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ORIGINAL PAPER

A high-throughput *Agrobacterium*-mediated transformation system for the grass model species *Brachypodium distachyon* L.

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Abstract In the ongoing process of developing *Brachypodium distachyon* as a model plant for temperate cereals and forage grasses, we have developed a high-throughput *Agrobacterium*-mediated transformation system for a diploid accession. Embryogenic callus, derived from immature embryos of the accession BDR018, were transformed with *Agrobacterium tumefaciens* strain AGL1 carrying two T-DNA plasmids, pDM805 and pWBV-Ds-Ubi-bar-Ds. Transient and stable transformation efficiencies were optimised by varying the pre-cultivation period, which had a strong effect on stable transformation efficiency. On average 55% of 17-day-old calli co-inoculated with *Agrobacterium* regenerated stable transgenic plants. Stable transformation frequencies of up to 80%, which to our knowledge is the highest transformation efficiency reported in graminaceous species, were observed. In a study of 177 transgenic lines transformed with pDM805, all of the regenerated transgenic lines were resistant to BASTA®, while the *gus*A gene was expressed in 88% of the transgenic lines. Southern blot analysis revealed that 35% of the tested plants had a single T-DNA integration. Segregation analysis performed on progenies of ten selected T_0 plants indicated simple Mendelian inheritance of the two transgenes. Furthermore, the presence of two selection marker genes, *bar* and *hpt*, on the T-DNA of pWBV-Ds-Ubi-bar-Ds allowed us to characterize the developed transformation protocol with respect to full-length integration rate. Even when not selected for, full-length integration occurred in 97% of the transformants when using bialaphos as selection agent.

Keywords *Brachypodium distachyon* · Model plant · *Agrobacterium* · Transformation · Optimization

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
GUS	Glucuronidase
MG/L liquid medium	Garfinkel and Nester (1980) medium

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Introduction

The establishment and implementation of Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) as model plants has greatly accelerated research in plant biology. However, the value of these plants as models for temperate cereals and turf and forage grasses has been questioned (Devos et al. 1999; Tikhonov et al. 1999; Keller and Feuillet 2000; Vogel et al. 2006). Recently, the wild grass *Brachypodium distachyon* has been proposed as an alternative model species for this purpose (Draper et al. 2001). Although this species has no agricultural significance, it offers many advantages over current model systems for plant genetic, cellular and molecular biology studies in grasses. In addition other desirable features, *B. distachyon* boasts a simple genome, small size and a relatively rapid lifecycle (Draper et al. 2001). The importance of this proposed model is increasing as knowledge of the genetics and biology of *B. distachyon* broadens with respect to tissue culture and transformation techniques (Bablak et al. 1995; Christiansen et al. 2005; Vogel et al. 2006), plant-pathogen interactions (Draper et al. 2001; Routledge et al. 2004; Jenkins et al. 2005), cytogenetics (Hasterok et al. 2004), mutagenesis (Engvild 2005), BAC library construction (Hasterok et al. 2006) and the development of homogeneous inbred lines (Vogel et al. 2006).

One of the first, and most important, prerequisites in developing a model plant is the availability of an efficient transformation protocol. By utilizing the tissue culture and regeneration system previously described by Bablak et al. (1995), both microprojectile bombardment (Draper et al. 2001; Christiansen et al. 2005) and *Agrobacterium*-mediated transformation (Vogel et al. 2006) have been developed for Brachypodium. These protocols provide transformation frequencies of up to 15%. However, for large-scale genomics projects, for example applying T-DNA insertion mutagenesis, higher transformation frequencies are desired.

Callus quality is considered one of the most important parameters for an efficient tissue culture and in vitro transformation system. Brachypodium, like other species, produces a range of morphologically distinct callus types and embryogenic callus formation is genotype dependent (Bablak et al. 1995; Vogel et al. 2006). Immature embryos isolated from selected ecotypes of Brachypodium developed type II embryogenic callus at a rate between 45% (Draper et al. 2001) and 91% (Christiansen et al. 2005), with the tetraploid ecotypes apparently giving higher percentages.

Another major factor influencing the frequency of stable plant transformation is the efficiency of T-DNA transfer from *Agrobacterium* into the plant genome. Actively dividing cells, and particularly those in the *S*-phase of the cell division cycle, are necessary for *Agrobacterium* T-DNA transfer and/or integration (Binns and Thomashow 1988; Kartzke et al. 1990; Villemont et al. 1997; Gordon-Kamm et al. 2002). Metabolically active cells produce *vir*-inducing compounds essential in the early phase of transformation (Stachel et al. 1985; Spencer and Towers 1991). Stimulation of cell division in callus tissue by auxin analogues, such as 2,4-D, enhances *Agrobacterium*-mediated transformation in forage grasses (Bettany 2003) and pre-cultivation of Arabidopsis explants, prior to inoculation, on a medium rich in auxins greatly increased the number of competent cells for *Agrobacterium* transformation (Sangwan et al. 1992). Moreover, phytohormone pretreatment can overcome the recalcitrance of several Arabidopsis genotypes to *Agrobacterium*-mediated transformation (Chateau et al. 2000).

Considering these studies, we aimed to develop a highly efficient *Agrobacterium*-mediated transformation protocol for the diploid *B. distachyon* accession BDR018. We focussed on improving the efficiency of transformation by varying the pre-cultivation period on hormone-containing medium, thereby maximizing the amount of embryo-derived callus tissue amenable to transformation and subsequent regeneration. The optimized transformation procedure reported here is expected to facilitate large-scale Brachypodium projects in the future.

Results

Optimization of stable transformation frequency through variation of pre-cultivation period

A protocol previously developed for the tetraploid *B. distachyon* accession BDR030 (Ingo Lenk, unpublished data), using *Agrobacterium tumefaciens* strain AGL1 carrying the vector pDM805 (Tingay et al. 1997), was modified to develop a transformation

system for the diploid accession BDR018. Immature BDR018 embryos were pre-cultivated for 3 days on plain callus induction media and co-cultivated with *Agrobacterium* for 5 days. Low levels transient GUS expression were observed and no stable transgenic lines were recovered. A comparison of tissue culture behaviour of these two accessions revealed that the formation of embryogenic callus from scutellar tissue was remarkably slower in BDR018 (data not shown).

It is generally assumed that actively dividing cells are most amenable to genetic transformation by *Agrobacterium* (Kartzke et al. 1990; Villemont et al. 1997). Therefore, we decided to optimize the transformation procedure for BDR018 by varying the pre-cultivation period, during which immature embryos develop into callus-like tissue. Moreover, the co-cultivation period with *Agrobacterium* was restricted to 3 days in all experiments in order to avoid bacterial overgrowth of the callus tissue.

When pre-cultivation period was varied between 1 and 8 days transient GUS expression, measured in explants 5 days after exposure to *Agrobacterium*, reached its maximum at 4 days of pre-culture with ~450 GUS-positive foci mg^{-1} (explant dry weight). Transient GUS activity was measured on half of the explants; the remaining explants were maintained on selective medium to recover stable transgenic plants. The stable transformation frequency was 17% after 7 days of pre-cultivation. No apparent correlation between transient GUS expression and stable transformation frequency was evident. Instead, stable transformation frequencies in this experiment implied a positive correlation with pre-cultivation time.

Subsequent experiments were performed where the pre-cultivation period was varied between 1 and 58 days (Fig. 1, Supplemental Table 1). Transient GUS activity reached its maximum after 4 days, when 330 GUS-positive foci mg^{-1} were observed. After this time-point, no specific trend of transient GUS activity was apparent. Variation of the pre-culture period strongly influenced the frequency of stable transformation. Stably transformed lines were only recovered from explants pre-cultured for at least 9 days, with a maximum frequency of 55% obtained after 17 days of pre-culture. Using longer periods resulted in a gradual decrease in the frequency of stable transformation.

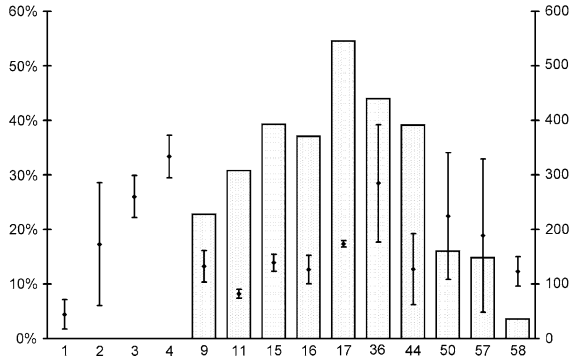

Fig. 1 Influence of pre-culture period on transient and stable transformation frequency of *B. distachyon* BDR018. Percent stable transformation (*bars*; *left Y-axis*) is given as recovered lines per 100 inoculated explants; transient GUS expression (*diamonds*; *right Y-axis*) is given as number of GUS positive foci per mg explant dry weight ± standard deviation of two duplicate experiments. Duration of pre-culture is given in days (*X-axis*)

Regeneration and molecular analysis of transgenic T_0 plants

Following selection in tissue culture three plantlets per callus line were transferred to soil. Once established in soil, putative transgenic plants were tested for GUS activity and resistance to BASTA®. All plants surviving herbicide treatment were grown to maturity in the greenhouse. In general, transgenic plants developed normally, and only in rare cases, reduced fertility or albinism was observed. A total of 177 putative transgenic pDM805 lines with up to three clones each were regenerated. All lines but one were BASTA® resistant and 83% of all tested plants displayed GUS activity in leaves (Table 1). In a few lines, single plants could be found lacking either BASTA® resistance or GUS activity. Neither GUS activity nor BASTA® resistance was observed in wild-type plants.

In order to confirm the presence of the *bar* and *gus*A genes, and to examine their clonal origin, Southern blot analysis with probes for the *bar* and *gus*A genes was performed on 80 individuals from 42 lines, and the number of T-DNA integrations was assessed (Supplemental Fig. 1, Supplemental Table 2). In the case of full-length integration of the transgene, minimum fragment sizes of 0.85 and 5.9 kb were expected upon hybridization with the *bar* and *gus*A probes, respectively. However, when using

Table 1 Transgene activity in established T_0 plants of *B. distachyon* BDR018 transformed with pDM805

pDM805 T_0 lines	GUS activity in leaves			BASTA® resistance		
	+[a]	±[b]	−[c]	+[a]	±[b]	−[c]
177	146	9	22	176	1	0
100%	82.5	5.1	12.4	99.4	0.6	0

[a] Lines showing transgene activity in all three plants

[b] Lines having plants both with and without transgene activity

[c] Lines lacking transgene activity in all three plants

the *gus*A probe, fragments smaller than 5.9 kb were detected, suggesting integration of truncated T-DNA copies into the genome. For copy number determination, bands of different size were considered independent integration events, and the transgene copy number was estimated from the number of bands exhibiting comparable hybridization intensity. Bands with relatively stronger hybridization signals were scored to reflect more than one copy of the transgene. In plants lacking GUS activity, no bands hybridizing to the *gus*A probe were found. All plants revealing at least one copy for *gus*A in the Southern blot analysis also showed GUS activity. This indicates that no transgene silencing had occurred. A summary of this analysis is presented in Table 2. More than 50% of all transgenic events were predicted to be either single or double copy integrations. As expected from the direction of T-DNA transfer, the average copy number of the *bar* gene (3.0) was higher than the average copy number of the *gus*A gene (2.2).

Transgene inheritance in the T_1 generation

Inheritance and segregation of the transgenes in the T_1 generation was investigated using a combined histological and molecular approach, allowing the estimation of the number of independent active loci in the T_0 lines. A segregation analysis was performed by monitoring activity of the *bar* and *gus*A genes in ~1,000 offspring of ten self-crossed T_0 pDM805 lines (Table 3). All T_0 lines produced viable seeds and germination rates were above 90%, with a mean value of 98%. A segregation ratio of 3:1, indicating a single functional *gus*A and *bar* gene locus, was observed in seven of the ten lines, despite three of these lines having apparent double integration patterns in the T_0 Southern blot analysis. In these seven lines, the *bar* and *gus*A loci co-segregated and were both active. In two lines, showing three copies of the transgene in the Southern blot analysis of the T_0 generation, a segregation ratio close to 15:1 indicated

Table 2 Summary of pDM805 integration events in the *B. distachyon* BDR018 genome in the T_0 generation

Number of integrations (Southern)	Number of lines showing bands for		Percentage of all lines showing bands for		Total number of insertions obtained[a]		Average copy number	
	bar	*gus*A	*bar*	*gus*A	*bar*	*gus*A	*bar* (Σ_2/Σ_1)	*gus*A (Σ_3/Σ_1)
0	n.a.	5	n.a.	6	n.a.	n.a.		
1	29	26	36	33	29	26		
2	16	21	20	26	32	42		
3	11	12	14	15	33	36		
4	7	7	9	9	28	28		
5	4	3	5	4	20	15		
6	3	1	4	1	18	6		
7	4	2	5	3	28	14		
8	2	–	3	–	16	–		
9	–	–	–	–	–	–		
10	1	1	1	1	10	10		
11	1	–	1	–	11	–		
12	1	–	1	–	12	–		
Unclear	1	2	1	3	n.a.	n.a.		
	$\Sigma_1 = 80$				$\Sigma_2 = 237$	$\Sigma_3 = 177$	3.0	2.2

[a] Number of integrations × number of lines with positive bands

Table 3 Segregation analyses and transgene inheritance in the T_1 generation

Line #[a]	Transgene copy number in T_0 (Southern blot) *bar*/*gus*A	Expected ratio[b]	Observed ratio	BASTA® r/s	Ratio BASTA® r/s (*p*-value)[c]	GUS±	Ratio GUS ± (*p*-value)[c]
4-1A	1/1	3:1	3:1	76/24	3.17 (0.9254)	76/24	3.17 (0.9254)
16-1A	1/1	3:1	3:1	70/22	3.18 (0.9188)	70/22	3.18 (0.9188)
23-1A	1/1	3:1	3:1	76/23	3.30 (0.8670)	76/23	3.30 (0.8670)
24-1B	1/1	3:1	3:1	77/20	3.85 (0.6649)	77/20	3.85 (0.6649)
3-1A	2/2	15:1	3:1	71/28	2.54 (0.7706)	71/28	2.54 (0.7706)
14-1A	2/2	15:1	3:1	71/29	2.45 (0.7244)	71/29	2.45 (0.7244)
7-1B	2/2	15:1	3:1	73/27	2.70 (0.8570)	73/27	2.70 (0.8570)
11-1B	3/3	63:1	15:1	93/5	18.60 (0.4039)	91/7	13.00 (0.5791)
17-1B	3/3	63:1	15:1	94/5	18.80 (0.3808)	96/3	32.00 (0.0027)
18-1A	5/4	255:1	n.a.	91/0	n.a.	91/0	n.a.

[a] A and B indicate from which experiment the line was obtained

[b] Theoretical expected ratio for the given transgene copy number in case of independent segregation

[c] Chi-square test comparing experimentally observed and closest theoretical ratio

the presence of two functional loci. However, different segregation ratios for the *bar* and the *gus*A loci were observed in both lines, suggesting that the transgenes were segregating partially independently, or that segregation ratios were altered by e.g. transgene rearrangement, partial integration or silencing. A Chi-square test ($p < 0.05$) confirmed that segregation followed Mendelian rules in these nine lines. In one line, with five *bar* and four *gus*A copies, no null segregants were observed.

A Southern blot analysis is exemplified for 19 T_1 progeny plants of line 11-1B (Fig. 2, compare also with Suppl. Fig. 1, lane 11-1). When probing with *gus*A, eleven progenies of line 11-1B showed an identical banding profile with the parental T_0 plant, whereas three plants had only the upper band and five plants had two (central and lower) bands. Hybridization with a *bar* probe revealed bands completely co-segregating with the bands for *gus*A. An identical analysis for line 17-1B gave a similar result (data not shown).

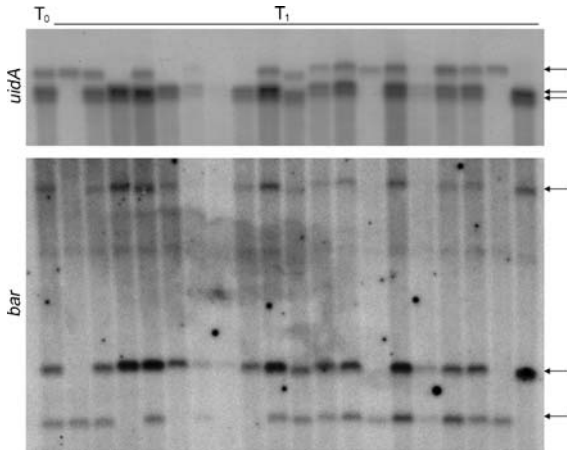

Fig. 2 Southern blot analysis of parental (T_0) and progenies (T_1) of the line 11-1B. Genomic DNA (3.5 µg) extracted from the parental T_0 line (*left lane*) and 19 T_1 progenies (following lanes) was digested with *Pst*I and separated by agarose gel electrophoresis. After blotting, the filter was hybridized with the probes indicated to the *left*, washed and exposed to X-ray films. *Arrows* indicate the fragments as to be expected from the Southern blot analysis performed on ten T_0 lines (see Supplemental Fig. 1). Approximate fragment sizes were (from *top* to *bottom*): 4.7, 4.2 and 4.0 kb for *gus*A, and 4.2, 1.8 and 1.2 kb for *bar*

Efficient selection of full-length T-DNA integration events using bialaphos

Based on the results above, an optimized transformation protocol was employed to generate transgenic *B. distachyon* lines using the vector pWBV-Ds-Ubi-bar-Ds (Zhao et al. 2006, Fig. 3). The presence of the two selection genes *bar* and *hpt* on the T-DNA allowed us to further characterize the developed transformation protocol with respect to full-length integration rate. As T-DNA transfer and integration

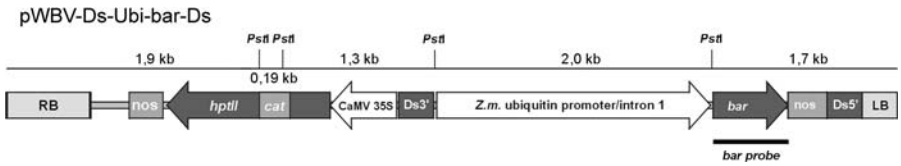

Fig. 3 T-DNA region of the T-DNA region of pWBV-Ds-Ubi-bar-Ds. Relevant fragment sizes upon digestion with *Pst*I are indicated above; approximate location of the probe fragments are indicated below; for further information see Zhao et al. (2006)

progresses from the right border (close to *hpt* on this T-DNA) towards the left border (close to *bar*), we could distinguish partial from full-length integrations by testing plantlets for *bar* gene activity using BASTA® spraying.

Immature embryos were pre-cultured for 12–21 days prior to inoculation with AGL1 pWBV-Ds-Ubi-bar-Ds. Callus selection was carried out for 8 weeks (with 2 weeks sub-cultivation intervals) as follows: 1/3 of the explants were selected only on medium containing bialaphos, 1/3 only on medium containing hygromycin B and 1/3 were first selected on bialaphos containing medium for 4 weeks and subsequently transferred to hygromycin B-containing medium for another 4 weeks. Subsequent selection on regeneration and rooting medium was performed using hygromycin B for all three selection regimes to assure the presence of *hpt* in all selected lines. The efficiency of the different selection regimes was assessed by scoring the total number of lines recovered from tissue culture and the number of lines resistant to the 100 mg l^{-1} BASTA® treatment (Table 4). The different selection regimes had different efficiencies, both in terms of stable transformation frequency and full-length integration rate. Callus selection exclusively on bialaphos gave the highest number of recovered lines (85 lines/150 embryos inoculated = 57% transformation frequency) and full-length integrations, with all 85 lines being BASTA® resistant. A lower total average transformation rate of 35% was obtained when solely using the potent antibiotic hygromycin B as selection agent. However, 2.6% of the regenerated lines were susceptible to bialaphos, indicating partial T-DNA integration. The resulting full-length integration rate was 33%. The combined selection regime (2B2H; each 4 weeks on bialaphos and subsequently on hygromycin B) resulted in the lowest average transformation rate (27%) observed in this experiment, and an intermediate rate of 1.3% of truncated T-DNA integration. Consequently, a full-length integration rate of 26% was obtained. Statistical analysis of variance revealed that the frequency of stable transformation for treatment 4B was significantly higher

Table 4 Influence of selection regime on transformation frequency and assessment of T-DNA integrity in transgenic plants of *B. distachyon* pWBV-Ds-Ubi-bar-Ds

Selection regime[a]	Total inoculated embryos	Number of replicates[b]	Regenerated lines[c] (%)		BASTA® resistant lines (%)		
			Total	Average[d]	Total	Average[d]	Range[e]
4B	150	6	85	56.7[a]	85	56.7[a]	28–80
2B2H	150	6	41	27.3[b]	39	26.0[b]	12–48
4H	150	6	53	35.3[b]	49	32.7[b]	24–48

[a] Callus phase selection regime:

4B, 4 × 2 weeks bialaphos

2B2H, 2 × 2 weeks bialaphos + 2 × 2 weeks hygromycin

4H, 4 × 2 weeks hygromycin

[b] With 25 embryos each

[c] Number of T_0 *Ds* lines regenerated in this experiment

[d] Frequencies of stable transformation; A and B assign significance classes ($p < 0.05$)

[e] Range of transformation frequency

than for the treatments 2B2H and 4H ($p < 0.05$), both with respect to the total number of lines recovered and the number of lines showing resistance to BASTA®. However, frequencies of stable transformation for the treatments 2B2H and 4H were not significantly different from each other. Interestingly, in one of the replicates using the 4B selection regime, 20 of 25 calli were successfully transformed giving an 80% transformation frequency (Table 4).

Discussion

Determination of the optimal callus age for transformation

The influence of pre-cultivation period, on transient and stable transformation frequencies, in *B. distachyon* BDR018 was investigated by varying the pre-cultivation period of immature embryos between 1 and 58 days prior to inoculation with *Agrobacterium*. Rapidly growing tissue undergoing the phase transition from embryo to embryogenic callus was best suited for efficient transformation and regeneration. Following transformation of pre-cultured immature embryos or embryogenic callus, with *A. tumefaciens* AGL1 carrying the vector pDM805, we recovered 177 transgenic lines with a maximal stable transformation frequency of 55% obtained at 17 days of pre-culture. A pre-cultivation period of around 17 days coincided with the appearance of strongly proliferating embryogenic callus cells. These results are consistent with previous reports that the two tested diploid accessions of *B. distachyon* develop callus at the surface of the scutellum typically after ten to 15 days of culture on callus induction media (Bablak et al. 1995; Draper et al. 2001). The high frequencies of stable transformation reported here suggest that after this period of hormone-induced callus growth, cells are highly competent for both transformation and subsequent regeneration.

The role of pre-culture treatment in increasing transformation frequency has been observed in various *Agrobacterium*-mediated transformation systems such as Arabidopsis (Valvekens et al. 1988; Sangwan et al. 1992), *Petunia hybrida* (Villemont et al. 1997), wheat (Cheng et al. 1997; Wu et al. 2003) and *Populus* sp. (Han et al. 2000). To date, transformation frequencies of typically 5–15% have been reported for different monocots, such as barley, wheat, rye, zoysiagrass, ryegrass and Brachypodium (Tingay et al. 1997; Hu et al. 2003; Popelka and Altpeter 2003; Ge et al. 2006; Bajaj et al. 2006; Vogel et al. 2006). Only a few reports on *Agrobacterium*-mediated transformation of rice indicate higher transformation frequencies of e.g. 41% (Lucca et al. 2001). Upon optimization, the protocol reported here transformed *B. distachyon* BDR018 with a mean transformation frequency of ~50% of all inoculated embryos regenerating transgenic lines. Single replicates gave stable transformation rates of up to 80%. This clearly demonstrates that upon adoption of this protocol to other accessions of Brachypodium, such as Bd21, large-scale genomics will soon be feasible at similar efficiencies as achieved in rice.

Transient GUS expression and stable transformation frequency

In our experiments, no correlation was found between transient expression of the reporter gene and stable transformation frequency. Instead, two major peaks of transient GUS expression were found at four and 36 days of pre-cultivation, whereas the maximum rate of stable transformation was obtained using 17 days of pre-cultivation. It has been suggested that increased "cell activity" (cells undergoing division, with voluminous nuclei and dense cytoplasm) can facilitate T-DNA transfer (Montoro et al. 2003). Taking these findings into account, the first peak of transient GUS expression in our experiments might be interpreted as the onset of strong and highly synchronized cell division. At this stage, cells are able to express the T-DNA-delivered *gus*A gene, either transiently present or stably integrated into the genome, but apparently have a low or even no regeneration capacity. Beyond 4 days of pre-cultivation, transient GUS expression decreased to a level remaining relatively constant up to 17 days, with larger variation observed towards the end of the experiment. The strong increase in stable transformation up to day 17 is possibly due to the callus tissue becoming regenerative, enabling the production of transgenic plants from transformed cells. Beyond this point, the frequency of stable transformation decreased constantly, indicating progressive loss of regeneration capacity, while transient GUS expression remained relatively constant.

Taken together, these results suggest that in our tissue culture system, Brachypodium, as cottonwoods (Han et al. 2000), undergoes a dedifferentiation phase during which regeneration competence reaches an optimum, at which transformed cells most efficiently regenerate transgenic plants.

In some instances, association between stable and transient transformation efficiency has allowed use of transient reporter gene activity for optimization of transformation protocols (Hiei et al. 1994; Cao et al. 1998; Suzuki and Nakano 2002). In other instances, there was no apparent correlation between transient and stable transformation (Han et al. 2000), underpinning the risk of making a link between the two. Furthermore, Trifonova et al. (2001) reported that the experimental conditions could influence whether a positive or a negative correlation was obtained in barley. This suggests that a positive correlation is only obtained when the transient transformation and the regeneration capacities coincide. Consequently, for protocol optimization one should aim at finding the optimal combination between these two parameters.

Aiming at high transformation efficiency may increase transgene copy number

We investigated whether the increased transformation efficiency in our protocol is associated with a change of T-DNA integration pattern compared to results previously reported. In these experiments, Southern blot analysis of the transgene copy number showed that the percentage of single copy lines (between 26 and 36%) was comparable to rates reported for other monocots such as rice (33 and 27% single-copy for *bar* and *gus*A, respectively, Vain et al. 2003) and wheat (35% single-copy, Cheng et al. 1997), but significantly lower than those obtained in maize (60–70% single-copy, Ishida et al. 1996).

The lowest percentage of single integration plants of 26%, obtained after transformation with pWBV-Ds-Ubi-bar-Ds, was associated with the highest average copy number (3.5, data not shown), but also with the highest stable transformation frequency. For transgenic pDM805 lines, the average copy number was 2.2 for the *gus*A and 3.0 for the *bar* gene. The relatively high values of average copy number reported here, compared to other monocot studies (Vain et al. 2003; Sallaud et al. 2003), may be due to increased transformation efficiency in the optimized protocol, which enhances the overall T-DNA transfer from *Agrobacterium* to Brachypodium cells. This potential relationship is important to bear in mind if this efficient protocol that can lead to a transformation frequency as high as 80%, is used. To our knowledge, this is the highest reported transformation frequency for a monocot species.

Efficient recovery of full length T-DNA integration lines through bialaphos selection

We observed that the selection regime has an influence on the frequency of stable transformation, as well as on full-length integration rate. The three tested selection regimes are not equally efficient, neither is the rate of full-length integration. The rather slow selection properties of bialaphos might allow transformed cells to reach the phase of high regeneration capacity, and plants can be regenerated with the support of cross-feeding from surrounding non-transformed tissue (D'Halluin et al. 1992). At the same time, bialaphos-based selection in our experimental design favoured integration of the complete T-DNA and resulted in complete absence of escapes. On the other hand, the absence of escapes raised the question whether selection pressure was too strong, i.e. whether a lower selection pressure might have resulted in an even higher total transformation frequency. This question should be addressed by future experiments.

A lower total transformation rate was obtained with the potent antibiotic hygromycin B as the only selection agent. This could be the result of high selection pressure early in the selection process. As a consequence, non-transformed cells are rapidly killed and cannot cross-feed transformed cells with metabolites. Moreover, dead tissue may poison the selection medium, thereby preventing transformed cells from efficient proliferation.

Conclusions and perspectives

The optimized transformation protocol developed in this study offers a transformation efficiency opening up new perspectives for a T-DNA tagging project in diploid *B. distachyon*. This type of insertional

mutagenesis requires a large number of transformed lines. The reliable transformation method described here, with transformation frequencies of up to 80% and around 30% single copy integrations, is not only suited for small-scale experiments aiming at a few dozen transformants, but provides the tool for generating thousands of transgenic lines in a high-throughput transformation program. Therefore, our results are pivotal for developing Brachypodium into a widely accepted model for temperate grasses and cereals, and open the door for large-scale transgenic approaches in *B. distachyon.*

Materials and methods

Plant material and growth conditions

Seeds of *B. distachyon* BDR018 were sown in 12 cm pots containing Pindstrup Substrate no. 2 peat (Pindstrup, Denmark) as previously described (Christiansen et al. 2005). After germination, plants were grown for 3 to 4 weeks in the greenhouse at 24°C under natural daylight, with long day (LD) 16/8 h light/dark supplementary lighting in winter. In order to support synchronous induction of flowering and embryo development, plants were subsequently vernalized for 2 weeks at 5°C, 8/16 h light/dark, followed by growth under LD conditions.

Pre-conditioning of immature embryos

Immature seeds of *B. distachyon* BDR018 were harvested as described by Draper et al. (2001). Immature embryos were isolated aseptically and placed with the scutellar surface in contact with callus induction medium as previously described (Christiansen et al. 2005). Embryos were pre-cultured in the dark at 22°C for one to 58 days, with additional subculture steps after 15 and 24 days for callus pre-cultured for 36 and 44 days, respectively.

Strains and constructs

For optimization experiments, we used the super-virulent *A. tumefaciens* strain AGL1 harbouring the standard binary vector pDM805 (Tingay et al. 1997). Cells of AGL1, rescued for pDM805, were re-transformed with pWBV-Ds-Ubi-bar-Ds (Fig. 3, Zhao et al. 2006) by electroporation. Standard inoculums for plant transformation were prepared as described previously (Tingay et al. 1997). To obtain *Agrobacterium* culture for co-inoculation, standard inoculums of *A. tumefaciens* AGL1 pDM805 were grown in MG/L liquid medium (Garfinkel and Nester 1980) supplemented with 1 mg l^{-1} biotin, 20 mg l^{-1} rifampicin and 10 mg l^{-1} tetracyclin. For AGL1 pWBV-Ds-Ubi-bar-Ds, MG/L liquid medium was supplemented with 1 mg l^{-1} biotin, 20 mg l^{-1} rifampicin and 100 mg l^{-1} spectinomycin. The culture was incubated at 28°C for 18 h with continuous shaking (225 rpm). For co-inoculation, the bacterial suspension was adjusted to an OD_{660} of 1.0 using MG/L liquid medium containing 1 mg l^{-1} biotin.

Explant transformation and selection

Explants were inoculated by total immersion in a suspension of *Agrobacterium* overnight culture supplemented with 0.01% Silwet L-77, for 15 min. To avoid bacterial outgrowth during subsequent co-cultivation steps, excess bacteria was removed by briefly draining explants on sterile filter paper. The embryos were immediately transferred onto plain callus induction medium with the scutellar in contact with the medium. Co-cultivation was carried out for 3 days at 24°C under LD conditions.

After co-cultivation, embryos were briefly rinsed twice in a solution containing 250 mg l^{-1} augmentin (amoxycillin Na/K [5:1] clavulanate, Duchefa) and 0.01% Silwet L-77, briefly drained on sterile filter paper and transferred to callus selection medium containing either 5 mg l^{-1} bialaphos or 60 mg l^{-1} hygromycin B. Further bacterial growth was inhibited by addition of 250 mg l^{-1} augmentin to the culture media. Selection was performed for a period of 2–8 weeks on callus induction medium, ~3 weeks on shoot induction medium and ~3 weeks on root induction medium in tissue culture containers as described (Christiansen et al. 2005). Five to seven centimetres tall plantlets with healthy roots were trimmed, washed and transferred to eight cm pots with a soil mixture composed of peat, perlite and vermiculite (16:1:1) with 0.2% Osmocote®Exact®. Transferred plantlets were acclimated to low humidity

conditions by cultivation in resealable plastic bags and grown at 24°C under LD conditions, with light supplied by cool-white fluorescent lights. Once established, the plants were grown as described above.

Transgene activity assays and analysis of transgenic lines

Transient *gus*A expression was assayed histochemically by staining the explants with X-Gluc [5-bromo-4-chloro-3-indolyl β-D glucuronic acid] as described previously (Jefferson et al. 1987). Chlorophyll was removed by washing with 70% ethanol until the solution remained colourless. GUS-positive foci were counted under a stereomicroscope. For evaluation of transient GUS activity, the destained, scored explants were kept under vacuum for 3 days to ensure all liquid was completely evaporated. Dried explants were weighed using an analytical scale and GUS activity was expressed as the number of GUS-positive foci mg^{-1} explant dry weight. For semi-quantitative evaluation of GUS expression in young shoots and leaf samples of primary regenerants, tissue was stained as described above, and staining intensity was assessed visually.

To test for activity of the *bar* gene, young established putative transgenic pDM805, pWBV-Ds-Ubi-bar-Ds and non-transgenic (control) plants were sprayed every second day for 1 week with 100 mg l^{-1} BASTA® (AgrEvo) supplemented with 0.1% Tween X-100 and survival of the plantlets was recorded.

Genomic southern blot analyses were performed according to standard procedures (Sambrook et al. 1989) using 3.5 µg genomic DNA per sample and experimental parameters as described previously (Christiansen et al. 2005).

Statistical analysis

Statistical analysis of variance was performed using the SAS procedure PROC GLM (SAS Version 8.02, Copyright 1999–2001 SAS Institute). Distribution of residuals was checked visually by graphical means.

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