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# Evaluation of rye (*Secale cereale* L.) inbred lines and their crosses for tissue culture response and stable genetic transformation of homozygous rye inbred line L22 by biolistic gene transfer

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**Abstract** The efficient and reproducible production of stably expressing transgenic rye plants is described. Analysis of the genotype-specific callus culture-response of 21 rye inbred lines, single crosses and a population variety resulted in the identification of the highly responsive inbred line L22. Biolistic transformation experiments were performed using line L22 and the impact of different selection agents on the regeneration capacity was analyzed. Using the selectable marker gene *npt*II and corresponding paromomycin selection resulted in transformation efficiencies of up to 4.0% of the bombarded explants. A total of 17 independent transgenic rye plants were produced, and their stability and level of transgene expression was analyzed. The majority of these lines showed stable transgene expression. In contrast a few transgenic lines with multiple transgene inserts, provided evidence of transcriptional and post-transcriptional gene silencing.

**Keywords** Genetic transformation · Genotype screening · In-vitro response · Rye (*Secale cereale* L.) · Transgene expression · *npt*II

## Introduction

Genetic transformation is currently complementing conventional plant breeding in the development of advanced germplasm.The commercially important monocotyledonous crops proved to be more recalcitrant in tissue culture and genetic transformation than most of the dicotyledonous species. Genetic transformation systems were developed in the last decade for corn, rice, wheat, barley, oat and other monocot species.

Rye (*Secale cereale* L.) is one of the most recalcitrant plant species for tissue culture and genetic transformation. To-date two transgenic rye plants derived from a single experiment have been reported (Castillo et al. 1994). This important milestone in cereal transformation is motivating the development of a reproducible rye transformation protocol. Increased efficiency of stable transformation and reproducibility of results require the integration of multiple optimised factors. These include genotype-specific tissue culture (Shimada 1978) and transformation response (Iser et al. 1999; Rasco-Gaunt et al. 2001), quality and the developmental stage of the explant at the time of culture initiation (Armaleo et al. 1990), culture medium composition (Barro et al. 1998) and culture conditions, culture period prior and after gene transfer (Rasco-Gaunt et al. 1999), osmotic treatment of the tissue cultures to reduce tissue damage during biolistic gene transfer (Vain et al. 1993), transgene expression cassettes (Li et al. 1997), gene transfer system and its specific parameters (Altpeter et al. 1996), and the selection system and its parameters (Christou and Ford 1995).

Rye is a strictly cross-pollinating species and the identification of inbred lines displaying a good regeneration response from tissue cultures could significantly increase the reproducibility and efficiency of transformation experiments in rye. Therefore, this study includes the identification of inbred lines with superior regeneration potential, the optimisation of selection parameters with respect to the regeneration capability of the rye tissue cultures and transformation frequency, and the characterization of transgenic rye plants derived from alternative selection protocols using the selectable agent paromomycin sulphate.

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## Materials and methods

### Plants and explants

Plants were grown under controlled environmental conditions. Seedlings were vernalized 2 weeks after germination for a period of 50 days (8 h light, 4 °C). Subsequently, plants were kept at 12 h light, 10 °C, followed by a stepwise adaptation to 20 °C and a 16-h photoperiod before flowering. Spikes were covered with cellophane bags before anthesis to prevent cross-pollination. Surface-sterilization of immature caryopses and the preparation of immature embryos were prepared as previously described (Popelka and Altpeter 2001). Selective agents and transfer of the *npt*II gene were investigated with the spring-rye inbred line L22, which requires no vernalization.

### Culture media, callus culture and selection of transgenic events

The callus-induction medium consisted of MS salts (Murashige and Skoog 1962), 30 g/l of sucrose, 100 mg/l of casein hydrolysate, 500 mg/l of glutamine, 2.5 mg/l of 2,4-D, 3.0 g/l of phytagel and was supplemented with 72.9 g/l of mannitol for osmotic pre-culture before bombardment. For callus selection, 50 mg/l of paromomycin sulphate were added and phytagel was replaced by 6.0 g/l of agarose Type I. Media for regeneration were identical except that 2,4-D was omitted. Selective-regeneration medium was supplemented with 100 mg/l of paromomycin sulphate. The pH of all media was adjusted to 5.8 prior to autoclaving at 121 °C and 1.5 bars for 20 min. Immature embryos were placed with their scutellum side-up on callus-induction medium and cultured in the dark at 25 °C. To analyse the genotype-specific regeneration potential a 3-week and a 9-week callus-induction period with two 3-weekly subcultures where compared before transfer to regeneration medium for 4 weeks under a 16 h (light)/8 h (dark) photoperiod and approximately 300 $\mu$ Em$^{-2}$ s$^{-1}$ of illumination. Pre-cultured scutellar calli were transferred to callus-induction media (14 days after culture initiation) or directly to regeneration medium (21 days after culture initiation), both media containing the selective agent.

For transformation experiments immature embryos were pre-cultured for 5 days, osmotically pre-treated during 5 h prior to bombardment, and transferred to fresh callus-induction medium 12 to 16 h after bombardment using two different selection treatments. After 3 weeks on regeneration medium calli were transferred into culture-containers for shoot elongation.

### 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 the first intron (Christensen and Quail 1996) and the 35-S terminator, inserted in the pPZP111 vector (Hajdukiewicz et al. 1994), was introduced into rye tissue-cultures by biolistic gene transfer.

DNA coating of gold particles was prepared using equal amounts of 0.6 and 1.0 $\mu$m microparticles from BIO-RAD and 6 $\mu$g of DNA (containing Vector DNA) per precipitation reaction according to Sanford et al. (1991). A total of 35 $\mu$g of microparticles were used per bombardment using the Biolistic Particle Delivery System (PDS)-1,000/He, and 1.100 psi rupture disks from Bio-Rad.

### Evaluation of tissue-culture response

Evaluation of the tissue-culture response was repeated independently by two operators. Induced embryogenic calli were counted 3 weeks after culture initiation. The regeneration response was quantified 3–4 weeks after transfer on regeneration medium by counting the number of regenerating calli (regeneration frequency) and the number of regenerated plantlets. In biolistic experiments the regeneration response and the transformation efficiency (NPTII-ELISA positive plants in the % of bombarded explants) were recorded.

### Experimental design and statistical analysis

For the genotype-screening donor plants were grown in two replications in separate environments, each with three completely randomised blocks. Per environment and callus-culture length, 25-embryos were cultured resulting in 12 × 25 explants per genotype. Per selective agent and concentration, two Petri dishes with 12 calli each were cultured. Each transformation experiment was repeated three times with 150 explants per selection protocol and 25 explants per bombardment, selection and regeneration control. For statistical data analysis the SAS System (1990) was used and the GLM procedure with the *t*-test applied. Segregation patterns were analysed with the chi-square test ($\chi^2$) against the expected ratio of 3:1.

### Southern-blot analysis

Genomic DNA from transgenic plants was extracted according to Dellaporta et al. (1983); restriction, digested with *Bam*HI that cuts the pJFnptII plasmid once in the multi-cloning site downstream of the transgene expression cassette, was separated on an 0.8% (w/v) agarose gel and blotted on Hybond-N membrane (Amersham) using an alkaline transfer buffer (0.4 M NaOH, 0.6 M NaCl). Probes for hybridisation were prepared by PCR and the amplification product was purified on 1.0% (w/v) agarose gel. The resulting 800-bp fragment, which included the sequence of the promoter and the coding region, was extracted using the QIAEX II gel-extraction kit and [$^{32}$P]$\alpha$-dCTP labeled using the random primer labeling kit from GIBCO-BRL. Hybridisation was performed using Rothi-Hybri-Quick solution (Roth) for 24 h 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 visualized by using the phosphor-imaging system (Storm).

### Immunodetection of transgene expression

For NPTII ELISA (5′Prime → 3′Prime, Inc.; Boulder, Calif.) 40 $\mu$g per microwell of protein in crude extracts (0.25 mM Tris-HCl, pH 7.8 and 0.1 mM PMSF) was used, following the manufacturers instructions. For NPTII Western blotting, 20 $\mu$g of crude protein per transgenic rye plant were separated by 12% (v/v) SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham). After overnight blocking with dried milk the membrane was incubated for 1 h each, with 1:5000 biotinylated NPTII-antibody and 1:5000 streptavidin-conjugated alkaline phosphatase (both from 5′Prime → 3′Prime). The signal was visualized after incubation with ProtoBlot II (Promega) for several minutes.

### Screening of segregating progeny

Genomic DNA from segregating progeny plants was used as a template for transgene integration-analysis by PCR. Oligonucleotide primers were derived from the ubiquitin promoter (5′-GTC TGG TTG GGC GGT CGT TCT AG-3′) and the *npt*II coding region (5′-GTG CCC AGT CAT AGC CGA ATA GC-3′). PCR conditions were as follows: 4 min pre-denaturation at 94 °C followed by 30 cycles of 25 s denaturation at 94 °C, 30 s annealing at 60 °C and 50 s extension at 72 °C each, and a final 5-min extension step at 72 °C. PCR amplification products were separated by agarose-gel electrophoresis (1% w/v) and visualized by ethidium-bromide staining. To analyse *npt*II gene expression, plants

were evaluated 1 week after leaf painting of the seedlings with 1% (w/v) paromomycin solution with 0.2% w/v Tween-20.

## Results

### Genotypic in vitro response

Callus induction and regeneration response were evaluated from 11 genetically divergent homozygous inbred lines, 11 crosses between inbred lines ($F_1$) and the population cultivar Wrens Abruzzi. Plants of different genotypes differed in phenotypic appearance and flowering time. 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 (Fig. 1H for L22) was developed within 3 weeks after culture initiation from 81% (L20) to 99% (L17 × L20) of the cultured explants with no significant difference between $F_1$ crosses (95.4%) and inbred lines (91.2%) (Table 1).

Regeneration of callus tissue to plants (Fig. 1I and J for L22) was observed in most of the genotypes with a considerable genotypic difference in the regeneration rate (the % of explants forming calli with plant regeneration) ($Pr > F = 0.0001$). After a short callus-induction period of 3 weeks the highest regeneration rates were observed from L15 (91.65%), L22 (86.87%) and L22 × L15 (85.80%), not differing significantly from each other but from the other tested genotypes (Table 1). On average over all genotypes, single crosses regenerated more plants per regenerating callus (4.43) than inbred lines (3.03) or the population variety Wrens Abruzzi (1.33). However, the inbred line L22 regenerated a significantly higher number of shoots per callus (6.93; Table 1; Fig. 1C) than all other genotypes. The regenerated plants (Fig. 1K to M) were normal and fertile (Fig. 1N) after transfer to soil, encouraging gene-transfer experiments.

After extending the callus-induction period from 3–9 weeks the regeneration rate decreased, on average of all genotypes, from 47.22% to 14.12% with distinct genotypic interactions ($Pr > F = 0.0001$ for regeneration rate

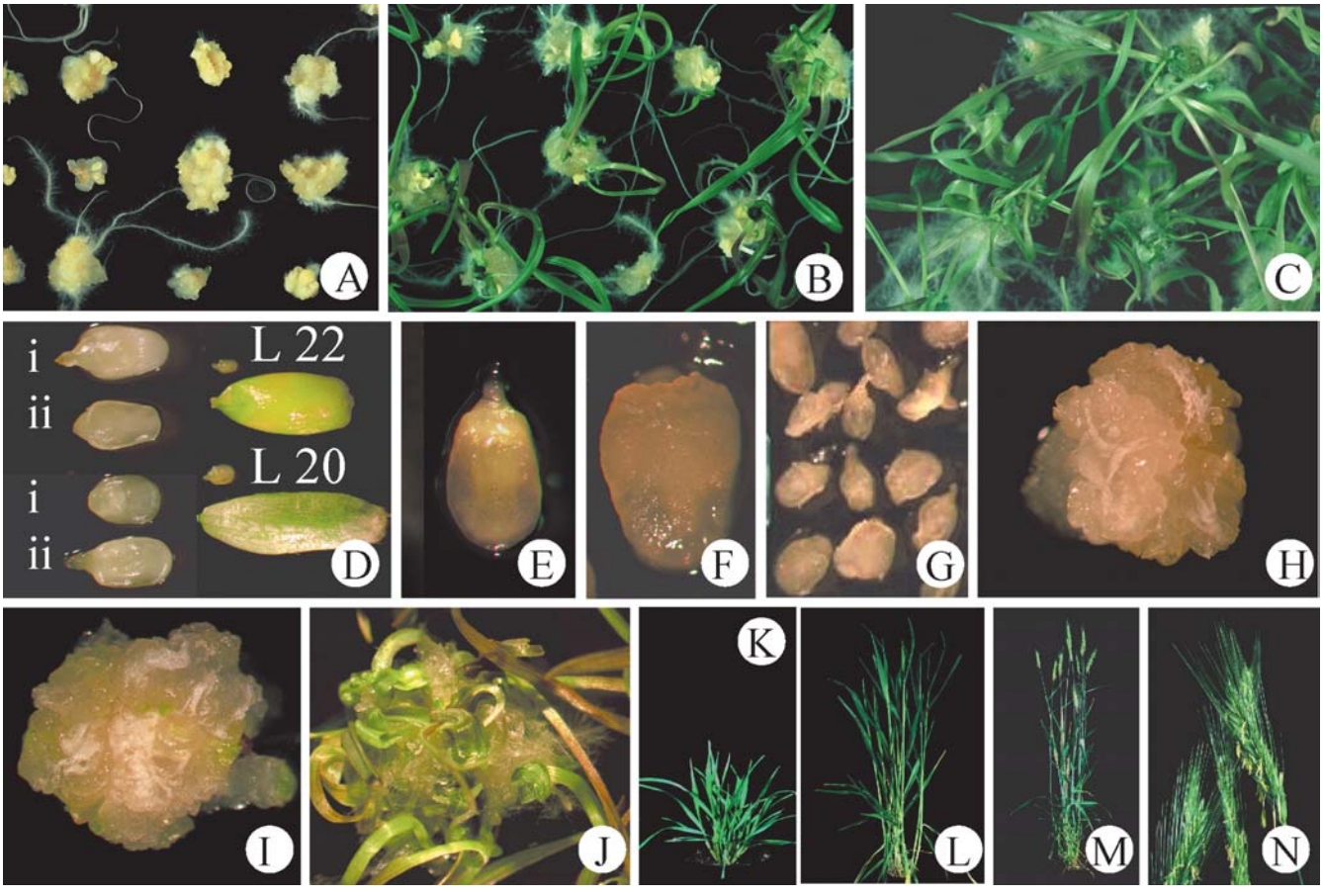


**Fig. 1A–N** Genotypic variation for in vitro culture response of rye (*S. cereale* L.): regeneration of fertile plants from scutellar callus of immature embryos. **A–C** Genotypic variation in regeneration response: low (L16), medium (L20) and high (L22) responding genotypes after 21 days of regeneration (**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 1- (E) and 5-days (F and G) culture on callus-induction medium. Embryogenic scutellar callus (L22) 21 days after culture initiation (**H**). **I–N** Callus regeneration: callus (L22) 3 (**I**) and 21 days (**J**) after transfer to regeneration conditions. Regenerated rye plants (L22) were transferred to soil and showed a normal development during tillering (**K**), heading (**L**) and flowering (**M and N**)

**Table 1** Tissue-culture response in rye (*S. 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

| Genotype | 21 Days callus culture before regeneration[a] | | | | 3 × 21 Days callus culture before regeneration[a] | | |
|---|---|---|---|---|---|---|---|
| | Replication[b] (total) | Callus induction Embryogenic calli (% of explants) | Regeneration response | | Replication[b] (total) | Regeneration response | |
| | | | Regenerating calli (% of calli) | Shoots per reg. Callus (mean) | | 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 |

$^{a, b, c, d, e, f, g, h, i, j}$ 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 the low number of replications

[a] Callus induction was followed by 4-weeks culture on regeneration medium before regeneration responce was recorded

[b] 25 Embryos were cultured per replication

and $Pr > F = 0.0048$ for regenerated shoots per callus; Table 1).

## Selection system

The selection efficiency of two different selective agents at different concentrations (0.5–4 mg/l of phosphinothricin; 10–100 mg/l of paromomycin sulphate) on tissue-culture performance and regeneration was evaluated after application during callus induction or regeneration. Both agents suppressed callus embryogenesis. This was more pronounced on phosphinothricin-containing medium where calli were soft and structureless even at a concentration of 1.0 mg/l and shoot regeneration was strongly suppressed. Only 25% of calli grown on callus-induction medium containing 4 mg/l of phosphinothricin regenerated shoot primordia. 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 phosphinothricin was applied exclusively during rye-callus regeneration a strong suppression of shoot and root regeneration was not observed. In contrast to the results observed using 0.5 to 4 mg/l of phosphinothricin, 100 mg/l of paromomycin fully suppresses shoot and root formation if applied exclusively during the regeneration process of rye tissue cultures (Table 2). This allows developing a selection protocol that will not interfere with callus embryogenesis and should realize the full regeneration potential of transgenic calli.

## Transformation

Line L22 was chosen for transformation experiments due to its superior in vitro response. Four to five days after pre-culture of immature embryos, calli were bombarded with micro-particles coated with a constitutive *npt*II expression cassette (Fig. 2A), and paromomycin sulphate selection was used for the identification of transgenic events. Either a 2-week callus selection and selection during the regeneration period or a selection exclusively during regeneration were applied. On an average of three independent experiments a higher transformation efficiency was observed if selection was exclusive during

**Table 2** Selection effect of the herbicide phosphinotricin and the antibioticum paromomycin sulphate on the regeneration potential of scutellar callus of rye (*S. cereale* L.)

| Type | Concentration (mg/l) | Selection during callus induction | | Selection during callus regeneration | |
|---|---|---|---|---|---|
| | | Regenerating calli (% ± SD) | Shoot primordia (mean ± SD) | Regenerating calli (% ± SD) | Shoot primordia (mean ± SD) |
| Paromomycin sulfate | 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 |
| Phosphino-thricin | 0.5 | 95.84 ± 5.9 | 12.39 ± 0.2 | 100.00 ± 0.0 | 12.00 ± 0.4 |
| | 1 | 87.50 ± 5.9 | 7.87 ± 1.7 | 100.00 ± 0.0 | 9.75 ± 0.1 |
| | 2.5 | 66.67 ± 0.0 | 2.69 ± 0.1 | 80.00 ± 14.1 | 9.00 ± 0.3 |
| | 4 | 25.00 ± 11.8 | 3.13 ± 0.5 | 90.00 ± 7.1 | 5.78 ± 0.3 |
| Control cultures without selective agent: | | | | 80.00 ± 0.0 | 10.82 ± 0.8 |

**Table 3** Selection of transgenic NPT II-positive rye (*S. cereale* L.) plants, after selection at different culture stages (means of three independent replications)

| Experiment | Explants | Days on medium[a], post-bombardment | | | Regenerated plants | | Transformationfrequency (%)[b] |
|---|---|---|---|---|---|---|---|
| | | IM | IMP50 | RMP100 | Total number | ELISA positive | |
| 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 |

[a] IM and IMP50: callus-induction medium without and with 50 mg/l of paromomycin sulphate, respectively; RMP100: regeneration medium with 100 mg/l of paromomycin sulphate
[b] Number of independent transgenic rye plants expressing NPTII, in percent of bombarded explants

**Table 4** Segregation pattern for the expression of the selectable marker gene *npt*II in the T1 progeny. Abbreviations: R = resistant, S = sensitive, * = for segregation 12:18 after PCR analysis

| Transformants | Copy number (Approx.) | R | S | Segregation ratio of 3:1 ($\chi^2$-value, $p$) | | Number of loci |
|---|---|---|---|---|---|---|
| | | | | Expression | Integration (PCR) | |
| p1 | 1 | 15 | 8 | 1.17, >0.50 | | 1 |
| p6 | 1 | 18 | 6 | 0.00, >0.90 | | 1 |
| p14 | 2 | 13 | 9 | 2.97, >0.05 | | 1 |
| p16 | 2 | 33 | 15 | 1.00, >0.25 | | 1 |
| p15 | 3 | 29 | 7 | 0.59, >0.25 | | 1 |
| p8 | 3–4 | 5 | 7 | 7.11, >0.005 | | 1 |
| p12 | 4 | 27 | 8 | 0.09, >0.75 | | 1 |
| p13 | 4 | 11 | 7 | 1.85, >0.10 | | 1 |
| p17 | 4 | 10 | 6 | 1.32, >0.25 | | 1 |
| p2 | 5 | 9 | 10 | 7.74, >0.005 | | 1 |
| p11 | 5 | 0 | 18 | No expression | 0.67, > 0.25 * | 1 |

regeneration (2.2%) compared to a selection during callus induction and regeneration (1.1%; Table 3). Most calli cultured on selective-induction medium showed growth retardation, browning and no regeneration. All transgenic plants were confirmed by Southern-blot analysis and by the NPTII-Western-blot and NPTII-ELISA (Fig. 2B and C).

## Transgene integration, expression and stability

Transgenic plants with single-copy inserts (p1, p6, p10), few transgene copies (p4, p7) and high copy numbers (p2, p11, p13) where identified (Fig. 2B). Transgene expression levels, as detected with the semi-quantitative Western blotting, appear to correlate with the copy number. Highest expression levels were observed when two or few copies were inserted (p4, p7, p17) (Fig. 2B). Lowest transgene expression was detected in p1, p6 and p10, all with single-copy inserts (Fig. 2B). In several plants with high copy numbers (p2, p8, p11) no expression was found. However, high copy number did not always lead to gene silencing, as observed in p12 with about four copies.

The transgenes were stably transmitted and expressed in the subsequent generation of most of the analysed lines (Table 4). Line p11 with at least four transgene copies

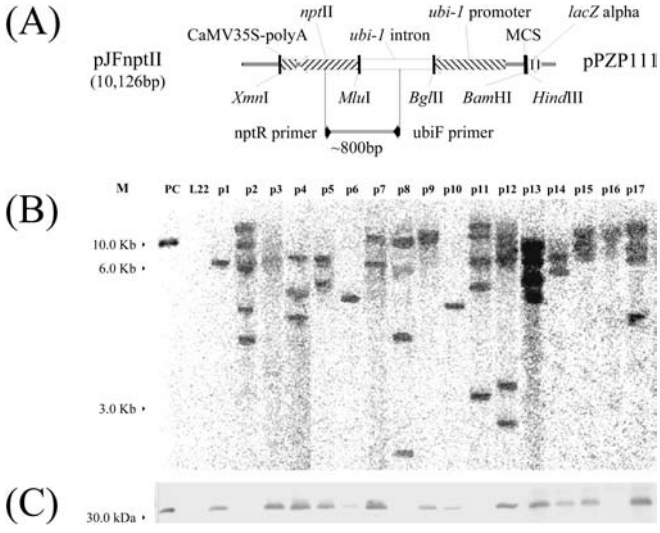


**Fig. 2A–C** Integration and expression analysis of the selectable marker-genes *npt*II in transgenic rye (*S. cereale* L.) plants. **A** Schematic representation of the pJFnptII vector: the expression cassette of the *npt*II gene under control of the maize *ubi* promoter and the *35*S terminator was cloned into the pPZP111 vector (Hajdukiewicz et al. 1994). PJFnptII contains a single *BamH*I restriction site, located in the multi-cloning site (MCS). Specific primers were designed for the amplification of an approximately 800-bp DNA fragment of the *ubi::npt*II region which was used as probe for Southern-blot analysis. **B** Southern blot of the *npt*II gene: individual lanes represent 10 to 15 $\mu$g of genomic DNA of transgenic $T_0$ plants (p1–p17) in comparison to a non-transformed wild-type plant (L22) and 25 pg of pJFnptII plasmid DNA (PC) after restriction digest with *Bam*HI and hybridization with an approximate 800-bp DNA fragment of the transgene coding region. **C** Western-blot analysis: individual lanes represent 20 $\mu$g of leaf protein extract of plants corresponding to (**A**) and 0.1 ng of purified NPTII protein (PC). Proteins were separated by 12% (v/v) SDS-PAGE and conjugated to primary and secondary antibodies from 5′Prime → 3′Prime (Inc.; Boulder, Calif.)

showed non-detectable expression levels in the subsequent generation. Segregation of the *npt*II gene in this line followed the Mendelian pattern as a single locus; however no NPTII could be detected (Fig. 2C; Table 4). However, a reactivation of the NPTII expression could be observed (data not shown) after culturing shoot axes of line p11 on medium containing 5-azacytidine, a de-methylating agent (Kumpatla et al. 1997).

## Discussion

The presented results describe the efficient and reproducible production of stably expressing transgenic rye plants. Integration of multiple optimised factors was essential for successfully transforming this recalcitrant crop. These included: (1) the identification of homozygous inbred lines with a uniform and superior tissue-culture performance in contrast to earlier used heterozygous open-pollinated population cultivars (Castillo et al. 1994); (2) the adjustment of parameters that influence the regeneration capacity of bombarded and selected tissues, like osmotic treatment prior to bombardment, low amount of gold particles for biolistic gene transfer, a short callus-culture period and an emphasis with the selection on the regeneration period; and (3) the use of a selective agent that allows the control of most of the escapes if applied exclusively during the regeneration of transgenic plantlets.

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: Rakoczy-Trojanowska and Malepszy 1993 and 1995) and up to 100% (with Karlshulder: Zimny and Lörz 1989) have been described. However, the number of regenerated rye plantlets per callus was not presented in most of the earlier studies. Rakoczy-Trojanowska and Malepszy (1995) reported an average of 1.9 green plants per callus for the best responsive genotype (L318). In our study, the inbred line L22 regenerated a significantly higher number of shoots per callus (6.93; Table 1; Fig. 1C), compared to all other tested genotypes and single crosses. The high reproducibility of this in vitro system, resulting from the use of defined homozygous inbred lines or their $F_1$ crosses as starting material for tissue culture, is an essential component for the establishment of a reproducible transformation protocol. However, the regeneration rate of all genotypes including L22 decreased significantly when the callus-induction period was extended (Table 1). Similar responses were observed in bread wheat (Fennel et al. 1996; Varshney and Altpeter 2002) and suggest minimizing the tissue-culture phase in a transformation protocol. In the presented study donor plants from growth chambers were used and little variation in callus-induction capacity of the tested genotypes was observed. Genotypic variations in callus induction observed in earlier studies (Rybczynski and Zdunczyk 1986; Rakoczy-Trojanowska and Malepszy 1995) might therefore be attributed to genotypic differences in flowering time associated with different environmental conditions during maturation.

Based on this in vitro culture system biolistic transformation experiments were performed. For this purpose transient reporter-gene activity has been frequently used to optimise biolistic parameters, such as particle density per bombardment, distance to target cells and particle acceleration pressure (Chibbar et al. 1991; Finer et al. 1992). Although transient expression frequency is positively correlated with the amount of particles per bombardment, it seems necessary to identify conditions that support the introduction of DNA with minimal tissue damage. Castillo et al. (1994) used 2500 $\mu$g of microparticles/bombardment, in some cases in two bombardments, which might have resulted in a considerable reduction of the regeneration potential. Consequently, the majority of transgenic calli did not regenerate into transgenic plants in these experiments. Osmotic treatment

of calli prior to particle bombardment (Vain et al. 1993) is a widely applied procedure to reduce tissue damage and consequently enhanced stable-transformation frequencies in the presented study (Table 3) in comparison to previous attempts in rye (Castillo et al. 1994).

After several subcultures on Basta- or phosphinothricin-containing medium the majority of transgenic rye calli had lost their regeneration potential (Castillo et al. 1994). To reduce the negative impact of a selective agent on embryogenesis of transgenic callus, we analysed phosphinothricin and paromomycin sulphate for their effect on tissue performance and regeneration. Paromomycin fully suppressed shoot and root formation if applied exclusively during the regeneration process of rye tissue cultures, and this allowed a selection protocol with no interference with callus embryogenesis. Paromomycin was also the superior selective agent for the transformation of oat (Torbert et al. 1995) and perennial ryegrass (Altpeter et al. 2000).

Due to its superior in vitro response, line L22 was chosen for transformation experiments involving the selectable marker gene *npt*II and paromomycin sulphate as a selectable agent. In contrast to Castillo et al. (1994) a long-term callus-selection period was replaced by a selection exclusively during regeneration, obtaining transformation efficiencies of up to 4.0%. The total period from culture initiation of the explants until the transfer of transgenic plants to soil was less than 3 months, while in the earlier report more than 6 months were required (Castillo et al. 1994). Transgenic plants developed like wild-type control plants (data not shown).

Three independent transformation experiments compared two different selection protocols and resulted in 15 transgenic rye plants (Table 3). These 15 transgenic rye plants and two from an additional experiment showed an independent transgene integration pattern in the Southern-blot analysis (Fig. 2B) demonstrating the efficiency and consistency of the presented transformation protocols.

Transgenic plants were confirmed by Southern blots and further analysed with expression assays. Expression levels of the selectable marker gene appear to depend on the copy number, being highest when two or few copies were inserted. A similar trend was also observed in transgenic wheat plants (Stoger et al. 1998). Most of the transgenic rye lines showed stable transgene expression and Mendelian segregation in sexual T1 progenies. Transgene silencing, which could be reversed by 5-azacytidine, a de-methylating agent (Kumpatla et al. 1997), was observed in Line p11 with approximately five inserts (Table 4). This suggests transcriptional silencing by cytosine methylation of the transgene promoter, as described earlier by Matzke and Matzke (1995). Transgenic lines p8 and p2 showed a segregation of expressing and non-expressing progeny plants of 1:1 after self-pollination (Table 4). This suggests a threshold sensitive gene-silencing in the homozygous offspring commonly associated with post-transcriptional gene-silencing (Baulcome and English 1996). However, stable Mendelian segregation and stable expression were even observed in a transgenic line with four inserts (p17, Table 4), suggesting that additional factors beside the transgene copy number substantially contribute to the level of expression and its stability in rye.

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