Elimination of selectable marker genes via segregation of uncoupled T-DNAs in populations of doubled haploid barley

Dissertation
zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)

vorgelegt der
Mathematisch-Naturwissenschaftlich-Technischen Fakultät
(mathematisch-naturwissenschaftlicher Bereich)
Martin-Luther-Universität Halle-Wittenberg

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geboren am 25. Februar 1978 in Szeged, Ungarn

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Halle (Saale), den 22.04.2010

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List of abbreviations

bar	phosphinothricin acetyltransferase
CCM	Co-culture Medium
CIM	Callus Induction Medium
d35S	doubled enhanced promoter of the cauliflower mosaic virus
DH	doubled haploid
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
GMO	Genetically Modified Organism
GOI	Gene of Interest
gus	ß-glucuronidase
hpt	hygromycin phosphotransferase
IE	Immature Embryo
KBP	Kumlehn´s Barley Pollen Medium
KBPD	Kumlehn´s Solid Barley Pollen Medium
MCS	Multiple Cloning Site
nos	nopaline synthase
npt	neomycin phosphotransferase
ORI	Origin of Replication
pat	phosphinothricin acetyltransferase
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
PRM	Plant Regeneration Medium
SM	Selectable Marker gene
SMB	Starvation Medium for Barley
T-DNA	transfer DNA
uidA	ß-glucuronidase
X-gluc	5-bromo-4-chloro-3-indolyl glucuronide

Bacteria

E. coli	*Escherichia coli*
A. tumefaciens	*Agrobacterium tumefaciens*
A. rhizogenes	*Agrobacterium rhizogenes*

1. INTRODUCTION

The main goal of the present study was the generation of homozygous selectable marker-free transgenic barley plants via segregation in populations of doubled haploid (DH) plants derived from embryogenic pollen cultures, following co-transfer of selectable marker (SM) and gene-of-interest (GOI) as mediated by *Agrobacterium*. The selectable marker was the *hygromycin phosphotransferase* (*hpt)* gene, directly coupled with *green fluorescent protein (gfp*) and the model gene-of-interest was *ß-glucuronidase (gus)*.

1.1. Agroeconomic importance of barley

Barley *(Hordeum vulgare)*, a member of the grass family *Poaceae*, was domesticated about 10 000 years ago in the Near East. It is ranked as the world´s fourth crop in terms of acreage and production, and is probably the oldest crop grown by man. Barley is mainly used for animal forage and malting, but can be found in special areas as well, e.g. human consumption (muesli, flour, malt coffee) and renewable raw material for bioethanol production.

It is one of the most widely distributed crops. In 2008, 149.5 million tons of barley were harvested worldwide, more than half in the countries of the European Union. The main producers are Russia, Canada and Germany (11 million tons) *(*source: *FAO*). One of its main advantages is that it can be grown in regions where other cereals grow poorly due to low rainfall, altitude or soil salinity. Barley is a major food resource in some regions of the world, including North Africa, the Near East, central Asia, in the Horn of Africa, in the Andean countries and the Baltic States.

There are two forms of barley spikes, the two-rowed and the six rowed varieties. Six-rowed barley, which evolved from the two-rowed variant through a mutation, is better for dry or short growing season, but for other conditions the two-rowed barley gave better yield. Furthermore the carrying stem of two-rowed barley is stronger with superior grain formation and overall grain count. Despite these facts, the six-rowed form is far more widely cultivated all over the world.

The main research on barley deals with fodder and brewing quality improvement to alter the structural grain constituents (starch, proteins, lipids, cell walls) or the enzyme activities that mobilise storage reserves in the seed. Barley seed is a good resource of two components, which are in the focus of attention: tocols (E vitamin) and β-glucans.

Tocols (tocopherols and tocotrienols) have antioxidant activity (Kamal-Eldin and Appelvist 1996) and reduce serum LDL-cholesterol (Wang et al. 1993).

Barley-derived ß-glucan rich diet has several health benefits, among them reduced rate of sugar absorption, a decrease of postprandial glucose, an attenuated glycemic response and reduced risk of cardiovascular diseases (reviewed by Goedeke et al. 2007).

(1→3)(1→4) mixed linked β-glucans (β-glucans) of the non-starch polysaccharide family are the major components of barley endosperm cell walls. Its viscosity enhancing property may cause problem in brewing, to modify malting quality by decreasing the soluble β-glucan content in the wort, the gene 1,4-β-glucanase is transferred into the plant to break down the glucan thus improving the filtration rate of beer. Modified grains can also be fed to animals which have difficulty to digest the long chain glucans in the barley cell walls (Nuutila et al. 1999; Xue et al. 2003).

1.2. Genetic transformation of barley

Various transgenic commodity crops, such as herbicide resistant canola, soya and pest resistant maize, are currently grown on millions of hectars around the world. The first transgenic cultivar arrived on the market approximately 15 years ago. Although efficient transformation methods were established first for dicotyledonous plants, the first stable transformation methods for "non-host" monocotyledonous barley appeared in the middle of the 1990s and since then many reports have been presented by various labs (Jähne et al. 1994; Ritala et al. 1994; Wan and Lemaux 1994; Salmenkallio-Marttila et al. 1995; Funatsuki et al. 1995; Tingay et al. 1997; Cho et al. 1998; Wu et al 1998; Nuutila et al. 1999; Zhang et al. 1999; Holm et al. 2000; Patel et al. 2000; Carlson et al. 2001; Matthews et al. 2001; Wang et al. 2001; Trifonova et al. 2001; Fang et al. 2002; Stahl et al. 2002; Murray et al. 2004; Travella et al. 2005; Kumlehn et al. 2006; Holme et al. 2006; Hensel et al. 2007). Up till now, transformation technology of barley is confined to efficiently regentrating cell culture systems of totipotent cells and tissues. Plant regeneration from differentiated tissues, such as leaf and root cells, is not established so far (reviewed by Goedeke et al. 2007)

1.2.1. Methods of gene transfer

Lazzeri et al. (1991) were the first to report about stable transformation of barley immature embryo derived protoplasts. The PEG-induced DNA uptake resulted stable expression of the introduced genes, but no transgenic regenerants were obtained. Later on plants were produced with a similar method (Funatsuki et al. 1995), but with a very low efficiency.

When it comes to efficient barley transformation technology, the spring cultivar "Golden Promise" is the model genotype. Biolostic gene transfer to pre-cultured immature embryos and microspore derived embryos has led to the establishment of stable transformation of the cultivar (Wan and Lemaux 1994). Tingay et al. (1997) were the first to conduct *Agrobacterium*-mediated gene transfer to immature barley embryos. This method served as a basis for the further improvement in *Agrobacterium*-mediated DNA transfer methods (reviewed by Goedeke et al. 2007). Matthews et al. (2001) further improved the protocol of Tingay and co-workers directly co-culturing immature embryos with agrobacteria, and without prior biolistic wounding.

Gene transfer to cultivars other than "Golden Promise" is also possible, but because of poor reproducibility, efficiency and expression, scientists trying to introduce foreign genes to elite barley cultivars are still facing a challenge. However, a growing number of reports have been

published about genetic transformation of cultivars other than "Golden Promise", albeit often associated with poor regeneration efficiency (Ritala et al. 1994; Wu et al. 1998; Cho et al. 1998; Nobre et al. 2000; Trifonova et al. 2001; Wang et al. 2001; Manoharan and Dahleen 2002; Murray et al. 2004; Hensel et al. 2008).

Kumlehn et al. (2006) established a method of *Agrobacterium*-mediated gene transfer to embryogenic pollen, where 1.3-8.9 transgenic Igri plants were obtained per 10^6 cultivated immature pollen .

Holme et al. (2006) used isolated barley ovules for *Agrobacterium tumefaciens*-mediated gene transfer, with transformation efficiency comparable to the methods routinely used.

1.2.2. Vector systems

Several strategies exist for the introduction of foreign genes with the use of binary vector systems. *Agrobacterium tumefaciens* can be used for the transfer of heterologous genes. In these systems T-DNA is located *trans* on a separate replicon, while the disarmed Ti plasmid contains the Vir elements necessary for the gene transfer (*vir* helper) (Hellens et al. 2000). Additional copies of the VirB, VirC1 and VirG genes are responsible for the hypervirulence of some *Agrobacterium* strains or so called superbinary vectors (Komari et al. 1996).

Table 1-1.
Agrobacterium strains and binary vectors used for stable transformation of barley

Strain	Biovar	Ti-plasmid	Opine
AGL-1	Biovar l.	pTiBo542ΔT-DNA	Nopaline
AGL-0	Biovar l.	C58 pTiBo524	Nopaline
LBA4404pSB1	Biovar l.	pAL4404	Octopine
GV3101	Biovar l.	pMP90 (pTiC58ΔT-DNA)	Nopaline
EHA101	Biovar l.	pEHA101 (pTiBoΔT-DNA	Nopaline

For the *Agrobacterium*-mediated stable transformation of barley, several binary vectors have been used (see table 1-1), which consist of the T-DNA flanked by the left and right border seqences, multiple cloning sites, origin of replication (ORI) for *E. coli* and *A. tumefaciens*. The ORIs used in binary vectors of different agrobacteria strains are to our knowledge

confined to pVS1, RK2 and pSP72. Except for GV3101, which resulted in comparatively low transformation efficiency (Kumlehn et al. 2006), all *Agrobacterium* strains shown in table 1-1 are hypervirulent.

1.2.3. Integration of T-DNA sequences

The first report about the integration patterns of T-DNA into the barley genome was written by Stahl et al. (2002), who expressed human genes in transgenic plant tissue. The genomic and T-DNA junctions were determined with biotinylated primers specific for target sequences of the vector border regions. The flanking genomic DNA was cut with a restriction enzyme and ligated to an adapter. The two DNA strands were separated and PCR was carried out. The integration patterns proved to be similar to that observed in dicotyledonous plants. Linkage groups in one single locus occurred with high frequency (50%) in inverted (head-to-tail) configuration, which means that the right border is always attached to a left border and the vice versa, but never two right or left borders are adjacent to each other (head-to-head or tail-to-tail). Deletions from the border sequence were also observed. Right border region flanking the T-DNA seems to be highly conserved, but on the other hand the left border shows greater variability (Fang et al. 2002)

A study aiming at the comparison between biolistic and *Agrobacterium*-mediated T-DNA transfer revealed a difference in the number of integrated copies (Travella et al. 2005), although the results are not supported with statistical analysis. *Agrobacterium* integrated rather few copies of the transgene with minimal rearrangements in all cases, while 60% of the transgenic barley lines derived from particle bombardment integrated more than eight copies of the transgene with extensive DNA rearrangements and multiple integration events. Furthermore, transgene silencing only occurred in lines obtained by biolistic transformation. The results published by Lange et al. (2006) also support the data on *Agrobacterium*-mediated gene transfer. Genomic DNA of 52% of the regenerants obtained by immature barley embryo transformation contained single copy, 33% had two to three copies, and 15% of the independent lines had four inserions. Furthermore, unprecise DNA integration was determined, deletions and integration of backbone vector pieces might occur. Similarly, Hensel et al. (2008) obtained 50% of the plants with single copy, 30% with two, 10% with three and 9% had more than three transgenic fragments integrated in their genome. Holme et al. (2006) analysed the integration pattern of the transgene in independent regenerants obtained by gene transfer to isolated ovules. 37% of the lines contained single copy, 53% two

or three copies and 10% four copies, displaying comparable results to those reported on plants derived from gene transfer to immature embryos.

An important issue is the location of T-DNA in the plant genome. Choi et al. (2002) identified integration loci with three or more copies. No preferred integration site was detected, but a trend was shown towards the distal end of the chromosomes. Salvo-Garrido et al. (2004) conducted physical and genetic mapping of T-DNA integration sites in 19 independent transgenic barley lines obtained by particle bombardment of immature embryos. A total of 23 transgene integration sites were detected in five (2H, 3H, 4H, 5H and 6H) of the seven barley chromosomes. The integration pattern shows a rather non-random distribution and the genes are integrated preferably in the gene rich telomeric and subtelomeric regions. In specific regions of the choromosomes 4H and 5H clusters of transgenes were observed

1.2.4. Selectable markers and reporter genes

The most commonly used marker genes are antibiotic or herbicide resistance genes. They make the modified cells able to detoxify substances which would otherwise be fatal to them (Miki and McHugh 2004). Usually, selectable markers have no impact on plant growth or development in the absence of selective conditions. Expression of selectable marker genes enables an efficient production and identification of transgenic cells. They are usually introduced in a linked position to the target gene, which confers the desired new trait, and render transgenic cells, tissues and plants resistant to selective pressure.

The first selectable marker used for stable barley transformation was neomycin phosphotransferase (*nptII*) isolated from *E. coli*, providing transgenic cells with kanamycin resistance (Lazzeri et al. 1991; Funatsuki et al. 1995; Ritala et al. 1994; Zhang et al. 1999; Nobre et al. 2000).

Later on, other selection systems were introduced, two of which have been extensively used in barley; the phosphinothricin acetyltransferase (*bar/pat*) genes from *Streptomyces* species (Wan and Lemaux 1994; Jähne et al. 1994; Tingay et al. 1997; Wu et al. 1998; Cho et al. 1998; Kumlehn et al. 2006) that confer resistance to the herbicide phosphinothricin and its derivatives, and the *E.coli* derived hygromycin phosphotransferase gene (*hpt*) confering resistance to the antibiotic hygromycin B. The *hpt*-system proved to be the most efficient in barley transformation (Hagio et al. 1995; Wu et al. 1998; Cho et al. 1998; Matthews et al. 2001; Manoharan and Dahleen 2002; Murray et al. 2004; Coronado et al. 2005; Holme et al. 2006; Kumlehn et al. 2006; Hensel et al. 2008).

A conditional negative selection system was developed by Koprek et al. (1999) for barley, where a naturally non-toxic substrate triggers phytotoxic properties in transgenic cells. Use of a positive selection system does not lead to the death of non-transgenic cells, but provides the transformed ones with beneficial properties, so they can thrive on medium unsuitable for the cells lacking the transgene. This method does not require the use of herbicide or antibiotic.
Reed et al. (2001) successfully introduced the phosphomannose isomerase (*pmi*) gene, which converts mannose-6-phosphate into fructose-6-phosphate, in barley among other plants with 3% transformation frequency. Cells expressing the *pmi* gene thrive on medium containing mannose solely as carbon source, non-transformed cells cannot grow on this medium.
Reporter genes are used to indicate their expression or the expression of the target gene in the target cell, tissue, organ or in the entire organism. They must be integrated into transcriptionally active regions in the genome. Regeneration efficiency of transgenic barley calli expressing different reporter genes might differ (Murray et al. 2004).
The reporter function of the green fluorescent protein (*gfp*) gene (Chiu et al. 1996; McCormac et al. 1998; Ahlandsberg et al. 1999; Wang et al. 2001; Carlson et al. 2001; Fang et al. 2002; Murray et al. 2004; Holme et al. 2006; Kumlehn et al. 2006) and the ß-glucuronidase (uidA, *gus*) gene (Lazzeri et al. 1991; Jähne et al. 1994; Ritala et al. 1994; Wan and Lemaux 1994; Hagio et al. 1995; Tingay et al. 1997; Cho et al. 1998; Cho et al. 1999; Zhang et al. 1999; Nobre et al. 2000; Manoharan and Dahleen 2002; Murray et al. 2004; Kumlehn et al. 2006) are commonly used to monitor gene expression in transgenic barley tissue. The disadvantage of the *gus* reporter system is that it is a destructive technique, thus leading to the death of the tissue sample analysed, in contrast to the application of the green fluorescent protein gene, which encodes a relatively small protein with various available derivatives of different emission spectra.
There are reports on the expression of the firefly (*Photinus pyralis*) luciferase reporter gene in barley after particle bombardment of immature embryos (Schledzewski and Mendel 1994; Harwood et al. 2002).

1.2.5. Generation of marker-free lines

Selectable markers are used for the recovery of transgenic plants in the presence of selective pressure, since they provide the plants with antibiotic or herbicide resistance. On the other hand, these markers are useless once transgenic plants are produced, and might prevent the use of the same marker gene for further transformation of that plant. Moreover, the presence

of selectable markers raises environmental and consumer concerns as well. Since 2002, the EU Deliberate Release Directive, which has been in effect, requires "the phasing out of the use of antibiotic-resistance markers in GMOs which may have a harmful impact on human health or the environment".

The removal of unwanted transgenes after *Agrobacterium*-mediated plant transformation was reviewed e.g. by Yoder and Goldsborough (1994), Hohn et al. (2001) and Shrawat et al. (2006). Several strategies exist to eliminate the selectable marker, such as use of intra-genomic relocation of the transgenes using site-specific recombination systems and transposable elements; homologous recombination; and co-integration of transgenes in an unlinked manner, followed by segregation of the T-DNAs in the T_1 generation.

Recombinases are used, among other purposes, for the elimination of undesired DNA sequences. The Cre protein, from P1 bacteriophage, is a site-specific DNA recombinase, which is applied to delete a segment of DNA flanked by *lox* recognition elements in the genome, if the *lox* repeats are in a direct orientation. The selectable marker, flanked by specific recognition sites and integrated together with the gene-of-interest in a transgenic unit, is excised by the enzyme, thus selectable marker-free transgenic plants are obtained. Selectable marker genes were successfully excised from tobacco (Odell et al. 1990; Dale and Ow 1991; Gleave et al. 1999) *Arabidopsis thaliana* (Russel et al. 1992), rice (Hoa et al. 2002), maize (Zhang et al. 2003), and wheat (Srivastava and Ow 2003) using the Cre/*lox* recombinase system.

Other recombinase systems are used as well for the elimination of unwanted DNA sequences. The FLP/FRT system of the 2 μ plasmid of *Saccharomyces cerevisiae* was used to get rid of the selectable marker in both dicots, such as tobacco and *Arabidopsis thaliana* (Kilby et al. 1995), and monocots, e.g maize (Lyznik et al 1996).

The elimination of the selectable marker using the R-RS system of the pSR1 plasmid of *Zygosaccharomyces rouxii* was established for *Arabidopsis thaliana* (Onouchi et al. 1995), tobacco (Sugita et al. 2000) and rice (Endo et al. 2002).

An irreversible site-specific recombination system is the integrase-*att* from *Streptomyces* phage phiC31 (Thorpe and Smith 1998), which is used for the elimination of selectable markers (Ow 2007; Kittiwongwattana et al. 2007) from transgenic plants. Unlike, Cre/*lox* and FLP/FRT systems, the phiC31 integrase (that mediates recombination between bacterial *attB* and phage *attP* attachment sites) alone cannot reverse the recombination reaction.

The recombinase is not necesseraly included in the T-DNA unit, it might also later be delivered into the plant by secondary transformation (Odell et al. 1990; Dale and Ow 1991; Lyznik et al. 1996), transient expression (Gleave et al. 1999; Kopertekh et al. 2004; Jia et al. 2006) or sexual crossing with a plant expressing the protein (Russel et al. 1992; Bayley et al. 1992; Kilby et al. 1995; Kerbach et al. 2005). The transgenic T-DNA sequence to be evicted and the gene-of-interest, originally coupled to each other in one transgenic unit, are thus separated from each other.

Transposases are also suitable for the production of selectable marker-free transgenic plants, such as the maize *Ac*/*Ds* elements, which consist of two essential components, the transposase coding gene (*Ac*) and the inverted repeat termini (*Ds*). The transgenes are incorporated within *Ds* elements and its maize transposon in the genomic DNA. *Ds* elements are stable in the absence of *Ac* and lack transposase function. Transgenic sequences integrated between the *Ds* elements can be mobilised to new genomic locations in the presence of the *Ac* transposase gene (Lassner et al. 1989; Masterson et al. 1989; Kunze 1996). The advantage of this, is that after the relocation of the transgene to new chromosomal region, altered expression level might occur, caused by "position effect" (Yoder and Goldsbrough 1994).

Transposable elements retain their transposition competence when introduced in other plant species. The selectable marker was removed from tobacco, aspen (Baker et al. 1986; Ebinuma et al. 1997), tomato (Goldsborough et al. 1993) and rice (Cotsaftis et al. 2002), with the use of transposable elements, where the excision does not necessarily lead to their reintegration (Belzile et al. 1989; Gorbunova and Levy 2000).

The occurrence of a DNA deletion in tobacco is described through intra-chromosomal recombination between two homologous regions (Puchta 2000; Zubko et al. 2000) However, up till now this system is not eligible for the efficient elimination of the selectable marker.

An other way to obtain selectable marker-free transgenic plants is the co-integration of the selectable marker and the gene-of-interest, followed by segregation of the two uncoupled T-DNAs in the T_1 progeny (Depicker et al. 1985; McKnight et al. 1987; De Block and Debrouwer; 1991 Komari et al. 1996). *Agrobacterium*-mediated co-transformation is preferred, because it leads to unlinked T-DNA integration events with higher probability than particle gun mediated bombardment of the transgenes.

Different ways of *Agrobacterium* mediated T-DNA transfer exist to obtain co-transgenic plants: the use of a mixture of strains (mixture methods) or delivery of T-DNAs from a single strain (single-strain methods). The two plasmids/one strain method means mixture of the same *Agrobacterium* strain (e.g LBA4404) harbouring plasmids with different T-DNAs

(McKnight et al. 1987; De Block and Debrouwer 1991; Komari et al. 1996). Coronado et al. (2005) mixed two different *Agrobacterium tumefaciens* strains (LBA4404 and AGL-1) to obtain co-transgenic barley cv. "Golden Promise" (two plasmids in two different strains method), giving rise to selectable marker free, homozygous T_1 progeny. Single strain methods, with the introduction of two transformation plasmids in one *Agrobacterium tumefaciens* clone (De Framond et al. 1986; Komari et al. 1996; Daley et al. 1998,) and use of a binary plasmid, which contains two T-DNAs (Komari et al. 1996; Matthews et al. 2001; Stahl et al. 2002) are also applied for co-transformation experiments.

Co-transformation methods, followed by meiotic segregation of the selectable marker and the gene-of-interest in the T_1 progeny, were succesfully established in monocots, with rice (Komari et al. 1996), and barley among them (Matthews et al. 2001; Coronado et al. 2005). The study published by Coronado et al. (2005) is based upon *A. tumefaciens*-mediated co-transformation of independent T-DNAs for selectable marker and effector gene followed by the identification of selectable marker-free, homozygous T_1 barley plants among populations of doubled haploids generated though embryogenic pollen cultures.

Mattews et al. (2001) introduced a Twin binary vector (two T-DNAs adjacent to each other on the same vector) into *A. tumefaciens* (strain AGL-0 and AGL-1) to infect immature barley embryos. The obtained co-transformation efficiency in the T_0 generation was 66%, 24% of which showed segregation of the GOI in the T_1 population, which equals an overall segregation efficiency ca.16%.

Holme et al. (2006) successfully obtained 0.8 stable transgenic barley plants per 100 isolated barley ovules, without use of any selective conditions.

1.3. Haploid technology

Haploid technology uses haploid cells to produce plants via callus or embryo formation. The originally haploid genome of the regenerants could be doubled either autonomously or by chemical treatment to obtain instantly homozygous plants. For the release of new cultivars this method is routinely applied to accrelerate the breeding procedure (Pickering and Devaux 1992). The particular value of this technology lies in the fact that every indiviual DH-line is a product of random meiotic recombination, but identically reproducible. Moreover, the technique is also widespread to produce mapping populations used in basic and applied research.

1.3.1. Generation of doubled haploid barley

The major techniques to produce DH barley are anther (Clapham 1973) and ovary culture (Dunwell 1985), interspecific hybridization with *Hordeum bulbosum* L., the use of the haploid initiator gene (Hagberg and Hagberg 1980; Kasha and Reinbergs 1982), and the culture of immature pollen rendered competent to undergo embryogenic development. Embryogenic pollen cultures offer great potential for the generation of DH populations. Sunderland and Xu (1982) were the first to induce callus formation in barley pollen cultures and by now several reports exist on successful production of immature pollen derived plants (Köhler and Wenzel 1985; Hunter 1987; Ziauddin et al. 1990; Olsen 1991; Hoekstra et al. 1992). Moreover, isolated late uninucleate microspores have been considered a valuable material for genetic transformation as well, either by particle bombardment (Jähne et al. 1994), electroporation of protoplasts of microspore-culture origin (Salmenkallio-Martilla et al. 1995a) or *Agrobacterium*-mediated gene transfer (Wu et al. 1998; Kumlehn et al. 2006).

Much effort was done to improve embryo/callus formation and regeneration efficiency of barley embryogenic pollen cultures (Modhorst and Lörz 1993; Kao 1993; Scott and Lyne 1994; Salmenkallio-Martilla et al. 1995b; Cistue et al 1995; Ritala et al 2001; Kasha et al. 2001), e.g. through phenylacetic acid treatment (Ziauddin et al. 1992), and co-cultivation with ovaries (Li and Devaux 2001).

In barley, several common types of inductive treatments exist in order to provide the signal the microspores need to be switched from the gametophytic to the sporophytic development pathway (Sunderland et al. 1978). The common pre-treatment methods are cold shock to anther cultures (Hunter 1987), spikes (Coronado et al. 2005), isolated microspores (Mejeza et al. 1993; Indrianto et al. 1999); starvation (Olsen 1991; Gustafson et al. 1995, Touraev et al. 1997; Kumlehn and Lörz 1999); heat (Touraev et al. 1996) and gametozide-like substances (Zheng et al. 2001).

In barley the prevailing methods are the application of cold stress (+ 4 ^{o}C) to anther cultures, spikes and isolated pollen grains in dark, and nutrient starvation (Li and Devaux 2003; Coronado et al. 2005), followed by co-culture of immature pistils, which is likely to provide the developing embryogenic culture with signal molecules (Koehler and Wenzel 1985; Coronado et al. 2005)

The ability of isolated immature pollen to form embryogenic calli and plantlets is highly cultivar dependent (Ziauddin et al. 1990; Li and Devaux 2001). In order to fulfil the major

requirement of producing a sufficient number of plants from embryogenic pollen cultures, the isolation and regeneration protocol must be very efficient.

1.3.2. Simplified segregation of transgenes in populations of doubled haploids

The working hypothesis of the present study is that doubled haploid technology can be used for the rapid and efficient production of selectable marker free transgenic T_1 barley plants. Primary plants containing the selectable marker and the gene of interest are obtained from co-transformation. If the T-DNAs are integrated in the plant genome in an unlinked manner, they segregate during the meiotic phase of pollen formation. Isolated microspore cultures of such segregating lines produce selectable marker-free transgenic doubled haploid progeny plants, which are easily identified among individuals of the relatively small T_1 population, without further need of segregation analysis of their offspring. Moreover, the desired plants are instantly homozygous for the transgene, which is of great benefit for scientific and breeding purposes.

1.4. Scope of the thesis

Primary transgenic barley plants (T_0) were generated via *Agrobacterium*-mediated gene transfer to immature embryos using separate T-DNAs, one for the selectable marker (SM) gene hygromycin phosphotransferase *(hpt)*, directly coupled with a green fluorescent protein gene (*gfp*) to be used as additional screenable marker, and the other for the model gene of interest (GOI) ß-glucuronidase *(gus)*, without any selectable marker coupled. Different *Agrobacterium* strain-vector combinations were compared to ultimately identify the most efficient way of uncoupled co-integration of the T-DNAs. To facilitate the generation of homozygous transgenic SM-free lines in a novel approach, co-transformation was combined with haploid technology. Uncoupled T-DNAs present at hemizygous state in primary transformants are randomly and independently distributed to the pollen grains during male meiosis. Thus, homozygous transgenic selectable marker-free plants can be instantaneously produced and identified amongst doubled haploid (DH) plants generated from embryogenic cultures of segregating pollen populations (Fig. 1-1).

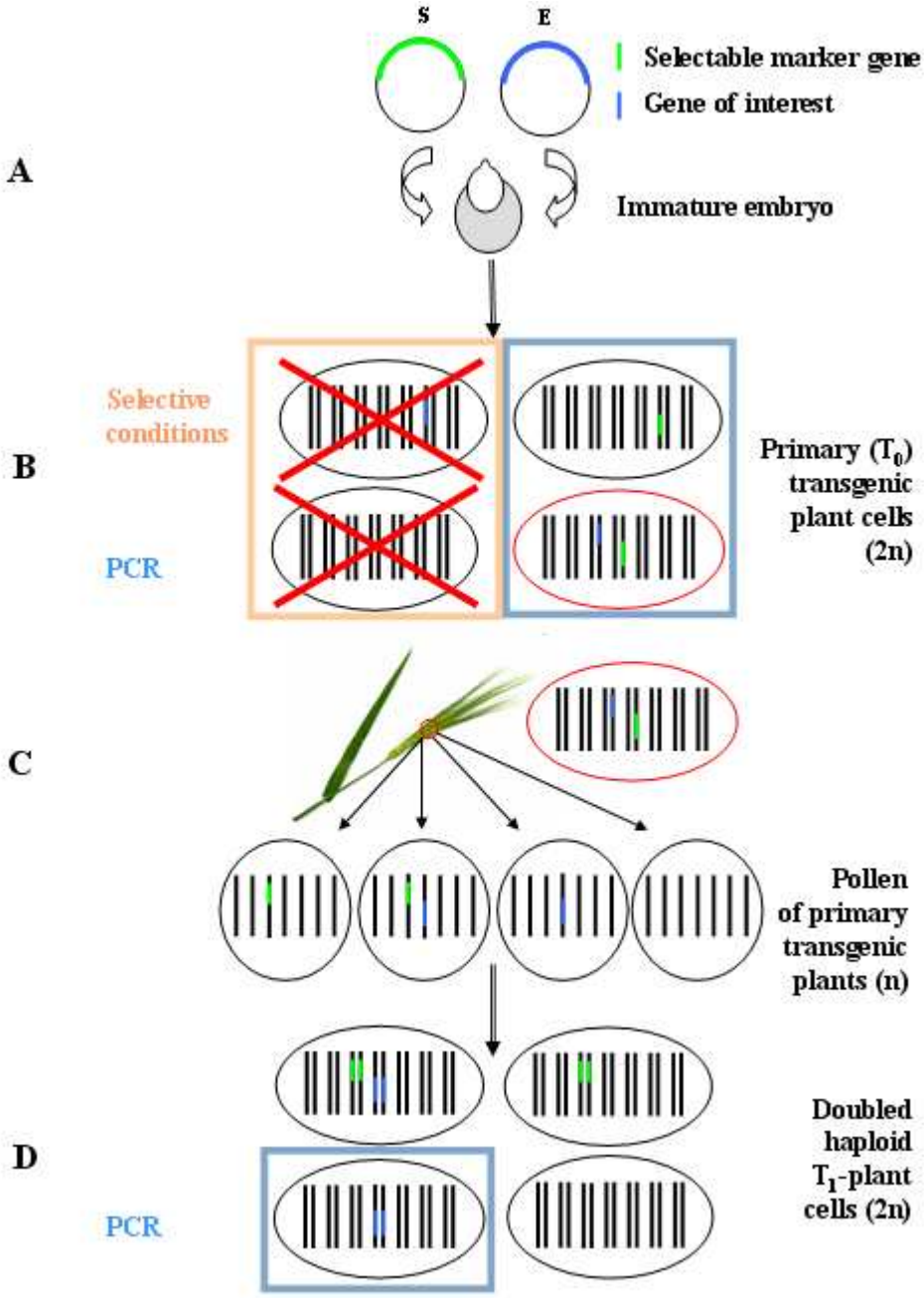


Figure 1-1.
Immature embryos were used as target for *Agrobacterium*-mediated gene transfer of the selectable marker gene (*hpt* coupled with *gfp)*, and the gene-of-interest (*gus*) in co-transformation experiments (A). After about 2 months first plantlets appear, which are delivered to the greenhouse. Co-transformed plants can be identified by PCR using gene-specific primers (B). The production of selectable marker-free homozygous transgenic barley plants from primary co-transgenic plants is carried out from embryogenic pollen cultures (C), which result in entirely homozygous progeny of T_0 plants. Within these populations, selectable marker-free T_1 plants can be identified (D).

2. MATERIALS AND METHODS

Agrobacterium-mediated transformation of barley is not genotype-independent. The first stable transformation system of immature barley embryos developed for the model cultivar "Golden Promise" was established by Tingay et al. 1997. This two kernel row barley with diploid genome (2n=14 chromosomes) is a gamma-ray induced semi-dwarf mutant of the cultivar "Maythorpe". It contains the recessive mutation *GPert,* which results short stiff straw and reduced awn length, and also has pleiotropic effects on yield and grain size. The cultivar shows considerable salt tolerance (Forster et al. 1994), but is susceptible to powdery mildew (*Erysiphe graminis* sp. *hordei*).

"Golden Promise" was used in Northern Britain and Scotland as malting barley from the late 1960s to the 1980s. It was favoured by maltsters and distillers, who used two-row barley for malt whisky, because of the unique combination of highly desirable agronomic characters such as earliness (ripening time in August), short stiff straw, easy combining ability, good resistance to grain and ear loss and good malting quality. However, "Golden Promise" became neglected as many farmers moved to other strains of barley that provided them with higher yield.

2.1. Bacterial strains and vectors

Two *Agrobacterium* strains (LBA4404pSB1, AGL-1, table 2-1) and four binary vectors were used in strain mixture (two binary plasmids in two clones of the same *Agrobacterium* strain and two plasmids in two different *Agrobacterium* strains methods) and single strain (two plasmids in one *Agrobacterium* clone, and one Twin-vector harboring two T-DNAs in one *Agrobacterium* clone) co-transformation methods. A total of 14 different variants were applied for immature embryo (gene transfer target) inoculation, table 2-3.

The cloning steps were conducted in DH5α and DH10B strains of *Escherichia coli* (Sambrook et al. 1989), table 2-1.

Table 2-1.
Bacterial strains used for cloning and gene transfer to immature barley embryos

Strain	Genotypic specification	Reference
E. coli DH5α	deoR, endA1, gyrA96, hsdR17 (r_k^-, m_k^+), recA1, relA1, λ^- supE44, thi-1, Δ(lacZYA-argFV169), Φ80 Δ lacZ Δ M15, F^-	Sambrook et al. 1989
E. coli DH10B	endA1, recA1, galE15, galK16, nupG rpsL ΔlacX74, Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^- , F^-	Sambrook et al. 1989
A. tumefaciens LBA4404pSB1	AGL0 recA::bla pTiBo542ΔT Mop+Cb$_R$	Lazo et al. 1991
A. tumefaciens AGL-1	Ach5 pTiAch5ΔT	Hellens et al. 2000

Plasmids, used for cloning purposes to produce binary vectors applied for the *Agrobacterium*-mediated barley transformation, are presented in table 2-2.

Table 2-2.
Plasmids used for cloning

Vector	Bacterial marker	Origin of replication
pSB227	Sm/Sp^r	pVS1
pGUSi-AB	Amp^r	ColE1
pD35S-Nos-AB	Amp^r	ColE1
p6U	Sm/Sp^r	pVS1

The binary vector pSB227 (provided by Sylvia Broeders, a former member of the group, unpublished) includes the selectable marker gene, hygromycin phosphotransferase, driven by the maize Ubiquitin promoter, and directly coupled with a Synthetic *gfp*-S65T gene (Chiu et al. 1996) driven by the rice Actin1 promoter to be used as additional screenable marker, see figure 2-1.

The binary vector containing the gene-of-interest, without plant selectable marker was obtained by removing the *hpt* expression cassette from the p6U vector (DNA Cloning Service, Hamburg, Germany), see figure 2-2. Then the *E. coli* ß-glucuronidase gene including the StLS1 intron (Vancanney et al. 1990) *gus*i was cut out from pGUS-AB (DNA Cloning Service, Hamburg) vector using restriction enzymes SalI and NotI and inserted in the pd35S-

Nos-AB backbone vector. Finally, the d35S promoter-*gus*i cassette was inserted in the *hpt*-free p6U binary vector with the help of SfiI restriction enzyme.

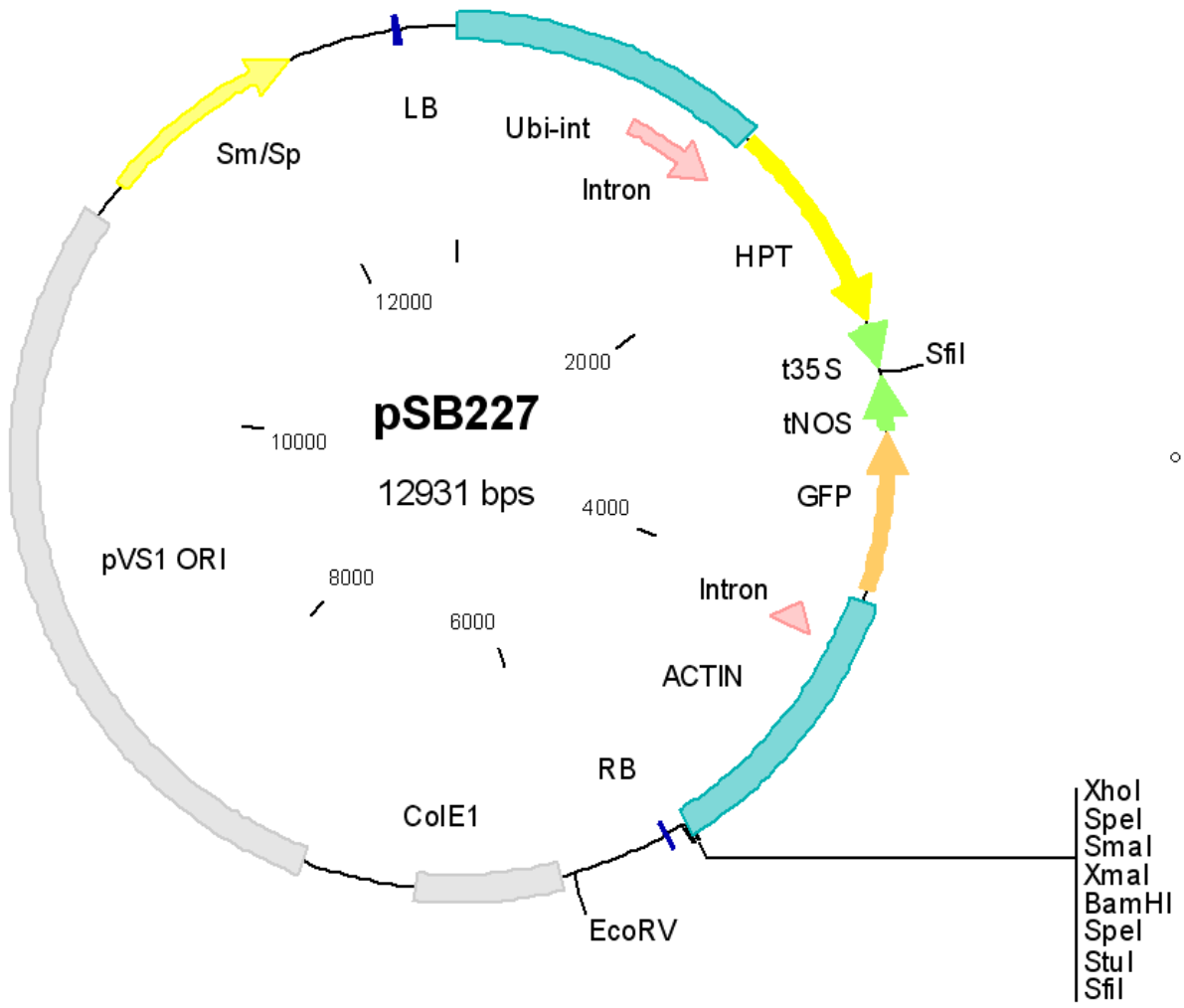


Figure 2-1.
Binary vector pSB227 containing the selectable marker gene (*hpt*) coupled with the *gfp* reporter gene

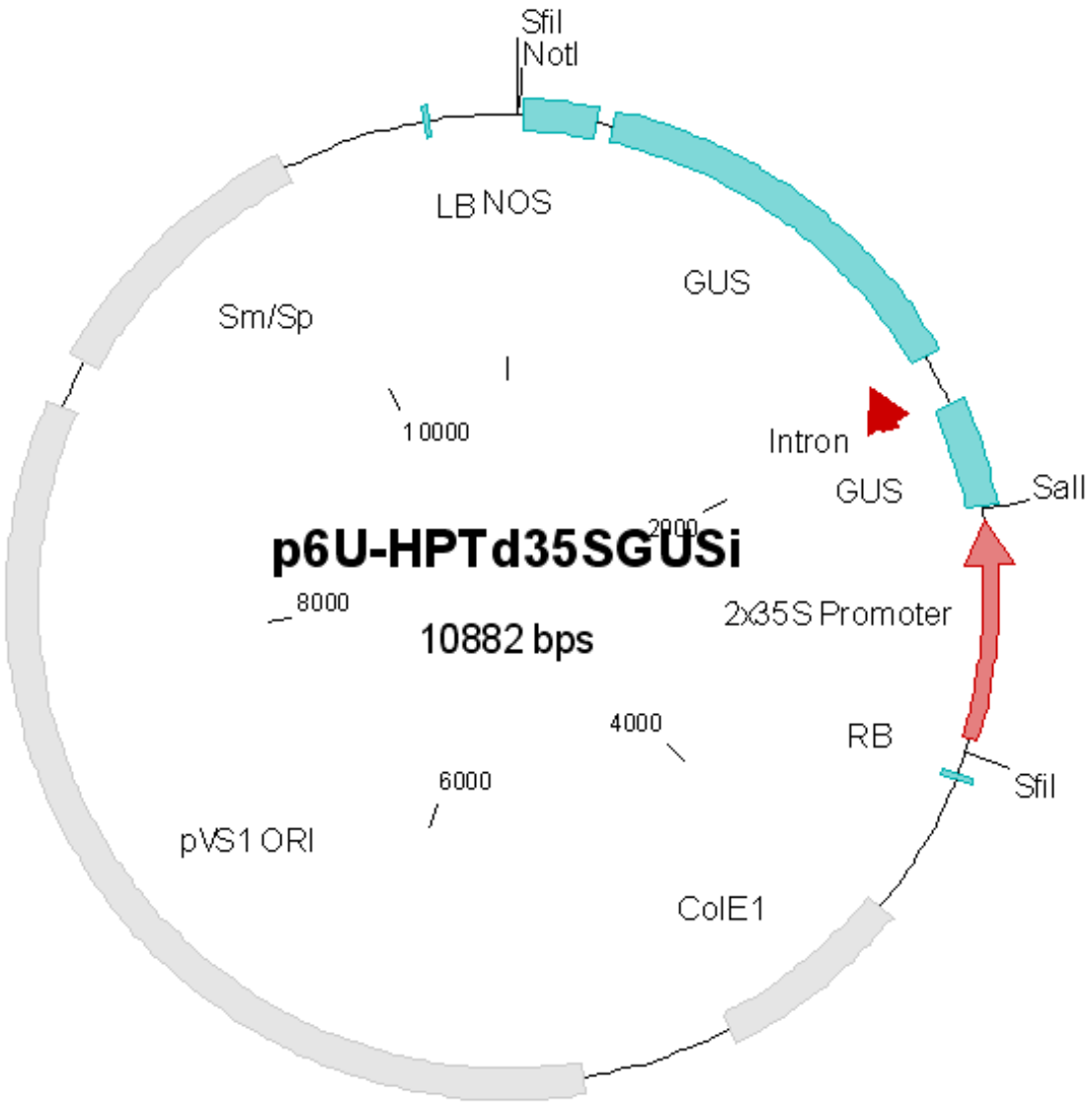


Figure 2-2.
Binary vector containing the gene-of-interest (*gus*i) without the selectable marker gene (*hpt*).

The Twin binary vector pair harbouring the two T-DNAs separated by left and right border sequences was prepared from the modified pSB227 vector.

The cloning steps are the following:

1. Modification of the pSB227 vector.

The SpeI and StuI restriction sites were removed by digesting the pSB227 vector with the two enzymes followed by 5'-3' exonuclease treatment and religation. Then the SfiI resticion enzyme site was removed from this modified pSB227 vector via SfiI digestion followed by 3'-5'exonuclease treatment and relegation, see figure 2-3.

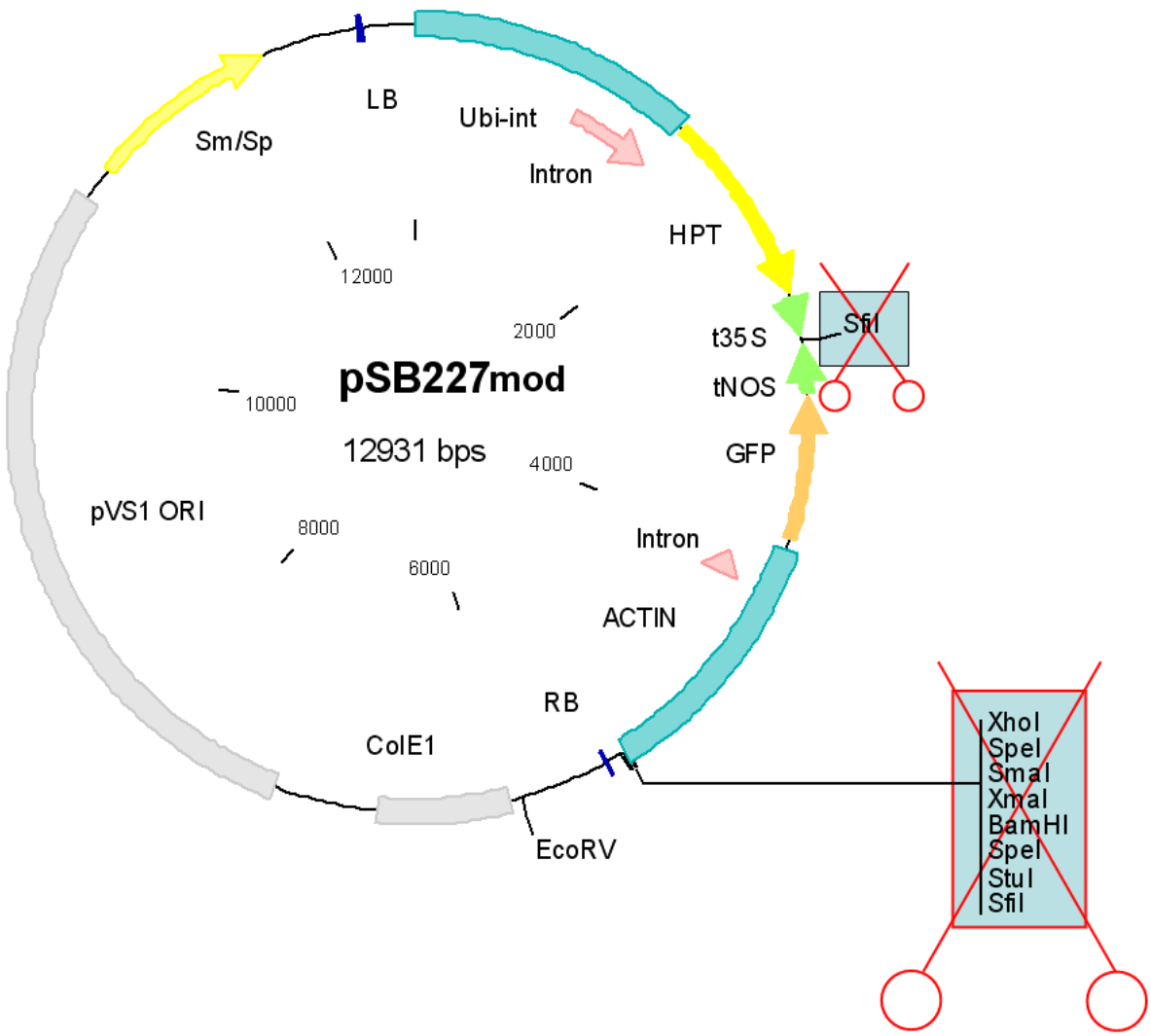


Figure 2-3.
Modification of the pSB227 vector

2. Amplification of the left border-multiple cloning site-right border cassette (LB-MCS-RB) by PCR

The LB-MCS-RB fragment was amplified by PCR using two specific primers (flanked by restriction sites for the EcoRV endonuclease) and the *hpt*-free p6U vector as template, see figure 2-4. The fragment was cloned with the help of the TOPO-Cloning Kit (Invitrogen).

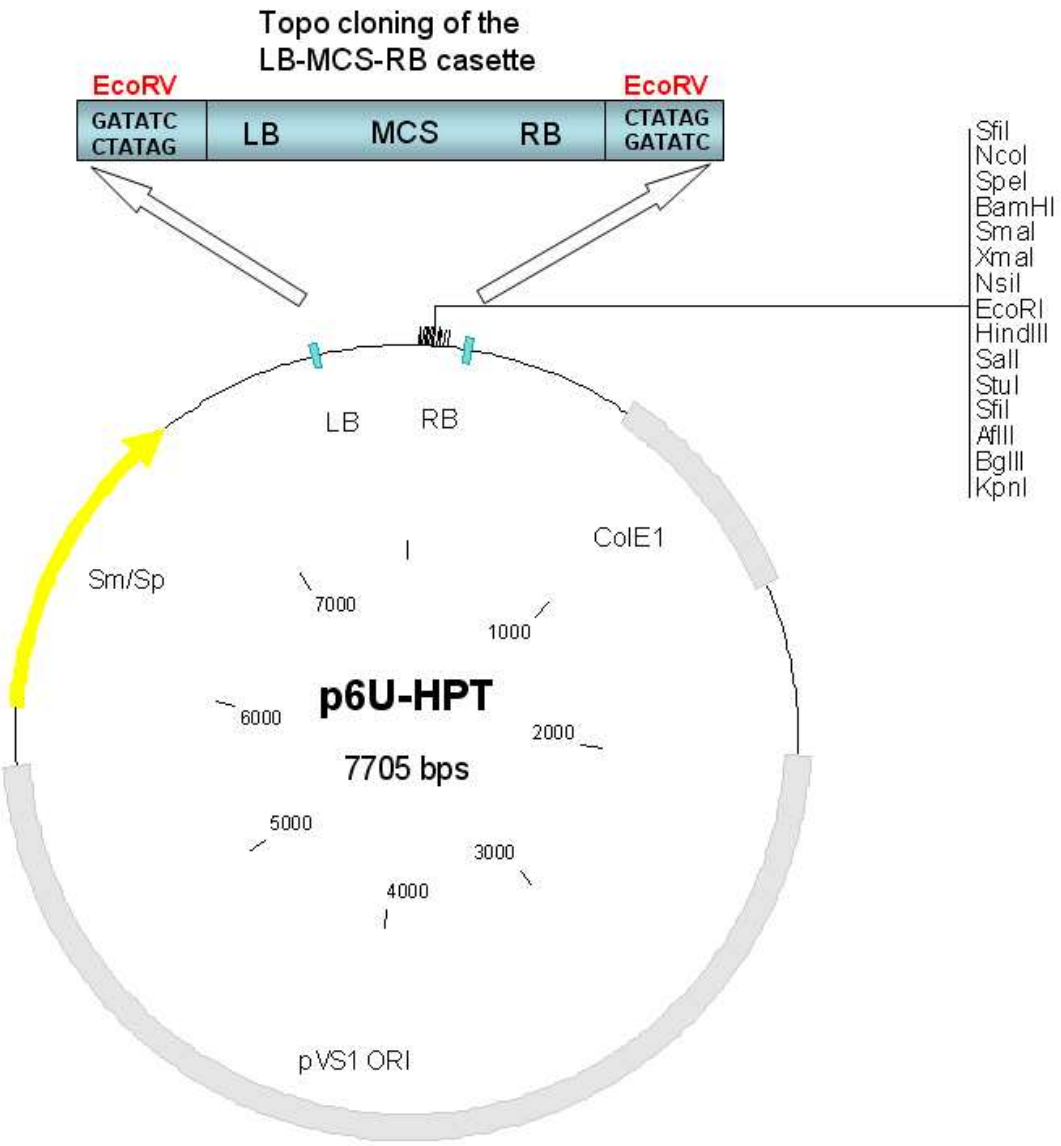


Figure 2-4.
PCR amplification of the left border-multiple cloning site–right border cassette from the p6U binary vector lacking the selectable marker (*hpt*)

3. Insertion of the LB-MCS-RB fragment into the modified pSB227 vector

The TOPO and the modified pSB227 vectors were digested with the EcoRV restriction enzyme and the LB-MCS-RB fragment was ligated in the vector. The LB-MCS-RB sequence was introduced in two different orientations in the modified pSB227 vector, due to blunt end ligation (figure 2-5).

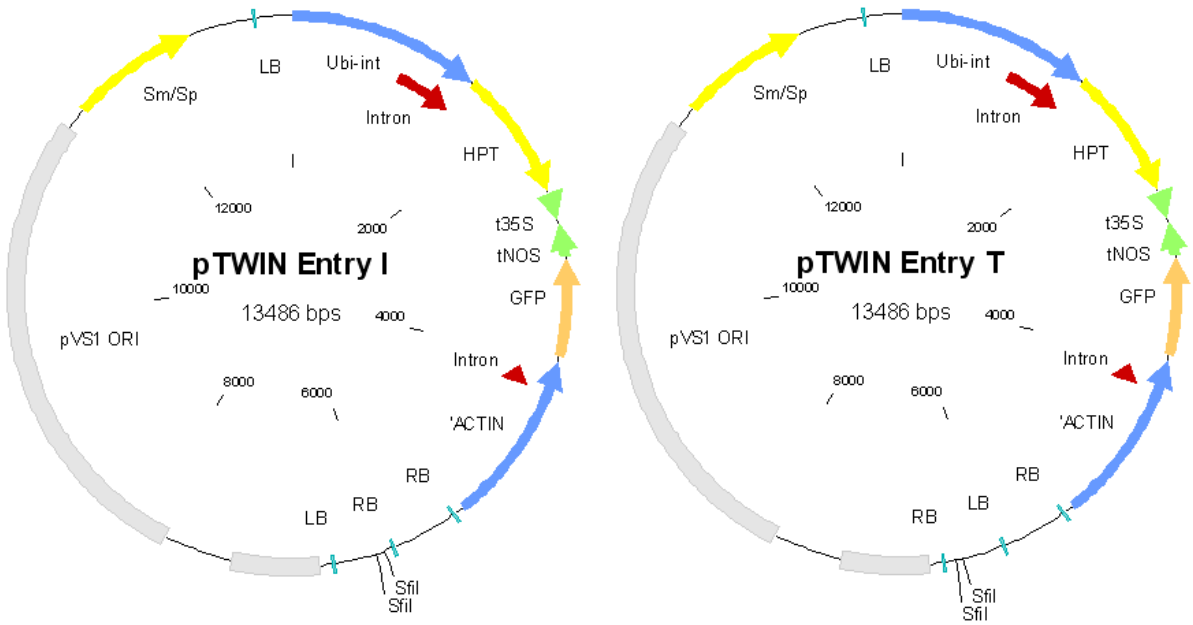


Figure 2-5.
Entry vectors for the introduction of the d35S*gus*i cassette.

4. Insertion of the d35S-*gus*i-nos cassette into the Twin Entry vectors

Using the SfiI restriction enzyme the d35S*gus*iTnos cassette was cut out from the *hpt*-free p6Ud35S*gus*i (figure 2-2) vector and inserted into the pSB227 vector containing the multiple cloning site fragment flanked by the border sequences (Twin Entry I and T). This cloning step resulted two types of binary vector, where the orientation of the *gus*i gene is different related to the *gfp* gene (tandem or inverted), figure 2-6.

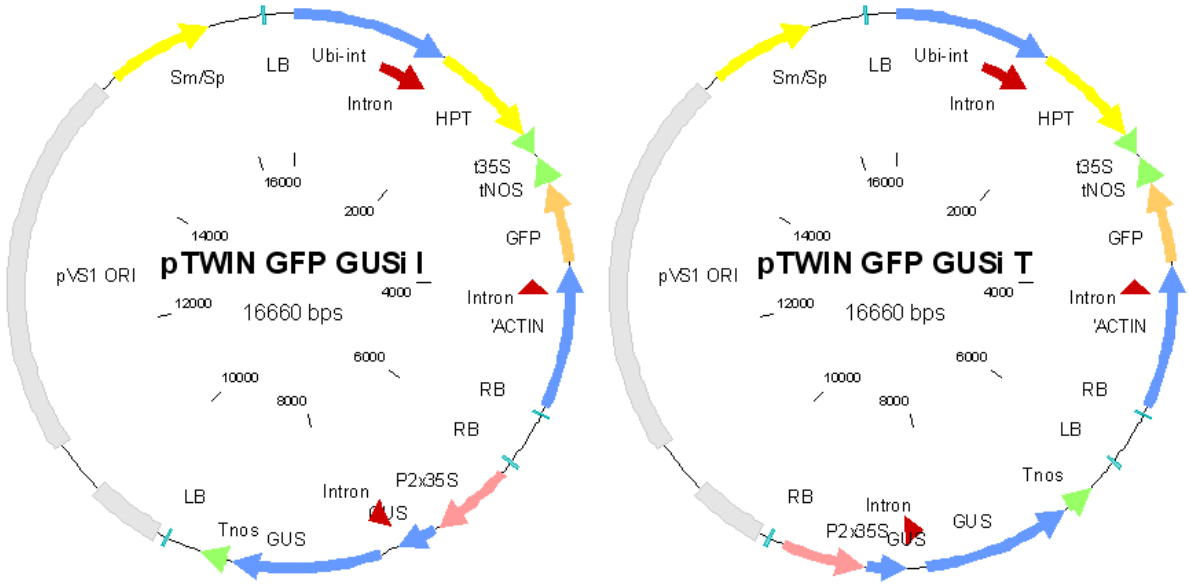


Figure 2-6.
Twin vector pair containing two separate T-DNAs, one is the selectable marker (*hpt*) coupled with the *gfp* gene, and the other is the model gene-of interest (*gus).* The letters T (tandem) and I (inverted) refer to the different orientations of the *gus* gene related to the *gfp* gene within the vector.

Binary vectors were transformed into *Agrobacterium tumefaciens* strains AGL-1 and LBA4404pSB1 (Lazo et al. 1991; Hellens et al. 2000) by electroporation (see "Transformation of *E. coli* and *A. tumefaciens*") LBA4404pSB1contains additionally the acceptor vector pSB1 (Japan Tobacco Inc., Higashibara, Japan), which confers hypervirulence with the help of accessory alleles VirB, VirC and VirG (Komari et al. 1996).

The 14 different combinations and proportions of the derivative clones of the two *Agrobacterium* strains (AGL-1 and LBA4404pSB1) applied for co-culture and their binary vectors- grouped in four methods- are summarized in table 2-3. The different variants were always compared to a control, because of the highly variable amenability of the donor material due to the environmental conditions which cannot be entirely controlled.

Table 2-3. *Agrobacterium*- binary vector combinations used in the comparative co-transformation approach

	Method	Variant no.	Mixture proportions	*A. tumefaciens* strain/ binary vector combination
Mixture methods	two binary plasmids in two clones of the same *Agrobacterium* strain	1	50% 50%	AGL-1 p*hpt::gfp* AGL-1 p*gus*
		2	50% 50%	LBA4404pSB1p*hpt::gfp* LBA4404pSB1p*gus*
	Two plasmids in two different *Agrobacterium* strains	3	70% 30%	AGL-1 p*hpt::gfp* LBA4404pSB1p*gus*
		4	50% 50%	AGL-1 p*hpt::gfp* LBA4404pSB1p*gus*
		5	30% 70%	AGL-1 p*hpt::gfp* LBA4404pSB1p*gus*
		6	30% 70%	LBA4404pSB1p*hpt::gfp* AGL-1 p*gus*
		7 control	50% 50%	LBA4404pSB1p*hpt::gfp* AGL-1 p*gus*
		8	70% 30%	LBA4404pSB1p*hpt::gfp* AGL-1 p*gus*
Single strain methods	Two plasmids in one A*grobacterium* clone	9	-	AGL-1 (p*hpt::gfp*+p*gus*)
		10	-	LBA4404pSB1 (p*hpt::gfp* +p*gus*)
	Two T-DNAs in one binary vector in AGL-1	11	-	AGL-1 pTwin T
		12	-	AGL-1 pTwin I
	Two T-DNAs in one binary vector in LBA4404pSB1	13	-	LBA4404pSB1 pTwin T
		14	-	LBA4404pSB1 pTwin I

2.1.1. Bacterial media and culture conditions

Bacterial culture media, which were prepared either in liquid and solid form, were autoclaved at 120 ^{o}C for 20 minutes. Solid media were prepared with 0.8% agar.

E. coli strains were grown on LB-Medium (Silhavy et al. 1984).

MG/L culture medium (Garfinkel and Nester 1980) was used for the growth of AGL-1, and CPY for strain LBA4404pSB1 (Komari et al. 1996) see table 2-4.

Table 2-4.
Culture medium applied for the growth of *Agrobacterium* strains AGL-1 and LBA4404pSB1

Components		**MG/L**	**CPY**
Sugars g/l	Mannitol	5	-
	Sucrose	-	5
Amino acid (g/l)	L-Glutamic acid	1	-
Macro elements (mg/l)	KH_2PO_4	250	-
	NaCl	100	-
	$MgSO_4 \cdot 7H_2O$	100	500
Vitamin (µg/l)	Biotin	1	-
Miscellanous (g/l)	Peptone	-	5
	Tryptone	5	-
	Yeast extract	2.5	1
pH		7.0	7.2

Antibiotics were added after autoclaving in following concentrations: carbenicillin 100 µg/ml, rifampicin 50 µg/ml, spectinomycin 100 µg/ml, and tetracycline 10 µg/ml.

E. coli strains were grown at 37 ^{o}C, while *A. tumefaciens* strains at 28 ^{o}C. Liquid media were shaken at 180 rpm in Erlenmeyer flask.

Cryostock cultures of *A. tumefaciens* strains were prepared with 7% glycerol and stored at -80 ^{o}C. For the co-culture with immature embryos the content of the tube was thawed and put in 10 ml medium without antibiotics and shaken (180 rpm) at 28 ^{o}C for about 24 hours, optical density (OD) was set at 0.2-0.25.

2.1.2. Isolation of plasmid DNA from bacteria

Bacterial plasmid DNA was isolated from 2 ml liquid culture, using Qiagen Spin Miniprep Kit (Quiagen/Germany).

2.1.3. Restriction of plasmid DNA

Restriction enzymes from Fermentas were used with appropriate reagent buffer. For cloning purpose, 15-20 µl restriction digest reactions contained 2-4 U endonuclease at incubation temperature given by the producer. Successful Southern blot analysis required the digest of greater amount of DNA (10-30 µg) with 10-20 U enzyme in 50 µl end volume.

2.1.4. Agarose gel electrophoresis of plasmid DNA fragments

DNA fragments were run in 1.2 % agarose gel containing ethidium-bromide (0.3 µg/ml), in order to make DNA visible under UV light. Gel electrophoresis was done in 0.5xTBE buffer (45 mM Tris, 45 mM Borsäure, 1 mM EDTA).

2.1.5. Isolation of DNA fragments

DNA fragments were isolated using Qiagen Gel Extraction Kit (Qiagen/Germany).

2.1.6. Ligation of plasmid DNA

Ligation of DNA with overhanging ends was carried out by Sambrook et al. (1989) using ligase from Fermentas, blunt ends were treated with phosphatase (Antarctic phosphatase, Biolabs New England) in order to prevent self-ligation.

2.1.7. Sequencing

Sequencing was carried out by AGOWA genomics services according to their protocol.

2.1.8. Transformation of *E. coli* and *A. tumefaciens*

For the production of electrocompetent cells 800 ml appropiate medium was inoculated with 0.8 ml fresh culture of *E. coli* or *A. tumefaciens* strains AGL-1, LBA4404pSB1. The liquid culture was shaken (37 ^{o}C *E. coli*, 28 ^{o}C *A. tumefaciens*, 200 rpm) for 4-5 hours until it reached 0.5-0.8 OD_{600} value. This was followed by a centrifugation (5000 rpm) step at 4 ^{o}C.

The cells were washed with 800 ml ice-cold distilled water and then centrifuged for 20 minutes at 4500 rpm, this was followed by an other washing step in 400 ml ice-cold distilled water. The pellet was washed with 25 ml 10 % glycerine and centrifuged (10 minutes, 4000 rpm). The cells were resuspended in 1.5-3.0 ml 15 % glycerine and 50 µl aliquots were stored at -80 ^{o}C (storage life 6-12 months) in 1.5 ml tubes.

Transformation of bacterial cells was conducted in an electroporation cuvette, containing an aliquot thawed on ice with 2 µl added DNA solution. After electroporation (4-5 msec, 25 µF, 2,5 kV, 200 Ohm; BioRad/USA) 1 ml SOC medium (table 2-5.) was added quickly, then *E. coli* cells were incubated and shaken (180 rpm) at 37 ^{o}C for one hour, *A. tumefaciens* cells at 28 ^{o}C for 3 hours. Transformed bacteria were plated (100-200 µl) on solid medium containing antibiotics and grown (*E. coli* overnight, *A. tumefaciens* 2 days).

Table 2-5. Composition of SOC medium

SOC medium components	
Bacto-Trypton	2 %
Yeast extract	0.5 %
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose (added after autoclaving)	20 mM

2.2. *Agrobacterium*-mediated transformation of barley cv. "Golden Promise"

The constructs were transformed into barley cv. "Golden Promise" immature scutella (embryo axis was removed) according to Tingay et al. (1997), but without prior biolistic wounding.

Hygromycin selection was used instead of bialaphos, due to increased efficiency of the previous system. The further improved immature barley embryo transformation protocol presented in this work is based on the method established by G. Hensel and J. Kumlehn, 2004.

2.2.1. Production of donor plants and growth conditions

Seeds of spring barley (*Hordeum vulgare*) cultivar "Golden Promise" were germinated in substrate mix (Specialmischung Petuniensubstrat) in growth chamber under controlled condition (14/12 ^{o}C day/night, 12h light, 20 000 lux, relative humidity ca. 80%) for 10-12 weeks. The plants were fertilised at the beginning of tillering with Osmocote (40g/7.5 l), a long-term fertilizer, containing 19% Nitrogen, 6% Phosphorus and 15% Potassium. In the period of every two weeks the plants were watered with 0.3% Hakaphos Blau (Compo, Germany), a general fertilizer containing 15% Nitrogen, 10% Phosphorus and 15% Potassium, until the stems started to elongate. The plants were placed in a greenhouse cabin (18/14 ^{o}C day/light, min. 25 000 lux for 16 h) immediately after the spikes emerged from the leaf sheath.

Donor plant conditions highly influence the outcome of the experiment (Kasha et al. 1989; Kuhlmann and Foroughi-Wehr 1989)

2.2.2. Plant tissue culture media

Tissue culture media protocols (table 2-6), used for *Agrobacterium*-mediated immature barley embryo transformation and regeneration of the primary transgenic plants, are based on the publication by Tingay et al. 1997.

Table 2-6.
Barley immature embryo derived tissue culture media

Components		**CCM (Tingay et al. 1997)**	**CIM (Hensel and Kumlehn 2004)**	**PRM (Hensel and Kumlehn 2004)**
Macroelements (mg/l)	NH_4NO_3	1650	1650	320
	KNO_3	1900	1900	3640
	KH_2PO_4	170	170	340
	$CaCl_2 \cdot 2H_2O$	441	441	441
	$MgSO_4 \cdot 7H_2O$	331	331	246
Microelements (mg/l)	H_3BO_3	6.2	6.2	3.10
	$MnSO_4 \cdot 4H_2O$	22.4	22.4	11.20
	$ZnSO_4 \cdot 7H_2O$	8.6	8.6	7.20
	KI	0.83	0.83	0.17
	$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.12
	$CuSO_4 \cdot 5H_2O$	0.025	1.275	0.13
	$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.024
	$Na_2FeEDTA$	36.70	36.70	36.70
Vitamins (mg/l)	B5 Vitamins (Duchefa)	-	-	112
	Thiamine-HCl	1.0	1.00	10
Amino acids (mg/l)	L-Cysteine	800	-	-
	L-Glutamine	-	-	146
	L-Proline	690	690	-
Sugars (g/l)	Maltose monohydrate (Duchefa)	30	30	36
Growth regulators (mg/l)	DICAMBA	2.50	2.50	-
	6-BAP	-	-	0.225
Miscellaneous (g/l)	Acetosyringone	0.098	-	-
	Casein Hydrolysate	1.00	1.00	-
	Myo-Inositol	0.25	0.25	-
	Timentin	-	0.15	0.15
	Phytagel (Sygma, Germany)	-	3.0	3.0
pH		5.8	5.8	5.8

2.2.3. Gene transfer to immature embryos

The experiments were numbered according to their succession. 90 immature embryos were isolated, the embryos axis was removed and each experiment was repeated three times. Consequently, a total of 270 embryos were inoculated per variant. The control variant was applied six times, which means that 1620 embryos were inoculated with the 1:1 mixture of LBA4404pSB1 p*hpt:gfp* and AGL-1 p*gus*.

All of the below mentioned immature embryo transformation steps were carried out under sterile conditions in a sterile bench.

Developing caryopses of donor barley plants were harvested at around 12 days after pollination for immature embryo isolation and surface sterilized in order to avoid infection of the tissue cultures. Seeds were stirred in 70% ethanol for 3 minutes, followed by a 20 minutes washing step in 5% NaOCl solution, to which 0.5 ml Tween was added. Finally, seeds were stirred 5 minutes in double distilled autoclaved water, rinsed 5 times and stored, if necessary overnight at 4 ^{o}C.

Immature barley embryos were excised from the caryopses by using forceps and lanzette needle at a stereo microscope under sterile conditions (Tingay et al. 1997). The stadium of the embryos highly determines their capability for transformation, the best are the ones which are transparent in the middle, but white on the side with a diameter of 1.5-2mm. The embryonic axes were dissected and 30 embryos were put in each well of a 6-well-plate (Greiner Bio-One Gmbh, Austria) filled with 2.5 ml co-culture medium (CCM) supplemented with 9.8 mg/l acetosyringone, see table 2-5.

CCM was removed using a sterile pipet from the plates and 600 µl *Agrobacterium* suspension (OD range 0.2-0.25) was added. The 6 well-plate was vacuum infiltrated for 1 minute at 500 mbar (diaphragm pump MP 201 E from Ilmvac, Ilmenau, Germany) and incubated covered for 10 minutes. The *Agrobacterium* suspension was removed and the embryos were washed with 2.5 ml CCM medium and incubated for another 15 minutes. After another washing step with 2.5 ml CCM medium the plates were transferred to 21 ^{o}C in the dark.

2.2.4. Generation of transgenic plants

After 60 hours the embryos, later the derived developing calli, were fortnightly transferred first onto modified Callus Induction Medium (CIM), described by Trifonova et al. 2001, and supplemented with 20, later 50 mg/l Hygromycin B (Boehringer, Germany) and 150 mg/l timentin. 10 embryos/ calli were placed on each 9-cm petri dish (Greiner Bio-One Gmbh, Austria) with the scutellum side facing the medium (Hensel et al. 2008). Calli were kept in an incubator at 25 ^{o}C in dark. After the induction phase the obtained calli were then transferred onto fresh Plant Regeneration Medium (PRM), supplemented with 25mg/l Hygromycin B, in every two weeks (table 2-6.), incubated under light (24 ^{o}C, 16/8h light/dark photoperiod, 10 000 lux).

Solid media were prepared from a mixture of the components (table 2-6), filter sterilized in fourfold-concentration, and the diluted Phytagel (previously solved in double-distilled water and autoclaved for 20 min at 120 ^{o}C) used for solidification. During the regeneration process the developing calli produced plantlets.

Table 2-7.
Summary of callus transfer periods, induction and regeneration conditions

	Medium	**Time period**	**Conditions**
Co-culture	CCM	60h	21 ^{o}C, dark
Callus induction	CIM_{solid} + 20mg/l Hygromycin B	2 weeks	24 ^{o}C, dark
	CIM_{solid} + 50mg/l Hygromycin B	2 weeks	24 ^{o}C, dark
Plant regeneration	PRM_{solid} + 25mg/l Hygromycin B	2x 4 weeks	24 ^{o}C, light

The primary regenerants were transferred into glass tubes (Height 100 mm, outer diameter 25mm, Schütt, Germany) containing solid PRM medium supplemented with 25mg/l Hygromycin B. After the roots have appeared, the small barley plants were transferred to the greenhouse (14/12 ^{o}C day/night, 12h photoperiod, 20 000 lux, relative humidity ca. 80%) in small pots with 6cm diameter. Later on selected lines to be brought to maturation were put in big pots with 16cm diameter. 1 m^2 greenhouse area is required to store 120 small plantlets or 20 mature barley plants.

2.2.5. Analysis of transgenic plants

After the plants were transferred to soil, their genomic DNA was tested for the presence of the gene-of-interest (*gus*) and the selectable marker gene (*hpt::gfp*). Based on the PCR results the co-transformed primary transgenic (T_0) lines containing both *gus* and *hpt::gfp* were selected. Among the positively tested ones further analysis was carried out, ploidy level and copy number of the integrated T-DNAs were determined as well.

2.2.5.1. Isolation of genomic DNA from plant material

Genomic DNA was isolated from leaves of the primary regenerants according to the method established by Palotta et al. (2000). Fresh leaf material (200-400 mg) was put in a 2 ml Eppendorf tube with two metal beads (diameter 4.1 mm) and stored in liquid nitrogen or at -80 ^{o}C until extraction. The frozen leaves were comminuted using a mixer mill for 2 minutes, 1/27 s (Retsch Mixer mill MM301). 800 µl extraction buffer (1% N-Lauryl-Sarcosin, 100mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 100 mM NaCl) was added to each tube and vortexed until the clumps were dissolved. 800 µl Phenol/Chlorophorm/Isoamyl alcohol (25:24:1, Roti) was added and suspended by thorough vortexing, which was followed by a centrifugation step at room temperature (5000 rpm, 3 minutes). The upper layer containing dissolved DNA was transferred in clean 1.5 ml Eppendorf tubes. 1/10 vol. (80 µl) 3M Na-Acetate (pH 5.2) and 1 vol. (800 µl isopropanol) was added and mixed until white DNA precipitate appeared. After the centrifugation step at 4 ^{o}C 13 000 rpm for 10 minutes the supernatant was discarded and the pellet was washed with 1 vol. (800 µl) 70% ethanol. The supernatant was discarded after the final centrifugation step (1 minute, 13 000 rpm) and the pellet was air dryed for ca. 30 minutes. The plant genomic DNA was resolved in 100 µl R40 (40 µg/ml RNase in TE buffer, 10:1 pH 8.0) and incubated at 37 ^{o}C for one hour. The samples were stored for shorter time period at 4 ^{o}C, longer storage is possible at -20 ^{o}C.

2.2.5.2. PCR

Standard PCR reaction was carried out using specific primers for *gfp* (linked with the selectable marker) and *gus*, in order to confirm the absence/presence of the T-DNAs (table 2-8). Oligos were ordered from Metabion AG (Germany).

The PCR amplification program (Eppendorf thermocycler) started with an initial denaturing step at 95 ^{o}C for 5 minutes, then ran 35 cycles: 30 seconds at 95 ^{o}C /45 seconds at 60 ^{o}C/

1 minute 15 seconds at 72 ^{o}C. After the final extension step, 7 minutes at 72 ^{o}C, it cooled to 4 ^{o}C .

PCR was conducted in one reaction solution, usually in a 96-well plate, including a negative and plasmid controls. Product size for *gus* fragment was 730 bp, and 450 bp for *gfp*. The amplified fragments were run in 1.2 % agarose gel to which 4 µg/100 ml ethidium bromide was added.

2.2.5.2.1. Oligo-nucleotides

Table 2-8.
Forward and reverse primers used for the identification of the gene-of-interest (*gus*) and the selectable marker (*hpt* coupled with *gfp*) integrated in the genome of barley plants.

Sequence	Name
5'-CCGGTTCGTTGGCAATACTC-3'	GH-GUS F1
5'-CGCAGCGTAATGCTCTACAC-3'	GH-GUS R1
5'-GGTCACGAACTCCAGCAGGA-3'	GH-GFP F1
5'-GACCACATGAAGCAGCACGA-3'	GH-GFP R1

2.2.5.3. Ploidy level analysis

The ploidy level of the primary co-transgenic plants was determined using a flow cytometer (Partec), precisely measuring the total DNA content of individual nuclei, in order to confirm if the plants are in a haploid, diploid or tetraploid state. This information is especially useful when analysing T_1 populations.

2.2.5.4. Southern blot

DNA gel-blot hybridization was used to confirm the selectable marker-free status and characterize the segregation pattern of the insertions (Sambrook et al. 1989) using the non-radioactive fluorescence DIG labelling method. A blot was probed first with the *gus* probe, then stripped and reprobed with the *hpt* probe.

About 30 µg genomic DNA was digested with HindIII at 37 ^{o}C, overnight. As negative control isolated genomic DNA from wild type "Golden Promise" plants was taken. 80 pg of pSB227 and p6U-HPTd35sGUSi plasmids served as positive control.

2.2.5.4.1. Blotting of separated barley DNA fragments

DNA fragments were separated on 0.8% (w/v) agarose gel by electrophoresis at 25V overnight. According to the manufacturers instructions (Roche Diagnostics, Mannheim, Germany) the DNA fragments were transferred to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany.

2.2.5.4.2. DIG labelling and hybridisation

DNA fragments, blotted on a positively charged nylon membrane, were hybridized with DIG-dUTP labeled probes, with the use of PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The applied primers were the same as that of used for PCR reactions. Hybridisation steps, signal visualisation by CDP-Star and detection on chemiluminescent detection film were done according to the DIG Application Guide for Filter Hybridization Manual (Roche Diagnostics, Mannheim, Germany).

2.2.5.5. Histochemical analysis of *gus* reporter gene expression

The *gus* reporter gene system is generally used in molecular biology (Jefferson 1987). X-Gluc is the substrate of the ß-glucuronidase enzyme, which converts it to glucuronic acid and 5-bromo-4-chloro-indoxyl. The latter is then oxidised to 5,5'-dibromo-4,4'-dichloro-indigo, a blue coloured product. Embryogenic callus and leaf tissue *gus* expression was detected by incubation at 37 ^{o}C overnight in X-Gluc (5-bromo-4-chloro-3-indonyl-D-glucuronide) solution, see table 2-9. The tissue material was previously vacuum infiltrated (ILMVAC, Laboratory Vacuum System, LVS 301 Zp). When testing leaves, the chloroplasts, which disturb the blue tone, were extracted by alcohol (60 ^{o}C, 2 hours in water bath).

Table 2-9.
Composition of X-Gluc solution

Components	Final concentration	Comment
X-Gluc	1 mg/ml	
Methanol	20%	dissolve X-Gluc in methanol
0.5 M Na_2HPO_4/NaH_2PO_4 solution	100 mM	
0.5 M NaEDTA	10 mM	
Triton X-100	0.1%	
K-Hexacyanoferrat (II)	1.4 mM	
K-Hexacyanoferrat (III)	1.4 mM	Set pH at 6.2-7.2 Store at -20 ^oC

2.2.5.6. Microscopic detection of GFP expression

GFP expression was screened in callus tissue and root tips, using a Leica MZFLIII fluorescence microscope with a filter set for GFP Plant (Leica Microsystems, Wetzlar, Germany).

Reporter gene expression is shown in figure 3-2/C.

2.2.5.7. Leaf assay for hygromycin resistance

Wang and Waterhouse (1997) recommend an easy assay for the detection of *hpt* and *bar* marker genes in transgenic barley leaves.

The leaves were sprayed with 70% ethanol and stabbed in a 9 cm Petri dish containing PRM medium supplemented with 200 mg/l Hygromycin B. The dish was kept in a light chamber (24 ^oC, 16h photoperiod, 20 000 lux) for one week. The leaf pieces of the plants possessing hygromycin resistance stay green after this period, while those lacking this trait look almost or completely bleached (figure 3-9).

2.3. Production of doubled haploids from co-transgenic T_0 plants

2.3.1. Plant tissue culture media

Descriptions of media prepared for barley microspore starvation treatment (table 2-10) and embryogenic callus formation (table 2-11) are based on the publication of Kumlehn et al., 2006. PRM medium (see 2.2.2.) was used for the regeneration of doubled haploid barley plants from microspore derived calli.

Table 2-10.
Composition of microspore starvation medium (SMB)

Components		**Final concentration**
Macroelements (mg/l)	NH_4Cl	53.40
	$CaCl_2$	110.80
Microelements (mg/l)	$MnSO_4 \cdot H_2O$	5.25
	H_3BO_3	3.10
	$ZnSO_4 \cdot 7H_2O$	7.20
	$Na_2MoO_4 \cdot H_2O$	0.123
	$CuSO_4 \cdot 5H_2O$	0.025
	$CoCl_2 \cdot 6H_2O$	0.020
	KI	0.166
Sugar (g/l)	Maltose (Sigma)	144
Growth regulator (mg/l)	BAP	0.9
Antibiotic (mg/l)	Cefotaxime	250
Miscellaneous (mg/l)	MES	426
	pH	5.5

Table 2-11.
Composition of KBP liquid and KBPD solid media

Components		**Final concentration**
Macroelements (mg/l)	NH_4NO_3	80
	KNO_3	101
	KH_2PO_4	136
	$CaCl_2 \cdot 2H_2O$	110.8
	$MgSO_4 \cdot 7H_2O$	246.3
Microelements (mg/l)	$MnSO_4 \cdot H_2O$	5.25
	H_3BO_3	3.10
	$ZnSO_4 \cdot 7H_2O$	7.20
	$Na_2MoO_4 \cdot H_2O$	0.123
	$CuSO_4 \cdot 5H_2O$	0.025
	$CoCl_2 \cdot 6H_2O$	0.020
	KI	0.166
	NaFeEDTA	27.5
Sugar (g/l)	Maltose (Sigma)	90
Amino acid (mg/l)	Glutamine	438
Growth regulator (mg/l)	BAP	0.9 (KBP) 0.225 (KBPD)
Vitamin	Kao and Michayluk Vitamin Solution (Sigma)	1x
Antibiotic (mg/l)	Cefotaxime Timentine	250 (KBP) 150 (KBPD)
Miscellaneous (g/l)	Phytagel (Sygma, Germany)	3.0 (KBPD)
	pH	5.9

2.3.2. Stress treatment of microspores

2.3.2.1. Cold treatment of harvested spikes

Spikes, 6 cm from the first knot and covered by the leaf sheath, were harvested from each plant separately. The spikes were surface sterilized with 70% ethanol, the awns were removed and pre-treated at 4 ^{o}C for 3-4 weeks in 9-cm Petri dishes kept humid by wet filter paper (Hunter 1987; Mordhorst and Loerz 1993). Five-six spikes were placed in each dish, which was sealed by Parafilm (Pechiney Plastic Packaging, Menasha, WI 54952, USA). The following steps were carried out under aseptic conditions in a sterile bench.

2.3.2.2. Stravation treatment of isolated microspores

SMB liquid medium (table 2-10) was used for starvation stress of the freshly isolated microspores. The optimal time span of the stress treatment is two days.

2.3.3. Embryogenic pollen cultures

Isolation of microspores in the late uninucleate stage from co-transgenic To "Golden Promise" barley plants was done according to the protocol established by Coronado et al. 2005.

For the isolation of the microspores 10-15 spikes were cut to ca. 1cm long pieces and put in a sterile, pre-cooled Micro Container. Twenty milliliters of ice cold 0.4 M mannitol (Duchefa) was added and blended using a Waring Blendor power unit (Eberbach, Ann Arbor, MI, USA) for 2x 10 seconds at "low" speed. The suspension was poured into a vessel on ice through a 100 µm nylon sieve (Winson, Nottingham, UK) and rinsed with 10 ml 0.4M mannitol. The rest was pressed by a sterile forceps to obtain more suspension and put back in the blender. Ten milliliters 0.4M mannitol was added and homogenised for another 2x 10 seconds. The suspension was poured onto the 100 µm sieve and the Micro Container rinsed again with 10 ml 0.4M mannitol. The rest was gently pressed by a sterile forceps and then removed together with the sieve. The suspension was poured in a 50 ml srew-cap tube. Five milliliters 0.4M mannitol was taken to rinse the vessel and poured in the srew-cap tube. The suspension was centrifuged at 4 ^{o}C, 705 rpm. The supernatant was removed with a sterile 10 ml pipet and using a new sterile pipet the pellet was resuspended in 5 ml ice-cold 0.55M maltose (Sigma) and transferred into a 12 ml tube (Greiner Bio-One Gmbh, Austria). The 50 ml srew-cap tube was rinsed with 1.5 ml 0.4M mannitol and carefully overlayed on the suspension in the 12 ml

tube. After the next gradient-centrifugation step (4 °C, 705 rpm) the interphase containing the viable microspores was removed and resuspended in 0.4 ml Mannitol in a new 50 ml srew-cap tube setting the final volume at 20 ml. These cells are in a vacuolated mononucleous stadium, able for cell division and production of doubled haploid regenerants. Debris of dead cells is found in the pellet. Twenty microliters of the suspension was pipetted on a haemocytometer in order to count the number of microspores. The supernatant was removed after the following centrifugation step, the pellet was dried by a sterile 1 ml pipet tip, which was pressed to the bottom of the srew-cap tube and the mannitol rest was carefully sucked out without removing the microspore cells. The pellet was resuspended in starvation medium (SMB, table 2-10) and the concentration set between 100.000-400.000 microspores/ml aliquoted in 35-mm Petri dishes (1 ml culture per dish, Greiner Bio-One Gmbh, Austria) and incubated in dark at 21 °C for two days.

After 2 days SMB medium was removed with a 1 ml disposable pipet and KBP medium (Kumlehn`s Barley Pollen medium, table 2-11), including cefotaxim as antibioticum, and 5 immature wheat pistils (pre-incubated for one day in KBP medium with a maximum number of 20-30 pieces per 2 ml medium in a 35 mm petri dish) were added (Koehler and Wenzel 1985; Hu and Kasha 1997; Li and Deaux 2001) The cultures were incubated at 25 °C in dark. After one week an additional milliliter of fresh KBP (incl. cefotaxim) medium was added, and the cultures were put on a rotary shaker (ca. 50 rpm) at 25 °C in the dark. After two weeks the calli were transferred on filter paper containing solid KBPD medium (table 2-11) in 9-cm dishes (Greiner Bio-One Gmbh, Austria) and incubated at 25 °C in dark. After another two weeks calli were transferred to PRM medium (see table 2-6, Kumlehn et al. 2006), kept in dark at 25 °C for 5 days and then put in a light chamber (24 °C, 16h photoperiod, 20 000 lux). Calli were transferred to fresh medium in periods of 3x2 weeks. Regenerants were put in sterile boxes containing fresh PRM medium. After the small plantlets developed roots they were transferred to a phytochamber in the greenhouse (14/12 °C day/night, 12h photoperiod, 20 klx, relative humidity ca. 80%).

2.3.4. Identification of selectable marker-free, transgenic segregants

PCR, Southern Blot, *gus* assay and hygromycin leaf test were carried out the same way as explained by “2.2.5. Analysis of transgenic plants”.

2.3.5. Colchicine treatment of haploid plants

Colchicine is a toxic chemical, first extracted from, *Colchicum autumnale* (Autumn crocus). It is a "mitotic poison", inhibiting mitosis by binding to tubulin, a crucial component. Because colchicine is inhibiting chromosome segregation during cell division, it is applied to induce polyploidy in plant cells, to double the chromosome numbers during cell division (Luckett 1989; Takamura and Miyajima 1996).

Selected haploid barley plants, grown in the greenhouse (14/12 ^{o}C day/night, 12h photoperiod, 20 klx, relative humidity ca. 80%), were put overnight in a dark chamber at 4 ^{o}C before the colchicine treatment, in order to induce mitotic cell division in the plant tissue. Soil was removed from between the roots by washing with tap water. Roots were cut to 3 cm and leaves to 5 cm long pieces. Plants were put in 50 ml screw cap tubes filled with 25 ml colchicine solution (1g/l) and incubated for 6 hours in a light chamber. Plants were then carefully washed, repotted and put in the greenhouse.

2.4. Analysis of sexually generated lines

When no plants of embryogenic pollen culture origin were obtained, sexual lines of self-pollination were germinated from the seed reserve of T_0 plants and analysed. Usually 20 plants were analysed, but more material might be needed in case of high copy numbers of the transgene(s).

Barley cv. "Golden Promise" seeds were put on PRM medium without antibiotics, and germinated in a light chamber (24 ^{o}C, 16h photoperiod, 20 000 lux). Leaf material of small plantelets was taken and genomic DNA isolated.

Embryo rescue provides the possibility of reducing the time span needed to obtain the following generation and thus enables an earlier timepoint for their analysis. This way the time required for the ripening of seeds can be saved using immature embryos (dissected from surface sterilised seeds and put on CMR medium) to form small plantlets.

PCR, Southern Blot, *gus* assay and hygromycin leaf test were carried out the same way as explained by "2.2.5. Analysis of transgenic plants"

2.5. Statistical evaluation of the data

The obtained data were analysed by parameter-independent Kruskal-Wallis One Way Analysis of Variance on Ranks (SigmaStat 3.0, SPSS Inc.,Chicago, IL, USA). Pairwise comparisons of the variants against the control repetitions applied in parallel in the same experiment were performed. Differences with P-values <0.05 were considered statistically significant.

Average absolute deviation values were calculated across the experimental repeats of each variant according to the formula given below, so as to intelligibly visualize the variation within treatments in the diagrams:

$$\frac{1}{n}\sum_{x_i=1}^{n} /x_i - \overline{x_q} /$$

where,

n is the sample size

x_q is the mean value

3. RESULTS

3.1. Binary vectors

Four types of binary vectors (pSB227, p6U-HPTd35SGUSi, Twin T and Twin I) were used for the *Agrobacterium* based transformation experiments.

The production of the binary vector containing the *gus* reporter gene (p6U-HPTd35SGUSi) was carried out through integration of the *E. coli ß-glucuronidase* gene, including the *StLS1* intron driven by the d35S promoter, into the *hpt*-free p6U binary vector.

The substrate for the production of the two Twin binary vectors, each harbouring two T-DNAs separated by left and right border sequences, was the modified pSB227 binary vector, in which two restriction sites were removed from its multiple cloning site (MCS). The d35S*gus*iTnos cassette was cut out from p6U-HPTd35SGUSi vector and inserted into the modified MCS of the pSB227 plasmid. Two types of binary vector were obtained by this latter cloning step, where the orientation of the *gus*i gene is different related to the *gfp* gene resulting tandem (pTwin T) and inverted (pTwin I) fragments.

Figure 3-1 presents derivatives of two *Agrobacterium tumefaciens* strains, AGL-1 and LBA4404pSB1, harbouring different binary vectors, which carry the gene-of-interest and/or the selectable marker gene.

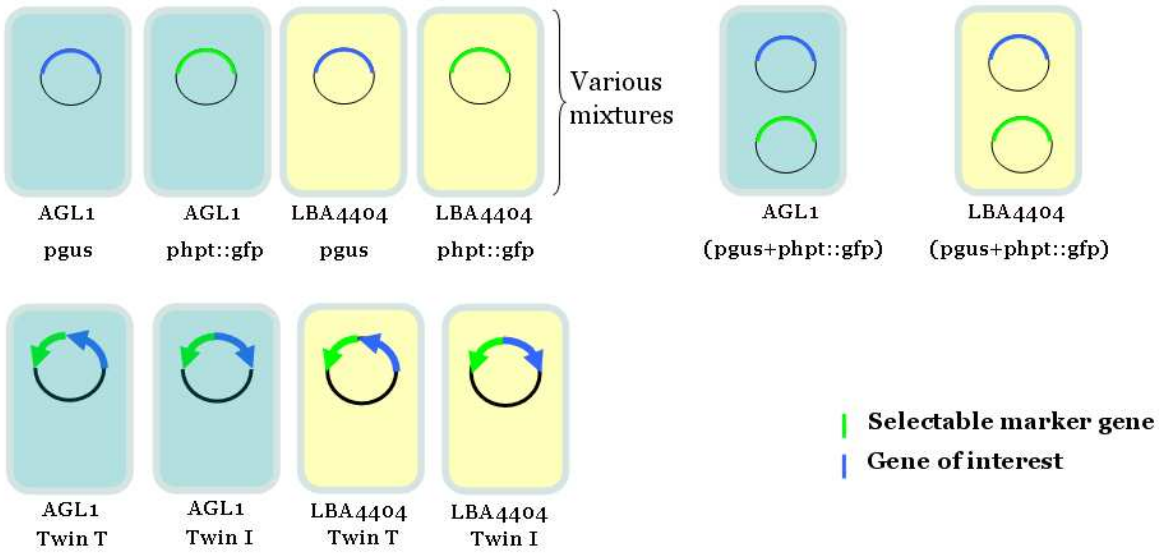


Figure 3-1.
Four different types of binary vectors were transferred in two *Agrobacterium* strains resulting in a total of 14 variants. For the sake of simplicity, the bacterial genetic background is not indicated.

3.2. Primary transgenic (T_0) plants

Barley genetic transformation was carried out through infection of immature embryos using two *Agrobacterium* strains (LBA4404pSB1 and AGL-1) according to the different experimental variants applied (table 2-3). The embryo co-cultures comprised 14 different modes to employ the diverse *Agrobacterium* clones or mixtures. For these 14 different variants (including methods with two plasmids/one strain, two plasmids in different strains, two plasmids in one *Agrobacterium* and Twin vectors harboring two T-DNAs) four types of binary vectors were used, in order to identify the most efficient combination(s) and mixture proportion(s) in terms of co-transformation and independent integration. The aim was to find the best variant(s) that will enable us to efficiently produce co-transformed primary T_0 plants with transgenes introduced in an unlinked manner, giving rise to selectable marker-free DH progeny which are instantly homozygous for the gene-of-interest.

The inoculated 5130 embryos produced 616 regenerants in total, out of which 606 proved positive by PCR for the presence of the selectable marker gene (diagram 3-1), which was coupled with the additional reporter gene (*hpt::gfp*, see figure 3-2). Those plants which were able to grow under selective pressure conditions despite lacking the resistance gene are called escapes. Their proportion among the regenerants was 1.78%.

However, the 616 regenerants were produced by altogether 206 calli, because more than one plant was often obtained per callus, with numbers per callus being as high as fifteen. These so called sister plants might either be genetically identical clones or represent independent lines. Initially only one plant per callus was considered independent, since molecular analysis was needed to determine the relationship among the progeny of the same callus.

Among the 606 transgenics 129 co-transgenics were identified, where our model gene-of-interest was integrated in the genome along with the selectable marker, see figure 3-3. These plants were produced by 50 calli in total. In those cases where more than one co-transgenic sister plants were obtained from one callus, further molecular tests were carried out, in order to find out whether their analysis is worth to be conducted in future applications of the method.

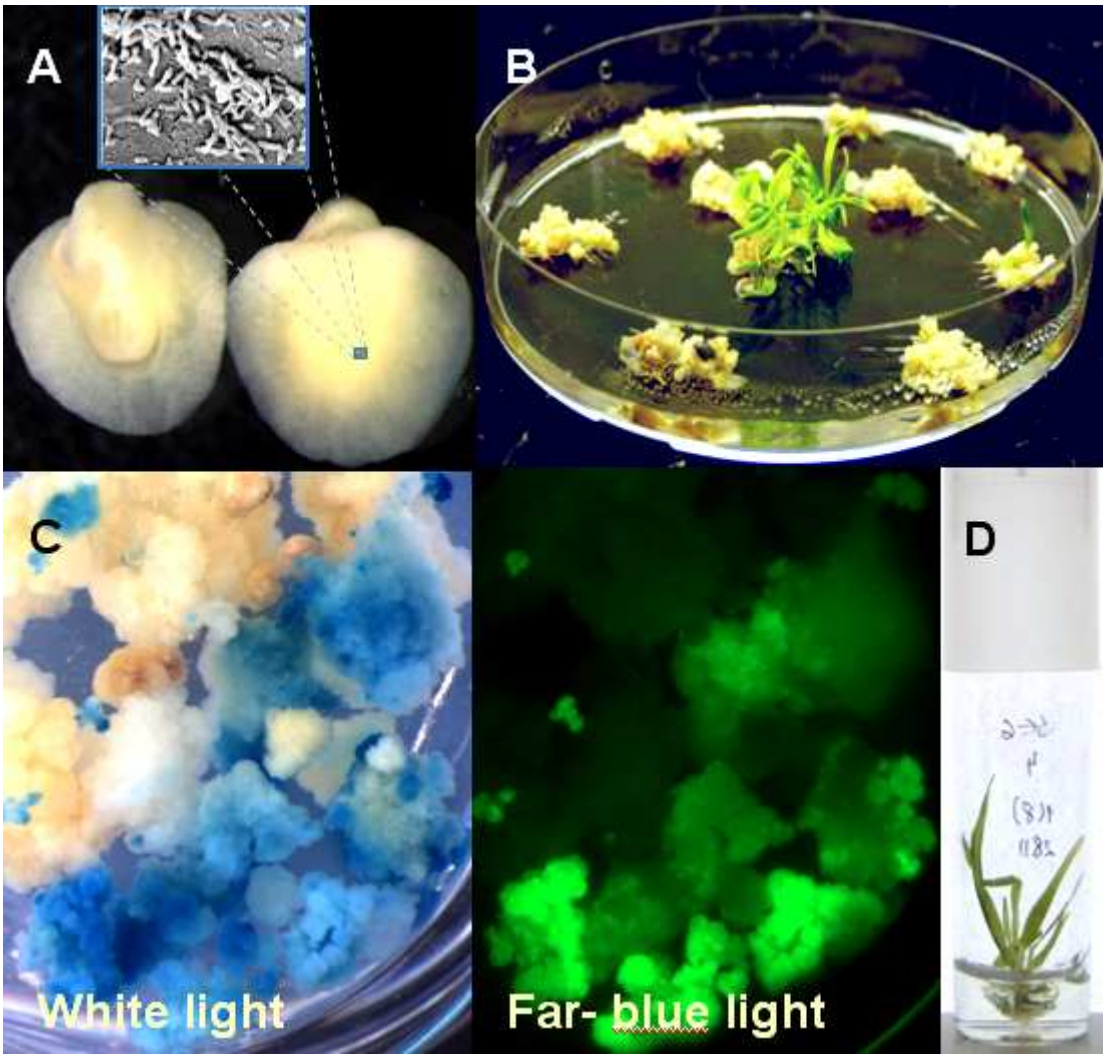


Figure 3-2.
Co-transformation of immature embryos

A. Immature barley cv. "Golden Promise" embryos were used for *Agrobacteium*-mediated gene transfer
B. Regenerating calli on selective medium (PRM+25 mg/l Hygromycin B)
C. Co-transformed calli showing expression of the reporter genes GUS and GFP
D. Primary regenerant (T_0)

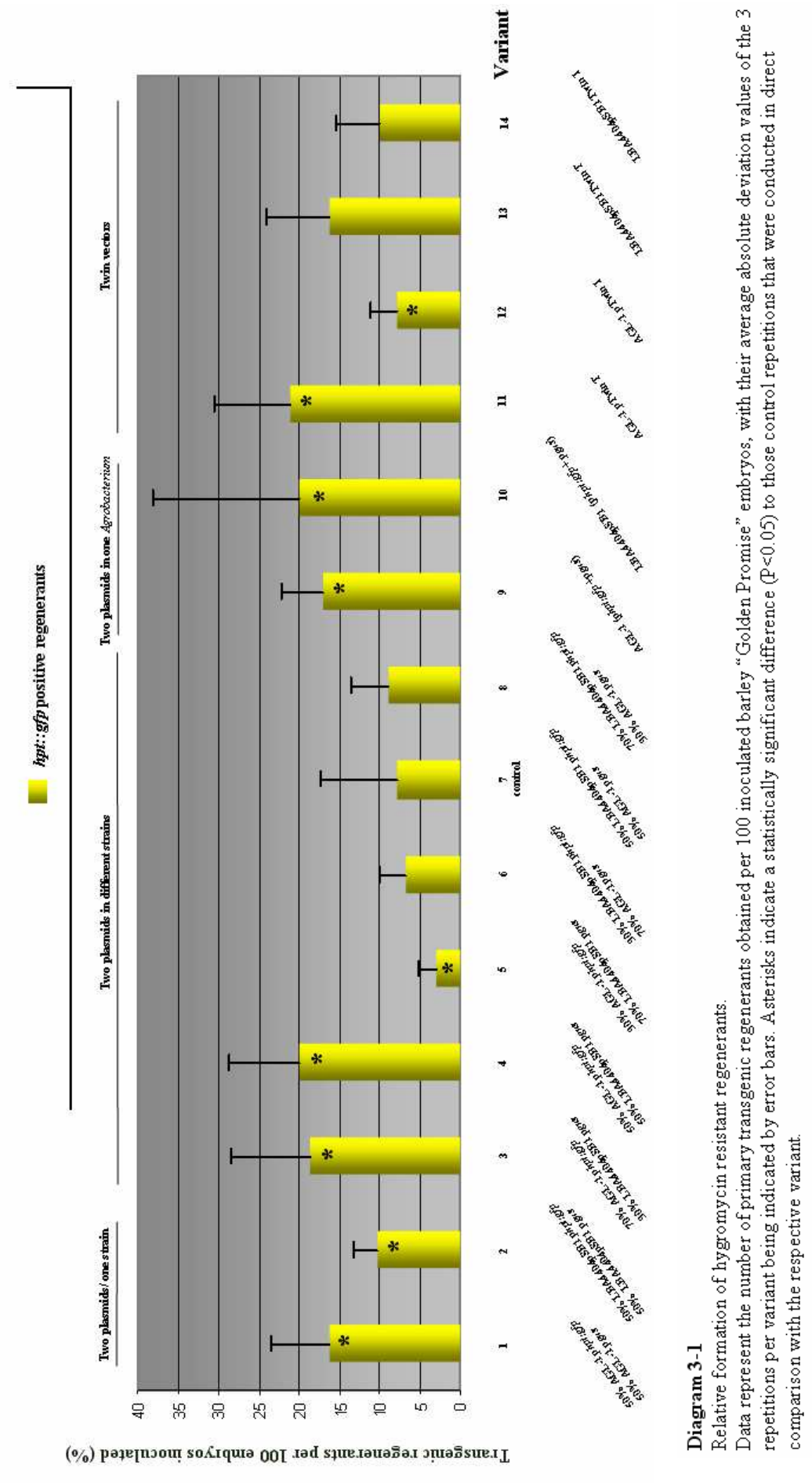


Diagram 3-1
Relative formation of hygromycin resistant regenerants.
Data represent the number of primary transgenic regenerants obtained per 100 inoculated barley "Golden Promise" embryos, with their average absolute deviation values of the 3 repetitions per variant being indicated by error bars. Asterisks indicate a statistically significant difference ($P<0.05$) to those control repetitions that were conducted in direct comparison with the respective variant.

3.2.1. Evaluation of sister plants derived from the same embryo

As mentioned previously, occasionally several sister plants were produced per one callus, their clonal state can be determined by molecular analysis. In view of the PCR results co-transgenic sister plants were subject to Southern blot analysis, in order to identify, whether they represent genetically identical clones or independent multiple lines. Taking only independent lines into consideration was a prerequisite for an appropriate evaluation of co-transformation and segregation of the transgenes.

The regeneration pattern of the calli was followed through the experiments, in order to identify clones and independent lines derived from the same callus. Each of the calli which produced regenerants was given a number. The sister plants of a callus were numbered in alphabetical order, which means that plants indicated with the same number, but different letters stem from the same callus. Examples can be seen in figure 3-3 and 3-4, e.g. 3a-d are four primary transgenic plants regenerated from callus number 3. As plant 3b is different from 3a, 3c and 3d it can already be deduced from the PCR test results that the regenerants produced by the same callus are not necesseraly clones. In principle the same applies to the two plants derived from callus number 4. By contrast, callus number 5 has produced three plants, all of which tested positive for both T-DNAs by PCR. These co-transgenic lines had to be further analysed by Southern blot to find out their individual integration pattern and transgene copy numbers in order to decide if they are to be considered clones or if there are independent transgenic lines among them.

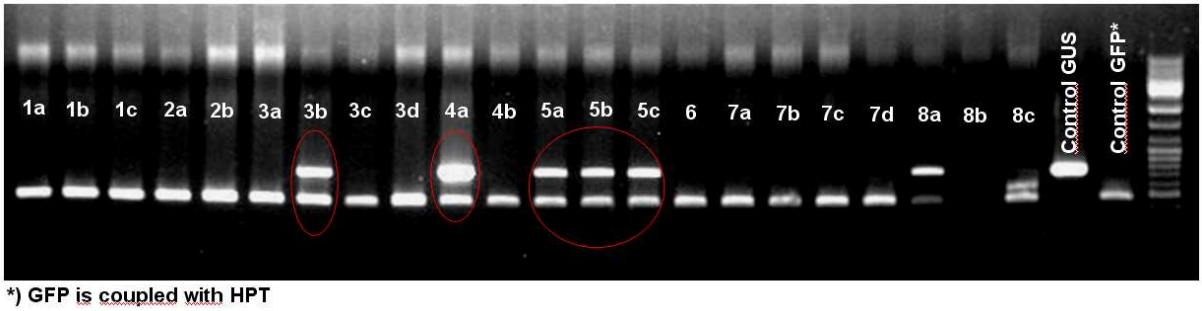


Figure 3-3.
PCR analysis of the primary transgenic (T_0) plants. Numbers indicate different calli and letters the deriving regenerants. Co-transgenics referred to in the text are encircled in red.

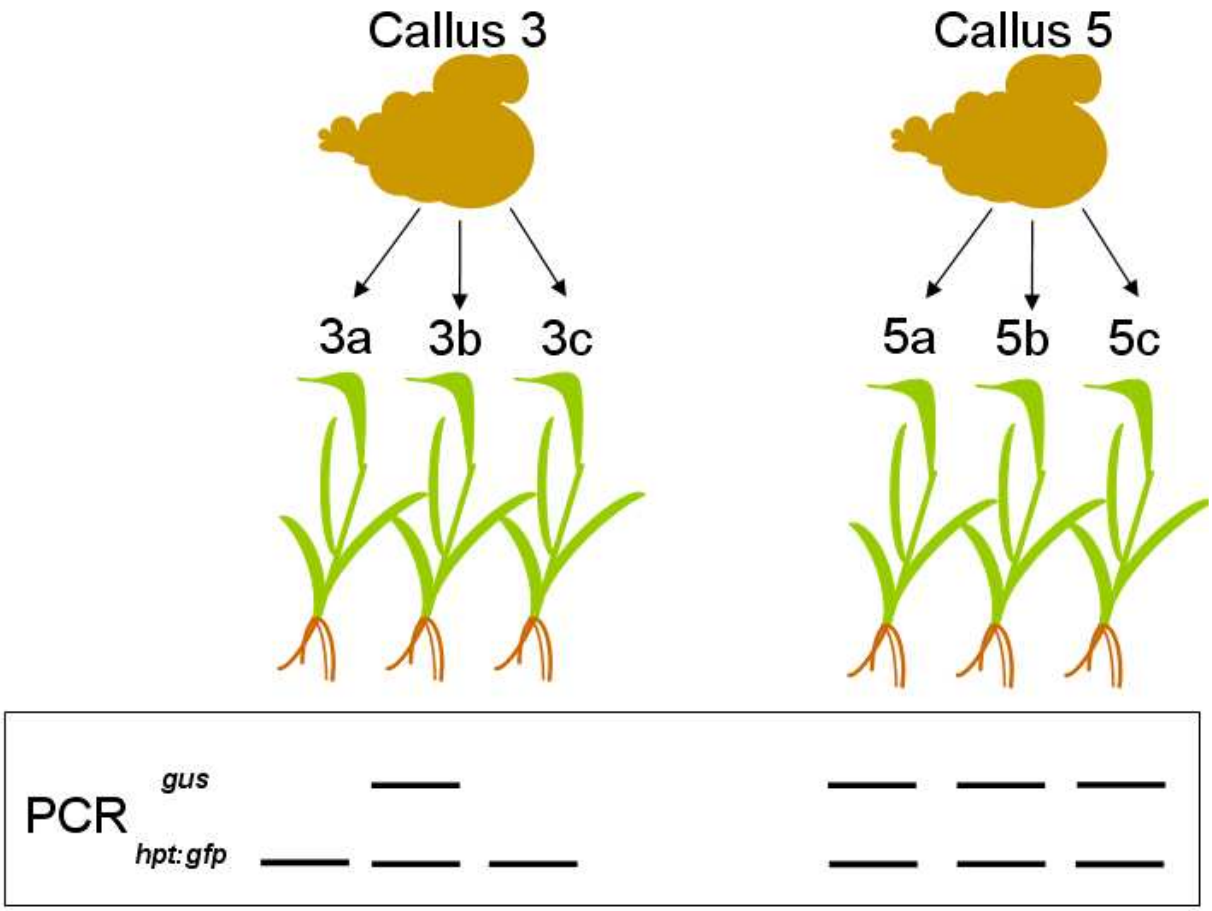


Figure 3-4.
Comment on figure 3-3. PCR carried out on genomic DNA of plants obtained from co-transformation experiments might confirm genomic differences between regenerants coming from the same callus (callus 3). In other cases, the question of clonal state can only be answered with the use of other methods, such as Southern blot (callus 5).

The plantlets were put in glass tubes on regeneration medium containing hygromycin. In some cases it happened that in a tube containing one single plantlet, two or more other shootings appeared. The resultant plants were indicated by additional arabic numerals, e.g. 10a1 and 10a2, and were expected to be clones. However, it turned out from the PCR and Southern blot results that individual shoots can be escapes or might even stem from independent transformation or „supertransformation" (containing extra copy/copies) events (figure 3-5).

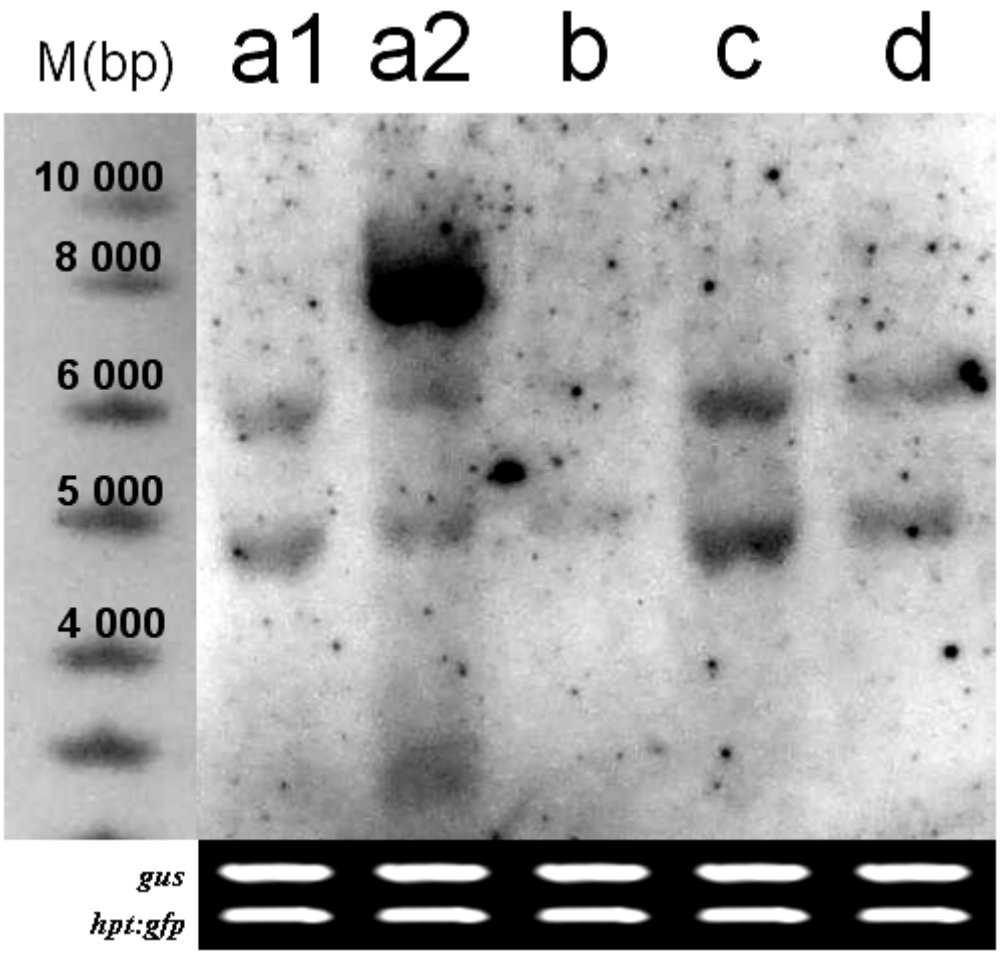


Figure 3-5.
Southern blot revealing genetic differences among regenerants derived from the same callus. The bands represent copies of the selectable marker detected in five sister plants. Respective PCR results are shown below the Southern blot. It can be clearly seen that regenerant a1, b, c and d are clones, while plant a2, which grew together with a1 in one tube has at least two extra copies of the resistance marker. Note that the strong intensity of the uppermost band of plant a2 indicates a concatamer of multiple T-DNAs integrated.

Diagram 3-1 shows the distribution of the total number of primary transgenic regenerants among the 14 different variants, obtained per 100 inoculated embryos.

Co-transgenic sister plants regenerating from the same callus and later proven not to be clones by Southern blot were considered as additional indpendent transgenic lines in the calculation of the efficiencies, since these lines constituted additional candidates for the eventual obtention of marker-free lines. 30 % of the analysed co-transgenic sister families (6 out of 20) produced multiple independent lines (table 3-1)

Typically there were many not co-transgenic sister regenerants, which were not individually analysed for their integration pattern and transgene copy number, because they were no candidate progenitors for marker-free lines. As a consequence, it was impossible and not necessary to determine a total efficiency in independent transgenic plant generation. In those cases, where one callus produced more than one primary transgenic plants lacking the gene-

of-interest (*hpt+,gus-),* the regenerants were considered as one single line. This means that the number of transgenic lines obtained from 100 inoculated embryos is underestimated by ca. 15%. Hereinafter, regenerants are considered as plants obtained from calli derived from *Agrobacterium*-mediated genetic transformation of immature embryos, without any further indication about their clonal state or relations. An independent line always refers to the sum of genetically identical clones or a single transgenic individual without any additional sister plants.

Table 3-1. Regeneration pattern of clones and independent lines of different variants.

Variant	No. of isolated IEs	Callus no.	No. of co-transgenic sister plants stemming from one callus	Any non-identical?	No. of lines proved independent per callus	No. of co-transgenic sister plants proved clones
2	270	1	**5**	yes	**2**	2+3
4	270	2	**5**	no	**1**	5
5	270	3	**4**	no	**1**	5
6	270	4	**5**	yes	**3**	1+2+2
7 control	1620	5	**3**	no	**1**	3
		6	**3**	no	**1**	3
		7	**8**	no	**1**	8
		8	**2**	no	**1**	2
		9	**4**	yes	**3**	1+1+2
9	270	10	**5**	yes	**2**	1+4
		11	**5**	no	**1**	5
		12	**2**	no	**1**	2
10	270	13	**2**	yes	**2**	1+1
		14	**4**	no	**1**	4
11	270	15	**6**	no	**1**	6
		16	**11**	no	**1**	11
		17	**5**	yes	**2**	1+4
12	270	18	**2**	no	**1**	2
13	270	19	**5**	no	**1**	5
14	270	20	**15**	no	**1**	15

3.2.2. Genetic transformation and co-transformation

There are various possibilities for presenting the effectiveness of the *Agrobacterium*-mediated gene transfer system to produce selectable marker-free transgenic barley lines. Three points of reference are mentioned hereinafter:

1. Efficiencies correlate with the *number of inoculated embryos*, e.g the transformation efficiency of a variant reveals the number of identified independent transgenic lines per hundred embryos.
2. Frequencies apply to the number of obtained *primary transgenic T_0 lines*, e.g. a co-transformation frequency represents the proportion of independent co-transgenic lines among the primary transgenic independent lines of the same variant.
3. Rates bear reference to *co-transgenic T_0 lines*, e.g doubled haploid production rate represents the proportion of co-transgenic T_0 lines producing doubled haploids in a variant.

Genetic transformation efficiencies were highly variable, ranging from 1.5 to 9.6%.

The results indicate that the most efficient variant from this aspect was variant 4 (1:1 mixture of AGL-1 p*hpt:gfp* and LBA4404pSB1p*gus*). In this case 26 independent lines out of 270 inoculated immature embryos have been tested positive for the T-DNA of hygromycin resistance, which means a 9.6% transformation efficiency (indicated by green colour in diagram 3-2). However, from the aspect of co-transformation efficiency this variant was not among the best (0.7 co-transgenic lines per 100 inoculated immature embryos).

A control variant was added to each embryo transformation experiment, because donor material quality cannot be equally provided over time. In the statistical analysis, the variants were compared only to those control cultures grown in the very same experiment. Considering all 14 variants in terms of transformation efficiency, statistically significant differences ($P<0.05$) between the control and variants 2, 5, 9, 10 and 11 were determined (diagram 3-2). However, because of the cumulative representation of all control data, the diagram does not necessarily show if a variant is significantly better or worse than its particular control. All of the variants, except for number 5, proved to be more efficient than their control in the pairwise comparison.

The highest co-transformation efficiency among all *hpt*-positive regenerants occurred in variant 9 (two plasmids in one *Agrobacterium* clone method), where 8 out of 100 isolated immature embryos (3.0%) gave rise to independent co-transgenic lines. Six lines positive for both the selectable marker and the gene-of-interest were obtained in variants 10 and 11.

However, a statistically significant difference was determined only in the latter case, which was due to different numbers of co-transgenics obtained in the respective parallel controls.

Co-transformation frequency presents the proportion of co-transgenics related to the total number of obtained transgenic lines. Both T-DNAs were integrated in the plant genome in 40.0% of the lines in both variant 10 (two plasmids in LBA4404pSB1) and variant 11 (AGL-1 pTwin T). In both cases 6 lines out of 15 turned out to be co-transgenic for the selectable marker and the gene-of-interest (diagram 3-3). On the whole the proportion ranged from 7.7% to 40.0%, but no statistical difference to the control was determined from the available data.

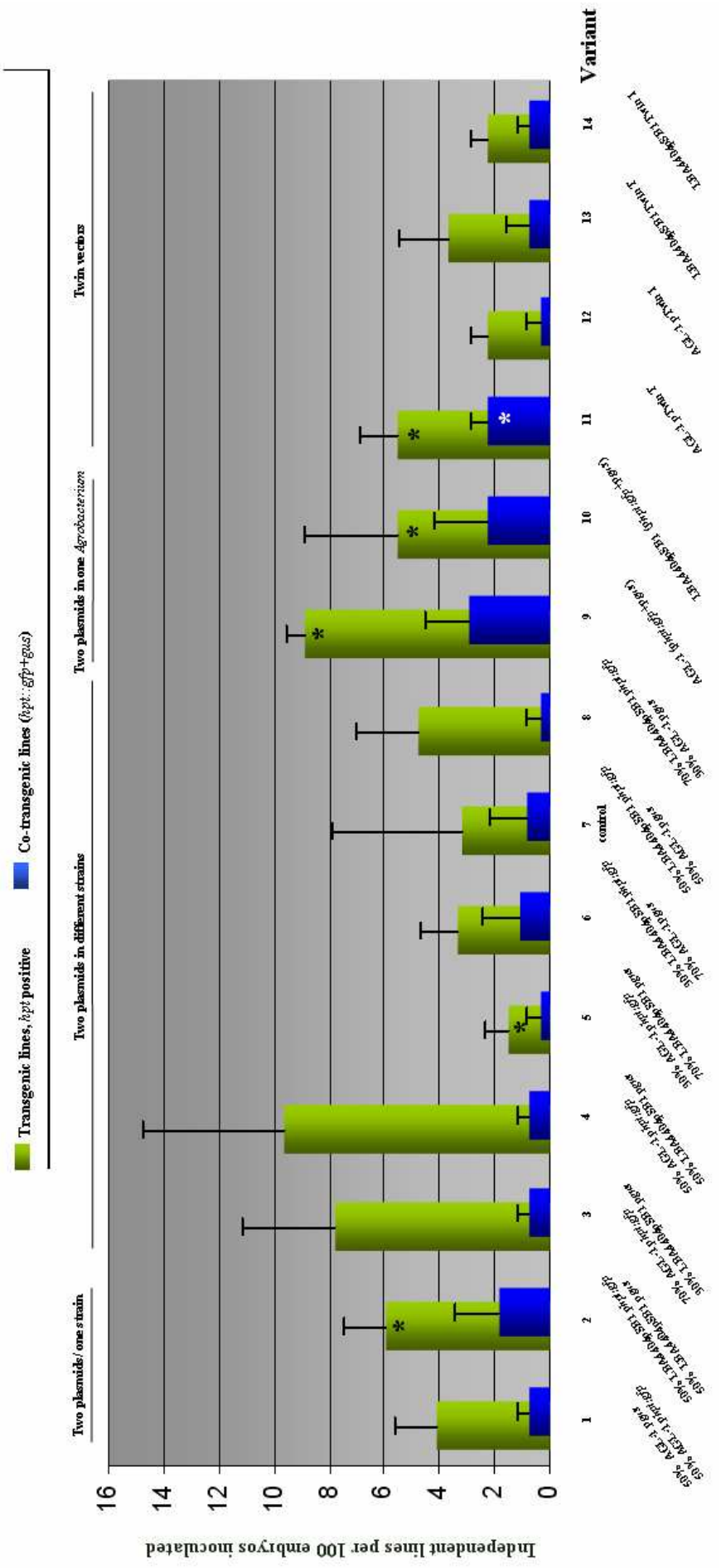


Diagram 3-2.
Relative formation of hygromycin resistant and co-transgenic lines
Data represent the number of primary transgenic and co-transgenic independent lines obtained per 100 inoculated barley "Golden Promise" embryos, with their average absolute deviation values of the 3 repetitions per variant being indicated by error bars. Asterisks indicate a statistically significant difference ($P<0.05$) to those control repetitions that were conducted in direct comparison with the respective variant.

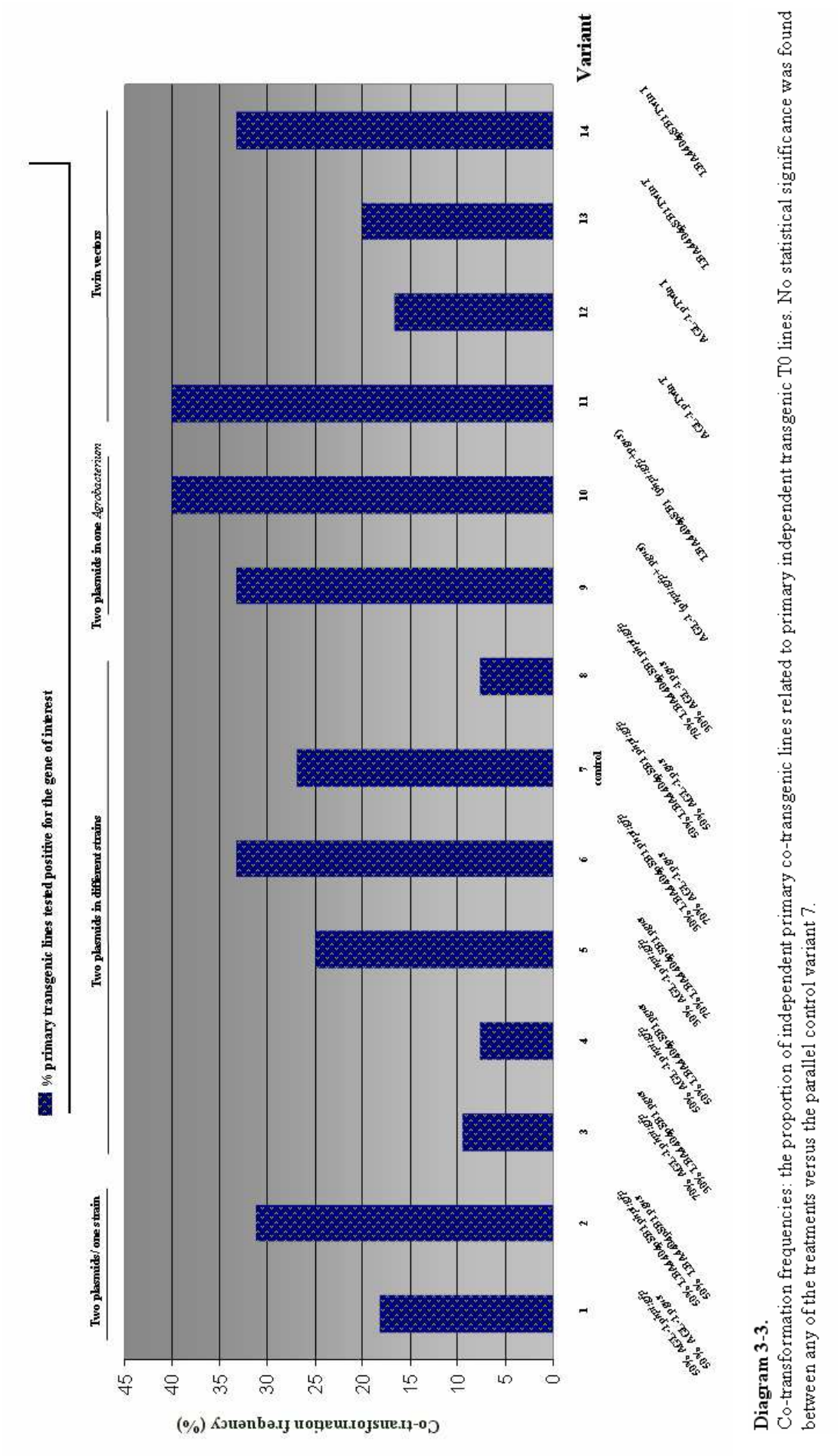


Diagram 3-3.
Co-transformation frequencies: the proportion of independent primary co-transgenic lines related to primary independent transgenic T0 lines. No statistical significance was found between any of the treatments versus the parallel control variant 7.

3.2.3. Transgene copy numbers in primary co-transgenic lines

Stable integration of the the selectable marker gene and the gene-of-interest in the plant genome was detected and their copy numbers were analysed by Southern blot in 91 primary co-transgenic regenerants. Table 3-2 summarises data on transgene integration in all analysed co-transgenic plants including sister plants. In view of the DNA hybridisation results, the final number of genetically independent lines was determined, depending on the clonal state of sister plants.

The gene-of-interest was present as single copy in 48.8% of the independent lines analysed. Two copies were found in 17.1%, 34.1% had three or more copies.

One copy of the selectable marker gene was detected in 22.0% of the primary co-transgenics, 19.5% possessed two copies, and 58.5% three or more copies (table 3-2 and diagram 3-4).

The variants did not markedly differ from each other. Lines carrying single copies of both T-DNAs were found in variants 2, 7, 11 and 12. On the other hand, occasionally very high numbers were detected, e.g. in one line of variant 7 more than ten copies of *gus* and six copies of the selectable marker were integrated in the plant genome.

However, using one enzyme for the detection of transgenic fragments bears the risk of underestimation of these data. Stronger band signals might represent two or more copies, which by chance have resulted in equal sizes.

Table 3-2.
T-DNA copy numbers as detected by Southern blot in genomic DNA of co-transgenic T_0 plants including all sister lines. The plants identified as clones are considered together as a single independent line, which are represented by consecutive Roman numerals and placed in columns according to their copy numbers.
+) indicate if no doubled haploids were obtained from a line, and sexual T1 individuals were analysed
T) tetraploid line

Variant	Proportion/ strain/ binary vector	No. of analysed (T_0) regenerants	Identified independent lines	T-DNA	No. of T-DNA copies determined by Southern blot (% of lines analysed)		
					1	2	>3
2	50% LBA4404pSB1p*hpt::gfp* 50% LBA4404pSB1p*gus*	6	3	*hpt::gfp*	I^{+} (33.3%)		II,III (66.7%)
				gus	I^{+},III (66.7%)	II (33.3%)	
3	70% AGL-1 p*hpt::gfp* 30% LBA4404pSB1p*gus*	1	1	*hpt::gfp*		I (100%)	
				gus	I (100%)		
4	50% AGL-1 p*hpt::gfp* 50% LBA4404pSB1p*gus*	5	1	*hpt::gfp*			I (100%)
				gus			I (100%)
5	30% AGL-1 p*hpt::gfp* 70% LBA4404pSB1 p*gus*	4	1	*hpt::gfp*			I (100%)
				gus	I (100%)		
6	30% LBA4404pSB1p*hpt::gfp* 70% AGL-1 p*gus*	5	3	*hpt::gfp*		II (33.3%)	I, III (66.7%)
				gus			I,II,III (100%)
7 control	50% LBA4404pSB1p*hpt::gfp* 50% AGL-1 p*gus*	13	9	*hpt::gfp*	II,III VIII,IX (44.4%)	IV (11.2%)	I,V,VI VII (44.4%)
				gus	II,III,IV VI,VIII IX (66.7%)		I,V,VII (33.3%)
8	30% AGL-1 p*gus* 70% LBA4404pSB1p*hpt::gfp*	1	1	*hpt::gfp*			I(T) (100%)
				gus	I(T) (100%)		
9	AGL-1 (p*hpt::gfp* +p*gus*)	16	8	*hpt::gfp*	II,VII(T) (25%)	I,IV,VIII (37.5%)	III,V VI(T) (37.5%)
				gus	I,III,IV V (50%)	II,VII(T) (25%)	VI(T) VIII (25%)
10	LBA4404pSB1(p*hpt::gfp* +p*gus*)	9	6	*hpt::gfp*		II,IV(T) (33.3%)	I^{+},III, V VI(T) (66.7%)
				gus		I^{+},II IV(T) (50%)	III,V VI(T) (50%)
11	AGL-1 pTwin T	23	5	*hpt::gfp*	I (20%)		II,III,IVV (80%)
				gus	I,II,III,V (80%)		IV (20%)
12	AGL-1 pTwin I	2	1	*hpt::gfp*	I (100%)		
				gus	I (100%)		
13	LBA4404pSB1pTwin T	6	2	*hpt::gfp*			I,II (100%)
				gus		I (50%)	II (50%)

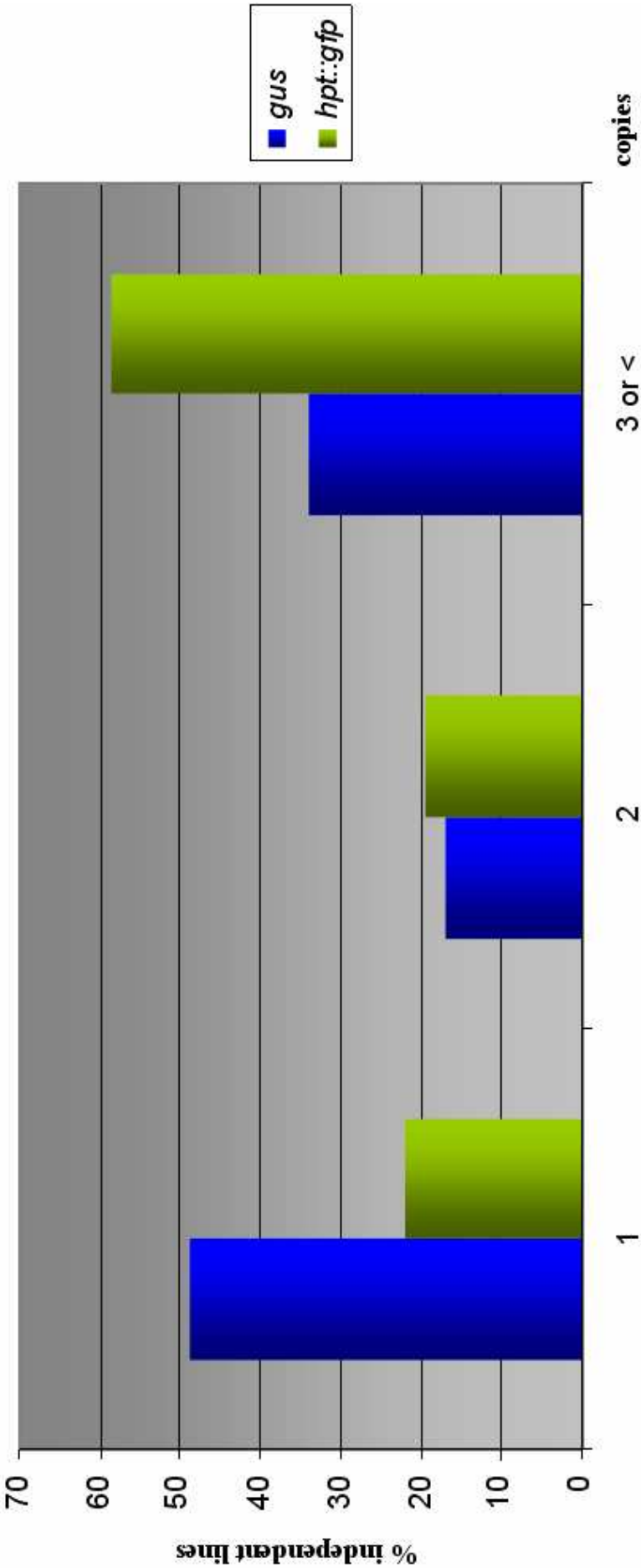


Diagram 3-4. Frequency of the two T-DNAs in the independent lines of the fourteen variants.

3.2.4. Spontaneous genome duplication in primary transgenic lines

Agrobacterium-mediated gene transfer to immature barley embryos typically resulted in diploid pimary transgenic plants. However, occasionally some regenerants showed a deviating phenotype, i.e. a relatively tall stature with long, wide leaves, but weak and sear overall appearance (figure 3-6). They mature ca. one month later than their normal counterparts. The grain set in these plants is highly variable, though some produce good yield. Flow cytometry analysis revealed that such plants were tetraploid.

Microspore isolation from the spikes of tetraploid barley plants is possible and embryogenic pollen cultures can result in ample green regenerants. Progeny of such tetraploid T_0 individuals were dihaploid (2x, note the difference to doubled haploids) or, if spontaneous genome doubling happened, tetraploid (doubled dihaploids). Among all of the 14 variants producing a total of 606 primary transgenic regenerants, altogether 20 were tetraploid, which belonged to 6 independent lines. Among the pollen embryogenesis-derived T_1 plants of three independent tetraploid T_0-lines (ten plants) the *gus* gene segregated independently, producing transgenic selectable marker-free barley plants in diploid and tetraploid state. Although those diploids are generated through haploid technology, further segregation of the GOI is still possible in the following generations, because the spontaneous genome doubling that has resulted in their 4x mother plants had been initiated from hemizygous somatic cells. Consequently, the 4x plants were also hemizygous and thus producing dihaploid pollen and respective pollen-derived dihaploid plants which expectedly segregate into 50% hemizygous, 25% homozygous transgenic and 25% azygous individuals (see table 3-3).

Table 3-3.
Distribution of tetraploid primary co-transgenic lines

Variant	Method	No. of tetraploid, independent co-transgenic lines	No. of the tetraploid lines producing SM-free transgenic progeny from pollen cultures
7	Two plasmids in two different *Agrobacterium* strains	1	0
8		1	1
9	Two plasmids in one *Agrobacterium*	2	1
10		2	1

The true number of tetraploid T_0 plants might be even higher than given here, because the occurrence of the phenomenon was not realised and followed from the beginning of the experiments and only co-transgenic regenerants were tested with regard to ploidy.

Figure 3-6.
Comparison of barley plants of the same age, having diploid (on the left) and tetraploid (on the right) genomes. Tetraploid individuals tend to grow and mature slowly and produce larger grains.

3.3. Doubled haploids derived from primary co-transgenic lines

Spikes of the potentially independent primary co-trangenic (T_0) lines were harvested, embryogenic pollen development was induced so as to generate doubled haploid (DH) populations, among which homozygous transgenic recombinants lacking the redundant selectable marker gene can be identified. In order to determine whether the model gene-of-interest segregated independently from the selectable marker in the T_1 generation, different analysis methods were carried out (PCR, hygromycin leaf assay and Southern blot).

3.3.1. Embryogenic pollen cultures

Of all the independent co-transgenic lines selected to generate embryogenic pollen cultures 43 lines (78.2 %) produced doubled haploid T_1 progeny. Clones facilitated the generation of DH lines from one independent line, since more material was available. The average number of green DH regenerants was 1.4 per harvested spike, but it showed great variability ranging between 0.09 and 4.26. Generation of ca. 15 doubled haploid regenerants are statistically expected per transgenic barley plant, if we take into consideration that on average 10.7 spikes were harvested for the preparation of embryogenic pollen cultures (figure 3-7).

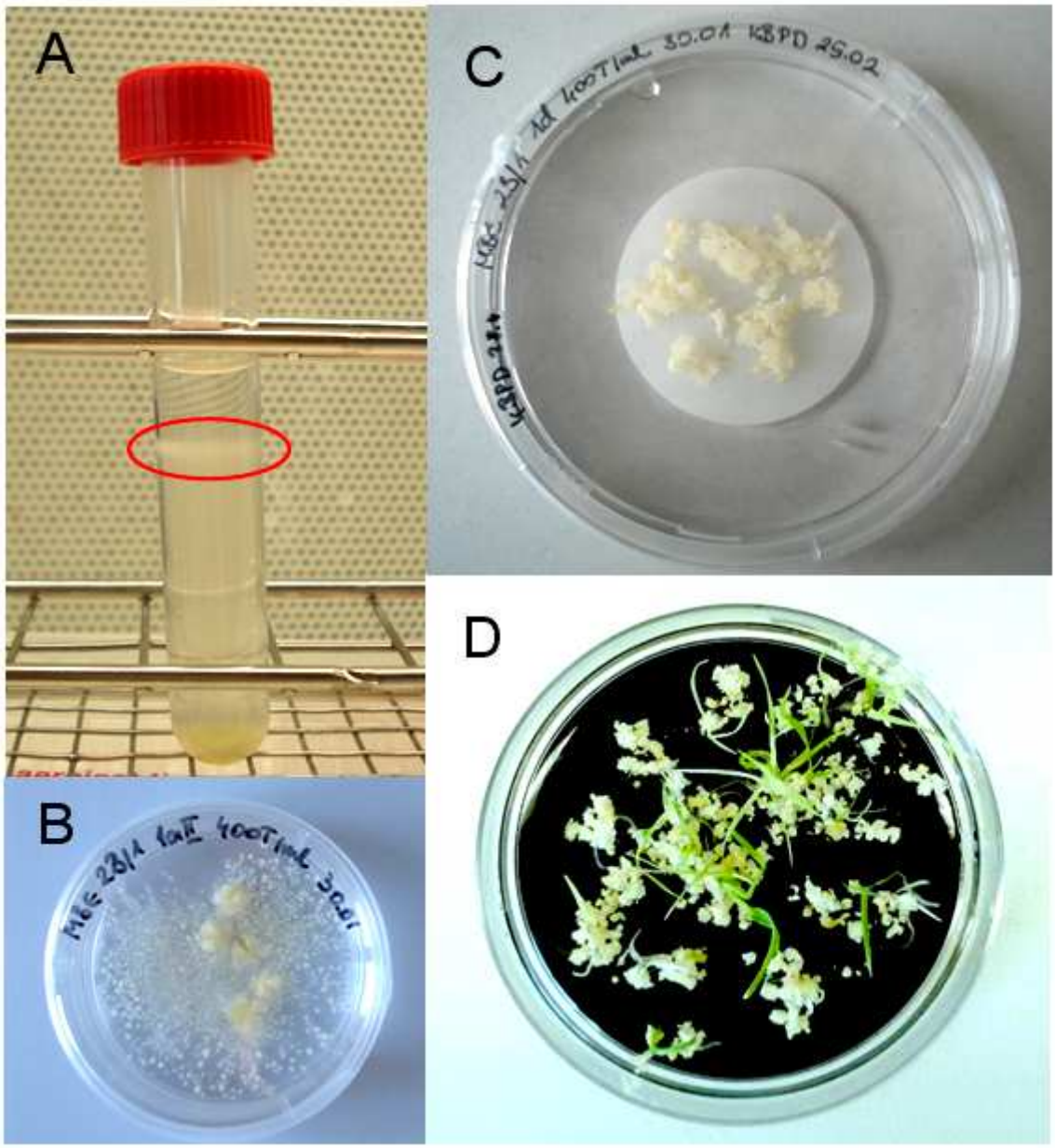


Figure 3-7.
Isolation and pollen embryogenesis of barley microspores.

A. Interphase containing the viable microspores
B. Calli in KBP medium with immature wheat pistils
C. Developing calli on solid KBP medium
D. Regenerating calli producing haploid and doubled haploid plants

Diagram 3-5 presents green doubeld haploid production efficiencies of the 14 variants. Values range between 0.37 and 2.22%. The highest efficiency was performed by variant 9, because of the relatively high number of independent co-transgenic lines that were succesful in regenerating plants from embryogenic pollen cultures. Although several variants produced a reasonal number of independent co-transgenics giving rise to green DH plants, only variant 11 was significantly better as compared to its control.

On average, the proportion of green DH producing co-transgenic lines in relation to the number of independent co-transgenics of the same variant (doubled haploid production rate) ranged between 50 and 100% (diagram 3-6). The variants do not significantly differ from their respective controls. In six variants, all of the primary co-transgenic plants gave rise to green doubled haploid progeny (4, 5, 6, 7, 8, 12).

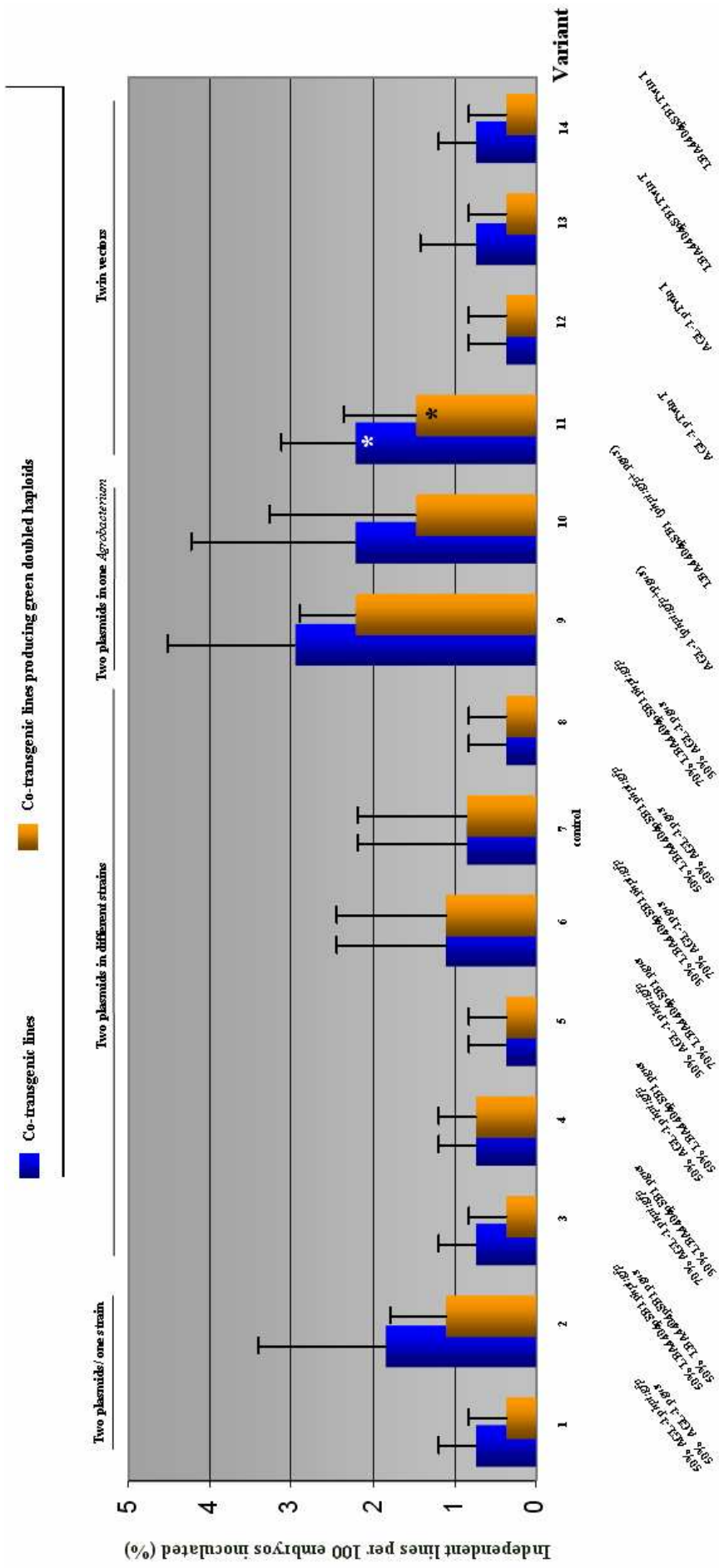


Diagram 3-5.
Green doubled haploid production efficiency. The columns represent the number of independent co-transgenic lines (blue) and their portion producing green doubled haploid progeny (orange) per hundred inoculated embryos with the average absolute deviation across the 3 repetitions of a variant being shown by error bars. Asterisks indicate a statistically significant difference ($P<0.05$) to those control repetitions that were conducted in direct comparison with the respective variant, according to parameter-independent Kruskal-Wallis ANOVA on Ranks.

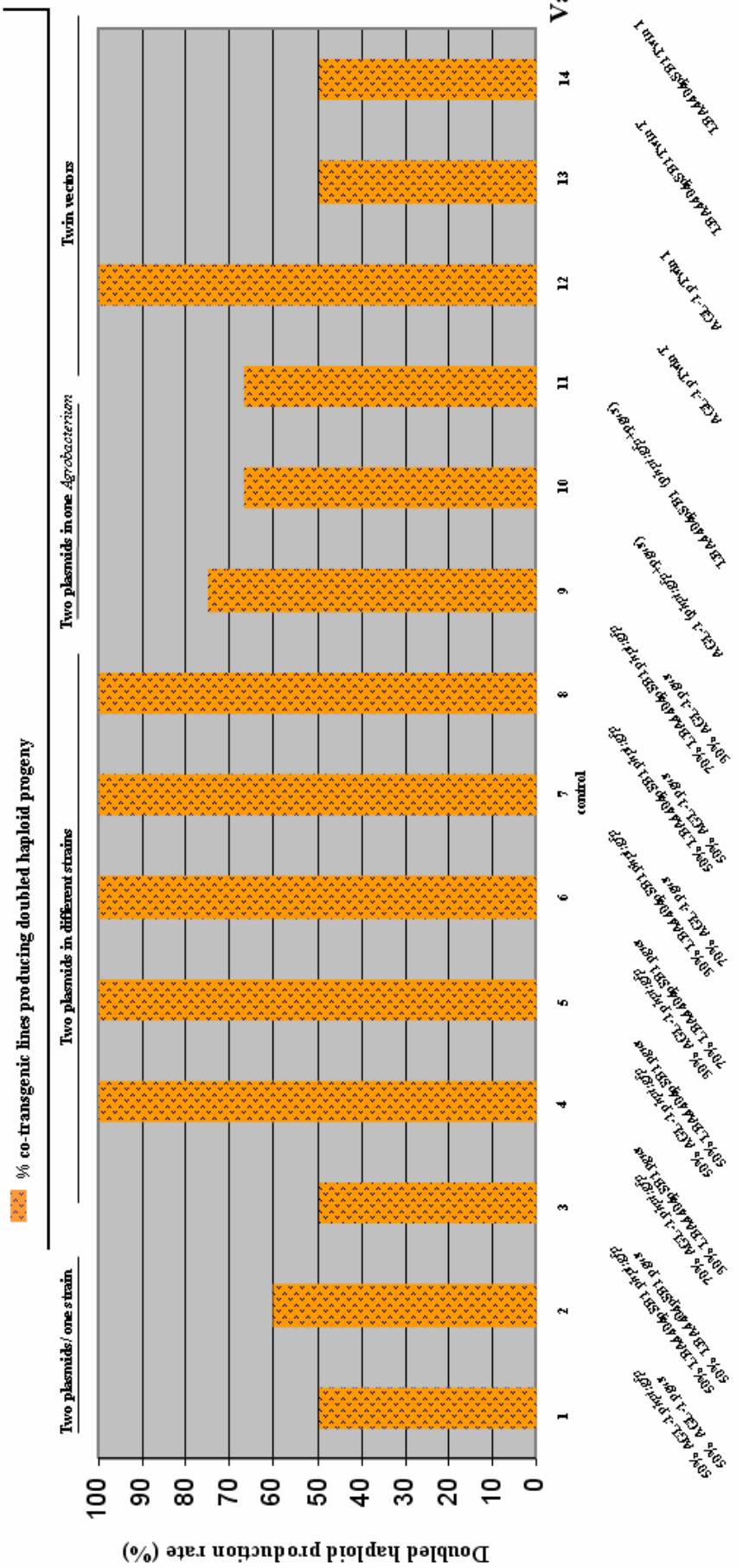


Diagram 3-6.
Green doubled haploid production rate. Data represent the proportion of independent co-transgenic lines producing green doubled haploids related to the total number of primary independent co-transgenic lines per co-transformation variant.

3.3.2. Environmental influence on the formation of doubled haploids

The outcome of immature barley embryo genetic transformation is highly dependent on environmental conditions. The obtained T_0 regenerants show phenotypic variability as well, e.g. in height, maturity, yield etc. A difference can also be seen when examining embryogenic pollen cultures even if they derive from the same barley genotype. Some cultures produce many microcalli, but in other cases hardly any cell division is observed. The number of green and albino regenerants is also highly variable.

It is difficult to determine which factor has greater influence on the DH production capacity of the embryogenic pollen cultures: seasonal variability or phenotypic deviations in the T_0 generation. Diagram 3-7 presents the average number of haploid and doubled haploid barley green and albino regenerants produced per spike during the four seasons of a year. Although the highest number of plants is produced in spring, the ANOVA on Ranks test shows no statistically significant difference between the median values among the four groups.

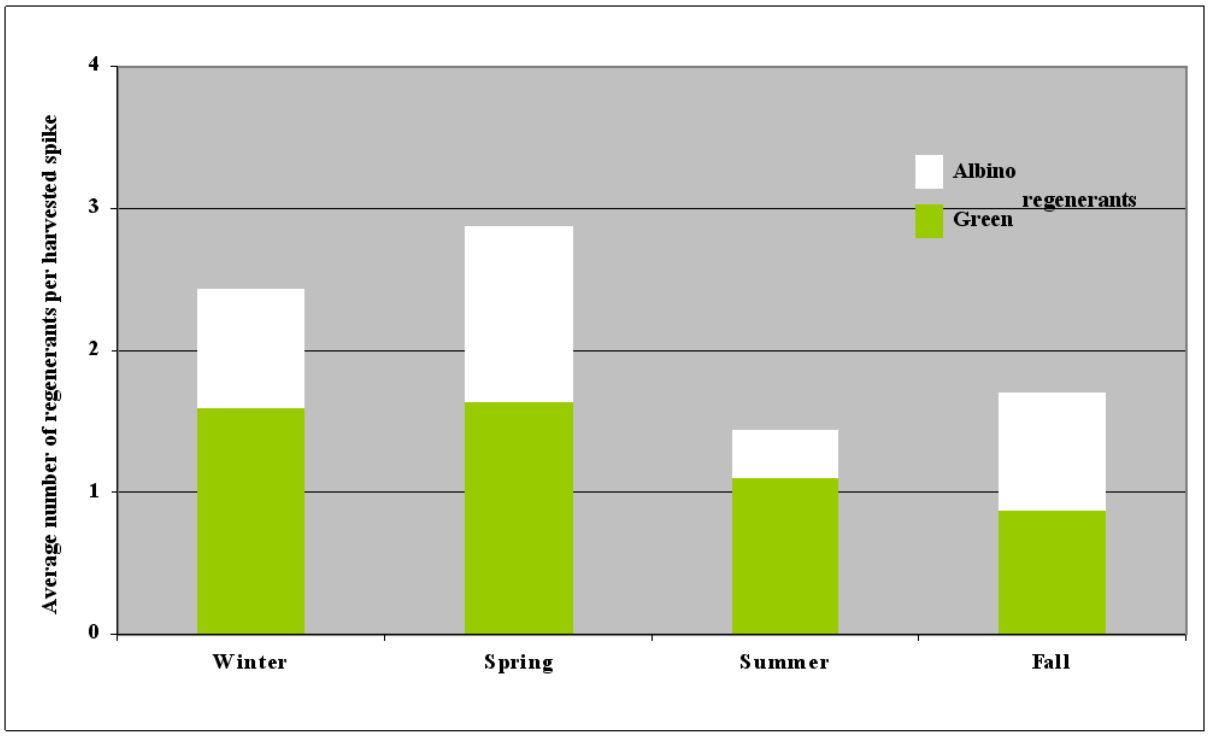


Diagram 3-7.
Seasonal distribution of average numbers of green and albino regenerants per spike

3.3.3. Analysis of individual doubled haploid plants

A total of 55 co-transgenic independent lines were obtained. Only two of them proved to be sterile and produced neither doubled haploids nor seeds. Segregation was detected in 31 DH families derived from independent co-transgenics, but since both types of T-DNA can co-segregate this does not mean that all in those cases both T-DNAs segregated from each other. The latter case was observed only in DHs derived from four independent co-transgenic lines.

Selectable marker-free doubled haploid T_1 progeny containing only our model gene-of-interest, *gus*, were produced by 14 independent lines, which is 45.2% of the lines showing segregation (figure 3-8 and figure 3-9)

Diagram 3-8 summarises the proportion of independent co-transgenic lines in the different variants producing embryogenic pollen culture-derived doubled haploids containing the gene-of-interest, but lacking the selectable marker.

No selectable marker-free transgenic individuals were identified among the DH families obtained from the two binary plasmids in two clones of the same *Agrobacterium* strain method (variant 1 and 2), variants 3 and 5 from the two plasmids in different strains method and the Twin variants (11, 12, 13, 14), although co-transgenic lines producing GOI-free, *hpt*-transgenic progeny were found among them. The reason for the phenomenon is that the two T-DNAs were integrated in linkage groups, and only an additional unlinked copy of the selectable marker gene segregated in the T_1 generation. Three or more copies of the *hpt::gfp* were also found frequently in such lines.

From the aspect of efficient production of homozygous selectable marker-free transgenic barley cv. “Golden Promise”, the two plasmids in one *Agrobacterium* clone method proved to be the best, both variants showed statistically significant differences to their control variant.

In variant 9, three out of eight primary co-transgenic lines gave rise to SM-free and *gus* positive doubled haploid lines, that is 1.1 lines per 100 embryos. In variant 10 (LBA4404pSB1 p*hpt::gfp*+p*gus*), 4 of the 6 co-transgenic lines, 1.5 lines per 100 inoculated embryos, gave rise to SM-free plants carrying the gene-of-interest

Two plasmids in different strains was the only other method that generated GOI positive homozygous doubled haploids lacking the SM, namely variants 4, 6, 7 and 8.

Selectable marker-free transgenic doubled haploid production rate presents the proportion of co-transgenic T_0 lines producing hygromycin sensitive, GOI positive regenerants per number of independent co-transgenics (diagram 3-9). Values ranged between 14.3% and 100%.

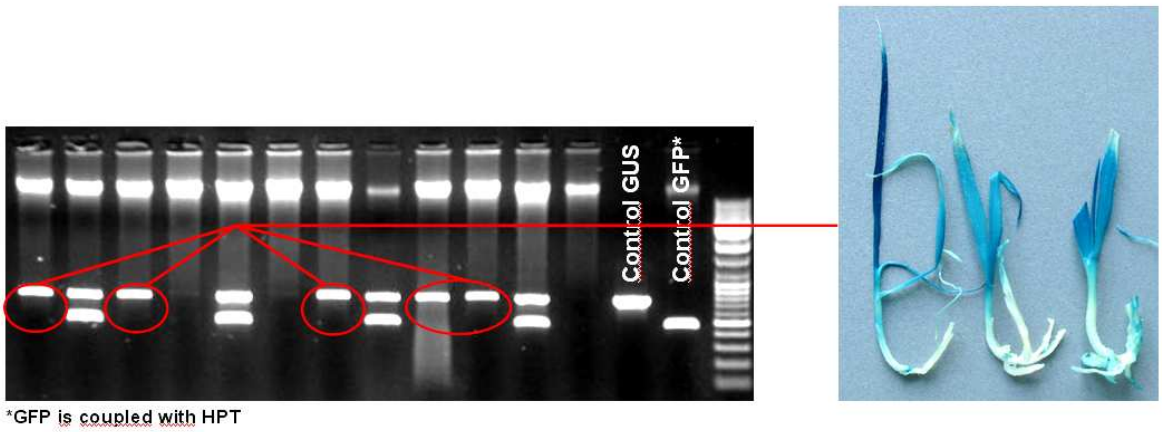


Figure 3-8.
Identification of selectable marker-free transgenic progeny

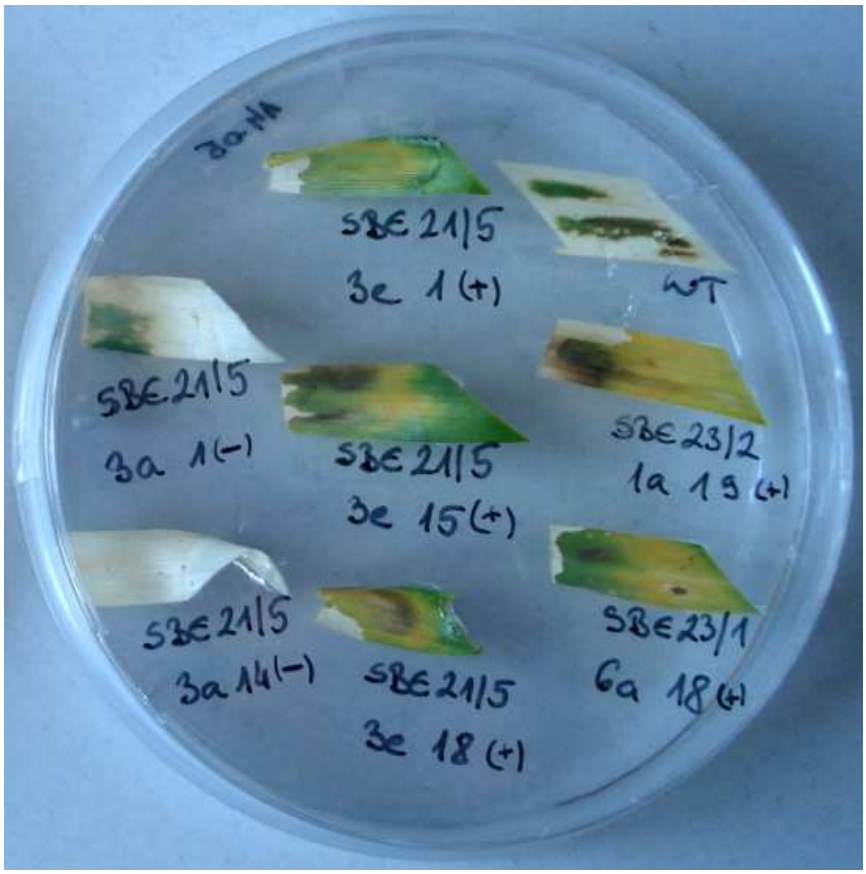


Figure 3-9.
Leaf assay showing presence or absence of hygromycin resistance gene expression (-) PCR *hpt* negative, (+) PCR *hpt* positive, WT- wildtype

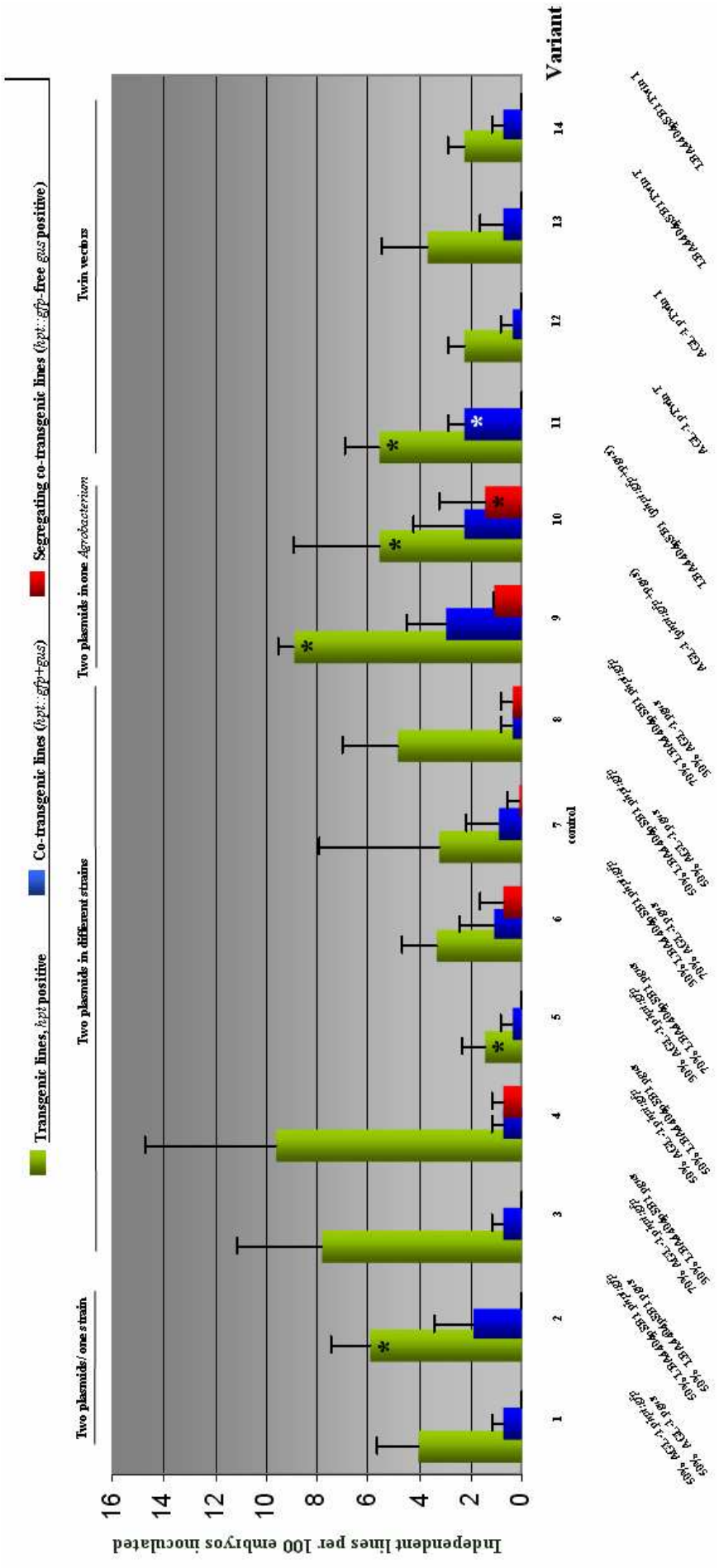


Diagram 3-8.
Formation of primary transgenic plants, independent co-transgenic and independent selectable marker-free transgenic lines
Data represent the number of independent primary transgenic and co-transgenic lines as well as those lines producing independent selectable marker-free GOI positive green doubled haploid progeny as quantified per hundred inoculated embryos with their average absolute deviation indicated by error bars. Asterisks indicate a statistically significant difference ($P<0.05$) to the respective control repetitions according to the parameter-independent Kruskal-Wallis ANOVA on Ranks.

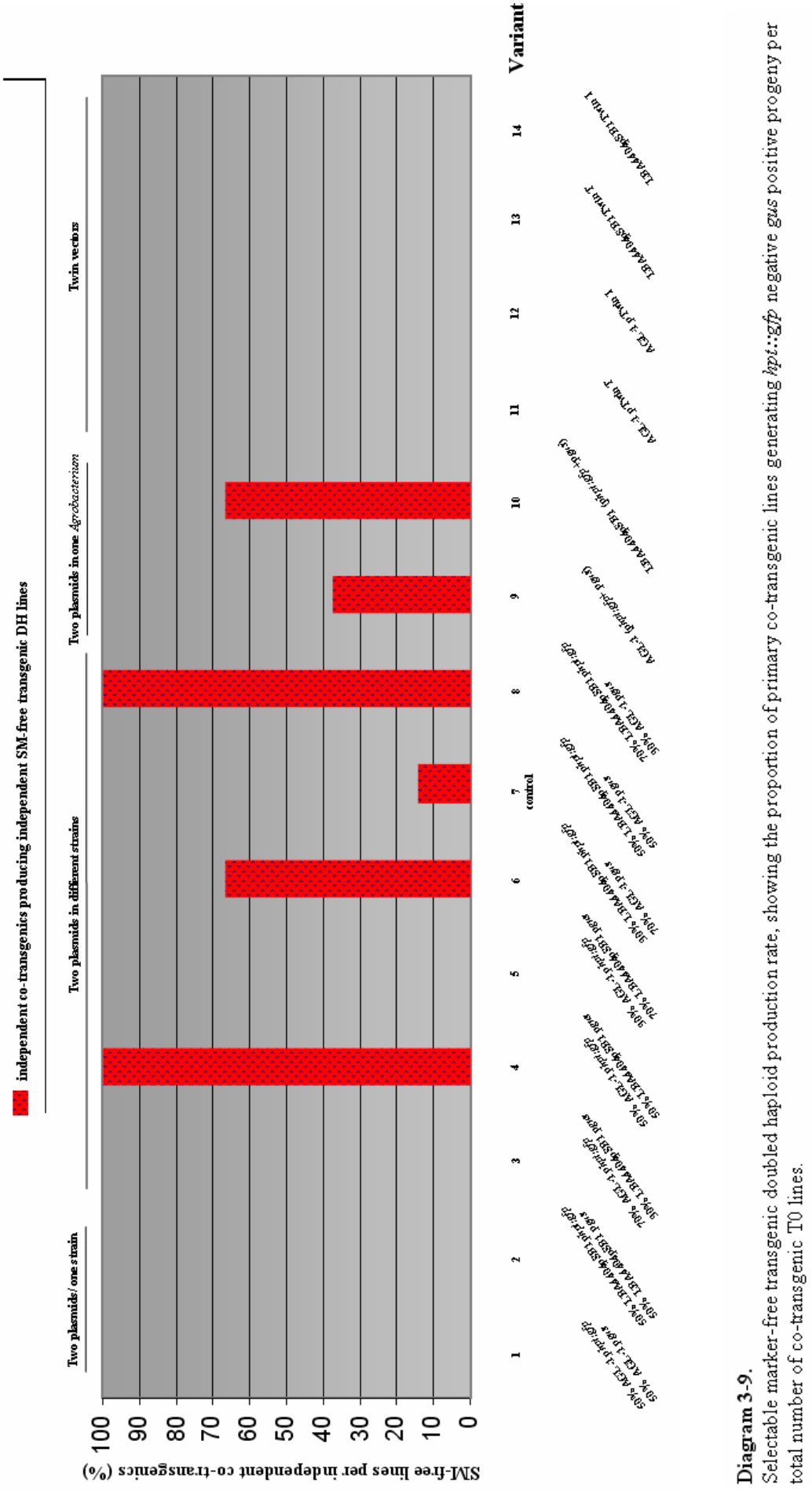


Diagram 3-9.
Selectable marker-free transgenic doubled haploid production rate, showing the proportion of primary co-transgenic lines generating *hpt::gfp* negative *gus* positive progeny per total number of co-transgenic T0 lines.

3.3.4. T-DNA segregation in populations of doubled haploids

Southern blot analysis not only provided us with results on the copy number of the two types of T-DNAs in the primary co-transgenic T_0 plants (table 3-2), analysis of the T_1 generation gave us hints about the relation of the integrated DNA fragments in the plant genome, i.e. whether they are coupled in linkage groups, or located on different chromosomes.

Usually only one of the two transgenes was unlinked from all copies of the other and produced DH regenerants carrying only one type of T-DNA, as was the case in 49.1% of the independent co-transgenic lines. In such case, there are often multiple copies of the two T-DNA types integrated in linkage groups coupled to each other. If just one of the copies is unlinked from all others, segregants exclusively carrying the respective T-DNA type are obtained, but no plants tested positive only for the other type. This means, that a given co-transgenic line did not produce progeny, among which both only GOI and only SM positive genotypes were found.

However, in 43.6% of the independent co-transgenic lines, the transgenic fragments were integrated in linkage groups, without any uncoupled one, during the *Agrobacterium*-mediated gene transfer. In such cases the chance for separation decreases sharply. The likelihood that closely linked T-DNAs segregate through recombination during meiosis is very low.

Linked integration of the transgenes can clearly be followed by Southern blot analysis of T_1 populations obtained either from doubled haploid or sexual progeny (figure 3-10).

Table 3-4 summarizes the segregation pattern of the selectable marker and the model gene-of-interest in several lines. Among the four independent DH lines, where segregation of both T-DNAs occurred, two lines contained one copy of the selectable marker and one copy of the GOI in their genome. In one case 1 GOI and 2 SMs were detected, but the latter were coupled to each other as it turned out from the Southern blot results, and one co-transgenic plant had two GOIs and 1 SM.

In case of one T_0-plant (variant 8, 70% LBA4404pSB1 p*hpt::gfp* and 30% AGL-1 p*gus*) the *hpt::gfp* construct segregated in non-Mendelian fashion. Only selectable marker-free GOI positive progeny were found in the doubled haploid T_1 population, despite the fact that five copies (possibly linked at the same insertion locus) of the resistance gene were detected in the T_0 plant by Southern blot analysis.

In the Twin variants only segregation of the *hpt::gfp* T-DNA was observed, and mostly the detected number of copies was high.

Interestingly, even if there are many copies detected by Southern blot, most of them were integrated linked to each other. These linkage groups will likely segregate as one locus in the

T_1 generation. The highest number of "actually" segregating loci was detected in variant 13, LBA4404pSB1 pTwin T, where 8 copies of the *hpt:gfp* T-DNA appeared to be integrated in five loci. Hygromycin resistant homozygous DH progeny lacking the GOI were obtained, but no marker-free *gus* positive ones were found in the doubled haploid T_1 population. Probably the two *gus* T-DNA copies were integrated in a linked manner to at least one of the selectable marker gene copies.

Due to the frequent linkage of both types of T-DNA, the vast majority of DH lines were either co-transgenic for both T-DNAs or lacking any transgenic fragments.

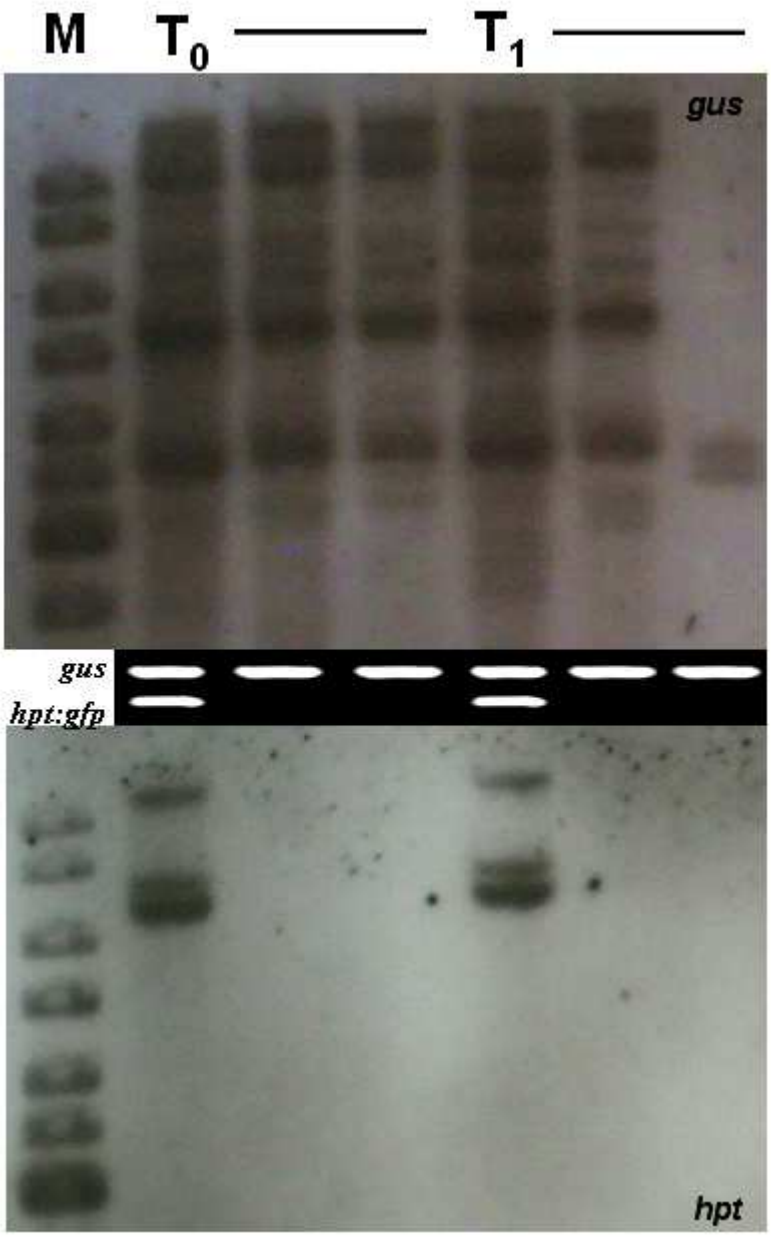


Figure 3-10.
Example for the segregation pattern of both types of T-DNAs among doubled haploid T_1 progeny of a co-transgenic T_0 plant using PCR and Southern blot. *Gus* T-DNAs were integrated in two linkage group loci, one of which proved not to be coupled with the one *hpt* linkage group.

Table 3-4. Segregation pattern of the two T-DNAs in the T_1 generation
*) Plants that segregated in the T_1 for the respective T-DNA are indicated by an asterisk
+) indicate if no doubled haploids were obtained from a line, and sexual T_1 individuals were analysed
T-DNAs introduced in a linked manner in one locus are indicated by L, with the number of copies in the linkage group, e.g 2L means two copies of the transgene in a linkage group. Additional single copies are indicated by S.

Variant	Proportion/ strain/ binary vector	Identified independent lines	T-DNA	No. of T-DNA copies determined by Southern blot		
				1	2	>3
2	50% LBA4404pSB1p*hpt::gfp* 50% LBA4404pSB1p*gus*	3	*hpt::gfp*	I*$^{+}$		II*(3L+1S) III*(4L+1S)
			gus	I*$^{+}$,III	II	
3	70% AGL-1 p*hpt::gfp* 30% LBA4404pSB1p*gus*	1	*hpt::gfp*		I*(2L)	
			gus	I		
4	50% AGL-1 p*hpt::gfp* 50% LBA4404pSB1p*gus*	1	*hpt::gfp*			I(3L)
			gus			I* (4L+1S)
5	30% AGL-1 p*hpt::gfp* 70% LBA4404pSB1 p*gus*	1	*hpt::gfp*			I*(2L+1S)
			gus	I		
6	30% LBA4404pSB1p*hpt::gfp* 70% AGL-1 p*gus*	3	*hpt::gfp*		II(2L)	I(2L+1S),III
			gus			I*(>3S) II*(>3S) III(5L)
7 control	50% LBA4404pSB1p*hpt::gfp* 50% AGL-1 p*gus*	9	*hpt::gfp*	II*,III VIII,IX	IV	I,V,VI*VII
			gus	II*,III*, IV,VI VIII,IX		I,V VII*
8	70% LBA4404pSB1p*hpt::gfp* 30% AGL-1 p*gus*	1	*hpt::gfp*			I
			gus	I*		
9	AGL-1 (p*hpt::gfp*+ p*gus*)	8	*hpt::gfp*	II*,VII	I*(2L),IV* VIII	III*,V*,VI*
			gus	I*,III,IV V	II*,VII*	VI,VIII
10	LBA4404pSB1(p*hpt::gfp* + p*gus*)	6	*hpt::gfp*		II(2L) IV(2L)	I^{+},III(2L+1S) V(4L),VI
			gus		I^{+},II* IV*(2S)	III* V*(3L+1S) VI
11	AGL-1 pTwin T	5	*hpt::gfp*	I		II*,III*, IV,V*
			gus	I,II,III,V		IV
12	AGL-1 pTwin I	1	*hpt::gfp*	I		
			gus	I		
13	LBA4404pSB1 pTwin T	2	*hpt::gfp*			I*(3L+2L+ 3S) II
			gus		I	II

3.4. Sexually generated T1 lines

No doubled haploid regenerants were obtained from the embryogenic pollen cultures of 12 primary co-transgenic independent lines, belonging to variants 1 (one line), 2 (two lines), 3 (one line), 9 (two lines), 10 (two lines), 11 (one line), 12 (one line), 13 (two lines). Among them only one line (variant 2, 50% LBA4404pSB1 p*gus* and 50% LBA4404pSB1 p*hpt:gfp*) was identified, where the resistance gene and the gene-of-interest were integrated in an unlinked manner, giving rise to transgenic selectable marker-free sexual T_1 plants which need to be further analysed in T2 and probably T3 to generate and identify a respective homozygous line (figure 3-11).

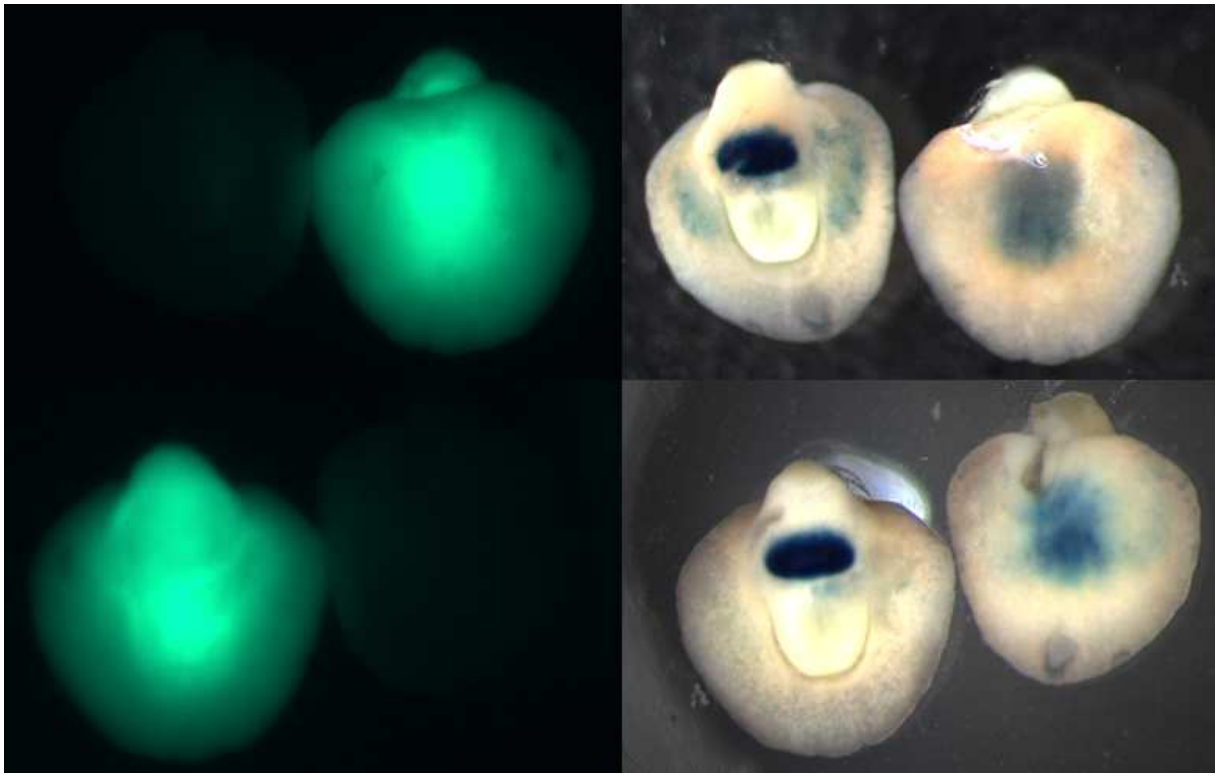


Figure 3-11.
Embryos of a co-transgenic plant showing independent segregation of the *hpt::gfp* T-DNA and the *gus* T-DNA.

3.5. Time frame of the established method

Table 3-5 gives a summary about the time intervals needed for the generation of doubled haploid T_1 from which homozygous transgenic SM-free individuals can be selected. It takes about 43 weeks to conduct the entire method, starting with immature embryo inoculation and finishing with the plant regeneration from embryogenic pollen culture.

Table 3-5.
Time plan for the production of doubled haploid transgenic barley cv. Golden Promise from embryogenic pollen culture of co-transgenic T_0 plants generated by *Agrobacterium*-mediated gene transfer to immature embryos

Steps in succession	**Time interval**
***Agrobacterium*-mediated transformation of immature embryos**	
Co-culture of immature embryos with agrobacteria	60 hours
Callus induction	2x2 weeks
Plant regeneration	4x2 weeks
Growth and maturation of the primary co-transgenic T_0 plants	ca. 17 weeks
Production of doubled haploid T_1	
Spike harvest from co-transgenic plants and cold treatment	3 weeks
Starvation treatment of isolated immature pollen	2 days
Callus formation in liquid KBP medium	3 weeks
Callus development on solid KBPD medium	2 weeks
Plant regeneration and identification of selectable marker-free transgenic individuals	3x2 weeks

4. DISCUSSION

4.1. Efficiency of the established method

Monocotyledonous plants do not naturally belong to the hosts of *Agrobacterium tumefaciens*, but efficient protocols were developed for genetic transformation of such non-hosts, with barley among them (Tingay et al. 1997; Wu et al. 1998; Patel et al. 2000; Wang et al. 2001; Trifonova et al. 2001; Fang et al. 2002; Stahl et al. 2002; Murray et al. 2004; Travella et al. 2005; Kumlehn et al. 2006; Hensel et al. 2008). The pioneer protocols used *bar* and *hpt* as selectable marker. In the present study we used *hpt*, according to the protocol of Hensel and Kumlehn (2004), because due to its effectiveness in contrast to other selection systems the proportion of non-transgenic escapes (1.78% in this study) can be significantly reduced.

In the thesis efficient co-transformation of barley "Golden Promise" with selectable and non-selectable T-DNAs using *Agrobacterium tumefaciens* for the gene transfer and identification of a most successful variant where fragments separated in successive generation with comparatively high frequencies are presented. For the successful application of the method several important parameters must be taken into consideration: a very efficient genetic transformation protocol is crucial, where high frequency of co-transformation events with unlinked T-DNA integration is optimised, thus the proportion of segregating (*gus* positive antibiotics sensitive) progeny must be high.

Agrobacterium-mediated barley genetic transformation using four binary vector constructs in 14 different variants were conducted, including methods using mixtures of *Agrobacterium tumefaciens* strains (McKnight et al. 1987; De Block and Debrouwer 1991; Komari et al. 1996; Coronado et al. 2005), two plasmids in one *Agrobacterium* clone (De Framond et al. 1986; Komari et al. 1996; Daley et al. 1998) and a vector, Twin, harboring two T-DNAs (Komari et al. 1996; Matthews et al. 2001; Stahl et al. 2002). The latter method seemed very promising for the purpose of establishing the method presented in this thesis. Komari et al. (1996) co-transformed rice and tomato plants using a super binary vector, where the two T-DNA regions were separated by a large, at least 15 kb section, on a huge 50-55 kb plasmid obtained by homologous recombination. The advantage of this system is that it gives the possibility for the substitution of various GOIs, opposed to the conventional vector systems. On the other hand, the vector is very large and cumbersome to work with. Co-transformation frequency was between 47-85%, more than half of the lines were SM-free *gus* positive. They

also conducted experiments with *Agrobacterium tumefaciens* strain mixtures, but co-transformation efficiency was lower.

Two adjacent T-DNAs, separated by left and right border regions, integrated in a standard binary vector were transformed by Matthews et al. (2001) into barley. The transgenes were divided only by a small plasmid region (850 bp). Transgenic lines were produced with 2-12% transformation efficiency, 66% co-transformation frequency, 24% of the co-transgenics produced selectable marker-free progeny containing the GOI, resulting in 16% useful independent co-insertions events.

Table 4-1 summarises previous techniques for the generation of co-transformants, in which the GOI and SM gene can be segregated in the successive generation. Co-transformation frequency values and the proportion of selectable marker-free lines with regard to the number of co-transgenics showed great variability among the reports, but it must be mentioned that different species were subject to the co-transformation experiments. It has been widely discussed (Depicker et al. 1985; Komari et al. 1996; Matthews et al. 2001) that using single strain methods (two T-DNAs in one *Agrobacterium* clone, Twin vectors), the co-transformation efficiency is much higher than applying mixture methods (two plasmids/one strain, two plansmids in different *Agrobacterium* strains). However, this hypothesis was contradicted by McKnight et al. (1987).

Table 4-1. Delivery of T-DNAs to plants using *Agrobacterium*-mediated co-transformation for the generation of selectable marker-free transgenic lines.
n.d. not determined

Species	**Proportion of co-transgenic lines per all transformants (%)** Co-transformation frequency	**Selectable marker-free lines per co-transgenics (%)** SM-free transgenic production rate	**Method**	***Agrobacterium* strain**	**GOI**	**SM**	**Reference**
Nicotiana tabacum	49	19	Mixture method: Two plasmids/one strain (1:1)	*A. rhizogenes* A4	*nos*	*npt*	McKnight et al. 1987
Brassica napus	39-85	n.d. linked integrations	Mixture method: Two plasmids/one strain (1:1)	*A. tumefaciens* C58	*bar*	*npt*	De Block and Debrouwer 1991
Brassica napus	62	40	Single strain method: Two plasmids in one *Agrobacterium* clone	*A. tumefaciens* LBA4404	*gus*	*npt*	Daley et al. 1998
Nicotiana tabacum and *Oryza sativa*	47-85	50	Single strain method: Two plasmids in one *Agrobacterium* clone	*A. tumefaciens* LBA4404	*gus*	*hpt* *npt*	Komari et al. 1996
	0-35	ca. 50	Mixture method: Two plasmids/one strain (1:1 and 3:1)	*A. tumefaciens* LBA4404	*gus*	*hpt* *npt*	
Hordeum vulgare cv. "Golden Promise"	66	24	Twin vector	*A. tumefaciens* AGL-1 and AGL-0	α-amylase α-glucosidase	*hpt*	Matthews et al. 2001
Hordeum vulgare cv. "Golden Promise"	34.6	5.6	Mixture method: Two plasmids/two strains	*A. tumefaciens* AGL-1 and LBA4404	*gus*	*hpt*	Coronado et al. 2005
Hordeum vulgare cv. "Golden Promise"	7.7-40	67 (Variant 10)	Mixture methods Single strain methods	*A. tumefaciens* AGL-1 and LBA4404	*gus*	*hpt*	Present study

Barley cv. “Golden Promise” genetic transformation efficiencies presented in this study varied between 1.5-9.6% obtained independent *hpt*::*gfp* positive lines per 100 inoculated immature embryos, among the different variants (diagram 3-2). It is comparable to the up-to-date existing *Agrobacterium*-mediated barley cv. “Golden Promise” immature embryo genetic transformation techniques (reviewed by Goedeke et al. 2007). Transgenic and co-transgenic plants were obtained from each variant. It was not possible to carry out the immature barley embryo genetic transformation of all the variants at once, division of the experiments was necessary. Due to this fact, a control variant (1:1 mixture of AGL-1 p*gus* and LBA4404pSB1p*hpt::gfp*) was applied, which makes transformation and co-transformation efficiencies, frequencies and rates comparable to a standard, and suitable variants can be selected.

Two percent of the inoculated embryos gave rise to more than one regenerant. A detailed analysis of the co-transgenic sister plants regenerating from the same callus revealed that they were mostly clones. According to the obtained data summarized in table 3-1, the probability that two regenerants randomly chosen from such multiple plant producing calli belong to different independent lines is 9.3% Due to the tremendous additional effort necessarily required for their analysis, it is recommended to discard those lines in future applications.

The range of transformation efficiencies did not correlate with the co-transformation efficiencies, only variant 11 (AGL-1 pTwin T) differed significantly from its control variant in both aspects (diagram 3-2). Variants 3 and 4 of the two plasmids in different strains method performed well in the number of independent transgenic T_0 lines per 100 immature embryos. In the same time, high number of *hpt* positive lines were obtained from the parallel control variant, which suggests that the quality of the donor material was better than usual, thus the two variants did not prove to be outstanding in transformation efficiency. However, their co-transformation efficiency values were not among the best. The lack of association between genetic transformation and co-transformation of barley cv. “Golden Promise” is probably due the fact, that presence of the second T-DNA, the gene-of-interest, does not provide the plants in the regeneration process with any benefit, therefore there is no selection for it.

An other way to test the effectiveness of the system is to show, what proportion of the lines were co-transgenic in comparison to the total number of obtained antibiotic resistant lines. This is expressed by the co-transformation frequency (diagram 3-3). Variants 10 (two plasmids in LBA4404pSB1) and 11 (AGL-1 pTwin T) reached the highest value (40%). This is lower than the 66% co-transformation frequency result published by Matthews et al. (2001). Although the mean values showed a range from 7.7% up to 40.0%, the high average

absolute deviation values indicate that the data is not necessarily conclusive. Further repeats of the experiments would be needed to increase the statistical power of the calculations.

In the different variants, all together 55 primary co-transgenic independent lines were identified, 72.7% of these selected plants gave rise to successful embryogenic pollen cultures.

DH production efficiency was expectedly unaffected by the co-transformation method, and thus the most DH populations were obtained from variants with a high output of co-transgenics thus the highest green doubled haploid production efficiency, belonged to variant 9 (diagram 3-5).

Doubled haploid production rates, presenting the proportion of primary co-transgenic lines successfully producing green DH progeny, ranged between 50% and 100% (diagram 3-6). However, the proportion of independent lines producing green DHs per 100 isolated immature embryos must be taken into consideration as well. Only one primary co-transgenic plant was obtained in each of three variants (5, 8, and 12), all of which produced green doubled haploids (100% green DH production rate), while 270 embryos have to be inoculated to obtain only one of such co-transgenic line, and thus 0.4% of the embryos produced green DHs.

T-DNA segregation events were observed in more than half of the primary co-transgenic T_0 plants. However, only every second of these segregating lines produced *gus* positive plants lacking the selectable marker, because frequently the two types of T-DNA were integrated in linkage groups with at least one extra copy of the selectable marker unlinked from all others. In such case, the segregants carrying exclusively the hygromycin resistance gene are obtained, but no *gus* positive, SM-free plants were found. All but one of the GOI segregating T_0 regenerants generated homozygous DH populations of embryogenic pollen culture origin. In only a single case, a selectable marker-free *gus*-postitve segregant was exclusively obtained through sexual reproduction.

Great variability is shown among the different variants of the applied methods for the number of SM-free transgenic lines per 100 embryos inoculated (diagram 3-8). Only 6 out of the 14 variants gave rise to hygromycin sensitive *gus* positive doubled haploids, the two binary plasmids in two clones of the same *Agrobacterium* strain method (mixture method) and the Twin variants completely failed to produce such lines. The most likely reason for the lack of such plants in these variants is the comapratively high number of integrated selectable marker T-DNAs, which increases the probability that one or several of them are linked to the gene-of-interest.

The results of the two plasmids in different strains method were diverse, up to 0.7 selectable marker-free transgenic lines per 100 isolated immature barley embryos (variant 4 and variant 6).

The single method that yielded outstanding numbers of SM-free transgenic DH lines was the two plasmids in one *Agrobacterium* clone (single strain methods), irrespective of whether AGL-1 or LBA4404pSB1 was used. The number of selectable marker-free transgenic doubled haploids obtained per 100 inoculated embryos showed statistically significant difference only by the latter variant, when compared to the parallel control variant.

Selectable marker free transgenic DH production rates, the proportion of co-transgenic lines giving rise to hygromycin sensitive and *gus* positive plants, were 100% in variants 4 and 8. However, a total of only one co-transgenic line had been obtained by each of theses variants (diagram 3-9).

Variant LBA4404pSB1 (p*hpt:gfp*+p*gus*) is more favourable from the aspect of selectable marker free transgenic DH production rate, in comparison to AGL-1 (p*hpt:gfp*+p*gus*), because the former variant had 66.7% of the primary co-transgenic plants (4 out of 6) producing DH progeny lacking the selectable marker, but tested positive for the model GOI, while in the latter this ratio was only 37.5% (3 out of 8).

The advantage of the here presented *Agrobacterium*-mediated gene transfer system is that it provides a comparatively efficient method to generate selectable marker-free plants which are instantly homozygous for the gene-of-interest. They are produced as early as in the T_1 generation, which is a faster way when compared to the conventional selection of homozygous sexual plants in the T_2 generation, and the whole generation process can be conducted within eleven months. This can be demonstrated through the comparison of the most effective method (two plasmids in one *Agrobacterium*, variants 9 and 10), from the aspect of producing *hpt-free, gus* positive progeny using haploid technology, and the theoretical outcome of the same setup applying conventional selection of homozygous plants from sexual populations (diagram 4-1 and 4-2).

Altogether 39 independent primary transgenic lines were identified using the two plasmids in one *Agrobacterium* clone method, out of which fourteen were tested positive for both transgenes. Eventually, seven primary co-transgenic lines generated doubled haploid progeny lacking the selectable marker, but containing the gene-of-interest. The major reason for the high proportion of SM-free transgenic plants appears to be the predominantly uncoupled integration of the two different types of T-DNA. Even if the T-DNA copy number was high,

the same T-DNA type was mostly integrated in linkage groups and behaved as one single locus often segregating independently from the other type of T-DNA.

The advantage of the doubled haploid technique is that after identification of the selectable marker-free transgenic regenerants, there is no need to further test the plants with regard to their homozygosity, because the regenerants (through spontaneous or induced genome doubling) are for sure instantly homozygous for the transgene. This means a significant reduction in the required greenhouse area in comparison to the conventional method using sufficiently high numbers of sexually produced T_2 lines to select SM-free true-breeding T_1 plants.

Furthermore, the doubled haploid technique provides ample green regenerants. Even if six copies of the selectable marker integrated in different barley chromosomes and an additional unlinked single gene-of-interest are assumed, there is a statistical chance to obtain one homozygous, antibiotic sensitive plant tested positive for the GOI among the regenerants.

In comparison, in sexual T_1 populations with Mendelian segregation of unlinked T-DNAs, most of the plants tested positive for the GOI and lacking the selectable marker are hemizygous for the transgene, which requires a follow-up selection of homozygous lines that are ultimately needed for breeding purposes. To select the homozygous ones among the others the T_2 generations must be tested for the presence or absence of segregation event in each line. Not only remarkably more greenhouse space is needed in this case, but an enormous number of individuals need to be analysed to obtain the comparatively rare events of selectable marker free lines which are homozygous for the gene-of-interest.

It must be emphasized as well, that the established method must be robust and reproducible, with high proportion of unlinked T-DNA integration. However, agrobacteria rather prefer the integration of transgenes in linkage groups. An alternative to span this problem would be the introduction of only the gene-of-interest without use of any selectable marker, as reported by Holme et al. (2006). They obtained 0.8 stable transgenic barley cv. “Golden Promise” plants per 100 isolated ovules inoculated with agrobacteria without selective conditions.

Another aspect showing the relevance of this system, is that the European Deliberate Release Directive (2001/18/EC) requires the phasing out antibiotic resistance markers in GMOs, thus only transgenic lines free of the selectable marker are allowed to be used in field-test trials. Haploid technology not only provides homozygous transgenic lines, but is able to enhance the process of generating selectable marker-free progeny in the same time.

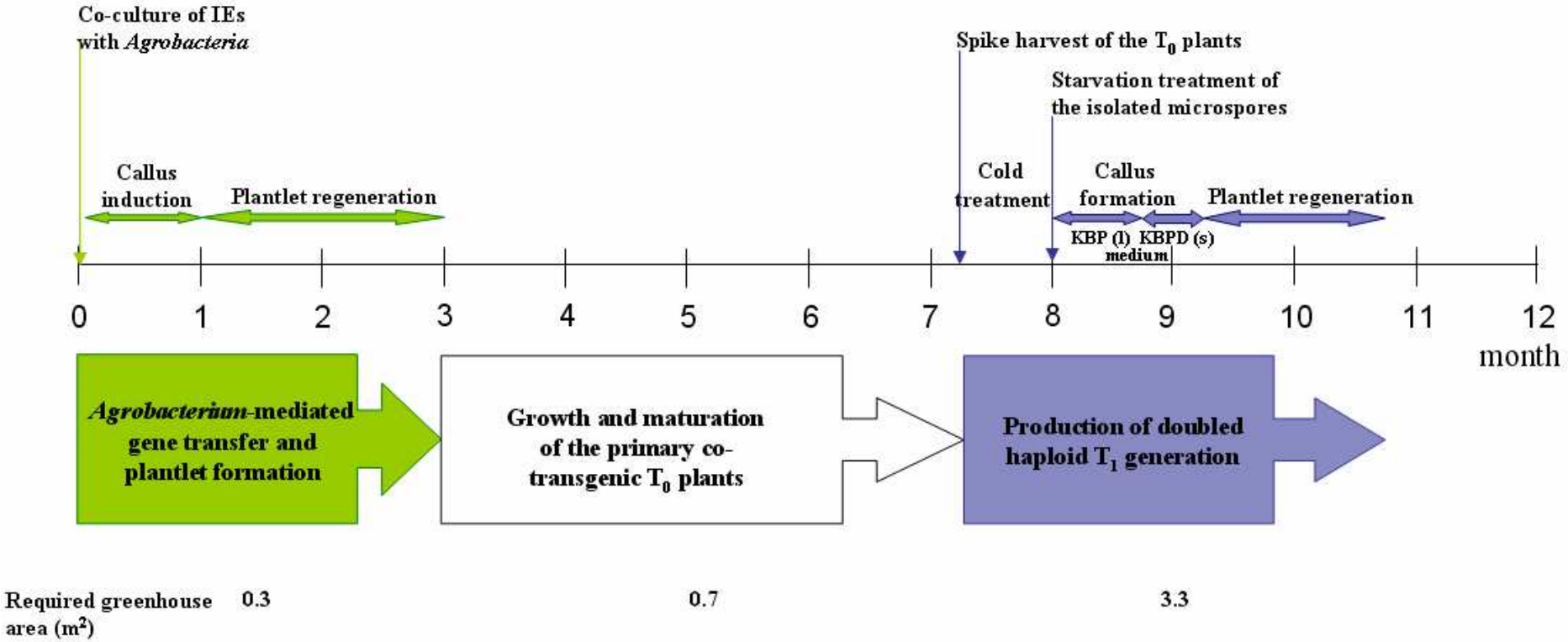
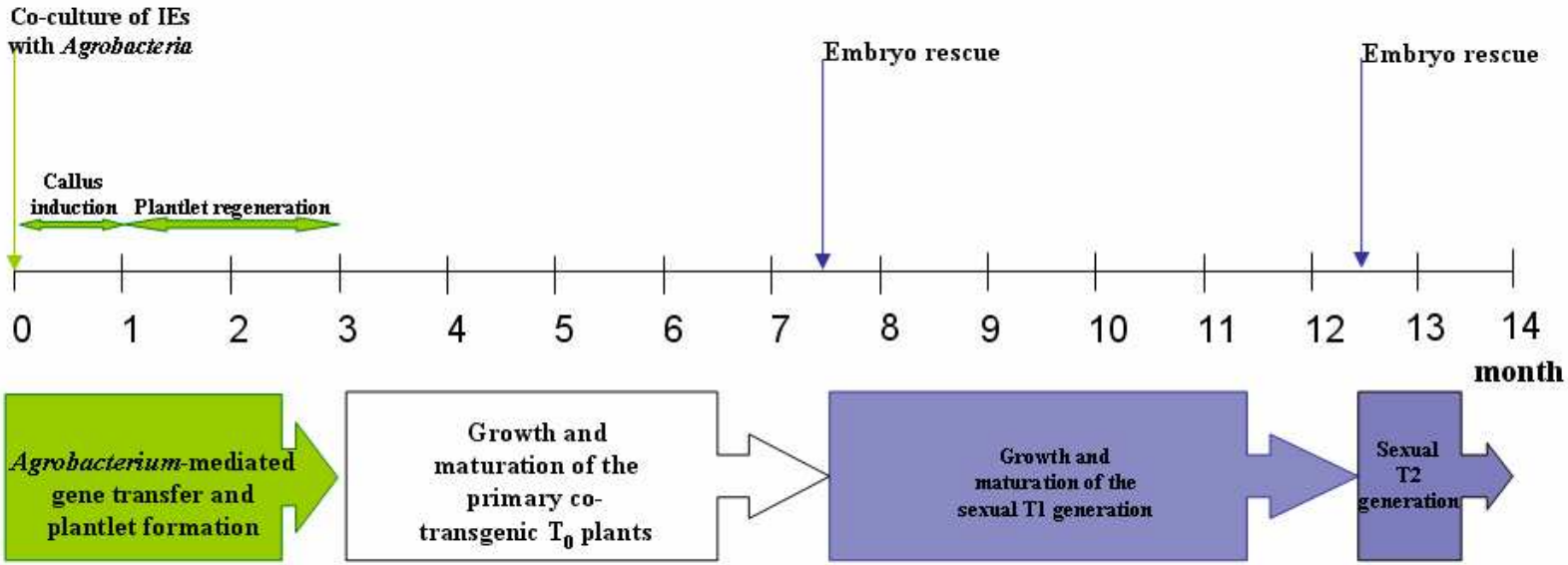


Diagram 4-1.
Timeline and effectiveness of the production of SM-free homozygous transgenics by application of the two plasmids in one *Agrobacterium* clone method along with the implementation of haploid technology.



Required greenhouse area (m^2)	**0.3**	**0.7**	**2.5**	**10.3**

No. of plants to test	**1348**
Homozygous transgenic SM-free T1 lines	**ca.13**

Diagram 4-2.
Timeline and effectiveness of the production of SM-free sexual T1 homozygous transgenics by application of the two plasmids in one *Agrobacterium* clone method along with segregation analysis using T2 populations.

4.2. Integration of recombinant DNA in the barley genome

The reason why *Agrobacterium*-mediated gene transfer was chosen for the introduction of T-DNAs in the plant genome, was that the method results in transgenic plants of higher quality with regard to copy number and integrity of the transgene sequence as compared to direct DNA-transfer (Stahl et al. 2002; Travella et al. 2005; Kumlehn et al. 2006; Lange et al. 2006; Hensel et al. 2008).

According to literature when conducting *Agrobacterium*-mediated embryo transformation the typical number of introduced T-DNA falls between 1 and 3 in barley (Travella et al. 2005; Lange et al. 2006; Hensel et al. 2008). Only small proportion (9-15%) of the transgenic lines are reported to contain 4 or more insertions (Lange et al. 2006; Hensel et al. 2008), although in 50% the T-DNA copies were integrated at the same locus (Stahl et al. 2002).

Considering all 14 variants the gene-of-interest was present in low (1-3) copy numbers in the vast majority (ca. 75%) of the primary co-transgenic plants (diagram 3-4), whereas for the selectable marker rather high copy numbers were typical.

The T-DNA copy number itself does not give any information about the number of integration loci, e.g. a high number of transgene copies is not necessarily associated with many integration loci. More conclusions can be drawn by following the pattern of transgene segregation in the DH and sexual T_1 populations. This can indicate if there are loci where more than one transgene copy was integrated in the genomic DNA of the plant cell by the gene transfer apparatus of agrobacteria. In fact, multiple T-DNAs were often integrated linked to each other in the plant genome and behaved like a single locus (Hensel et al. 2008), such linkage groups were frequently found among the variants. The highest number of independently behaving integration sites was found in variant 13, LBA4404pSB1 pTwin T, where eight transgenic fragments were integrated at five independent loci (table 3-4). The chance for the segregation of the GOI might be very low if there were many unlinked SMs integrated at separate loci. However, it is unlikely that seven or more unlinked copies of the resistance gene and only one single independent GOI T-DNA are integrated in the genome, in which case the chance to identify an embryogenic pollen culture derived, doubled haploid SM-free transgenic line would be very little.

The phenomenon that segregation of both of the selectable marker and the gene-of-interest occurred only in four lines (that is 7.3% of the identified primary co-transgenic barley lines, table 3-4) among all variants, indicates that linked integration of transgenes is to be regarded as the typical phenomenon rather than uncoupled integration.

4.3. Further characteristics of the immature barley genetic transformation and DH production

The number of transgenic regenerants varied strongly between the three repeats of the experiments. The type of construct integrated in the plant genome might highly influence the transformation efficiency. Fang et al. (2002) reported that strong GFP expression reduced regeneration. Murray et al. (2004) compared *Agrobacterium*-mediated transformation of four barley genotypes and found that cells expressing GFP produced fewer regenerants than those expressing *gus*.

De Block and Debrouwer (1991) suggested that the type of *Agrobacterium* strain used in the co-transformation experiment might influence the arrangement of T-DNA insertions in the plant genome, namely nopaline-derived strains like AGL-1 favour linked co-insertions, while octopine-derived strains, such as LBA4404 tend to make unlinked co-insertions. Matthews et al. (2001) failed to conduct genetic transformation of immature barley embryos using a Twin construct in LBA4404. The results presented in this thesis suggest, that T-DNA integration pattern seems to depend both on the applied construct and the *Agrobacterium* strain. When using strain mixtures and the two plasmids in one *Agrobacterium* method, segregation of the gene-of-interest was frequent. Twin variants were prone to integration of high numbers of the selectable marker, as no event of GOI segregation was observed. The two T-DNAs, integrated in the Twin binary vector to be used for the experiments presented in this work, might be too close to each other, separated only by a short 500 bp sequence. As already mentioned by Matthews et al. (2001), a comparatively lower segregation frequency might be due to the proximity of the two T-DNAs on the transformation plasmid, so the selectable marker gene might be often integrated in a linked manner to the model gene-of-interest in the Twin variants.

Plants regenerating from the same callus are not by all means clones. Sallaud (2003) already reported that lines may arise from independent transformation events in a single co-cultued callus in rice. The results of the present study on gene transfer to immature embryos of barley show that copy numbers and fragment sizes detected by Southern blot might differ in „sister" plants, which indicates that the lines were independent. About one third of the analysed calli (6 out of 20) produced multiple independent lines.

Occurrence of somaclonal variation due to transformation and tissue culture processes was reported by Choi et al. (2001), reviewed by Lemaux et al. (1999). Barley genetic transformation conducted with particle-bombardment frequently produces tetraploid

regenerants, which look abnormal and were partially sterile (Choi et al. 2002, Choi et al. 2003; Manoharan and Dahleen 2002).

At least 10% of the primary co-transgenic regenerants obtained in the present study proved tetraploid. Among them, segregating individuals were identified, which generated GOI positive hygromycin sensitive progeny. Microspore isolation from the spikes of such tetraploid barley plants was possible and successful cultures producing ample green regenerants were obtained. Embryogenic pollen culture derived progeny from the tetraploids were diploid, but not necesseraly homozygous. Tetraploid (doubled dihaploids) plants were obtained as well, when spontaneous genome doubling happened. These T_1 regenerants could be tested in a super-transformation approach in order to analyse their amenability for *Agrobacterium*-mediated gene transfer.

In case of one plant stemming from the two plamids/two strains method, 70% LBA4404pSB1p*hpt::gfp* + 30% AGL-1 p*gus* (variant 8), an unstable transformation event occurred. Five copies of the selectable marker were detected in the primary co-transgenic plants, whereas none of them were found in the DH progeny. The integrated T-DNAs were probably integrated in a linkage group in one single locus. Possible interpretations for this phenomenon are: that the T_0 plant was chimeric with regard to transgenicity (Marcotrigiano 1986), or the integration site was a telomeric region of one of the chromosomes, where the loss of genetic information occures with a higher probability (Choi et al. 2002).

4.4. Identification of factors influencing the DH production efficiency

There were various conditions which have huge influence on the quality of callus growth, regeneration, the obtained number of transgenic plants and hereby the transformation frequency. Obviously, transgenic regenerants show phenotypic variability. Environmental conditions, quality of the donor plants and seasonal variability, transferred T-DNA sequence were factors which have a major effect on the outcome of plant genetic transformation. (Hensel et al. 2008).

In the present study, the highest number of green regenerants was obtained in spring time, i.e. 1.6 doubled haploid lines were obtained per spike. However, it must be mentioned that the proportion of albinos, which are useless for any application, display a considerable sum (43.1%). Throughout a whole year, between 9.3-17.6 doubled haploid T_1 plants are to be expected on average per 10.7 harvested spikes. The proportion of albino regenerants in the embryogenic pollen cultures was the lowest in summer (23.8%), highest in fall (49.1%). Their number might be lowered by optimisation of the culture conditions (Coronado et al. 2005). Genotype has a major impact on albino formation e.g embryogenic pollen cultures of the barley cultivar "Igri" produce predominantly green regenerants.

On the other hand, quality of the donor material is the most influential on the outcome of the genetic transformation experiment and the number of regenerants of the embryogenic pollen cultures, e.g. in case of one of the the most successful variants, LBA4404pSB1(p*hpt::gfp* +p*gus*), all the primary co-transgenic plants producing GOI positive antibiotic sensitive progeny are obtained from one single experiment. Matthews et al. (2001) reported about similar observations, in their case eight of their selectable marker free transgenic lines resulted from two experiments and already suggested that various factors, e.g. bacterial concentration and tissue culture conditions, might influence transformation and co-transformation efficiency.

5. SUMMARY

Barley represents one of the economically most important and widely distributed crops worldwide, and genetic engineering is expected to play a crucial role in its further improvement. However, once transgenic plants are obtained, the selectable marker gene is not necessary anymore or even unwanted, and thus viable means to circumvent the use of selectable markers or to remove them from transgenic plants are required.

In the present study, a novel strategy has been pursued to generate selectable marker-free transgenic lines instantly homozygous for the transgene. Primary co-transgenic plants (T_0) containing both the selectable marker gene (*hygromycinphosphotransferase*) and a model "gene-of-interest" (*ß-glucuronidase*) were produced via *Agrobacterium*-mediated gene transfer using two independent T-DNAs. Both removal of the selectable marker and establishment of instant homozygosity of the gene-of-interest was then achieved at a time through segregation of uncoupled T-DNAs in populations of doubled haploid (DH) lines produced from the (T_0) plants via embryogenic pollen culture.

Two *Agrobacterium* strains (LBA4404pSB1, AGL-1) and six binary vectors were used in co-transformation methods employing either *Agrobacterium* mixtures (two binary plasmids in two clones of the same *Agrobacterium* strain, or two plasmids in two different *Agrobacterium* strains) or single *Agrobacterium* clones (two plasmids in one *Agrobacterium* clone, or a Twin-vector harboring two T-DNAs in one *Agrobacterium* clone). A total of 14 different variants were applied for the inoculation of immature embryos which were used as gene transfer target. The comparison of these variants revealed considerable differences in terms of various aspects of transgenicity.

The highest number (9.6) of independent primary transgenic (T_0) plants per 100 inoculated embryos was obtained by a 1:1 mixture of AGL-1 p*hpt:gfp* and LBA4404pSB1p*gus*, whereas co-transformation was most efficient (3.0) when the "two plasmids in one A. *tumefaciens* AGL-1 clone" variant was used.

The integration of the gene-of-interest in the barley genome occurred physically unlinked to the selectable marker gene in 25% of the independent co-transgenic lines. The co-transformation method based on two binary vectors in one *Agrobacterium* clone proved to be the most efficient as to the generation of selectable marker-free, homozygous transgenic lines,

irrespective of whether *Agrobacterium* strain AGL-1 or LBA4404pSB1 was utilized. Using LBA4404pSB1 along with this method, per 100 inoculated embryos ca. 1.5 co-transgenic T_0 lines were generated that eventually gave rise to independent selectable marker-free, homozygous transgenic doubled haploids. In two thirds of the co-transgenic lines obtained by this variant the gene-of-interest segregated uncoupled from the selectable marker in the doubled haploid T_1 generation. By contrast, no marker-free transgenic line could be generated by the four variants using Twin vectors.

Across the co-transformation variants, neither the overall transformation efficiency was associated with co-transformation efficiency nor was co-transformation efficiency associated with the efficiency of uncoupled T-DNA integration.

Two percent of the inoculated embryos gave rise to more than one regenerant, with as many as 15 co-transgenic plants being obtained per embryo. A detailed analysis of co-transgenic sister plant families derived from the same callus revealed that sister plants mostly constitute clones, and only every 9th be different from other co-transgenics of the same family in terms of transgene integration sites. In total, two additional independent co-transgenic lines giving rise to true-breeding, selectable marker-free lines were identified among the accessory sister plants obtained in this study. However, due to the tremendous additional effort ultimately required for the analysis of accessory co-transgenic sister plants, it is recommended to consider not more than one co-transgenic regenerant per embryo in future applications.

With regard to effort, cultivated area and time, the implementation of haploid technology renders co-transformation approaches considerably more efficient in producing true-breeding, selectable marker-free transgenic barley.

6. ZUSAMMENFASSUNG

Gerste ist eine der ökonomisch bedeutendsten und am weitesten verbreiteten Kulturpflanzen weltweit, und es wird erwartet, dass gentechnische Ansätze bei der weiteren züchterischen Bearbeitung dieser Art eine entscheidende Rolle spielen werden. Sobald jedoch eine transgene Pflanze generiert worden ist, wird der dazu verwendete Selektionsmarker in der Regel nicht mehr benötigt oder ist für die weitere Bearbeitung oder Nutzung des Materials sogar hinderlich. Daher ist die Entwicklung von praktikablen Methoden zur Herstellung selektionsmarkerfreier transgener Pflanzen erforderlich.

In der vorliegenden Studie wurde eine neuartige Strategie verfolgt um selektionsmarkerfreie, unmittelbar homozygot transgene Pflanzen herzustellen. Dazu wurden zunächst per Agrobakterien-vermitteltem Gentransfer primäre co-transgene Pflanzen (T_0) erzeugt, die nach Verwendung von zwei unabhängigen T-DNAs ein Selektionsmarkergen (*hygromycinphosphotransferase*) und ein Modell-Effektorgen (*ß-glucuronidase*) in ihrem Genom integriert haben. Die Eliminierung des Selektionsmarkergens und die damit einhergehende Herstellung homozygot transgener Linien wurde daraufhin durch Segregation ungekoppelt integrierter T-DNAs in Populationen doppelhaploider (DH) Linien erreicht, die mittels embryogener Pollenkultur aus den T_0-Pflanzen generiert wurden.

Es wurden zwei Agrobakterienstämme (LBA4404pSB1 bzw. AGL-1) und sechs Binärvektoren für unterschiedliche Methoden der Co-Transformation verwendet, die entweder auf der Mischung unterschiedlicher Agrobakterien (zwei Binärvektoren in zwei Klonen des gleichen Agrobakterienstammes bzw. zwei Binärvektoren in zwei verschiedenen Agrobakterienstämmen), oder auf der Verwendung einzelner Agrobakterienklone (zwei Binärvektoren zusammen in einem Agrobakterienklon bzw. ein 'Twin'-Vektor mit zwei T-DNAs in einem Agrobakterienklon) beruhen. Insgesamt ergaben sich dabei 14 verschiedene Varianten der Übertragung von jeweils zwei unterschiedlichen T-DNAs in unreife Gerstenembryonen, die als Zielgewebe des Agrobakterien-vermittelten Gentransfers verwendet wurden. Der Vergleich dieser Varianten ergab beträchtliche Unterschiede bezüglich diverser Aspekte der Transgenität der generierten Pflanzen.

Die höchste Anzahl (9.6) unabhängiger primär transgener (T_0) Pflanzen pro 100 inokulierter Embryonen wurde nach Verwendung einer 1:1 Mischung von AGL-1p*hpt:gfp* und LBA4404pSB1p*gus* erzielt, wohingegen die Co-Transformation am effizientesten war (3.0),

wenn zwei Binärvektoren zusammen in einem Klon des Agrobakterienstammes AGL-1 verwendet wurden.

Die Integration des Effektorgens erwies sich bei 25% aller unabhängigen co-transgenen T_0 Pflanzen bezüglich des Selektionsmarkergens als ungekoppelt. Unabhängig vom verwendeten Agrobakterienstamm (AGL-1 bzw. LBA4404pSB1) war die Herstellung selektionsmarkerfreier, homozygot transgener (DH-) Linien am effizientesten, wenn zwei Binärvektoren zusammen in einem Agrobakterienklon verwendet wurden. Anhand dieser Co-transformationsmethode unter Verwendung von LBA4404pSB1 wurden pro 100 inokulierter Embryonen etwa 1,5 co-transgene T_0 Pflanzen erzeugt, die unabhängige, selektionsmarkerfreie homozygot transgene (DH-) Linien hervorbrachten. Bei zwei Dritteln der mit dieser Variante generierten unabhängigen co-transgenen Pflanzen segregierte das Effektorgen in den resultierenden DH-Populationen ungekoppelt bezüglich des Selektionsmarkergens. Im Gegensatz dazu konnte nach Anwendung aller vier auf Twin-Vektoren beruhenden Varianten der Co-Transformation keine einzige selektionsmarkerfreie transgene Linie gewonnen werden.

Über die verwendeten Co-Transformationsvarianten waren sowohl die Gesamteffizienz der Transformation und die Co-Transformationseffizienz als auch die Co-Transformationseffizienz und die Effizienz der Integration ungekoppelter T-DNAs nicht miteinander korreliert.

Etwa zwei Prozent der inokulierten Embryonen ergaben mehr als ein Regenerat, wobei pro Embryo bis zu 15 co-transgene Pflanzen erhalten wurden. Die detaillierte Analyse der aus jeweils einem Embryo resultierenden Regenerat-Familien ergab, dass co-transgene Schwesterpflanzen bezüglich der Transgenität in den meisten Fällen identisch sind und sich nur etwa jede 9. Pflanze von co-transgenen Individuen der gleichen Familie bezüglich der Integrationsorte unterschied. Unter den zusätzlichen Schwesterpflanzen wurden in dieser Studie insgesamt zwei co-transgene Linien identifiziert, die unabhängige selektionsmarkerfreie (DH-) Linien hervorbrachten. Aufgrund des immensen Aufwandes, der für die Analyse zusätzlicher co-transgener Schwesterlinien erforderlich ist, wird für zukünftige Anwendungen der hier entwickelten Methode empfohlen pro Embryo nicht mehr als eine co-transgene Pflanze zu verwenden.

Bei der Herstellung selektionsmarkerfreier, homozygot transgener Gerste mittels Co-Transformation erwies sich die Implementation von Haploidentechnologie als geeignete Maßnahme den erforderlichen Umfang an Arbeit, Anbaufläche und Zeit erheblich zu reduzieren.

7. ACKNOWLEDGMENTS

This work was done in the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben.

I am grateful to my supervisor, Jochen Kumlehn, and to my mentor, Helmut Bäumlein, for the support during the time of my PhD studies.

I acknowledge the following people, who helped in different ways: Götz Hensel and David Köszegi for general advice about molecular work, Armin Meister for helping in statistical evaluation of the data, and Isolde Saalbach for valuable discussions.

I am especially thankful to Cornelia Marthe, Ingrid Otto and Sandra Wolf for excellent technical assistance.

I acknowledge to Jochen Kumlehn, Götz Hensel and David Köszegi for critical reading of the manuscript and for useful comments.

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9. APPENDIX

Variant No.	No. of inoculated embryos	No. of transgenic regenerants (SM+)	Transgenic independent lines (SM+)	Escapes	No. of co-transgenic regenerants (SM+,GOI+)	Co-transgenic independent lines (SM+,GOI+)	Independent co-transgenic line(s) producing green DH progeny	Independent co-transgenic line(s) producing GOI positive marker free DH progeny	Sexual line(s) producing GOI positive marker free DH progeny
1	270	44	11	1	2	2	1	0	0
2	270	28	16	1	8	5	3	0	1
control 1,2	270	9	5	0	7	3	3	1	0
3	270	50	21	0	2	2	1	0	0
4	270	54	26	0	6	2	2	2	0
5	270	8	4	0	4	1	1	0	0
control 3,4 ,5	270	24	15	2	5	4	4	1	0
6	270	18	9	0	5	3	3	2	0
8	270	24	13	0	1	1	1	1	0
control 6,8	270	28	13	3	5	4	4	0	0
9	270	46	24	3	16	8	6	3	0
10	270	54	15	0	9	6	4	4	0
control 9,10	270	24	5	0	9	2	2	0	0
11	270	57	15	2	24	6	4	0	0
12	270	21	6	0	2	1	1	0	0
control 11 ,12	270	6	2	0	0	0	0	0	0
13	270	44	10	0	6	2	1	0	0
14	270	27	6	0	16	2	1	0	0
control 13,14	270	40	12	0	2	1	1	0	0
control sum	1620	131	52	5	28	14	14	2	0

ERKLÄRUNGEN
GEMÄß § 5 ABS. 2 DER PROMOTIONSORDNUNG VOM 17.09.1998

Ich erkläre hiermit, daß ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe. Ich habe die Arbeit selbständig und ohne fremde Hilfe verfaßt, und nur die von mir angegebenen Quellen und Hilfsmittel benutzt. Wörtlich oder inhaltlich entnommene aus anderen Werken habe ich Stellen als solche kenntlich gemacht.

Halle, 22. October, 2009

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