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Development of a genetic transformation protocol for rye (*Secale cereale* L.) and characterisation of transgene expression after biolistic or *Agrobacterium*-mediated gene transfer

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Abbreviations

35S= *Cauliflower mosaic virus* (CaMV) 35S promoter

BAP= 6-benzylaminopurine

bar= phosphinothricin acetyltransferase gene

2,4-D= 2,4-dichlorophenoxyacetic acid

dicamba= 3,6-dichloro-2-methoxybenzoic acid

DNA= deoxyribonucleic acid

ELISA= enzyme-linked immunosorbent assay

ferr= ferritin gene

gfp= green fluorescent protein gene

GLM= general linear model

GUS= β-glucuronidase

HMW-gs= High Molecular Weight-glutenin subunits

LMW-gs= Low Molecular Weight-glutenin subunits

L= line

*lac*Z= β-galactosidase gene

LSD= least significant difference

M_r= molecular mass

MS= Murashige and Skoog (1962) medium

nos= nopaline synthase promoter

NPT II= neomycin phosphotransferase enzyme

*npt*II= neomycin phosphotransferase gene

P<= probability of alpha < as

PAT= phosphinothricin acetyltransferase

PAGE= polyacrylamide gel electrophoresis

PCR= polymerase chain reaction

PEG= polyethyleneglycol

picloram= 4-amino-3,5,6-trichloropicolinic acid

pmi= phospho mannose isomerase gene

PMSF= phenolmethylsulphonyl fluoride

PPT= phosphinothricin

Pr>*F* = critical value for analysis of significance

RNA= ribonucleic acid

rcf= relative centrifugal force

SAS= statistic analysis system

SD = standard deviation

SDS= sodium dodecyl sulphate

γ-*tmt*= gamma-tocopherol methyltransferase gene

*uid*A= β-glucuronidase gene

vs= versus

Index

Index of Tables

Index of Figures

0 Summary

Rye (*Secale cereale* L.) is known as one of the most recalcitrant species in tissue culture and genetic transformation. So far two transgenic rye plants have been reported after biolistic gene transfer (Castillo *et al.*, 1994), both expressing the selectable marker gene *bar*. After this pioneering work no other reports in rye transformation were published and no useful gene has as yet been expressed in transgenic rye. The main hurdle for the establishment of a reproducible transformation protocol in rye is the non-availability of an efficient *in-vitro* culture system, a basic requirement for genetic transformation. The aim of this project was the development of an efficient and reproducible system for the production of heterologous transgenic rye plants.

For this, the main factors determining the tissue culture response in rye were analysed and optimised. Inbred lines with superior regeneration potential from tissue cultures were identified out of a wide collection of rye genotypes. Optimal culture media composition, media preparation procedure and *in-vitro* growth conditions for individual, genetically divergent rye inbred lines, were identified in a multifactorial experiment which allowed the estimation of interactions between genotypes and different tissue culture components.

On the basis of the established tissue culture system different selection strategies were evaluated for their selection effect in rye callus cultures, including the use of phosphinothricin, paromomycin sulphate and hygromycin B as selection agents. Tissue culture and selection parameters with respect to regeneration response, reasonable agent concentrations and suitable developmental stages for their application were identified. Based on PCR analysis of *in-vitro* regenerated plants a selection system for the “direct” production of transgenic plants, free of selectable marker gene, was developed.

Based on an efficient tissue culture protocol, biolistic transformation parameters such as tissue age during bombardment and micro-projectile density, were compared in a multi-factorial experiment design. A suitable combination of interactive factors allowed biolistic gene transfer and recovery of transgenic events without compromising on the regeneration potential of this recalcitrant cereal. Different selection strategies using the selectable marker genes *bar* or *npt*II resulted in similar transformation efficiencies in the range of 2 to 4% of the

bombarded explants. A total of 37 independent transgenic rye plants were produced by biolistic gene delivery.

For *Agrobacterium tumefaciens*-mediated gene delivery, different influencing factors specific for the plant-tissue as well as for the *Agrobacterium*, were combined. The co-cultivation of immature rye embryos with *A. tumefaciens* led to morphologically normal and fertile transgenic plants at a frequency of 3.87% of the inoculated explants. The evidence for *Agrobacterium*-mediated T-DNA transfer was based on molecular and genetic studies of transgenic rye plants and the analysis of T-DNA::rye DNA boundary sequences.

The high-molecular-weight glutenin subunit genes *Ax1*, *Dx5* and *Dy10* from wheat (*Triticum aestivum* L.), known to be associated with good bread-making quality, were introduced into rye by biolistic gene transfer. The stable expression of the Ax1, Dx5 and Dy10 protein subunits was demonstrated in endosperms of primary transformants of rye and in their segregating progeny. This is the first report of the expression of a useful gene in rye.

The molecular analysis of the transgenic plants confirmed the integration and expression of the transferred selectable marker genes and its transmission and expression in the sexual progeny of transgenic rye. Stability and levels of transgene expression were analysed with regard to the transgene copy number.

0 Zusammenfassung

Roggen (*Secale cereale* L.) ist als eine der widerspenstigsten Pflanzenarten bezüglich Gewebekulturfähigkeit und Transformierbarkeit bekannt. Bisher wurde nur von zwei transgenen Roggenpflanzen berichtet (Castillo *et al.*, 1994), die nach biolistischem Gentransfer das selektierbare Markergen *bar* exprimierten. Nach dieser Pionierarbeit gab es keine weiteren Berichte über erfolgreiche Roggentransformation und noch nie wurde ein Nutzgen in transgenem Roggen exprimiert. Die grösste Hürde bei der Etablierung eines reproduzierbaren Transformationsprotokolls in Roggen ist das fehlende *in-vitro* Kultursystem, eine Grundvoraussetzung für genetische Transformation. Es war daher das Ziel dieser Arbeit, ein effizientes und reproduzierbares System zur Herstellung von heterologen transgenen Roggenpflanzen zu entwickeln.

Hierfür wurden die wesentlichen Faktoren, welche die Gewebekultur beeinflussen, analysiert und optimiert. Aus einem weitem Spektrum verschiedener Roggengenotypen konnten Selbstungslinien mit herausragendem Regenerationspotential identifiziert werden. Durch eine mehrfaktorielle Optimierung konnten die Kulturmedienzusammensetzung, -zubereitung und die *in-vitro* Kulturbedingungen auf individuelle, genetisch divergente Selbstungslinien abgestimmt und Interaktionen zwischen Genotypen und verschiedenen Gewebekulturparametern quantifiziert werden.

Auf der Basis dieses *in-vitro* Kultursystems wurden unterschiedliche Selektionsstrategien im Hinblick auf den Selektionseffekt von Phosphinothricin, Paromomycin-Sulfat und Hygromycin B in Kalluskulturen untersucht. Gewebekultur- und Selektionsparameter wurden bezüglich Regenerationsfähigkeit, Konzentration und Anwendungszeitpunkt der Selektionsagenzien optimiert. Ein Selektionssystem zur „direkten“ markergen-freien Herstellung von transgenen Pflanzen, basierend auf PCR-Analye von *in-vitro* regenerierten Pflanzen, wurde erstellt.

Auf der Grundlage eines effizienten Gewebekulturprotokolls wurden biolistische Parameter, wie Gewebevorkultur und Mikropartikeldichte, in einem multifaktoriellem Experiment untersucht. Durch die Kombination geeigneter interaktiver Faktoren war der biolistische Gentransfer und die Identifizierung transgener Ereignisse möglich, ohne Beeinträchtigung der Regenerationsfähigkeit der Roggenkulturen. Unterschiedliche Selektionsstrategien unter

Verwendung der selektierbaren Markergene *bar* oder *npt*II führten zu Transformationseffizienzen zwischen zwei und vier Prozent der beschossenen Explantate. Insgesamt konnten 37 unabhängige transgene Roggenpflanzen durch biolistischen Gentransfer hergestellt werden.

Für *Agrobacterium tumefaciens*-vermittelten Gentransfer wurden verschiedene beeinflussende Faktoren, die spezifisch für das Pflanzengewebe oder für die *Agrobacterium*-Kultur sind, kombiniert. Durch Co-Kultur von unreifen Roggenembryonen mit *A. tumefaciens* konnten morphologisch normale und fertile, transgene Roggenpflanzen aus 3.87% der inokulierten Explantate gewonnen werden. Molekulare und genetische Untersuchungen der transgenen Pflanzen sowie die Analyse der T-DNA-flankierenden Roggen-DNA-Sequenzen bestätigen den *Agrobacterium*-vermittelten T-DNA Transfer.

Die Gene der hochmolekulargewichtigen Gluteninuntereinheiten *Ax1*, *Dx5* und *Dy10* von Backweizen (*Triticum aestivum* L.), bekannt durch ihren positiven Einfluss auf die Backqualität, wurden durch biolistischen Gentransfer in Roggen übertragen. Die stabile Expression der Proteinuntereinheiten Ax1, Dx5 and Dy10 konnten im Endosperm transgener Roggenpflanzen und deren Nachkommenschaft gezeigt werden. Dies ist der erste Bericht über die Expression eines transgenen Nutzgenes in Roggen.

Durch eine molekulare Analyse der transgenen Pflanzen konnten Integration und Expression der übertragenen selektierbaren Markergene sowie deren Übertragung und Expression in spaltenden Nachkommenschaften nachgewiesen werden. Stabilität und Expressionsniveau wurden bezüglich der Transgenkopienzahl analysiert.

1. General introduction and aim of study

Rye (*Secale cereale* L.) is one of the most recently domesticated cereals. Its likely origin is the Caucasus region from where it spread out as a weed in wheat, representing a so called “secondary crop” (Miedaner 1997). There are five species within the genus *Secale*: *S. silvestre, S. vavilovii, S. montanum, S africanum* and *S. cereale*, with all cultured forms seeming to be originated from the latter (Jones and Favell, 1982). Rye was also used to develop the most important artificial amphidiploid cereal crop, *Triticale*, a hybrid between *Triticum* and *Secale*. Rye is mainly used as bread cereal and is prized due to the high nutritional value of its proteins, the characteristic taste and its long fresh-keeping properties compared to wheat bread. On the other hand, the technical property of pure rye dough is low, and is usually mixed with gluten rich wheat flours for bread preparation. It is further used for feeding purposes, especially in northern areas, where corn (*Zea mays* L.) cultivation is limited. Rye can also be used as a coffee substitute, for distillation (Franke 1989) and even as raw material for the insulation in constructions (http://www.cerealith.de).

Compared to other cereal crops rye is the most adaptable. Due to its high cold resistance it can be grown at higher latitude and altitude than any other winter cereal. It tolerates well adverse soil conditions like acidity or alkalinity, can be grown on poor and marginal soils and gives reasonable yield even in regions where no other cereals grow. Although rye is cultivated all over the world, it is an important crop only in central Europe, in countries such as Poland, Germany, countries of the Russian Federation or in the Ukraine. Fifty years ago rye was the most cultivated cereal in middle and northern Europe. With a reported decrease of more than 50% during the last 40 years (Hoffman *et al.*, 1985), the cultivated area was estimated to be only 9,759 thousand hectar in 2000 (FAO 2000). The main reason for this drastic decrease is the low breeding advance of rye, being the only cross-pollinated native cereal species, compared to the self-pollinated wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) or oat (*Avena sativa* L.). Classical breeding methods have been successfully employed for rye improvement but the breakthrough came late, with the discovery of the male sterility inducing cytoplasm by Geiger and Schnell (1970). With the releasing of the first hybrid varieties in 1984 (Miedaner 1997) the productivity could be increased and the original importance of rye may be recovered partially.

It is now generally agreed that bio-techniques, including genetic transformation, can complement traditional breeding programmes. Genetic transformation provides a powerful method to introduce novel and useful genes into crops, even from unrelated species and organisms. The use of such technologies requires the development of reliable and efficient methods for DNA delivery into plant cells and the regeneration of normal and fertile transgenic plants from *in-vitro* cultures. During the last decade genetic transformation has been increasingly used for the successful improvement of dicot and monocot crops (Rajesh *et al.*, 1997; Dunwell 2000).

Rye is known as one of the most recalcitrant species for regeneration and genetic transformation. Only one successful transformation experiment of rye has been reported so far (Castillo *et al.*, 1994). The authors obtained six transgenic callus lines and were able to regenerate two independent transgenic rye plants derived from a single experiment. After this pioneering work no other reports in rye transformation were published and no useful gene has as yet been expressed in rye. The main hurdle for the establishment of a reproducible transformation protocol in rye is the non-availability of an efficient *in-vitro* culture system, a basic requirement for genetic transformation.

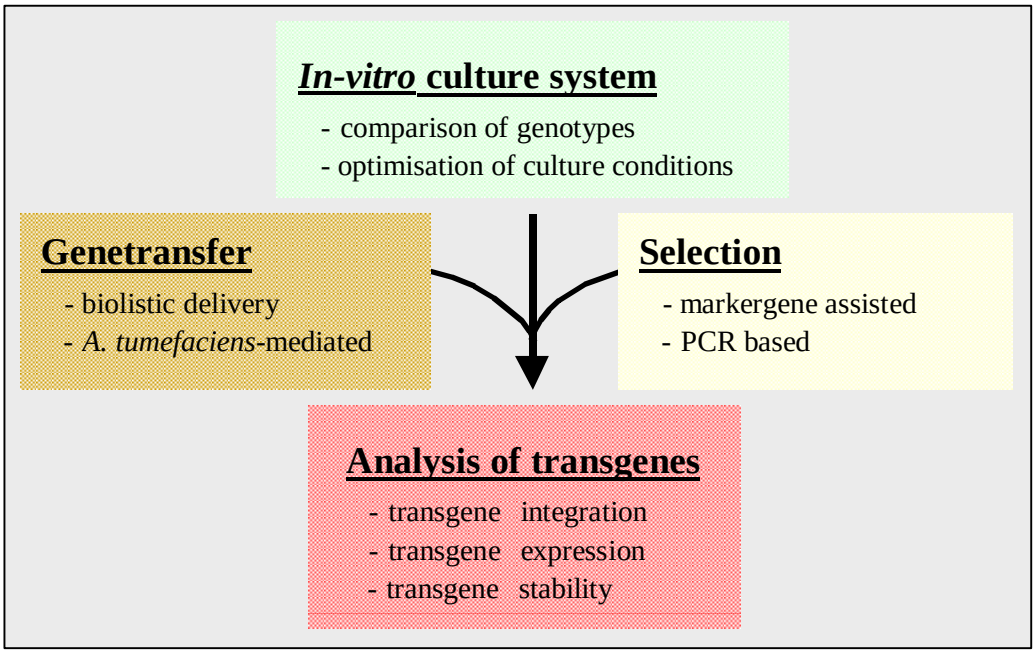


Figure 1: Development of a reproducible transformation system for a recalcitrant species like rye (*Secale cereale* L.).

The aim of this project was to develop an efficient and reproducible system for the production of heterologous transgenic rye plants. The establishment of such a transformation system can be divided in four major areas (Figure 1). The presented project is based on an efficient tissue culture system, established by screening diverse rye genotypes for *in-vitro* culture suitability and optimisation of culture conditions for selected genotypes. On the basis of an efficient *in-vitro* culture system biolistic and *Agrobacterium* mediated delivery strategies were applied, and selection protocols to identify transgenic events in rye were developed. Putative transgenic plants needed a detailed molecular analysis to confirm both integration and expression of the transgenes. Segregation and expression stability as well as functional properties of the transgene are further criteria to characterise transgenic lines.

2. Establishment of an *in-vitro* culture system for rye

2.1. Summary

To establish an efficient *in-vitro* culture system for rye (*Secale cereale* L.) a comparison of genotype specific culture response and an optimisation of culture media and culture conditions were performed. Eleven rye inbred lines, 11 single crosses and the population variety Wrens Abruzzi were compared for their tissue culture response after short and long term callus culture, showing a high variation for regeneration potential. Genotypes with superior tissue culture suitability were identified. A multi-factorial designed experiment allowed the development of genotype-specific *in-vitro* culture protocols, maximising the plant regeneration response from rye tissue culture. Precocious germination of explants, callus induction and callus maintenance (formation of rhizogenic and caulogenic callus) as well as regeneration response (regenerating calli and number of regenerated plants per callus) were significantly influenced by genotype, carbohydrate and auxin sources and their respective concentrations and interactions. Genotypes differed in the callus response to media sterilisation procedure, basic salt composition, gelling agent, $CuSO_4$ complementation and illumination. The selection of inbred lines with superior tissue culture response and the development of genotype-specific culture protocols provide an important basis for the development of a genetic transformation protocol for rye.

2.2. Introduction

Rye is known as one of the most recalcitrant species for tissue culture. The lack of an efficient *in-vitro* culture system limits the development of a reproducible genetic transformation protocol for this crop. The main factors determining the tissue culture response in rye and other recalcitrant crops include genotype, donor plant quality, developmental stage of the explant at the time of culture initiation, culture medium composition and culture conditions. Different strategies to establish *in-vitro* culture systems for rye have been attempted and are summarised in Table 1.

Tissue culture response has been shown to be highly genotype dependent for many cereal crops (Vasil 1987) including rye (Krumbiegel-Schroeren *et al.*, 1984; Rybczynski and Zdunczyk, 1986; Zimny and Lörz, 1989; Linacero and Vazquez, 1990; Rakoczy-Trojanowska and Malepszy, 1993). However, in contrast to most cereals for which highly responsive

genotypes have been identified (rice: Taipei 309, IRRI 54; barley: Golden Promise; wheat: Bobwhite) and reproducible tissue culture systems are available, in rye no such genotypes have as yet been described. In addition, their conservation is difficult in strictly cross-pollinating species like rye and this strongly influences the reproducibility of an *in-vitro* culture system. Genotypes with common genes from *S. vavilovii* have been described to be more suitable for anther cultures than other rye species like *S. cereale* (Friedt *et al.*, 1983; Flehinghaus *et al.*, 1991). However, *S. vavilovii* is of poor agronomic value mainly due to its fragile and brittle spikes (Miedaner 1997).

It is generally agreed that donor plant quality plays a key role in the establishment of regenerable tissue cultures and transformation success. Donor plants should be healthy and vigorously growing with great importance of the temperature during seed setting.

There is no universally applicable method of culture and regeneration for all species, as tissues from different sources will differ in their response to tissue culture. A protocol to produce morphogenic callus from one cultivar may be very different from that for another cultivar, even within the same species. Different tissues have been described for tissue culture initiation in rye (Table 1). Apart from anthers, which are important for production of double haploid lines (Wenzel and Thomas, 1974), immature embryos and immature inflorescences were the two most frequently used explants. Since different genotypes and media compositions were applied in early reports (Table 1) callus induction frequencies and regeneration response of different explants can hardly be compared. Unexpectedly Rakoczy-Trojanoska and Malepszy (1993) describe immature embryos as less suitable as starting material than immature inflorescences (Rakoczy-Trojanoska and Malepszy, 1995). However, the developmental stage of the explants has been found to be a crucial factor in the establishment of totipotent cultures (Vasil 1987). Heritabilities of 0.8-0.93 and 0.36-0.5 were described for regeneration of embryogenic callus derived from immature embryos and immature inflorescences, respectively (Rakoczy-Trojanoska and Malepszy, 1993 and 1995). This supports the importance of genetical aspects for this tissue culture factor, but could also be explanatory for non-genetic factors like the stage of maturation of the explants. Donor plants for immature inflorescences can be harvested at a relatively early developmental stage and might therefore escape unfavourable environmental conditions that apply to those that produce immature embryo explants. But immature inflorescences are difficult to prepare and

Table 1: Summary of strategies to establish an *in-vitro* culture system for the genus *Secale*.

Genotypes	Explants	Analysed media components	Culture conditions	Reference
F_1 hybrids of *S. cereale* and *S. vavilovii*	anthers	NN and MS-medium; 2,4-D; mesoinositol; glutamine; serine;		Thomas *et al.* (1975)
M.A.C.S. 898	I. embryos	MS-medium; LS-vitamins; sucrose; agar; 2,4-D; 2,4,5 T; 2,4,5-Cl_3 POP; PCPA; NOA; NAA; IAA; IBA; Kn; BA; 2,I-P; Z.	950 lux	Eapen and Rao (1982)
1922, Lms, Cms, CG and 1514	I. inflores. I. embryos	MS-medium with thiamine; Monnier medium with glutamine and casein hydrolysate; 2,4-D; NAA; PCPA; BA; Z.		Krumbiegel-Schroeren *et al.* (1984)
Wrens Abruzzi	I. embryos	MS-medium; 2MS; 2,4-D; MCPA; 2,4,5-T; sucrose; coconut milk; casein hydrolysate; αNAA; βNAA; IAA IBA; IPA.	light/dark	Lu *et al.* (1984)
Not specified	I. inflores.	MS-medium; LS-vitamins; sucrose; agar; 2,4-D; BA; coconut water; IAA; NAA	950 lux	Eapen and Rao (1985)
Ailes, Merced, Elbon and D. Enrique	leafs	MS-medium; 2,4-D; NAA; BA	light/dark	Linacero and Vázquez (1986)
Wild species of the *Secale* genus	I. embryos	MS-medium; 2,4-D; Difco-Bacto agar; yeast extract; casein hydrolysate		Rybczynski and Zdunczyk (1986)
$S_1D_4$83; POP SMH 49; S6D67; Karlshulder, Sorom and Rogo	I. embryos	MS-, N6-, CC-, and B5-medium; sucrose; Dicamba; 2,4-D; Picloram; coconut water; media pH-value; Kn; agarose	light/dark	Zimny and Lörz (1989)
Elbon, D. Enrique, Ailes and Merced	I. inflores.	MS-medium; 2,4-D;		Linacero and Vázquez (1990)
DH5, TEKU (Tetraploid), SC35 and DH lines with common genes from *S. vavilovii*	anthers	N6-medium; maltose; sucrose; 2,4-D; Kn; agarose; gelrite;	post-plating Temperature	Flehinghaus *et al.* (1991)
SC35, L201, L301 and Carokurz	anthers	MS-medium; 2,4-D; Picloram; gelrite;		Daniel (1993)
10 inbred lines including L318 and H363, 6 F_1 crosses and resp. F_2 and F_3 generations.	I. inflores.	MS-medium; 2,4-D; sucrose; Difco agar; BAP; IAA; folic acid.	light/dark	Rakoczy-Trojanowska and Malepszy (1993)
7 inbred lines including L318 and 5 + 2(R) F_1 crosses and resp. F_2 and F_3 generations	I. embryos	Rakoczy-Trojanowska and Malepszy, 1993		Rakoczy-Trojanowska and Malepszy (1995)
L9, L318, Dw28 and one synthetic hybrid (F_1(5)), all common genes from *S. vavilovii*.	anthers	N6 and P2 (with modifications)-medium; sucrose, 2,4-D; Kn; Picloram; gelrite; Difco-agar; glutamine.		Rakoczy-Trojanowska *et al.* (1997)

Abbreviations: BA = benzyladenin; B5 = Gamborg *et al.* (1968); 2,4,5-Cl_3 POP = 2,4,5-trichlorophenoxypropionic acid; CC = Potrycus *et al.* (1979); 2,4-D = 2,4-dichlorophenoxyacetic acid; Dicamba = 3,6-dichlorophenoxyacetic; 2,i-P = 6-γ-γ-dimethylallylamino purine; I = immature; IAA = indolacetic acid; IBA = indolbutyric acid; IPA = indolpropionic acid; Kn = kinetin; LS = Lin and Staba (1961); MS = Murashige and Skoog (1962); NAA = naphthalene acetic acid; NOA = naphtoxyacetic acid; NN = Nitsch and Nitsch (1969); N6 = Chu *et al.* (1975); P2 = with potato extract, considered by Wenzel *et al.* (1977); PCPA = *p*-chlorophenoxyacetic acid; Picloram = 4-amino-3,5,6-trichloropicolinic acid; 2,4,5 T = 2,4,5-trichlorophenoxyacetic acid; Z = zeatin.

explants usually show a wide range of maturation stages. With some experience, on the other hand, immature embryos can be produced in large amount, obtaining explants at a similar maturation stage. This increases the reproducibility of experiments and the reliability of results. Lu *et al.* (1984) and Zimny and Lörz (1989) described developmental stages of immature embryos for callus initiation of rye. Embryogenic callus can also be initiated from mature embryos as demonstrated for wheat by Özgen *et al.* (1998) and could be an economic alternative to tedious donor plant cultivation for immature explants.

Different media compositions have been described for rye tissue cultures (Table 1) and significant influence on tissue culture response was observed (Eapen and Rao, 1982; Lu *et al.*, 1984; Zimny and Lörz, 1989; Flehinghaus *et al.*, 1991). In these reports only single factors were compared and no statistical data analysis was carried out in any of these investigations. Consequently, interactions between different factors could not be examined. An in-depth study of such interactions would enable the development of genotype-specific culture protocols to better exploit regeneration potential, which might enhance the tissue culture response of recalcitrant crops like rye.

One further important factor is the culture length, which can vary according to the requirements and final use of the cultured tissues. In general, regeneration response is lower the longer cultures are maintained under inductive conditions. Especially high auxin levels decrease the regeneration potential and should therefore be reduced and kept as low as possible. In extended long-term callus cultures, genetic variability can also be a serious impediment for biotechnological applications (Vasil 1987). Several other culture factors like temperature during *in-vitro* culture (Flehinghaus *et al.*, 1991) or callus culture under dark or light conditions (Eapen and Rao, 1984; Lu *et al.*, 1984; Zimny and Lörz, 1989) also influence the tissue culture performance and should be taken in account when establishing an *in-vitro* culture system.

To overcome the limitations in tissue culture ability of rye we analysed the regeneration response of a wide range of *S. cereale* genotypes after short and long term callus cultures. A culture media optimisation experiment with multi-factorial experimental design allowed a thorough statistical analysis, including the estimation of interactions between genotypes and media components as well as interactions between different media components

and their concentrations. Multiple parameters describing the tissue culture response were analysed.

2.3. Comparison of Genotypes

2.3.1. Materials and Methods

Plants and explants

Two weeks old seedlings of 11 inbred lines, 11 single crosses and the population cultivar Wrens Abruzzi were vernalised for 50 days (8 h light, 4°C) and transferred to a controlled environment chamber (12 h light, 10°C). Temperature and duration of illumination was stepwise increased to 20°C and 16 h light at the time of flowering. Spikes were isolated in cellophane bags to prevent cross-pollination. Immature caryopses were surface-sterilised for 3 min in 70% (v/v) ethanol and for 20 min in sodium hypochlorite solution (2.4% active Cl) containing approximately 0.1% (w/v) of Tween-20 followed by five washes with sterile Millipore water. Immature embryos, in developmental stage 3, as suggested by Zimny and Lörz (1989) and approximately 2 mm in size, were excised aseptically from the caryopses and placed on culture medium (Figure 2D).

Culture media and callus cultures

Callus induction medium consisted of MS (Murashige and Skoog, 1962) as basic salts, supplemented with 30g/l sucrose, 100mg/l casein hydrolysate, 500mg/l glutamine and 3.0mg/l 2,4-D. Regeneration medium composition was similar to callus induction medium, without phytohormones. After adding 0.3% (w/v) phytagel for solidification and adjusting the pH to 5.8, media were autoclaved at 121°C and 1.5 bar for 20 min. Immature embryos (Figure 2E) were placed scutellum side up on callus induction medium. For the comparison of genotypes short term cultures were transferred to regeneration medium after three weeks of callus induction, whereas long term cultures were sub-cultured two times (three weeks each) on fresh callus induction medium before transfer to regeneration medium for four weeks.

Evaluation of tissue culture ability

Evaluation of callus response was repeated independently by two persons. Induced embryogenc calli were counted three weeks after culture initiation (Figure 2H). The

regeneration response was quantified by counting the calli with regenerating shoot structures (Figure 2I and J) three to four weeks after transfer on regeneration medium.

Experimental design and statistical analysis

Donor plants were grown in two replications in separate environments with three completely randomised blocks, each. Per environment and callus culture length 25 embryos were cultured resulting in 12×25 explants per genotype. For statistical data analysis the SAS System (SAS 1990) was used and the General Linear Model (GLM) procedure with the *t*-test applied.

2.3.2. Results and Discussion

Twenty-three rye genotypes were compared for their tissue culture suitability. For this callus induction and regeneration response after short and long time of callus culture were evaluated. The 11 single lines, 11 single crosses (F_1) and the population cultivar Wrens Abruzzi were highly divergent in phenotypic appearance e.g. height (Wrens Abruzzi: more than 150 cm and inbred line L22: less than 60 cm), earliness (from 60 up to 95 days between vernalisation and anthesis) or shape of spikes.

Potential for callus induction

Induction of scutellar callus was observed in all cultured genotypes. Different genotypes showed only minor differences in callus colour, texture and callus development. Embryogenic callus (Figure 2H) developed within three weeks after culture initiation from 81% to 99% (L17×L20) of the cultured explants with no significant difference between F_1 crosses (95.37%) and inbred lines (91.21%) (Table 2). Using donor plants from growth chambers might result in a more homogenous explant quality and callus induction response compared to field material. The latter was used in earlier studies and suggests that the reported genotypic variations (Rybczynski and Zdunczyk, 1986; Rakoczy-Trojanowska and Malepszy, 1995) might be attributed to genotypic differences in flowering time resulting in different environmental conditions during maturation.

Potential for regeneration

Callus regeneration to plants was observed in most of the genotypes (Figure 2B, C, I and J) with a considerable genotypic difference in the regeneration rate (% of explants forming calli with plant regeneration) ($Pr>F$ = 0.0001).

After short term callus culture of three weeks the highest regeneration rates were observed from L15 (91.65%), L22 (86.87%) and L22×L 15 (85.80%), not differing significantly from each other but from the other tested genotypes (Table 2 and Figure 2C). A genotypically influenced regeneration response has been reported earlier for cereals (Maddock *et al.*, 1983; Hanzel *et al.*, 1985) including rye (Eapen and Rao, 1982; Rybczynski and Zdunczyk, 1986; Rakoczy-Trojanowska and Malepszy, 1995). Callus regeneration rates of 17% (with M.A.C.S. 898; Eapen and Rao, 1982), 27% (with Wrens Abruzzi; Lu *et al.*, 1984), 60% (with L318 using immature inflorescences and immature embryos; Rakoczy-Trojanowska and Malepszy, 1993, 1995) and up to 100% (with Karlshulder; Zimny and Lörz, 1989) have been described. However the number of regenerated rye plantlets per callus were not presented in most of the earlier studies. Rakoczy-Trojanowska and Malepszy (1995) reported an average of 1.9 green plants per callus of the best responsive genotype (L 318). In this study single crosses regenerated on average of all genotypes more plants per regenerating callus (4.43) than inbred lines (3.03) or the population variety Wrens Abruzzi (1.33). However, the inbred lines L22 regenerated a significant higher number of shoots per callus (6.93; Table 2; Figure 2C) than all other genotypes. The high reproducibility of this *in-vitro* culture system results from the use of defined homozygous inbred lines or their F_1 crosses as starting material for tissue cultures and is an essential component of a reproducible transformation protocol. The regenerated plants (Figure 2K to M) were normal and fertile (Figure 2N) after transfer to soil, encouraging for gene transfer experiments.

After long term callus culture of nine weeks the regeneration rate over all genotypes differed significantly decreasing from 47.22% (after three weeks) to 14.12% (in average over all genotypes), with distinct genotypic interactions ($Pr>F$ = 0.0001 for regeneration rate and $Pr>F$ = 0.0048 for regenerated shoots per callus; Table 2). After long term callus culture the highest regeneration rate was observed in L22 (38.70%) followed by L17×L 21 (38.50%) and L20 (37.96%). Several genotypes, L12, L13×L 15, L16, L21, L14 and L13, lost their regeneration ability completely. Similar responses were observed in bread wheat (Fennell *et al.*, 1996; Varshney and Altpeter, 2002) and suggest to minimise the tissue culture phase in a transformation protocol.

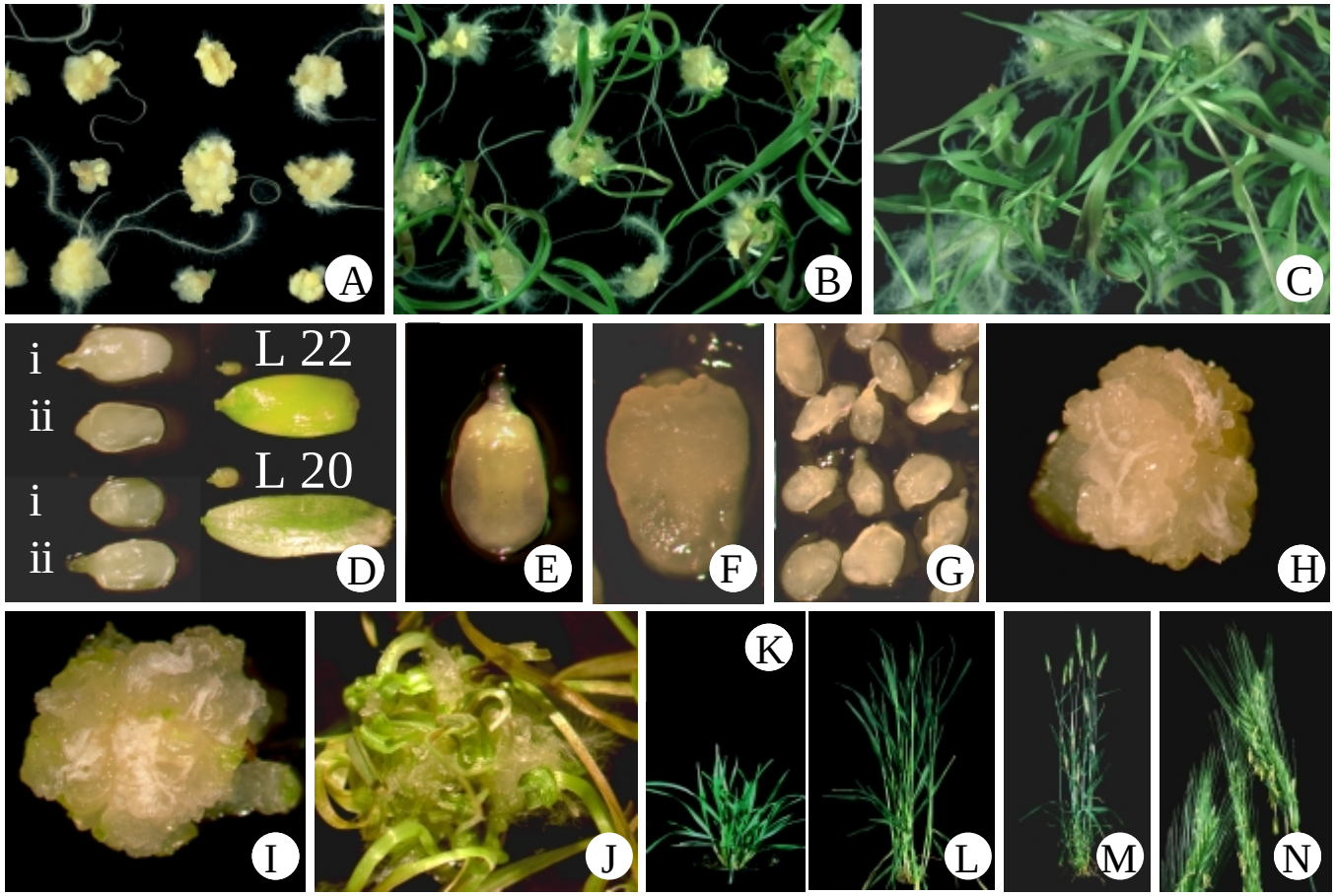


Figure 2: Genotypic variation for *in-vitro* culture response of rye (*Secale cereale* L.): regeneration of fertile plants from scutellar callus of immature embryos.

(**A-C**) Genotypic variation in regeneration response: low (L16), medium (L20) and high (L22) responding genotypes after 21 days of regeneration (A, B, and C, respectively). (**D**) Immature rye caryopses: of L22 and L20 and their respective dissected embryos with scutellum side up (i) and down (ii). (**E-H**) Callus induction: development of scutellar L22 callus after a one (E) and five days (F and G) culture on callus induction medium. Embryogenic scutellar callus (L22) after 21 days of culture on callus induction medium (H). (**I-N**) Callus regeneration: Callus (L22) 3 (I) and 21 days (J) after transfer to regeneration conditions. Regenerated *in-vitro* rye plants (L22) were transferred to soil and showed normal development during tillering (K), heading (L) and flowering (M and N).

Table 2: Tissue culture response in rye (*Secale cereale* L.): callus induction and regeneration potential of immature embryos from different inbred lines, single crosses, and the winter rye cultivar Wrens Abruzzi, after short and long term callus culture.

	21 days callus culture before regeneration [1]				3 x 21 days callus culture before regeneration [1]		
		callus induction	regeneration responce			regeneration responce	
Genotype	Replication[2] (total)	compact calli (% of explants)	regenerating calli (% of calli)	shoots per reg. callus (mean)	Replication[2] (total)	regenerating calli (% of calli)	shoots per reg. callus (mean number)
L 17 * L 20	6	98.67 a	68.87 bc	5.52 bcd	6	30.05 abc	5.10 ab
L 17 * L 21	6	87.33 bdc	42.62 efg	4.37 defg	6	38.50 a	4.33 abc
L 22 * L 15	6	94.67 ab	85.80 a	6.85 ab	6	6.77 bcd	1.27 bc
L 12 * L 14	6	93.28 abc	24.03 hi	4.02 efgh	6	4.70 bcd	2.00 abc
L 12 * L 15	6	98.00 a	31.62 gh	3.05 ghi	6	4.13 a	2.25 abc
L 12 * L 17	6	97.33 a	41.10 fg	3.57 fgh	6	16.48 abcd	2.78 abc
L 13 * L 15	6	95.33 ab	52.63 def	3.12 fghi	6	2.92 cd	0.67 bc
L 13 * L 16	6	95.33 ab	62.40 bcd	3.72 efgh	6	0.80 d	0.17 c
L 15 * L 17	6	97.33 a	39.65 fg	4.08 efg	6	25.85 abcd	3.35 abc
L 15 * L 19	5	95.80 ab	38.86 fg	4.46 def	4	9.30 bcd	2.38 abc
L 16 * L 17	6	96.00 ab	71.57 b	6.02 abc	6	33.92 ab	3.35 abc
L 12	6	92.00 abc	10.52 j	1.23 j	5	0.86 d	0.20 c
L 13	5	98.24 a	8.60 j	0.72 j	5	0.00 d	0.00 c
L 14	5	95.20 ab	51.38 def	4.02 efgh	5	0.00 d	0.00 c
L 15	6	87.58 bcd	91.65 a	5.00 cde	6	4.12 cd	0.83 bc
L 16	6	94.97 ab	55.65 cde	2.72 hi	6	0.00 d	0.00 c
L 17	2	95.35 -n.i.-	54.90 -n.i.-	2.60 -n.i.-	0	--- -n.i.-	--- -n.i.-
L 18	6	95.20 ab	12.05 j	1.30 j	4	3.55 cd	1.25 bc
L 19	2	92.00 -n.i.-	24.75 -n.i.-	3.00 -n.i.-	1	8.00 -n.i.-	2.00 -n.i.-
L 20	5	81.40 d	60.34 bcd	3.96 efgh	5	37.96 a	1.48 abc
L 21	6	84.72 cd	8.32 j	1.83 ij	1	0.00 -n.i.-	0.00 -n.i.-
L 22	6	86.63 bcd	86.87 a	6.93 a	6	38.70 a	6.07 a
Wrens Abruzzi	3	92.47 abc	37.27 gh	1.33 j	2	26.00 abcd	3.25 abc
Means / LSD		93.25 / 9.65	47.22 / 13.85	3.78 / 1.36		14.12 / 27.63	2.04 / 4.64

[1] :Callus induction was followed by four weeks culture on regeneration medium before regeneration response was recorded

[2] :25 Embryos were cultured per replication

a. b. c. d. e. f. g. h. I. j :Means followed by the same letters are not significantly different from each other according to the t-tests (LSD) at the 5% level of significance

-n.i.- : data were not included in the statistical analysis due to low number of replications

2.4. Media optimisation

2.4.1. Materials and Methods

Plants and explants

One spring inbred line from the Petkus gene pool (L 22), one winter inbred from the Carstens gene pool (L 20) and the Polish winter inbred line L318 derived from Dankowske Słote (Figure 3A) were compared regarding tissue culture response. For plant growing and preparation of immature embryo explants see Section 2.3.1.

Culture media and callus cultures

Culture media consisted of MS basic salts (Murashige and Skoog, 1962) supplemented with 100mg/l casein hydrolysate and 500mg/l glutamine. Carbohydrate and auxin sources and varying concentrations thereof as well as different preparation procedures are given in Table 3. The composition of the regeneration media was similar to that of callus induction media, with 30g/l sucrose and no phytohormones. After adding 0.3% (w/v) phytagel (for solid media) and adjustment of the pH to 5.8, the media were autoclaved at 121°C and 1.5 bar for 20 min. Alternatively, components were dissolved in water at double concentration and the pH adjusted to 5.8; the media were then filter-sterilised through 0.1µm filter and mixed with double concentrated phytagel suspended and autoclaved in Millipore water. Explants were placed scutellum side up on callus induction medium and cultured in the dark at 25°C for three weeks. Calli were subcultured on the same or a modified medium (Table 3) for two weeks, followed by a four-week culture period on regeneration medium at 25°C under 16 h (light)/8 h (dark) photoperiod cycle and 1,800 lx illumination.

Evaluation of callus induction, -quality and regeneration response

For evaluation of callus induction the total number of precocious germinating explants (Figure 3B) and the number of compact calli (Figure 3F) were counted three weeks after culture initiation. After the second subculture the number of rhizogenic (Figure 3C) and caulogenic calli (Figure 3D) were recorded. Four weeks after transfer to regeneration medium the regeneration response was quantified by counting the number of calli regenerating shoots (Figure 3G and Figure 3H) as well as the number of shoots regenerated per callus. Ten

regenerated plants from each genotype and from all different media were transferred to soil and evaluated under greenhouse conditions for phenotypic abnormalities. The number of regenerated albino plantlets (Figure 3E) was also recorded.

Table 3: Composition of 24 media and culture conditions in a multi-factorial design and six media as single factors with respect to the optimisation of callus induction potential, callus maintenance ability and regeneration response of rye (S*ecale. cereale* L.).

Media components in factorial experiment[1]	Callus induction[2]	Callus maintenance[2]
Carbohydrate source / concentration (g/l)	Sucrose / 30	Sucrose / 30
	Sucrose / 30	Sucrose / 60
	Maltose / 30	Maltose / 30
	Maltose / 30	Maltose / 60
Auxin source / concentration (mg/l)	2,4-D / 2.5	2,4-D / 2.5
	2,4-D / 2.5	2,4-D / 1.5
	Dicamba / 2.5	Dicamba / 2.5
	Dicamba / 2.5	Dicamba / 1.5
	Picloram / 2.5	Picloram / 2.5
	Picloram / 2.5	Picloram / 1.5
Factors analysed in single experiments	**Concentration**	
Sucrose autoclaved[3]	30g/l	
Sucrose filter sterilised[3]	30g/l	
Maltose autoclaved[4]	30g/l	
Maltose filter sterilised[4]	30g/l	
CC-medium[5*)] as salt base[4]	with 10% coconut water	
Agarose Type I as gelling agent[4]	0.6%	
$CuSO_4$ addition[4]	MS (0.1µ*M*) + 50 µ*M*/l	
$CuSO_4$ / callus induction in low light[4]	MS (0.1µ*M*) + 50 µ*M*/l / 700 lux	

[1]: Supplemented with MS-salts, 100mg/l casein hydrolysate, 500mg/l glutamine and 0.3% phytagel.
[2]: Callus induction (3 weeks) and maintenance (2 weeks) in dark and 25°C.
[3] and [4]: replacing respective component in above medium and supplemented with 2.5mg/l 2,4-D and 30g/l sucrose or 30g/l maltose, respectively.
[5]: Potrykus *et al.* (1979).

Experimental design and statistical analysis

Donor plants of all evaluated inbred lines were raised in the same environment. Within one replication, explants from the same batch of donor plants were cultured on all media combinations and evaluated together as a set. Six to ten replications were cultured per genotype, with ten explants per medium combination representing one replication. For statistical data analysis the SAS System (SAS 1990) was used and the GLM procedure with the *t*-test applied.

2.4.2. Results and Discussion

The tissue culture response of immature embryos of three genetically divergent homozygous rye inbred lines L22, L20 and L318 (Figure 3A) was evaluated on 24 different culture media. The multi-factorial design allowed the statistical analysis of main factor and interaction effects (Table 4) with respect to the parameters investigated: callus induction, callus maintenance, regeneration rate and number of regenerated plants per callus (Table 5). In addition, six single-factor experiments comparing the mode of media sterilisation, alternative basic salt composition, gelling agents, supplementation of MS salts with 50µ*M* $CuSO_4$ and illumination conditions during callus induction were carried out (Table 6).

Callus induction

The germination of explants during callus induction (Figure 3B) is undesirable since it reduces the callus formation and callus quality. Genotype, auxin source and their interaction significantly influenced germination (Table 4). Explants of genotypes L22 and L20, germinated at a higher frequency than explants of L318. The carbohydrate source did not influence the precocious germination of immature embryos. Media supplemented with 2,4-D suppressed the germination of explants for all genotypes significantly better than picloram or dicamba. This is in accordance with the observations of Lu *et al.* (1984). Zimny and Lörz (1989) also reported a higher germination rate of immature embryo explants when low concentrations of dicamba were used instead of 2,4-D. In addition, we observed a significant interaction between genotype and auxin source. In contrast to genotypes L22 and L20, genotype L318 increased its germination frequency following culture on media containing picloram relative to those containing dicamba. An interaction between carbohydrate and auxin source was also observed for the genotype L22 ($Pr>F$ = 0.0008). Filter sterilisation of maltose-containing medium significantly increased the germination of immature embryos of the genotypes L22 and L20, and of L20 on sucrose containing medium. The use of CC-10 basis medium (Potrykus *et al.*, 1979) for genotype L22 also increased the number of germinated explants (Table 6). Under low light conditions the germination rate of L22 and L20 explants was significantly less ($P<0.01$) than in the dark Removal of the embryo axis from the scutellum prior to culturing also prevented germination in rye (data not shown). However, since this is a time-consuming procedure, optimising media composition to prevent germination, as shown in this study, is the preferred strategy.

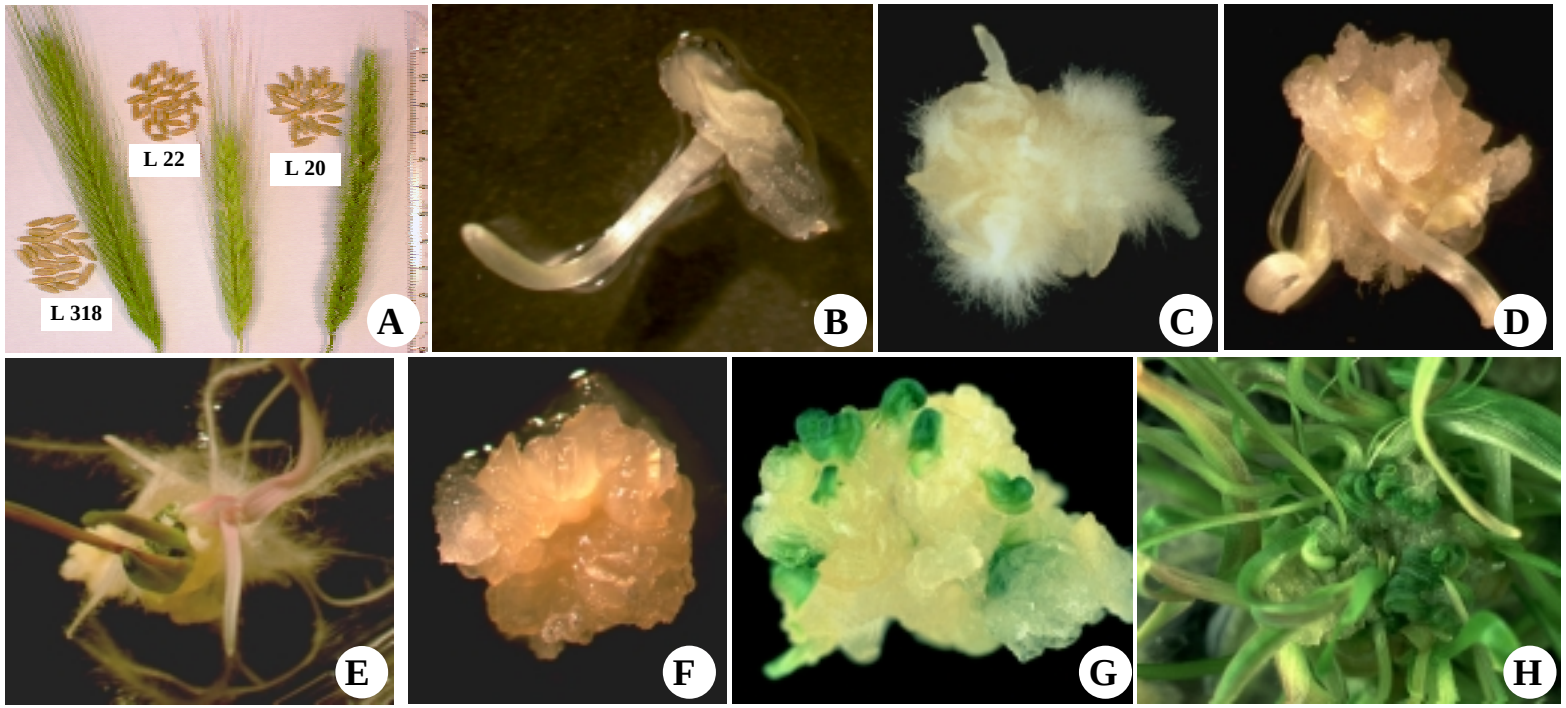


Figure 3: Rye (*Secale cereale* L.) tissue culture in response to genotype, culture media composition and callus growing conditions.

(**A**) Spikes of three genetically diverse genotypes used to initiate rye tissue culture and their mature caryopses. Difficulties in rye tissue culture: (**B**) germinating explant of L22 after one week of callus induction on CC-10 medium; (**C**) rhizogenic callus of L318 after 21 + 14 days of callus induction on MS medium supplemented with sucrose and picloram; (**D**) caulogenic callus of L22 after 21 + 14 days of callus induction on MS medium supplemented with sucrose and dicamba; (**E**) regenerated albino plant from L20 callus. Optimised rye tissue culture: (**F**) embryogenic callus from L22 after 21 + 14 days of callus induction on MS medium supplemented with sucrose and 2,4-D; (**G**) regenerating L20 callus after low light callus induction on Maltose and 2,4-D containing MS medium, complemented with 50µ*M* $CuSO_4$; (**H**) regenerated L22 callus after induction on medium containing sucrose and 2,4-D.

Table 4: Analysis of variance of genotype and medium components influencing the *in-vitro* response of rye (*Secale cereale* L.).

Source of Variance	*df*	germ. E.	comp. C.	rhizog. C.	caulog. C.	regen. C.	shoots per regen. C.
Error	546	367.4	126.8	168.4	120.2	261.6	14.7
Genotype (Genot)	2	50770.6**	476.8*	13009.3**	9646.7**	59824.7**	2991.9**
Carbohydrate source (C.H.)	1	16.6	4004.8**	4524.4**	2387.9**	42371.2**	348.3**
Auxin source (Aux.)	2	24863.4**	4.7	16236.7**	13337.1**	19816.7**	15.4
Variation of C.H. Concentration (V. of. Con.)	1	152.8	248.8	661.5*	142.6	1923.8**	66.8*
Variation of Aux. Concentration (V. of Con.)	1	70.1	111.3	13397.0**	807.0**	14.8	0.8
Genot. × C.H.	2	323.9	1577.0**	4316.5**	341.7^{+}	2691.3**	59.9*
Genot. × Aux.	4	2923.0**	70.0	3529.2**	9265.1**	20609.2**	343.4**
Genot. × V. of C.H. Con.	2	43.8	102.2	2196.6**	106.4	504.2	30.2
Genot. × V. of Aux. Con.	2	441.4	545.9*	3654.6**	226.8	921.4*	52.4*
C.H. × Aux.	2	750.8	77.2	2255.0**	137.4	1042.5*	201.3**
C.H. × V. of C.H. Con.	1	105.3	32.2	941.5*	34.6	176.0	5.5
C.H. × V. of Aux. Con.	1	77.1	0.4	0.5	72.0	20.5	11.9
Aux. × V. of C.H. Con.	2	151.2	6.1	167.3	21.0	110.9	18.0
Aux. × V. of Aux. Con.	2	406.3	84.5	3232.2**	56.8	945.4*	84.2**
V. of C.H. Con. × V. of Aux. Con.	1	1012.6^{+}	219.7	32.4	3.1	11.2	0.3
Genot. × C.H. × Aux.	4	1003.4*	148.3	1625.0**	482.1**	2635.9**	84.0**
Genot. × C.H. × V. of C.H. Con.	2	198.2	84.8	747.9*	193.2	335.1	22.9
Genot. × C.H. × V. of Aux. Con.	2	173.5	212.8	11.1	228.4	286.3	32.8
Genot. × Aux. × V. of C.H. Con.	4	100.8	48.3	785.1**	15.0	253.6	21.9
Genot. × Aux. × V. of Aux. Con.	4	140.4	70.0	976.1**	53.1	454.4	6.8
Genot. × V. of C.H. Con. × V. of Aux. Con.	2	1635.2*	579.0*	1525.8**	123.9	55.2	39.8^{+}

Mean square for analysis of significance = $^{+}$, * and ** = differences with $P<0.10$, $P<0.05$ and $P<0.01$ of significance, respectively. *df* = degrees of freedom; comp. C. = compact Callus; germ. E. = germinating explants; rhizog. C. = rhizogenic Callus; caulog. C. = caulogenic Callus; regen. C. = regenerating Callus.

Callus growth (Figure 3F) was induced from immature embryo explants of all genotypes and on all of the media evaluated. The effect of media components on callus induction was genotype-specific, with significant effects of the carbohydrate source and Genotype×carbohydrate source interaction (Table 4). A statistically higher callus induction rate with maltose (93.99%) compared to sucrose (82.27%) was observed for L22. Similar effects of maltose have been described by Flehinghaus *et al.* (1991), who reported a fourfold increase in the callus induction rate in rye anther cultures with considerable genotypic differences. Auxin sources did not affect the callus induction frequency in any of the genotypes, but the callus growth rate (fresh weight) was significantly increased in L22 ($Pr>F$ = 0.0001) and L318 ($Pr>F$ = 0.0001) in the order 2,4-D, picloram and dicamba (data not shown). Carman *et al.* (1987a, b) and Barro *et al.* (1998) also described similar efficiencies for callus formation of immature wheat embryos in which media comtaining 2,4-D and dicamba and 2,4-D and picloram, respectively were compared. In case of filter sterilisation of sucrose-containing medium, a positive effect on the callus induction rate of L22 was found, while no negative effect was present when maltose was the carbohydrate source (Table 6). We observed that the composition of the basal media (MS or CC-10) did not significantly influence the induction of embryogenic callus, which is in contrast to the observations of Zimny and Lörz (1989) with different genotypes. Replacing phytagel by agarose significantly reduced the callus induction frequency of L20 (Table 6).

Precocious morphogenesis

Formation of rhizogenic callus (Figure 3C) was influenced by all of the factors investigated, and interactions between genotypes and all other main factors were observed (Table 4). L318 calli formed significantly more rhizogenic calli on sucrose-containing medium. Following an increase in the maltose concentration L22 and L20 developed significantly more rhizogenic calli (0.62 vs 4.07 and 7.60 vs 23.58 respectively). These observations demonstrate a significant genotype×carbohydrate interaction ($Pr>F$ = 0.0001) with respect to the development of rhizogenic callus. The use of 2,4-D suppressed best the formation of rhizogenic callus in all genotypes analysed, even when the 2,4-D concentration was decreased during callus culture. The use of dicamba or picloram led to significantly increased root formation in L318, while a decreased concentration increased the number of rhizogenic calli in all genotypes. The effect was significant for L20 and L318 on picloram-containing medium

and for L22 and L20 on dicamba-containing medium (Table 5). Beside this genotype×media interaction, carbohydrate and auxin sources interacted significantly in L20 with respect to the formation of rhizogenic callus ($Pr>F$ = 0.0061). Out of all single factors tested only CC-10 medium affected the callus maintenance significantly in all genotypes, but the most pronounced effect was in L20 for the number of rhizogenic calli: 2.25% (MS salts) to 76.20% (CC-10 medium) (Table 4).

The formation of caulogenic callus (Figure 3D) was influenced by genotype, carbohydrate and auxin source and auxin concentration. Genotype×auxin source interactions were also significant (Table 4). The use of maltose positively influenced callus maintenance in two genotypes. The number of caulogenic calli of L22 and L20 decreased from 19.72% and 7.86% to 12.94% and 3.56%, respectively, when maltose replaced sucrose as the carbohydrate source. The precocious regeneration of calli might be caused by stress factors, such as accumulation of toxic products, as described by Scott *et al.* (1995) for barley microspore cultures. The authors found that in cultures on sucrose, in comparison to those growing on maltose, the initial rate of metabolism was faster but that it declined rapidly due to an accumulation of ethanol and a lower adenylate energy. Maltose is metabolised slower than sucrose and, subsequently, sufficient oxygen remains available to allow the cells to survive in culture (Scott *et al.*, 1995). In the present experiment, the callus growth (fresh weight) of all genotypes was decreased when calli were cultured on medium with increased sucrose concentration (interaction of carbohydrate source×carbohydrate concentration, $Pr>F$ =0.0909); this decrease was significant for L22 ($Pr>F$ = 0.0001, $Pr>F$ = 0.0007 and $Pr>F$ =0.0077 for carbohydrate source, increase of carbohydrate concentration and their interaction, respectively) (data not shown). The development of rhizogenic calli after an increase in the maltose concentration could in fact be due to a carbohydrate oversupply and, therefore, a toxic accumulation of metabolites. Auxin sources influenced the callus maintenance of all tested genotypes ($Pr>F$ = 0.0001) and also interacted significantly with them ($Pr>F$ = 0.0001). The use of dicamba significantly supported premature morphogenesis of L22 and L318, while no or only few calli from these genotypes developed premature regenerating structures on 2,4-D containing media. Zimny and Lörz (1989) also reported that dicamba stimulated the rapid formation of somatic embryoids. Carbohydrate and auxin sources interacted significantly in all the tested genotypes with respect to the development of caulogenic callus ($Pr>F$ = 0.0573, $Pr>F$ = 0.0270, $Pr>F$ = 0.0035 for L20, L22 and L318,

Table 5: Multi-factorial analysis of carbohydrate and auxin sources and concentrations on tissue culture response[1] of three homozygous inbred lines of rye (*Secale cereale* L.)

		callus induction		precocious morphogenesis		regeneration response	
Genotype (replications)[1]	Media components (conc. I / conc. II)[2]	germination (% of expl.)	compact calli (% of expl.)	rhizogenic calli (% of calli)	caulogenic calli (% of calli)	regenerating calli (% of calli)	shoots per reg. callus (mean number)
L 22 (9)	sucrose (30mg/L / 30mg/L)	38.48 a	82.37 b	1.49 b	17.36 ab	77.89 a	14.71 a
	sucrose (30mg/L / 60mg/L)	42.14 a	82.16 b	2.02 ab	22.09 a	80.77 a	13.59 ab
	maltose (30mg/L / 30mg/L)	38.62 a	93.15 a	0.62 b	12.64 b	64.17 b	11.84 b
	maltose (30mg/L / 60mg/L)	38.33 a	94.82 a	4.07 a	13.23 b	60.10 b	11.21 b
	Least Significant Difference	9.80	6.32	2.35	7.11	7.38	2.42
	2.4-D (2.5mg/L / 2.5mg/L)	23.07 b	86.11 a	0.00 c	0.28 b	73.02 ab	12.58 ab
	2.4-D (2.5mg/L / 1.5mg/L)	24.44 b	90.00 a	0.00 c	2.04 b	72.05 ab	12.85 a
	dicamba (2.5mg/L / 2.5mg/L)	44.44 a	87.50 a	1.39 bc	37.65 a	78.56 a	13.14 a
	dicamba (2.5mg/L / 1.5mg/L)	49.26 a	87.16 a	3.71 ab	44.31 a	78.84 a	10.28 b
	picloram (2.5mg/L / 2.5mg/L)	48.77 a	88.52 a	1.42 bc	5.39 b	64.35 bc	14.11 a
	picloram (2.5mg/L / 1.5mg/L)	46.39 a	89.44 a	5.78 a	8.30 b	59.58 c	14.07 a
	Least Significant Difference	10.10	5.60	2.36	9.42	9.03	2.37
L 20 (10)	sucrose (30mg/L / 30mg/L)	36.50 a	84.67 a	14.47 bc	8.51 a	52.84 a	6.92 a
	sucrose (30mg/L / 60mg/L)	38.79 a	83.21 a	16.71 ab	7.21 ab	44.91 b	6.45 a
	maltose (30mg/L / 30mg/L)	41.24 a	85.59 a	7.60 c	2.67 b	41.88 bc	7.28 a
	maltose (30mg/L / 60mg/L)	39.00 a	87.33 a	23.58 a	4.45 ab	36.70 c	4.94 b
	Least Significant Difference	17.91	5.55	7.10	4.73	6.58	1.34
	2.4-D (2.5mg/L / 2.5mg/L)	19.25 b	86.75 a	3.35 b	4.71 bc	58.31 b	7.81 b
	2.4-D (2.5mg/L / 1.5mg/L)	20.00 b	84.75 a	5.03 b	9.55 ab	68.20 a	10.29 a
	dicamba (2.5mg/L / 2.5mg/L)	46.00 a	88.00 a	9.90 b	5.77 abc	50.35 c	6.26 c
	dicamba (2.5mg/L / 1.5mg/L)	43.25 a	83.25 a	37.15 a	10.41 a	45.14 c	5.45 c
	picloram (2.5mg/L / 2.5mg/L)	57.30 a	84.95 a	6.30 b	1.11 c	16.08 e	3.54 d
	picloram (2.5mg/L / 1.5mg/L)	47.50 a	83.50 a	31.81 a	2.70 c	26.42 d	5.01 c
	Least Significant Difference	18.88	5.73	9.13	5.29	7.79	1.46
L 318 (6)	sucrose (30mg/L / 30mg/L)	3.89 ab	86.36 a	20.98 b	2.57 a	49.31 a	7.40 a
	sucrose (30mg/L / 60mg/L)	5.83 ab	82.22 a	29.54 a	1.24 a	47.76 a	5.64 ab
	maltose (30mg/L / 30mg/L)	4.72 ab	85.56 a	5.29 c	0.66 a	24.27 b	5.26 b
	maltose (30mg/L / 60mg/L)	1.67 b	86.30 a	10.29 c	0.81 a	23.06 b	4.81 b
	Least Significant Difference	3.75	6.04	8.39	2.22	7.64	2.13
	2.4-D (2.5mg/L / 2.5mg/L)	0.00 c	81.62 a	0.00 c	0.00 c	11.68 c	3.85 b
	2.4-D (2.5mg/L / 1.5mg/L)	0.00 c	86.99 a	0.00 c	0.00 c	14.55 c	4.08 b
	dicamba (2.5mg/L / 2.5mg/L)	3.33 bc	83.29 a	22.64 b	3.59 a	60.53 a	8.00 a
	dicamba (2.5mg/L / 1.5mg/L)	5.00 ab	88.75 a	42.79 a	2.93 ab	53.49 a	7.33 a
	picloram (2.5mg/L / 2.5mg/L)	7.92 a	84.58 a	13.94 b	0.88 bc	38.41 b	5.39 ab
	picloram (2.5mg/L / 1.5mg/L)	7.92 a	85.42 a	19.79 b	0.52 bc	37.92 b	6.02 ab
	Least Significant Difference	4.06	8.42	10.76	2.70	9.84	2.75

[1]: 10 Embryos were cultured per media combination and replication
[2]: Conc. I: concentration during culture initiation (3 weeks); Conc. II: concentration during callus maintenance (2 weeks). Calli were additionaly regenerated for 3.5 weeks
[a, b, c]: Means with the same letters are not significantly different from each other according to *t*-tests (LSD) with 5 % alpha level

Table 6: Single factorial analysis of culture media composition[1], media preparation and culture conditions[2] on tissue culture response of three homozygous inbred lines of rye (*Secale cereale* L.)

		callus induction		precocious morphogenesis		regeneration response	
Genotype (replications)[3]	Treatment	germination (% of explants)	compact calli (% of explants)	rhizogenic calli (% of calli)	caulogenic calli (% of calli)	regenerating calli (% of calli)	shoots per reg. callus (mean number)
L 22 (9)	Sucrose, autoclaved	24.44 ^{ab}	74.44 ^{c}	0.00 ^{b}	0.00 ^{b}	83.58 ^{a}	17.98 ^{a}
	Sucrose, filter-sterilised	27.78 ^{ab}	93.33 ^{ab}	0.00 ^{b}	0.00 ^{b}	84.68 ^{a}	16.13 ^{a}
	Maltose, autoclaved	12.22 ^{bcd}	92.22 ^{ab}	0.00 ^{b}	0.00 ^{b}	68.94 ^{b}	9.47 ^{c}
	Maltose, filter-sterilised	37,78 ^{a}	94.44 ^{ab}	0.00 ^{b}	0.00 ^{b}	86.05 ^{a}	12.81 ^{b}
	CC- salt basis[4]	37.78 ^{a}	90.00 ^{b}	6.67 ^{a}	7.31 ^{a}	63.40 ^{b}	9.70 ^{bc}
	Agarose	15.56 ^{bcd}	98.89 ^{a}	0.00 ^{b}	0.00 ^{b}	45.93 ^{c}	7.18 ^{c}
	$CuSO_4$-supplemented MS	21.11 ^{abc}	94.44 ^{ab}	0.00 ^{b}	0.00 ^{b}	59.17 ^{b}	8.22 ^{c}
	$CuSO_4$ / low light induction (700 lx)	2.22 ^{d}	97.78 ^{ab}	0.00 ^{b}	0.00 ^{b}	64.82 ^{b}	8.29 ^{c}
	Least Significant Difference (LSD)	18.29	9.37	4.77	12.49	14.62	3.78
L 20 (10)	Sucrose, autoclaved	23.00 ^{bcd}	89.00 ^{ab}	7.47 ^{cb}	10.17 ^{b}	80.53 ^{ab}	8.35 ^{b}
	Sucrose, filter-sterilised	49.44 ^{a}	79.78 ^{bc}	0.00 ^{c}	1.25 ^{b}	60.90 ^{cd}	7.55 ^{cb}
	Maltose, autoclaved	22.00 ^{cd}	85.00 ^{abc}	2.25 ^{cb}	1.25 ^{b}	46.75 ^{ed}	7.47 ^{cb}
	Maltose, filter-sterilised	41.11 ^{ab}	91.11 ^{a}	0.00 ^{c}	2.35 ^{b}	60.19 ^{cd}	8.47 ^{b}
	CC- salt basis[4]	32.00 ^{abc}	88.00 ^{ab}	76.2 ^{a}	39.28 ^{a}	66.36 ^{bc}	5.38 ^{c}
	Agarose	13.00 ^{de}	77.00 ^{c}	1.43 ^{c}	0.00 ^{b}	39.10 ^{e}	5.46 ^{c}
	$CuSO_4$-supplemented MS	25.00 ^{bcd}	93.00 ^{a}	10.56 ^{b}	6.72 ^{b}	70.70 ^{bc}	12.75 ^{a}
	$CuSO_4$ / low light induction (700 lx)	2.00 ^{e}	87.00 ^{abc}	2.11 ^{bc}	3.65 ^{b}	88.57 ^{a}	11.38 ^{a}
	Least Significant Difference (LSD)	29.02	14.04	17.01	11.58	20.69	3.89
L 318 (6)	Sucrose, autoclaved	0.00 ^{a}	83.15 ^{a}	0.00 ^{b}	0.00 ^{b}	16.11 ^{bc}	6.42 ^{ab}
	Sucrose, filter-sterilised	0.00 ^{a}	83.33 ^{a}	0.00 ^{b}	1.85 ^{a}	26.16 ^{ab}	6.63 ^{ab}
	Maltose, autoclaved	0.00 ^{a}	80.00 ^{a}	0.00 ^{b}	0.00 ^{b}	6.02 ^{cd}	4.50 ^{abc}
	Maltose, filter-sterilised	0.00 ^{a}	93.33 ^{a}	0.00 ^{b}	0.00 ^{b}	36.67 ^{a}	8.24 ^{a}
	CC- salt basis[4]	0.00 ^{a}	80.00 ^{a}	2.08 ^{a}	0.00 ^{b}	3.70 ^{cd}	2.00 ^{abc}
	Agarose	0.00 ^{a}	85.00 ^{a}	0.00 ^{b}	0.00 ^{b}	2.08 ^{d}	0.83 ^{bc}
	$CuSO_4$-supplemented MS	0.00 ^{a}	88.33 ^{a}	0.00 ^{b}	0.00 ^{b}	5.42 ^{cd}	7.50 ^{a}
	$CuSO_4$ / low light induction (700 lx)	0.00 ^{a}	86.67 ^{a}	0.00 ^{b}	0.00 ^{b}	2.08 ^{d}	0.50 ^{bc}
	Least Significant Difference (LSD)	6.28	13.45	15.67	4.66	16.92	4.61

[1]: Variations from the standard medium: MS-salts, 30 g/l carbohydrate source, 500 mg/l casein hydrolysate, 100 mg/l glutamine, 2.5 mg/l 2,4-D, pH (KOH) 5.8 and 0.3% phytagel

[2]: Callus induction in dark, relative to low-light induction

[3]: Ten embryos were cultured per media combination and replication

[4]: Potrykus et al. (1979)

[a, b, c, d, e]: Means followed by the same letters are not significantly different from each other according to *t*-tests (LSD) at the 5% level of significance

respectively). The replacement of MS salts by the CC-10 basic medium significantly affected the callus maintenance of L22 and L20 cultures: the number of caulogenic L20 calli increased by more than 30-fold when the former was replaced by the latter.

Regeneration response

Regeneration rate of calli (% regenerating calli) was significantly influenced by genotype and carbohydrate sources (Table 4), and interactions between carbohydrate and auxin sources were observed for all genotypes (L 20: $Pr>F$ = 0.0001, L22: $Pr>F$ = 0.0008 and L318: $Pr>F$ = 0.0035). The regeneration rate of calli induced on sucrose-containing medium, compared to maltose-containing medium, was significantly higher for all of the genotypes tested. Maltose-containing medium was found to be superior for the production of embryogenic callus in alfalfa (*Medicago sativa* L.) compared to 13 other carbohydrate sources, including sucrose (Strickland *et al.*, 1987). Several monocotyledons also responded positively to callus induction on maltose-containing medium. Altpeter and Posselt (2000) also reported significant genotype×carbo-hydrate source interactions in long term cultures of perennial ryegrass (*Lolium perenne* L.). Maltose has been described as being beneficial for initiation of rye anther cultures (Flehinghaus *et al.*, 1991); high genotype×carbohydrate interactions were described. An increase in the sucrose concentration during callus culture reduced the regeneration rate of L20 from 52.84% to 44.91%. The auxin sources influenced the regeneration response significantly in all three genotypes ($Pr>F$ = 0.0048, $Pr>F$ = 0.0001 and $Pr>F$ = 0.0001 for L22, L20 and L318, respectively); auxin source and genotypes interacted significantly for this factor ($Pr>F$ = 0.0001). The regeneration rate of L22 was highest when dicamba or 2,4-D were used; both supported significantly higher regeneration rates than picloram-containing medium. In contrast the preferred auxin source for L20 was 2,4-D, followed by dicamba and picloram (regeneration rates: 63.20%, 47.75% and 21.25%, respectively), with significant differences between all auxin sources. L318 showed lowest regeneration rate on 2,4-D containing medium (Table 5). Based on a study in which different genotypes were used, Zimny and Lörz (1989) reported picloram to be the auxin that induced the lowest response but describe dicamba rather than 2,4-D as the best suited auxin source for the induction of regenerable rye callus cultures. Barro and coworkers (1998, 1999) found that picloram in the callus induction medium for wheat and barley gave rise to more regenerative cultures than 2,4-D. Daniel (1993) also arrived at that result after culturing rye anthers on

picloram-containing media. In the presented experiments, decreasing the 2,4-D or picloram concentration significantly reduced the regeneration response of L20 calli. Additional single factors influenced the regeneration efficiency of L22, L20 and L318. Filter-sterilisation significantly increased the regeneration efficiency of L22 and L318 when they were cultured on maltose-containing medium. With respect to the callus induction and regeneration of genotype L20 (Table 6), the significant positive effect of autoclaving sucrose should be further investigated by an analysis of the media composition following autoclaving. The use of agarose instead of phytagel as gelling agent had a negative effect on all genotypes, that was significant for L22 and L20 cultures. $CuSO_4$ complementation as well as callus induction under low light significantly increased the regeneration rate of L20 but was not significant for other genotypes. With respect to L20, Zimny and Lörz (1989) observed that callus induction under low light gave rise to slow-growing but highly embryogenic callus.

The number of regenerated plants per callus was significantly influenced by genotype and carbohydrate source (Table 4). The number of plants per callus was higher for media containing sucrose than for those containing maltose, and the effect was significant for L20 and L318. An increase in the maltose concentration significantly reduced the number of plants per regenerating L20 callus from 7.28 to 4.94. Callus induction on picloram-containing medium gave the highest regeneration response of the L22 cultures, followed by dicamba and 2,4-D. For L20, the best response (9.05 plants) was observed on 2,4-D containing media, whereas media containing dicamba and picloram resulted in the regeneration of 5.86 and 4.28 plants per callus, respectively. In contrast, L318 showed the highest regeneration response (7.67 plants) when cultured on dicamba-containing medium. For this genotype, a significantly lower response was observed when grown on media containing picloram (5.71 plants) and 2,4-D (5.47 plants). A reduced auxin concentration led to a lower number of regenerated L22 plants per callus when dicamba was used and to an increased number of L20 plants when 2,4-D or picloram was used. Carbohydrate×auxin source interaction was observed for L22 ($Pr>F$ = 0.0001). On sucrose-containing media, the best regeneration response was observed with 2,4-D (significantly higher than dicamba). However this auxin led to the lowest number of regenerated plants (significantly lower than picloram) when maltose was the carbohydrate source. Filter-sterilisation of sucrose-containing media did not influence the number of shoots per regenerating callus of any genotype. Filter-sterilisation of maltose-containing media resulted in a significant increase in the number of regenerated plants per callus in L22 cultures

(12.81 vs 9.47 plants), but regeneration was highest after culture on sucrose-containing medium (17.06 plants) with no effect of the sterilisation procedure on the regeneration potential. $CuSO_4$ supplementation showed a genotype-specific effect. In contrast to L22 and L318, cultures of L20 showed the highest number of regenerating plantlets on $CuSO_4$ containing medium (12.75 and 11.38 plantlets, respectively), as shown in Figure 3G. This number was significantly more than that observed on all other media tested. Several authors have suggested that copper content of standard culture media should be revised. Purnhauser (1991) reported an eight-fold increased number of regenerated shoots of wheat cultures after elevating the $CuSO_4$ concentration to 10µ*M* (MS = 0.1µ*M*). Similar results have been reported for *Triticale* and tobacco (*Nicotiana tabacum* L.) cultures. However, no or an inhibitory effect was observed for grape (*Vitis vinifera* L.) (Purnhauser and Gyulai, 1993). As in the present study, Dahleen (1995) described genotypic differences with respect to the response of two barley cultivars on different $CuSO_4$ concentrations. Callus induction under low light conditions significantly decreased the regeneration potential of L318 cultures.

Several authors have supplemented the regeneration media with cytokinins to stimulate regeneration of somatic embryoids (Eapen and Rao, 1982; Zimny and Lörz, 1989). In this experiments, embryogenic calli from L22 were regenerated on media containing zeatin, zeatin riboside and BAP. Except for a faster elongation of regenerated shoots, no increase in the number of regenerating calli and regenerated plants per callus was observed (data not shown). No genotype or medium composition resulted in the regeneration of a considerable number of albino shoots (Figure 3E), although L20 produced significantly more albino plantlets than L22 and L318 with 0.54%, 0.13% and 0.00% regenerated shoots respectively. Eapen and Rao (1982) observed no albino plants in rye cultures, while Lu *et al.* (1984) reported approximately 5% of the regenerated plantlets as chlorophyll variants derived from prolonged culture. A few tissue culture-derived plants showed delayed heading in comparison to their respective genotypic average. This could not be assigned to any particular media component or combination. Zimny and Lörz (1989) observed also somaclonal variation influencing plant hight or double spike formation in some of the regenerants. The vast majority of the regenerated plants from the presented experiments were phenotypically normal. In conclusion, genotypic interactions with many media components and culture parameters were observed in rye. Genotype-specific adjustments of these components and factors are essential to release the full regeneration potential of this recalcitrant crop.

3. Gene transfer

3.1. Summary

The efficient and reproducible production of stably expressing transgenic rye plants is described for the first time. This development was supported by the identification and combination of interactive factors which allowed biolistic gene transfer and recovery of transgenic events, without compromising on the regeneration potential of this recalcitrant cereal. A selection during the callus growth or regeneration of shoot primordia was not required for the efficient identification of transgenic rye. A total of 21 independent transgenic rye plants were produced with transformation frequencies of up to 2.2% of the bombarded explants.

Agrobacterium tumefaciens-mediated gene transfer allowed also the production of a total of 35 transgenic rye plants. Morphologically normal and fertile transgenic plants were obtained out of 3.87% of the inoculated explants. The evidence of *A. tumefaciens*-mediated T-DNA transfer is based on Southern blot analysis, the analysis of T-DNA::plant DNA junction sequences and segregation studies.

3.2. Introduction

The development of a transformation system requires the availability of a reproducible and efficient *in-vitro* culture protocol. Depending on the species and the tissue culture system available different tissues were target of genetic transformation. Until the 1990th the *Agrobacterium tumefaciens* mediated plant transformation (De Block *et al.*, 1984; Horsch *et al.*, 1984, 1985) was thought to be specific for dicotyledonous plant species (Potrykus 1990). Therefore, in cereals first attempts where performed on protoplast transformation by direct gene transfer using polyethylene glycol (PEG) (Lörz *et al.*, 1985; Potrykus *et al.*, 1985; Vasil *et al.*, 1988; Datta *et al.*, 1990, 1992) and electroporation (Fromm *et al.*, 1985; Hauptmann *et al.*, 1987; Rhodes *et al.*, 1988; Vasil *et al.*, 1988; Shimamoto *et al.*, 1989) but efficiencies were low and protoplast culture techniques limited to few species like rice (*Oriza sativa* L.), maize (*Zea mays* L.) or guineagrass (*Panicum maximum* Jacq.). The invention of the particle bombardment technique (Sanford *et al.*, 1987) was a major break through in plant transformation as it has enabled the genetic engineering of species not amenable to *Agrobacterium* or protoplast based transfer techniques. Based on acceleration (Klein *et al.*,

1988; Vain *et al.*, 1993a) microscopic tungsten (Russel *et al.*, 1992b) or gold particles coated with DNA can be propulsed into practically all kind of tissue (callus: Vasil *et al.*, 1992; Zhong *et al.*, 1993; Ritala *et al.*, 1994; suspension culture: Klein *et al.*, 1988; Gordon-Kamm *et al.*, 1990; inflorescences: Barcelo *et al.*, 1994; shoot apices: Zhong *et al.*, 1996; microspores: Jähne *et al.*, 1994; leaves: Tomes *et al.*, 1990; roots: Seki *et al.*, 1991; pollen grains: Stoeger *et al.*, 1995) and stable transgenic cereal plants were reported for wheat (Vasil *et al.*, 1992), oat (Somers *et al.*, 1992), barley (Wan and Lemaux, 1994) as well as for rye (Castillo *et al.*, 1994). But efficiency and reproducibility was low in this early reports were callus tissues were bombarded and regeneration potential was limited by long term callus cultures. Vasil *et al.* (1993) bombarded shortly pre-cultured immature embryos and could reduce the required tissue culture time by 50%. The trend to reduced tissue culture periods was continued by other authors (Weeks *et al.*, 1993; Wan and Lemaux, 1994; Altpeter *et al.*, 1996a), improved reproducibility of genetic transformation and accelerated the production process of transgenic wheat plants to two month (Altpeter *et al.*, 1996a). Several factors have been described to influence the applicability and efficiency of biolistic gene transfer. On the tissue culture side genotype (Koprek *et al.*, 1996), type and age of bombarded explants (Armaleo *et al.*, 1990), culture period prior and after gene transfer (Rasco-Gaunt *et al.*, 1999) culture medium composition (Barro *et al.*, 1998) and osmotic pre-treatment (Vain *et al.*, 1993b) have been shown to be crucial. Concerning the biolistic device the applied acceleration pressure (Koprek *et al.*, 1996; Rasco-Gaunt *et al.*, 1999), the adjustable distances between rupture disc, macrocarrier, stopping screen and target plate (Koprek *et al.*, 1996; Rasco-Gaunt *et al.*, 1999), the vacuum pressure in the bombardment chamber (Rasco-Gaunt *et al.*, 1999), number of bombardments (Lonsdale *et al.*, 1990) as well as size and density of micro-particles (Altpeter *et al.*, 1996a; Rasco-Gaunt *et al.*, 1999), DNA/micro-particle mixing protocols (Perl *et al.*, 1992) and physical configuration of transforming DNA (Nandadeva *et al.*, 1999; Fu *et al.*, 2000) are factors to be optimised. Several attempts to establish or improve transformation protocols focused on transient GUS expression without consideration of the regeneration response of the bombarded tissues (Chibbar *et al.*, 1991; Perl *et al.*, 1992; Bilang *et al.*, 1993). However, particle bombardment, especially of recalcitrant species, can have severe effects on the regeneration capability of cultures. Optimised protocols for generating transgenic plants should therefore not be based exclusively on transient gene expression assays (Nandadeva *et al.*, 1999); an adjustment of bombardment parameters to maintain the

shoot regeneration ability and allow the recovery of stable transformants is recommended (Altpeter *et al.*, 1996a).

Agrobacterium-mediated gene transfer offers potential advantages, including preferential integration of the transgene into transcriptionally active regions of the chromosome (Czernilofsky *et al.*, 1986; Koncz *et al.*, 1989) with exclusion of vector DNA (Hiei *et al.*, 1997), unlinked integration of co-transformed T-DNA´s (McKnight *et al.*, 1987; De Block and Debrouwer 1991; Komari *et al.*, 1996) and transfer of large DNA fragments (Hamilton *et al.*, 1996; Hamilton 1997; Liu *et al.*, 1999). After the first report of gene transfer in plants mediated by the soil pathogen *Agrobacterium tumefaciens* (De Block *et al.*, 1984; Horsch *et al.*, 1984) the success was limited to *Solanaceae*, tobacco in particular. Several reports presented early attempts to genetically transform *Gramineae* with *A. tumefaciens* (Raineri *et al.*, 1990; Gould *et al.*, 1991; Mooney *et al.*, 1991). Substitutive confirmation was then accumulated that stably transformed cereal plants can be produced using *Agrobacterium* as transformation vector (Chan *et al.*, 1992, 1993; Hiei *et al.*, 1994; Ishida *et al.*, 1996; Rashid *et al.*, 1996). Recent reports gave evidence for stable *Agrobacterium* mediated gene-transfer into rice (Hiei *et al.*, 1994; Dong *et al.*, 1996), wheat (Cheng *et al.*, 1997) and barley (Tingay *et al.*, 1997; Fang *et al.*, 2002). So far, a report on *Agrobacterium*-mediated rye transformation is missing.

Several factors were identified to influence the transfer of T-DNA and its integration in the plant genome which could be divided in plant and *Agrobacterium* specific factors.

Three *Agrobacterium* specific components are required (Sheng and Citovsky 1996). The first is the suite of *chromosomal virulence* (*chv*) genes, which are involved in bacterial chemotaxis toward and attachment to the wounded plant cell. The second component is the T-DNA, which will be transported from the bacterium into the plant cell. It is delineated by two 25bp imperfect direct repeats, known as the left and the right T-DNA borders. Further, the virulence (*vir*) region, a 35kb region composed of several major loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG* and *virH*) which protein products, the virulence proteins, respond to specific compounds secreted by the wounded plant. They generate a copy of the T-DNA and mediate its transfer into the host cell (reviewed by Gheysen *et al.* 1998).

On the other side plant specific factors, such as compounds (phenolics) that induce the expression of *Agrobacterium vir* genes are necessary (Stachel *et al.*, 1985). The attachment of virulent *Agrobacterium* to plant cells is a two-step process (Sheng and Citovsky, 1996; Gelvin

2000). First, the bacteria loosely bind to the host cell surfaces, a process that is likely mediated by a cell-associated polysaccharide (Reuhs *et al.*, 1997). This step is reversible because sheer forces, e.g. those generated by vortexing or washing, are sufficient to dislodge the bacteria (Gelvin 2000). In the second step the bound bacteria synthesise cellulose fibrils that stabilise the initial binding and results in a tight association between *Agrobacterium* and plant cell and enmeshes large numbers of bacteria at the wound surface (Matthysse 1983, 1987). Virulence inducing phenolic compounds were first described by Bolton *et al.* (1986) and are limited to dicolyledonous plants (Smith and Hood 1995). Gheysen *et al.* (1998) and Gelvin (2000) review the still not fully understood T-DNA transfer and integration process.

Several key factors involved in *Agrobacterium* mediated transfer to cereals have been described (Hiei *et al.*, 1997) and different strategies were applied to increase the transformation efficiency. A large number of *Agrobacterium* strains have been isolated and several of them were modified (Klee 2000) for use in transformation of recalcitrant species. An important step was the separation of *vir* and T-region from the large Ti-Plasmid in two separate, smaller plasmids. This so called binary plant vector system (Hoekema *et al.*, 1983) requires only easy cloning work in *Escherichia coli*, transformation of *Agrobacterium* with the T-DNA containing plasmid and allows the introduction of the manipulated T-DNA into plants. The *Agrobacterium* strain EHA101 for instance (Gordon-Kamm *et al.*, 1990; Hiei *et al.*, 1994; Rashid *et al.*, 1996), carries a dis-armed version of p*TiBo542* (Hood *et al.*, 1986), the Ti plasmid of the super-virulent A281 strain (Komari *et al.*, 1986). Another system is the ´super-binary` vector, where a fragment containing the *virB*, *virC* and *virG* genes (from p*TiBo542*), known to increase the virulence, were introduced into the T-DNA containing plasmid of a binary vector system (Komari 1990). Multiple T-DNAs were delivered to plant cells either from a mixture of strains or from a single strain and segregation of one T-DNA from others observed in various occasions (De Block and Debrouwer, 1991; De Framond *et al.*, 1986; McKnight *et al.*, 1987). In an other approach Komari *et al.* (1996) co-transformed tobacco and rice with unique plasmids carrying two separate T-DNAs and were able to separate them in successive generations by mendelian seggregation.

The induction of the *Agrobacterium vir* genes was greatly enhanced by addition of phenolic compounds, particularly acetosyringone (Vijayachandra *et al.*, 1995) during bacteria/plant co-cultivation, and was recognised as a key for successful transformation of rice (Hiei *et al.*, 1994). Further inducing factors are a low pH (Alt-Mörbe et al 1989; Godwin *et al.*, 1991;

Turk *et al.*, 1991), temperature (Alt-Mörbe *et al.*, 1989; Dillen *et al.*, 1997) and high osmotic pressure (Usami *et al.*, 1988). In presence of 100µ*M* acetosyringone, addition of certain carbohydrates did not have any significant synergistic effect (Hiei *et al.*, 1997) or was even lower than on conventional low sugar-medium (Enríquez-Obregón *et al.*, 1997). Bidney *et al.* (1992) enhanced *Agrobacterium* transformation frequencies by microprojectile bombardment as a wounding mechanism prior to co-cultivation. Uzé *et al.* (1997) however, reported that plasmolysis alone followed by *Agrobacterium* inoculation gave an even better transient expression than the combination of plasmolysis and bombardment. The genotype and type of tissue to be transformed, composition of culture media and elimination of *Agrobacterium* after co-cultivation (Nauerby *et al.*, 1997) further influence the efficient production of stable transformed plants.

Other DNA delivery protocols like macroinjection (Soyfer 1980; Zhou *et al.*, 1983), the use of silicon carbide whiskers (Wang *et al.*, 1995; Petolino *et al.*, 2000), ultrasound (Joersbo and Brunstedt, 1990) or laser mediated gene-transfer (Weber *et al.*, 1988) are of rather theoretical importance and were reviewed by Barcelo and Lazzeri (1998).

In order to develop an efficient transformation system for rye biolistic and A*grobacterium* mediated DNA-delivery strategies were applied on inbred lines with superior regeneration potential. Biolistic delivery parameters such as tissue age during bombardment and micro-projectile density were optimised in a multi-factorial experiment design with respect to the regeneration response and the transformation frequency. For *Agrobacterium* mediated gene delivery, different influencing factors specific for the plant-tissue as well as for the *A. tumefaciens*, were combined.

3.3. Biolistic gene transfer

3.3.1. Materials and Methods

Plants and explants

The inbred lines L22, L20 and L318 were used in experiments concerning optimisation of biolistic parameters. Lines L20 and L318 were grown with, L22 without previous

vernalisation and immature embryos were prepared and cultured as mentioned in Section 2.3.1.

Culture media and callus cultures

Based on a basic medium consisting in MS salts (Murashige and Skoog, 1962), 30g/l sucrose, 100mg/l casein hydrolysate and 500mg/l glutamine, callus induction medium (2.5mg/l 2,4-D; 3.0g/l phytagel) and osmoticum medium (2.5mg/l 2,4-D; 3.0g/l phytagel; 72.9g/l mannitol) were prepared. Medium for regeneration was prepared as callus induction medium but without 2,4-D.

Immature embryos were pre-cultured for one to seven days (L22) or one, three, five and seven days (L20 and L318) on callus induction medium and transferred on osmotic medium for 4 to 6 hours plasmolysis before bombardment. Bombarded calli were transferred back on fresh callus induction medium 12 to 16 h after bombardment, 14 days later on regeneration medium and after further three weeks into culture containers (Green Box from Duchefa, D 1604) for shoot elongation. Regenerated plantlets were sprayed with filtersterilised 0.05% Basta®–solution and resistant plants transferred to soil after approximately 3 further weeks *in-vitro*. For details related to the media preparation procedure and culture conditions during callus induction and regeneration, see Section 2.4.1.

Vectors and biolistic parameters

In this experiment the plasmid vectors pJFbar (Figure 4), containing the selectable marker gene *bar* (Thompson *et al.*, 1990) under control of the maize ubiquitin promoter with first intron and the 35-S terminator inserted in the pPZP111 vector (similar to pJFnptII: Altpeter and Xu, 2000) was used.

The Gold-DNA coating was prepared using equal amounts of 0.6 and 1.0 micron gold particles from BIO-RAD and 6µg DNA (containing the Vector pJFbar) per precipitation reaction according to Sanford *et al.* (1991). Each of the pre-culture treatments were bombarded with 150, 50 and 25µg gold particles/shot (adjusted by dilution 1:3 and 1:6 in Ethanol, respectively) using the PDS-1000/He BIOLISTIC Particle Delivery System and 1.100 psi rupture disks, both from Bio-Rad.

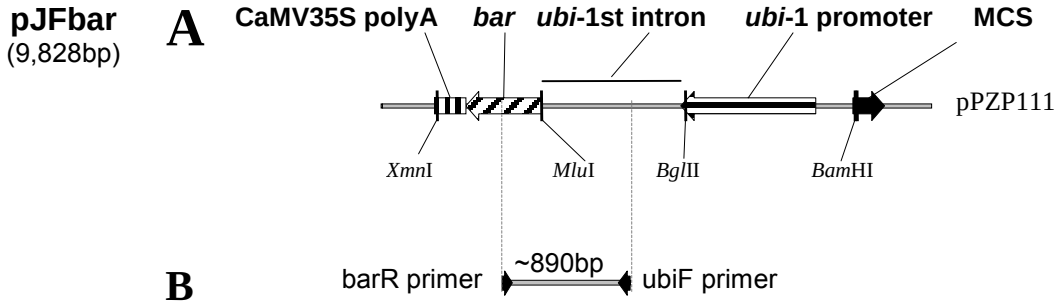


Figure 4: Schematic representation of the pJFbar vector.
(**A**) The expression cassette of the *bar* gene (Thompson *et al.*, 1987) under control of the maize ubiquitin promoter (*ubi*) and the *35S* terminator was cloned into the pPZP111 vector (Hajdukiewicz *et al.*, 1994). (**B**) Specific primers were designed for detection of *ubi*::*bar* in transgenic plants. (MCS: multiple cloning side)

Evaluation of tissue culture ability

To quantify the impact of the micro-particle bombardment on the tissue culture response of callus cultures the callus induction ability and the regeneration potential of bombarded calli was recorded. To evaluate the transformation efficiency PAT (*bar*) positive plants (see Section 5.2.4) were counted (in % of bombarded explants).

Experimental design and statistical analysis

Callus cultures were initiated on subsequent days, allowing to carry out the simultaneous bombardment of cultures differing in their pre-culture time in a set. This allows to eliminate the effects created by variable micro-projectile coating with DNA. Four replications were carried out, with 3×20 bombarded explants per combination of callus pre-culture time and particle density. For statistical data analysis the SAS System (SAS 1990) was used and the GLM procedure with the *t*-test applied.

3.3.2. Results and Discussion

Optimisation of biolistic parameters

Transient reporter-gene activity has been used to optimise biolistic parameters, including particle density per bombardment, distance to target cells and particle acceleration pressure (Chibbar *et al.*, 1991; Finer *et al.*, 1992). Transient expression frequency is positively correlated to the amount of particles per bombardment (Figure 5) but higher particle densities

reduce the regeneration response of the cultures, most probably due to tissue damage caused by the micro-projectiles. It seems therefore necessary to identify conditions that support the introduction of DNA with minimal tissue damage or interference with the callus regeneration potential.

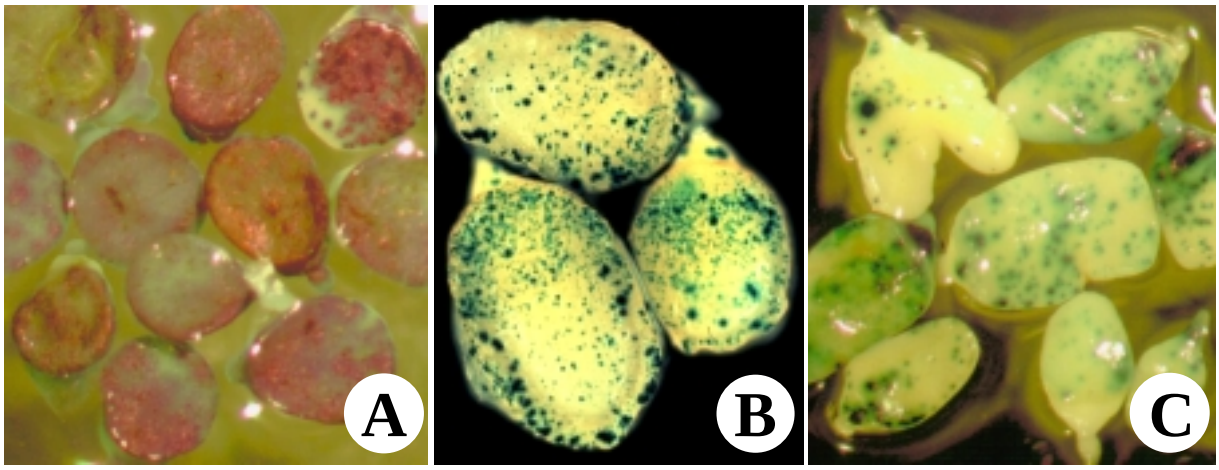


Figure 5: Transient GUS expression of immature rye embryos bombardment with different micro-particles amount, coated with the pHC25 GUS expression vector (Christensen and Quail, 1996). After bombardment with approximately 200µg (**A**), 100µg (**B**) and 30µg (**C**) micro-particles per bombardment.

Callus induction was significantly increased after bombardment with 25 to 150µg micro-particles per bombardment in comparison to the non-bombarded control (Table 8). Callose formation in the wound area might support callus induction. This is in accordance to cytological observations after particle bombardment of corn suspension cultures (Hunold *et al.*, 1994). However, the regeneration potential of the bombarded callus significantly decreased with increasing particle density. The number of regenerating calli and the number of regenerated shoots per callus were significantly reduced in all genotypes when 150µg micro-particles/bombardment were delivered, compared to non-bombarded cultures and 25µg to 50µg particles/bombardment (Table 8). Calli bombarded with 25µg or 50µg particles/bombardment did not differ in the number of regenerated shoots for any of the genotypes. Compared to non-bombarded calli only the bombardment with 25µg particles resulted in no significant difference in the number of regenerated shoots (Table 8). Castillo *et al.*, (1994) used 2500µg micro-particles/bombardment, in some cases in two bombardments, which might have resulted in a considerable reduction of the regeneration potential and consequently the majority of transgenic calli did not regenerate to transgenic plants in their

experiments. The presented data in this study suggest not to repeat bombardments as proposed by (Lonsdale *et al.*, 1990).

Osmotic treatment of calli prior particle bombardment (Vain *et al.*, 1993b) is a widely applied procedure to reduce tissue damage and consequently enhances stable transformation frequencies. The pre-culture time of the target tissue and the micro projectile density seem to be additional critical parameters (Altpeter *et al.*, 1996a; Takumi and Shimada, 1996). In this study, we therefore analysed the interaction of pre-culture time and micro projectile density on the callus induction and regeneration potential of different rye genotypes in a factorial designed experiment. Significant interactions between genotype×pre-culture time ($Pr>F$ = 0.000 and $Pr>F$ = 0.000) and genotype×particle density ($Pr>F$ = 0.005 and $Pr>F$ = 0.004) for callus induction ability and number of regenerated plants per callus respectively (Table 7 and Table 8), were observed.

Table 7: Influence of biolistic parameters on *in-vitro* response of rye (*Secale cereale* L.).

Source of Variance	*df*	Callus induction	Callus regeneration	Regenerated plants per callus
Error	322	23.9	247.7	1.0
Genotype	2	104.4^{+}	28240.5**	153.4**
Particle density	3	134.2**	12815.7**	44.1**
Pre-culture time	6	72.6*	1429.1**	11.6**
Genotype × Pre-culture time	6	258.3**	353.6	5.7**
Genotype × Particle density	6	75.1*	318.3	3.3*
Pre-culture time × Particle density	18	64.6**	256.3	1.1
Genotype × Pre-culture time × Particle density	18	112.9**	276.4	0.7

Critical F-values for analysis of significance: $^{+}$, * and ** = differences at $P<0.10$, $P<0.05$ and $P<0.01$ levels of significance, respectively. *df* = degrees of freedom.

The pre-culture time before bombardment had little effect on the callus induction ability which was in all treatments higher than 97% (Table 8). However, explants pre-cultured prior bombardment had a significant higher regeneration capacity then explants bombarded with no or short pre-culture time, indicating that pre-cultured explants are less sensitive to tissue damage. The number of regenerated shoots per callus was significantly lower on average of the three genotypes when explants were bombarded after a pre-culture period of less than 5 days (Table 8). Genotype L22 showed the highest regeneration response if tissues were

Table 8: Effect of microparticle density and callus induction time pre bombardment[1] on tissue culture response of immature embryo explants of rye (*Secale cereale* L.) and transformation frequency.

Biolistic Parameters		Response over all three tested Genotypes (means per treatment)				Response of genotype L22 (means per treatment)			
		callus induction	regenerating calli (% of calli)	shoots per reg. callus (mean)	transgenic plants	callus induction	regenerating calli (% of calli)	shoots per reg. callus (mean)	transgenic plants
Particle density (µg/shot)	C	96.22 ^{b}	81.09 ^{a}	4.96 ^{a}	0	96.54 ^{b}	88.94 ^{a}	5.75 ^{a}	0
	25	98.35 ^{a}	65.74 ^{b}	4.65 ^{ab}	6	99.34 ^{a}	73.05 ^{b}	5.54 ^{a}	4
	50	99.27 ^{a}	57.57 ^{c}	4.37 ^{b}	14	99.79 ^{a}	65.84 ^{c}	5.31 ^{a}	14
	150	99.76 ^{a}	39.23 ^{d}	3.92 ^{c}	1	99.68 ^{a}	43.72 ^{d}	4.71 ^{b}	1
Means / LSD		98.78 / 1.51	57.80 / 6.34	4.40 / 0.42		99.35 / 1.55	64.55 / 7.09	3.50 / 0.45	
Callus preculture (days)	1	97.50 ^{b}	47.67 ^{c}	3.15 ^{c}	0	99.36 ^{ab}	60.42 ^{bc}	3.77 ^{f}	0
	2	n.a.	n.a.	n.a.	0	100.00 ^{a}	68.98 ^{ab}	4.64 ^{e}	0
	3	99.37 ^{ab}	58.13 ^{ab}	3.81 ^{b}	3	100.00 ^{a}	67.04 ^{ab}	4.85 ^{de}	3
	4	n.a.	n.a.	n.a.	5	99.75 ^{a}	56.65 ^{c}	5.30 ^{cd}	5
	5	98.65 ^{ab}	54.95 ^{b}	4.36 ^{a}	7	97.85 ^{b}	62.82 ^{bc}	5.60 ^{bc}	6
	6	n.a.	n.a.	n.a.	2	98.50 ^{ab}	62.92 ^{bc}	5.98 ^{b}	2
	7	98.50 ^{ab}	62.52 ^{a}	4.77 ^{a}	4	100.00 ^{a}	74.86 ^{a}	6.58 ^{a}	3
/ LSD		/ 1.97	/ 6.34	/ 0.42		/ 1.89	/ 8.67	/ 0.55	

[1]: After bombardment, calli were cultured 14 days on callus induction medium and four weeks on regeneration medium

[a, b, c, d, e, f]: Means followed by the same letters are not significantly different from each other according to *t*-test (LSD) at the 5% alpha level of significance

n.a.: not analysed

bombarded after a 7 days pre-culture period (Table 8). Fourteen days after biolistic transfer of a constitutive *bar* expression cassette the calli were transferred to regeneration conditions.

The regenerated plants were sprayed with Basta® without any prior selection in tissue culture. A total of 21 transgenic rye plants with independent transgene integration pattern, as indicated by Southern blot analysis (Section 5.3.1) were obtained from three genotypes (L22, L20 and L318) (Table 8, Figure 15). Expression of the phosphinothricin acetyltransferase (PAT) was confirmed by tolerance to Basta® and PAT assay (Table 8, Figure 15B). Optimum biolistic gene transfer parameters for high regeneration and high transformation efficiencies were in accordance (Table 8). Highest transformation frequencies (2.22%) were observed when explants were pre-cultured for 4 to 5 days before bombardment with 50µg micro-particles. Without pre-culture prior to biolistic gene transfer, no transgenic plants were recovered at any density of micro-particles. The bombardment of calli with high micro-projectile densities of 150µg resulted only in one transgenic but sterile plant (b17, Figure 15A) and required a pre-culture period of 7 days. From genotype L22, a total of 19 transgenic lines were obtained from a range of conditions. In contrast, from genotype L20 and L318 only one transgenic plant each was obtained after bombardment with the lowest micro-particle amount (25µg). The presented data (Table 8) demonstrate the importance of a detailed analysis of the gene-transfer parameters with respect to the tissue culture performance and regeneration potential of the individual genotype. The negative impact of the bombardment is more pronounced in genotypes with poor response in tissue culture. An adjustment of gene-transfer parameters was also suggested by Altpeter *et al.* (1996a) and Takumi and Shimada (1996) and genotypic differences in the regeneration and transformation response were accordingly reported for barley (Koprek *et al.*, 1996) and wheat (Rasco-Gaunt *et al.*, 2001; Varshney and Altpeter, 2002).

Most transformation protocols use the combination of selectable markers with corresponding selective agents to avoid overgrowth of transgenic by non-transgenic callus and to identify the transgenic events during the tissue culture process.

This study demonstrated that no tissue culture selection step is necessary for the regeneration of transgenic rye plants. Spraying the regenerated plantlets with 0.05% commercial Basta® solution before transfer to soil resulted in the reproducible identification of transgenic rye with no interference of the selective agent with the regeneration process. This indicates that

with the appropriate timing of bombardment and regeneration of calli, the overgrowth of transgenic tissues by non-transgenic tissues is not significant. Consequently, transgenic rye plants can also be produced without any selectable marker gene and could be identified by PCR (Section 4.4).

3.4. *Agrobacterium tumefaciens*-mediated gene transfer

3.4.1. Materials and Methods

Plants and explants

Donor plants of the inbred line L22 were grown in the greenhouse at approximately 20°C and 16h light at the time of flowering. Surface sterilisation of immature caryopses and preparation of immature embryos were previously described (Section 2.3).

Culture media and callus cultures

Based on a basic medium (BM) consisting of MS salts (Murashige and Skoog, 1962), 100mg/l casein hydrolysate and 500mg/l glutamine, different culture media were prepared and autoclaved at 121°C and 1.5bar for 20min. Immature embryos were placed scutellum side up on callus induction medium (BM; 30g/l sucrose; 2.5mg/l 2,4-D; pH 5.8; 3.0g/l phytagel) and pre-cultured in the dark at 25°C during 5 days before transformation. In a second treatment calli were directly cultured in liquid callus induction medium (callus induction medium without gelling agent). For transformation, immature embryos either pre-cultured or not, were placed into 6×macroplates (Greiner Cellstar) and suspended in 10ml liquid callus induction medium. For osmotic treatment liquid medium was exchanged by 10ml osmotic medium (BM; 30g/l sucrose; 6.0mg/l 2,4-D; 72.9g/l mannitol; pH 5.8) and explants were plasmolysed for 4-6h. Osmotic medium was removed and calli were inoculated with approximately 300µl *Agrobacterium* suspension, vacuum treated at 500mbar for 1min and finally kept for 10min in the laminar hood. Explants were gently washed in 10ml infection medium (BM; 15g/l sucrose; 15g/l glucose; 6.0mg/l 2,4-D; pH 5.2; 200µ*M* acetosyringone) two times and co-cultured overnight at 22°C. After 14-16h, explants were washed again several times in infection medium and finally transferred to solid co-culture medium (infection medium supplemented with 3.0g/l phytagel) with scutellum-side up. Explants were co-cultured for further two days and then transferred on callus culture medium containing

150mg/l timentin for inhibition of *Agrobacterium* growth. After 14 days calli were transferred on selective regeneration medium (BM; 30g/l sucrose; pH 5.8; 5.0g/l agarose type I; 150mg/l timentin; 30mg/l paromomycin sulphate) and after further three weeks into culture containers containing selective regeneration medium with 50mg/l paromomycin sulphate (Green Box from Duchefa, D 1604) for shoot elongation

Vector and *Agrobacterium* pre-culture

The vector pYFnptII (Figure 6), containing the selectable marker gene *npt*II under control of the maize ubiquitin promoter with first intron and the 35-S terminator inserted in the pPZP111 vector (Figure 6) was introduced into the *Agrobacterium tumefaciens* strain AGLO (Lazo *et al.*, 1991) by electroporation (Mersereau *et al.*, 1990). An AGLO (pYFnptII) culture was grown overnight in LB medium supplemented with 50mg/l up to saturation (OD_{660} 2-2.5), 2ml were centrifuged at 5000rcf for 5min and the pellet dissolved in 1ml LB medium and 1ml infection medium. Before infection of explants bacteria were incubated again for approximately two hours (OD_{660} 1.5-2.0).

Evaluation of tissue culture ability

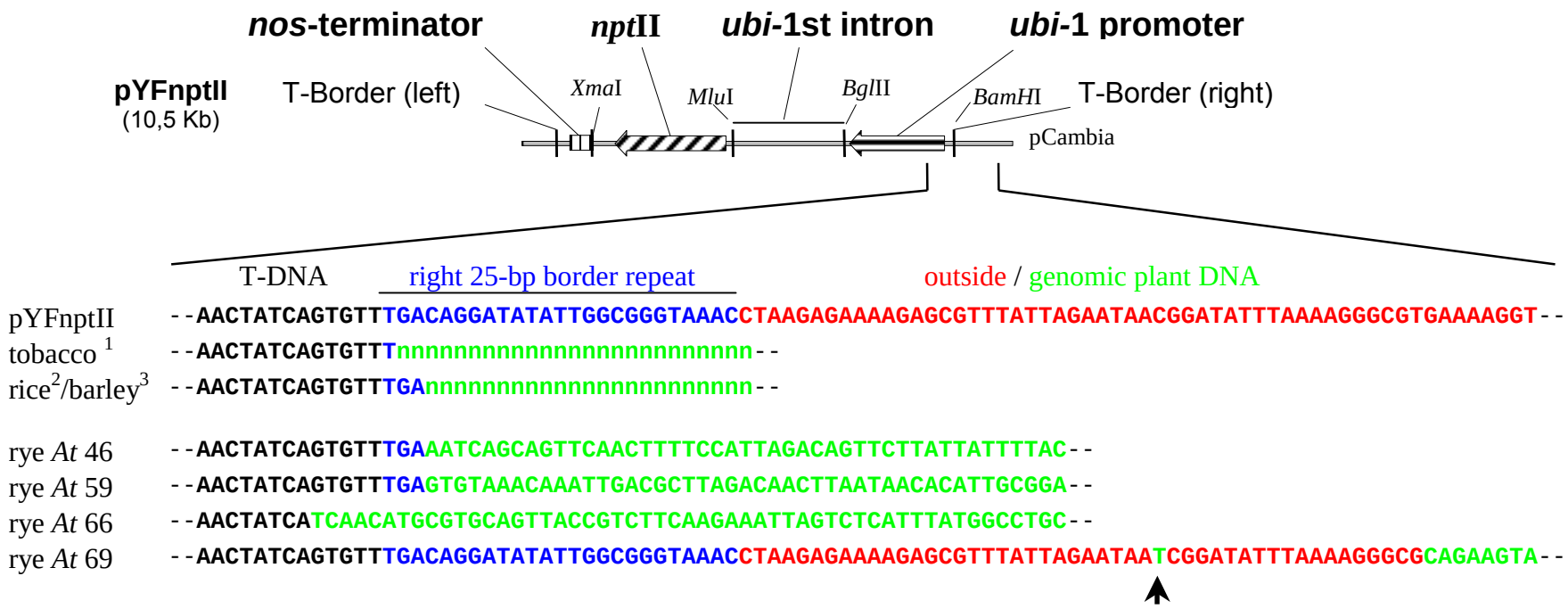
The transformation efficiency was recorded by counting the number of NPTII-ELISA (*npt*II) positive plants (in % of inoculated explants).

Experimental design and statistical analysis

Per treatment 800 explants were cultured and inoculated with *Agrobacterium* and 25 explants each as regeneration and selection control, were cultured.

Analysis of T-DNA::plant DNA junction sequences

The junction region of the T-DNA boundary and the rye genome were amplified using inverse-PCR (Ochman *et al.*, 1990). For this, the DNA of transgenic rye plants was digested with *Bam*HI or *Bgl*II, circularised by T4 DNA-ligase and then used as template for PCR. Nested DNA-amplification was performed with the GeneAmp-PCR System 9700 (Perkin Elmer) with reaction conditions according to the manufacturers advice; using 200ng template DNA in the first reaction and 0.5µl of the first reaction as template for the second, with a respective final volume of 25µl.



[1] = Yadav *et al.* (1982) and Zambryski *et al.*(1982); [2] = Hiei *et al.* (1994); [3] = Fang *et al.* (2002)

Figure 6: Sequences analysis of T-DNA::plant DNA junctions of transgenic rye (*Secale cereale* L.) plants

Sequences of the right border junctions are shown below the sequences of the T-DNA borders of pYFnptII vector plasmid. Sequences presumably originated from rye genomic DNA are shown green letters (Abbreviations: n= any nucleotide).

For the right border (RB) the RB1R 5´- CTG AAT GGC GAA TGC TAG AGC AG -3´ (LacZ region) and UBIF 5´- CTG CAG TGC AGC GTG ACC CG -3´ (3´-region of the maize ubiquitin promoter) primer were used for the first (28 cycles of 94°C for 30s, 48°C for 60s and 72°C for 2min), and the RB2R 5´- CGT TTC CCG CCT TCA GTT TAA AC -3´ and UBIF primer for the second reaction (32 cycles of 94°C for 30s, 52°C for 60s and 72°C for 2min). PCR amplification products with blunt ends were obtained by adding Pwo DNA-polymerase to the second reaction mixture and were cloned into the PCR vector (Invitrogen, San Diego, CA) followed by sequence analysis.

3.4.2. Results and Discussion

For the first time transgenic rye (*Secale cereale* L.) plants were generated after co-culivation with *Agrobacterium tumefaciens* (Table 9). The presented data demonstrate the advantages of the described protocol including the production of normal and fertile plants with predominantly single trangene inserts (Section 5.3.1, Figure 16) with defined T-DNA borders (Figure 6) segregating in mendelian fashion (Section 5.3.2, Table 16). Also a large number of independent tansgenic rye plants could be produced with a transformation efficiency of 3% of the cultured explants, resulting in transgenic plants (Table 9). One commonly observed problem with *Agrobacterium*-mediated gene transfer is the over-infection of explants. Minimising the risk of over-infection with minimal effort was achieved in the presented protocol with a liquid culture system and allowed to carry out large-scale experiments.

Table 9: Stable *npt*II expression after co-cultivation of immature rye (*Secale cereale* L.) embryos (genotype L22) with *Agrobacterium tumefaciens*.

Explants		Callus appearance (in %)		Regenerated plants		
Pre-culture	Number	Necrotic	Shoot primordia	Total number	ELISA positive	Transformation frequency[1]
No	800	42.1	25.7	0	0	0
5 days	800	72.5	26.7	145	31	3.87
5 days	500	n.d.	n.d.	19	4	0.80

[1]: in percent of inoculated explants; n.d. : not determined

The regeneration response of cultures pre-cultured for one day on liquid medium did not differ significantly from cultures grown on solid medium particularly when the explants

were cultured with the coleoptyle axis in contact with the culture medium (Table 10). The regeneration response was reduced if the pre-culture on liquid callus induction medium was extended to three days or more and was particularly affected when the explants were afterwards cultured with the scutellum in contact with the medium (Table 10). In this case germination of the explants reduced the callus induction frequency. Removing of the embryonic axis did not increase the induction of embryogenic callus. Tissue developing from the coleoptyle side of the scutellum was white, soft and aqueous in appearance.

Table 10: Influence of pre-culture conditions on regeneration response immature embryo explants of rye (*Secale cereale* L.) genotype L22.

Pre-culture on liquid medium (days)	Scutellum in contact with medium[1]		Coleoptile in contact with medium[1]	
	Regen. calli (% of calli)	Shoots per reg. callus (mean)	Regen. calli (% of calli)	Shoots per reg. callus (mean)
0	33	5.2	68.0	2.9
1	30	6.7	90	5.3
3	10	6.3	40	6.0
5	0	0	40	5.8

[1]: pre-cultured embryos were transferred on solid callus induction medium with scutellum side up or down and cultured for three weeks before transfer to regeneration medium.

Several factors were applied that might have a positive effect on the gene transfer efficiency. Explants were plasmolysed on a 2,4-D rich osmotic medium (6mg/l) for four to six hours prior to inoculation with *Agrobacterium*. The high auxin concentration might increase the cell growth rate during the co-culture process and the high osmotic condition might enhance the plant-*Agrobacterium* contact (Uzé *et al.*, 1997). Explants were inoculated with the *Agrobacterium* suspension under a vacuum, which might also enhance the contact between plant tissue and bacteria. The rye cultures were transferred to medium containing sucrose, acetosyringone and high level of 2,4-D and explants were rinsed two times before overnight incubation at 22°C. Rinsing resulted to be crucial to avoid overgrowth of tissue cultures with *Agrobacterium*. The attachment of virulent *Agrobacterium* to plant cells is a two-step process (Sheng and Citovsky, 1996; Gelvin 2000) where the bacteria first bind loosely to the host cell surfaces by a cell-associated polysaccharide (Reuhs *et al.*, 1997) and then synthesise cellulose fibrils that stabilise the

initial binding and results in a tight association between *Agrobacterium* and plant tissue (Matthysse 1983, 1987). Therefore explants could be rinsed intensively after the overnight liquid co-culture before transfer on solid infection medium. Plant specific phenolic compounds that induce the expression of *Agrobacterium vir* genes are important (Stachel *et al.*, 1985). In monocots, where such compounds are normally not synthesised, addition of phenolic compounds like acetosyringone after inoculation with *Agrobacterium* during bacteria/plant co-cultivation supports the gene-transfer (Vijayachandra *et al.*, 1995). This was recognised as a key to successful transformation of rice (Hiei *et al.*, 1994). However, acetosyringone was not necessary for *Agrobacterium*-mediated barley transformation (Tingay *et al.*, 1997). Also a comparatively low pH (Alt-Mörbe et al 1989; Godwin *et al.*, 1991; Turk *et al.*, 1991), temperature during co-cultivation (Dillen *et al.*, 1997), and micro-projectile bombardment as a wounding mechanism (Tingay *et al.*, 1997; Bidney *et al.*, 1992) have been described as factors influencing *Agrobacterium*-mediated gene-transfer. The tissue was not damaged in this study neither by removing the embryonic axis nor by bombardment since regeneration potential of rye is negatively impacted by these procedures. Three days after co-cultivation at 22°C, explants were transferred on callus culture medium with 100-150mg/l. This strongly inhibited *Agrobacterium* overgrowth which otherwise interferes with the efficient callus development and regeneration of stable transformed plants.

Calli were transferred on regeneration medium containing 50mg/l paromomycin sulphate for suppression of non-transgenic plants. Only calli derived from explants pre-cultured prior gene transfer, regenerated plantlets and a total of 31 rye plants expressing the selectable marker gene *npt*II were identified by ELISA (Table 9). This corresponds to a transformation efficiency of 3.9% related to the number of pre-cultured embryos that were co-cultured with *Agrobacterium* and is similar to previous reports in other cereal crops (Ishida *et al.*, 1996; Cheng *et al.*, 1997; Tingay *et al.*, 1997). Southern blot hybridisation confirmed the independent nature of all analysed transgenic plants (Section 5.3.1; Figure 16).

The boundary regions of genomic rye DNA and the integrated T-DNA's were amplified and nucleotide sequences were determined (Figure 6). The right boundaries of lines *At*46 and *At*59 showed that defined T-DNA´s were inserted with exclusion of vector

sequences, similar to the reports in tobacco (Yadav *et al.*, 1982; Zambryski, *et al.*, 1982), rice (Hiei *et al.* 1994) and barley transformants (Fang *et al.*, 2002), supporting the hypothesis that the molecular mechanisms involved in T-DNA transfer to monocots is similar to those in dicot plants. This further supports the hypothesis that the *vir*-D2 protein nicks the 25bp right border repeat and nicks covalently with the 5´-end of the T-strand to protect the right border from degradation (Herrera-Estrella *et al.*, 1988). In contrast to previous reports examples were also found were either five bases of the T-DNA were deleted next to the right border (*At*66, Figure 6) or vector backbone sequences of at least 29 bases were inserted beyond the 25bp right border repeat region (*At*69, Figure 6). This stresses the importance of detailed analysis of the transgene insert. The whole T-DNA transfer process is still not fully understood and needs further investigations. Integration of defined T-DNA´s with exclusion of vector DNA might be an important regulatory issue for the field release of transgenic crops and might support stability of transgene expression (Iglesias *et al.*, 1997).

In the previous report on rye transformation the total time required from inoculation to the establishment of transgenic plants in soil was more than six months (Castillo *et al.*, 1994) while in the here described protocol only 42 days from culturing the immature embryos until transfering the regenerated plantlets to soil where required. Transgenic plants obtained in this experiment were phenotypically normal and fully fertile which might be due to a short *in-vitro* culture period and selection exclusively during regeneration.

4. Selection of transgenic rye

4.1. Summary

Suitable selection parameters, with respect to the regeneration response of rye tissue cultures were analysed for three widely used selection agents for transformation of cereal crops. Appropriate selection agent concentrations and preferred culture period for the selection process were determined. On the basis of an efficient tissue culture system (Chapter 2) and optimised biolistic DNA delivery conditions (Section 3.3), transformation experiments were carried out and two selection strategies were applied. One protocol, based on the selectable marker gene *npt*II, allowed the control of most of the escapes if applied only during the regeneration period. Transformation frequencies of up to 2.0% were achieved and a total of 17 NPTII expressing plants were identified. Applying a second selection strategy based on PCR-analysis of regenerated plantlets, 17 transgenic rye plants free of any kind of selectable or screenable marker gene were produced.

4.2. Introduction

Generally, successful genetic transformation requires not only efficient gene delivery but also an efficient selection system to distinguish transgenic from non-transgenic events. Different selection strategies have been proposed and successfully applied in plant transformation (Wilmink and Dons, 1993).

A number of selective agents and suitable resistance genes were investigated concurrently with studies on gene transfer and tissue culture. In cereal transformation the most widely used selectable marker genes include *bar* (Thompson *et al.*, 1987), *hph* (Waldron *et al.*, 1985) and *npt*II (Mazodier *et al.*, 1985), encoding phosphinothricin acetyl transferase (PAT), hygromycin phosphotransferase (HPT) and neomycine phosphotransferase (NPT), that confer resistance to phosphinothricin (PPT), hygromycin B and kanamycin and other related aminoglycosides (G418 and paromomycin sulphate), respectively. In cereals mainly the *bar* gene has been used (Vasil *et al.*, 1992, 1993; Weeks *et al.*, 1993; Castillo *et al.*, 1994; Becker *et al.*, 1994; Nehra *et al.*, 1994; Altpeter *et al.*, 1996a; Koprek *et al.*, 1996), but *npt*II (Torbert *et al.*, 1995; Davey *et al.*, 1991; Nehra *et al.*, 1994) and *hph* (Christou and Ford, 1995; Hagio *et al.*, 1995; Ortiz *et al.*,

1996; Li *et al.*, 1997) are also reported to be suitable for selection of cereal transformants. In these systems transformed cells are able to survive while non-transformed cells are killed by the selective agent (Joersbo and Okkels, 1996; Hansen and Wright, 1999).

The use of selective agents may have negative effects not only on non-transformed cells, but could also suppress the regeneration of transgenic tissue. Therefore, a second selection strategy, using selectable marker genes based on physiological advantage rather than detoxification has been described and is called positive selection. Mannose was recently reported as a novel selectable agent and the phosphomannose isomerase gene (*pmi*) as its suitable selectable marker (Joersbo and Okkels, 1996). In contrast to herbicides or antibiotics, mannose has no direct adverse effects on plants as the toxicity is not mediated by the compound per se. The addition of sucrose can alleviate the effect of mannose on growth and germination. Genetically transformed cells are able to utilise mannose as carbon source and acquire a growth advantage on mannose-containing media. It is therefore called positive selection (Joersbo and Okkels, 1996). Successful genetic transformation with *pmi* as selectable marker resulted in a tenfold increase in transformation frequencies over kanamycin selection in sugar beet (*Beta vulgaris* L.) (Joresbo *et al.*, 1998), which was as high as 30% (Negrotto *et al.*, 2000) in protoplast cultures of maize and over 40% in callus cultures of rice (Lucca *et al.*, 2001) and maize (Wright *et al.*, 2001).

Beside the selectable agent, the timing of selection and culture practices influence the selection success and appeared to be more critical for the recovery of transgenic callus and plants than the level of selection used (Christou and Ford, 1995).

Once an intact transgenic line has been established the selectable marker gene is generally superfluous and does not generally contribute positively to the final product. The presence of antibiotic and herbicide resistance marker genes in transgenic crop plants has generated consumer concerns (Zechendorf 1994). Although risk assessment reports have argued that there are no scientific, health or safety reasons to restrict the use of *npt*II as selectable marker (Malik and Saroha, 1999), this might not apply to all selectable markers. Regardless of the assurances provided in risk assessment reports it is consumer acceptance of a product that governs its market performance (Gleave *et al.*, 1999). In addition, the presence of a selectable marker gene in a transgenic plant

precludes using the same marker in subsequent transformations. Therefore, elimination of marker genes would be a desired aim. Several strategies have been employed to generate marker-free transgenic plants. These include: transposition mediated repositioning of the marker gene (Goldsbrough *et al.*, 1993), co-transformation of two T-DNA molecules (McKnight *et al.*, 1987; De Block and Debrouwer 1991; Komari *et al.*, 1996) and site-specific recombination (Dale and Ow, 1991; Russel *et al.*, 1992a). The construction of respective transformation vectors is complicated and tedious sexual crosses and segregation studies are necessary to get plants free of marker gene. Beside these, transient expression of the *cre/lox*P site-specific recombination system (Dale and Ow, 1990; Gleave *et al.*, 1999) in combination with a conditional lethal dominant gene *cod*A (Perera *et al.*, 1993) facilitated the elimination of marker genes from transgenic plants without sexual crossing. The efficiency of such systems is reduced compared to protocols that use selectable marker genes. A recent approach demonstrated in tobacco by Sugita *et al.* (2000) showed the use of the chemically inducible GST-II-27 promoter (Bridges *et al.*, 1993) fused to the R recombinase (Sugita *et al.*, 1999) to control an excision of the isopentenyltransferase (*ipt*) selectable gene (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984). Recently, transgene removal systems denoted as Multi-Auto-Transformation (MAT) vectors have been developed to generate marker-free transgenic plants through a single step transformation (Ebinuma and Komanine, 2001; Ebinuma *et al.*, 2001).

On the basis of the established tissue culture system (Chapter 2) different selection strategies were evaluated for their selection effect in rye callus cultures. Using the selective agents phosphinothricin, paromomycin sulphate and hygromycin B, we optimised tissue culture and selection parameters with respect to regeneration response and identified reasonable concentrations and suitable developmental stages for their application. Further the use of mannose/sucrose as possible agent for positive selection in rye was assessed. On the basis of suitable biolistic parameters (Section 3.2) and selection parameters, transformation experiments were performed with the selectable marker gene *npt*II. Based on PCR analysis of *in-vitro* regenerated plants a selection system for the "direct" production of transgenic plants, free of selectable marker gene, was developed.

4.3. Optimisation of selection process

4.3.1. Materials and Methods

Explants and tissue culture

To identify suitable selection agents suitable for selection in rye callus cultures three selection agents, the herbicide phosphinothricin (0.5 to 4mg/l), and the antibiotics hygromycin B (1 to 60mg/l) and paromomycin sulphate (10 to 100mg/l) were compared for their selection effect. Also the selection effect of mannose/sucrose (0/10, 0/15, 0/30, 1.24/0, 1.24/10, 1.24/15, 1.24/30, 1.75/0, 1.75/10, 1.75/15, 1.75g/30g per Liter) as positive selection strategy was tested. For this, the pre-cultured scutellar calli of line L22 were transferred on selective callus induction media (14 d after culture initiation) or directly on selective regeneration medium (21 d after culture initiation). For preparation of selective media, selective agents were filter-sterilised and added to the lukewarm autoclaved callus induction or regeneration medium (Section 2.2.1). Paromomycin sulphate containing media were prepared with 0.5% Agarose Type I instead of phytagel, since this chemical precipitates in presence of gelrite or phytagel, as also observed for kanamycin by Chauvin *et al.* (1999).

Evaluation of tissue culture ability and experimental design

Per selective agent and -concentration two petridishes with 12 calli each were cultured and the regeneration response quantified by counting the calli with regenerating shoots three to four weeks after transfer on regeneration medium, as well as the number of regenerated shoots per callus. Callus evaluation was repeated independently by two persons and means per experimental unit were calculated. From each treatment several representative calli were selected and photographily documented with a Nikon SMZ800 stereomicroscope and the Lucia Image Analysis System (V. 4.21). By this way, the development of single calli could be followed during the callus induction and regeneration process.

4.3.2. Results and Discussion

The effect of three commonly used selection compounds the herbicide PPT, and the antibiotics hygromycin B and paromomycin sulphate was documented in different

culture media on representative calli (Figure 7, Figure 8 and Figure 9, respectively), after application of different concentrations in the callus induction media. The influence of selection agent on regeneration efficiency after selection during callus induction and regeneration or exclusively during callus regeneration is shown in Table 11. All agents suppressed callus embryogenesis. This was more pronounced on PPT containing medium where calli were soft and structureless even at a concentration of 1.0mg/l (Figure 7) and shoot regeneration was strongly suppressed.

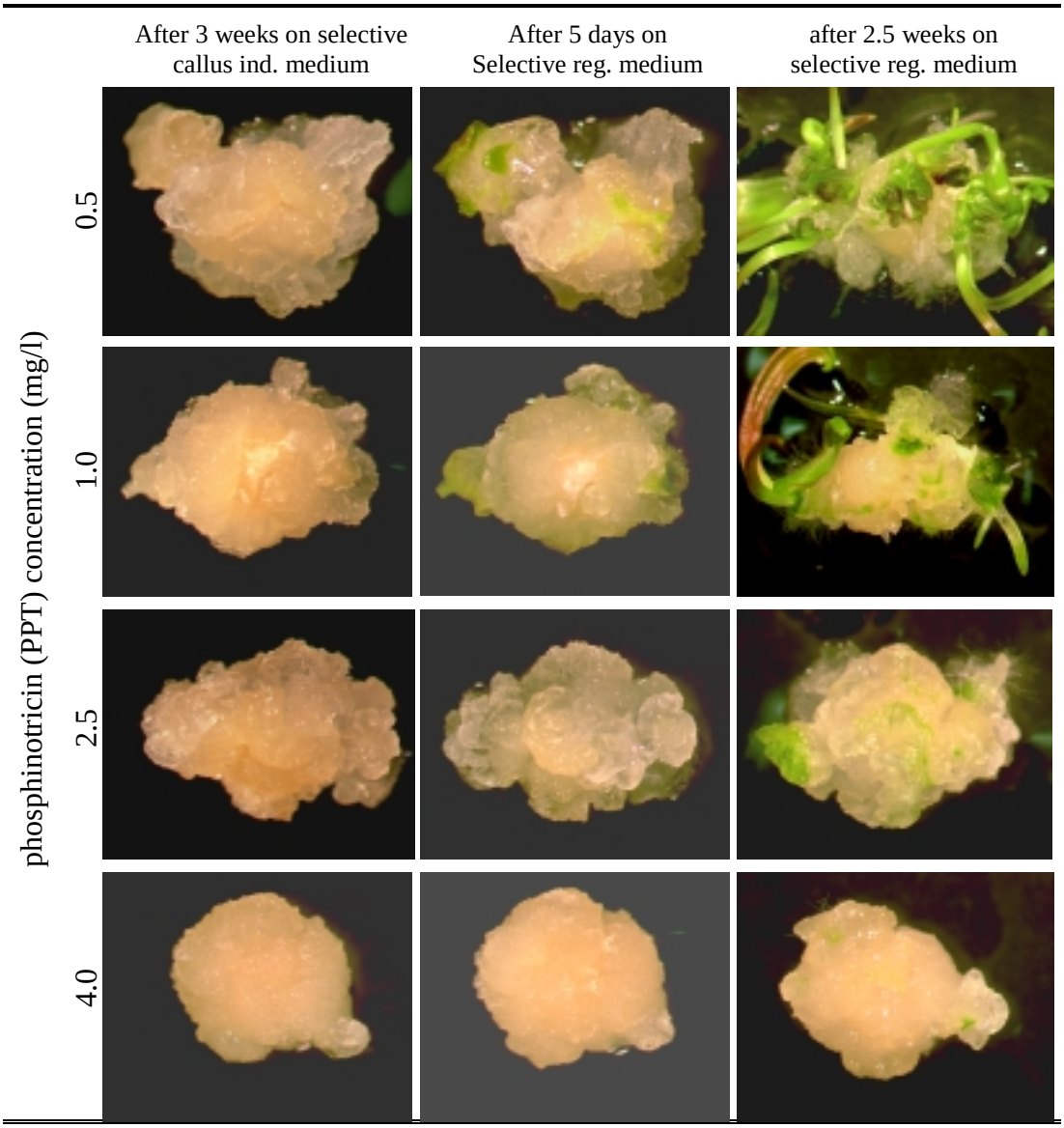


Figure 7: Effect of the herbicide phosphinothricin (PPT) on growth and regeneration response of callus from rye (*Secale cereale* L.) inbred line L22 (callus-ind. = callus induction; reg. = regeneration)

Only 25% of calli grown on selective callus induction medium with 4mg/l PPT regenerated shoot primordia. After several sub-cultures on Basta® or PPT containing medium the majority of transgenic rye calli had lost their regeneration potential (Castillo *et al.*, 1994). Also the number of shoot primordia were lower when PPT selection was applied during both, callus induction and regeneration phase. Application of the selective agent exclusively during the regeneration process might be desirable if a selective agent negatively impacts embryogenesis of transgenic calli. This selection strategy requires a strong suppression of shoot and root development of the non-transgenic escapes. If PPT was applied exclusively during rye callus regeneration a strong suppression of shoot and root regeneration was not observed.

In other reports on cereal transformation 1 to 5mg/l PPT was described as adequate to select transformed cells (De Block *et al.*, 1987; Gordon-Kamm *et al.*, 1990; Jähne *et al.*, 1994; Koprek *et al.*, 1996; Tingay *et al.*, 1997; Zhang *et al.*, 1999b) but concentrations up to 50mg/l Bialophos were also applied (Christou and Ford, 1995).

It has been shown that other media components, mainly amino acids influence the selective effect of PPT. Glutamine, proline and arginine for instance, allowed growth of non-transformed cells in presence of the herbicide (Dekeyser *et al.*, 1989). Carbohydrate source as well as light conditions applied were also found to influence the sensitivity to PPT (De Block *et al.*, 1995), demonstrating the influence of the metabolic status of the tissue.

Callus quality was less affected by hygromicin B and embryogenic structures were observed even at relatively high concentrations of 40 and 60mg/l (Figure 8). However, hygromicin B drastically suppressed the regeneration potential of the callus cultures and the average number of shoot primordia was drastically reduced even at relatively low concentration of 10mg/l (Table 11). Hygromycin B has been successfully used in monocot transformation at the concentrations between 25mg/l (Datta *et al.*, 1992; Ortiz *et al.*, 1996) and 100mg/l (Christou and Ford, 1995).

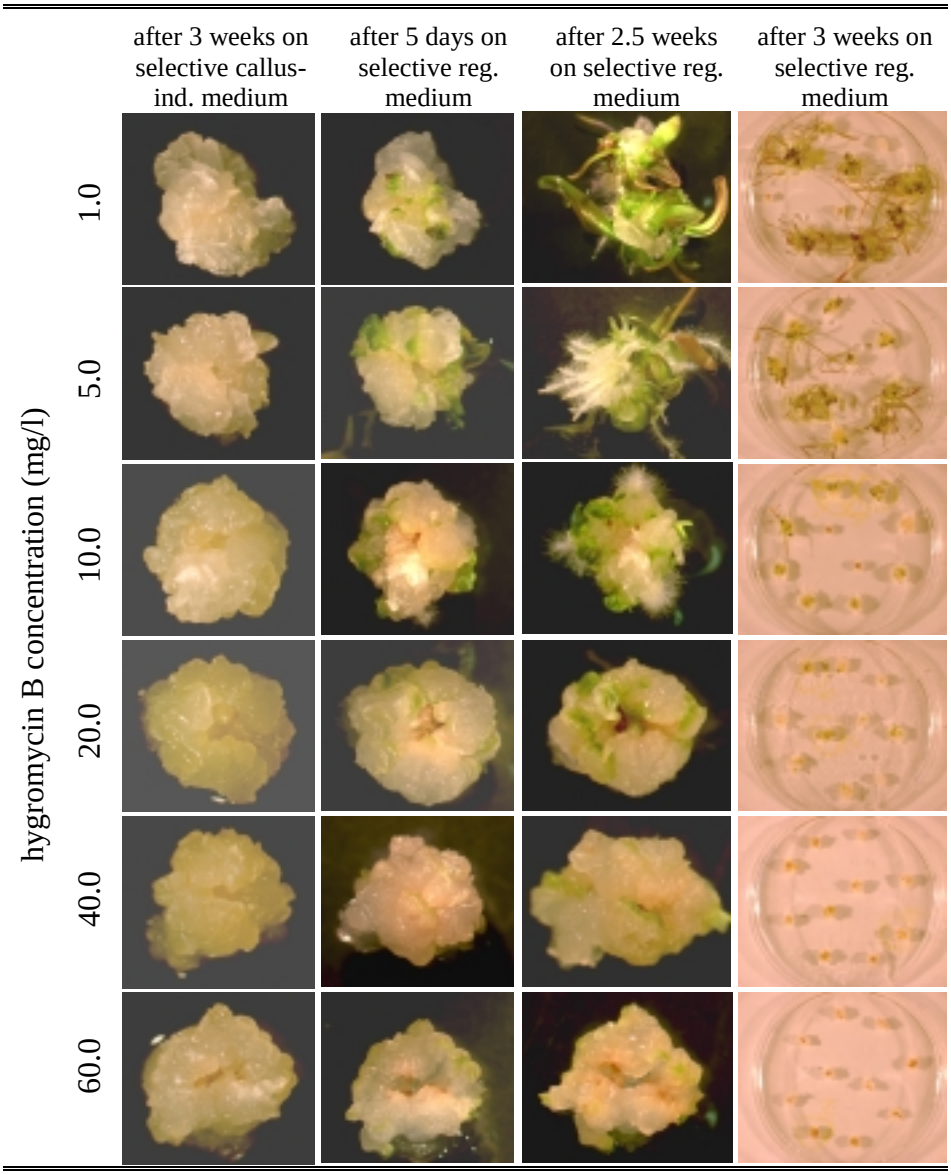


Figure 8: Effect of the antibiotic hygromycin B on growth and regeneration response of callus from rye (*Secale cereale* L.) inbred line L22 (callus-ind. = callus induction; reg. = regeneration)

In transformation experiments on dicotyledonous species based on the *npt*II selectable marker gene, kanamycin is a commonly used selection agent. However, it has been shown that this compound has no clear selection effect in cereals (Dekeyser *et al.*, 1989). The same selectable marker gene also confers resistance to paramomycin sulphate which caused toxicity effects on rye callus that were characterised by browning at concentrations higher than 50mg/l. Callus appeared to be hard and woody with no embryogenic structures (Figure 9). As on PPT containing medium, its effect on regeneration was strongest when applied during callus induction (Figure 7). However,

100mg/l paromomycin fully suppressed shoot and root formation if applied exclusively during the regeneration process of rye tissue cultures (Table 11). This allows to develop a selection protocol that will not interfere with callus embryogenesis and should realise the full regeneration potential of transgenic calli. Paromomycin sulphate was also the superior selective agent for the transformation of oat (Torbert *et al.*, 1995) and turfgrass (*Lolium pereene* L. and *Festuca rubra* L.) (Altpeter *et al.*, 2000).

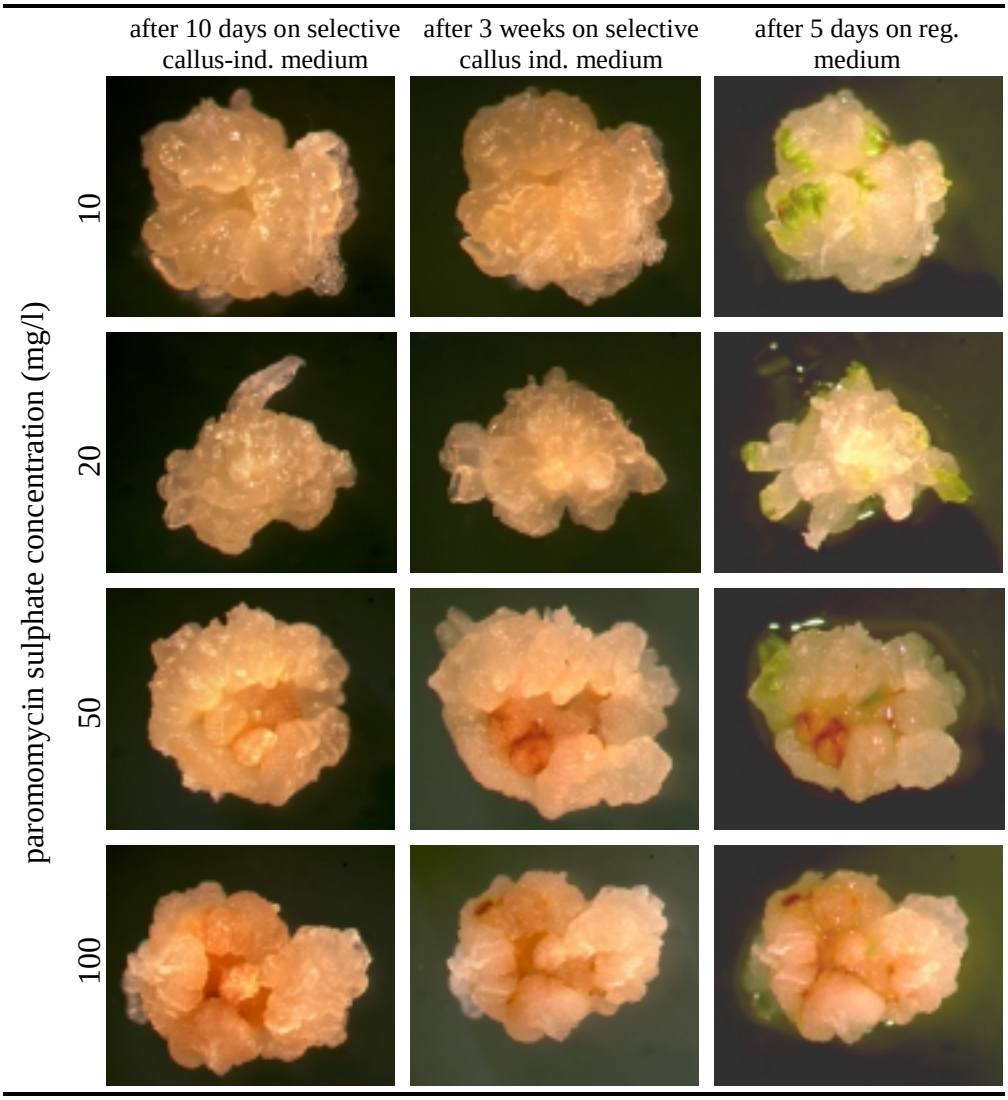


Figure 9: Effect of the antibiotic paromomycin sulphate on growth and regeneration response of callus from rye (*Secale cereale* L.) inbred line L22 (callus-ind. = callus induction; reg. = regeneration)

While in selection protocols based on herbicides or antibiotics non-resistant cells are killed, protocols for positive selection are based on physiological advantage and wild

type cells vegetate rather than die. Mannose is converted to mannose-6-phosphate and accumulates with a concomitant consumption of the phosphate and ATP pools resulting in severe growth retardation (Ferguson *et al.*, 1958). If other carbohydrate sources are limited only cells able to convert the derivatised selective agent to fructose-6-phosphate could maintain their energy status. Therefore, in selection protocols based on mannose the availability of other carbon sources such as sucrose should be considered. To analyse the endogenous ability of rye to use mannose as energy source, pre-cultured calli were cultured on media containing different amounts of sucrose and mannose. Callus development (Figure 10) and regeneration response were quantified (Table 12).

Table 11: Selection effect of the herbicide phosphinothricin, the antibiotics paromomycin sulphate and hygromycin B on the regeneration potential of scutellar callus of rye (*Secale cereale* L.).

	Concentration (mg/l)	Selection during callus induction and regeneration		Selection exclusively during callus regeneration	
		regenerating calli (% ± SD)	shoot primordia (mean ± SD)	regenerating calli (% ± SD)	shoot primordia (mean ± SD)
phosphino-thricin	0.5	95.84 ± 5.9	12.39 ± 0.2	100.00 ± 0.0	12.00 ± 0.4
	1.0	87.50 ± 5.9	7.87 ± 1.7	100.00 ± 21.2	9.75 ± 0.1
	2.5	66.67 ± 0.0	2.69 ± 0.1	80.00 ± 14.1	9.00 ± 0.3
	4.0	25.00 ± 11.8	3.13 ± 0.5	90.00 ± 7.1	5.78 ± 0.3
hygromycin B	1	70.00 ± 0.0	14.7 ± 1.9	-	-
	5	95.00 ± 7.1	7.7 ± 0.8	-	-
	10	70.00 ± 15.6	4.8 ± 1.7	-	-
	20	60.00 ± 12.7	2.3 ± 0.6	55.00 ± 7.1	5.2 ± 1.6
	40	30.00 ± 14.1	1.3 ± 0.4	0.00 ± 0.0	0.00 ± 0.0
	60	0.00 ± 0.0	0.0 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
paromomycin sulphate	10	95.84 ± 5.9	5.22 ± 1.2	95.00 ± 7.1	12.19 ± 2.1
	25	75.00 ± 0.0	3.06 ± 0.5	65.00 ± 7.1	5.48 ± 2.6
	50	20.83 ± 17.7	1.13 ± 0.2	70.00 ± 14.1	3.94 ± 0.8
	100	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Control cultures without selective agent				80.00 ± 0.0	10.82 ± 0.8

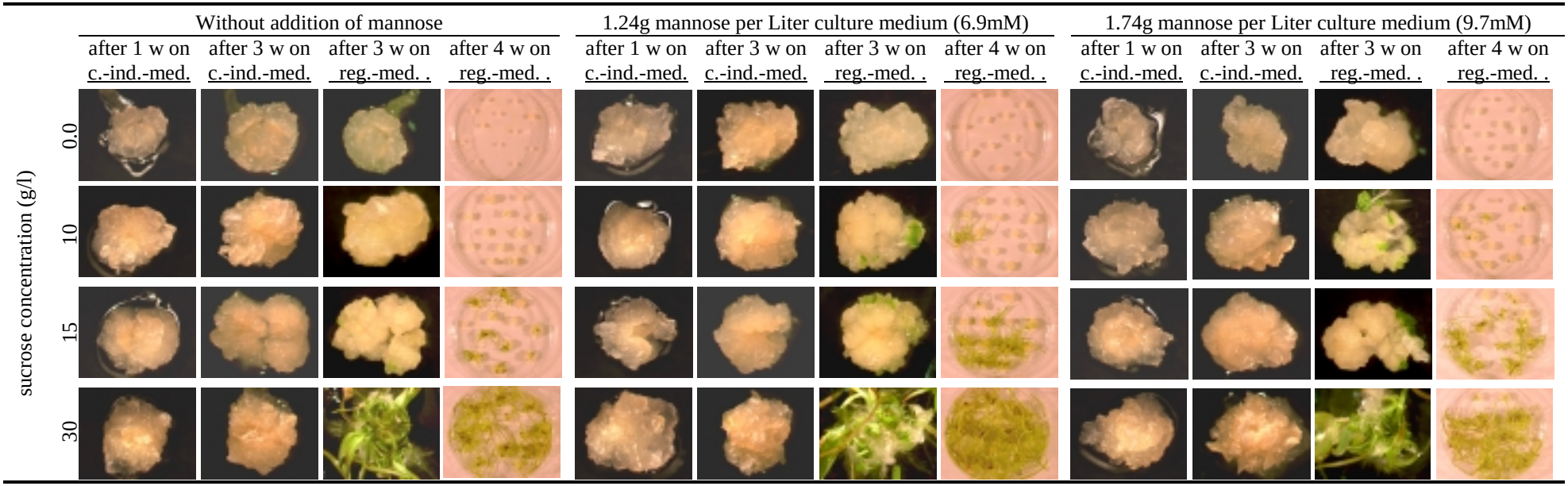


Figure 10: Effect of the mannose/sucrose concentration on growth and regeneration response of callus from rye (*Secale cereale* L.) inbred line L22 (w = week(s); c.-ind.-med. = callus induction medium; reg.-med. = Regeneration medium).

Table 12: Effect of mannose in dependence of the sucrose concentration on the regeneration potential of scutellar rye (*Secale cereale* L.) callus.

mannose (mg/l)	mannose addition during callus induction							
	regenerating calli; sucrose (g/l) (% ± SD)				shoot primordia; sucrose (g/l) (mean ± SD)			
	0	10	15	30	0	10	15	30
0	0.00 ± 0.0	41.67 ± 11.8	83.34 ± 11.8	100.00 ± 0.0	0.00 ± 0.0	1.88 ± 0.5	5.64 ± 0.9	10.71 ± 3.4
1.24	0.00 ± 0.0	79.17 ± 5.9	70.84 ± 5.9	91.67 ± 0.0	0.00 ± 0.0	2.17 ± 0.2	7.14 ± 2.5	12.59 ± 0.6
1.75	0.00 ± 0.0	79.17 ± 5.9	91.67 ± 11.8	75.00 ± 11.8	0.00 ± 0.0	3.89 ± 0.3	8.00 ± 1.4	11.62 ± 1.4
	mannose addition during callus regeneration							
0	-	-	-	10.82 ± 0.8	-	-	-	80.00 ± 0.0
1.24	-	-	7.4	7.38	-	-	100.00	80.00
1.75	-	-	6.38	6.83	-	-	80.00	60.00

In the applied concentration range of mannose/sucrose (0-0.75mg/l / 0-30g/l) the embryogenecity was drastically reduced and regeneration was observed only if at least a minimal sucrose concentration was supplied. Increased mannose concentration slightly supported callus growth (Figure 10) but did not affect the regeneration potential in any sucrose treatment (Table 12). The reason for this non-selective effect could be the low mannose concentration supplied. For this, a second experiment with mannose concentrations as high as 30g/l mannose and no sucrose were performed but also no clear selection effect could be observed (data not shown). It appears that mannose can be metabolised by rye cultures to some extent. However, it can not be certainly deduced whether putative transgenic plants would have a significant advantage compared to non-transgenic plants.

4.4. NPT II based selection

4.4.1. Materials and Methods

Plants and explants

Donor plants of the spring inbred line L22 were grown without vernalisation and immature embryos were prepared and cultured as mentioned above (Section 2.2.1).

Culture media and callus cultures

Based on a basic medium consisting in MS salts (Murashige and Skoog, 1962), 30g/l sucrose, 100mg/l casein hydrolysate and 500mg/l glutamine, different culture media were prepared: callus induction medium (2.5mg/l 2,4-D, 3.0g/l phytagel), osmoticum medium (callus induction medium with 72.9g/l mannitol) and selective callus induction medium (2.5mg/l 2,4-D, 50mg/l paromomycin sulphate, 6.0g/l agarose Type I). Media for regeneration were prepared as callus induction media but without 2,4-D. Selective medium was supplemented with 100mg/l paromomycin sulphate. The pH was adjusted in all media to 5.8 prior autoclaving at 121°C and 1.5 bar for 15min. Immature embryos (Figure 1D and E) were placed scutellum side up on callus induction medium and cultured in the dark at 25°C. Immature embryos were pre-cultured on callus induction medium for five days, osmotically pre-treated for 4 to 6 hours, bombarded, transferred on fresh callus induction medium after 12 to 16 hours and two different selection treatments (Table 13). After three weeks on regeneration medium, calli were transferred into culture containers for shoot elongation

(Figure 12B and C). Details related to the media preparation procedure and culture conditions during callus induction and regeneration are mentioned in Section 2.3.1.

Vectors and Biolistic parameters

The vector pJFnptII (Altpeter and Xu, 2000), containing the selectable marker gene *npt*II under control of the maize ubiquitin promoter, with first intron, and the 35-S terminator inserted in the pPZP111 vector was used (Figure 11). The Gold-DNA coating was prepared and the bombardment performed as mentioned in Section 3.2.1, with 6µg DNA (containing the pJFnptII vector) per precipitation reaction; a total amount of 35µg micro-particles was used per particle bombardment.

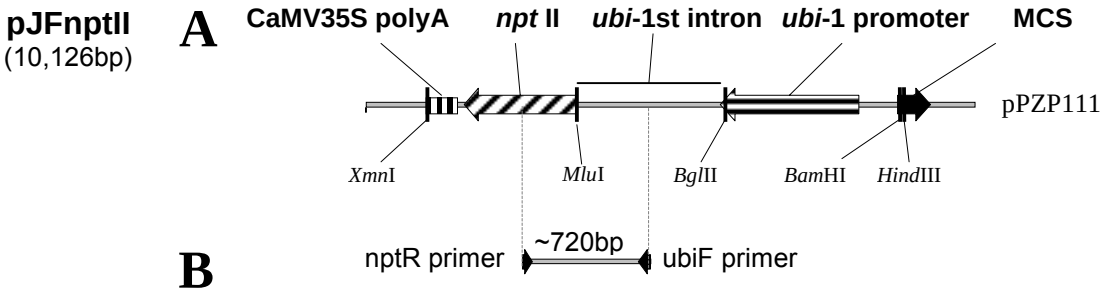


Figure 11: Schematic representation of the pJFnptII vector (Altpeter and Xu, 2000).
(**A**) The expression cassette of the *npt*II gene (Mazodier *et al.*, 1985) under control of the maize ubiquitin promoter (*ubi*) and the *35S* terminator was cloned into the pPZP111 vector (Hajdukiewicz *et al.*, 1994). (**B**) Specific primers were designed for detection of *ubi*::*npt*II in transgenic plants. (MCS = multiple cloning side)

Experimental design and statistical analysis

This transformation experiment was carried out in three replications, each containing 6×25 explants per treatment and 25 explants each, as bombardment, regeneration and selection control. The transformation efficiency was recorded by counting NPTII-ELISA positive plants (Section 5.2.4) in percent of bombarded explants.

4.4.2. Results and Discussion

Line L22 was chosen for transformation experiments due to its superior *in-vitro* response (Section 2.3). After four to five days of pre-culture of immature explants, calli were bombarded with micro-particles coated with a constitutive *npt*II expression cassette. Paromomycin sulphate selection was used for the identification of transgenic events. In

contrast to Castillo *et al.* (1994) a long-term callus selection period was replaced by either a two weeks callus selection and a two weeks regeneration period or a selection exclusively during the regeneration. In three independent experiments a higher transformation efficiency was observed if selection was exclusively during regeneration (4.0 and 2.7%) compared to a selection during callus induction and regeneration (2.0 and 1.3%; Table 13). Most calli cultured on selective induction medium showed growth retardation, browning and no regeneration (Figure 12A). All transgenic plants (Figure 3C) were confirmed by NPTII-ELISA (Figure 12D) and Southern blot analysis (Figure 15C; Section 5.3.1) giving rise to a total of 5 and 10 transgenic plants per treatment, respectively (Table 13). The total period from culture of the explants until transfer of transgenic plants to soil was less than three month, while in the earlier report more than six months were required (Castillo *et al.*, 1994). Transgenic plants developed like wild type control plants. Similar transformation efficiency were obtained after selection with paromomycin during the regeneration of *npt*II expressing plants or after regeneration of *bar* expressing plants by application of Basta® (Section 3.3). The availability of independent selectable marker/selection systems is desirable when pyramiding transgenes into crops.

The presented results describe for the first time the efficient and reproducible production of stabile transgenic rye plants. The integration of multiple optimised factors was essential for successful transformation of this recalcitrant crop, including (1) the identification of homozygous inbred lines (Section 2.3) in contrast to earlier used heterozygous open pollinated populations cultivars (Castillo *et al.*, 1994); (2) genotype specific adjustment of tissue culture parameters, like osmotic treatment prior bombardment, pre-culture period before bombardment, low amount of particles for biolistic gene transfer, a short callus culture period with an selection emphasis on the regeneration period (Section 3.3; (3) use of a selective agent that allows the control of most of the escapes if applied only during or after regeneration of the transgenic plantlets (Section 4.4).

Table 13: Selection of transgenic, NPT II positive rye (*Secale cereale* L.) plants, after selection at different culture stages (means of three independent replications).

Experiment		Days on medium[1], post bombardment			Regenerated plants		Transformation frequency
replication-treatment	Explants	IM	IMP50	RMP100	Total number	ELISA positive	(%)[2]
1-1	150				23	3	2.0
2-1	150	4	12	28	0	0	0.0
3-1	150				10	2	1.3
1-2	150				2	0	0.0
2-2	150	16	0	28	25	6	4.0
3-2	150				10	4	2.7

[1]: IM and IMP50 = callus induction medium without and with 50mg/l paromomycin sulphate respectively; RMP100 = regeneration medium with 100mg/l paromomycin sulphate

[2]: number of independent transgenic rye plants expressing NPTII, in percent of bombarded explants.

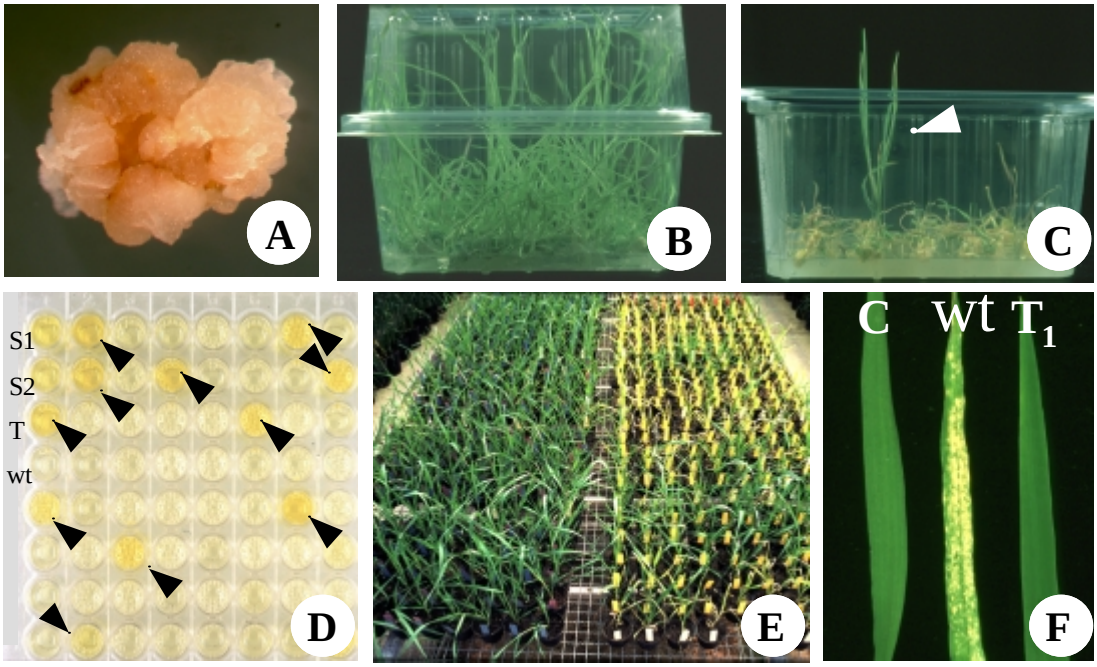


Figure 12: Selection of transgenic rye (*Secale cereale* L.) after biolistic transformation with the selectable marker gene *npt*II.

(**A**) Callus, three weeks after subculture on selective callus induction medium containing 100mg/l paromomycin sulphate. (**B, C**) *In-vitro* regeneration in non-selective treatment (**B**) and on selective regeneration medium (100mg/l paromomycin sulphate) (**C**) with one regenerated transgenic rye plant (arrow). (**D-F**) Transgene expression: NPTII-ELISA of regenerated plants: 40µg total protein extract per well were added. Arrows indicate NPTII positive plants (**D**). Segregating generation (**E**) and leaf painting assay with 1% (w/v) paromomycin sulphate solution, for *npt*II expression (**F**). (S1 and S2 = NPTII protein standards with 150pg and 100pg per well respectively; C = control; wt = wild type; T = transgenic, *npt*II positive plant).

4.5. Production of transgenic rye plants without use of selectable marker genes

4.5.1. Materials and Methods

Explants and callus culture

Immature embryos of the rye inbred lines L22 and L20 were obtained, pre-cultured and osmotically treated as mentioned in Section 2.3 and 3.3 Bombarded embryos were cultured on

callus induction medium for three weeks and than transferred on regeneration medium for another three weeks. A final three-week culture in containers (Green Box from Duchefa) allowed further shoot elongation and collection of leaf tips for analysis of transgene integration by Polymerase Chain Reaction (PCR).

Vectors and biolistic parameters

Two physical DNA configurations were coated to gold micro-particles. The first vector type consisted of a circular plasmid, pPZP200 (Hajdukiewicz *et al.*, 1994) with a coding sequence of the γ-tocopherol methyltransferase (*γ-tmt*) gene (Shintani and DellaPenna, 1998) or of the ferritin (*ferr*) gene (Van Wuytswinkel *et al.*, 1995) under control of the maize ubiquitin promoter (*ubi*) and the *nos* terminator. Plasmids were denominated as pJFtmt and pJFferr, respectively (Figure 13). The second DNA configuration consisted of a linear DNA fragment containing exclusively the transgene expression cassette including the *ubi* promoter with first intron, the *γ-tmt* or *ferr* coding region and the *nos* terminator with exclusion of the pPZP200 backbone. These minimal expression cassettes were obtained by PCR amplification with PWO DNA polymerase (proof reading) under use of specific primers annealing at the 5´ end of the promoter (ubi-primer = 5´- CTG CAG TGC AGC GTG ACC CG -3´) and 3´ the terminator (nos-primer = 5´- GAT CTA GTA ACA TAG ATG ACA CCG CG -3´) (Figure 13). Micro-particle coating was prepared with equal number of DNA molecules (2×10^{11} molecules). The equimolarity was calculated assuming that 1µg of a 1kb segment of double stranded DNA contains 9.48×10^{11} molecules (Sambrook *et al.*, 1989). Per coating reaction 2µg or 0.77µg of plasmid-DNA or minimal fragment-DNA respectively, were used. A total amount of 35µg of micro-particles were used per bombardment.

Evaluation of putative transgenes

Leaf-tips of regenerated plantlets of single culture containers were harvested together as pools (container pools) and the DNA extracted. PCR analysis with specific primers ((Figure 13A and B) for respective expression cassettes was performed in two replications per pool and DNA target (for DNA extraction and PCR conditions see Paragraph 5.3). Regenerating calli of PCR positive container pools were then transferred into fresh culture containers for further leaf growth. Leaf-samples were taken from all regenerating plantlets of individual calli (callus pools) and DNA extraction and PCR analysis were performed as before. In a third and final

selection step single plantlets of each PCR-positive callus pool were analysed and PCR-positive (transgenic) plants transferred to soil.

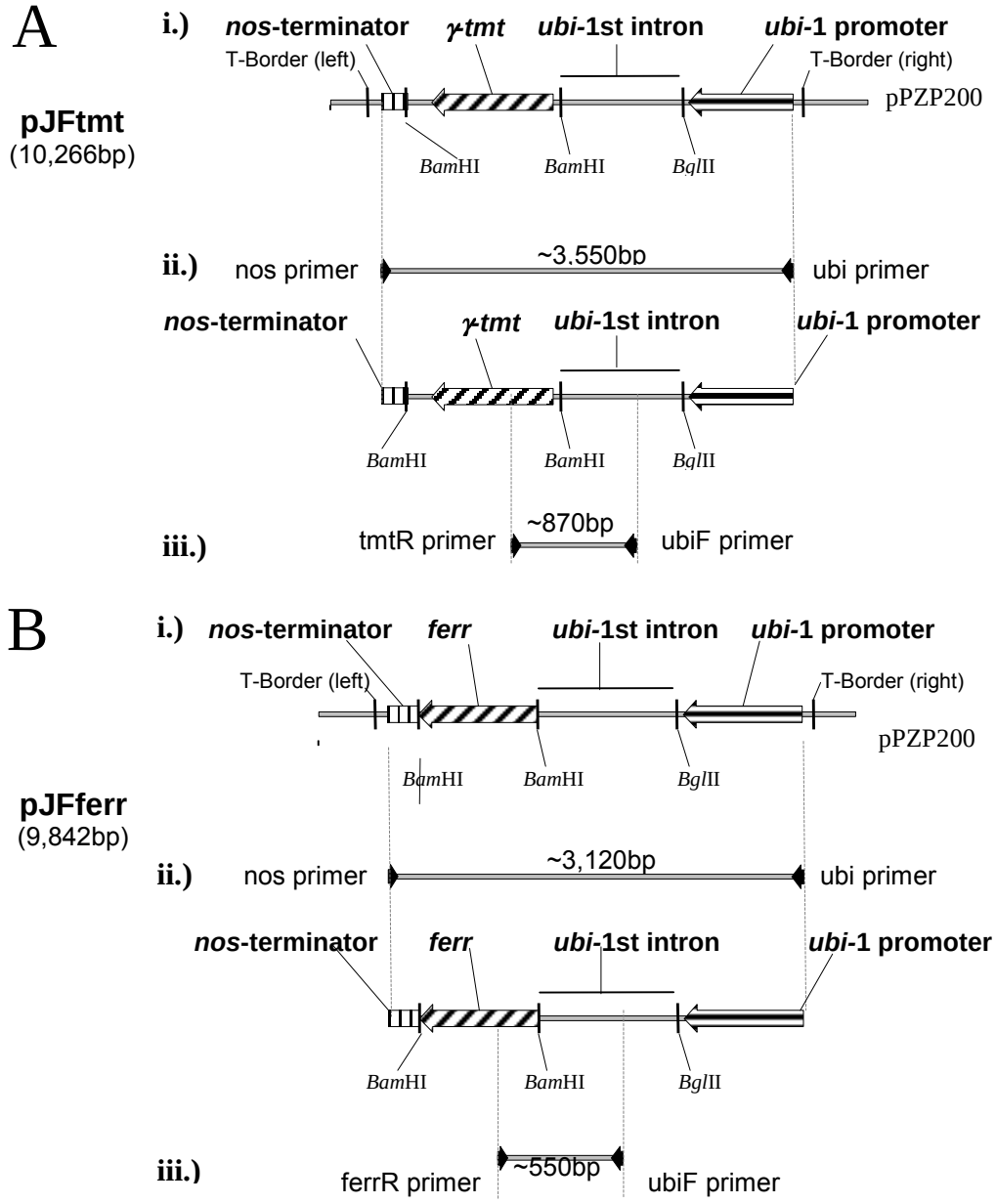


Figure 13: Schematic representation of the pJFtmt (**A**) and pJFferr (**B**) vectors and respective DNA fragments

(i.) The expression cassette of the γ-tocopherol methyl-transferase (*γ-tmt* = Shintani and DellaPenna, 1998) and the ferritin gene (*ferr* = Van Wuytswinkel *et al.*, 1995, under control of the maize ubiquitin promoter (*ubi*) and the *nos* terminator was cloned into the pPZP200 vector (Hajdukiewicz *et al.*, 1994). (ii.) DNA fragments containing the whole expression cassette were amplified by specific primers annealing at the right border of the ubiquitin promoter and the left border of the nos terminator. (iii.) Specific primers were designed for detection of *ubi*::*γ-tmt:nos* and *ubi::*ferritin:nos in transgenic plants.

The absence of the vector backbone DNA was analysed by PCR with the aadA-F primer (5´-CCG AAG TAT CGA CTC AAC TAT CA -3´) and the aadA-R primer (5´- TTT GCC GAC TAC CTT GGT GAT CT -3´), specific to the aminoglycoside adenyltransferase gene (*aad*A) located on the pPZP200 plasmid (Hajdukiewicz *et al.*, 1994).

Experimental design and analysis

For each of the two genotypes two separate replications, with 400 immature embryos per DNA type each, were carried out.

4.5.2. Results and Discussion

Transgenic events are usually identified by expression of selectable marker genes conferring a growth advantage under selective conditions. These selectable marker genes are usually co-transformed with the gene of interest. Desired transgenic plants need to carry both, the selectable marker gene as well as the gene of interest. In Section 3.3 it was demonstrated that spraying regenerated rye plantlets with a commercial herbicide solution before transfer to soil as exclusive selection process, results in the reproducible identification of transgenic rye plants. With the appropriate timing of tissue culture and gene delivery, the overgrowth of transgenic- by non-transgenic tissue is not significant and can be neglected. In the presented experiment it was possible to effectively produce transgenic rye plants without use of any selectable or screenable marker gene. The gene of interest could be identified via conventional PCR analysis of the regenerated plants (Table 14 and Figure 14) before transferring them to soil.

In a first selection step leaves of regenerated plantlets were pooled in growth containers and 219 individual container pools were analysed. Out of these, 36 container pools gave a positive PCR result (Table 14) for at least one of the two analysed transgenes (Figure 14). In a second selection step regenerating plantlets of PCR positive container pools were pooled per originated callus (callus pool). A total of 206 callus pools were analysed, resulting in 24 callus pools with positive PCR signals (Table 14). Leaf samples of single regenerated plantlets of positive callus pools were analysed in a third selection step. Out of 158 single plants 17 PCR positive plants could be identified as potentially transgenic. Nine of the selected plants were regenerated after bombardment of calli with the full DNA plasmid vector and 8 with the minimal expression cassette, showing no effect of DNA configuration on

transformation efficiency. In both cases the co-integration frequency of unlinked and co-transformed vectors was 100% (Figure 14D). The absence of the pPZP200 vector backbone DNA was demonstrated by a backbone specific PCR reaction (Figure 14E).

Table 14: Localised PCR positive pools, calli and plants, after PCR assisted selection for the production of transgenic rye plants (*Secale cereale* L.) free of selectable marker gene.

(Sum of two replications)		Analysed and selected samples per selection step					
Genotype	Explants	Container -pools	PCR +	Callus-pools	PCR +	Individual plants	PCR +
L 20	800	125	9 P 6 F	101	2 P 2 F	4	2 P 2 F
L 22	800	94	9 P 12 F	105	9 P 11 F	154	7 P 6 F

Abbreviations: P = bombarded with plasmid DNA; F = bombarded with DNA fragment.

Nandadeva *et al.* (1999) observed a two- fold increase in integrative transformation efficiency when linearised DNA was used, but admitted difficulties to generate statistical relevant data. The transformation frequencies (percentage of marker-free transgenic plants relative to the total number of bombarded explants) in the presented study were 0.5% and 1.6% for the genotypes L20 and L22, respectively (Table 14). At first glance this frequency seems in a normal range. However, to compare the efficiency with conventional selection protocols the co-transformation frequency of desired- and marker gene needs to be taken into account as well as the effort to eliminate the marker gene. Since a high range of variation, from 10% to 90%, can be found in the frequency of co-transformation, the theoretical frequency of marker-free transgenic plants can be much lower than the primary transformation frequency. In systems based on co-transformation it is required that respective T-DNAs with selectable marker gene and the gene(s) of interest integrate into unlinked loci. Otherwise independent segregation can not be expected. Advantages and limitations of systems for the removal of selection markers have been reviewed by Ebinuma *et al.* (2001).

By the here presented selection system PCR positive plants from two divergent genotypes could be recovered (Table 14). The higher transformation efficiency with genotype L22 (1.6%) compared with L20 (0.5%) corresponds to the difference of the regeneration potential of the individual genotype. The number of required DNA extractions and PCR analyses are the limiting factors for the production of large numbers of transgenic plants.

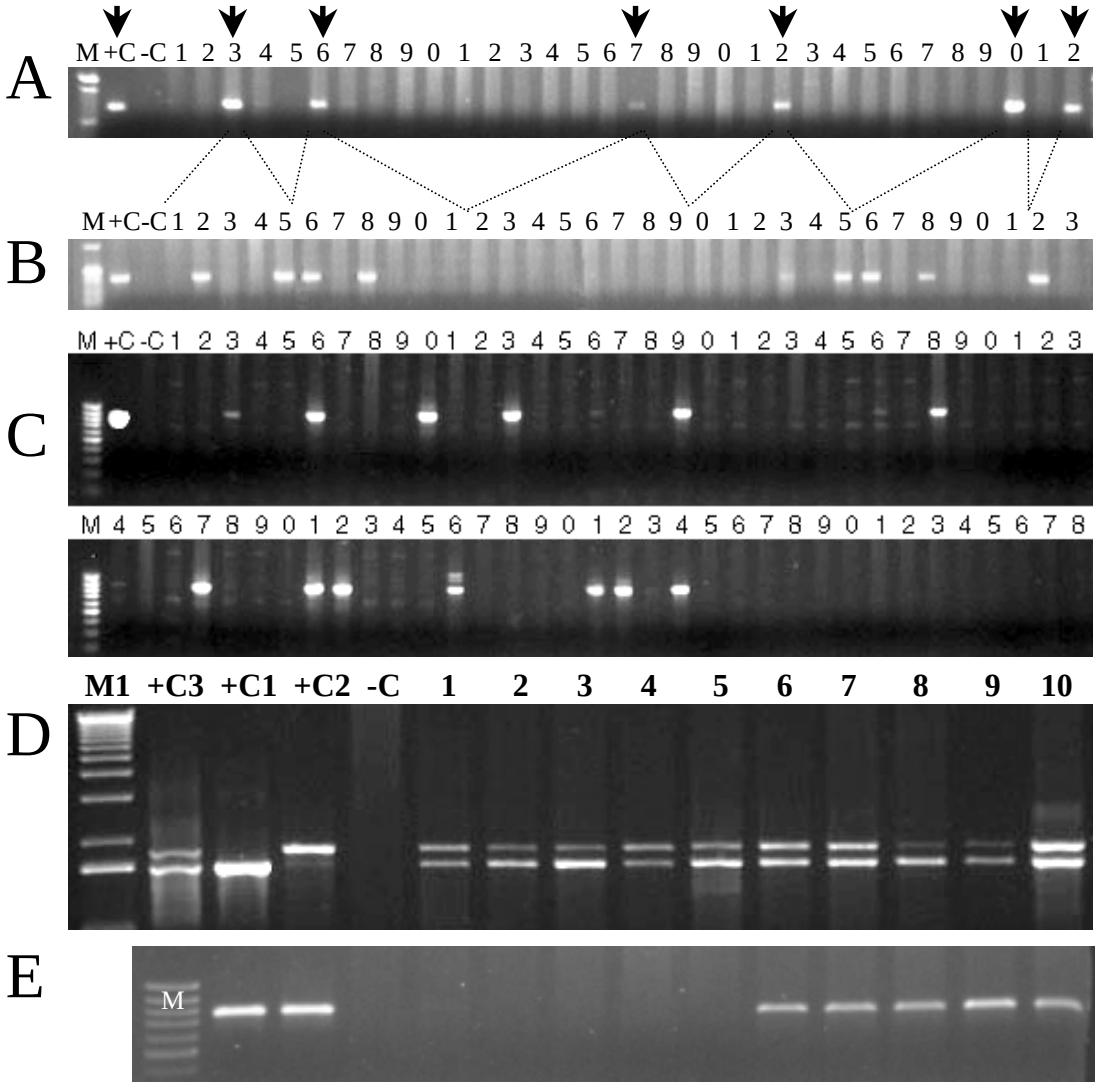


Figure 14: PCR based selection of transgenic, marker gene free rye (*Secale cereale* L.) plants.

Agarose gel electrophoresis of PCR reactions in the first (**A**: container pools), second (**B**: callus pools) and third (**C**: individual plants) selection step using specific primer combinations to detect the transgene of interest. (**D** and **E**) Agarose gel electrophoresis of PCR reactions of selected plants showing amplification products of the transgene (**D**) and the *aad*A gene (**E**). (M = 100bp DNA ladder; M1 = 1kb DNA ladder; +C = positive control; -C = Wild type DNA, negative control; +C1 = positive control for pJFferr; +C2 = positive control for pJFtmt; +C3 = positive control both, pJFferr and pJFtmt)

Considering the labour and time required for analysis, the low regeneration potential of L20 leads to a similar efficiency compared to L22 (1.8% and 3.6% transgenic plants relative to the number of analysis reactions, respectively), since only two selection steps were required in

L20 (Table 14). This suggests that the presented protocol is especially beneficial for recalcitrant species or cultivars in contrast to species with very high regeneration potential.

Taking into account that DNA extraction and PCR procedures are being more automated this selection strategy for the direct production of marker gene-free transgenic plants is a real alternative to other described protocols. For instance, the Company QIAGEN is offering high-throughput Extraction Kits where one person can extract more than two times 96 samples of genomic DNA (http://www.quiagen.com) in less than two hours. Full automated systems, e.g. by Robot-assisted pipetting and PCR-analysis are available and would speed up the analysis even more. Finally, facilities for analysis of DNA by PCR procedures are anyway available in most middle-sized biotech or plant breeding companies.

It was possible to select transgenic rye plants free of any selectable marker gene by application of a PCR based selection strategy. This “direct” production of marker-free plants can be achieved without complicated vector construction, as needed in most reported selection systems (Goldsbrough *et al.*, 1993; Dale and Ow, 1991; Akiyoshi *et al.*, 1984).

5. Analysis of transgenic rye plants

5.1. Summary

Transgenic rye plants were analysed for transgene integration and its functional integrity. Southern- and Western blot analyses, as well as enzyme activity assays confirmed the presence and expression of the transferred selectable marker genes *bar* and *npt*II and their transmission and expression in the sexual progeny of transgenic rye. In contrast to earlier reports, the presented biolistic gene transfer protocol resulted in low transgene copy number in most of the transgenic plants and 40% of the transgenic plants had a single copy insert. After *Agrobacterium*-mediated gene transfer plants with single and multiple copy insert were observed. Stablility and level of transgene expression were analysed with regard to the transgene copy number. Indication of transcriptional and post-transcriptional gene silencing was observed in few transgenic lines with multiple transgene inserts after biolistic transfer. The co-expression and co-segregation of High-Molecular-Weight-glutenin subunits was demonstrated in transgenic rye with 55.6% of the analysed lines expressing at least one glutenin subunit beside the selectable marker gene.

5.2. Introduction

In early developing stages of a transformation system qualitative expression assays of the β-glucoronidase (GUS) or the green fluorescent protein (GFP), encoded by the *uid*A and *gfp* reporter-genes, respectively, have been successfully used to monitor transgene delivery to plant tissue. However, resulting transgenic plants and their progeny, need to be carefully evaluated, concerning (1) transgene integration, (2) transgene expression and (3) transgene expression stability.

(1) DNA analysis for transgene integration and integration patterns are basic requirements to evaluate transgenic plants. Two standard molecular techniques are widely used for this purpose. The Polymerase-Chain-Reaction (PCR; Mullis and Faloona, 1987) has become a standard procedure to screen putative transgenic plants at an early developmental stage, when plant material would be limiting for other assays. In this method a defined DNA fragment, unique for the transgene is amplified *in-vitro* by elongation of specific oligonucleotide sequences and can be visualised after DNA separation by gel electrophoresis. Its high

sensitivity, specificity and low reaction-costs are some properties of this qualitative analytical method. However, in most cases further information is required concerning number of copies of the inserted trangene copies, which is usually obtained by Southern blot analysis (Southern 1975; Sambrook *et al.*, 1989). In this method genomic DNA is restriction digested, separated by gel electrophoresis, transferred and linked to a membrane and hybridised with labelled DNA strands complementary to the transgene that can visualise specific DNA fragments. DNA analysis by Southern blot is more time-consuming than PCR. However, it reveals information concerning the independence of transgenic plants and is therefore crucial for the evaluation of the efficiency of a transformation protocol.

(2) The functionality of a transgene can be evaluated at the RNA, Protein and bio-analytical levels. The procedure of RNA analysis is similar to the Southern blot technique. The method is called Northern blot and differs basically due to the fact that RNA secondary structure has to be denatured for size fractionation during gel electrophoresis. Usually complementary DNA strands are used for hybridisation on Northern blot filters. The presence of transgenic RNA demonstrates that the transgene is actively transcribed. Translation of transgene RNA into the encoded Protein can be demonstrated with SDS page, western blot or enzyme –linked immunosorbent assay (ELISA). These methods differ in the detection limit and specificity. With enzyme activity assays, the functional integrity of the transgenic protein can be evaluated.

(3) The stability of the transgene in consecutive meiotic generations is the final requirement and of central importance if the transgene is going to be introduced into a breeding programme. A molecular and genetical screening is necessary to identify individual transformants with desired characteristics, such as single inserts, desired expression level and single locus integration, etc., along with the desired agronomic performance. This can reduce the number of useful transgenic events and explains the need for the production of large numbers of transgenic plants per program.

In this chapter the conceptual tools of applied technical procedures are presented. Transgenic rye plants produced in the presented project were analysed for integration and functionality of respective transgenes. The segregation pattern and stability of the transgenic progeny was analysed.

5.3. Material and methods

5.3.1. Reportergene expression assays

Histochemical GUS assay

By this assay qualitative data concerning the specificity of the *uid*A gene expression, encoding β-glucuronidase (GUS), in tissue or single cells are obtained (Jefferson 1987). Transient as well as stable expression can be studied.

Fresh plant organs were infiltrated in GUS-staining solution (0.1 $NaPO_4$ pH 7.0, 10% methanol, 0.5mM $K_3[Fe(CN)_6]$, 0.5mM $K_4[Fe(CN)_6]\times 3H_2O$, 10mM EDTA($Na_2$) pH 8.5, 1mg/ml X-Gluc) by applying vacuum (ca. 200mbar) and were incubated at 37°C over night. First staining was often seen after a few hours. Adding 70% ethanol and incubating at room temperature bleached chlorophyll rich tissues. The ethanol solution was changed several times. GUS expression was examined under the stereomicroscope.

Histological GFP assay

The Green fluorescent protein (GFP), encoded by the *gfp* reporter-gene from the jellyfish (*Aequorea victoria*) is providing a useful, safe and easy detectable marker system for the development of transgenic plants. The utility and uses of GFP in plant biology and transformation have been reviewed in numerous articles (Haseloff and Amos, 1995; Leffel *et al.*, 1997; Stewart 2001). In the presented experiments GFP was visualised using a stereomicroscope with a 50W-mercury lamp, a BP470/20nm excitation filter and a BP505-530 barrier filter.

5.3.2. DNA protocols

Several extraction protocols were applied and results compared concerning DNA yield quantity, quality and labour intensity. In the following the two most suitable DNA extraction protocols are given and differ mainly in the required labour time and the quality of DNA.

Fast isolation of genomic DNA from rye for PCR analysis (after Tinker)

This DNA extraction procedure is a so-called fast protocol with a high throughput of samples and low costs. DNA quality and quantity is appropriate for PCR analysis but not useful when high molecular weight DNA is required.

Leaf samples (ca. 0.1g) were collected directly in reaction tubes and shock-frozen in liquid nitrogen. After homogenisation with DSTROY-S® (BIOzym) 500µl CTAB extraction buffer (1.4M NaCl, 20mM EDTA, 100mM Tris-HCl pH 8.0, 2% (w/v) CTAB) was added, vortexed and incubated at 65°C. After 1-1.5h 5µl RNAse A buffer (10mg RNAse A/ml, 10mM Tris-HCl pH 7.5, 15mM NaCl) were added and incubated at 37°C for a further 30min. 200µl Phenol:Chlorophorm:Isoamylalkohol (25:24:1) were added and mixed well for 2-3min. After 10min centrifugation at 12,000rcf the supernatant was transferred to a fresh tube and mixed with 1Volume Isopropanol. After 10-15min of precipitation, samples were centrifuged for 10min at 12,000rcf and the supernatant discarded. The DNA pellet was washed with 70% Ethanol, dried and dissolved in 50-150µl water or EB buffer (10mM Tris-HCl pH 8.0).

Isolation of high molecular genomic DNA from rye for Southern blot analysis

This extraction procedure yields large amounts of high molecular genomic DNA and is appropriate for use in Southern blot analysis.

Plant material (0.5 to 1g) was homogenised in liquid nitrogen with pestle and mortar and transferred into 50ml centrifugation tubes. The plant powder was vigorously mixed with 10ml SDS extraction buffer (100mM Tris-HCl pH 8.0, 20mM EDTA, 500mM NaCl, 1.5% (w/v) SDS) and incubated at 60°C. After 60 to 90min, 10ml Chloroform: Isoamylalcohol: Ethanol (80:4:16) was added and solutions mixed by gentle invertion. After 5-10min the samples were centrifuged at 3,500rcf for 15min and the supernatant transferred to a fresh tube. If necessary, the Chloroform extraction was repeated. For DNA precipitation, 1Volume of pre-cooled Isopropanol was added to the clean supernatant and both solutions mixed by gentle invertion. Precipitated, high molecular DNA was removed with a glass hook, washed with 70% Ethanol and dried in a reaction tube. DNA was dissolved in 270µl TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) and 30µl RNAse solution (1mg/ml) was added. After 10-15min incubation at 37°C DNA was precipitated by adding 1/10volume of NaAc solution (3M NaAc pH 5.2 with Acetic acid) and 2volumes of ice cooled Ethanol and incubating at –20°C for 20min. DNA was removed with a glass hook, washed in 70% Ethanol and dried in a fresh reaction tube. DNA was finally dissolved in 300-500µl water or TE buffer. DNA concentration was quantified by spectrometer at 260A-320A and quality determined by gel electrophoresis (0.6-0.8% Agarose).

Polymerase-Chain-Reaction, PCR

The PCR (Mullis and Faloona, 1987) specifically amplifies DNA sequences between defined synthetic primers, designed according to the target DNA sequence. Usually oligonucleotide primers of 19 to 22bp in length were designed for the amplification of 500-1000bp long PCR products. In combination with the ubiquitin forward primer (ubi F = 5´- GTC TGG TTG GGC GGT CGT TCT AG -3´) different reverse primers were designed and used to identify respective transgenes: for pJFbar the barR primer (5´- AGT CGT AGG CGT TGC GTG CC -3´), for pJFnptII the nptR primer (5´- GTG CCC AGT CAT AGC CGA ATA GC -3´), for pJFferr the ferrR primer (5´- CAC CAT TTC CAG AAA CAG G -3´) and for pJFtmt the tmtR primer (5´- CGC CTC AGT GGA TGT AGC A -3´). Due to the enormous amplification potential of PCR reactions, small levels of DNA contamination can result in false positive results. Special attention is therefore required to minimise cross contamination.

Southern blot analysis

In order to evaluate the individual nature of transgene integration and to estimate the transgene copy number, genomic DNA is restriction digested with enzymes that cut the introduced plasmid DNA only once. Frequently cutting enzymes such as *Hind*II, *Bam*HI or *Bgl*II are preferentially used. The insertion of non-truncated expression cassettes can be estimated with a restriction digest of such enzymes that flank the expression cassette. Normally this approach will not allow confirmation of the independent nature of the events.

High molecular weight genomic DNA was digested overnight with corresponding restriction enzymes, for the pJFbar, pJFnptII and pYFnptII plasmids preferably *Bgl*II or *Bam*HI. For respective controls, corresponding plasmid DNA (positive control) and DNA of a wild type plant (negative control) were digested in the same way. After digestion DNA was precipitated by adding 1/10Volume of NaAc solution (3M NaAc pH 5.2 with Acetic acid) and 2Volumes of ice cooled Ethanol and incubated at –20°C for 20min. After centrifugation at 12,000rcf for 20min the DNA pellet was washed with 70% Ethanol and dried. DNA was dissolved in TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0), mixed with 6×Loading buffer III (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and electrophoresed 12-24h on 0.8% (w/v) Agarose gel at approximately 20V. Preferentially DNA amounts, in the range of 10-15µg digested DNA and 20-30pg vector DNA give best results and less background. DNA containing Agarose gel was denatured by soaking in freshly

prepared denaturation buffer (1.5M NaCl, 0.5M NaOH) for 45min with constant and gentle agitation. The gel was transferred on the TurboBlotter™ system (Schleicher&Schuell) using several layers of Whatman 4MM paper to allow blotting with alkaline transfer buffer (0.4M NaOH, 0.6M NaCl). By this method DNA was completely transferred on Hybond-N membrane within 3h. Nylon membrane was washed in 6*SSC buffer (20*SSC = 300mM sodium citrate, 3M NaCl, pH 7.6) for 5min to remove any adhering Agarose. For final fixation, the membrane was exposed to UV light (302nm) for several minutes. Hybridisation was performed with Rothi®-Hybri-Quick solution (Roth) for 24h at 65°C in a rotating hybridisation oven. The membrane was washed at 65°C with 4×SSC for 30 min followed by 15 to 30 min washing with 2×SSC, 0.1% (w/v) SDS and 1×SSC, 0.1% (w/v) SDS. Hybridisation signals were visualised by a phosphor imaging system (Storm).

Probes for hybridisations were prepared by PCR as mentioned above and eventually restriction digested. Required products and fragments were electrophoresed on 1.0% (w/v) Agarose gel, the corresponding fragment extracted using the QIAEX®II gel extraction kit and [^{32}P]-dCTP labelled using the random primer labelling kit from GIBCO-BRL.

5.3.3. Protein protocols

Different protein extraction buffers and protocols were compared in regard to the required protein fraction. In the following the most suitable protein extraction protocol for phosphinothricin acetyltransferase (PAT), neomycin phosphotransferase II (NPT II) and High-molecular-weight (HMW) glutenin subunuts (gs) are given.

Protein extraction from leaves

Plant leaves (ca. 0.1g) were homogenised in reaction tubes using DSTROY-S® (BIOzym) in approximately 150µl protein extraction buffer (For PAT assay = 0.25M Tris-HCl pH 7.5, 2mM EDTA pH 8.0, 5mM DTT, 7.5mg/l Leupeptin, 300mg/l BSA, 0.2mM PMSF. For NPTII assays = 0.25M Tris-HCl, pH 7.8 and 0.1mM PMSF). If necessary, autoclaved sea sand and polyvinyl-pyrrolidone (PVP) was added. Samples were centrifuged at 11,000rcf and supernatant transferred to a fresh reaction tube. Protein concentration was quantified according to Bradford (1976) and extracts used for immunodetection or SDS-PAGE separation.

Protein extraction from mature rye seeds (Altpeter *et al.*, 1996b)

Mature dry seeds were grinded individually with pestle and mortar. Ten to 14mg of the resultant rye flour was vortexed with 200µl sample buffer (2% SDS, 5% β-mercaptoethanol, 0.001% pyronin Y, 10% glycerol, 0.063M Tris-HCl pH 6.8) for 2min and incubated for 2h on a rotary shaker at 250rpm. Extracts were centrifuged at 12,000rcf for 10min and the supernatant boiled for 5 minutes before SDS-PAGE separation.

SDS-polyacrylamide gel electrophoresis (PAGE) of proteins

The strongly anionic detergent SDS in combination with a reducing agent β-mercaptoethanol were used in the loading buffer (2% SDS, 5% β-mercaptoethanol, 50mM Tris-HCl pH 6.8, 10% glycerol, 0.1% bromophenol blue). Heating the crude protein samples together with the loading buffer dissociates the proteins and polypeptides bind SDS to become negatively charged. Since the amount of bound SDS is proportional to the molecular weight of the polypeptide, SDS-polypeptide complexes migrate in accordance with their size. The sieving properties of SDS-PAGE gels are determined by the size of the pores, which is a function of the absolute concentration of acrylamide and bisacrylamide used to cast the gel. Chains of polymerised acrylamide that are cross-linked by bifunctional agents such as *N,N´*-methylenebisacrylamide add rigidity and tensile strength to PAGE gels and form pores through which the SDS-polypeptide complexes must pass. For SDS-PAGE gel preparation see Sambrook *et al.* (1989).

For separation of HMW-gs, 20-30µl of each sample were loaded on 10% SDS-PAGE gel and run (electrophoresis buffer = 25mM Tris, 250mM glycine pH 8.3, 0.1% SDS) until the HMW-subunits were separated. Gels were then stained with Roti®-Blue colloidal coomassie staining solution according to the manufacturer`s instructions. Protein bands were visualised by destaining in aqueous solution of 25% methanol until a clear background was obtained and single bands could be identified.

For NPTII western blotting 20µg crude protein per transgenic rye plant and 0.1ng purified NPTII protein were separated by 12% (v/v) SDS-PAGE.

Western blot analysis

For western blotting, the electrophoretic transfer of proteins from the SDS-PAGE gel to a Hybond™ ECL™ nitrocellulose membrane (Towbin *et al.*, 1979) was performed in a transfer

tank with transfer buffer (1×electrophoresis buffer, 20% Methanol in water) and appropriately placed wire electrodes. During transfer at 200-250mA 2-2.5h the buffer was cooled. For immunodetection, the membrane was blocked overnight with dried milk–membrane blocking buffer (Amersham) and then successively incubated for one hour each with 1:5000 biotinylated antibody to NPTII and 1:5000 streptavidin conjugated alkaline phosphatase (both from 5´Prime→3´Prime; 1:5000) with several washing steps in Tris-buffered saline Tween buffer (TBST = 20mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween 20) between each incubation. The signal was visualised by incubation with ProtoBlot II (Promega) for several minutes.

Enzyme activity assay for phosphinothricin acetyltransferase (PAT)

This assay detects the PAT activity, encoded by the *bar* gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987) in crude plant protein extracts (De Block *et al.*, 1987). It is based on the thin layer chromatographic (TLC) separation of the reaction products resulting from the incubation of the plant protein extract with labeled [^{14}C] PPT and acetyl-coencyme A. This assay has been used to detect PAT activity in different transgenic plant species, e.g. tabacco, rice, maize and wheat.

For each sample 50µg crude protein in 20µl volume, 1.5µl acetyl-coencyme A (1mg/124µl Tris-Hcl pH 7.5), 1.2 µl [^{14}C] PPT (500bq/µl) were added into a reaction tube, was well mixed well and incubated at 31°C for 30min. The samples were boiled for 5min, cooled on ice and centrifuged at 11,000rcf for several minutes. Six µl of each sample were applied on TLC plate and dried carefully with a hair dryer, if necessary. The TLC was run in a chromatography tank with TLC running buffer (200ml Ammonia solution and 300ml 1-Propanol 99,5%) for approximately 2-2.5h. Finally, the TLC plate was dried and exposed to an X-ray film for at least 12h.

ELISA assay for neomycin phosphotransferase II (NPT II)

This ELISA detects the enzyme neomycin phosphotransferase II (NPT II), encoded by the *nptII* gene in crude protein extract and is based on the NPT II ELISA Kit from 5´Prime→3´Prime, Inc.® (Boulder, CA). The assay is a sandwich immunoassay were a rabbit polyclonal antibody specific to NPT II is coated onto polystyrene microwells. During the incubation NPT II of the sample is captured and bound to the microwells. Biotinylated

secondary antibody to NPT II then binds to the immobilised primary antibody/NPT II complex. Biotinylated antibody can then be quantitated colorimetrically by incubation with streptavidin conjugated alkaline phosphatase and substrate. The resultant colour development is proportional to the NPT II concentration in each microwell. Per microwell 40µg of crude protein extracts were used following the manufacturers instructions. Using the Tecan Spectra photometer and the easyWin kinetics software from Tecan (V. 4.0a) a quantitation of the NPTII assay was possible. Several other protocols have been reported for analysis of NPT II activity in transgene plants (Reiss *et al.*, 1984; McDonnell *et al.*, 1987; Platt and Yang, 1987; Cabanes-Bastos *et al.*, 1989).

5.4. Results and Discussion

5.4.1. Transgene integration and expression

Selectable marker genes

A total of seven biolistic transformation experiments with use of selectable marker genes resulted in 38 transgenic rye plants with independent transgenic integration pattern (Figure 15) demonstrating the efficiency and consistency of the presented transformation protocols. A total of 21 transgenic plants with independent transgene integration pattern, as indicated by Southern blot analysis were obtained from three divergent genotypes (L22, L20 and L318) (Figure 15A) after selection with commercial Basta® solution exclusively during the regeneration phase (Section 3.3). After transformation with a constitutive *npt*II expression cassette (Figure 11) transgenic plants were selected during different developmental stages and give rise to 17 independent transgenic plants (Section 4.3) as demonstrated by Southern blot (Figure 15C). Expression of the phosphinothricin acetyltransferase (PAT) and neomycine phosphotransferase (NPT) were confirmed by tolerance to Basta® and paromomycin sulfate and PAT-activity assay or Western blot (Figure 15B and D), respectively.

Transgenic plants with single copy inserts (b1, b4, b5, p1, p6, p10, and others), few transgene copies (b3, b9, p4, p7, and others) and high copy number (b2, b6, b10, p2, p11, p13) where identified (Figure 15A and C). Transgene expression levels (PAT and NPTII-Western blot) correlated with the copy number. Highest expression levels where observed when two or few copies where inserted (b3, b9, p4, p7, p17) (Figure 15B and D).

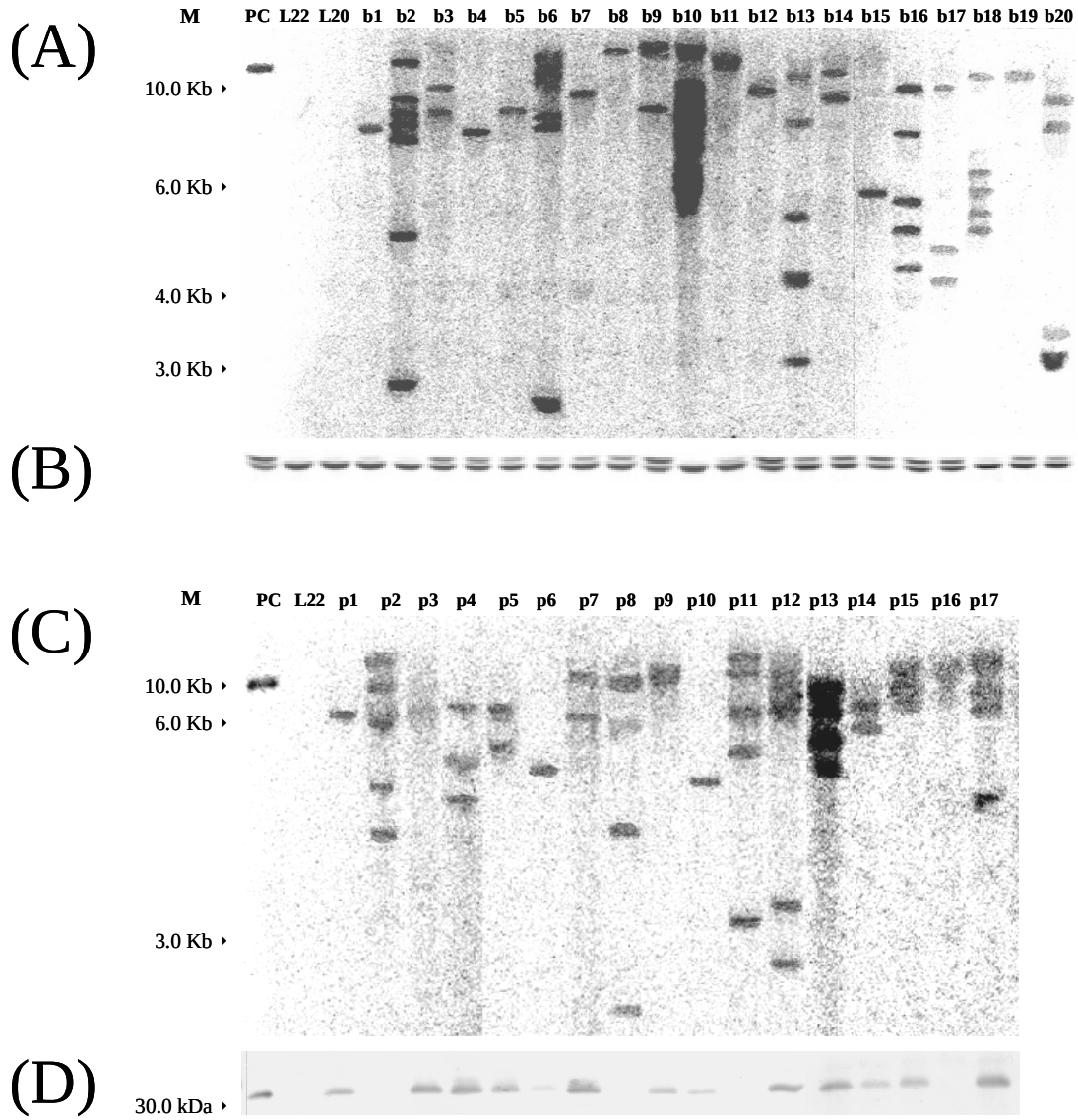


Figure 15: Integration and expression analysis of the selectable marker genes *bar* and *npt*II in transgenic rye (*Secale cereale* L.) plants after biolistic gene transfer.

(**A, C**) Southern blot of the *bar* (A) and the *npt*II (C) gene: individual lanes represent 10 to 15µg genomic DNA of transgenic T_0 plants (b1-b20 and p1-p17) in comparison to wild type plant (L22 and L20) and 25 pg pJFbar or pJFnptII plasmid DNA (PC) after restriction digest with *BamH*I and hybridisation with an approximately 800bp DNA fragment of the transgene coding region. (**B**) PAT enzyme activity assay: Individual lanes (corresponding to (A)) represent 6µl of PAT reaction sample separated on Thin-Layer-Chromatography plates. (**D**) Western blot analysis: Individual lanes represent 20µg leaf protein extract of plants corresponding to (C) and 0.1ng purified NPTII protein. Proteins were separated by 12% (v/v) SDS-PAGE and conjugated to primary and secondary antibodies from 5´Prime→3´Prime (Inc.; Boulder, CA).

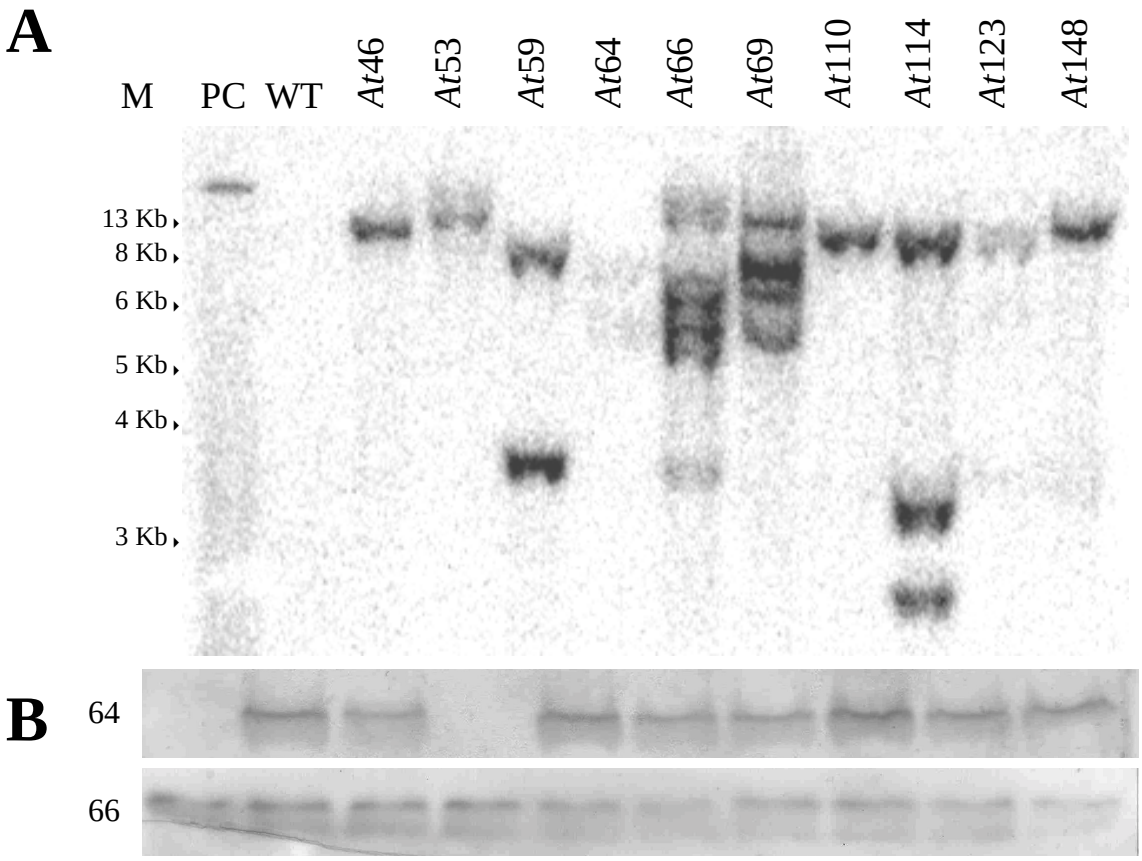


Figure 16: Integration and expression analysis of the selectable marker gene *npt*II in transgenic rye (*Secale cereale* L.) plants after *Agrobacterium* mediated gene transfer.

(**A**) Southern blot analysis for the *npt*II gene: individual lanes represent 10 to 15µg genomic DNA of transgenic T_0 plants in comparison to wild type plant (WT) and 25 pg pYFnptII plasmid DNA (PC) after restriction digest with *BamH*I and hybridisation with an approximately 800bp DNA fragment of the transgene coding region. (**B**) Western blot analysis of transgenic T_1 progeny: Individual lanes represent 20µg leaf protein extract of plants. Proteins were separated by 12% (v/v) SDS-PAGE and conjugated to primary and secondary antibodies from 5´Prime→3´Prime (Inc.; Boulder, CA).

The lowest, but detectable transgene expression was observed in transgenic lines b1, p1, p6 and p10, all with single copy inserts (Figure 15A to D). A similar trend was also observed in transgenic wheat plants (Stoger *et al.,* 1998). In several plants with high copy numbers (b2, b10, p2, p8, p11), no expression could be detected. However high copy number did not always lead to gene silencing after transfer of plants to soil, as observed in b6, b13, b16, and p12, all with five or more copies. It has been frequently stated that one negative aspect of biolistic DNA delivery is the low frequency of single insert events, as compared to *Agrobacterium* mediated delivery (Cheng *et al.,* 1997). In the presented biolistic gene transfer

experiment, surprisingly 40% of the transgenic plants showed single inserts, and only 7 out of 20 lines had a considerable high copy number. This might be due to the comparably low particle density used in this study and consequently low transgene DNA amount introduced into the cells.

A protocol for *Agrobacterium tumefaciens* mediated transformation of rye was also developed in this study, giving rise to a total of 35 rye plants expressing the selectable marker gene *npt*II as identified by ELISA (Section 3.4, Table 9). Southern blot hybridisation confirmed the independent nature of all analysed transgenic plants (Figure 16). The majority of transgenic lines had a single transgene insert (*At*46, *At*53, *At*110, *At*123 and *At*123). Lines with up to four transgene copies were also observed (*At*66 and *At*69). Expression in T_0 plants of all analysed lines was stable regardless of the number of transgenic copy number.

Genes of interest

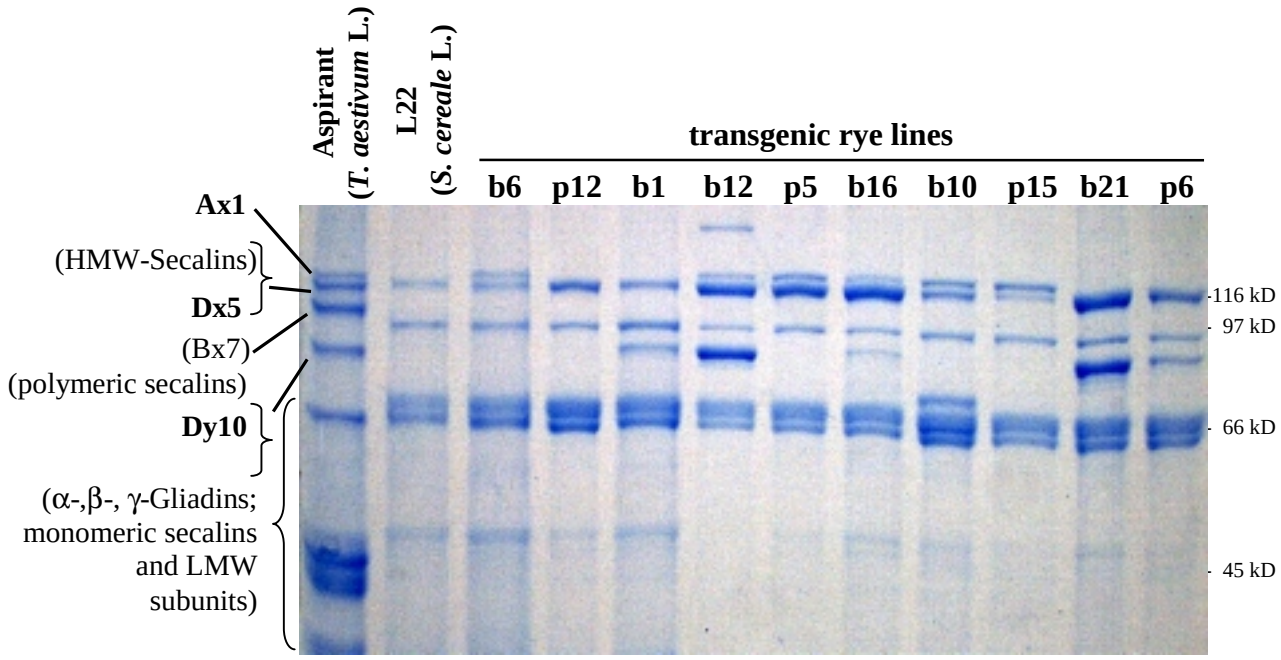


Figure 17: Protein extracts from transgenic rye (*Secale cereale* L.) seeds expressing HMW-gs of wheat (*Triticum aestivum* L.).

Protein separation on SDS-PAGE reveals the expression of one (b6, p12, b1, b10, p15), two (p5, b21, p6) and three (b12 and b16) HMW-gs in transgenic rye, compared to the rye wild type L22 and Aspirant, a bread wheat variety expressing Ax1, Dx5 and Dy10 (HMW-gs = High Molecular Weight glutenin subunits).

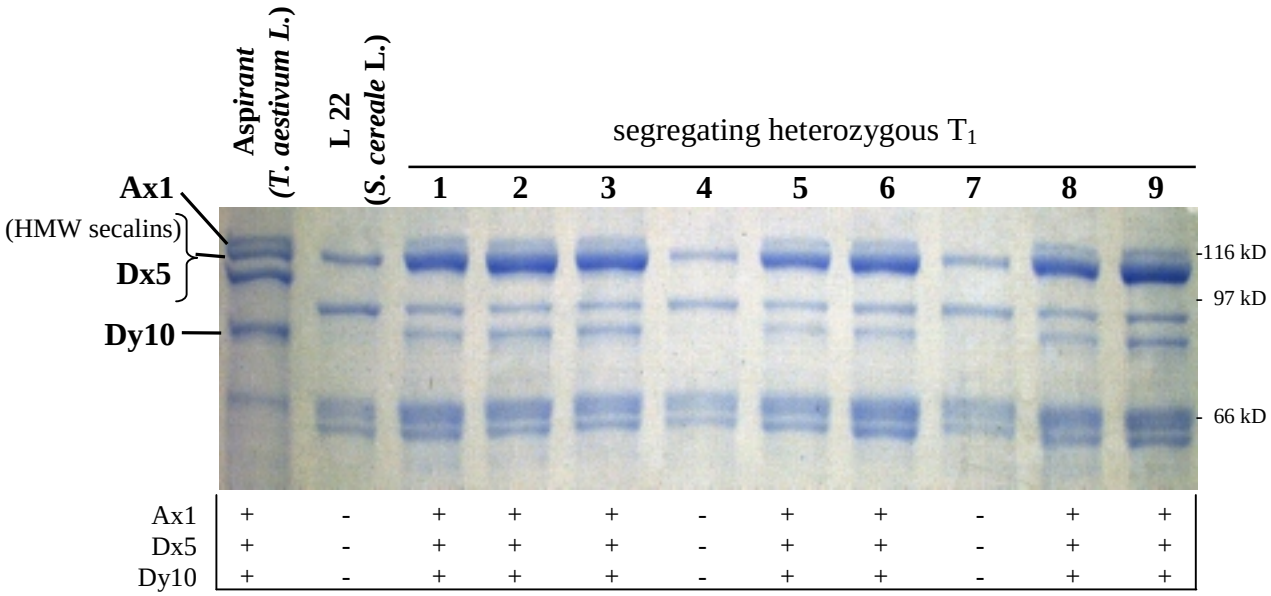


Figure 18: Co-segregation of co-expressed HMW-gs Ax1, Dx5 and Dy10 in transgenic rye (*Secale cereale* L.) line b16.

Protein separation on SDS-PAGE reveals co-segregation as a single dominant locus of transgenic rye line b16 (lanes 1, 2, 3, 5, 6, 8 and 9) in comparison to null-segregants (lanes 4 and 7), the rye wild type L22 and the bread wheat cultivar Aspirant.

Beside the selectable marker genes, three genes of interest, *1Ax1* (Halford *et al.*, 1992), *1Dx5* and *1Dy10* (Anderson *et al.*, 1989), encoding for the High-Molecular-Weight (HMW) glutenin subunits (-gs) Ax1, Dx5, Dy10 of bread wheat (*Triticum aestivum* L.) were co-transformed into rye with the respective marker genes in the biolistic experiments. Transgenic rye lines expressing one (p12, p15, b1, b6, b10), two (p5, p6, b21) and three HMW-gs (b12, b16) at levels detectable in SDS-PAGE gels are shown in Figure 17. A higher expression level for Dy10 was observed in line b21 and b12 compared to line p6, b1 and b16 while the expression level of Ax1 and Dx5 subunits are similar between respective lines. Novel, not further characterised protein fractions are present in line b12 and b10 (Figure 17).

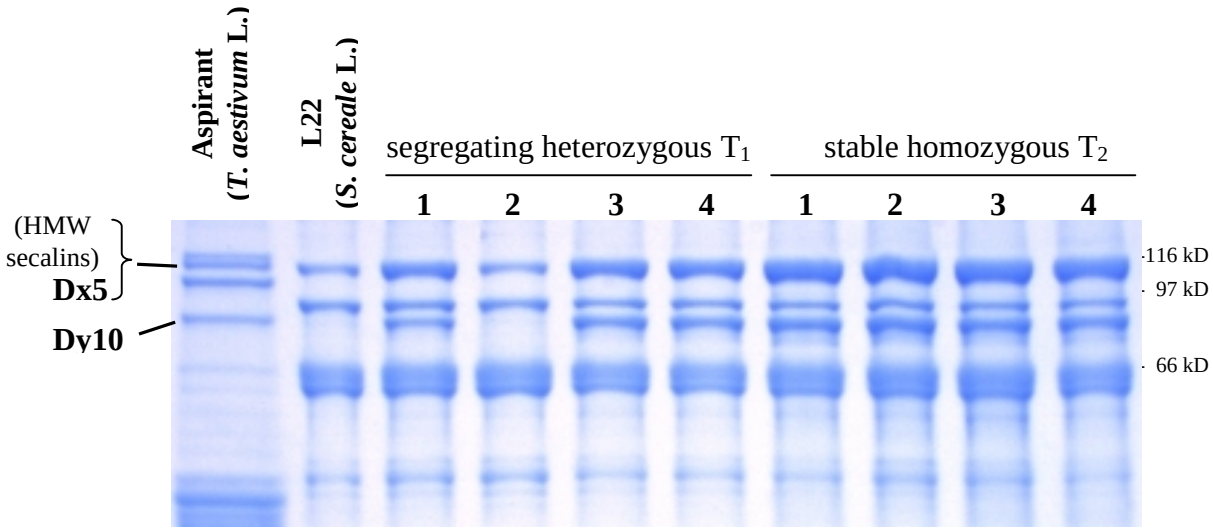


Figure 19: Elevated HMW-gs expression level of 1Dx5 and 1Dy10 in homozygous seeds (T_2) of transgenic rye (*Secale cereale* L.) line p6.

Protein of respective homozygous- (T_2) and segregating heterozygous (T_1) seeds was separated on SDS-PAGE and compared to the rye wild type L22 and the wheat cultivar Aspirant.

5.4.2. Transgene stability

The transgenes, selectable marker genes as well as genes of interest, were stably transmitted and expressed in subsequent sexual generations of most of the analysed lines (Table 15 and Table 16). Several of the lines obtained by biolistic genetransfer with four or more transgene copies (e.g. p11, b13, b18) showed non-detectable expression levels in subsequent generations. Segregation of the *npt*II gene in line p11 for instance, followed a mendelian integration pattern as a single locus, however no NPTII could be detected (Table 15). A reactivation of the NPTII expression could be observed after culturing shoot axes of line p11 on medium containing 5-azacytidine, a demethylating agent (Kumpatla *et al.*, 1997) (data not shown). This suggests transcriptional silencing by cytosine methylation of the transgene promoter in line p11. Transgenic line b10 on the other hand, showed a segregation of expressing and non-expressing progeny plants of 1:1 after self pollination. This suggests a threshold sensitive gene silencing of the homozygous offspring commonly associated with post-transcriptional gene silencing (Baulcome and English, 1996) (Table 15). A segregation pattern typical for multiple loci insertion was observed in transgenic lines b2 and b5 for three and two independent loci, respectively (Table 15).

Table 15: Integration and segregation pattern of the selectable marker genes *npt*II and *bar* in transgenic progeny of rye (*Secale cereale* L.) depending on the copy number of transgenic inserts after biolistic gene transfer.

Transformants	Copy number (Approx.)	R	S	Segregation ratio (χ^2-value, *p*)		Number of loci
				Expression	Integration (PCR)	
p1	1	15	8	3:1 (1.17, *p*>0.50)		1
p6	1	18	6	3:1 (0.00, *p*>0.90)		1
b1	1	66	19	3:1 (0.32, *p*>0.50)		1
b5	1	47	3	15:1 (0.01, *p*>0.90)		2
b8	1	2	2	3:1 (1.32, *p*>0.25)		1
b12	1	27	11	3:1 (0.32, *p*>0.50)		1
b19	1	4	0	3:1 (1.32, *p*>0.25)		1
p14	2	13	9	3:1 (2.97, *p*>0.05)		1
p16	2	33	15	3:1 (1.00, *p*>0.25)		1
p15	3	29	7	3:1 (0.59, *p*>0.25)		1
p8	3-4	5	7	3:1 (7.11, *p*>0.005)		1
p12	4	27	8	3:1 (0.09, *p*>0.75)		1
p13	4	11	7	3:1 (1.85, *p*>0.10)		1
p17	4	10	6	3:1 (1.32, *p*>0.25)		1
b20	4	1	1	3:1 (0.67, *p*>0.25)		1
p2	5	9	10	3:1 (7.74, *p*>0.005)		1
p11	5	0	18	---	3:1 (0.67, *p*>0.25)*	1
b16	5	40	19	3:1 (1.63, *p*>0.10)		1
b2	7	41	1	63:1 (0.18, *p*>0.50)		~3
b6	7	27	10	3:1 (0.08, *p*>0.75)		1
b10	>10	9	15	1:1 (1.50, *p*>0.10)		≥1

Abbreviations: R = resistent, S = sensitive, *=for Seggregation 12:18 after PCR analysis

The stability of transgene expression was negatively affected in most of the lines with high transgene copy number as described earlier by Matzke and Matzke (1995). However, examples of stable mendelian segregation and expression were also observed in transgenic lines with four (p17) and seven inserts (b6) (Table 15). Additional factors beside the transgene copy number significantly contribute to the level of expression and its stability in rye.

The selfed progeny of ten transgenic plants after *Agrobacterium*-mediated transformation was evaluated for *npt*II expression. Out of ten lines, eight segregated 3:1 for resistance:paromomycin sensitivity which confers to Mendelian segregation for a single dominant locus (Table 16). Transgenic line *At*66 showed a segregation pattern revealing an integration pattern typical for at least two independent dominant loci (Table 16; Figure 16) and expressed stably in the sexual progeny. On the other hand lines *At*114 and *At*53 showed a segregation patern of 1:1 for NPTII expression (Table 15) suggesting a threshold sensitive

gene silencing of the homozygous offspring, as also described for the lines b16 and b10 after biolistic transformation (Table 15).

Table 16: Integration- and segregation pattern of the selectable marker gene nptII in transgenic T_1 progeny after *Agrobacterium*-mediated transfer in rye (*Secale cereale* L.) genotype L22.

Transformants	Copy number (approx.)	Segregation ratio (χ^2-value, *p*) Expression R	S		Number of loci
At 46	1	14	9	3:1 (2.45, *p*>0.10)	1
At 53	1	9	9	1:1 (0.00, *p*>0.99)	1
At 59	2	16	6	3:1 (0.06, *p*>0.25)	1
At 64	1	16	8	3:1 (0.89, *p*>0.25)	1
At 66	4-5	25	0	64:1 (0.40, *p*>0.50)	≥3
At 69	4	8	4	3:1 (0.44, *p*>0.50)	1
At 110	1	15	4	3:1 (0.16, *p*>0.50)	1
At 114	3	11	10	1:1 (0.15, *p*>0.50)	≥1
At 123	1	18	6	3:1 (0.00, *p*>0.99)	1
At 148	1	20	5	3:1 (0.33, *p*>0.50)	1

Abbreviations: R= resistent, S= sensitive

Multiple HMW-gs co-segregated as a single dominant locus in mendelian fashion (Figure 18 and 19 for line b16 and p6, respectively). Homozygous lines, stably expressing multiple HMW-gs in the T_2 at an elevated level compared to the heterozygous T_1 generation, were identified (Figure 19, for line p6).

The presented results describe for the first time the efficient and reproducible production of stably expressing transgenic rye plants. The integration of multiple optimised factors was essential for successful transformation of this recalcitrant crop, including (1) the identification of homozygous inbred lines with a uniform and superior tissue culture performance (Chapter 2) in contrast to earlier used heterozygous open pollinated population cultivars (Castillo *et al.*, 1994); (2) genotype specific adjustment of parameters that influence the regeneration potential of bombarded and selected tissues, such as osmotic treatment prior to bombardment, pre-culture period before bombardment, low amount of particles for biolistic gene transfer, a short callus culture period and an emphasis on selection during the regeneration period (Chapter 3); (3) use of a selective agent that allows the control of most of the escapes if applied only during or after regeneration of the transgenic plantlets (Section 4.4).

6. Application of the gene-transfer technology

In modern plant breeding classical methods are more and more complemented by biotechnological techniques, such as selection on cell and tissue level, DNA and genome analysis for marker-assisted selection and transfer of novel genes into elite genotypes. Advances in the development of gene-transfer techniques make it now possible to overcome biological hurdles such as the inability to sexually cross different species. In principle, genes from any species, and even synthetic genes, can be introduced into any transformable genome. By integration of one or few genes, defined features can be ameliorated. Genetic transformation can expand the range of genetic variation in both additive and subtractive ways. Using sense and anti-sense suppression it is possible to create dominant loss-of-function mutants in a single generation. During recent years many new genetically modified crops have been generated, some field-tested or even commercially released. Different achievements were realised:

(1) Enhancing the resistance of plants: (a) to herbicides like PPT (De Block *et al.*, 1987) and glyphosate (Comai *et al.*, 1995); (b) to insects by expressing toxic proteins of *Bacillus thuringensis* (Nayak *et al.*, 1997; Delannay *et al.*, 1989; Wilson *et al.*, 1992), α-amylase inhibitor (Shade *et al.*, 1994) and an thrypsin inhibitor (Altpeter *et al.*, 1999); (c) to viral pathogens by protein mediated (Dempsey *et al.*, 1998) or RNA mediated strategies (Pang *et al.*, 1993; Xu *et al.*, 2001), the use of antibodies (Tavladoraki *et al.*, 1993) or the use of naturally ocurring plant-resistance genes (Salmeron and Vernooij, 1998); (d) against bacterial and fungal pathogens expressing chitinase and glucanase genes (Zhu *et al.*, 1994; Jach et al 1995), expression of a grapevine phytoalexin (Hain *et al.*, 1993) and detoxifying genes (Zhang *et al.*, 1999a) and expression of antifungal proteins (Clausen *et al.*, 2000).

(2) Artificial induced male sterility to produce hybrid seeds: (a) by anther-specific expression of ribonucleases (Mariani *et al.*, 1990, 1992); (b) tapetum specific expression of endo-β-1,3-glucanase (Tsuchiya *et al.*, 1995).

(3) Improvement of plant micronutrient content for nutritional health and well being of humans and animals (DellaPenna 1999): (a) by enrichment of β-carotene (Ye *et al.*, 2000) (b) increase of iron content (Goto *et al.*, 1999).

(4) Modification of storage carbohydrates and proteins: (a) increase of starch production by ADP glucose pyrophosphorylase (Stark *et al.*, 1992); (b) cold storable potatoes by ectopic

expression of invertase-inhibitor-like proteins (Greiner *et al.*, 1999); (c) production of soft, starchy endosperm (*fluory2* phenotype) by expression of a *α-zein* mutant gene (Coleman *et al.*, 1997); (d) improvement of breadmaking quality by expression of high-molecular-weight glutenin subunits (Altpeter *et al.*, 1996b; Barro *et al.*, 1997; Rooke *et al.*, 1999; He *et al.*, 1999; Vasil *et al.*, 2000).

(5) Production of vaccines: (a) by expression of antigens (Curtiss and Cardineau, 1990; Haq *et al.*, 1995); (b) expression of antibodies used for passive immunotherapy (Hiatt *et al.*, 1989) or after purification (Ma *et al.*, 1998).

(6) Understanding plant genomes (Gordon-Kamm *et al.*, 1999).

Since a transformation protocol was missing, so far there is no report for integration of a useful transgene in rye. Possible applications of such a system are theoretically endless and limited only by available gene sequences and existing copyrights.

6.1. Biotechnology of bread-making: an example

Breadmaking is one of humankind's oldest technologies. The ability to make leavened bread depends largely on the visco-elastic properties conferred to wheat dough by the gluten proteins. These allow the entrapment of carbon dioxide released by the yeast, giving rise to a light and porous bread structure. Wheat gluten proteins exhibit physical properties, which are usually described as a combination of elasticity with extensibility or viscous flow. Elasticity is an unusual property of proteins, and no other example of elastic protein derived from plants is known (Shewry *et al.*, 1995). Gluten is not a single protein, but a complex mixture of components with over 50 individual proteins (Shewry *et al.*, 1995) that are classified according to their solubility in aqueous alcohol into two groups: the soluble gliadins and the insoluble glutenins. The gliadins are present as monomers, since they either lack cysteine or have only intra-chain disulphide bounds. In contrast, glutenins consist of high molecular (M_r) polymers stabilised by inter-chain disulphate bounds. Both fractions have functional significance, as the glutenins are responsible for gluten elasticity and the gliadins for the viscosity. Treatment of the glutenin fraction with a reducing agent converts the polymers in to soluble monomers, resulting in a loss of elasticity (Shewry *et al.*, 1995). The cysteine residues in the HMW-gs are concentrated in the two terminal domains and are necessary for the formation of these glutenin polymers. Today there is little doubt that all the individual proteins contribute in some way to the functional properties of the whole gluten.

One group of proteins, however, appears to have a particular importance in determining dough elasticity. This group, the high molecular weight (HMW) subunits of gluten (-gs), also called the “flour power“ proteins (Wrigley 1996) which represent approximately 0.5% of the total seed dry weight have been extensively studied because of their effect on elasticity and hence the bread-making quality of wheat dough. HMW-gs form high molecular polymers together with the monomeric, S-rich gliadins and polymeric low-molecular-weight (LMW) glutenin subunits (Figure 20).

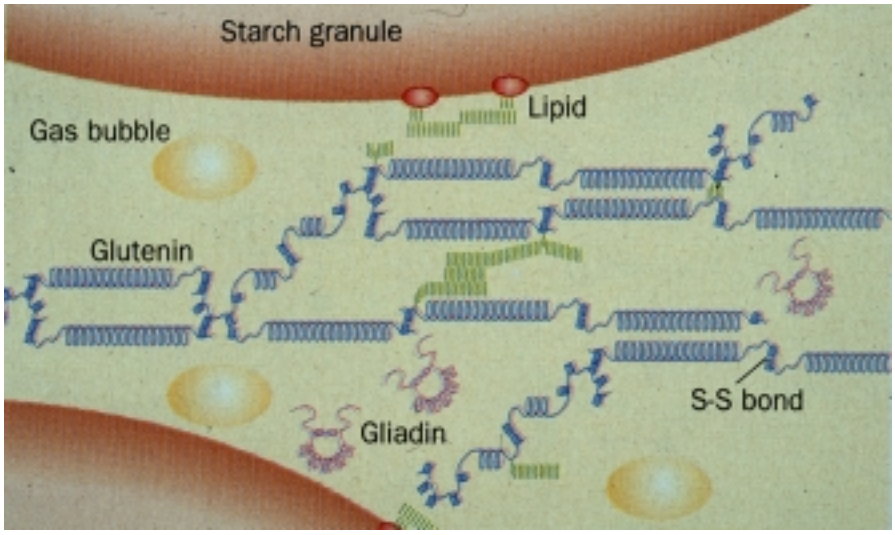


Figure 20: HMW-gs as main factor for bread-making quality of wheat dough: protein matrix formation by disulphide-boundings (Wrigley 1996; original drawing by R. Appels).

These gluten polymers with M_r values of over 10 million are some of the largest protein molecules found in nature (Wrigley 1996), giving wheat dough its superiority for making bread, pasta and other food products. Six HMW-gs genes are present in each cultivar of hexaploid bread wheat, but because of specific gene silencing only three, four or five subunits are expressed in different cultivars (Vasil and Anderson, 1997) and are explanatory for their polymorphism. Thirteen HMW-gs genes have been isolated and sequenced from bread wheat and ancestor genomes, including pairs of alleles correlated with good and poor dough-processing characteristics (Shewry *et al.*1992). The major subunits 1Ax1, 1Ax2*, 1Bx7, 1By9, 1Dx5 and 1Dy10 have been associated with increased dough strength.

In rye only one HMW subunit, the polymeric HMW secalins and mainly S-poor prolamins (ω -secalins) are present (Table 17). Rye does not form gluten, and its dough is, in difference to bread wheat, characterised by high viscosity and lack of elasticity. HMW subunits of wheat

and rye have been analysed and compared, showing high similarities concerning number of subunits, molecular masses, peptide composition and partial sequences, with approximately 80% peptide similarity (Wieser *et al.*, 1996; Kipp and Wieser, 1999). Rye HMW-subunits are more hydrofoil and the cysteine content is increased by the factor 2-3 in comparison with HMW-subunits of wheat (Kipp and Wieser, 2002). The authors assume that an intra-molecular disulphide bound might terminate the polymerisation of rye subunits (Kipp and Wieser, 2002), and therefore be responsible for the poor dough-processing characteristics of rye flour. Instead of the protein fraction the main determinant for the baking performance of rye is therefore the starch content. Cultivars with high bread making quality require the resistance to pre-harvest sprouting and the main breeding goal related to baking performance is therefore a low level of α-amylase activity. However, climatic conditions remain the main determinant for baking quality of the harvest.

Table 17: Prolamin groups present in rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.).

Protein fraction	Rye	ca. M_r (D)	Wheat	ca. M_r (D)
HMW prolamins (polymeric)	HMW secalins	90,000	HMW glutenins	65,000 to 90,000
S-poor prolamins (monomeric)	ω-secalins	39,000	ω-gliadins	50,000 to 60,000
S-rich prolamins				
Ancestral γ-type (monomeric)	γ-secalins	40,000	γ-gliadins	30,000 to 50,000
Derived γ-type (polymeric)	γ-secalins	75,000	---	
α-type (monomeric)	---		α-gliadins	30,000 to 50,000
Aggregated type (polymeric)	---		LMW glutenin subunits	?

Abbreviations: M_r = molecular mass; HMW = High-Molecular-Weight; LMW = Low-Molecular-Weight.

Considering the three classes of glutenin polymer formation (Figure 21), (A) large polymers of a mixture of HMW-gs and LMW-gs, (B) shorter polymers containing HMW-gs and LMW-

gs and (C) polymers exclusively formed of LMW-gs, different targets for genetic engineering were suggested (Vasil and Anderson, 1997) and are shown in Figure 21.

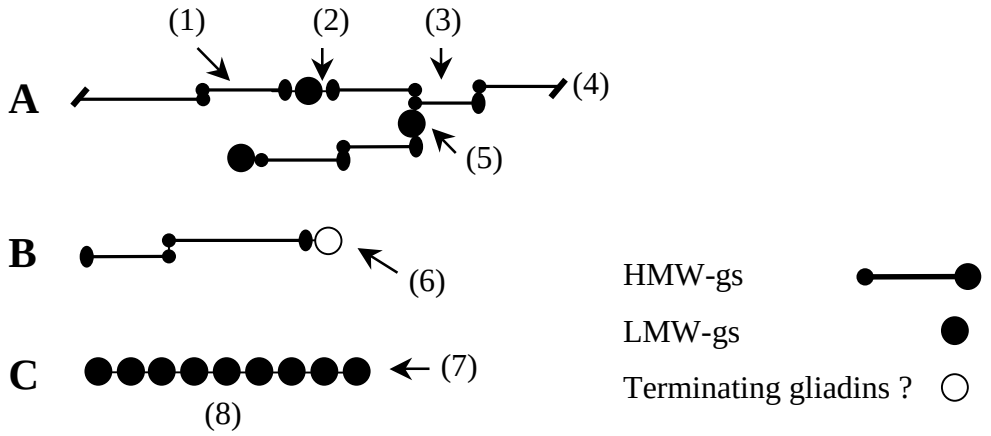


Figure 21: Formation of glutenin polymer and targets for genetic engineering (Vasil and Anderson, 1997).

Structure and function of HMW-gs could thereafter be engineered by (1) increasing the amount of HMW-gs with respect to the total seed protein, (2) altering the number and position of cysteine residues, (3) modification and alteration of the repetitive peptide domain, (4) altering the length of the glutenin macro-polymer, e.g. considering the specificity of disulphide crosslinks and (5) favouring branching instead of linear polymer formation. Further, transformation technologies could help to approach other important questions in gluten functionality, such as (6) the role of LMW-gs with only one cysteine for intermolecular bonds (Kasarda 1989) and similarly of gliadins with one available cysteine, that might function as polymer chain terminators, (7) the role of LMW-gs in baking quality and (8) the organisation, size and cross-link specificity and the functional role of LMW-gs polymers.

Transformation of HMW-gs genes into wheat led to stable expression of transgenic 1Ax1 (Altpeter *et al.*, 1996b) and Dx5 and Dy10 (Blechl *et al.*, 1996) subunits of glutenin. Functional properties under greenhouse (Barro and Anderson, 1997) and field conditions (Vasil *et al.*, 2000) were improved and expression of transgenic HMW-gs in *Triticum turgidum* L. var. *durum* (He *et al.*, 1999) and *Tritordeum* (Rooke, *et al.*, 2000) also resulted in significant improvement in dough strength and stability.

Introducing genes encoding HMW-gs into rye could reduce the environmental sensitivity of the baking quality and provide the basis to analyse the interactions between HMW and LMW-subunits in rye. In the presented work the expression of the HMW-gs genes encoding 1Ax1, 1Dx5 and 1Dy10 in rye was demonstrated and transgenic rye lines expressing one, two and three transgenic HMW-gs were produced (Figure 17) with co-segregation as a single dominant locus in mendelian fashion (Figure 18).

By sexual crossing of lines with independent transgene integration (Figure 17), lines with multiple transgenic loci can be obtained and could result in over-expression of determined HMW-gs. Their stable expression will facilitate further investigation of their effect on bread making quality in rye. For this purpose, seeds were multiplied and mixing properties (mixograph) are currently being analysed.

6.2. Outlook

Efficient transformation systems for rye are now available by biolistic and *Agrobacterium*-mediated approaches. However, our understanding of the molecular factors that influence the molecular mechanisms of transgene DNA integration are still limited. Different hypothesis of transgene integration during biolistic gene transfer were stated after fluorescent *in-situ* hybridisation (FISH) and three-dimensional confocal microscopy on metha-and interphase wheat chromosomes (Abranches, *et al.*, 2000). The availability of an *Agrobacterium*-mediated gene transfer system is encouraging for attempts to transfer high-molecular-weight T-DNA constructs into rye. Transferring fragments as large as 150kb by the binary-BAC system (Hamilton 1997; Frary and Hamilton, 2001) would facilitate the engineering of whole metabolic pathways in a single transformation step. Characterisation of transgenic lines by molecular marker assisted localisation of the inserted transgene is a further task. For this, a dense molecular linkage map of rye is available (Börner and Korzun, 1998; Korzun *et al.*, 2001).

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1996-97: European Spelt (*Triticum spelta*) Network at Landes-satzuchtanstalt Hohenheim/Germany

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Publication list

Research Papers:

Popelka JC, Schubert S, Schulz R and Hansen AP (1996) Cadmium uptake and translocation during reproductive development of peanut (*Arachis hypogaea* L.). Angew Bot 70:140-143

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Patents:

"Verfahren zur raschen Herstellung von transgenen, einkeimblättrigen Pflanzen", Dtl.DE 10115761.4; PCT/DE 01/01209, including

„Verfahren Zur Herstellung von transgenen, markergen-freien Pflanzen",Prio 29.3.2000

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Erklärung

Hiermit erkläre Ich, daß diese Arbeit von mir abgefertigt wurde. Es wurden keine anderen als die in der Literatur zitierten Hilfsmittel benutzt. Diese Arbeit wurde weder für vergebliche Promotionsversuche verwendet noch irgendwo sonst zur Promotion eingereicht.

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