DEVELOPMENT OF A SELECTION SYSTEM FOR TRANSGENIC PLANT SUSPENSION CULTURES BASED ON DICISTRONIC VECTORS

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SUMMARY

This study aimed to explore the possibility to select transgenic high expressing cells in plant cell cultures by using dicistronic transformation vectors. The coexpression of a target and a selectable marker gene (SMG) was mediated by an internal ribosome entry site (IRES). In contrast to traditional plant transformation vectors carrying only one SMG and a target gene under control of different promoters, the newly designed dicistronic transformation vectors carry a second SMG controlled by the same promoter as the target gene. With these vectors it was investigated whether selection on the SMG under the same promoter or under a distinct promoter leads to higher and more stable protein expression of the reporter gene *luciferase*, which was taken as a model target gene because of easy expression monitoring by chemiluminescence detection.

An *Agrobacterium tumefaciens* mediated transformation and selection system for *Nicotiana tabacum* strain Bruessel suspension cultures (DSMZ No. PC-120) was established. The direct selection of high expressive transgenic cell cultures was much faster than the establishment of transgenic plant derived cell cultures and provided probably the highest diversity of independent transformation events. The tobacco cell cultures were transformed with either a construct carrying genes for antibiotic and herbicide resistance on two different expression cassettes, or another construct carrying genes for herbicide and putative salt tolerance on two different expression cassettes.

Transgenic cell cultures carrying the first construct expressed the *luciferase* reporter gene reliably in high quantities when they were treated with the herbicide Phosphinotricin; in this case selection took place on the SMG which is coexpressed with the *luciferase* gene.

Selection with NaCl on the SMG coexpressed with the reporter gene had no effect on transgenic cell cultures carrying the second construct.

Selection with PPT, mediated by the *bar* gene, was more efficient in respect to Luciferase expression than selection with G418 or NaCl, mediated by the *nptII* and *PR10a* genes respectively, regardless if the *bar* gene was under control of the same or another promoter than the *luciferase* gene. However, treatment with PPT also reduced considerably the initial proliferation of transgenic cell cultures.

Remarkably the Luciferase activity of transgenic cell cultures carrying the *PR10a* gene was always higher compared to transgenic cell cultures harbouring other constructs.

The potential of the dicistronic transformation vectors to produce recombinant proteins in a plant system was demonstrated. In this study the tuberculosis antigen HSPX was detected after transient expression in tobacco leaves.

Keywords: IRES, coexpression, expression instability, suspension cultures, PR10a

ZUSAMMENFASSUNG

In dieser Studie wurde die Möglichkeit der Verwendung dicistronischer Transformationsvektoren zur Selektion transgener hoch exprimierender Zellen in Planzenzellkulturen untersucht. Die Koexpression eines Ziel- und selektiven Markergens (SMG) wurde durch eine interne Ribosomeneintrittsstelle (IRES) vermittelt. Im Gegensatz zu traditionellen Pflanzentransformationsvektoren, die ein einzelnes SMG und ein Zielgen unter der Kontrolle verschiedener Promotoren tragen, enthalten die neu konstruierten dicistronischen Vektoren ein weiteres SMG, welches durch denselben Promotor wie das Zielgen gesteuert wird. Anhand dieser Vektoren wurde untersucht, ob die Selektion auf das SMG unter demselben Promotor oder unter einem anderen Promotor zu einer höheren und stabileren Expression des Reportergens *luciferase* führt. Das *luciferase* Gen wurde als Modell-Zielgen ausgewählt, weil sich dessen Proteinexpression durch den Nachweis der Chemilumineszenz leicht beobachten lässt.

Für die *Nicotiana tabacum* Suspensionskulturen des Stammes „Brüssel“ (DSMZ Nr. PC120) wurde ein *Agrobacterium tumefaciens* vermitteltes Transformations- und Selektionssystem etabliert. Die Selektion auf hoch exprimierende transgene Zellkulturen nach direkter Transformation der Suspensionskulturen ersparte viel Zeit im Vergleich zur Etablierung einer Zellkultur aus transgenen Pflanzen und erbrachte vermutlich die höchste Diversität an unabhängigen Transformationsereignissen. Die Tabakzellkulturen wurden entweder mit einem Konstrukt transformiert, das Gene für Antibiotika- und Herbizidresistenz auf zwei unterschiedlichen Expressionskassetten bereitstellt oder mit einem anderen Konstrukt, das auf zwei unterschiedlichen Expressionskassetten Gene für Herbizidresistenz und eine mögliche Salztoleranz trägt.

Transgene Zellkulturen, die mit dem ersten Konstrukt transformiert wurden, exprimierten das *luciferase* Reportergen zuverlässig in großen Mengen, wenn sie mit dem Herbizid Phosphinotricin behandelt wurden. Das für die Herbizidresistenz zuständige SMG war bei diesem Konstrukt unter Kontrolle desselben Promotors wie das Reportergen.

Bei den transgenen Zellkulturen, die das zweite Konstrukt trugen, hatte die Selektion mit NaCl auf das SMG, das mit der Luciferase koexprimiert wurde, keinen Effekt.

Die Selektion mit PPT auf die durch das *bar* Gen vermittelte Herbizidresistenz war in Bezug auf die Luciferase Expression effizienter als die Selektion mit G418 auf die durch das *nptII* Gen vermittelte Antibiotikaresistenz oder die Selektion mit NaCl auf die durch das *PR10a* Gen vermittelte Salztoleranz gleichgültig, ob das bar Gen von demselben oder einem anderen

Promotor als das *luciferase* Gen reguliert wurde. Allerdings reduzierte die PPT-Behandlung auch das anfängliche Zellkulturwachstum erheblich.
Bemerkenswerterweise wurde in Zellkulturen, die das *PR10a* Gen eingebaut hatten, in der Regel eine höhere Luciferase Aktivität gemessen als in transgenen Zellkulturen, die mit verschiedenen anderen Konstrukten transformiert worden waren.
Weiterhin wurde in dieser Studie gezeigt, dass es mit Hilfe von dicistronischen Vektoren gelang, transient hoch exprimierende Bereiche zu identifizieren und ein rekombinantes Protein aus diesem Material zu isolieren. Hierbei konnte im speziellen die transiente Expression des Tuberkulose-Antigens HSPX in Tabakblättern nachgewiesen werden.

Schlüsselworte: IRES, Koexpression, Expressionsinstabilität, Suspensionskulturen, PR10a

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LIST OF ABBREVIATIONS

A.tumefaciens	*Agrobacterium tumefaciens*
Bar	Bialophos resistance gene
Bp	base pairs
°C	degree Celsius
CMV	Cauliflower Mosaic Virus
Cm	centimetre
DNA	deoxyribonucleic acid
dNTP	deoxynuclosidetriphosphate
DW	dry weight
E. coli	*Escherichia coli*
FW	fresh weight
g/l	gram per litre
GFP	green fluorescent protein
GMP	good manufacturing practice
GUS	glucuronidase
h	hour(s)
HSPX	Heat Shock Protein X
IRES	internal ribosome entry site
kb	kilo base
kD	kiloDalton
l	litre
LB	left border
LB medium	Luria Broth medium
LS medium	Linsmaier and Skoog medium
LUC	Luciferase
M	Molar
mA	milliAmpere
MAS	mannopine synthase
mg	milligram
mg/l	milligram per litre
ml	millilitre
mM	milli Molar

mRNA	messenger RNA
MS	Murashige and Skoog medium
ng	nanogram
N. benthamiana	*Nicotiana benthamina*
N. tabacum	*Nicotiana tabacum*
nm	nanometre
NOS	nopalin synthase
nptII	neomycin phosphotransferase
OD	optical density
ORF	open reading frame
PCR	Polymerase Chain Reaction
pMAS	mannopin synthase promoter
pNOS	nopalin synthase promoter
PPT	Phosphinotricin
PR-proteins	pathogenesis related proteins
PTGS	posttranscriptional gene silencing
RB	right border
RLU	relative light units
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SMG	selectable marker gene
SDS-PAGE	Sodium dodecyl sulfate polyacrylamid gelelectrophoresis
s	second(s)
t-CaMV	Cauliflower mosaic virus terminator
T-DNA	transferDNA
TGS	transcriptional gene silencing
Ti-Plasmid	tumour inducing plasmid
t-NOS	nopalin synthase promoter
µ1	microlitre
µM	microMolar
V	volume
vir	virulence

wpt	weeks post transformation
WT	wild type

1 INTRODUCTION

1.1 Overview

For nearly 20 years, efforts have been made to use plant cell cultures for recombinant protein production because they combine the merits of whole-plant systems with those of microbial and animal cell cultures (for a review see Hellwig et al., 2004). In addition, plant cells already have an established track record for the production of valuable secondary metabolites. Many proof-of principle studies have been published, most of them using well-characterised tobacco cell lines. In 2006, the first vaccine made from plant cell cultures received approval from the US Department of Agriculture (USDA) (Vermij and Waltz, 2006; Floss et al., 2007). This vaccine fights the Newcastle disease, a contagious and fatal viral disease affecting poultry.

However, production of recombinant proteins in plant cell culture still presents several challenges. The major difficulty is the instability of transgene expression due to chromosome loss (Kononowicz et al., 1990; Winfield et al., 1993), gene silencing (Chandler and Vaucheret, 2001; Dorokhov, 2007) or cell death (Weld et al., 2001).

As long as the efficiency of the currently available stable transformation methods is not 100%, selection and screening procedures are a major part of the regeneration of transgenic cells or plants (Gasparis et al., 2008). In a positive selection system the critical concentration of the selective agent promotes the growth of the cells carrying the selective marker gene and hinders the growth of non transgenic cells (Miki and McHugh, 2004). The precondition for a positive selection of transgenic cells is the stable expression of the marker gene during the whole selection process, because otherwise the growth of the cell culture would be limited. If the selectable marker gene (SMG) and the target gene are driven by different promoters, there is no correlation between the expression level of the selectable marker and the target gene. Expression instabilities could affect only the target gene even under selective pressure and continuous cell proliferation. Since the expression of the newly incorporated target gene is in most cases not essential for the plant cell, the target gene could be silenced without being detected if the marker gene expression is not affected. Gene Silencing occurs frequently in transgenic plant cell cultures. High transcription of transgenes either caused by integration of multiple copies of transgenes (Baulcombe and English, 1996; Depicker and Van Montagu, 1997; Vaucheret et al., 1998) or by high expression of a single transgene (Elmayan and Vaucheret, 1996) was often observed to be associated with posttranscriptional gene silencing

(PTGS). Another form of gene silencing was investigated by Fojtova et al. (2003). They reported that tissue culture leads to a switch from PTGS to TGS (transcriptional gene silencing), correlated with hypermethylation of the promoter.

The intention of this study was to investigate strategies aiming to overcome the challenge of expression instability by developing a model transformation system for the optimisation of protein production in plant cell cultures. To avoid different expression levels of selectable marker and target gene, dicistronic plant transformation vectors for coexpression of both genes were constructed and applied to establish transgenic tobacco cell cultures.

These newly designed vectors carry dicistronic transgene constructs which link a target gene with a reporter or selectable marker gene by an internal ribosome entry site (IRES element) in one expression cassette resulting in the coexpression of both genes under the control of the same promoter.

To investigate the functionality and applicability of our approach and for comparison of different selection modes and selective agents, two selectable marker genes were used instead of “real target genes” as first cistrons in front of an IRES-element in two distinct vectors. The two dicistronic transformation vectors harboured either the *bar* gene, providing resistance to Phosphinotricin, or the *PR10a* gene, providing increased salt tolerance. Both constructs carried another selectable marker gene under control of a NOS-promoter and the *luciferase* reporter gene as second cistron behind the IRES element. With these vectors it was investigated whether selection on the SMG under the same promoter or under a distinct promoter leads to higher and more stable protein expression of the reporter gene. The reporter gene, the light emitting *luciferase* gene, allowed not only easy expression monitoring but also enabled manual selection of highly expressing clones. The effect of chemical selection could therefore be compared to manual selection.

Finally, the potential of the IRES based constructs for the production of valuable proteins was tested with the approach to express the HSPX protein of *Mycobacterium tuberculosis* in tobacco cell cultures. HSPX, produced in *E. coli*, is already used in ELISA based diagnostic kits.

Objectives

The main objective of this study was

- To investigate the possibility to select high expressing cells in plant cell cultures based on the coexpression of target and selectable marker gene linked by IRES elements.

With the final aim

- To establish transgenic tobacco suspension cultures which express reliably a target protein in high quantities.

This was carried out by

- The establishment of a simple plant transformation system for the immediate transformation of plant suspension cultures,

- The construction and application of dicistronic model transformation vectors:
  In contrast to traditional plant transformation vectors carrying only one SMG and a target gene under control of different promoters, the newly designed transformation vectors carry a second SMG controlled by the same promoter as the reporter/target gene.

- The comparison of different selection strategies for the optimisation of production yield.
  To do so, transgenic cell cultures were exposed to different selective agents and characterized by measuring fresh and dry weight, protein content and Luciferase expression as an indicator for target gene production.

1.2 Plant cell cultures

Callus and suspension cultures consist of unorganised cell clusters, which can be achieved by exposing differentiated plant tissue to media containing plant growth regulators, also called plant "hormones" (Schieder in: Odenbach, 1997). Suspension cultures are prepared by agitation of friable callus tissue in shaker flasks to form single cells and small aggregates. Such cultures are widely used for fundamental research and have also been investigated as production sources of plant metabolites and – more recently – of recombinant proteins.

The German collection of microorganisms and cell cultures (DSMZ) in Braunschweig maintains presently more than 700 different plant cell lines from more than 80 different plant families for distribution. The priority of plant cultures in the collection are dicotyledonous angiosperms. In addition to crop plant cultures, the collection consists of a large number of cultures derived from plant species containing secondary metabolites of pharmaceutical importance. Most of the cultures are maintained for distribution in a living state as calli – some also as suspension cultures.

1.2.1 History of plant cell cultures

Attempts to cultivate isolated plant cells can be traced back to the early twentieth century, when in 1902 Haberlandt was the first to try to obtain experimental evidence of plant cell totipotency. The development of improved nutrient solutions, the informed choice of plant material and the appreciation of the importance of aseptic culture led to unlimited growth of plant tissues such as carrot root and tobacco stem tissues (Gautheret, 1939; Nobécourt, 1939 and White, 1939). Today, the most widely used medium for plant tissue culture is the MS medium, a completely defined nutrient solution (Murashige and Skoog, 1962).

The initial success in obtaining unlimited growth of cultured plant tissues by Gautheret and others had been limited to the use of explants containing meristematic cells. However, with the discovery of plant growth substances such as naturally occurring auxin (indole-3-acetic acid, IAA), synthetic auxin (2.4-dichlorophenoxyacetic acid, 2.4-D) and cytokinins such as kinetin, continued cell divisions could also be obtained in non-meristematic tissues (Kögl et al., 1934; Skoog and Tsui, 1948; Miller et al., 1955). Skoog and Miller (1957) contributed to understanding the role of plant growth substances in plant morphogenesis by demonstrating

the chemical regulation of growth and organ formation (shoots and/or roots) in cultured callus tissue.

Even more emphasis was put on the development of techniques for the propagation of plant cells in the 1950s, when it was realised that plant cell cultures had the potential to synthesize a variety of useful and valuable molecules (Gamborg, 2002).

Today, plant cells as well as tissues can be cultivated *in vitro* for many purposes in plant breeding, plant propagation and molecular biology. They are used for a broad range of applications in fundamental research such as the investigation of biochemical pathways including the formation of plant stress associated metabolites or subcellular localisation and transport of molecules (Schumacher et al., 1987; Grill et al., 1991). In applied research, efforts were made to use plant suspension cultures as a production platform for rare and valuable secondary metabolites and also for recombinant proteins, as an alternative for the production in the more expensive animal cell cultures or microbial systems lacking certain eukaryotic protein modifications (for a review see Heine-Dobbernack et al. in: Reed, 2008 and Vasil, 2008).

1.3 Plant cell cultures as a production platform for secondary metabolites

For plants, secondary metabolites play a crucial role in the competition for survival since they include compounds which are highly poisonous to animals and microorganisms, attract insects for pollination, or have attractive flavours or colours for fruit dispersal. Humans also benefit from these plant derived compounds, for example by applying the anti-tumour alkaloid Vinblastin or the triterpene Diosgenin which is used as the steroid skeleton in the synthesis of oral contraceptives (Wilson in: Dix, 1990). The concept to produce such valuable compounds by growing cell cultures in fermentation vessels offers many advantages: the production is independent from specific climatic conditions and it can be carried out under defined and sterile conditions. Extractable raw material can be produced faster with cell cultures than with intact plants, especially for plants like ginseng where the harvest starts many years after planting and destroys the plants.

Shikonin, a red-coloured antimicrobial compound, and paclitaxel (Taxol) are two examples of secondary metabolites produced in plant cell cultures on commercial scale (Hellwig et al., 2004). In these examples high yielding strains could be obtained which reliably produced the secondary metabolites.

In many cases these high yielding strains have been obtained by the selection of highly producing cells via manual selection. The basis for manual selection has been the colour of fluorescence of the desired compound, like the red colour of Shikonin, the light blue fluorescence of alkaloids or the colour of anthocyanins (Dougall et al., 1980). These colours or fluorescence made it possible to identify highly producing cell clusters and to pick them manually. Alternative approaches have used the plating of cells on conditioned media to obtain cell colonies derived from single cells (Schulte and Zenk, 1977) or the cultivation of single isolated cells in small medium droplets (Koop et al., 1983).

Nevertheless in many other cases the difficulty occurred that cell lines selected for a high capacity to produce a certain product tended to result in production instability. Often the expression instability occurred so quickly, that it turned out to be even impossible, to grow a large volume culture from a small inoculum (Wilson in: Dix, 1990). Deus-Neumann and Zenk (1984) reported an example of the instability of alkaloid production in *Catharanthus roseus*. Loss of productivity occurred during the first few months of cultivation. Recovery of high alkaloid yielding strains was, however, possible at any time by repetition of the clonal selection procedure but the resulting strains were again unstable.

The observation of stable as well as instable cell culture strains caused a strong and finally unsolved debate in the 1980´s, as to whether the basis of the observed instabilities were caused by genetic mutations or by changes in gene expression.

At the same time evidence of the occurrence of mutations in *in vitro* cultures caused or increased by hormone application came from *in vitro* cultures of differentiated tissues and plantlets. In 1981, Larkin and Scowcroft observed the occurrence of aberrant plants among plants regenerated from *in vitro* culture and termed this phenomenon “somaclonal variation”. Later different types of genetic changes could often be shown to be the reason for somaclonal variation. More recently also epigenetic changes were demonstrated to cause somaclonal variants (Kaeppler et al., 2000). The question, whether in dedifferentiated cell cultures genetic or epigenetic changes dominate and to which extent, is still considered to be open.

1.4 Plant cell cultures as a production platform for recombinant proteins

The first recombinant protein produced in plant cells was human serum albumin reported by Sijmons et al. in 1990. Since this initial demonstration, over 20 different recombinant proteins have been produced in plant cell cultures, including antibodies, enzymes, hormones, growth factors and cytokines (for a review see Hellwig et al., 2004).

Plant cell cultures are inexpensive to grow and maintain and because of the experience with the production of secondary metabolites in cell cultures, the infrastructure and expertise already exists for growing, harvesting and processing of plant material. Plant cells also bear no risks concerning biosafety – such as the spreading of antibiotic resistance genes, because they are cultured in closed containers (Horn et al., 2004). Furthermore, they neither harbour human pathogens nor produce endotoxins which could contaminate the final product.

Post-translational modifications of plant proteins like the correct folding and activity generally resemble those of mammalian proteins. However, the glycosilation pattern differs and certain plant-specific protein-linked sugars are immunogenic in humans (Gomord et al., 2005; Decker and Reski, 2007).

The procedure for product isolation and purification in plant cells is simpler than in whole plants – especially when the product is secreted into the culture medium (Hellwig et al., 2004). Even if the target proteins are not excreted, the dark grown cell cultures lack photosynthetic proteins and pigments, which in plants can complicate downstream processing. Using fermentation vessels, good manufacturing practise (GMP) can be implemented throughout the whole production pipeline.

The challenges associated with suspension cells include the formation of aggregates, the tendency for cells to adhere to the walls of the fermenter vessel, somaclonal variation and gene silencing (Offringa et al., 1990; Meijer et al., 1994; Yu et al., 1996). Nevertheless, the two most important drawbacks of protein production in plant cell cultures are the low protein concentration in plant cells compared to plant storage organs and the genetic instability of the target protein expression, which in the most extreme case could even lead to the loss of protein productivity (James and Lee, 2005).

1.5 Genetic modifications of plant cell cultures

Three methods are frequently used for the introduction of foreign genes into plants (for a review see Vasil, 2008):

(1) Indirect or vector-based gene transfer, such as *Agrobacterium tumefaciens*-mediated transformation (Van Haute et al., 1983; Hoekema et al., 1983)

(2) Direct DNA delivery into protoplasts by osmotic or electric shock (Cocking, 1960; Takebe et al., 1971).

(3) Direct DNA delivery into intact cells or tissues by high velocity bombardment of DNA-coated microprojectiles (Sanford, 2000).

The latter two transformation methods were developed as an alternative to *Agrobacterium*-mediated transformation, because *A. tumefaciens* naturally infects only dicotyledonous plants. The absence of wound response and the associated activation of virulence genes was one of the major barriers to use *Agrobacterium* to transform monocots. This problem could be overcome by the addition of acetosysringone, a potent inducer of virulence genes, and led to the first successful *Agrobacterium*-mediated transformation of rice and then other cereals (Hiei et al., 1994).

1.5.1 *Agrobacterium* – mediated gene transfer

Since *Agrobacterium* – mediated gene transfer is the most promising method to achieve a simple transformation procedure which is applicable to a large volume of cell culture material, plant cell cultures in this study were genetically engineered by *Agrobacterium* - mediated transformation. In contrast, both other methods of direct gene transfer would have been applicable only to a small number of cells, because of the time-consuming and complicated preparation of protoplasts and samples for the gene gun method respectively.
Wild type *Agrobacterium tumefaciens* has the ability to transfer a particular DNA segment (T-DNA), located on extrachromosomal DNA and therefore it is interchangeable, into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (for reviews see Gelvin, 2003 and Citosky et al., 2007). The process of gene transfer from *Agrobacterium tumefaciens* to plant cells involves several essential steps. First wounding of the plant is necessary to allow entrance of bacteria and to make available compounds that induce the bacterial virulence system. The attachment of the bacteria to the plant cell wall is induced by small phenolic compounds such as acetosyringone. The processing and transfer of the T-DNA are mediated by products encoded by the *vir* (virulence) region, which in wild type bacteria is resident on the Ti-plasmid. The activation of the *vir* genes generates single-stranded (ss) molecules representing the copy of the bottom T-DNA strand. With these single stranded molecules other *vir* encoded proteins form a ss-T-DNA protein complex containing two plant nuclear location signals, so that this complex is translocated through the bacterial membrane, the plant cell wall and its membrane, cellular spaces and then is targeted to the plant nucleus crossing the nuclear membrane. Another *vir* gene product finally plays a role in the precise integration of the T-DNA strand into the plant chromosome.

1.5.2 Plant transformation vectors

Any DNA placed between the borders of the T-DNA of *Agrobacterium tumefaciens* is integrated into the plant genome. Plant transformation vectors based on the Ti plasmid were constructed after removing the genes for tumour formation and opine biosynthesis. Only the 25 bp imperfect repeats forming the boundaries for the T-DNA and the *vir* region remained. These two main components for gene transfer, the T-DNA borders and the *vir* region, can reside on separate plasmids.

1.5.2.1 Binary plasmid vectors of the pGreen-family

The plant transformation vectors used in this work were constructed based on the binary Ti vectors of the pGreen series (Hellens et al., 2000). The *vir* gene functions are provided *in trans* by a disarmed helper Ti plasmid resident in the *Agrobacterium tumefaciens* strain EHA 105 (An, 1985). The used vectors have the ability to replicate both in *E. coli* and *A. tumefaciens*. To reduce the plasmid size the *replicase* gene for *Agrobacterium* (*rep A*) is resident on a compatible plasmid (pSoup) and provides pGreen replication *in trans*. In the binary pGreen vector system, the pGreen vector itself contains a selectable marker and the minimum *cis*-acting sequences required for transformation, i.e. the T-DNA border sequences. Plasmid pGreen 0229 contains the *bar* gene (pGII 0229) and plasmid the pGreen 0029 the *nptII* gene (pGII 0029) under control of a nopalin-synthase-promoter (p-NOS) for selection in plants.

1.5.2.2 Dicistronic vectors and IRES-Elements

The transformation vectors used in this work are dicistronic vectors in which a reporter gene is coexpressed with a gene of interest, in our case a selectable marker gene, by cap-independent translation via an internal ribosome entry site (IRES). IRES-elements are specific sequences, usually of several hundred nucleotides, that can directly recruit ribosomes to internal positions within mRNAs and initiate translation in a cap-independent manner (Halpin, 2005). IRES-elements from different viruses have been tested and shown to function in plant systems (Urwin et al., 2000; Dorokhov et al., 2002). In this work the 148 nucleotide sized region upstream the coat protein gene of the crucifer-infecting Tobamo-Virus (TMV)

was used as IRES-element. The functionality of such constructs has been demonstrated to function in tobacco cells (Dorokhov et al., 2002) and tobacco plants (Ali, 2007).

1.5.3 Marker genes

1.5.3.1 Selectable marker genes (SMGs)

Despite the large number of selectable marker genes (SMGs) that exist for plants, only a few marker genes are preferentially used for plant research and crop development. Two of the most frequently used selectable marker genes are the Kanamycin resistance gene (*npt II*) and the Phosphinotricin resistance gene (*bar*), with which in most cases good results have been obtained (Miki and McHugh, 2004). Therefore these genes have also been chosen for the present study as selectable markers for construction of our dicistronic transformation vectors.

The *nptII* resistance gene confers resistance to the antibiotics Kanamycin or G418. Gentamicin derivative G418, also known as geneticin, belongs to the group of aminoglycoside antibiotics. These antibiotics inhibit protein biosynthesis by blocking the ribosomal subunits both in bacteria and eukaryotic cells. The *nptII* gene codes for neomycin phosphotransferase II, which catalyses the ATP-dependent phosphorylation of the 3´-hydroxyl group of the amino-hexose portion of certain aminoglycosides as G418 (Miki and McHugh, 2004).

The *bar* gene codes for the enzyme phosphinotricin acetyl transferase (PAT) and inactivates the herbicide Phosphinotricin (PPT) by acetylation (Botterman et al., 1991). L-Phosphinotricin is a glutamate analogue that inhibits glutamine synthase (Manderscheid and Wild, 1986). Glutamine synthase is the only enzyme that can catalyse the assimilation of ammonia into glutamic acid in plants. Inhibition of glutamine synthase ultimately results in the accumulation of toxic ammonia levels resulting in plant cell death.

As an alternative to antibiotic and herbicide resistance genes, the effect of the *PR10a* gene as selectable marker was tested. In *Brassica napus* expression of a recombinant pea *PR10a* gene was reported to enhance germination and growth in the presence of sodium chloride (Srivastava et al., 2004). Increased salt tolerance of tobacco and potato cell cultures overexpressing PR10a was also achieved in experiments of El Banna at DSMZ in 2008. Nevertheless, the mechanism of PR10a to provide salt tolerance is still unknown.

High salinity causes osmotic stress in plant cells because it influences photosynthesis and cell energy supply negatively by destroying the intracellular ion gradient (Wang et al., 2003).

PR10 proteins have been identified by induced expression in response to pathogen infection (Lamb et al., 1989). Later induction was also observed after abiotic stresses such as drought and salinity.

1.5.3.2 Reporter genes

Reporter genes are commonly used in molecular biology to visualize and study gene expression and activity of target proteins. In this study all selectable marker genes were linked to a reporter gene by an IRES-element and were compared in respect to the expression of this reporter gene. To the most commonly used reporter genes belong the genes encoding for the green fluorescent protein (GFP) or for the enzymes β-Glucuronidase (GUS) and Luciferase (LUC). Whereas destructive assays are necessary to measure the activity of GUS, Luciferase activity can be monitored in living tissue and is a more sensitive method than GFP. Furthermore, the lack of endogenous activity is an advantage of the Luciferase assay system.
So as reporter gene the *luciferase* gene from North American firefly (*Photinus pyralis*) was chosen. Luciferase catalyzes the ATP and Mg^{2+} dependent oxidation of the substrate luciferin to oxyluciferin. In this bioluminescent reaction light is emitted. The light emitting Luciferase reaction enables to manual selection by monitoring and picking of luminescent cells in addition to chemical selection by SMGs.

In the present study it has been investigated whether the described transformation tools can be applied to establish a cell culture expressing a recombinant protein, which forms the basis for selecting a high yielding strain. A recombinant protein is usually difficult to detect, because high producing cell lines mostly cannot be recognized due to any visible characteristics for manual selection. Therefore the coexpression with the *luciferase* gene should make high expressing cells visible. Even more the coexpression of the target protein with a selectable marker gene providing a certain resistance should allow a continuous selection on chemical basis. This concept would not only allow the establishment of high yielding strains, but also their stabilisation during a fermentation process.

2 MATERIALS AND METHODS

2.1 Material

2.1.1 Technical equipment

Balances:	IKA big-squid, IKA-Werke, Staufen, Germany
Centrifuges:	Biofuge 28RS, Heraeus Sepatech, Osterode, Germany
	Centrifuge 5415 C, Eppendorf, Hamburg, Germany
	Multifuge 1S-R, Heraeus, Osterode, Germany
	Table centrifuge Mini Spin Plus, Eppendorf, Hamburg, Germany
Electrophoresis equipment:	Ettan Daltsix electrophoresis unit, Amersham Biosciences
	LKB 2117 Multiphor II electrophoresis unit
	LKB Bromma 2197 Power supply
	Electrophoresis power supply EPS 301 and EPS 3501 X4
	each Amersham Biosciences AB,Uppsala, Sweden
Electroporator:	Equibio EasyjecT optima, promega, Madison, USA
Gyratory shaker:	TR-250, Infors AG, Bottmingen, Switzerland
Heater & stirrer:	Ikamag RH, Janke & Kunkel, IKA Labortechnik, Staufen, Germany
Hybridization incubator:	Biometra OV1, Göttingen, Germany
Incubators:	Memmert Universaltrockenschrank ULM 400 and U 600, Schwabach, Germany
Luminescence image analyzer:	LAS 3000, Fuji Germany, Düsseldorf, Germany
Luminometer:	Lumat LB9501, berthold technologies, Wildbad, Germany
Microscope:	Jenaval, Carl Zeiss AG, Oberkochen, Germany
Multifunctionreader:	Tecan Multifunctionreader GENios, Tecan GmbH, Crailsheim, Germany
pH-Meter:	pH-MV-Meter, PH522, WTW, Weilheim, Germany
Photometer:	Biophotometer, Eppendorf, Hamburg, Germany
	DPU-414 Thermal Printer, Eppendorf, Hamburg, Germany
Rocking platform:	WT 15, Biometra, Göttingen, Germany

Safety cabinet:	Herasafe, KS 18, Thermo fisher scientific, Waltham, USA
Thermoblock:	TB1, Biometra, Göttingen, Germany
Thermoshaker:	Thermomixer comfort 5355 R, Eppendorf, Hamburg, Germany
Thermocyclers:	Primus 96plus Thermocycler, MWG-biotech, Martinsried, Germany
	T-Gradient Thermoblock, Biometra, Göttingen, Germany
	T-Personal Thermocycler, Biometra, Göttingen, Germany
Vortexer	Vortex Genie 2, Scientific Industries, Bohemia, USA
Water bath	GFL 1083 Schüttel-Wasserbad, BLB, Braunschweig, Germany

2.1.2 Ready-to-use solutions and kits

2D-Protein Quant Kit	80-6483-56; Amersham Biosciences AB, Uppsala, Sweden
6x Loading Dye Solution	Fermentas R0611, St. Leon-Rot, Germany
Anti-Digoxigenin-AP Fab Fragments 150U	Roche 11093274910, Roche Diagnostics GmbH Mannheim, Germany
ChargeSwitch gDNA Plant Kit	CS18000, Invitrogen GmbH, Karlsruhe, Germany
CSDP	Roche Diagnostics GmbH, Mannheim, Germany
DIG Easy Hyb Granules	Roche Diagnostics GmbH, Mannheim, Germany
DIG-labeling probe PCR Kit (vial 2 PCR DIG synthesis mix),	Roche Diagnostics GmbH, Mannheim, Germany
DNA Extraction Kit	Fermentas K0513, St. Leon-Rot, Germany
Gene Ruler 100 bp DNA Ladder Plus	Fermentas SM0323, St. Leon-Rot, Germany
Gene Ruler 1 kB DNA Ladder	Fermentas SM0313, St. Leon-Rot, Germany
Luciferase Assay System E1500	Promega, Madison, USA
MinElute PCRPurification Kit	Qiagen, Hilden, Germany
StrataClean Resin	Stratagene 400714, La Jolla, USA

2.1.3 Chemicals

2.4-D (Dichlorphenoxyacetic acid)	M = 221.04 g/mol	Merck 820451
Acetic acid glacial	M = 60.05 g/mol	Roth 3738
Acetosyringone	M = 196.2 g/mol	Roth 6003
Acrylamide 4K-solution (30%) Mix 32:1		AppliChem A0947
Agar		Merck 1.01614
Agarose NEEO ultra quality		Roth 2267
Ammonium acetate	M = 77.08 g/mol	Merck 1116
Ampicillin sodium	M = 371.4 g/mol	Duchefa A0104;
APS, ammonium persulfate	M = 228.2 g/mol	AppliChem A2941
BCIP-T, 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt		Fermentas R0821
Bromphenol blue	M = 691.9 g/mol	Sigma B5525
Casein enzmatic hydrolysate NZ amine A		Sigma C7290
Chloroforme	M = 119.38 g/mol	JTBaker 7836
CTAB, cetyl trimethyl ammonium bromide		Serva 16530
DMSO, dimethylsulfoxide	M = 78.1 g/mol	Duchefa D1370
DTT, 1.4-dithiothreitol	M = 154.24	Merck 1.11474
Disodiumhydrogenphosphat heptahydrat	M = 268.03 g/mol	Roth X987.2
EDTA disodiumsalt dihydrate	M = 372.24 g/mol	Roth 80432
(Ethylenediamintetraacetic acid)		
Ethanol absolute	M = 46.07 g/mol	JTBaker 8006
Ethidium bromide solution 1%		Fluka 46067
G 418 disulfat	M = 692.7 g/mol	Duchefa G0175
Glycerol 86% p.a.	M = 92.1 g/mol	Roth 4043
Hydrochloric acid HCl 1N solution (1 mol/l)		Roth K025.1
Hydrochloric acid 37%	M = 36.49 g/mol	Roth 4625
IAA (Indole-3-Acetic acid)	M = 175.19 g/mol	Merck 353
Isoamyl alcohol	M = 88.15 g/mol	Merck 979
Kanamycinsulfate	M = 582.58 g/mol	Roth T832.2
Kinetin	M = 215.2 g/mol	Sigma K2875
LB Broth High Salt		Duchefa L 1704
LB Broth Low Salt		Duchefa L 1703

LS salt and vitamins		Duchefa L 0230
Luciferin, potassium salt		synchem bc 219
N-Lauroyl-Sarcosin	M = 293.4 g/mol	Fluka 61745
MES Monohydrat		Duchefa M 1503
MS salt and vitamins		Duchefa M 0222
Magnesium chloride hexahydrate	M = 203.3 g/mol	Merck 1.05833
Maleic acid	M = 116.07 g/mol	Roth K304
ß-Mercaptoethanol	M = 78.13 g/mol	Merck 12006
NAA (1-Naphtylessigsäure)	M = 186.21 g/mol	Merck 806862
NBT (nitro blue tetrazolium chloride)		
Ortho-phosphoric acid 85%	M = 98 g/mol	Fluka 79617
Potassium chloride	M = 74.56 g/mol	Fluka 60130
PPT, DL-phosphinotricin	M = 198.2 g/mol	Duchefa P0159
PVP 40, polyvinylpyrrolidine	M = 40 g/mol	Sigma
Plant agar		Duchefa P 1001
Saccharose	M = 342.3 g/mol	Duchefa S0809
SDS, sodium dodecyl sulphate	M = 288.38 g/mol	Sigma L4390
Sodium carbonate anhydrous	M = 105.99 g/mol	Roth A135.2
Sodium chloride	M = 58.44 g/mol	Roth 3957.1
Sodium hydroxide	M = 40.01 g/mol	Roth 6771
Sodiumdihydrogenphosphate monohydrate	M = 137.99 g/mol	Merck 63461000; l
Sodium phosphate	M = 268.1 g/mol	Sigma S9390
tri-Sodium citrate 2-hydrate	M = 294.1 g/mol	Roth 3580.1
Strata Clean Resin		Stratagene 400714-61
TEMED, N,N,N´,N´-tetramethylethylenediamine	M = 116.21 g/mol	AppliChem A1148
Ticarcillin disodium mixture 15:1		Duchefa T0190
Trizma base minimum	M = 121.14 g/mol	Sigma T1503-1KG
TRIS HCl	M = 157.5 g/mol	Roth 90902
Tryptone		Duchefa T1332
Tween 20	M = 1227.72 g/mol	AppliChem A4974
X-Glc A	M = 521.8 g/mol	Duchefa X1405

2.1.4 Plant material

For this study *Nicotiana tabacum* L. strain: Bruessel (PC 120, DSMZ) suspension cultures were used. 100 ml of the suspension cultures were cultivated in 300 ml Erlenmayer flasks on a gyratory shaker at 100 rpm (TR-250, Infors AG, Bottmingen, Switzerland) in liquid LS medium (Appendix I). Callus cultures were initiated by spreading an aliquot of suspension cultures on solid LS medium. Suspension and callus cultures were grown at 23°C. The suspension culture was subcultured weekly by transferring 50 ml of cell suspension to 50 ml fresh LS medium. Callus cultures were transferred to fresh medium at 4 week-intervals.
Nicotiana benthamiana D. plants were used in transient expression assays to test the vector functionality.

2.1.5 Bacteria

Cloning work was carried out with the *Escherichia coli* strain GM2163. For plant transformation the *Agrobacterium tumefaciens* strain EHA105 was used. This strain already contains a helper plasmid carrying the *vir*-region. Since we worked with the binary vector system of the pGreen vector family in this *Agrobacterium* strain a plasmid named pSoup was also incorporated. Plasmid pSoup harbours the gene for *Agrobacterium* replication and a tetracyclin resistance gene (Hellens, 2000).
Overnight cultures of both bacterial strains were inoculated from glycerol stocks (1 ml of bacterial suspension with OD (600 nm) 0.8 to 1.0 in 0.5 ml 87% glycerol) and grown in LB medium (Appendices II and III).

2.2 Methods

2.2.1 PCR based cloning

Table 1: DNA sequences of cloning primers containing the respective restriction sites

Cloning primers			
Target gene	Name	Sequence	Size of PCR product
bar gene from *Streptomyces hygroscopius*	bar XmaI forw	5´-AAA CCC GGG ATG AGC CCA GAA CGA CGC C-3´	552
	bar HindIII rev	5´-TTT AAG CTT TCA GAT TTC GGT GAC GGG C-3´	
nptII gene kanamycin resistence gene	nptII NotI	5´-AAA GCG GCC GCA TGA TTG AAC AAG ATG GAT TGC-3´	792
	t-nos SacI	5´-AAA GAG CTC TAT CAG CTT GCA TGC CGG-3´	
hspx gene from *Mycobacterium tuberculosis* behind MAS Promotor	hspx Kpn21 forw	5´-GGA TCC GGA ATG AAG ACT AAT CTT TTT CTC TTT C-3´	
	hspx HindIII rev1	5´-GAC AAG CTT TTA GTT GGT GGA CCG GAT C-3´	489
	hspx HDEL HindIII rev2	5´-GAC AAG CTT TTA AAG CTC ATC ATG GTT GG-3´	500
	hspx HindIII HIS rev3	5´-GAC AAG CTT TTA GTG GTG GTG GTG GTG GTG GTT GGA CCG GAT C-3´	528

The DNA sequences of the respective target genes were amplified via a polymerase chain reaction (PCR) by using primers containing the respective restriction sites (Table 1). For PCR the proof-reading enzyme High Fidelity Polymerase was used. All components and the amplification program of the PCR are listed in Tables 2 and 3. PCR fragment amplification was performed in Biometra thermocyclers. The PCR product was purified by the MinElute PCR-Purification Kit according to the manufacturer's manual.

Tables 2 and 3: Components and amplification programme for proof-reading PCR.

Cloning PCR by High Fidelity Polymerase (Fermentas)		
High Fidelity Puffer	5	µl
10 mM dNTPs	1	µl
Primer forward 100 pmol	1	µl
Primer reverse 100 pmol	1	µl
High Fidelity Polymerase	0.3	µl
H_2O	41	µl
Template DNA	1	µl
Total	50	µl

Cloning PCR programme		
94°C	5 min	
94°C	45 s	} 30 loops
58°C	45 s	
72°C	45 s	
72°C	5 min	
8°C	∞	

After ligation of the PCR fragment with the vector, the plasmid was incorporated by heat-shock-transformation into competent *E. coli* cells for amplification. Plasmid-DNA was obtained by making clear lysates and was tested and verified by control restriction digest or PCR. Plasmid-DNA of positive tested *E.coli* was transferred into electro-competent agrobacteria by electroporation. *E. coli* and *Agrobacterium* cells containing the different vectors were stored for long term purposes as glycerol stocks.

2.2.1.1 Restriction digest and fragment separation by agarose gel electrophoresis

DNA was digested using different restriction endonucleases with the buffers as recommended by the respective supplier. Afterwards the enzymes were inactivated by heating them for 15 min at a minimum of 65°C, depending on the enzyme.
The restricted DNA was separated and purified by agarose gel electrophoresis (1% agarose in 1x TAE buffer: 40 mM tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 7). The wanted DNA fragment was cut out of the gel and then extracted by using the DNA Extraction Kit (Silica K0513) according to the manufacturer´s manual.

2.2.1.2 Transfer of the transformation vector into bacteria cells

Preparation of heat-shock competent *E. coli*

For amplification and storage, the new transformation vectors were transferred into *Escherichia coli*, strain GM 2163. For the uptake of foreign DNA bacteria must be made competent. This was achieved by chemical treatment.
An *E. coli* culture was grown overnight in 1 to 5 ml LB medium (Appendix III). On the next morning the culture was diluted 1:50 in fresh LB medium and grown at 37°C until an OD (600 nm) of approximately 0.4 was achieved. The bacteria were harvested by centrifugation at 4°C and 4400 rpm and resuspended in ½ volume ice-cold 100 mM $MgCl_2$ and centrifuged again. The supernatant was discarded and the pellet was resuspended once again in ½ volume ice-cold 100 mM $CaCl_2$. The bacteria were again centrifuged to collect the pellet, which was resuspended in 1/10 volume ice-cold 100 mM $CaCl_2$. Then the bacterial suspension was kept at 4°C for one hour. The competent cells were either used immediately for heat shock transformation or were stored. For preparation of a storage culture, 86% sterile glycerol was added to reach a final concentration of 15% glycerol. Then 100 µl aliquots of the suspension

were transferred into 1.5 ml reaction tubes, which were filled with liquid nitrogen, and were afterwards stored at -80°C for further use.

Heat shock transformation

For heat-shock transformation a frozen aliquot of competent *E.coli* was put on ice for thawing. Then 2 µl of the ligation product were added to the bacterial suspension and both were mixed by pipetting the liquids up and down. The plasmid-cell-mixture was incubated for 20 min on ice. Then the cells were heated at 42°C for 40 s in a heat block and cooled down on ice immediately, where they were kept for two more minutes until 900 µl of sterile SOC medium (Appendix IV) were added. Under agitation the plasmid-cell mixture was then incubated for at least 90 min at 37°C on a shaker. Finally 50 µl and 400 µl of the cells were spread on LB plates containing the respective antibiotics for selective growth of the transformed bacteria cells. The bacteria culture was incubated at 37°C overnight.

Isolation of plasmid DNA

For plasmid isolation 2 ml of an overnight culture grown out of a single bacterial colony were centrifuged for 5 min in a table centrifuge at maximum speed (14500 rpm). Afterwards the supernatant was discarded and the cells resuspended in 200 µl of solution A (15 mM trisHCl, pH 8, 10 mM EDTA, 50 mM glucose) by pipetting them up and down in order to break the cell walls. Then the cells were kept for 15 min at RT. For cell lysis 400µl of solution B (0.2 M NaOH, 1% SDS) were added and mixed by inversion of the tube. Immediately, 300 µl of solution C (3 M NaOAc, pH4.8) were added and the tube was again mixed by inversion. The cell extract was then incubated on ice for 10 min. Afterwards the tubes were centrifuged for 10 min and the supernatant was transferred into a new 1.5 ml tube and if necessary the centrifugation step was repeated. 800 µl of the supernatant were then transferred to a new tube and mixed with 600 µl ice-cold isopropanole (stored at -20°C). The solutions were mixed by inversion to precipitate DNA and centrifuged immediately. The supernatant had to be removed completely and the remaining dry pellet was dissolved in 200 ml of solution D (0.1 M NaOAc, ph 7, 0.05 M trisHCl pH8). For purification of the DNA 400 µl of absolute ethanol were added. The pellet must float in the ethanol. After a 10 min centrifugation step, the ethanol was removed completely and the DNA pellet was dried at 37°C or in a speedvac.

Finally, the pDNA was resuspended in 50 µl TE-buffer (10 mM tris-HCl, pH 8.0, 1 mM EDTA) or pure water, substituted with 1 µl RNAse (1mg/ml).

Preparation of electro competent agrobacteria

An overnight *A. tumefaciens* culture was grown in 20 ml LB (Appendix II) supplemented with the respective antibiotics. On the next morning the overnight culture was transferred in 200 ml fresh LB medium and was grown at 28°C to an OD (600 nm) of 0.5 to 1. Then the bacteria were centrifuged twice at 4°C for 15 min at 3000 g and resuspended in 100 ml of ice-cold 10% glycerol. After another two centrifugations the pellet was resuspended twice in 10 ml ice-cold 10% glycerol and finally dissolved in 5 ml ice-cold 10% glycerol. Aliquots of 50 µl competent agrobacteria were frozen by using liquid nitrogen. The aliquots were stored at -80°C.

Electroporation of competent agrobacteria

50 µl suspension of the competent *Agrobacteria* were mixed with 1 µl of plasmid DNA on ice. The mix was transferred to a precooled cuvette (0.2 cm gap, peqlab biotechnology GmbH) and a brief electric impulse was applied in a BioRad electroporator adjusted to the following parameters 2500 V; 15 µF; 335 R; 5.0 ms. Immediately after the electric impulse 900 µl precooled SOC (Appendix IV) were added to the competent cells and plasmid. The mixture was incubated for at least three hours at 28°C on a shaker. 100 µl of pure, 1:10, 1:100 and 1:1000 diluted bacteria were spread on solid LB medium with the respective antibiotic selection. Plates were grown at 28°C and colonies were picked after 24-48 hours of incubation time.

2.2.2 Transformation of plant material

Prior to each transformation overnight cultures of the *Agrobacterium* strain containing the target plasmids were grown. The optimal OD (600 nm) lays in the growing phase of bacteria at a value of between 0.6 and 1. *Agrobacterium* suspension was harvested by centrifugation (10°C, 4.000 g for 10 min), thereafter the pellet was resuspended in the same volume of respective plant medium plus 100 µM acetosyringone and incubated for at least half an hour.

2.2.2.1 Leaf infiltration of *Nicotiana benthamiana* plants

For transient studies of vector functionality *Nicotiana benthamiana* plants were infiltrated with the *A. tumefaciens* strain EHA 105 carrying the respective transformation vector. The bacterial cell pellet was resuspended in MMA (Appendix V) plus 100 µM acetosyringone.
Then the *Agrobacteria* suspension was pulled up in a syringe without a needle. Plant leaves were mechanically infused by pressing the tip of the syringe against the lower surface of the leaf by applying only gentle pressure to avoid destroying the leaf tissue. To avoid desiccation during 2 days coculture time, plants were wrapped with aluminium foil. Then they were stored at 19°C in a dark incubation chamber.

2.2.2.2 Leaf disc transformation of *Nicotiana tabacum* strain SR1 plants

A single leaf was put in a glass petri dish, the midrib was removed and the rest of the leaf was cut into small pieces of around 1 cm² size. Leaf disks were incubated in 50 ml MS liquid (Appendix VI) and 500 µl *Agrobacterium* suspension in a Weck jar for 15 to 20 min with slight agitation.
Then leaf disks were dried on Whatman filter paper and put with their reverse side up (stomata facing up) on MS 1 (Appendix VII) solid medium without any selection. Leaf discs were incubated for 2 days in the culture room.
After two days the coculture period was finished by washing the leaf discs in a 50 ml falcon tube containing 40 ml MS liquid with 160 µl ticarcillin out of a stock of 200 mg/ml (final concentration: 800 mg/l ticarcillin). Leaf disks were dried shortly on Whatman filter paper, then placed on MS solid containing 400 mg/l ticarcillin, to get rid of agrobacteria. The wounded leaf margins were in direct contact with the medium. Leaf discs were incubated for one week in the culture room.
After one week leaf discs were transferred to MS 1 solid medium containing 400 mg/l ticarcillin and respective selective agents (for example PPT). From here on leaf discs were subcultured when necessary (around every two weeks) with a stepwise reduction of the ticarcillin concentration to a minimum of 100 mg/l.
As soon as callus of around 0.5 cm Ø was formed, calli were transferred to MS 2 medium (Appendix VIII) containing 200 mg/l ticarcillin and respective selective agents. Regenerated shoots and leaves were transferred on MS0 medium containing 100 mg/l ticarcillin and respective selection in Magenta boxes and later each single plantlet in Weck jars.

2.2.2.3 Transformation of *Nicotiana tabacum* strain Bruessel callus

Half of a four-week-old callus culture was incubated in 10 ml plant medium and 50 µl of agrobacteria suspension in a 90 mm petri dish for 5 to 15 min while shaking slightly. Then the coculture period was continued in the same petri dish for two days at room temperature in the dark without shaking.

For terminating the coculture period the calli were taken out of the liquid medium and dried on Whatman filter paper for one minute. Afterwards the calli were spread on solid plant medium containing 300 mg/l ticarcillin and other selective agents.

2.2.2.4 Transformation of *Nicotiana tabacum* strain Bruessel suspension cultures

50 ml (100 ml) of four-day-old suspension cells were transferred into a suction strainer (Buchner funnel) of 50 mm in diameter (60 mm), whose base was covered with a small-pored gauze. Liquid medium was removed by a vacuum pump. The now dried suspension cells were wetted with 3 ml (6 ml) plant medium (for control) or *Agrobacterium* suspension.

The Buchner funnel was covered with the lid of a 60 mm petri dish or aluminium foil and wrapped additionally with parafilm. The coculture period lasted for three days in the dark at room temperature.

For terminating the coculture, plant cells were washed three times with each 50 ml plant medium containing 150 mg/l ticarcillin. To obtain a homogeneous mixture of transformed and not transformed plant cells, the suspension culture was transferred into a sterile flask during washing and mixed by adding plant medium and shaking. To separate the washing medium from the plant cells, the mixture was poured back into the suction strainer.

For further subculture 100 mg (spade-full) cells were placed on solid medium with respective antibiotics, the rest of the suspension culture was transferred into a flask and a new suspension culture was started by adding a minimum amount of liquid plant medium – first without selection, but then selection was started by increasing the selective agent from 3 mg/l to 6 mg/l.

2.2.3 Maintenance and characterization of cell cultures

2.2.3.1 Maintenance

Callus cultures which were maintained in microtiterplates, were subcultured every four weeks by picking 1 to 2 mm sized pieces of callus and transferring them onto fresh medium (1 ml in each well of a 24-well microtiterplate). When the transgenic calli contained a *luciferase* gene, cell clusters of high Luciferase activity – screened with a Fuji Imager - were preferentially transferred.

Suspension cultures were subcultured weekly, by dividing the 100 ml cell suspension into two parts, by pouring 50 ml of a one-week old suspension culture into a new 300 ml flask which was refilled to 100 ml with fresh medium. Just the medium was changed, when a newly started suspension culture was too diluted to be separated. Therefore the one-week old medium was discarded after the cells had settled down and the flask was refilled with fresh medium.

2.2.3.2 Fresh and dry weight determination of cell material

For fresh weight determination, cells were transferred on preweighed filter papers in petri dishes. The petri dishes with the cell material were first weighed, then the cells were dried at 60°C. After 72 hours the cells on the preweighed filter papers were weighed again for determination of the dry weight. The water content of the cell culture was calculated as (FW – DW) / FW * 100%.

Statistical Analysis

Statistical data analysis was made for the calculation of the water content value of callus cultures. All transformations were performed twice and fresh and dry weight of eight samples was measured per transformation and subculture. Just the water content values of callus cultures transformed with the PR10a construct and calculated ten wpt are based upon only four samples. The data was analysed by one way ANOVA or ANOVA on ranks using the software Sigma Stat® 3.1. Multiple comparison procedures were made against a control group (non-selected callus cultures) using the Holm-Sidak or Dunn´s method. The term

significant was used to indicate differences for which P≤0.05. The boxplots were generated by using Sigma Plot® 9.0 software. The boxes mark the 25% and 75% percentile as well as the median. The whiskers mark the 5% and 95% percentile and the dots indicate the outliers.

2.2.3.3 Protein quantification

Protein concentration was determined by absorption measurement against a bovine serum albumin standard. Therefore the 2-D Quant Kit of Amersham Bioscience was used according to the manufacturer´s instructions (Amersham Biosciences AB, Uppsala, Sweden). Absorption measurement was done with duplicates or triplicates in 96 well microtiter plates in a Tecan Genios multiplate reader (Tecan GmbH, Crailsheim, Germany).

2.2.3.4 Monitoring of the Luciferase activity

Quantitative Luciferase assay

The functionality of the constructs used in this study was tested by monitoring the expression of the *luciferase* gene by a quantitative assay for Luciferase enzyme activity. The Promega Luciferase assay Kit (Promega, Madison, USA) was used. For measurement of Luciferase activity, plant material (leaves, cell suspension, callus) was harvested, frozen quickly in liquid nitrogen, ground to powder under liquid nitrogen using mortar and pestle. 100 mg of powdered cell material was resuspended in 300 µl of 1X lysis buffer (CCLR, Promega) by mixing thoroughly at room temperature (1x CCLR: 25 mM tris-phosphate pH 7.8, 2 mM DTT, 2 mM EDTA, 10 % glycerol, 1 % triton X-100) . The suspended plant material was incubated at 4°C for one hour. Pellet debris were removed by centrifugation at 17 000 rpm for 10 min at 4°C and the supernatant was transferred to a new tube. Measurement was carried out in a Berthold Luminometer (Lumat LB 9501, Berthold Technologies, Wildbad, Germany). 20µl of cell lysate was mixed automatically by the Luminometer with 100 µl of Luciferase Assay Reagent (LAR, Promega).

Qualitative Luciferase assay

Qualitative visual monitoring of Luciferase activity was executed using a Luminescence image analyzer (LAS 3000, Fuji Deutschland, Düsseldorf, Germany).
Leaf material was immersed in 5% Tween 20 for 3-5 min to reach uniform wetting, and then washed three times with water to remove the residual Tween, whereas cell suspension and calli were used directly for the analysis. A 1 mM Luciferin solution in the respective plant medium was sprayed or pipetted under sterile conditions over the leaves or the cells (Firefly luciferin potassium salt, bc 219, Synchem OHG, Felsberg/Altenburg, Germany). The materials were incubated at room temperature for 5 min and chemiluminescence was measured after 150 s exposure time with the image analyzer adjusted to the instrument setting “super”.

2.2.4 Molecular characterisation of plant material

Small amounts of genomic plant DNA were isolated with the chargeSwitch gDNA Plant Kit of Invitrogen (CS18000). Larger amounts of DNA, e.g. necessary for Southern blot analysis, were isolated by CTAB method.

2.2.4.1 DNA-Isolation by CTAB

This method was designed for up to 250 mg plant material (leaves, callus, suspension cells), using 2 ml reaction tubes at RT. Cell lysis took place *via* homogenisation of softer plant tissues (suspension cultures) by a homogeniser or *via* grinding of harder plant tissues like leaves by using liquid nitrogen, all under addition of CTAB-buffer at room temperature.
After homogenisation of up to 250 mg plant material 800 µl CTAB-buffer were added to each reaction tube (CTAB-buffer: 1.4 M NaCl, 20 mM EDTA, 100 mM tris-HCl pH 8.0,
0.5% PVP-40, 3% CTAB, 0.2% ß-mercapto-ethanol. (CTAB was added after autoclaving and ß-mercapto-ethanol just prior to use)).
Then plant material was incubated at 65°C in a shaker for 30 min up to 1 h and cell debris were removed by centrifugation at RT for 10 min at 14 000 rpm.
DNA was purified by chloroform and isopropanol mediated precipitation of DNA. Therefore 700 µl of supernatant were transferred into a new cap, 800 µl ice cold chloroform / isoamyl-alcohol-mix (CI-mix 24/1: 23 ml chloroform and 1 ml isoamylalcohol) were added and mixed by inversion. Work was done under a hood. DNA was found in the upper aqueous phase; denatured proteins and other organic material kept in the interphase or the lower organic layer. The centrifugation step was repeated. Afterwards 800 µl of supernatant were transferred into new caps, 600 µl ice cold isopropanol were added and mixed by inversion while working under a hood. The centrifugation step was repeated.
Then DNA was concentrated and precipitated by addition of alcohol and salt. The supernatant was discarded and the pellet washed with 200 µl washing buffer (WB: 76% ethanol absolute, 10 mM ammonium acetate) until the pellet was floating in the buffer. WB was removed and the completely dry pellet was dissolved in 200 µl TE-buffer plus 1 µl RNAse (TE-buffer: 10 mM tris-HCl, pH 8.0, 1 mM EDTA). The mixture was incubated for 30 min at 37°C. Then 100 µl 7.5 M NH_4-acetate and 750 µl of pure ethanol were added. For separation the centrifugation step was repeated. Finally the supernatant was discarded again and the DNA

pellet was dried for 10 min at 37°C. Later it was dissolved in 50 – 200 µl H_2O. The pellet was kept in a fridge overnight for better dissolving.

2.2.4.2 Transgene detection

Incorporation of the T-DNA was confirmed routinely by PCR. Check the tables below for the PCR components and program and the detection primers (Tables 4 to 6).

Table 4 and 5: Components and amplification programme for Immolase PCR

Detection-PCR with Immolase (Bioline)		
10x ImmoBuffer	2.5	µl
50 mM $MgCl_2$ Solution	1	µl
100 mM dNTP Mix	0.5	µl
Primer forward 100 pmol	1	µl
Primer reverse 100 pmol	1	µl
Immolase	0.2	µl
H_20	17.8	µl
Template DNA	1	µl
Total	25	µl

PCR programme for Immolase		
95°C	7 min	
95°C	45 s	} 30 loops
58°C	45 s	
72°C	45 s	
72°C	5 min	
8°C	∞	

Table 6: DNA sequences of internal primers for detection of target genes

Target gene	**Name**	**Sequence**	**Size of PCR product**
internal *hspx* primers	hspx 14 kD forw	5´-ATT ATC CTC GGC CGC CAC CA-3´	320
	hspx 14 kD rev	5´-AGC GAC ACC GTG CGA ACG AA-3´	
internal *bar* primers	bar 149	5´-GCA GGA ACC GCA GGA GTG GA-3´	233
	bar 382	5´-AGC CCG ATG ACA GCG ACC AC-3´	
internal *luc* primers	luc forw	5´-AAGCTATGAAACGATATGG-3´	531
	luc rev	5´-GGAACAACACTTAAAATCG-3´	
internal *gus* primers	uidA 10	5´-CCTGTAGAAACCCCAACCCG-3´	750
	uidA 762	5´-TTTGGCTGTGACGCACAGTTC-3´	

2.2.4.3 Southern Blot

The stable integration of target genes into genomic plant DNA was tested by Southern blot analysis (Southern, E.M., 1975). Southern blotting combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size-separated DNA to a filter membrane for probe hybridization. In this work the hybridization probe was labelled with Digoxigenin. The construction of the DIG-labelled probe was done by PCR and is described in the following tables (tables 7 to 9).

Construction of DIG-labelled probe by PCR

For construction of the DIG labelled probe a Biometra thermocycler was used.

Table 7 and 8: Components and amplification programme for construction of a DIG-labelled probe

solutions in µl	DIG-labelled probe	unlabelled control
10x immolase buffer	5	2.5
50 mM $MgCl_2$	2	1
10 mM dNTP mix	0	1
PCR DIG probe synthesis mix Roche vial 2	5	0
plasmid DNA 1:100	1	1
primer forward 10 pmol	1	1
primer reverse 10 pmol	1	1
immolase	0.5	0.5
H_2O	34.5	17
total	50	25

PCR-programme	**DIG probe**	
temperature	time	cycles
95°C	7 min	
94°C	40 s	} 20x
58°C	1 min	
72°C	40 s	
94°C	40 s	} 30x dt [2s]
58°C	1 min	
72°C	40 s	
total time	3 h 1 min	

Table 9: DNA sequences of internal primers for detection of target genes

Target gene	**Name**	**Sequence**	**Size of PCR product**
***luc* probe 1**	Luc 711 f1	5´-CGA TTC GGT TGC AGC ATT-3´	711
	Luc 711 r1	5´-CGA TCA AAG GAC TCT GGT ACA A-3´	
***luc* probe 2**	Luc 837 f2	5´-CCT TCC GCA TAG AAC TGC CT-3´	837
	Luc 837r2	5´-TCC AAA ACA ACA ACG GCG-3´	
***hspx* probe**	MAS-hspxSonde f	5´-TAC CCG CGA AAT TCA GGC-3´	608
	MAS-hspxSonde r	5´-ACC GTG CGA ACG AAG GAA-3´	

Digestion of DNA

5 to 30 µg of genomic DNA were cut into small DNA fragments by restriction endonucleases. Approximately one unit of restriction enzyme per µg of DNA was used. The restriction digest was executed overnight. Afterwards the digested genomic DNA was purified from remaining restriction enzymes and buffer by precipitation.
For precipitation 100 µl 7.5 M NH_4 acetate and 750 µl absolute ethanol were added. Centrifugation was performed for 10 min at 10000 g at room temperature. The supernatant was discarded and the pellet was resuspended in 50 µl TE-buffer, then 100 µl absolute ethanol for an additional washing step were added. After another centrifugation step the pellet was resuspended in as little TE-buffer as possible (e.g. 40 µl). For better dissolving the DNA was kept overnight in the fridge to continue on the next day.

Gel Electrophoresis

A 0.8% agarose gel containing 0.1% ethidium bromide was run overnight at low voltage (15 to 30 V) using 1x TAE as running buffer (40 mM tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 7). The samples and the DIG-labelled DNA molecular weight marker (Roche no. II) were loaded in the evening and diluted plasmid DNA as positive control the next morning.
To control if the genomic DNA was digested and separated properly, the gel was put under UV-light. Extra-staining in ethidium bromide solution (1x TAE-buffer plus 0.005% ethidium bromide) was occasionally necessary.

Gel preparations for Southern transfer

For a more efficient transfer of DNA from gel to membrane, the gel was treated with different acid and alkaline solutions to first break the DNA strand in smaller pieces and second to denature the double-stranded DNA fragments in single-stranded ones for later hybridization to the probe.
First the gel was placed for 10 min into 0.25 M HCl for depurination, second for 2x 15 min in denaturation solution (0.5 M NaOH, 1.5 M NaCl, pH 12-13), third for 2x 15 min in neutralization solution (0.5 M Tris base, 1.5 M NaCl, pH 7.5).
During the treatment of the gel, the material for the blotting procedure was prepared.

Capillary transfer of DNA

A tray was filled with 20x SSC (3M NaCl, 0.3 M sodium citrate, pH 7) and covered with a pane of glass carrying a paper bridge made of Whatman filter paper, with its ends hanging into the 20x SSC. Three layers of Whatman filter paper of the same size as gel and membrane were placed onto the filter paper bridge, onto which the gel was put, then a sheet of positively charged nylon membrane (Roche, 1 417 240) was placed on top. Finally the membrane was covered with three more soaked layers of Whatman paper and a stack of paper tissues. Pressure was applied evenly to the gel by placing a stack of paper towels and a weight on top of the membrane and gel to ensure good and even contact between gel and membrane.
Blotting took place overnight at room temperature.
Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) was used to move the DNA from the gel on to the membrane. Ion exchange interactions bound the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.

Fixation of DNA on membrane

After blotting, the DNA was fixed on the nylon membrane permanently either by UV-cross-linking or by baking the membrane for 30 min at 120°C in an oven. Before baking, the membrane was dipped shortly in 2x SSC and covered in a sheet of Whatman paper.

Prehybridisation and hybridisation

The membrane (DNA-side facing inside) was put carefully into a hybridization tube under addition of as much DIG Easy Hyb as needed to fully cover the membrane (10-20 ml for 10 x 10 cm blot). Prehybridization took place in the hybridisation oven at 37° - 42°C for at least 30 min.
The DIG labelled PCR-probe was denatured by heating it at 100°C for 5 min, then the probe was cooled immediately on ice. 2 µl of denatured probe were mixed per ml pre-warmed DIG Easy Hyb. Prehybridisation and hybridisation solution were exchanged. Hybridisation took place overnight at 37°-42° C with slight agitation in the hybridisation oven.
The first washing step took place manually in low stringency wash buffer (2X SSC plus 0.1% SDS) for twice 5 min at room temperature, high stringency wash buffers were preheated to

65°C in the hyb oven in this time. Then the membrane was washed for 15min in 0.5X SSC plus 0.1 % SDS using the hybridization oven and this step was repeated for 15 min in 0.1X SSC plus 0.1 % SDS.

Detection and stripping

Each of the following steps took place at room temperature and underwent slight agitation. The membrane was not allowed to run dry.

The membrane was equilibrated for two minutes in 1x washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3 % Tween20). Then the membrane was blocked for 30 min to 1 h in 1x blocking solution (1:10 dilution of 10x blocking solution stock from Roche and 1x maleic acid buffer; 1x maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, adjusted to a pH of 7.5 with > 15 g NaOH capsules).

The Anti-DIG-AP antibody (Roche 11093274910) was centrifuged for 5 to 10 min at top speed, for getting rid of antibody aggregates which could cause a strong background signal. Then the antibody was diluted 1:10 000 in 1x blocking solution (10 µl AB to 100 ml BS).

The antibody binding occurred for 30 min. Then the membrane was washed twice for 15 min in 1X washing buffer, before the membrane was equilibrated for 5 min in detection buffer.

1 ml of 1:100 diluted CSPD-solution was prepared (10 µl CSPD and 1000 µl 1x detection buffer: 100 mM tris HCl, 100 mM NaCl). The membrane was placed with the DNA-side up on a plastic sheet. 1 ml of diluted CSPD-solution was spread on the membrane and covered immediately with the plastic sheet. The substrate was spread carefully while avoiding formation of air bubbles. The membrane was incubated at room temperature for 5 min. To get rid of excess substrate, spreading of membrane was repeated. Detection occurred at the imager (instrument settings: Increment, 4 min, super).

As alternative permanent detection substrates NBT and BCIP were used. The substrate solution was prepared by mixing 40 ml substrate buffer (100 mM tris HCl, 100 mM NaCl, 5 mM $MgCl_2$), 264 µl of NBT stock (18.8 mg/ml in 67% DMSO) and 272 µl of BCIP stock solution (9.4 mg/ml in 67% DMSO). The membrane was incubated in this substrate solution at RT under slight agitation for several hours to overnight.

If a further hybridization was necessary the membrane was stripped and reprobed. Therefore the membrane was rinsed in Millipore H_2O, then it was incubated twice for 15 min in stripping buffer (0.2 M NaOH, 0.1% SDS) at 37°C in the hybridisation tube and oven,

followed by rinsing in Millipore H_2O again. Afterwards the membrane could be stored sealed in plastic and 2x SSC buffer at 4°C or was directly prehybridised again.

2.2.5 Protein analysis

2.2.5.1 Protein extraction

For protein extraction 1 g of plant material was ground in liquid nitrogen and resuspended in 1 ml of extraction buffer (50 mM $NaPO_4$ pH 7, 0.1% Triton X-100, 10 mM EDTA pH 8, 0.1% N-lauroyl-sarcosin, 10 mM ß-mercapto-ethanol); ß-mercapto-ethanol was added prior to use. The cell extract was incubated for at least 2 h at 4°C on a shaker. Afterwards proteins were separated from solid cell material and DNA by centrifugation for 20 min at 4°C at 12000 rpm. At last the supernatant was collected in a new tube.
The centrifugation step was occasionally repeated, to get rid of remaining cell material.

2.2.5.2 Protein quantification

Protein concentration was determined by absorption measurement against a bovine serum albumin standard. Therefore the 2-D Quant Kit of Amersham Bioscience was used according to the manufacturer´s instructions (Amersham Biosciences AB, Uppsala, Sweden). Absorption measurement was done with at least duplicates in 96 well microtiter plates in a Tecan Genios multiplate reader (Tecan GmbH, Crailsheim, Germany).

2.2.5.3 Sample preparation for 1-D gels

When small volumes of protein extract were loaded on 1-D SDS polyacrylamide gels, the concentration of protein extracts was up-scaled by adding StrataCleanResin (Stratagene 400714) to the protein extract. The proteins were absorbed by the resin and were dissolved again in a smaller volume. Circa 10 v/v % resin were given to the protein extract. Samples were vortexed and then separated by short centrifugation. The proteins binded to StrataCleanResin, which was dissolved in minor volumes of 2x sample buffer (100 mM tris HCl, pH 6.8, 200 mM DTT, 4 % SDS, 0.2% bromphenol blue, 20% glycerol). The samples were boiled in sample buffer for 5 min at 95°C and then loaded on a SDS-PAA gel.

2.2.5.4 One dimensional SDS PAGE

One dimension separation of proteins by SDS PAGE and later on western blot analysis were performed in laboratories of the Lionex company where staff kindly provided technical support and material like hspx specific antibodies.

SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis, is a technique widely used in molecular biology to separate proteins according to their electrophoretic mobility. Gels of different pore sizes were used. As stacking gel a large pore 4% polyacrylamide gel was used and as resolving gel a small pore 12% or 15% PA-gel (stacking gel: 0.5 M tris pH 6.8, 30% acrylamide, 10% SDS, 10% APS, TEMED; resolving gel: 1.5 M Tris pH 8.8, 30% acrylamide, 10% SDS, 10% APS, TEMED). Gels were run at 60 V until all samples reached the resolving gel, then currency was increased to 120 V (10 x electrophoresis buffer: 10% SDS, 250 mM tris base, 1.9 M glycine).

Staining was done overnight or over the weekend using 1.45 mM coomassie brilliant blue staining solution. If necessary, gels were destained by glacial acetic acid and ethanol.

2.2.5.5 Isoelectric focusing

The second dimension separation of proteins was performed at the DSMZ.

For IEF focusing different sizes of strips were used (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). A sample volume containing a specific amount of protein was diluted to a final volume of 250 µl with rehydration buffer according to 13 cm long strip (7 M urea, 2 M thiourea, 30 mM tris-HCl pH 8.5, 4 % CHAPS, 10 % glycerol, 0.002 % bromophenol blue, 2 % IPG buffer pI 3-10, 0.28 % DTT, IPG buffer and DTT was added prior to use). For coomassie staining and Western blot analysis 240 µg proteins were applied per strip (13 cm). Reagents, Immobiline DryStrips and IPG buffer were obtained from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. The 13 cm Immobiline DryStrips were rehydrated in 250 µl protein sample dissolved in rehydration buffer overnight at room temperature in the Amersham Reswelling Tray (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Rehydration was carried out according to the recommendation of GE Healthcare Bio-Sciences AB, Uppsala, Sweden. Rehydrated Immobiline DryStrips were rinsed with deionized water for a few seconds and slightly blotted to remove excess water. Isoelectric focusing was carried out in the IEF unit of the Multiphor II apparatus according to the manufacturer´s instructions

(GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at a temperature of 20 °C. IEF focusing was run according to the program recommended by GE Healthcare Bio-Sciences AB, Uppsala, Sweden for 13 cm IPG strips, pH 3-10 NL in gradient mode. The program is listed below:

1. 200 V, 0:01 h, 5 Watt, 0.5 mA per strip
2. 3500 V, 1:30 h, 5 Watt, 0.5 mA per strip
3. 3500 V, 0:35 -1:30 h, 5 Watt, 0.5 mA per strip

The total volt hours should be less than 60 kVh.

After IEF focusing the Immobiline DryStrips strips were either used directly for second dimension separation or stored in test tubes at -80 °C.

2.2.5.6 Second dimension separation (SDS-PAGE).

For the separation of proteins in the second dimension by SDS-Page electrophoresis the Immobiline DryStrips were equilibrated twice for 15 minutes in 2 x 100 ml equilibration buffer (50 mM tris-HCl pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS) on a shaker. In the first equilibration step 1 % DTT and in the second equilibration step 4.5 % iodoacetamide was added to the equilibration buffer. The equilibration was carried out in equilibration tubes (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 15 ml of the equilibration solutions for each equilibration step per Immobiline DryStrip in each tube. The equilibrated IPG gel strips were slightly rinsed and blotted to remove excess equilibration buffer and then positioned between the plates on the surface of the second dimension gel (12.5 % PAGel). The strips were fixed with 2 ml of 2 % warm agarose solution dissolved in running buffer (250 mM Tris base, 1.9 M glycine, 1% SDS). Electrophoresis was carried out in an Ettan DALTsix gel chamber (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 20 °C. Electrophoresis was started at 50 V and 400 mA for 30 min. Then the voltage and current were increased to 300 V and 400 mA. The electrophoresis was terminated when the bromophenol blue tracking dye had migrated off the lower end of the gel. Then it was used for staining or blotting.

2.2.5.7 Western blot analysis

For Western blot analysis, proteins from SDS-PAGE were semidry electroblotted to the PVDF membrane by using blotting buffer (39 mM glycine, 48 mM tris, 1.3 mM SDS and 20% methanol added prior to use). Electro-blotting took place for 30 min at 15 volts. Then the membranes were blocked for 2 h with a 1% solution of BSA in TBS-Tween (20 mM tris pH 7.4, 150 mM NaCl, 0.05% Tween 20). Before incubating the membrane with the first antibody (Anti-Hspx 1:10 000 in TBS-Tween) at RT for two hours or overnight in the fridge, the membrane was washed twice for 10 min in TBS-Tween. Afterwards the membrane was washed again, once for 15 min and twice for 5 min, before the second antibody was added. Incubation with the detection antibody took 30 min (Anti-mouse IgG with HRP 1:10 000). Then the membrane was washed again and the proteins were detected by application of 1 ml TMB (tetramethylbenzidin) ready-to-use solution on the membrane.

3 RESULTS

3.1 Establishment of a transformation and selection system

3.1.1 Comparison of different transformation systems

Transgenic suspension cultures can either be achieved by the cocultivation of plant cells and agrobacteria or they can be derived from transgenic plants. Initially we tested different plant materials with the objective to establish transgenic suspension cultures. Therefore tobacco leaf discs, callus or suspension cultures were cocultivated with *Agrobacterium tumefaciens*. With all transformation systems transgenic explants or cell cultures were obtained. Incorporation of the transgenes was tested by PCR, complemented with functional assays such as herbicide treatment ("Leaf Paint") and Luciferase Screening.

Soon an emphasis was put on the transformation of suspension cultures. In the present study different approaches for cocultivation of plant suspension cultures and agrobacteria were tested: petri dishes were used for small amounts of cells, larger scale transformation took place in a suction strainer (Buchner funnel). We obtained the best results in respect of transformation efficiency and practicable and rapid procedure by the immediate transformation of suspension cells in a suction strainer. Because of the time-consuming generation of callus material from transformed leaf discs, the direct transformation of callus or suspension culture –when available- saves time. In comparison to transformation of tobacco callus, the transformation of tobacco suspension cultures resulted in more transgenic clones per experiment. Compared to the transformation of suspension cells in other containers, in suction strainers more cells could be transformed simultaneously and coculture and washing procedure occured in the same container successively.

Distribution of transformation events in suction strainer (Buchner funnel)

Selection or screening procedures are an integral part of any programme of plant genetic manipulation, because the methods which are currently available for stable transformation are not 100% efficient (Lindsey and Jones in: Dix, 1990).

So it has to be assumed that each *Agrobacterium*-mediated transformation of a suspension culture results in a heterogeneous mixture of untransformed and transformed cells out of

different transformation events. Transformed cells can be separated from non-transformed cells by chemical or manual selection. Therefore aliquots of the cells were spread on solid medium containing different selective agents. In order to test whether the transformation events were distributed evenly throughout these aliquots, the distribution of transformation events in the suction strainer was investigated. For this purpose *Nicotiana tabacum* strain Bruessel and – due to the fact that the tobacco cells showed only weak staining - also *Solanum tuberosum* strain Desiree suspension cells were transformed with a gus IRES luciferase construct (Fig. 2). The transient transformation events were detected by X-Glc staining after three days of coculture.

As Fig. 1 illustrates the tobacco strain Bruessel cells were only slightly blue-coloured (blue spots marked by an arrow), whereas the Desiree cells showed a clearly visible blue staining. The transformation events could be detected spot wise in all cell layers above the holes of the suction strainer as well as spread over big areas both in the centre and the edge of the funnel.

The blue colour did not result from endogenous Glucuronidase because cells treated in the same procedure but cocultivated with plant medium lacking *Agrobacteria*, did not show any blue colour.

As a consequence of the described results the cells in the suction strainer were mixed after cocultivation during the washing steps to achieve an equal distribution of transformation events in the cell suspension material before starting selection experiments.

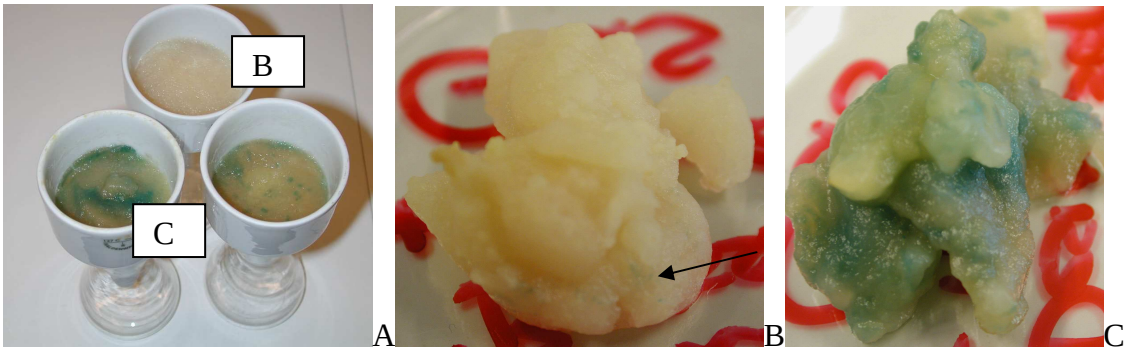


Fig. 1: Distribution of transgenic events detected by X-Glc-staining of cells transformed with the *uidA* gene.
A: 25 ml of tobacco cells (top row) and potato cells (bottom row) after cocultivation and detection reaction in a suction strainer.
B: tobacco strain Bruessel cells **C**: potato strain Desiree cells (enlarged photos).

Nevertheless, the cocultivation in the suction strainer turned out to be extremely useful to obtain a high number of transformed cells in a short time allowing an easy re-initiation of suspension cultures for further experiments.

3.1.2 Dosage of selective agents

In order to determine the selective conditions under which transgenic plant cells, harbouring constructs with different selectable marker genes, can be discriminated from non-transgenic cells, the transformed cells were spread on media supplemented with different dosages of the respective selective agents. These selective agents were Phosphinotricin (PPT) for selection on the *bar* gene, Gentamicin derivative G418 for selection on the *nptII* gene and NaCl for selection on the *PR10a* gene.

Table 10 illustrates the effect of selective conditions on callus cultures. Up to four weeks post transformation (pt) both wild type and transformed callus cultures showed an increment of callus mass, but the increment of transformed callus was higher under selective conditions. After four weeks pt the callus was transferred to fresh solid medium containing the same concentrations of selective agents. Only these dosages of selective agents were continued which negatively affected the growth of wild type cells. eight weeks pt 5 and 7 mg/l PPT, 6 mg/l G418 and 50 mM NaCl prooved to be the lethal doses for wild type cells, while transformed cells survived.

Table 10: Selection efficiency of different dosages of the selective agents PPT, G418 and NaCl on callus cultures.

Increment during selection process and efficiency of selective agents							
selectable marker gene	dosage of selective agents	2 weeks pt		4 weeks pt		8 weeks pt	
		Wild type	Transgene	Wild type	Transgene	Wild type	Transgene
	no selection	++	++	++	++	++	++
bar gene	PPT 3 mg/l	+	++	++	++	not continued	
	PPT 5 mg/l	+	++	+	++	-	++
	PPT 7 mg/l	+	++	+	++	-	++
npt II gene	G418 3 mg/l	+	++	+	+	not continued	
	G418 6 mg/l	+	++	+	++	-	++
bar gene and *npt II* gene	PPT 3 mg/l and G418 3 mg/l	+	++	+	+	not continued	
	PPT 3 mg/l and G418 6 mg/l	+	++	+	++	-	++
PR10a gene	50 mM NaCl	++	++	++	++	-	++
	100 mM NaCl	+	+	+	+	-	-
	150 mM NaCl	+	+	+	+	-	-
pt : post transformation; + or ++: different scales of increment of cell proliferation, -: no growth, death of cell cultures							

As a consequence in further experiments callus was maintained post transformation on solid medium supplemented with 6 mg/l PPT or 6 mg/l G418 or 50 mM NaCl. The concentration of 6 mg/l PPT was chosen as lethal dosage, as mean value between 5 and 7 mg/l PPT, to make sure to achieve a lethal effect, because 5 mg/l PPT were in other experiments observed to be too weak to hinder growth of wild type cells after a longer subculture period.

In suspension cultures only slightly different selective dosages were achieved. Here concentrations above 4 mg/l PPT and 4 mg/l G418 had already led to reduced growth, but lethal concentrations of selective agents for non-transgenic suspension cells were the same as for callus cultures: 6 mg/l PPT or 6 mg/l G418 and 50 mM NaCl. Because even transformed suspension cultures initially showed reduced growth when they were treated with selective agents, the dosages of PPT and G418 were increased in 1 mg steps from 3 mg/l to 6 mg/l. Only NaCl was added at the beginning of the selection of the cell culture with the final dose of 50 mM.

Also combinations of 3 mg/l PPT plus 6 mg/l G418 and 50 mM NaCl respectively were applied, because higher dosages of PPT frequently led to reduced growth in transgenic cell cultures up to a critical level.

3.2 Independent expression of a SMG and a reporter gene

3.2.1 Transformation vector pGII 0229 MAS gus IRES luc (GUS construct)

To investigate transgene expression stability in long term callus cultures, tobacco suspension cells were transformed with vector pGII 0229 MAS gus IRES luc, shortly named GUS construct. In this transformation vector the *bar* gene as the only selectable marker gene is controlled by a NOS-promoter, whereas the *glucuronidase (uidA)* and *luciferase* genes are controlled by a MAS promoter and are translationally linked by an IRES-element (Fig 2).

Fig. 2: T-DNA of transformation vector pGII 0229 MAS gus IRES luc, also named GUS construct. The selectable marker gene (*bar*) is driven by a nopalin-synthase promoter (p-NOS), whereas in the second expression cassette two reporter genes (*gus* and *luc)* are controlled by a mannopin-synthase promoter (p-MAS). This transformation vector was kindly provided by Ali, 2007.

From the cocultivated suspension cells independent transgenic callus lines were established. These callus cultures were maintained on LS medium containing 6 mg/l PPT and were screened monthly for their Luciferase activity. So these callus cultures were cultivated with the aid of herbicide selection provided by the p-NOS driven *bar* gene as well as with the aid of manual selection provided by the *luciferase* gene.

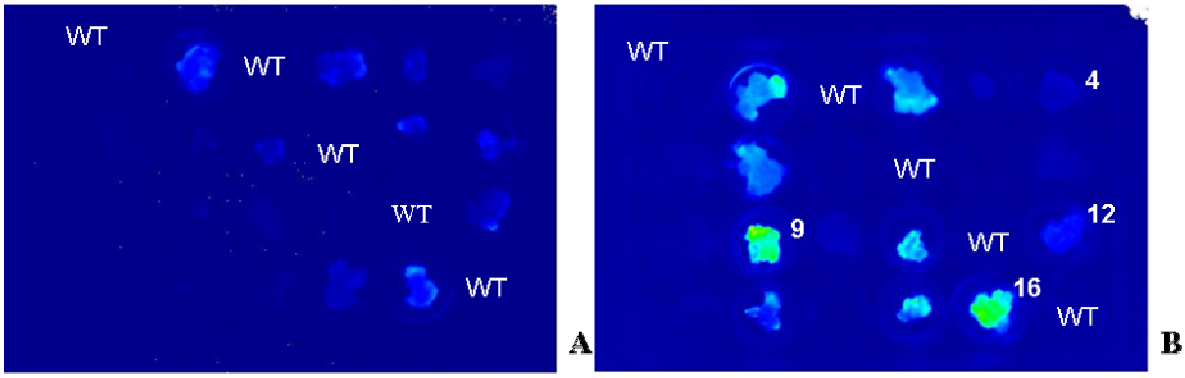


Fig. 3: Luciferase screening of tobacco callus lines transformed with the GUS construct and grown on solid LS medium containing 6 mg/l PPT. The pictures were taken four months (A) and 16 months post transformation (B). Here the pictures are presented in colour shift mode. Every four weeks only the brightest 1-2 mm small cell clusters were picked for proliferation. Numbers show the cell clusters described in table 11. WT: Wild type

Figure 3 illustrates the results of the Luciferase screening of these callus cultures four months post transformation (A) and 16 months post transformation (B). The pictures are presented in colour shift mode. A colour shift from bright blue to green indicates increasing chemiluminescence. Transgenic cells proliferated well on the PPT-containing medium for the whole selection process. The cells did show relative low Luciferase activity four months pt and after 16 months pt Luciferase activity was still detectable, but remained at a relative low level.

Parts of callus lines 4, 9, 12 and 16 were taken from the microtiterplate 16 months pt (Fig. 3B) and were propagated to obtain material for quantitative measurement of Luciferase activtiy. Even though Luciferase activity of the propagated calli could be detected by Luciferase screening on the imager (pictures not shown), only the cell extract of callus line 9 exceeded levels of background activity after quantitative measurement by the Berthold luminometer (Table 11).

Table 11: Luciferase activitiy of cell extracts of calli 4, 9, 12 and 16, which are indicated by their respective numbers in Figure 3.

callus number	**Luciferase activity [RLU/2s]**
4	55
9	198
12	44
16	60

3.3 IRES-mediated coexpression of a SMG with a reporter gene

3.3.1 Construction of dicistronic transformation vectors for coexpression of SMG and reporter gene

Two dicistronic transformation vectors with two selectable marker genes on distinct positions were constructed to investigate how the positions of the selectable marker genes would affect the stability of the *luciferase* gene expression. One selectable marker gene was expressed independently by the NOS-promoter. The other selectable marker gene was coexpressed with a *luciferase* gene mediated by an IRES-element under the control of the MAS-promoter. The measured level of chemiluminescence was taken as a parameter for the transgene expression stability.

Vector pGII 0029 MAS bar IRES luc combines antibiotic and herbicide selection and will be named BAR construct in the following chapters, vector pGII 0229 MAS PR10a IRES luc combines herbicide and salt selection and will be named PR10a construct.

3.3.1.1 Transformation vector pGII 0029 MAS bar IRES luc (BAR construct)

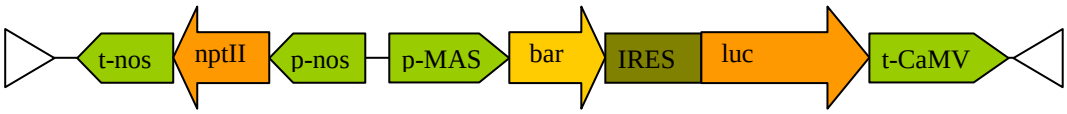


Fig. 4: T-DNA of the transformation vector pGII 0029 MAS bar IRES luc, also named BAR construct. The first selectable marker gene (*nptII*) is driven by p-NOS, whereas the second SMG (*bar*) and the reporter gene (*luc)* are located on a distinct expression cassette controlled by p-MAS.

In this vector the *nptII* gene under control of the NOS-promoter provides resistance to the kanamycin antibiotic family (G418, Kanamycin) (Fig. 4). In the second expression cassette the *bar* gene, which provides resistance to the herbicide Phosphinotricin and the *luciferase* reporter gene, are controlled by the MAS-promoter. Both cistrons in this expression cassette are linked by an IRES-element and therefore form one transcription unit, while two distinct proteins are translated.

For construction the *bar* gene was amplified by PCR using a pGII 0229 vector backbone as a template and oligonucleotides containing a *Xma*I or *Hind*III restriction site as primers (bar

XmaI forward and bar HindIII reverse, table 1). For fast and easy sequencing the *bar* gene was cloned into a pJet-Vector. After successful sequencing the bar gene was cut out by XmaI and HindIII via restriction digest and ligated into pGII 0029 MAS gus IRES luc by replacing the *gus* gene with the *bar* gene (kindly provided by Ali, 2007).

After incorporation into *E. coli* and *A. tumefaciens* the new vector pGII 0029 MAS bar IRES luc was verified by plasmid isolation and restriction digest with *Kpn*I and *Hind*III, resulting in the 1090 bp sized "MASbar"-fragment and the remaining 6700 bp sized vector (Fig. 5).

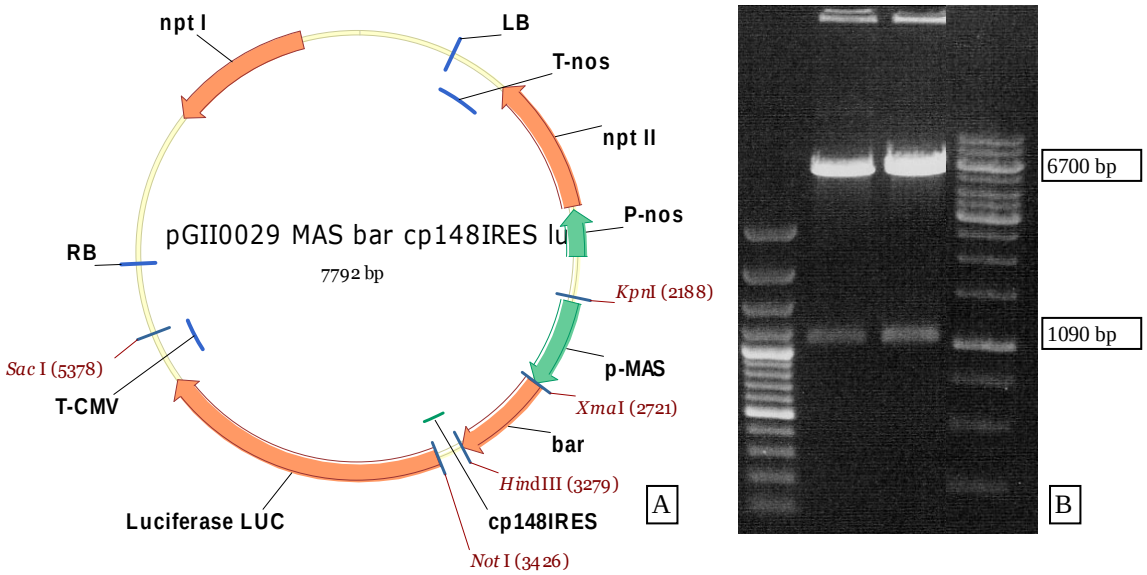


Fig. 5: A) Vector map of transformation vector pGII 0029 MAS bar IRES luc showing restriction sites important for cloning.
B) Agarose gel image of restricted plasmid: lane 1) 1 kB DNA ladder (Fermentas), lane 4) 100 bp DNA ladder plus (Fermentas), lane 2) and 3) pGII 0029 MAS bar IRES luc digested by KpnI and HindIII.

3.3.1.2 Transformation vector pGII 0229 MAS PR10a IRES luc (PR10a construct)

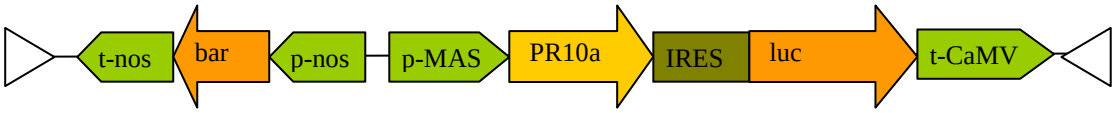


Fig. 6: T-DNA of the transformation vector pGII 0229 MAS PR10a IRES luc, also named PR10a construct. The first SMG (*bar*) is driven by the NOS-promoter, whereas the second SMG (*PR10a*) and the reporter gene (*luc)* are located on the same expression cassette controlled by the MAS-promoter. This transformation vector was kindly provided by El Banna, DSMZ.

The PR10a construct combines herbicide resistance provided by the NOS-promoted *bar* gene and increased salt tolerance provided by the *PR10a* gene, which was derived from *Solanum tuberosum* and was formerly known as *sth2* gene (Matton and Brisson, 1989) (Fig. 6). The *PR10a* gene is controlled by the MAS-promoter and is located on the same expression cassette as the *luciferase* gene in the described transformation vector.

3.3.2 Application of dicistronic transformation vectors for coexpression of SMG and target gene

To investigate the influence of the position of the selective marker on transgene expression *Nicotiana tabacum* strain Bruessel suspension cells were transformed with *Agrobacterium tumefaciens* carrying either the BAR construct or the PR10a construct. The experimental design, which is described in the following paragraph, is also illustrated in Fig. 7.

3.3.2.1 Experimental design

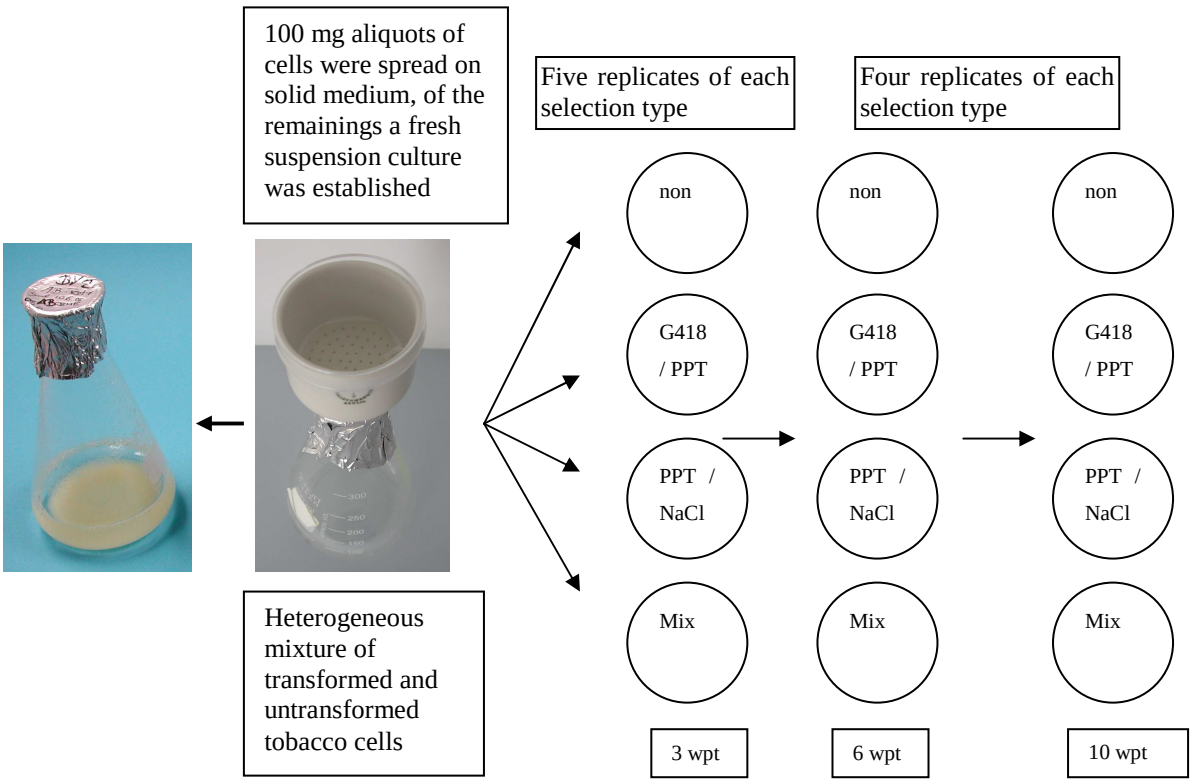


Fig. 7: Experimental design of the transformation and subculture process of tobacco cell cultures transformed with the BAR construct and the PR10a construct repectively. (wpt: weeks post transformation)

Tobacco suspension cells and *Agrobacteria* were incubated together in a suction strainer for three days. After terminating this coculture period by washing, the cell material was either recultivated in liquid or solid medium.

Callus cultures

Callus cultures were started by spreading an aliquot of 100 mg transformed cells on solid LS medium containing 100 mg/l Ticarcillin and the respective selective agent. For selection of cells transformed by the BAR construct the LS medium contained:

- 6 mg/l G418 for selection on the NOS-promoter driven *nptII* gene,
- or 6 mg/l PPT for selection on the *bar* gene,
- or a mixture of 6 mg/l G418 plus 3 mg/l PPT for selection of cells expressing both resistance genes.

In contrast, cells transformed by the PR10a construct were treated either with:

- 6 mg/l PPT for selection on the *bar* gene, which in this construct is located on its own NOS-expression cassette,
- or with 50 mM NaCl for selection on salt tolerance provided by the MAS-promoter driven *PR10a* gene,
- or a mixture of 50 mM NaCl plus 3 mg/l PPT for selection of cells expressing both resistance genes.

Control cells were cultivated without any selective agent for comparison.

20 petri dishes for each selection type were inoculated with aliquots of the initially transformed cell material. From 8 of these 20 petri dishes, data of fresh and dry weight were collected. For protein content and Luciferase activity measurements aliquots of cell material from four petri dishes were taken. From the remaining eight petri dishes the cell material of four was split into four parts and used to inoculate 16 new petri dishes. From the cell material of the other four petri dishes 16 small cell clusters were picked and transferred to 24 well plates containing 1 ml of solid medium. These 16 cell clusters were selected for high luminescence and subcultured routinely.

During the investigation period of ten weeks, subculturing and harvesting of cell cultures was done three, six and ten weeks post transformation (wpt).

Suspension cultures

All cell material which remained in the suction strainer after inoculation of the callus cultures was used for starting a new suspension culture in liquid LS medium with 150 mg/l Ticarcillin. After the first week of subculturing the fresh suspension culture was divided into three flasks for starting the selection process due to treatment with chemical agents: to the first flask no selective agent was added, the second and third flask were either treated with PPT or G418. In a second experimental series the second and third flask were treated either with PPT or NaCl. Antibiotic and herbicide treatment started with 3 mg/l selective agent and was increased in steps of 1 mg/l to a final concentration of 6 mg/l. For NaCl treatment a concentration of 50 mM was used. Suspension cultures were subcultured weekly by dividing the cell mass into two parts, and while dividing the cell cultures also samples for luminometer measurement were taken.

The experiments described above were carried out for two independent transformations for each of the two vectors pGII 0029 MAS bar IRES luc and pGII 0229 MAS PR10a IRES luc respectively. All following graphs regarding the chemical selection of callus cultures represent the mean values of both transformations. In contrast, pictures of manually and chemically selected callus clones and graphs about the chemical selection of suspension cultures represent results from one transformation. However, the latter results were reproduced at least once.

3.3.2.2 Effects of selection on cell cultures transformed with the BAR construct

The BAR construct combines an antibiotic and herbicide resistance. The *kanamycin* resistance gene is located on another expression cassette than the *bar* resistance gene which forms a transcription unit with the reporter gene *luciferase* (Fig. 4).

Water content of callus cultures under different selection regimes

The water content of transformed callus cultures during the selection phase was determined after three, six and ten weeks and was calculated as percentage of fresh weight after drying and weighing the callus material.

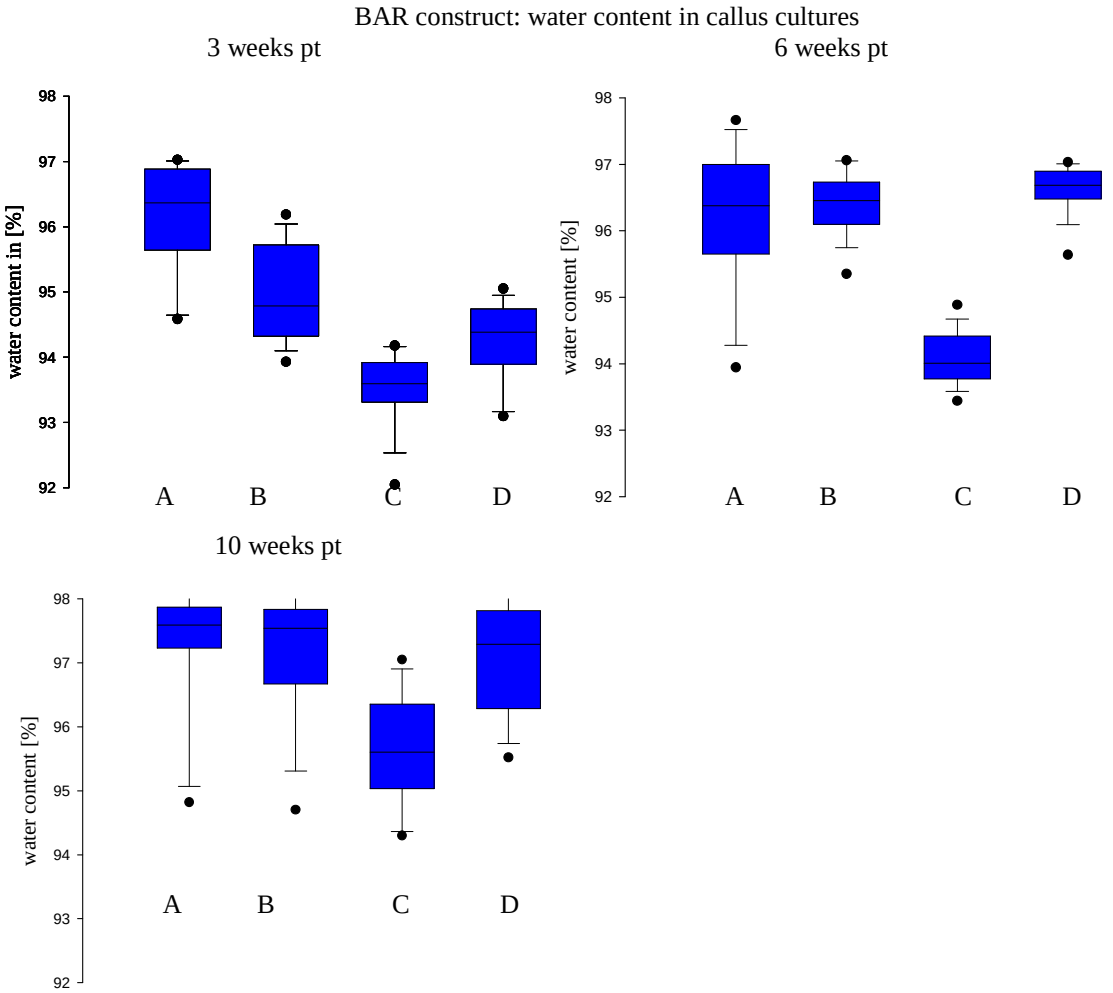


Fig. 8: Boxplots representing the water content in tobacco callus cultures transformed with the BAR construct and treated with the according selective agents: (A) no selection, (B) 6 mg/l G418 for the *nptII* gene, (C) 6 mg/l PPT for the *bar* gene and (D) a mixture of both selective agents (6 mg/l G418 and 3 mg/l PPT).

The callus cultures initially consisted of a mixture of transformed and untransformed cells. By application of chemical selection the growth of transformed cells was promoted, whereas the majority of untransformed cells died because of the exposure to lethal concentrations of the selective agents. Dead cells probably released their water, and the residual dry matter would decrease the water content measured for the whole callus culture.

Three weeks post transformation (pt) the water content measured in the selected callus cultures was significantly lower than the water content measured in the non-selected callus cultures. However, with continuous application of selective agents the water content of callus cultures measured six and ten weeks pt and selected with 6 mg/l G418 or both 6 mg/l G418 and 3 mg/l PPT increased to the same value as of the non-selected calli. Only the water content measured in PPT-selected callus cultures remained on a significantly lower level, but nevertheless increased during continuous selection from a level of 93.5% to 96%. That means the water content measured in selected transgenic callus obtained similar levels as the water content measured in transformed non-selected callus after a certain selection period, because the percentage of untransformed cells sensitive to the selective agents decreased.

Luciferase activity in callus cultures under different selection regimes

On the basis of the expression of the reporter gene *luciferase* it was investigated whether and how the position of the SMG affects the expression of a target gene. Figure 9 shows the Luciferase activity of tobacco callus cultures transformed with the BAR construct.

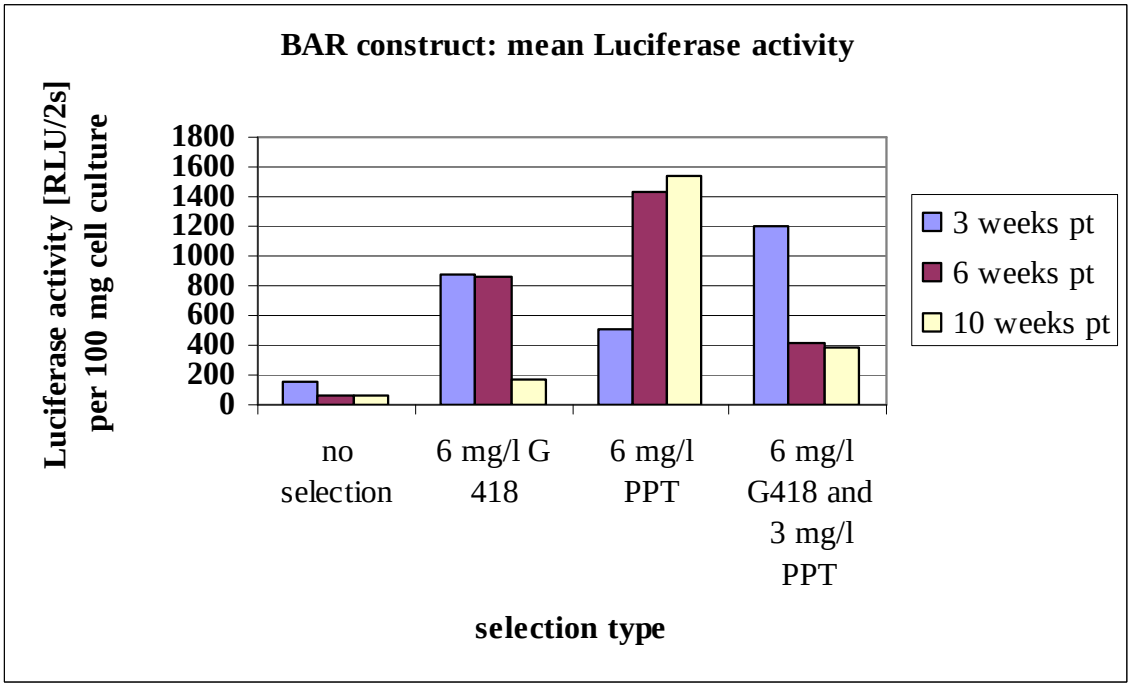


Fig 9: Mean Luciferase activity of tobacco callus cultures transformed with the BAR construct and treated with different selective agents according to the transgenic traits provided by the two SMGs: *nptII* and *bar*.

Callus cultures grown on LS medium lacking any selective agent initially showed only a low luminescence of 150 RLU/2s, which decreased continuously to a value of 60 RLU/2s ten weeks post transformation (Fig. 9). This Luciferase activity is not higher than the background activity of wild type cells which ranges usually between 30 and 100 RLU/2s. Callus cultures which were treated with G418 and therefore were selected for antibiotic resistance based on the *nptII* gene driven by a NOS-promoter showed a higher Luciferase expression than the non-selected cultures. The initial value was 850 RLU/2s. Within ten weeks post transformation the Luciferase activity decreased to 170 RLU/2s. The callus cultures treated with PPT showed the highest Luciferase expression over the whole investigation period Initially Luciferase was expressed by 500 RLU/2s and increased continuously to 1 500 RLU/2s. The *bar* gene which provides resistance to Phosphinotricin (PPT) is coexpressed with the *luciferase* gene and so controlled by the same promoter. Selection on both SMGs at the same time achieved luminescence of 1 200 RLU/2s after the first selection phase, but after six and ten weeks of subculture Luciferase expression decreased to 400 RLU/2s. When the callus cultures were treated with both G418 and PPT, instead of 6 mg/l only half of the concentration of PPT was used. 3 mg/l PPT was shown to be an insufficient dose of selective agent, although it was used in combination with the lethal dosage of G418.

In summary, selection using 6 mg/l PPT resulted in most successful reporter gene expression. So, in callus cultures transformed with the BAR construct the position of the SMG affected the stability of the *luciferase* gene expression during the investigation period of ten weeks: only when the SMG was coexpressed with the reporter gene Luciferase expression increased to a stable level significantly higher than the Luciferase expression of non-selected transformed callus cultures.

Luciferase activity in suspension cultures under different selection regimes

The same trends in Luciferase activity could be observed working with suspension cultures. The suspension cultures were started from the same material as the callus cultures three days after the transformation procedure in the suction strainer. The division of the initial suspension culture into three equal parts and the beginning of the selection process started ten days post transformation. Selection was started by adding either G418 or PPT or no selective agent to the three individual flasks. The suspension cultures were subcultured weekly and during subculturing an aliquot of the culture was taken to perform a Luciferase assay with the luminometer.

The PPT-treated suspension culture obtained a Luciferase activity of nearly 12 000 RLU/2s, ten times more than the callus culture maintained on PPT-containing solid medium. The PPT-treated suspension culture showed a high range of Luciferase activity in subsequent growth cycles, in which the lowest value varied around 1 000 RLU/2s and the highest around 6 000 to 12 000 RLU/2s. In these cultures the *luciferase* gene is coexpressed with the *bar* gene, while the *nptII* gene is controlled by another promoter in a distinct expression cassette. The curves, which illustrate the Luciferase activity of the other two suspension cell cultures, also oscillate, but on a lower range: the non selected suspension cell culture varied from 100 to 400 RLU/2s and the G418-treated suspension culture fluctuated from 1 000 to 6 000 RLU/2s.

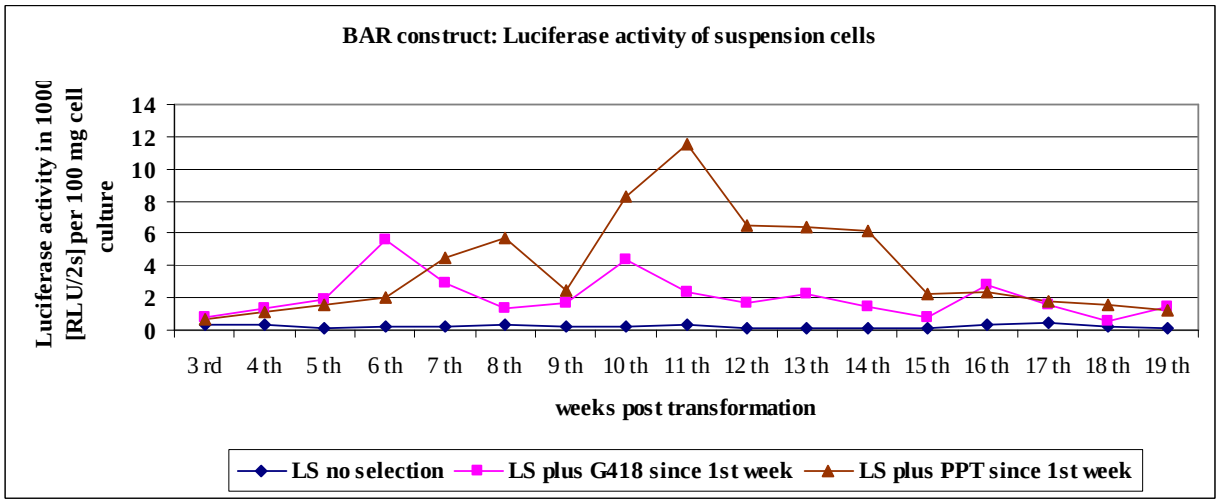


Fig 10: Weekly Luciferase activity of suspension cultures transformed with the BAR construct.

If the Luciferase activity, shown in Fig. 10, is compared to the weekly increment of growth of the suspension cultures, high Luciferase expression appears to be related to low cell proliferation. Fig. 11 illustrates the curve of the settled cell volume of the suspension cultures. The settled cell volume of each suspension culture was determined at the end of the seven day long growth period by sedimentation before subculturing.

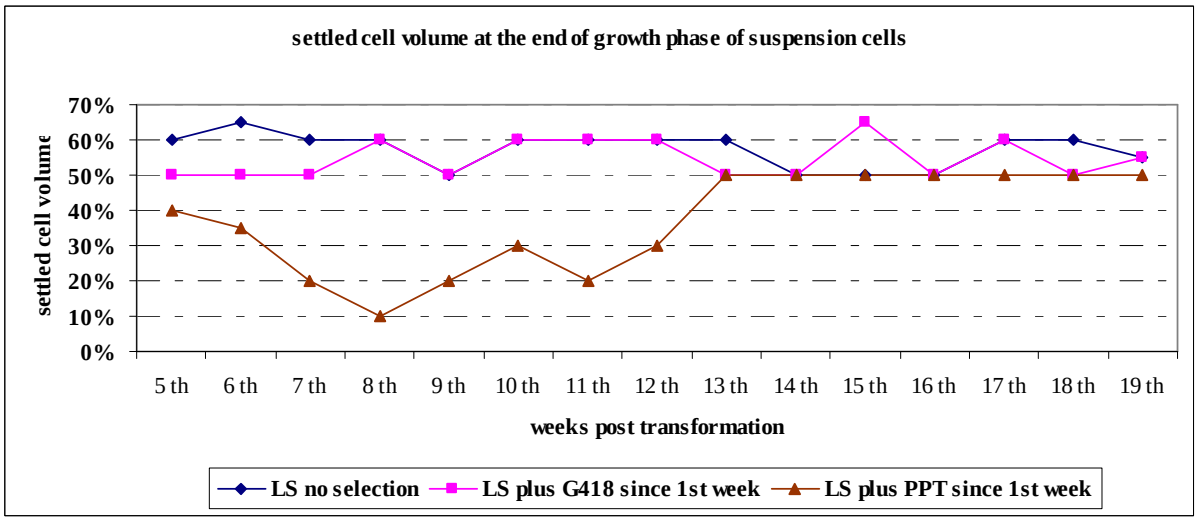


Fig 11: Weekly settled cell volume of suspension cells transformed with the BAR construct and treated with different selective agents.

The settled cell volumes of the non-selected and G418-treated suspension cells differed only slightly between 50 and 65% of the total suspension volume. Whereas the settled cell volume of the PPT-treated cells decreased from initially 40% to 10% in the eighth week post transformation, remained for four weeks on a low level of 10 to 30% and then increased to a settled cell volume of 50%. The peak of high Luciferase activity of around 12 000 RLU/2s in the 11th week post transformation corresponds to a low settled cell volume of 20% indicating a low proliferation rate during a period of high target gene expression.

Manual selection of high-expressing calli combined with chemical selection

In addition to the chemical selection provided by the different selectable marker genes, the *luciferase* reporter gene also provides a tool for manual selection. Manual selection is only possible for callus cultures by picking the brightest cell clusters for further propagation. By applying both chemical and manual selection, it was tested which selection regime led to the most luminescent and therefore highly expressive cell cultures. For selection the callus cultures were screened for their Luciferase activity by spraying a 1 mM Luciferin solution on top of the callus to visualise their luminescence with the Fuji LAS 3000 imager. After screening, app. 2 mm small cell clusters were picked according to their luminescence.

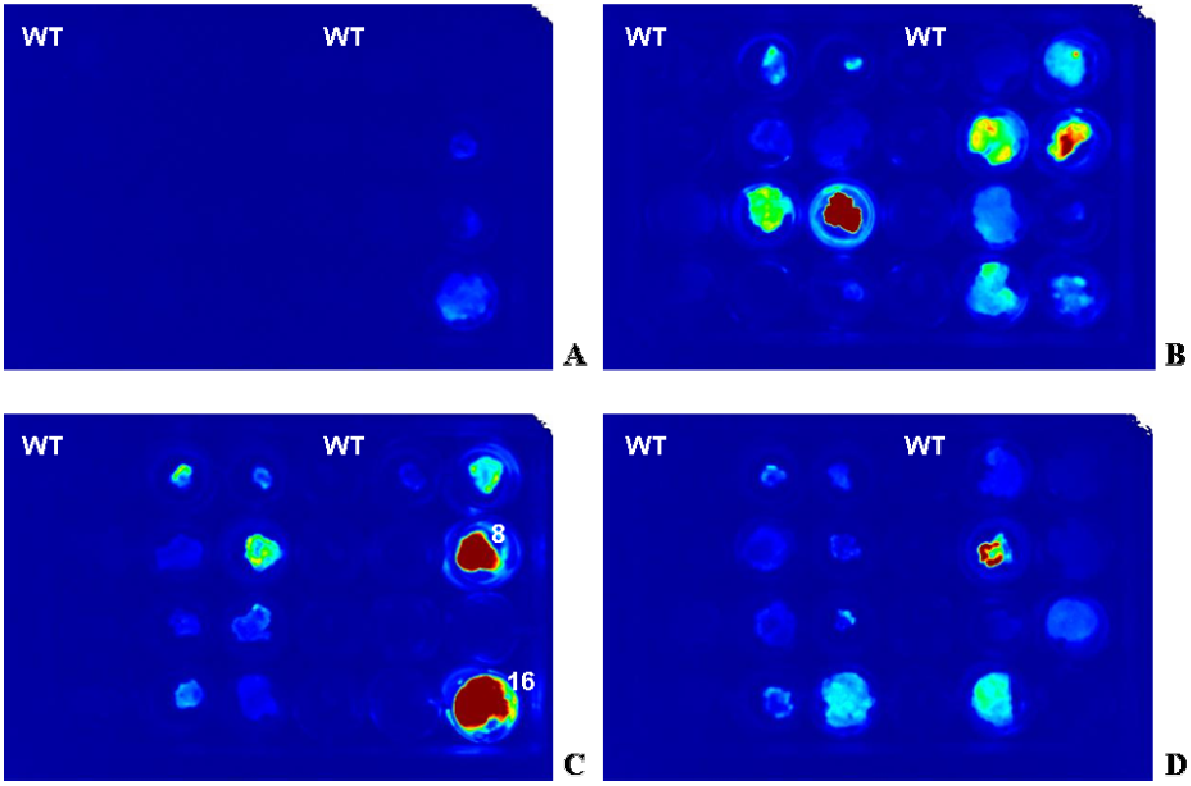


Fig. 12: Luciferase screening of tobacco cell clusters transformed with the BAR construct and grown on solid LS medium containing no selective agent (A), 6 mg/l G418 (B), 6 mg/l PPT (C) and 6 mg/l G418 plus 3 mg/l PPT (D). The pictures were taken 20 weeks post transformation and are presented in a colour shift mode. WT: Wild type

Figure 12 shows callus cultures maintained on different selection regimes screened for their Luciferase activity 20 weeks post transformation. The pictures are shown in a colour shift mode. A shift from bright blue over green to red indicates an increasing chemiluminescence intensity.

Nearly all 24 calli maintained on medium lacking selective agent lost their Luciferase activity during four months of subculture and only three cell clusters showed little expression. In contrast, the majority of cell clusters treated with selective agent showed a stable Luciferase expression over this time period. Callus cultures treated with G418 or PPT showed only slight difference in Luciferase expression. By application of G418 six highly illuminating cell clusters were obtained and by PPT-treatment four comparably high illuminating cell clusters (Fig. 12B and 12C). Here the effect of chemical selection was supported by manual selection, so that the number of highly illuminating cell clusters is individual per each experiment.

3.3.2.3 Effects of selection on cell cultures transformed with the PR10a construct

The PR10a construct provides herbicide resistance because of the *bar* gene (Fig. 6). It has been reported that the *PR10a* gene increases salt tolerance of transgenic plants. The *bar* resistance gene is situated on another expression cassette than the *PR10a* gene which forms a transcription unit with the reporter gene *luciferase*.

Cell cultures transformed with the PR10a construct were investigated under the same experimental design as the cell cultures transformed with the BAR construct.

Water content of callus cultures under different selection regimes

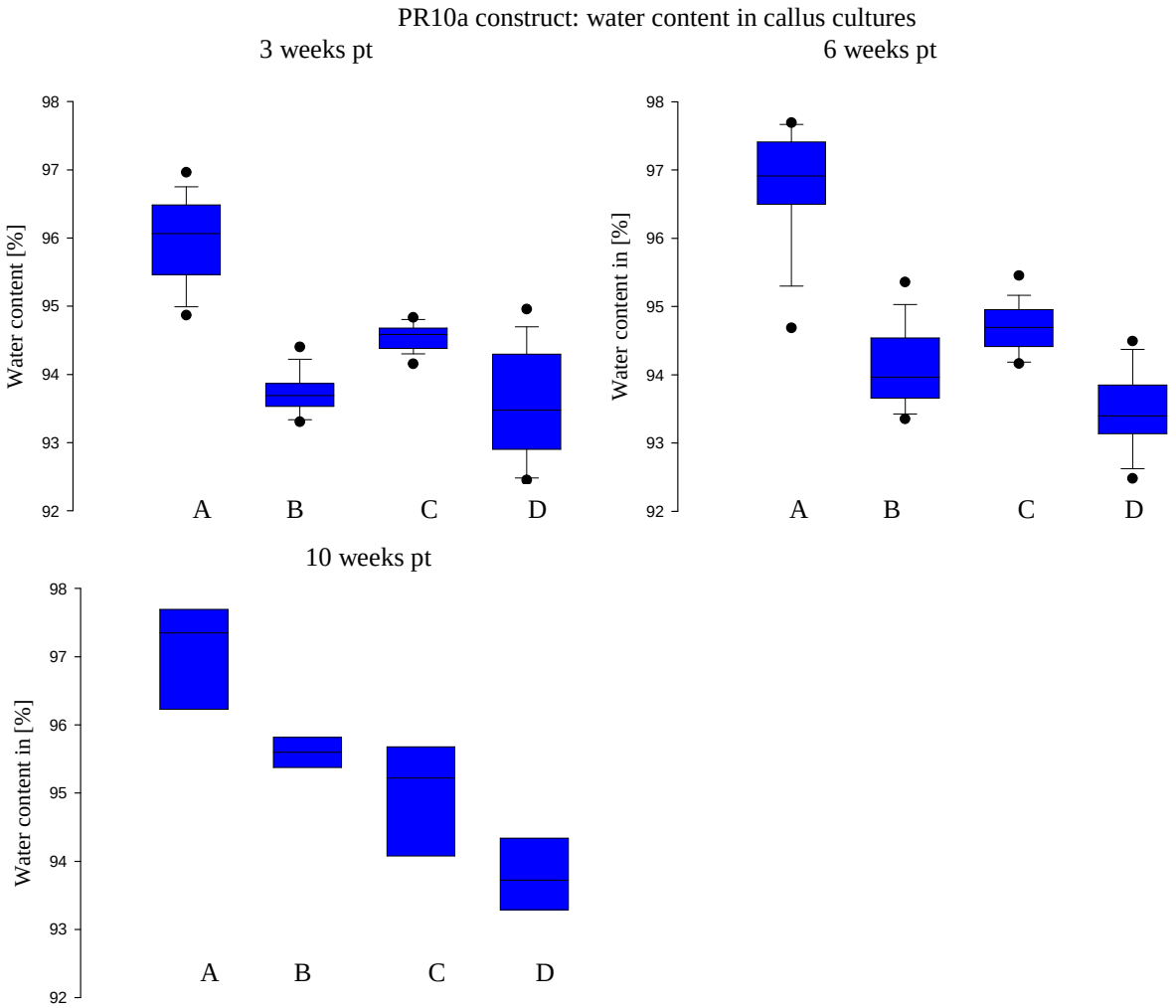


Fig. 13: Boxplots representing the water content in tobacco callus cultures transformed with the PR10a construct and treated with the following selective agents: (A) no selection, (B) 6 mg/l PPT for the *bar* gene, (C) 50 mM for the *PR10a* gene and (D) a mixture of both selective agents (50 mM NaCl and 3 mg/l PPT).

The water content of callus cultures transformed with the PR10a construct was measured by dry matter determination, calculated and expressed as percentage of the fresh weight three, six and ten weeks pt (Fig. 13). Over the whole investigation period the water content measured in the callus cultures treated with selective agents was significantly lower than the water content measured in the non-selected callus cultures. In all callus cultures which were treated with any selective agent values of water content lower than 96% were detected at the end of the investigation period. However, the water content measured in PPT-treated callus cultures increased to nearly the similar level of measured water content as the non-selected calli with the ongoing application of selective agents, whereas the water content measured in callus cultures treated with NaCl and both NaCl and PPT remained at a lower level. The addition of 50 mM NaCl to the plant medium increased the osmolarity of the LS medium: an osmolarity of 0.224 osmol/kg was measured in LS medium and an osmolarity of 0.315 osmol/kg in LS medium supplemented with 50 mM NaCl. In contrast, the addition of other selective agents like PPT or G418 did not change osmolarity of the applied plant medium. Nevertheless, the water content values for the cultures treated with PPT showed even lower levels than salt treated callus cultures growing in medium with increased osmotic value after three and six weeks.

The water content measured in the callus cultures transformed with the PR10a construct indicates that PPT treatment affected a decrease in the percentage of untransformed cells in the callus culture during the investigation period. That means that PPT was the selective agent causing the most prominent selective effect, which is emphasized by the initially highest decrease in the water content measured in the callus cultures. This could be observed in experiments with both the BAR construct and the PR10a construct. That a recovery effect could not be observed for application of NaCl as selective agent is probably due to the osmotic effect of NaCl which causes dehydration of plant cells.

Luciferase activity in callus cultures under different selection regimes

Here it was also investigated on the basis of the expression of the reporter gene *luciferase* whether and how the position of the SMG affects the expression of a target gene. Furthermore it was tested, whether NaCl works as a selective agent, as an alternative to the other, more toxic agents.

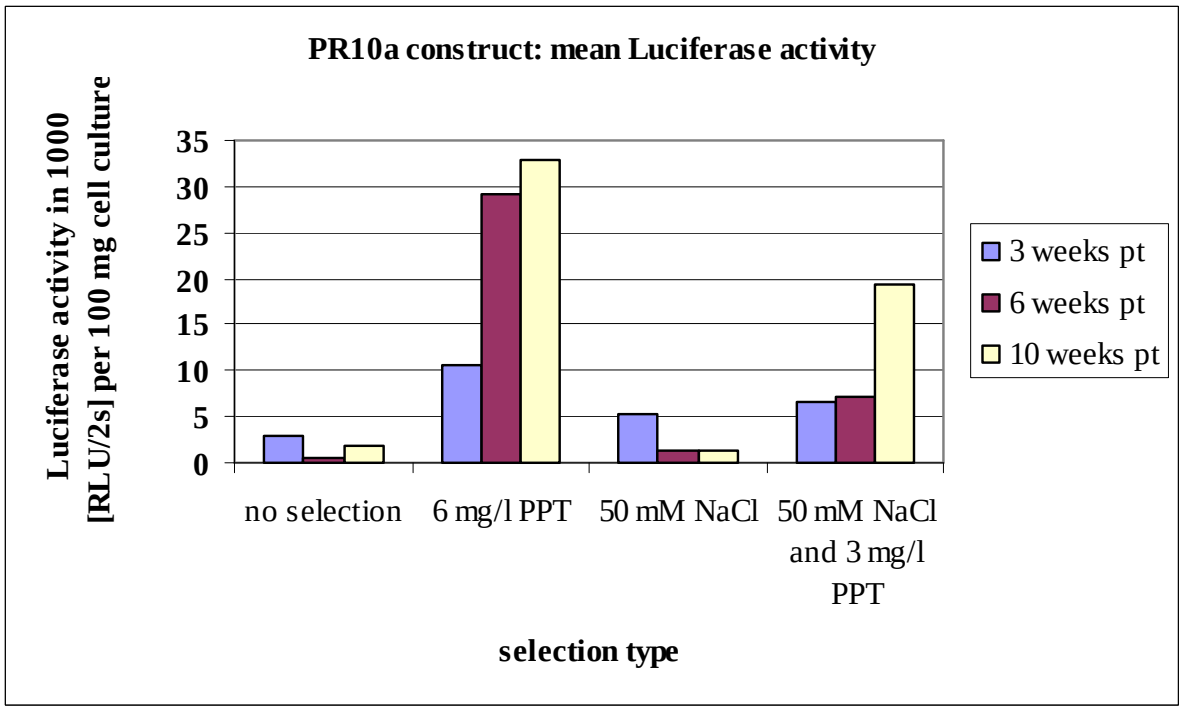


Fig 14: Mean Luciferase activity of tobacco callus cultures transformed with the PR10a construct and treated with different selective agents according to the transgenic traits provided by the two SMGs: *bar* and *PR10a*.

Relatively low Luciferase activity from initially 3 000 RLU/2s and less was shown by the transformed callus cultures which were not treated with selective agents. Callus cultures growing on 50 mM NaCl containing medium showed only initially a higher Luciferase activity of around 5 000 RLU/2s, but at six and ten weeks post transformation (wpt) the Luciferase activity did not exceed the values from the non-selected callus cultures. So the 50 mM salt concentration did not lead to a higher Luciferase expression. Callus cultures treated with PPT showed from the beginning the highest Luciferase expression which even increased from over 10 000 RLU/2s 3 weeks post transformation to more than 30 000 RLU/2s ten weeks post transformation. Callus culture treated with a selective mix of NaCl and PPT showed higher Luciferase activity than the cultures treated only with 50 mM NaCl, but lower than the PPT-treated ones, because in the combination of the selective agents only 3 mg/l PPT was used.

In transgenic callus cultures transformed with the PR10a construct stable and high Luciferase expression was obtained after PPT treatment, although the *bar* gene providing resistance against PPT is controlled by another promoter than the *luciferase* gene. Selection on 50 mM NaCl prooved to be inefficient.

Luciferase activity in suspension cultures under different selection regimes

The same results can be observed in suspension cultures transformed with the PR10a construct and treated with the respective selective agents. In comparison to Luciferase activity levels obtained by callus cultures, suspension cultures showed an up to ten fold higher Luciferase activity.

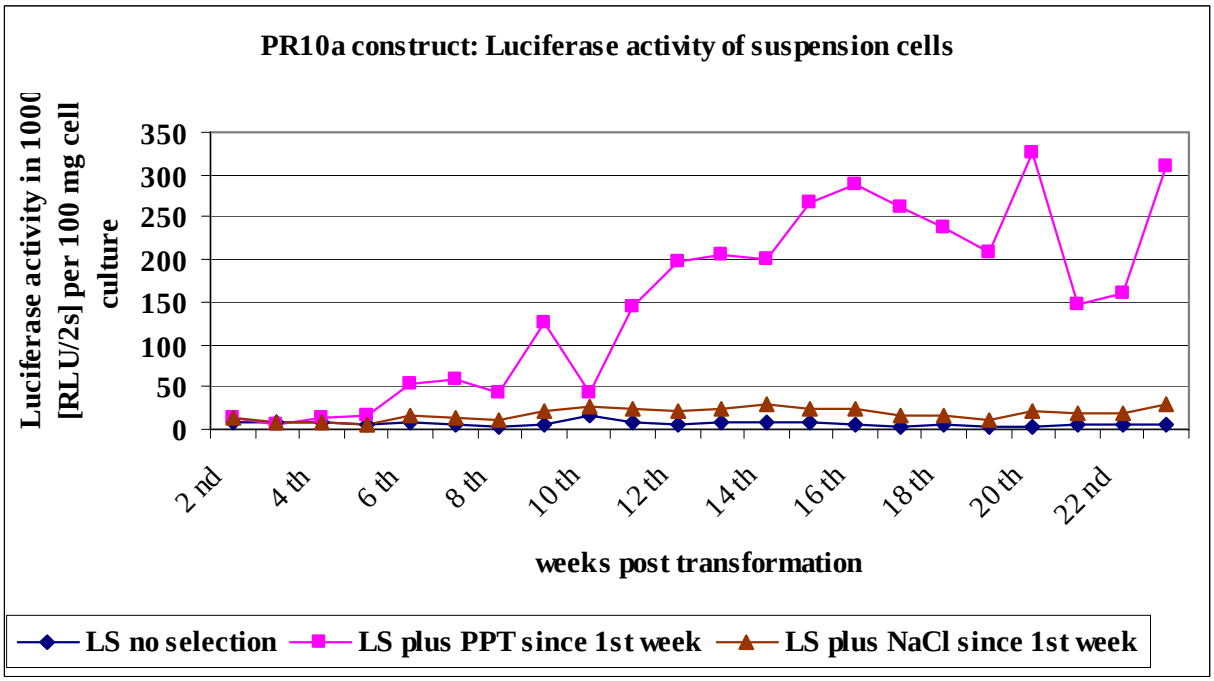


Fig 15: Weekly Luciferase activity of suspension cultures transformed with the PR10a construct.

The Luciferase expression of the non-selected suspension culture oscillated between 2 500 and 10 000 RLU/2s in subsequent growth cycles, whereas enzyme activity of the NaCl-treated suspension culture varied between 10 000 and 30 000 RLU/2s from subculture to subculture. Suspension cultures treated with PPT showed a steadily increasing luminescence from 20 000 to nearly 300 000 RLU/2s in the first 16 weeks of subculture. Then the Luciferase activity decreased to 200 000 RLU/2s in the next three weeks and after that oscillated between a maximum of 325 000 RLU/2s and 150 000 RLU/2s.

The suspension cell cultures transformed with the PR10a containing vector and treated with PPT showed a lower settled cell volume than the untreated suspension cells, but the increment of growth of the cell culture stabilized to a settled cell volume of 40 % towards the end of the investigation period (Fig 16).

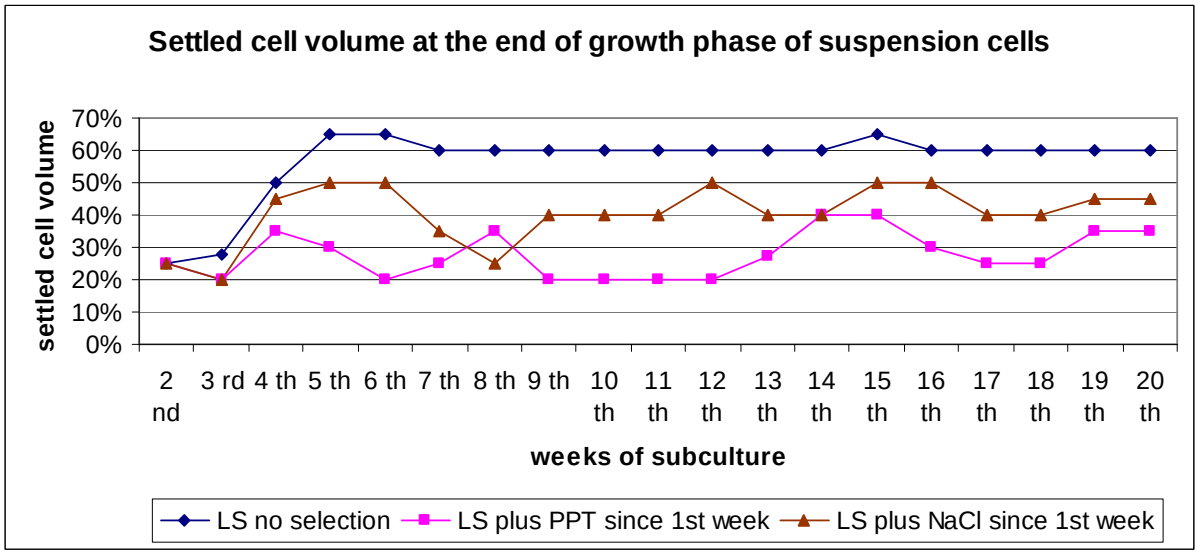


Fig 16: Weekly settled cell volume of suspension cells transformed with the PR10a construct and treated with the respective selective agents.

The relatively low settled cell volume of the NaCl-treated suspension culture was consistent to observations made by comparing the phenotype of a non-selected transformed tobacco suspension culture to one treated with NaCl. As visualised in Figure 17 D, NaCl-treatment resulted in a more compact cell shape and a cell culture of a comparatively denser consistence.

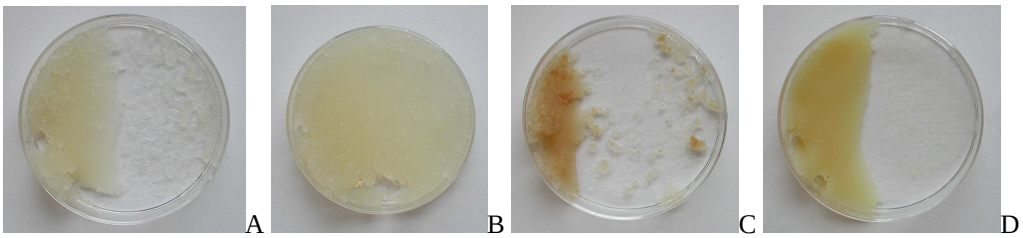


Fig. 17: Phenotype of transformed tobacco suspension cell cultures treated with no selective agent (A), treated with G418 (B), with PPT (C) or NaCl (D)

Suspension cells treated with G418 did not change their phenotype, while the other selective agents, NaCl and PPT, affected a change of phenotype in the suspension cell cultures.

Suspension cultures treated with PPT switched their colour from a light yellow to a reddish brown colour and formed dense cell clusters. NaCl treated suspension cell cultures consisted of very fine cells with no formation of aggregates, whereas non-selected or G418 treated suspension cultures contained loose aggregates in parallel to single cells.

Manual selection of high-expressing calli combined with chemical selection

Both chemical and manual selection were applied on callus cultures transformed with the PR10a construct to investigate which selection regime would lead to the highest increase in luminescence and therefore the most and highly expressive cell cultures.

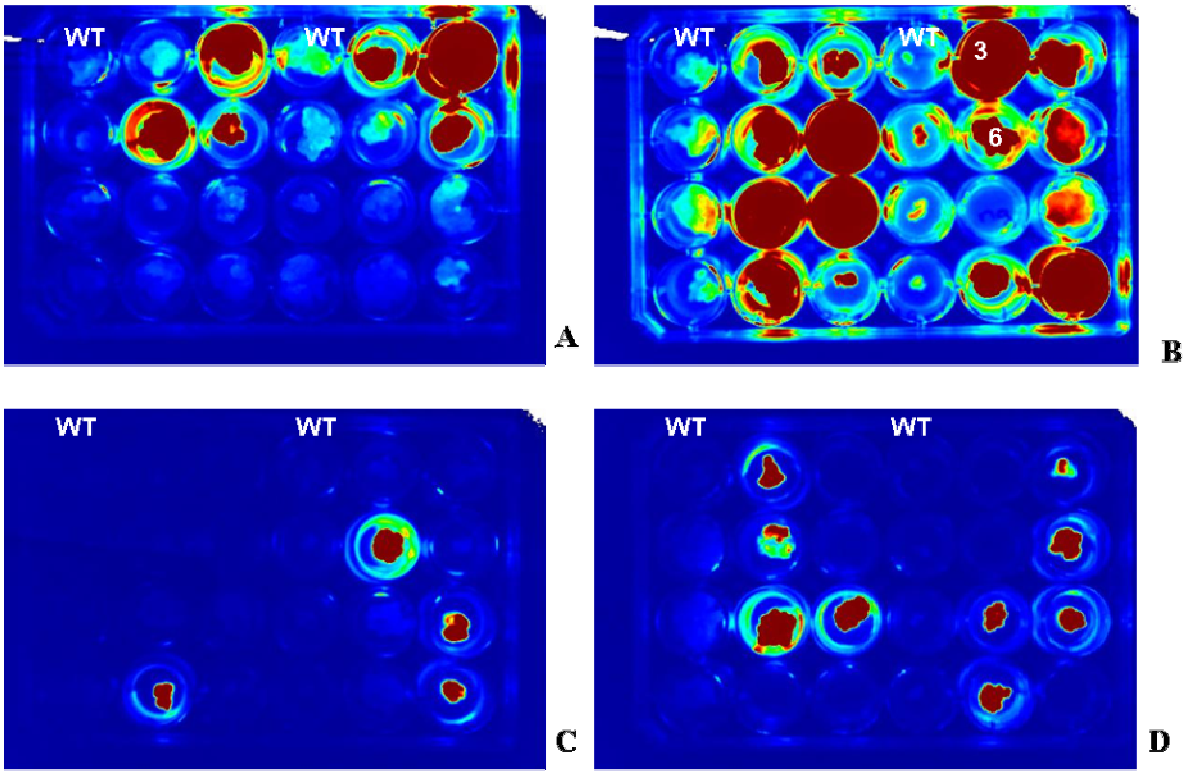


Fig. 18: Luciferase screening of tobacco cell clusters transformed with the PR10a construct and grown on solid LS medium containing no selective agent (A), 6 mg/l PPT (B), 50 mM NaCl (C) and 50 mM NaCl plus 3 mg/l PPT (D). The pictures were taken 20 weeks post transformation and are presented in colour shift mode. WT: Wild type

Since the pictures are presented in colour shift mode, the colour shift from green to red indicates increasing chemiluminescence intensity. The chemiluminescence of callus clusters transformed with the PR10a construct and screened by the Fuji LAS 3000 imager even exceeded the maximum detection level of the imager in one case when the callus cell lines

were not chemically selected and in five cases when the callus cell lines were treated with PPT (Fig. 18, wells completely red).

The cell clusters maintained on solid medium lacking selective agent showed in 6 out of 16 cell clusters extraordinarily high Luciferase activity. All manually selected cell clusters maintained on PPT containing medium showed high to extraordinary high Luciferase expression. 4 of 16 cell clusters highly illuminated when they were treated with NaCl, whereas 9 of 16 cell clusters showed high Luciferase expression when the cell cultures were not only treated with 50 mM NaCl but also with 3 mg/l PPT.

So again callus cultures, either non-selected ones or selected on Phosphinotricin showed more frequently and in higher ranges Luciferase expression than callus cultures selected on NaCl. Coexpression of the *PR10a* gene with the *luciferase* reporter gene resulted in high transgene expression, but not after treatment with NaCl.

3.3.2.4 Comparison of the selective effects of the BAR construct and the PR10a construct

One remarkable difference observed between the cell cultures transformed with the two transformation vectors described in the previous chapters is that the Luciferase activity obtained by cell cultures transformed with the PR10a construct was up to 20 fold higher even when the cell cultures were maintained on medium lacking any selective agent (Fig. 19 A).
When the cell cultures are selected on the distinct selectable marker gene driven by a NOS-promoter, the difference is even more obvious. Luciferase expression of the cell culture transformed with the BAR construct and treated with the antibiotic G418 resulted in only 900 RLU/2s, while Luciferase expression of the PR10a containing cell culture treated with the herbicide PPT reached over 30 000 RLU/2s (Fig. 19 B). The herbicide PPT seems to be the stronger selective agent. This observation was also confirmed by the Luciferase activities which are achieved when the selection took place by the selectable marker gene linked to the reporter gene. In this case the Luciferase expression achieved by the callus cultures transformed with the BAR construct showed the same range as the Luciferase expression of the PR10a transformed callus cultures selected by NaCl six and ten wpt (Fig. 19 C).

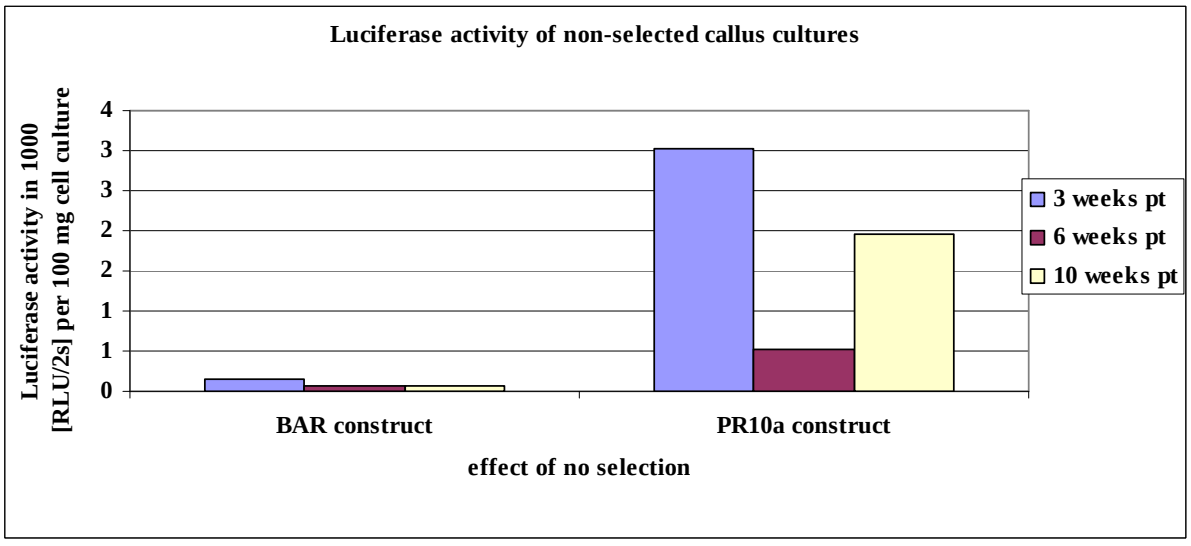
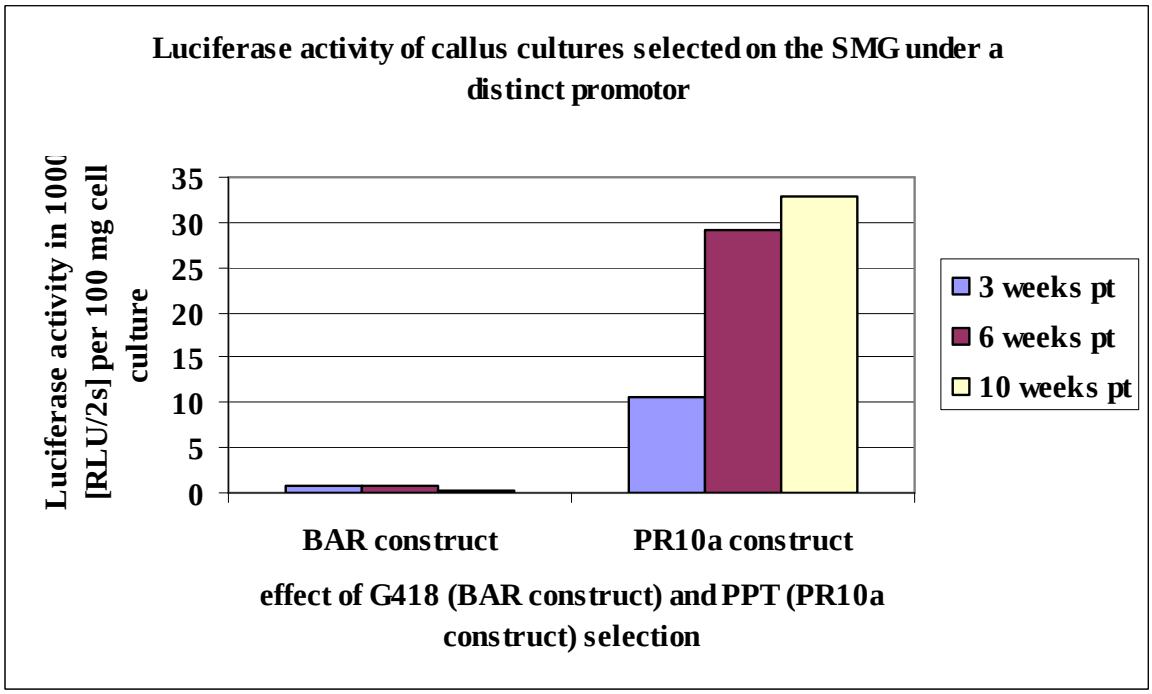
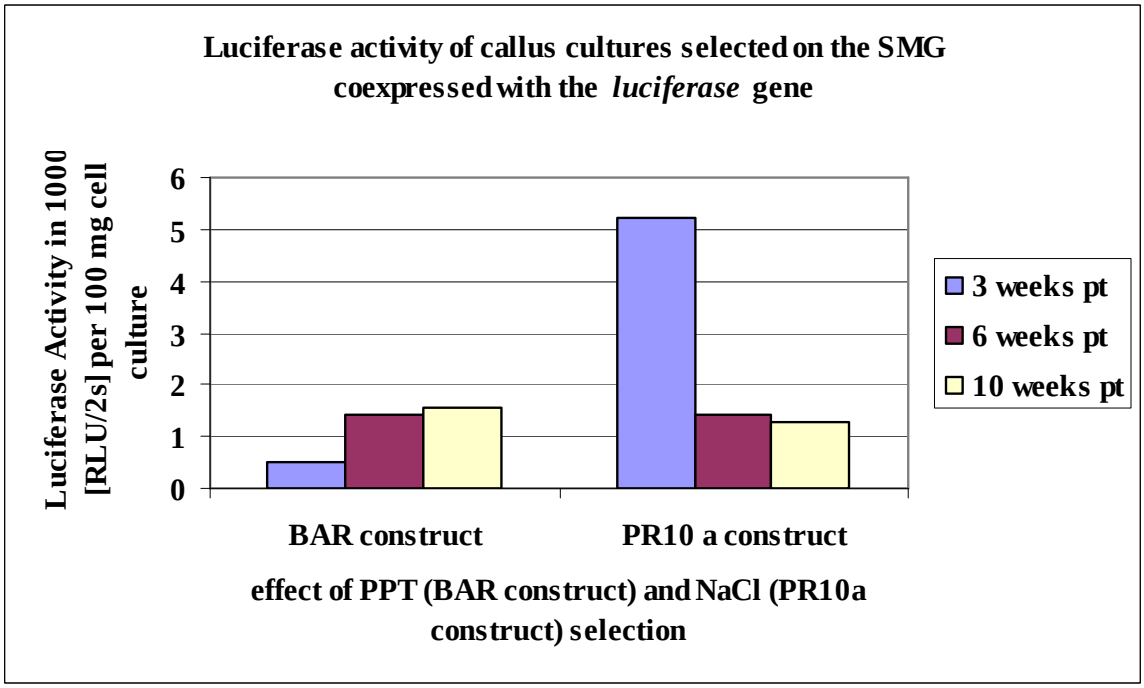


Fig 19A: Luciferase activity of non-selected callus cultures transformed with the BAR construct and the PR10a construct respectively.



B



C

Fig 19 B and C: Luciferase activity of selected callus cultures transformed with the BAR construct and the PR10a construct respectively.

3.3.2.5 Start of suspension cultures derived from manually and chemically preselected callus cultures

One possible reason for the high expression instability in suspension cultures could be that the suspension cultures directly started from the plant-bacteria-coculture probably still consist of a population of variant cells although chemical selection had been applied. This cell mixture may consist of untransformed and transformed cells, the latter expressing the target gene at different levels.

Hence it was tested, whether suspension cultures inoculated from manually and chemically preselected callus cultures express the *luciferase* gene more stably than suspension cultures started directly after the transformation.

Two clones were chosen from callus cultures transformed with one of the two distinct transformation vectors (Figure 10 and 16). The clones were maintained on PPT containing solid medium. The chosen callus cultures were preselected chemically and manually for high Luciferase expression for 5 subsequent Luciferase screenings within 5 months. The newly started suspension cultures were maintained in liquid LS medium containing 6 mg PPT per litre.

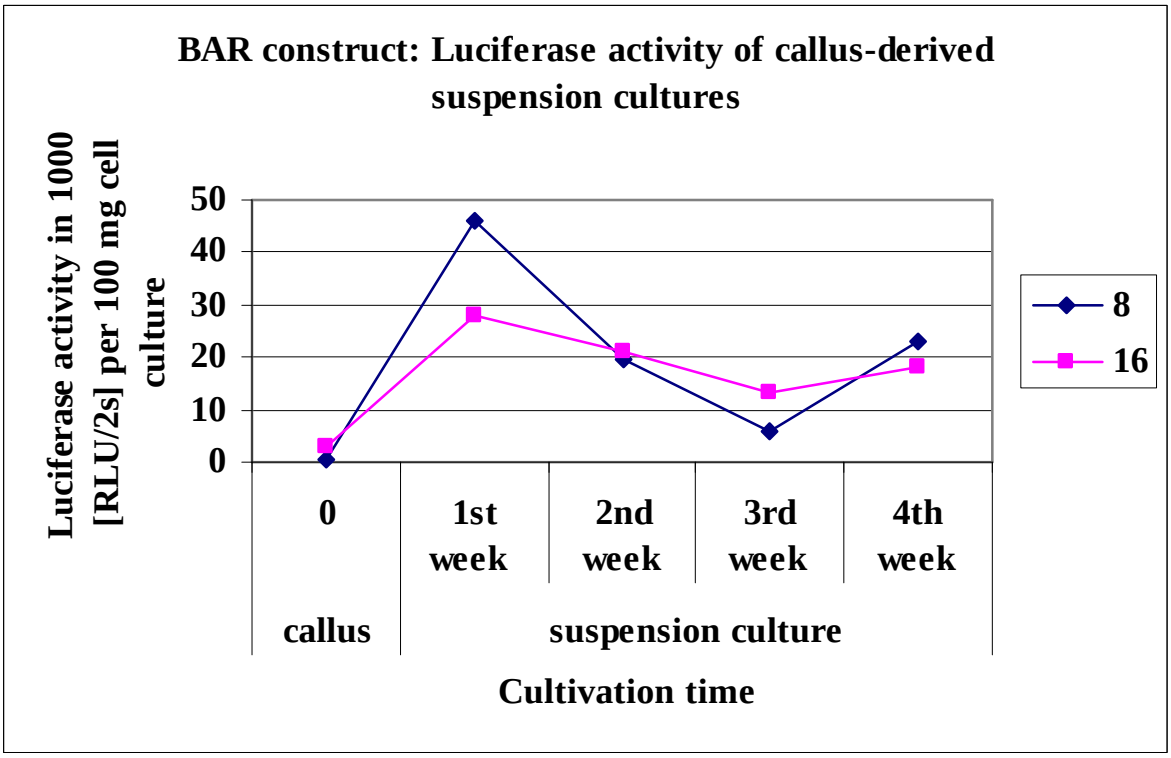


Fig 20: Luciferase activity of newly started suspension cultures derived from manually and chemically preselected callus cell lines transformed with the BAR construct. The callus lines refer to the callus lines shown in Figure 12.

All one week old suspension cultures transformed with either the BAR construct or the PR10a construct showed a Luciferase activity at least 10 fold higher than the callus cultures they had been started from (Figs. 20 and 21, data points 1 and 2). The expression stability of the cell lines transformed with both constructs was ambivalent. While the Luciferase activities of cell lines 8 (BAR) and 3 (PR10a) oscillated considerably after an initially high value, the other two cell lines 16 (BAR) and 6 (PR10a) expressed Luciferase quite stably.

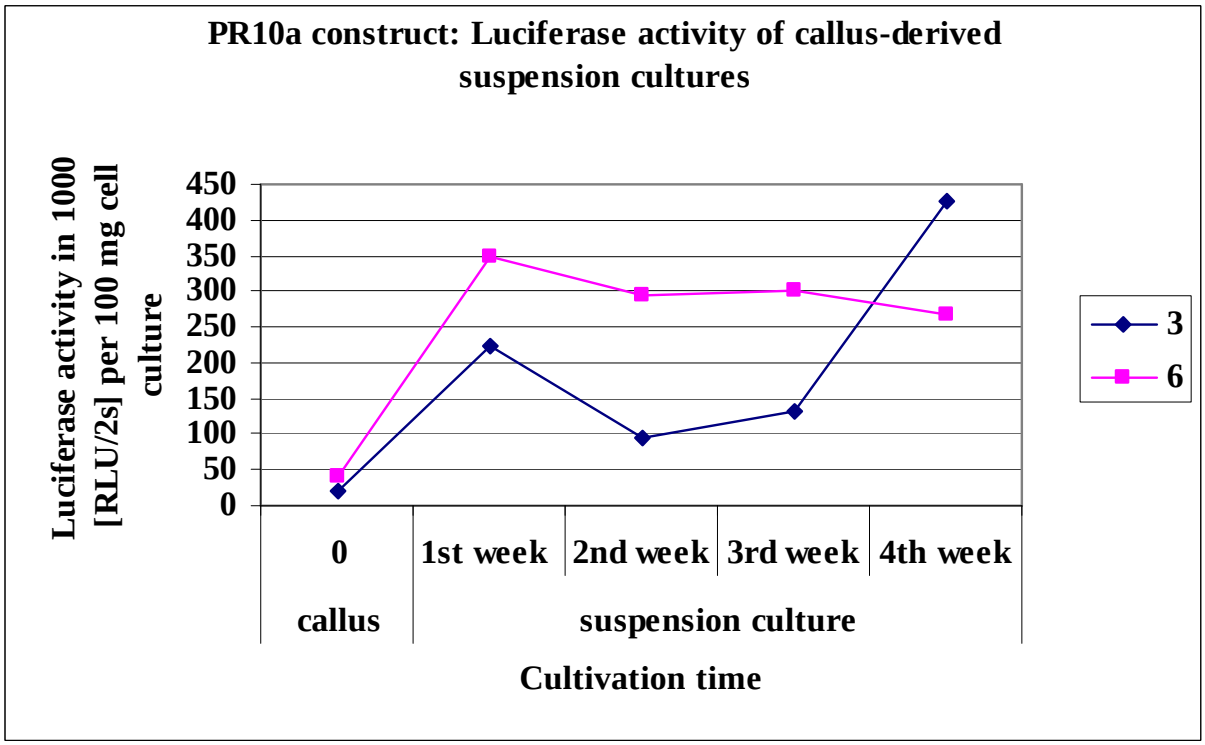


Fig 21: Luciferase activity of newly started suspension cultures derived from manually and chemically preselected callus cell lines transformed with the PR10a construct. The callus lines refer to the callus lines shown in Figure 17.

3.4 Application of a dicistronic transformation vector: enrichment of hspx-producing cells via reporter gene monitoring

To test the potential of the IRES based constructs for the production of recombinant proteins, tobacco cell cultures were transformed with vector pGII 0229 MAS hspx IRES luc, shortly named HSPX-construct. The functionality of the vector was tested by transient expression analysis of infiltrated tobacco leaves. Furthermore the enrichment of hspx expressing cell cultures was monitored via the *luciferase* reporter gene.

3.4.1 Construction of transformation vector pGII 0229 MAS hspx IRES luc (HSPX construct)

Fig. 22: T-DNA of the transformation vector pGII 0229 MAS hspx IRES luc, also named HSPX construct. The selectable marker gene (*bar*) is driven by the NOS-promoter (p-NOS), whereas *hspx* and *luc* are located on a distinct expression cassette controlled by the MAS promoter (p-MAS).

Many frequently used plant transformation vectors carry two reversely transcribed expression cassettes between the left (LB) and right border (RB) of their T-DNA. One expression cassette carries the SMG and the other the target gene or gene of interest. In this conventional design the HSPX construct was built. It carries a *bar* gene as selectable marker under control of the nopalin synthase promoter (p-NOS) in the first expression cassette. The second expression cassette, which was constructed in a dicistronic manner, consists of a mannopin synthase promoter (p-MAS), and *hspx* as target gene, which is linked to the *luciferase* reporter gene by an internal ribosome entry site (IRES) and ends with the CaMV-derived terminator sequence (t-CaMV) (Fig. 22).

Hspx is a gene derived from *Mycobacterium tuberculosis* which encodes a 16 kDa α-crystallin-like protein that is a major antigen (Hu et al, 2006). Recombinant Hspx protein produced in *E coli* is already used in ELISA-based kits for tuberculosis diagnostics.

Vector pGII 0229 MAS hspx IRES luc was constructed based on vector pGII 0229 MAS gus IRES luc (Ali, 2007). The *gus* gene was replaced *via Hind*III and *Cfr*9I / *Xma*I sites with *hspx*.

The last mentioned DNA sequence was generated by PCR using Primer hspxKpn21 and Primer hspxHindIII and vector pGII 0229 35S hspx as a template.

The transformation vectors were confirmed via control restriction digest with *Eco*RI and *Sac*I resulting in one 5 250 bp and one 2 250 bp band. The restriction enzymes *Kpn*I and *Hind*III cleaved a 1 000 bp-sized fragment (p-MAS and hspx) off the remaining 6 500 bp vector (Fig. 23). Additionally the transformation vectors were confirmed by sequencing. Table 12 illustrates the DNA sequences of the sequencing primers.

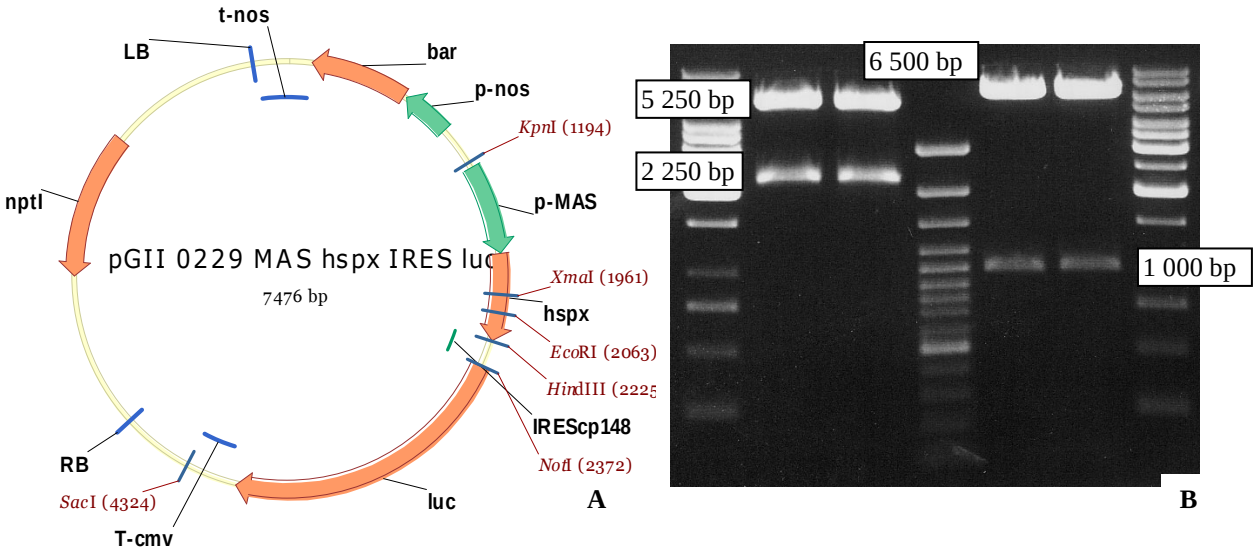


Fig. 23: A) Vector map of transformation vector pGII 0229 MAS hspx IRES luc showing some important restriction sites.
B) Agarose gel image of restricted plasmid: lane 1) and 7) 1 kB DNA ladder (Fermentas), lane 4) 100 bp DNA ladder plus (Fermentas), lane 2) and 3) pGII 0229 MAS hspx IRES luc digested by Eco RI and SacI, lane 5) and 6) same plasmid digested by KpnI and HindIII.

Table 12: DNA sequences of primers used for sequencing of the *hspx* gene

Sequencing primers		
Target gene	**Name**	**Sequence**
back part of *hspx* gene	hspx_sqterm	5´-AGG GCA TTC TTA CTG TGT CG-3´
complementary sequencing primer	hspx sq-r	5´-CGA ACA GCT CAG AAA ACT CG-3´
front part of *hspx* gene	Seq1	5´-TTC TCC TAT CAT TAT CCT CGG C-3´

3.4.2 HSPX detection in infiltrated tobacco leaves

The functionality of the HSPX construct was tested by infiltration of *N. benthamiana* leaves with *A. tumefaciens* suspension for transient expression of HSPX and Luciferase. Since both genes are located on one expression cassette, expression of the tuberculosis antigen HSPX is linked to Luciferase expression. Illuminating parts of tobacco leaves therefore also transcribed the *hspx* gene.

For detection of HSPX a western blot analysis was performed. Protein was extracted from tobacco leaves tested positive for Luciferase activity by screening for luminescence (Fig. 24).

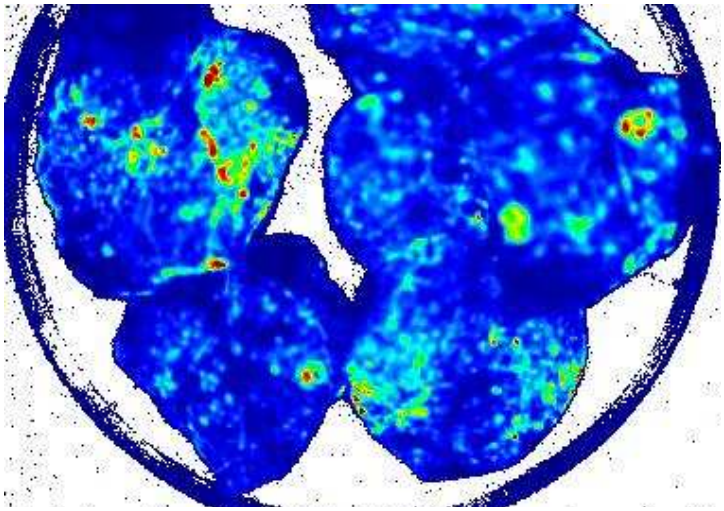


Fig. 24: Leaves of *N. benthamiana* plants were infiltrated with *A. tumefaciens* suspension carrying the HSPX construct. Illuminating parts of the leaves are green to red coloured.

Protein extract of leaves of *N. benthamiana* plants infiltrated with *Agrobacteria* carrying the GUS construct served as a negative control. Luciferase activity of the GUS-construct infiltrated leaves obtained a level of 75 000 RLU/2s, whereas samples of HSPX-infiltrated leaves obtained Luciferase expression of approximately 100 000 RLU/2s (Table 13). With a $NaSO_4$ based extraction method, a protein content of between 0.5 to 2 µg/µl for tobacco cell cultures and 2 to 10 µg/µl for tobacco plants was obtained.

Table 13: Luciferase activity and total protein content of leaves infiltrated with the HSPX construct or GUS construct (negative control).

Leaf Infiltration of *N. benthamiana* plants		
construct	**HSPX**	**GUS**
leaf material	2.6 g	1.25 g
protein extract	3 ml	2 ml
LUC-activity RLU/2s	106 142	74 919
total protein content	4.41 mg/g	4.84, mg/g

1-dimensional polyacrylamide gelelectrophoresis allowed only the application of a limited volume of protein raw extract. Therefore 2-dimensional gel electrophoresis was used for Western blot analysis, because a big volume of protein solution could be applied on the gel stripes. Since proteins were not only separated by size, but also by their isoelectric point, a higher amount of protein was concentrated on a single spot and was more easily detected by Western blot analysis.

As standard the 16kD HSPX protein extracted from *M. tuberculosis* was used. In Lionex lab it was observed that HSPX forms oligomers and dimers even after boiling and denaturing of protein samples for SDS-PAGE. Additionally the 16 kD monomer occurs in a degraded 14 kD sized form (Fig. 25). Both forms are immunologically active (personal communication, Lionex GmbH).

Detection of protein spots which corresponded in size (14 kD) and isoelectric point (app. 5.0) to the HSPX protein could be reproduced three times with different amounts of total protein extracted from tobacco leaves infiltrated with the HSPX-construct. Figure 25 shows the Western Blot of a gel on which 200 µg of total protein were applied and figures 26 A and B show the immunostained membranes of gels on which 240 µg total protein extract were loaded derived of tobacco leaves infiltrated with the GUS construct as negative control (A) and the HSPX construct (B).

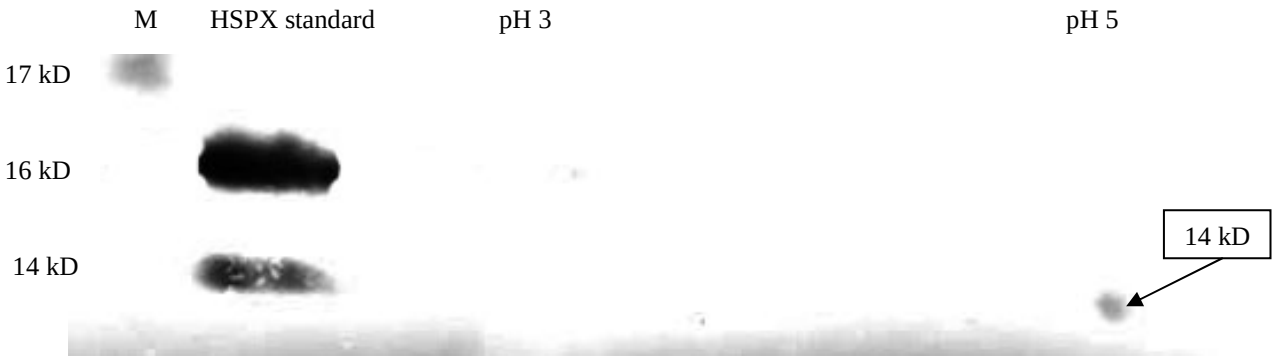


Fig. 25: Western Blot with HSPX-specific antibody after 2-D-gelelectrophoresis of 200 µg total protein extract of HSPX-construct infiltrated *N. benthamiana* leaves.

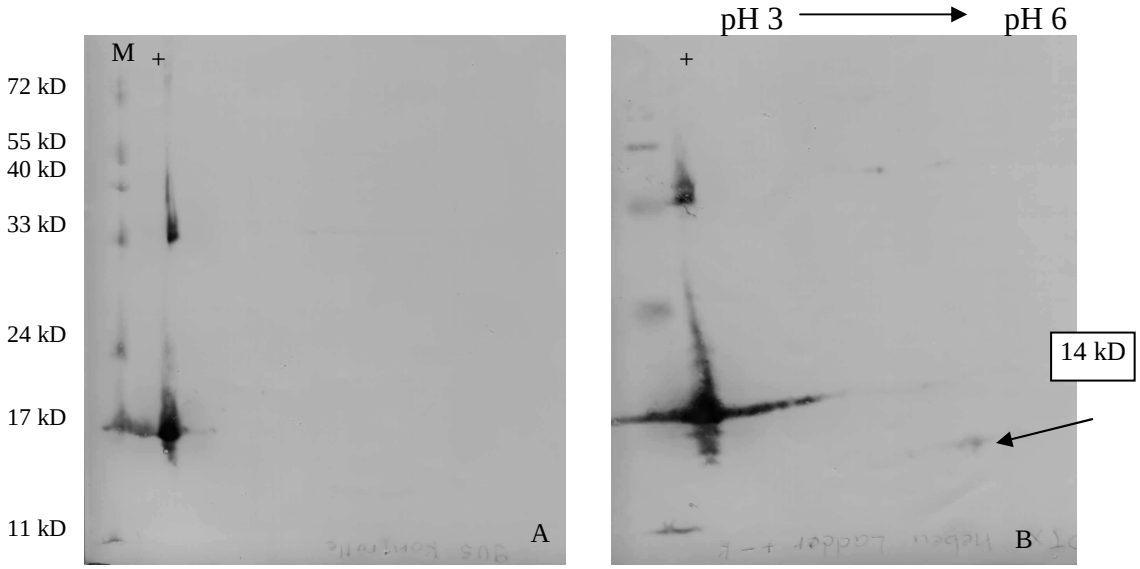


Fig. 26 A and B: Western Blot with HSPX-specific antibody after 2-D-gelelectrophoresis of 240 µg total protein extract of GUS-construct (A) and HSPX-construct infiltrated *N. benthamiana* leaves (B). M: Marker; + HSPX-standard

3.4.3 HSPX enrichment in tobacco cell cultures

To obtain a high producing hspx cell culture, tobacco cells transformed with the HSPX construct (Fig. 22) were not only selected chemically but also manually by selecting the illuminating *luciferase* expressing callus parts. This could be done because the HSPX construct provides not only herbicide resistance because of the NOS-driven *bar* gene, but also the possibility of monitoring the transgene expression of the IRES-driven *luciferase* translation. After transformation the cells were grown on solid medium, containing 6 mg/l PPT as selective agent. In the first four months of subculture only chemical selection was applied, because facilities for Luciferase screening were not available at this time. In the fifth month of subculture Luciferase screening with the Fuji LAS 3000 imager was started and ten illuminating cell clusters were chosen for further experiments. From these ten distinct cell clusters, ten callus cell lines as well as ten suspension cultures were initiated.

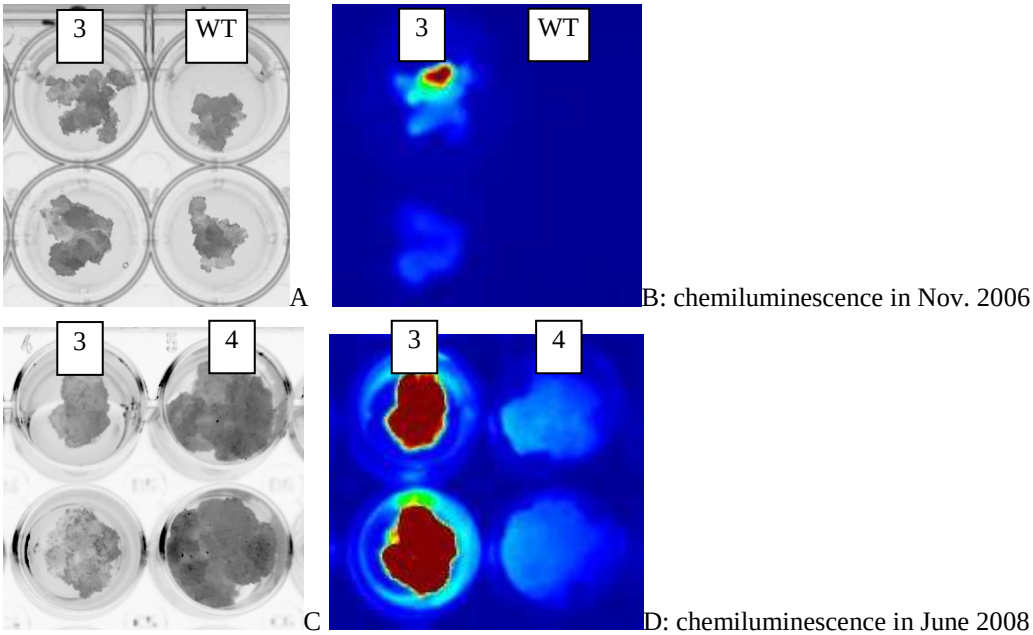
3.4.3.1 Comparison of chemical and manual selection under distinct promoters

To select highly expressive cells by chemical means, suspension cell lines were treated with 6 mg/l PPT. Seven of the originally ten suspension cultures could be grown continuously for 14 months. However, during this period the Luciferase expression of the suspension cultures decreased considerably and in some cases was even lost (Table 14). In parallel, the callus cell lines were subcultured on PPT containing medium every four weeks. According to the expression of the *luciferase* reporter gene the most illuminating parts of the cell clusters were manually selected. With this combination of chemical and manual selection high Luciferase expressing callus cell lines were achieved.

Table 14: Luciferase activity of suspension and callus cells transformed with the HSPX construct.
A: Luciferase activity in 2007. **B**: Luciferase activity in 2008.

Clone	plant material	RLU / 2s	plant material	RLU / 2s	plant material	RLU / 2s
wild type	suspension cells A	48	callus	36		
HSPX 3	suspension cells A	138	callus A	1 717	callus B	39 581
HSPX 7	suspension cells A	39	callus A	265	callus B	469
HSPX 10	suspension cells A	140	callus A	1367	callus B	207
A: LUC-activity in 2007; B: LUC-activity in 2008						

The background activity of wild type tobacco suspension and callus cells was between 30 and 50 RLU/2s. After twelve months selection on PPT containing medium, one suspension cell line showed no more Luciferase activity and two only very little. In contrast, after the same cultivation period the callus cell lines showed 4 to 34 times higher Luciferase activity than the wild type cells. Callus cell line 3 showed a Luciferase activity of nearly 40 000 RLU/2s – this is a 20 fold increase in comparison to the luminescence of the previous year. The increase of Luciferase expression of cell line 3 is also illustrated in Fig. 27.



A B: chemiluminescence in Nov. 2006

C D: chemiluminescence in June 2008

Fig. 27: Luciferase activity of tobacco callus lines transformed with the HSPX construct. The top pictures were taken in November 2006 and show callus line 3 and a wild type callus. The pictures at the bottom were taken in June 2008 and show callus lines 3 and 4.
Pictures B and D show the different intensities of luminescence.

Fig. 27 also shows that herbicide resistance and good growth on PPT containing medium were not necessarily linked to high Luciferase expression. Both cell lines 3 and 4 were started with 1 to 2 mm small cell clusters and showed considerably different cell mass accumulation after four weeks of growth under the same conditions. Cell line 4 proliferated better than cell line 3, but the latter cell line (3) illuminated to a much higher degree than cell line 4.

Since the here described HSPX cell lines were not established from single cells, they are mixed populations consisting of diverse cells which originated from distinct transformation events. Therefore in one cell line several cell "types" occur expressing the *luciferase* gene on different levels. The degree of transgene expression may be influenced by different factors such as the locus of transgene integration or the number of insertion points.

So stable integration and copy number of the T-DNA of different cell lines were characterised and compared to the Luciferase activity. Therefore the presence of the *luciferase* and *hspx* genes in transformed cell cultures was first confirmed by PCR and Luciferase screening. Then the established callus cell lines HSPX 3, HSPX 7 and HSPX 5 were further characterized by Southern Blot analysis.

An equal amount of KpnI digested genomic DNA from transgenic callus cultures was separated, blotted and finally hybridised with a DIG labelled luciferase-probe.

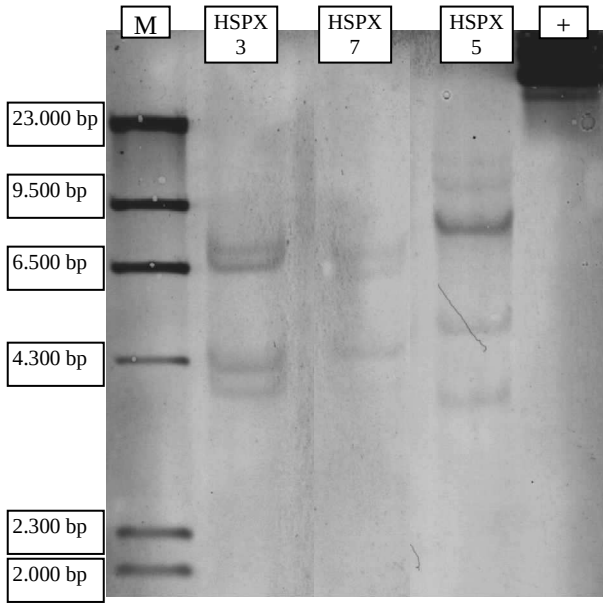


Fig. 28: Southern Blot analysis of genomic DNA from transgenic tobacco callus cultures, transformed with the HSPX construct and digested with KpnI
M: DIG-labelled DNA Marker Roche; +: plasmid DNA

Southern hybridization analysis confirmed the integration of T–DNA in the selected callus cultures (Fig. 28). Plasmid DNA was used as positive control. Three to five copies of the *luciferase* gene were detected for each of the cell lines. It could not be distinguished, in how many different cell "types" the copy numbers split up. In cell line HSPX 7 three insertion points were detected, the cell line could therefore consist of three cell "types", each carrying one copy of the luciferase gene, or of two cell "types", the first carrying two copies and the second carrying one copy.

The number and particularly the intensity of bands corresponded to the Luciferase activity (Table 15). The cell lines HSPX 3 and HSPX 5 with the highest Luciferase activity of around 40 000 and 23 000 RLU/2s respectively, showed four and five clearly visible bands of the *luciferase* gene. In contrast, cell line HSPX 7 showed three slightly visible bands and also the lowest Luciferase activity of only 469 RLU/2s.

The intensity of the bands might probably be taken as a measure of the percentage of transformed cells of which the specific callus culture consists at a given time.

Table 15: Luciferase activitiy and number of bands of established callus cell lines used for Southern Blot analysis.

callus cell line	**LUC-activity [RLU/2s]**	**Copy number**
HSPX 3	39 581	4
HSPX 7	469	3
HSPX 5	22 576	5

Western blot analysis was repeated with protein extracts from tobacco callus cell cultures transformed with the same HSPX construct. However, HSPX extracted by cell cultures could never be detected by Western blot.

4 DISCUSSION

Production of plant metabolites or recombinant proteins in plant suspension cultures offers many advantages. Nevertheless, for the efficient production of recombinant proteins two major challenges have to be overcome. One challenge is the relative low protein yield achieved in plant cell cultures. This problem is even more severe if the recombinant protein contributes only to a small percentage of the total protein content of the cells. Another challenge is the instability in expression of both native genes and transgenes shown in cell cultures (Schillberg, 2003; Hellwig, 2004; Ketchum and Gibson, 1996; James and Lee, 2005).

To increase and stabilize protein expression in plant suspension cultures in general different strategies were applied. One strategy was to enhance the protein content per cell by influencing the metabolism of the given cells. This could be achieved by either the enhancement of protein production or by minimizing protein degradation. Such approaches were demonstrated by Baur et al. (2005), who added or coexpressed stabilizing agents to enhance the recovery of a secreted recombinant human growth factor in *Physcomitrella* cell cultures, or by Soderquist and Lee (2005), who enhanced the production of recombinant proteins from plant cells by the application of osmotic stress and protein stabilization through the addition of mannitol and bovine serum albumin (BSA).

In the present work another strategy was investigated. By *Agrobacterium* – mediated transformation of a cell culture a high amount of genetically different cells were obtained. Starting with this heterogeneous cell culture, single cells should be selected which express the transgene at a high level and so produce the target protein at a high percentage of the total protein content.

By selecting these cells a high producing cell culture should be established. Even though the production rate of total protein would not be increased, the percentage of the recombinant protein in such a cell culture would increase. It has to be pointed out that this strategy is supplementary and not alternative to the previous strategies to increase the total protein production in plant cells. Possible reasons for high expression of the transgene could be due to: transgene incorporation in an active gene region, incorporation into cell types with either reduced protein degradation or higher metabolism rates or higher gene expression because of insertion of multiple copies.

By using the present approach cells were achieved expressing a recombinant target gene at a high level and accumulating in the cell culture either by means of chemical or in the case of callus by manual selection or a combination of these.

The investigated strategy to establish a high producing suspension culture involved the coexpression of a selectable marker and target/reporter gene under control of the same promoter. Therefore dicistronic transformation vectors were constructed and used for transformation. As a part of our efforts to optimise the process of recombinant protein expression in plant suspension cultures, we were also interested in gaining a broader perspective into the stability of transgenes and their expression through successive generations.

4.1 Establishment of a system for direct transformation of plant suspension cells

Different strategies are used to establish a transgenic suspension culture. One approach is to regenerate transformed plants and subsequently initiate transgenic cell cultures from these transgenic plants. In another approach wild type cell cultures are transformed to obtain a transgenic cell culture. In some other studies first transgenic plants have been established, selected and characterized and later cell cultures have been derived out of these plants (Firek et al., 1993; Girard et al., 2004). In the present study another concept was chosen. Here benefit should be made from a heterogeneous pool of different transformation events by inoculation of plant suspension cultures with agrobacteria. Subsequently these cells that showed high expression rates of the desired target gene were selected preferentially by chemical means.

In this work a cocultivation system for plant suspension cultures in a suction strainer was developed and successfully applied. The described cocultivation system provides the advantages of simultaneous transformation of a flexible volume of suspension culture cells and successive coculture and washing procedure in the same container minimising the risk of losing cell material in the washing process. In comparison to the establishment of suspension cultures derived from transgenic plants, the direct transformation of a suspension culture offers a more heterogeneous mixture of differently transformed cells.

Routinely we cocultivated 50 or 100 ml of suspension cultures in a suction strainer. The use of such a large number of cells could lead to very different environments in the strainer, influencing such things as the moisture of the cell culture or the availability of oxygen in different cell layers.

The distribution of transformation events in the suction strainer was analysed by X-Glc-staining of suspension cells transiently expressing a *glucuronidase* gene. Glucuronidase

activity could be detected spot wise in all cell layers above the holes of the suction strainer as well as spread over big areas of cells both in the centre and at the edge of the funnel (Fig. 1). Blue cells were discovered not only in the top layer of these wide spread areas, but also in some deeper layers

Colour formation of the X-GlcA substrate requires three separate reactions: after enzymatic turnover, the released indoxyl derivative dimerizes and is subsequently oxidized to the final indigo dye. That means the final step of the detection reaction requires oxygen for formation of the blue dye. The blue staining was most clearly visible on the surface of the cell culture or near the holes of the suction strainer. It must be assumed that there is an oxygen gradient in the suction strainer, and that the oxygen concentration was higher in the superficial cell layers of the cell culture or near the holes at the bottom of the suction strainer. This might indicate that at areas with higher oxygen supply the detection of Glucuronidase activity will be better, whereas the transformation rate will not be higher. So, even cells in deeper layers could have been transformed, but possibly this was not detected due to lack of oxygen. The heterogeneity of the distribution of transformed cells in the strainer could also be less pronounced than expected.

The conclusion drawn from the X-Glc staining was maintained, which is that the cells were mixed before continuing the cultivation process. A mixing of cells also guarantees that cells which suffered from the specific conditions in the suction strainer like for example low oxygen availability were properly mixed with more healthy cells and equally distributed over the samples used for further investigations.

4.2 Independent expression of a SMG and reporter gene

Vectors pGII 0229 MAS gus IRES luc (GUS construct) and pGII 0229 MAS hspx IRES luc (HSPX construct)

Reduction or loss of Luciferase expression was observed in the present study when the selectable marker gene was expressed under the control of another promoter than the reporter gene.

This was the case in tobacco callus cultures transformed with the GUS construct. Even though some transgenic cells proliferated well on the PPT-containing medium, these cells did not show high Luciferase activity. Furthermore Luciferase expression could not be increased despite picking the brightest part of the cell clusters each month (Fig. 3).

Similar observations were made with suspension cultures transformed with the HSPX construct: whereas the *bar* gene in these suspension cultures was active and provided herbicide tolerance to the well-proliferating transgenic cells, expression of the Luciferase gene dropped completely (Table 14).

Register et al. (1993) reported similar observations. This group transformed maize plants with vectors carrying two distinct expression cassettes – a non-selectable expression cassette containing the *uidA* gene for Glucuronidase expression and a selectable gene expression cassette containing the *bar* gene. Where there was no difference in the number of integrated bar and uidA expression cassettes, differences in transgene expression were reported. The non-selectable *uidA* gene was expressed in fewer plants than was the selectable transgene.

One possible reason for the loss of reporter gene activity could be the silencing of the reporter gene. However, a quantitative comparison whether such silencing events take place more often in dedifferentiated cell cultures than in differentiated tissues or plants cannot be decided from the present study.

Application of manual selection for enrichment of target gene production

Because all cell cultures in the present study were transformed with dicistronic vectors carrying the *luciferase* reporter gene as second cistron behind the IRES element, manual selection according to Luciferase expression was possible. By applying manual selection to callus cultures, which was always done additionally to chemical treatment, it was possible to establish cell lines showing high Luciferase activity. Via picking of high luminescent callus parts, the Luciferase activity of one Hspx and Luciferase expressing cell line could be increased to nearly 40 000 RLU/2s. These results are consistent to experiences made in classical production systems for secondary metabolites. In *Catharanthus roseus* cell suspension cultures high alkaloid production could only be achieved by manually picking fluorescent callus parts, but subsequent rapid loss of productivity occurred, when clonal selection was not applied permanently (Deus-Neumann B. and Zenk M., 1984). In former studies with secondary metabolites it has been observed that cell cultures lose their high production capacity during culture periods without selection pressure. To overcome this problem in our study, selection pressure was maintained through chemical selection during the growth phase of calli. Indeed this approach could stabilize the high productivity that was obtained in the selection steps. However, permanent manual selection is only possible with callus cultures and cannot be applied for suspension cultures, especially when suspension cells are cultivated in closed containers such as fermenters.

4.3 IRES-mediated coexpression of a SMG and a reporter gene

Growth of transgenic cell cultures

The value for water content of the transformed and selected callus material was taken as a parameter for the enrichment of transgenic cells in the cell culture due to chemical selection and was compared to the water content of unselected cells.

The water content percentage in cell cultures usually varies in different stages of the growth curve. Immediately after subculture, cells normally show a low degree of vacuolization and a relatively high dry matter percentage, which is the same as a low value of water content. Water uptake increases as well as the size and number of vacuoles during the growth phase and specifically in the late stationary phase. This is indicated by a general increase of the water content. In contrast, a comparatively low water content value at the end of the subculture may also represent a higher percentage of dead cells due to the selection process. The water content percentage of the transformed and selected callus material was therefore measured to see if differences could be observed which may be linked to the percentage of dead cells caused by the influence of the toxic selective agents.

As shown in Chapter 3.1, the cocultivated cell culture in the Buchner funnel consisted of a mixture of untransformed and transformed cells. Selective agents were therefore applied to select for the transgenic cells and to drive the untransformed cells to extinction. Dead cells should have released their water content whereas the insoluble parts of the dry matter should have persisted at least to some extent in the culture vessel thereby increasing the measured value for dry matter.

With the ongoing selection procedure, the water content of transgenic selected callus cultures shifts to the same level as the water content of transgenic non-selected callus cultures.

As Figures 8 and 13 illustrate, the values of water content of cell cultures transformed with one of both transformation vectors and selected on herbicides or antibiotics increased to a similar water content value as the non-selected transgenic callus cultures, which ranged between 96 and 98% after a period of ten weeks post transformation.

It has to be pointed out that any conclusion whether these effects were caused by the selection process have to be taken with care since the slight difference lies well within the fluctuation that a normal cell culture shows throughout different growth stages. The same caution has to

be applied for the conclusion that the differences are due to the percentage of dead cells in the culture. It may also be the consequence of unspecific stress exerted by the selective agents even for tolerant cells.
The necessary caution must also be applied for cell growth under salt selection. Here the water content values calculated for transgenic callus cultures treated with NaCl remained on a similar low level during the whole investigation period. Since NaCl has an osmotic effect on plant cells, dehydration of the cells is caused by NaCl. In contrast, PPT and G418 do not change osmolarity of the plant medium, so this effect could only be observed in cell cultures treated with NaCl.

Luciferase expression in transgenic cell cultures

To summarise the selection experiments with callus and suspension cultures carrying the BAR construct, it can be reported that chemical selection on the selectable marker gene (*bar* gene) which is under control of the same promoter as the reporter gene reliably yielded the highest expression of the *luciferase* gene. Whereas cell cultures selected on the other selectable marker gene (*nptII* gene) driven by a distinct promoter, obtained a comparably lower Luciferase expression. Luciferase activity of cell cultures treated with G418 also decreased during the investigation period. Without applying chemical selection, the cell cultures lost Luciferase expression.
In suspension cultures transformed with the BAR construct the same trends were observed. However, higher Luciferase activity levels than in callus cultures were obtained. We assume that usage of selective agents in suspension cultures is more efficient than in callus cultures, because in suspension cultures all cells (or cell clusters) are in direct contact to the surrounding medium and the selective agent.

Luciferase activity of cell cultures transformed with the PR10a construct and not treated with any selective agent was detectable over the whole investigation period. In manually preselected callus cultures two clones even exceeded the detectable level of Luciferase activity at the end of the investigation period. Chemical selection on the *PR10a* gene which is controlled by the MAS-promoter in the same expression cassette as the *luciferase* gene, did not lead to the highest Luciferase activity.
Highest Luciferase expression was measured after PPT treatment in cell cultures transformed with the PR10a construct.

Similar results were observed in suspension cultures transformed with the same construct and treated with the same selective agents. Suspension cultures showed an up to ten times higher Luciferase activity than the callus cultures.

4.4 Comparison of the efficiency of selection regimes

In the present study different types of selection regimes such as herbicides, antibiotics and salts were applied.

Against our expectations the highest Luciferase activity could be achieved in tobacco suspension cultures transformed with the PR10a construct and chemical selection exerted by PPT. The resistance against the herbicide PPT was provided by the *bar* gene which in these transgenic cell cultures was driven by a NOS-promoter, whereas expression of the *luciferase* gene was controlled by a MAS-promoter. So here the high expression of the model target gene *luciferase* was caused by selection through a selectable marker gene driven by a promoter different from the promoter controlling the target gene. In this case silencing effects should occur for both genes to the same degree. It also shows that in this case herbicide selection was more effective than antibiotic or osmotic selection.

Similar results are described by Register et al. (1994). They reported from experiments with transgenic maize plants that selection on bialophos (a precursor of PPT), mediated by the *bar* or *pat* genes, was more efficient, than the selection on kanamycin, mediated by the *nptII* gene.

While working with toxic substrates the transformation efficiencies might be suboptimal because dying untransformed cells may inhibit transformed cells from proliferating by secreting inhibitors or by preventing essential nutrients from reaching the living transformed cells (Haldrup et al., 1998).

The inhibition of glutamine synthetase by PPT results in an accumulation of ammonium to toxic levels for all plant cells. For non-photosynthetic tissues growing *in vitro*, it is not clear which biochemical mechanisms are responsible for the ammonium production. When photosynthesis and photorespiration are minimal, the remaining possible sources for ammonium are nitrate assimilation and metabolic processes. De Block et al. (1995) did not find a correlation *in vitro* between nitrate assimilation and ammonium production, but they reported that the mechanism of PPT toxicity *in vitro* is largely determined by the metabolic activity of the tissue. Tissues with a high metabolic activity are more sensitive to ammonium, while tissues with a low metabolic activity are more sensitive to glutamine deprivation.

Measured by the settled cell volume at the end of a seven day long subculture period, the suspension cultures showed the lowest increment of cell mass during PPT treatment. This could be due to the harmful effect of ammonium ions, but also due to the observation that high producing cells showed lower growth. That high-production cultures show growth inhibition upon subculture, whereas non-producing cultures show little growth inhibition was reported by Kim et al. (2004), who investigated the effect of subculture and elicitation on instability of taxol production in *Taxus* suspension cultures. They assumed that the growth inhibition may be an indication of cellular differentiation leading to cell death occurring in the high producing cells within the culture and that these cells emit chemical signals to switch cells into a high producing state. Such an explanation can probably be ruled out for our cell lines as well, where a recombinant protein is produced which was probably not participating in special differentiation processes of the plant cells.

4.5 Enhancing effect of the *PR10a* gene on transgene expression

Pathogenesis-related (PR) proteins are expressed by virtually all plants in response to pathogen infection and, in many cases, in response to abiotic stresses as well. The PR10 protein family consists of relatively diverse members and more than 100 PR10 or PR10-related sequences have been identified from various flowering plants. PR10 proteins are not only involved in plant defence, but display several additional functions, including a role in developmental processes and enzymatic activities in secondary metabolism (Liu and Ekramoddoullah, 2006). Srivastava et al. (2004) reported that the constitutive expression of a pea PR10 gene in *Brassica napus* enhances their germination and growth in the presence of NaCl. Recently salt tolerant potato cell cultures have been achieved at DSMZ by overexpression of the potato *PR10a* gene (El Banna, 2008). PR10a expressing calli derived from transgenic *Nicotiana tabacum* strain SR1 plants showed growth up to a level of 200 mM NaCl. This is a clearly higher salt tolerance than the 50 mM salt tolerance which could be obtained in the present study for *Nicotiana tabacum* strain Bruessel cell cultures expressing the same *PR10a* gene.

More remarkable than the moderate salt tolerance provided by the *PR10a* gene is the effect on transgene expression in cell cultures transformed with this construct. Even in non-selected cell cultures, calli transformed with the *PR10a* gene containing transformation vector showed an up to 20 fold higher Luciferase activity than calli transformed with the BAR construct. Whereas non-selected cell cultures transformed with this BAR construct showed an initial Luciferase activity of only 150 RLU/2s and then completely lost the transgene expression, Luciferase activity of non-selected cell cultures transformed with the PR10a construct ranged between 500 and 3 000 RLU/2s. Thus, incorporation of the *Solanum tuberosum* derived PR10a protein into tobacco cell cultures enhanced the expression of the *luciferase* gene. This enhancing effect was obtained in three different transformations. Although the expression of Luciferase was higher in these cell cultures, their total protein content remained similar to cell cultures containing the BAR-construct and did not increase (data not shown).

In general, functions of the PR10 proteins are still unknown, but some PR10 proteins may interact in plant cells with other proteins. It is possible that some PR10 proteins might have evolved the capacity for catalytic activities and/or ligand-binding activities due to selective pressure (Liu and Ekramoddoullah, 2006).

4.6 Experiments using the HSPX protein as an example for practical application

The final aim of the present study was the improvement of recombinant protein production by plant cell cultures. As a practical example of economic importance the HSPX protein from *Mycobacterium tuberculosis* was chosen. HSPX is already used by Lionex company for the production of ELISA kits for diagnosis of tuberculosis. The Lionex company is located in Braunschweig close to DSMZ and they provided the *hspx* gene as well as antibodies for the detection of the protein. Lionex is interested in alternative production systems, because the high hydrophobicity of HSPX causes problems for the production in transformed *E. coli* such as formation of inclusion bodies.

Dicistronic transformation vectors have been applied, in which the target gene *hspx* is coexpressed with a *luciferase* gene mediated by an IRES-element. Transient leaf infiltration assays for functionality testing of the vectors have been carried out as well as stable transformations of cell cultures.

The only case in which recombinant HSPX expression in tobacco was detectable by western blot analysis in this work, was transient HSPX expression in leaf-infiltrated *N. benthamiana* plants. In transgenic tobacco cell cultures co-expressing the *luciferase* and *hspx* genes HSPX was not detectable by Western blot analysis. This could be due to different reasons. First the total protein content of leaves is much higher than the total protein content of cell cultures. Since transgenic plant material usually expresses transgenes in low yield of an estimated range between 0.1 to 1% of the total protein content, the recombinant protein yield might have been beyond the detection level. Another reason could be the low number of producing cells in a suspension culture. Chemical selection on the selectable marker gene which in the HSPX construct was controlled in a separate expression cassette than the target protein did not necessarily result in high luciferase activity. In contrary, suspension cultures which were only chemically selected with Phosphinotricin lost their Luciferase activity after 14 months of subculture. Only in callus cultures was it probably possible to achieve a high transgene yield judged by Luciferase monitoring and manual selection on high luminescent callus parts. Just recently after a very long selection process in callus cultures comparably high levels of Luciferase activity could be achieved as by leaf infiltration (100 000 RLU/2s). Possibly in this high Luciferase expressing callus culture (40 000 RLU/2s) the HSPX is also expressed in a detectable amount.

Reasons for the difficult detection of the HSPX protein could come from incomplete extraction due to the high hydrophobicity of this protein. Such difficulties could occur in the extraction process but also hinder the electrophoretical separation of the protein. The possibility that production and glycosylation in plants may affect the antibody binding and thus handicap the immunological detection in the Western blot is unlikely since the detection was possible using *Agrobacterium*-infiltrated transient expressing tobacco leaves. Finally this problem could not be solved in the present study.

Callus cultures carrying the HSPX construct and enriched for Luciferase expression by manual selection were analysed by Southern hybridisation (Fig. 28). The two cell lines with the highest Luciferase activity show the highest number of bands which means the highest number of different integration loci. From this Southern blot it cannot be concluded whether these integration loci occur in different cells or several within the same cells. These cell lines show also the highest intensity of bands (Table 13). The intensity may be taken as measure for the percentage of cells carrying gene insertions in the cell culture. One cell line showed four bands inserted probably representing four different insertion loci, the other one five. It has to be pointed out again that it is not clear, how these insertion loci are distributed over the heterogeneous mixture of cells in the culture.

5 CONCLUSIONS

In transgenic cell cultures carrying the BAR construct the highest and most stable Luciferase expression was achieved after selection on the selectable marker gene which is coexpressed with the reporter gene; whereas non-selected cell cultures lost transgene expression.
However, in transgenic cell cultures carrying the *PR10a* gene the highest Luciferase activity was measured, no matter if and which selective agent was used.
Besides, it could be shown, that not only the position of the SMG is important but also its selection efficiency.
In respect to Luciferase expression selection on PPT was more efficient than selection on G418 or NaCl, regardless if the *bar* gene was under control of the same or another promoter than the *luciferase* gene.
It turned out that, the *PR10a* gene cannot be used in tobacco strain Bruessel cell cultures for selection on NaCl, because it provided only minor salt tolerance.

In summary, it was demonstrated that the newly designed dicistronic transformation vectors can be used
1) to increase expression stability of a target gene by its coexpression with a selectable marker gene; and
2) to enrich production of a target protein by coexpression with a detectable reporter gene such as *luciferase* as a tool for manual selection.

6 OUTLOOK

Dicistronic transformation vectors can be used as tools to express recombinant proteins in cell cultures on reliably high levels. By placing the selectable marker gene as second cistron behind the IRES element the efficiency of the dicistronic vector might even be exaggerated, because the IRES-mediated translation in general is lower compared to the cap dependent translation.

The functionality of the *bar* or *nptII* or *PR10a* genes as selective markers behind the IRES element needs to be demonstrated and an appropriate selection regime has to be established for different plant systems or species.

The *PR10a* gene might be a good option for a selectable marker gene in other plant species than *N. tabacum* strain Bruessel cells, because a non-toxic selective agent can be used. Recently, it could be shown in DSMZ that the *PR10a* gene provided high salt tolerance in potato cell cultures and plants and also in other tobacco species (El Banna, 2008).

Polycistronic constructs for parallel monitoring and selection of a target gene under control of the same promoter might also be applicable for research in plants, as they have long been used in mammalian cell culture.

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8 APPENDICES

8.1 Appendix I: LS plant medium for *Nicotiana tabacum* L. strain Bruessel cell cultures

LS and vitamins	(Duchefa L0230)	4.402	g/l
2,4-D		0.22	mg/l
1-NAA		0.186	mg/l
Sucrose		30	g/l

Adjust to a pH of 6.

For solid medium: add 9 g/l plant agar (Duchefa P1001).
For transgenic cell cultures, the same medium was supplemented with 150 mg/l Ticarcillin and other selective agents AFTER autoclaving.

8.2 Appendix II: LB Broth– Low Salt for *Agrobacteria* (Duchefa L1703)

Trypton	10	g/l
Sodiumchloride	5	g/l
Yeast extract	5	g/l
Total	20	g/l

For solid medium: add 15 g/l agar.

8.3 Appendix III: LB Broth – High Salt for *Escherichia coli* (Duchefa L1704)

Trypton	10	g/l
Sodiumchloride	10	g/l
Yeast extract	5	g/l
Total	25	g/l

For solid medium: add 15 g/l agar.

8.4 Appendix IV: SOC-Medium for heat shock transformation and electroporation

Tryptone	20	g/l
Yeast extract	5	g/l
Sodium chloride 10 mM	580	mg/l
Potassium chloride 2,5 mM	186.4	mg/l
Magnesium chloride sextahydrate 10 mM	2.033	g/l

For 250 ml SOC, add 5 ml **1 M sterile-filtrated glucose** after autoclaving the SOC.

8.5 Appendix V: MMA for tobacco leaf infiltration

MS salt and vitamins	Duchefa M 0222	4.6	g/l
Sucrose		20	g/l
MES		1.95	g/l
NAA		0.5	mg/l

Adjust the pH to 6.3.

8.6 Appendix VI: MS 0 for *Nicotiana tabacum* SR1 transformation

MS-salt and vitamins	(Duchefa M 0222)	4.4	g/l
MES Monohydrate	(Duchefa M 1503)	0.25	g/l
[solid: sucrose		20	g/l
plant-agar		8.5	g/l]

Adjust pH to 5.6 to 5.8 using 1 N KOH.

8.7 Appendix VII: MS 1 for callus and shoot formation

MS salt and vitamins	(Duchefa M0222)	4.4	g/l
MES Monohydrate		0.25	g/l
Sucrose		20	g/l
Plant-agar	(Duchefa P 1001)	8.5	g/l
NAA (α-Naphtalene acetic acid)		0.5	g/l
BAP (6-Benzylaminopurin)		1.0	g/l

Adjust to a pH of 5.6 – 5.8.

8.8 Appendix VIII: MS 2 for root generation

MS-salt and vitamins	(Duchefa M0222)	4.4	g/l
MES Monohydrate		0.25	g/l
Sucrose		20	g/l
Plant-agar		8.5	g/l
BAP		0.2	g/l

Adjust to a pH of 5.6 – 5.8.

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10 CURRICULUM VITAE

Date and place of birth: 14th July 1976, Nürtingen, Germany
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Academic Studies

2005 – 2008	PhD in Biology, Leibniz University, Hannover German Collection on Microorganisms and Cell Cultures (DSMZ), Braunschweig "Development of a selection system for transgenic plant suspension cultures based on dicistronic vectors
2001 – 2004	Advanced studies in biology, Leibniz University, Hannover Graduation as "Diplombiologin", marked "very good" Diploma thesis: „Distribution and adaption of *Fagus sylvatica* in Northwest Germany. Investigations with molecular markers and palynological data.
2000 – 2001	study abroad: Tropical Biology, Universidad de Heredia, Costa Rica
1999 – 2000	Advanced studies in biology, University of Heidelberg
1997 – 1999	Basic studies in biology, University of Ulm "Vordiplom", marked "good" (2.1)

Work experience

2004 - 2005	Customer Service, Bosch Siemens Hausgeräte, Hannover
1996 - 1997	Volunteer in Environmental Education, Friends of the Earth, BUND Mannheim (FÖJ)

School Education

1983 – 1996	Primary and Grammar School in Wendlingen, Baden-Württemberg

Trainining courses

September 2007	Gene Technology Security, government approved course § 15 GenTSV for project leaders, HZI, Braunschweig
2005 – 2006	Mentoring programme, Leibniz Universität, Hannover

Publications

Heidinger,B., Heine-Dobbernack,E., Singh,M., Schumacher,H.M. and Kiesecker,H. (2007) Production of recombinant proteins by plant cell cultures. Poster presentation at the 11th International Conference on Culture Collections (ICCC 11), 7-11th October 2007, Goslar, Germany

Heidinger,B., Singh,M., Schumacher,H.M. and Kiesecker,H. (2007) Process optimisation for the production of recombinant proteins in plant suspension cultures. Poster presentation at the International Conference on Plant Transformation Technologies, 4-7th February 2007, Vienna, Austria

11 DECLARATION OF SOURCES

I declare that I wrote this thesis with the topic “Development of a selection system for transgenic plant suspension cultures based on dicistronic vectors” independently.
I did not use other auxiliary material than indicated. All collaborative partners have been cited.

The work described is original and has not been submitted, in any form, for a higher degree at any other university or institution. All of the presented work was undertaken during my PhD candidature.

Hannover, 27.10.2008
Bettina Heidinger