investigate the effect of additional nitrogen supply on the intracellular M12 yields of the highly productive monoclonal cultures. Monoclonal cell lines selected after the first and second FACS round (3.3.4) were adapted to nitrate-enriched medium (MSN). Subsequent analysis of recombinant antibody production revealed enhanced amounts of M12 antibody with a highest yield of 276 ± 23 µg/g fresh weight for MTED#18msR2C6.5 compared to maximal 188 ± 24 µg M12 per g fresh weight that were accumulated during cultivation in standard MS medium (3.5.2). Interestingly, the degree of improved M12 yields varied broadly and MSN-induced productivity increases of initially less productive cultures were clearly more pronounced than for highly productive ones. More precisely, cultivation of the most productive monoclonal lines (MTED#18msR1C21 and MTED#18msR2C21.1) in nitrate-enriched medium resulted in 18-35% enhanced yields whereas even up to 100% more M12 antibody was reached in the initially less productive lines like MTED#18msR1C24 and MTED#18msR2C6.5. In comparison to the results obtained by Holland *et al.* (2010) the overall improvement in productivity of our cultures was strikingly lower. This might be due to the fact that the FACS selected cell lines already produce outstandingly high amounts of recombinant protein and further increases are limited. Alternatively, the differences in improved M12 and 2G12 productivity could also result from the different antibody targeting, i.e. ER-retrival versus secretion. Further investigations aiming at the identification of the mode of action of nitrate would be necessary to clarify this issue. So far, several references discuss the pleiotropic effects of nitrogen on plant gene expression (Gutierrez *et al.* 2007) and metabolism. Actually, nitrogen nutrition is considered to be a major factor associated with protein synthesis (Prinsi *et al.* 2009).

In summary, our strategies to further enhance M12 antibody levels of the FACS selected monoclonal cell lines by means of bioreactor cultivation or medium optimization were successful. The optimal cell growth in a stirred tank fermenter led to an 1.6-fold increase in M12 productivity and cultivation in nitrate-enriched MS medium brought up to 50% higher levels. As the next step, the two strategies should be combined to evaluate synergistic effects; however, this approach was beyond the scope of this work due to time constraints.

## 4.5 Flow sorting for the selection of highly productive BY-2 cultures early after transformation

In the first part of this PhD thesis, the successful use of flow cytometric sorting for the selection of highly productive polyclonal and monoclonal BY-2 suspension lines from the heterogeneous parental MTED#18 culture is described (3.3). Inspired by the clone selection

strategies applied for mammalian expression systems (DeMaria *et al.* 2007, Sleiman *et al.* 2008), we investigated in the second part, whether FACS is a suitable tool for high-throughput screening and selection of productive BY-2 cells shortly after *Agrobacterium*-mediated transformation (3.6). As only a small portion of the transformed cells produce high yields of recombinant protein (Carroll and Al-Rubeai 2004), their early identification is favorable. In addition, the FACS-based approach promises a faster establishment of transgenic cultures and reduced manual screening efforts compared to traditional procedures of transgenic plant cell regeneration and selection.

The experimental setup included the fluorescent marker proteins tGFP and DsRed both commonly been used for flow cytometric applications (Galbraith *et al.* 1999, Jach *et al.* 2001). Wild type BY-2 cells were transformed with a pDAB plant expression vector encoding the green fluorescent protein tGFP or the pTRAkc-MTED vector encoding the red fluorescent protein DsRed and the M12 antibody on one T-DNA, respectively. Half of the transformation mixture was processed according to the conventional procedure that included three working steps, i.e. (1) the plating of cells on selection medium, (2) the analysis of recombinant protein levels from callus tissue and (3) the establishment of suspension cultures from the best producers. The other half was used for the immediate establishment of a suspension culture, which served as starting material for FACS and the time span for the generation of first transgenic cultures was notably reduced to three weeks (3.6.1). According to our experience and to An (1985) and James and Lee (2006), the establishment of transgenic plant material by conventional strategies requires four to six weeks not including further culture optimization strategies like application of antibiotic pressure (Akashi *et al.* 2002) or manual cloning (Nocarova and Fischer 2009). In fact, the generation of highly productive plant or mammalian cell lines can easily cover additional 6-12 months of tedious and time-consuming screening processes without a guaranteed success (Hood *et al.* 2002, Sleiman *et al.* 2008).

Prior to FACS, the starting cultures were analysed with respect to tGFP or DsRed production and fluorescence intensity distribution to investigate the heterogeneity of the cultures. For both cultures, a broad distribution of the fluorescent protein was found and the portion of fluorescent cells within the culture was 60% for DsRed and 85% for tGFP, respectively (3.6.1). The obvious heterogeneity of the culture reflected a high amount of independent transformation events and confirmed that the two-week liquid cultivation of the transformation mixture had not led to an uneven enrichment of clonal cells. The two cultures were flow sorted and polyclonal cell depositions were conducted to avoid the interference of tGFP fluorescence with the strong autofluorescence of the wild type feeder cells. Problems caused by the non-distinguishability of GFP fluorescence and plant cell autofluorescence were previously observed by (Gupta and Ibaraki 2006) for transgenic tobacco cells.

The five highest fluorescent sorted cultures and the five highest fluorescent cultures originating form calli generated by the conventional transformation strategy were then compared regarding their recombinant protein production. Differences between these cultures became obvious in flow cytometric analyses of their fluorescence properties (3.6.2). On the one hand, the distributions of tGFP or DsRed in callus-derived cultures diversified broadly among and also within the cultures reflecting the heterogeneous production of the fluorescent marker proteins. These observations might be due to the mixed culture compositions consisting of high and low producing cells and/or differences in growth performances between the investigated cultures (Brezinsky *et al.* 2003). On the other hand, cultures originating from the FACS approach showed homogeneous and comparable high fluorescent signals for the respective marker protein. These tendencies were as well confirmed for the green fluorescent protein tGFP through the densitometric quantification of tGFP amounts detected via immunoblot and tGFP yields ranging between 0.7 and 3.2 µg/g fresh weight for the callus-derived cultures and between 2.7 and 5.1 µg/g fresh weight for the sorted cultures were determined. Moreover, the average tGFP amounts were 2.4-fold increased in cultures from FACS-selection. The potential of the new screening approach became also obvious during analysis of M12 antibody accumulation in the DsRed fluorescent cultures. Here, cultures derived from the conventional strategy produced between 33 and 85 µg antibody per gram fresh weight, whereas flow sorted cultures accumulated between 70 and 118 µg M12 per gram fresh weight corresponding to an average 1.6-fold improve.

Conclusively, the investigated FACS screening approach was superior to the conventional clone identification method with respect to duration and selection efficiency. The FACS-based approach enabled the faster establishment of transgenic cultures with reduced manual screening efforts and a time saving of two weeks compared to traditional procedures. It also resulted in the generation of cultures with significantly higher recombinant protein productivity. Finally, this work demonstrates the broad applicability of FACS as a tool for clone selection either from already established transgenic cultures (3.3) or from freshly transformed cells (3.6).

# 5 Outlook

The work presented in this PhD thesis proved two FACS-based selection strategies as effective approaches for productivity and homogeneity improvement of existing heterogeneous transgenic tobacco cell cultures (3.3). Applying the first selection strategy, three rounds of repetitive pool sorting were conducted that resulted in the generation of improved transgenic polyclonal cultures with up to 9.7-fold enhanced M12 antibody productivity (3.3.1). The alternative FACS-based selection strategy deployed a feeder-supported regeneration procedure of single cells (3.1.5) and let to the establishment of high-producing monoclonal suspension lines. Here, three repetitive rounds of single cell sorting enhanced the M12 productivity up to 13-fold (3.3.4). Even though the maximum yields of the polyclonal cultures were lower compared to the best monoclonal lines, the M12 yields of the pool sorted cultures increased steadily over the three repetitive FACS rounds, whereas the M12 productivity remained constant after the second sorting round for the monoclonal lines. These results indicate that the identification of highest producers based on the pool sorting strategy might demand more sorting rounds than the monoclonal sorting approach, but will finally result in equally high M12 productivities.

As initial medium optimization (3.5.2) and bioreactor cultivation (3.5.1) experiments performed in the present work indicated, the applied FACS procedure can still be replenished and/or combined with other commonly applied optimization strategies. In this context, the investigation of possible synergistic effects resulting from controlled bioreactor cultivation in optimized medium to further boost the yields of recombinant M12 antibody in the already high producing monoclonal cell lines is a promising approach. Specifically, the addition of nitrate-supplemented medium in combination with controlled batch fermentation had been recently reported to significantly increase BY-2 cell productivity (Holland *et al.* 2010). Beside this, numerous bioprocess or bioreactor engineering strategies (Huang and McDonald 2009) could be investigated to further boost the accumulation of recombinant IgG antibody production.

Within this work, the consistency of recombinant protein production was demonstrated over a time period of one year. However, continuous selection of high and stable producers might be beneficial to maintain high expression levels over longer time periods, because highly productive cells might be overgrown over time during routine cultivation as a consequence of the additional metabolic burden (Borth *et al.* 2000). In fact, this procedure is already commonly applied for optimization of mammalian host systems (Carroll and Al-Rubeai 2004, Ye *et al.* 2010).

For the genetic characterization of the polyclonal and monoclonal cultures, RT-qPCR experiments were performed resulting in ambiguous data that only reflect the relative transgene copy numbers. Therefore, the sound determination of the absolute transgene copy

numbers by Southern Blot analyses (Bubner and Baldwin 2004) is still needed to be conducted. These results might clarify whether the transgene copy numbers vary among the polyclonal cultures and monoclonal lines from the various flow sorting rounds.

Another important outcome of this thesis was the successful FACS-supported generation of transgenic BY-2 cultures shortly after transformation with a time saving of 2.5 months compared to the conventional transformation strategy. Based on these results, it might be worthwhile testing whether the sorting of freshly transformed single cells is feasible for the creation of monoclonal lines. As the generation of stable monoclonal lines takes less rounds of FACS, this would further shorten the time needed for the isolation of high producer lines.

The currently applied FACS strategy deploys the co-selection of the DsRed fluorescent protein and the M12 antibody, which are encoded as separate expression cassettes on one T-DNA. The linkage of one antibody gene with the fluorescent marker gene on transcriptional level by the introduction of an internal ribosomal entry site (IRES) could strengthen the correlative production of both recombinant proteins. Thus far, IRES-mediated translation initiation of marker and target proteins is mainly used as a tool in mammalian expression systems (Li *et al.* 2007) to optimize the selection of high producers. For plant cells, Urwin *et al.* (2000) showed the production of two functional proteins from a bicistronic transcript in tobacco plants using the Encephalomyocarditis virus (EMCV) IRES element and Jaag *et al.* (2003) confirmed the function of the Potato leafroll polerovirus (PLRV) IRES by successful GUS gene expression in potato protoplasts. According to the strategy of Urwin and colleagues, the design of a bi- or tricistronic expression construct - with heavy chain, light chain and DsRed flanked by IRES sequences - should tightly regulate the translation of the proteins resulting in a stringent correlation of M12 antibody and the fluorescence protein production.

During flow cytometric sorting highly productive plant cells are selected from a pool of hundreds of thousands cells according to their fluorescence signals and deposited in microtiter plates for regeneration. However, the monitoring and subsequent screening of a sufficient number of clones still remains time-consuming (DeMaria *et al.* 2007). Therefore, the implementation of an efficient and accurate image-based monitoring device such as the confocal microscopy imaging system Opera® would reduce the manual workload, streamline the screening process and increase the amount of analyzed cells to high throughput scale. The feasibility of this approach has already been demonstrated by Salomon *et al*. (2010) through the Opera® microscope-based high throughput quantification of transgenic *Arabidopsis* lines expressing fluorescently labelled proteins. Moerover, the combination of flow sorting and Opera® based imaging with a robotic handling system for the picking and transfer of microcallus tissue would also reduce the manual workload and further automize the process of clone selection and cell line establishment.

Finally, the knowledge aquired within this PhD work could be applied to other plant cell species for the development of suitable FACS-based screening and single cell regeneration procedures. As demonstrated, this technology offers the possibility of plant cell line optimization resulting in improved protein productivity and it could be deploited for other recombinant protein plant production systems such as Arabidopsis, carrot, rice or maize suspension cultures. Furthermore, the FACS-based cell selection of regenerable plant tissue, e.g. tobacco mesophyll cells, holds the opportunity to establish a “protoplast-to-plant system” that could enable the creation of intact plants from a single sorted cell.

## 6 Summary

Over the last 20 years, plant cells gained increasing interest as host systems for the production of human and animal pharmaceuticals. Transgenic tobacco suspension cultures are an especially promising production host because of their beneficial growth, low maintenance requirement and the possibility of contained cultivation in bioreactors under controlled conditions. The excellent product quality of plant-produced proteins was demonstrated for a multitude of proteins, but the heterogeneous and low protein productivity of transgenic plant suspension cultures is still a serious bottleneck that constrains their economic profitability and their competitiveness towards established host systems like mammalian cells or yeasts. To overcome this current drawback various strategies are followed to boost the recombinant protein productivity in plant suspension cultures. Currently, the optimization of bioreactor design, bioprocess control or medium composition is investigated on a broad range, but also the optimization of the plant host systems themselves are of major interest.

The objective of this work was the application of fluorescence activated cell sorting (FACS) technology for the selection of highly productive BY-2 cells from a heterogeneous tobacco suspension culture. The BY-2 cells co-produce the human antibody M12 and the selectable marker protein DsRed; the coding sequences of the recombinant proteins were present as separate expression cassettes on one T-DNA. The FACS strategy was based on the assumption that the amount of the fluorescent marker protein correlates with the amount of M12 antibody produced within a cell. Initial experiments on protoplast preparation and regeneration medium composition led to the reliable regeneration of BY-2 protoplasts, the sustained re-formation of cell walls and the efficient proliferation of plant cells at low densities ($2 \times 10^2$ per ml).

Starting from the heterogeneous transgenic BY-2 suspension culture MTED#18 the strict and consecutive flow cytometric sorting of highly DsRed fluorescent cells in pools of 20 cells resulted in the isolation of new polyclonal suspension cultures that showed increased levels of both recombinant proteins and the highest producing culture MTED#18psR3C11.5.8 accumulated almost 10-fold more M12 antibody than the parental MTED#18 culture. Similarly, it was demonstrated that flow sorting also resulted in a fourfold enrichment of DsRed fluorescent cells that showed a homogeneous distribution of the red fluorescence.

For the FACS-based generation of monoclonal cell lines a procedure using feeder cells was developed that enabled the regeneration of single BY-2 cells. In fact, this work describes for the first time, a straightforward procedure for single cell regeneration after flow sorting. Monoclonal tobacco cell lines with advanced homogeneity and up to finally 13-fold improved recombinant M12 productivity were established. For the best monoclonal suspension lines, i.e. MTED#18msR2C24.3 or MTED#18msR2C21.1, recombinant M12 antibody accumulation

levels of up to 182.9 ± 7.8 µg/g fresh weight were obtained that also remained stable over an observation period of one year.

The characterization of the improved polyclonal cultures and monoclonal lines regarding growth and M12 antibody production revealed that the growth was delayed and the overall biomass was reduced for the majority of flow sorted suspensions compared to the parental culture. Correspondingly, the time point of highest M12 accumulation was delayed with a retardation time of approx. 30 h.

The up-scaled cultivation of the monoclonal BY-2 line MTED#18msR2C21.1 in a 3 l stirred tank bioreactor was successful and a volumetric M12 antibody accumulation of 141 ± 3 mg/l was reached, a value that exceeded the one determined for the same suspension culture grown in shake flasks by 50%. Aiming at a further increase in M12 antibody accumulation the impact of cultivation in nitrogen-enriched medium was investigated for various monoclonal suspension lines. Indeed, the recombinant M12 antibody amounts were 18-100% higher upon cultivation in nitrogen-enriched medium compared to the standard BY-2 medium.

The FACS-supported generation of transgenic polyclonal cell lines at an early stage after a transformation resulted in the rapid generation of highly productive transgenic tobacco cultures with a time saving of 2.5 months. BY-2 cells were transformed with plant expression vectors encoding either the M12 antibody in combination with DsRed or the fluorescent protein tGFP. Transgenic cultures were simultaneously established by FACS-based selection of transgenic events and by the conventional callus-based strategy. Comparative immunological analyses confirmed that the majority of transgenic suspension cultures originating from the FACS selection were of superior homogeneity and produced 1.6-fold more M12 antibody or respectively 2.4-fold more tGFP compared to the amounts determined for the cultures derived from the conventional transformation procedure.

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# 8 Appendix

## 8.1 List of abbreviations

| | |
|---|---|
| *A. tumefaciens* | *Agrobacterium tumefaciens* |
| AP | Alcaline phosphatase |
| BCIP | 5-Bromo-4-chloro-3indolyl-phosphate |
| BSA | Bovine serum albumin |
| BY-2 | Bright Yellow 2 |
| °C | Degree Celsius |
| ca. | Circa |
| $CaCl_2$ | Calcium chlorid |
| CaMV | Cauliflower mosaic virus |
| Carb | Carboxycillin |
| CHO | Chinese hamster ovary cells |
| CHS | Chalconsynthase |
| cv. | Cultivar |
| DNA | Desoxy ribonucleic acid |
| dNTP | Desoxy nucleoside triphosphat |
| dpi | Days post inoculation |
| DsRed | Red fluorescent protein, reporter gene |
| EDTA | Ethylen-diamine-tetra-acetic acid |
| e.g. | *exempli gratiā*, for example |
| ELISA | Enzym linked immunosorbent assay |
| ER | Endoplasmatic reticulum |
| *et al.* | And other |
| FACS | Fluorescence activated cell sorter |
| FL-1, FL-2 | Fluorescence channel |
| FSC | Forward scatter channel |
| GBSS | granule bound starch synthase |
| x g | Gravitational acceleration |
| h | Hours |
| HC | Heavy chain of an antibody |
| $HNO_3$ | Nitric acid |
| i.e. | *Id est*, that means |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin class G |
| Kan | Kanamycin |
| kDa | Kilodalton |
| $KH_2PO_4$ | Potassiumdihydrogenphosphate |
| l | Liter |
| LC | Light chain of an antibody |
| LPH | Codon optimized signal peptide of the mAk24 heavy chain |
| M | Molar |
| Mab | Monoclonal antibody |
| mM | Millimolar |
| min | Minutes |

| | |
|---|---|
| mosmol/kg | Milliosmol per kilogram |
| MS | Murashige und Skoog |
| mg | Milligram |
| µg | Microgram |
| ml | Milliliter |
| µl | Microliter |
| $Na_2HPO_4$ | Dinatriumhydrogenphosphate |
| NBT | Nitrobluetetrazolium |
| ng | Nanogram |
| *N. tabacum* | *Nicotiana tabacum* |
| OD | Optical density |
| PAGE | Polyacrylamid-gelelectrophoresis |
| PBS | Phosphat buffered saline |
| PBS-T | Phosphat buffered saline with 0,05% (v/v)Tween-20 |
| PCR | Polymerase chain reaction |
| PCV | Packed cell volume |
| pH | Negative decimal logarithm of the hydrogen ion concentration |
| pNPP | p-nitrophenyl phosphate |
| pmol | Picomolar |
| Rif | Rifampicin |
| RNase | Ribonuclease |
| rpm | Generations per minute |
| RT | Room temperature |
| SAR | Scaffold attachment region |
| scFv | Single chain fragment of the variable region |
| SDS | Sodiumdodecylsulfat |
| Sec | Seconds |
| SSC | Side scatter channel |
| TEV | Tobacco etch virus |
| Tris | Tris-(hydroxymethyl)-aminoethan |
| UTR | Untranslated region |
| V | Voltage |
| v/v | Volume per volume, volume percentage of the total volume |
| WT | Wildtyp |
| w/v | Weight per volume, Mass percentage oft he total volume |

## 8.2 List of figures

### 8.3 List of tables

## 8.4 Plasmid maps

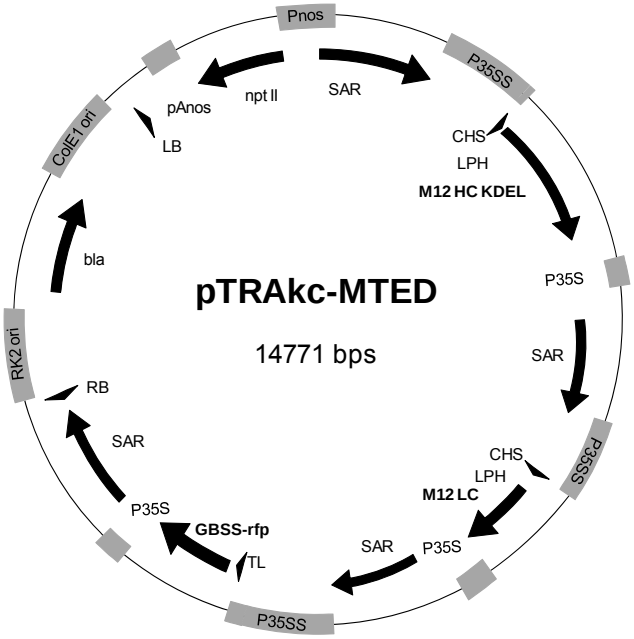


**Figure 23: Plant expression vector pTRAkc:MTED.**
LB: Left border for T-DNA integration; pA*nos:* promoter of the nopaline synthase gene; *nptII*: gene for kanamycin resistance in plants; p*nos:* polyadenylation signal of the nopaline synthase gene; SAR:scaffold attachment region of *N. tabacum*; p35SS: CaMV 35S promoter with duplicated transcriptional enhancer; CHS: Chalcone synthase 5' untranslated region; LPH: signal-peptide sequence from the murine mAb24 heavy chain; M12HC: heavy chain (HC) of the human M12 antibody; KDEL: Endoplasmic Reticulum (ER) retention signal; *p35S:* CaMV 35S polyadenylation signal; M12LC: human lambda light chain (LC) of the human M12 antibody; TL: 5`-region from *Tobacco etch virus;* GBSS: Granule Bound Starch Synthase transit peptide; rfp: red fluorescent protein (DsRed, *Discosoma* sp.); RB: Right border for T-DNA integration; RK2 ori: Ori for *A. tumefaciens* replication; *bla*: ampicillin/carbenicillin resistence; ColE1 ori: origin of replication (ori) for *E.coli* replication.

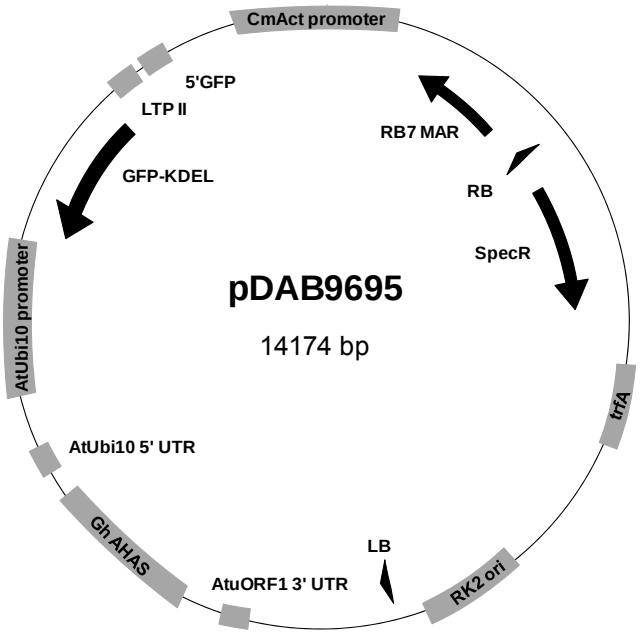


**Figure 24: Plant expression vector pDAB9695.**
RB: Right border for T-DNA integration; SpecR: *aadA* genes encoding resistance to streptomycin and spectinomycin; trfA: replication initiator protein for PK2; RK2ori: Origin of replication (ori) for *A. tumefaciens*; LB: Left border for T-DNA integration; AtuORF1 3`UTR: 3`UTR from *A. tumefaciens;* GhAHAS: Acetohydroxyacid Synthase from *Gossypium hirsutum*; AtUbi10 5`ÙTR: *A. thaliana* Ubiquitin10 promoter 5' untranslated region; Atubi10 promoter: Ubiquitine promoter from *A.thaliana;* GFP: Green fluorescent protein (tGFP, Evrogen); KDEL: Endoplasmic Reticulum (ER) retention signal; LTPII: Signal peptide; 5`GFP: 5`-region for GFP transcription; CmAct promoter: Melon Actin promoter from *Cucumis melo*; RB7 MAR: matrix attachment reagion (MAR) from *N.tabacum.*

## Patent and publications

### Patent application

**Kirchhoff J**, Schillberg S, Schiermeyer A, Schinkel H, Fischer R (2011)
Method for the generation of a monoclonal plant cell line. European Patent application. PCT/EP2011/002785

### Publications

**Kirchhoff J**, Raven N, Boes A, Roberts JL, Russell S, Treffenfeldt W, Fischer R, Schinkel H, Schiermeyer A, Schillberg S (2012)
Monoclonal tobacco cell lines with enhanced recombinant protein yields can be generated from heterogeneous cell suspension cultures by flow sorting. Plant Biotechnology Journal (in review)

Ullisch DA, Müller CA, Maibaum S, **Kirchhoff J**, Schiermeyer A, Schillberg S, Roberts JL, Treffenfeldt W, Büchs J
Comprehensive characterization of *Nicotiana tabacum* BY-2 cell growth leads to doubled GFP concentrations by media optimization. (2012) Journal of Bioscience and Bioengineering Vol. 113, No.2, pp. 242-248

Müller CH, Wenk P, **Kirchhoff J**
Online growth parameter measurement of *Nicotiana tabacum* cell suspension culture in microtiter plates. Electronic Journal of Biotechnology (in preparation)

### Book chapter

Raven N., Schillberg S., **Kirchhoff J**., Brändli J., Imseng N.and Eibl R. (2011).
Growth of BY-2 suspension cells and plantibody production in single-use bioreactors.
In: Eibl, R. E., Dieter (ed.) *Single-Use Technology in Biopharmaceutical Manufacture.* New York: John Wiley & Sons.

### Poster presentation

Raven N, **Kirchhoff J**, Schuster F, Stadlmann J, Altmann F, Fischer R, Schillberg S
Expression of the MUC-1 specific human antibody M12 in tobacco plants and BY-2 suspension cells
(Antibodies-Europe Conference, Vienna, Austria, November 2007)

Raven N, **Kirchhoff J**, Schuster F, Stadlmann J, Altmann F, Spiegel H, Schillberg S
Expression of the MUC-1 specific human antibody M12 in in plants
(PBVA 2007- 2nd International Conference Plant-Based Antibody & Vaccines, University of Verona, Italy)

**Kirchhoff J**, Schiermeyer A and Schillberg S
Flow cytometric sorting – a procedure for plant cell culture optimization and generation of high producing monoclonal tobacco suspension cultures
(DowAgroScience, Indianapolis, USA; 2009)

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**Lebenslauf**

| | |
|---|---|
| Name: | Janina Kirchhoff |
| Geburtsdatum: | 12. Juni 1983 |
| Geburtsort: | Dortmund |
| Nationalität: | Deutsch |

**Ausbildung und beruflicher Werdegang**

| | |
|---|---|
| 04/2012 - | **Wissenschaftliche Angestellte**<br>Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie (IME)<br>Abteilung Pflanzenbiotechnologie, Aachen |
| 10/2007 - 04/2012 | **Wissenschaftliche Angestellte/Promotion**<br>Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie (IME)<br>Abteilung Pflanzenbiotechnologie, Aachen<br>Titel der Promotionsarbeit: Generation of highly productive polyclonal and monoclonal tobacco suspension lines from a heterogeneous transgenic BY-2 culture through flow cytometric sorting |
| 11/2006 – 09/2007 | **Diplomarbeit**<br>Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie (IME)<br>Abteilung Pflanzenbiotechnologie, Aachen<br>Titel der Diplomarbeit: Produktion des Muzin-1 spezifischen Volllängenantikörpers M12 in Tabaksuspensionszellen |
| 10/2006 | **Projektleiterschein**<br>Westfälische Wilhelms-Universität Münster (WWU) |
| 2003-2006 | **Studentische Hilfskraftstelle**<br>Institut für Medizinische Mikrobiologie, Universitätsklinikum Münster |
| 07/2004 – 09/2007 | **Hauptstudium** Diplom -Biologie (modularisiert)<br>Westfälische Wilhelms-Universität Münster (WWU)<br>Abschluss: Diplom Biologin (Diplomnote/ECTS grade B) |
| 10/2002 – 07/2004 | **Grundstudium** Diplom -Biologie (modularisiert)<br>Westfälische Wilhelms-Universität Münster (WWU) |
| 1993-2002 | **Abitur**<br>Reinoldus-und Schiller- Gymnasium, Dortmund<br>Leistungskurse: Biologie, Erdkunde |

**Praktika**

| | |
|---|---|
| 08/09 2006 | Forschungspraktikum<br>Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie, Aachen, Abteilung Pflanzenbiotechnologie<br>Thema der Arbeit: Expression und Reinigung von rekombinanten Interferon-α2b nach Transformation von *Nicotiana tabacum* |
| 08/09 2005 | Forschungspraktikum<br>Institut für molekulare und klinische Herz-Kreislaufforschung, Universität Witten-Herdecke<br>Thema der Arbeit: Mutationsscreening in Exon 1, Exon 2, Exon 3.1, Exon 3.2, Exon 36, Exon 37.1, Exon 37.2, Exon 39 und Exon 40 des β-Myosin Schwereketten Gens (MYH7) bei Patienten mit hypertropher Kardiomyopathie |
| 07/ 2002 | Forschungspraktikum<br>Institut für Medizinische Mikrobiologie, Universitätsklinikum Münster<br>Thema der Arbeit: Analyse zu Verteilung des EspX-Gens in verschiedenen darmpathogenen *Escherichia coli* und Untersuchung der Expression und Sekretion des potentiellen Effektorproteins EspY |
| 02/ 1999 | Praktikum Blumen Risse, Dortmund |