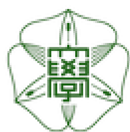

HOKKAIDO UNIVERSITY

Title	High frequency Agrobacterium-mediated transformation and plant regeneration via direct shoot formation from leaf explants in Beta vulgaris and Beta maritima
Author(s)	Hisano, H.; Kimoto, Y.; Hayakawa, H.; Takeichi, J.; Domae, T.; Hashimoto, R.; Abe, J.; Asano, S.; Kanazawa, A.; Shimamoto, Y.
Citation	Plant Cell Reports, 22(12), 910-918 https://doi.org/10.1007/s00299-004-0773-3
Issue Date	2004-07
Doc URL	http://hdl.handle.net/2115/16870
Rights	The original publication is available at www.springerlink.com
Type	article (author version)
File Information	PCR22-12.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

High frequency *Agrobacterium*-mediated transformation and plant regeneration via direct shoot formation from leaf explants in *Beta vulgaris* and *B. maritima*

H. Hisano · Y. Kimoto · H. Hayakawa · J. Takeichi · T. Domae · R. Hashimoto · J. Abe · S. Asano · A. Kanazawa · Y. Shimamoto

H. Hisano · Y. Kimoto · H. Hayakawa · J. Takeichi · T. Domae · R. Hashimoto · J. Abe · S. Asano · A. Kanazawa (Correspondence) · Y. Shimamoto
Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
e-mail: kanazawa@res.agr.hokudai.ac.jp
Fax: +81-11-706-4933

Present address:
Y. Shimamoto, Faculty of Bioindustry, Tokyo University of Agriculture, Abashiri 099-2493, Japan

Correspodence to:
Akira Kanazawa
Graduate School of Agriculture
Hokkaido University
Sapporo 060-8589
Japan

Tel: +81-11-706-3873
Fax: +81-11-706-4933
e-mail: kanazawa@res.agr.hokudai.ac.jp

Abstract We developed a new procedure for *Agrobacterium*-mediated transformation of plants in the genus *Beta* using shoot base as the material for *Agrobacterium* infection. The frequency of regeneration from shoot bases was analyzed in seven accessions of sugarbeet *(Beta vulgaris)* and two accessions of *B. maritima* to select materials suitable for obtaining transformed plants. The frequency of transformation of the chosen accessions using *Agrobacterium* strain LBA4404 and selection on 150-mg/l kanamycin was found to be higher than that in previously published methods. Genomic DNA analysis and GUS reporter assays showed that the transgene was inherited and expressed in subsequent generations. In our method, shoot bases are prepared by a simple procedure, and transformation does not involve the callus phase, thus minimizing the occurrence of somaclonal variations.

Keywords *Agrobacterium tumefaciens* · *Beta vulgaris* · Regeneration · Shoot-base tissue · Transformation

Introduction

Sugarbeet (*Beta vulgaris* L.) is a commercially important crop that produces sucrose in temperate climates and provides almost 30% of the world's sugar. The production of new sugarbeet varieties by conventional breeding is limited because sugarbeet is a highly heterozygous, naturally cross-pollinating biennial species. Despite its importance, genetic engineering of this plant by DNA transformation methods has been limited, mainly due to technical difficulties in obtaining regenerated plants from cells competent for transformation. Early attempts to transform sugarbeet using *Agrobacterium* have met with limited success. Hamill et al. (1987) obtained hairy root cultures by inoculation with *A. rhizogenes*. Harpster et al. (1988) and Krens et al. (1988) obtained transformed calli using *A. tumefaciens*-mediated transformation. However, they did not produce regenerated transformants. Lindsey and Gallois (1990) first reported the production of transformed sugarbeet plants via *A. tumefaciens* infection and regeneration from shoot bases, which enable relatively rapid and frequent regeneration, as compared with petioles or leaf tissue. Konwar (1994) also showed that shoot bases can be infected with *A. tumefaciens* to produce transgenic plants. Using the method of Konwar (1994), Mannerlof et al. (1997) made glyphosate-tolerant sugarbeets. D'Halluin et al. (1992) made transgenic sugarbeets via callus regeneration using tobacco suspension cells as a nutrient layer.

Sugarbeets have also been stably transformed by direct gene transfer to protoplasts. Lindsey and Jones (1989) transformed sugarbeet cells, but they experienced technical difficulties in regenerating plants from protoplasts. Hall et al. (1996) obtained transgenic plants by polyethylene glycol (PEG)-mediated transformation of protoplasts. To achieve high transformation frequencies, they enriched the protoplasts for a totipotent cell type from stomatal guard cells. This method was used to make transgenic sugarbeets with high levels of fructan (Sevenier et al. 1998).

For transforming higher plants, *Agrobacterium*-mediated methods are preferred

over direct gene-transfer methods (e.g., PEG- or liposome-mediated transformation, electroporation, or particle bombardment) because the former generally results in lower copy number, fewer rearrangements, and more stable expression of transgenes over generations (Dai et al. 2001; Gelvin 2003). We developed, therefore, an improved procedure for *Agrobacterium*-mediated transformation in the genus *Beta*. We first examined the regeneration ability of various lines of *B. vulgaris* and *B. maritima* to select materials suitable for genetic transformation. We then established a simple and efficient *Agrobacterium*-mediated transformation procedure involving the regeneration of plants from shoot bases. We also optimized the combinations of *Agrobacterium* strain and antibiotic to select transformed cells. The experiments involved the introduction of three genes of agronomical use into plants, thus demonstrating its reproducibility. Although *Agrobacterium*-mediated transformation of sugarbeet plants using shoot bases as explants has been reported (Lindsey and Gallois 1990; Konwar 1994), the analysis of transformants has been limited to the T0 generation. Thus, we examined both the transmission of the transgenes to subsequent generations and the stability of transgene expression using a *β-glucuronidase* (*GUS*) reporter.

Materials and Methods

Plant materials

Seven lines of sugarbeet (*B. vulgaris)* and two accessions of *B. maritima,* a close wild relative of sugarbeet, were used as plant materials for transformation (for the list of accessions, see Table 2). *B. vulgaris* and *B. maritima* seeds were sterilized first in 70% ethanol and then in 2% sodium hypochlorite with 0.1% Tween 20 and then were aseptically seeded onto an MS-based germination medium (Murashige and Skoog 1962) (Table 1). The pH of all media used in this study was adjusted with KOH to 5.8 before autoclaving. Germination occurred after two to seven weeks. After the cotyledons emerged, the hypocotyls were cut at the base and transferred onto shoot-formation

medium to induce leaf growth (Table 1). Then, leaf blades were cut from young plants and placed on the shoot-formation medium. Shoots were regenerated from the veins of the leaf blades. The shoots were removed, and the remainder of the leaf blades, on which the shoot bases were exposed, was used as explants for transformation. Cultures were done at 22ºC in light.

Bacterial strains and plasmid vectors

Agrobacterium tumefaciens strains EHA101 and LBA4404 were used. Binary plasmid vectors were introduced into the *Agrobacterium* strains by triparental mating (Bevan 1984). To transform beet plants with the *GUS* reporter, *Agrobacterium* strain EHA101 and the binary plasmid pGM221 were used. The binary vector contains the hygromycin resistance gene (hygromycin phosphotransferase; *HPH*), under the control of the nopaline synthase (NOS) promoter and NOS terminator, and the *GUS* reporter gene, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and NOS terminator. This vector was kindly provided by Dr. M. Murase (Plantech Research Institute, Japan). To transform beet plants with the insecticidal crystal protein genes from *Bacillus thuringiensis,* namely, *cryIA(b)* (Kondo et al. 1987) and *cryIC* (Sanchis et al. 1989), and the pumpkin chitinase gene (database accession number AB015655), we used *Agrobacterium* strain LBA4404 and binary vectors that contain these genes in place of *GUS* on the pBI121 vector (Clontech). The insertion of *cryIA(b)* into the vector was done in the following manner. The *GUS* sequence was removed from pBI121 by *Sma*I-*Sac*I digestion. After end-repair and ligation, a *Bam*HI-fragment containing the *cryIA(b)* sequence was cloned into the *Bam*HI site located downstream of the CaMV 35S promoter. This *Bam*HI-fragment was obtained by PCR-amplification of the coding sequence of *cryIA(b)* (Kondo et al. 1987) from *B. thuringiensis kurstaki* HD1 using primers that add a *Bam*HI site to each end of the *cryIA(b)* sequence and subsequent cloning of the amplified product into pGEM-T vector (Promega). Cloning of *cryIC* into the vector was done by

replacing the *Bam*HI-fragment that contains the *cryIA(b)* sequence with a *Bam*HI-fragment that contains the *cryIC* sequence (Sanchis et al. 1989), which was prepared from *B. thuringiensis aizawai* as described for *cryIA(b)*. The pBIC vector, which contains the pumpkin chitinase sequence in place of *GUS* between the *Xba*I-*Sac*I sites of pBI121, was kindly provided by Dr. R. Akashi (Miyazaki University, Japan).

Inoculation of sugarbeet tissues with *Agrobacterium* and plant regeneration

Agrobacterium containing the binary vector was cultured for two days at 28ºC on a rotary shaker at 180 rpm in liquid medium containing 50 mg/l kanamycin and 25 mg/l spectinomycin. The explants, on which shoot bases were exposed, were immersed in the *Agrobacterium* culture for 1 min, and excess liquid was removed by placing the explants on sterilized filter paper. Samples were transferred to a co-cultivation medium supplemented with 4 mg/l acetosyringone and cultured for three days. The media used for transformation are listed in Table 1. The explants were rinsed with washing medium to remove *Agrobacterium* from the surface and then transferred to selection medium. After two weeks, explants from which shoots were regenerated were transferred to growth medium. When shoots had grown 2-3 cm, they were cut and transferred to root-formation medium. After roots were generated, the plants were transferred to soil.

GUS assay

GUS assays were done according to Jefferson et al. (1987).

DNA isolation and PCR analysis

Total DNA was isolated from leaves as described in Doyle and Doyle (1990). The presence of the transgene was determined by PCR amplification, agarose gel electrophoresis, and gel blot analyses of the PCR products using transgene-specific probes. The PCR primers were chosen to amplify the coding sequence of the transgenes:

5'-GTTACGTCCTGTAGAAACCC-3' and 5'-TCGTCCTCCGTTTGTTACT-3' for a 1.9-kb portion of the *GUS* sequence; 5'-CCCGGTGCTGGATTTGTGTTAGG-3' and 5'-CACGCCCTGACCTAGTTGAGCA-3' for a 0.9-kb portion of *cryIA(b)*; 5'-CTCAAGCGGCCAATCTG-3' and 5'-CTACTCCTTCAACACCACG-3' for a 0.7-kb portion of *cryIC*; and 5'-GCTTAGCCTTTGCCTTCGT-3' and 5'-ATATCCCCACGCATAATGGGC-3' for a 0.4-kb portion of the chitinase gene. Each PCR cycle consisted of denaturation for 30 sec at 94ºC, annealing for 30 sec at 56ºC, and extension for 30 sec at 72ºC. This cycle was repeated 30 times. The PCR products were separated by electrophoresis in 1.5% agarose in TBE buffer followed by ethidium bromide staining and gel blot analysis.

DNA gel blot analysis

Plant genomic DNA (20 μg) was digested with restriction enzymes and separated by gel electrophoresis in 1% agarose in TBE buffer. The DNA was transferred to nylon membranes (Hybond N+; Amersham) and hybridized with probes labeled by AlkPhos Direct nucleic acid labeling and detection system (Amersham). Hybridization, membrane wash, and signal detection were carried out according to the manufacturer's instructions. Gel blot analysis of PCR-amplified DNA fragments was performed similarly. Hybridization probes were prepared by PCR amplification of portions of the coding sequences (for primers, see above) using the cloned DNA plasmids as templates.

RT-PCR

Total RNA was isolated from leaf tissues of sugarbeet plants using RNeasy Plant Mini Kit (QIAGEN). cDNA was synthesized from 1 μg total RNA using M-MLV reverse transcriptase (GIBCO BRL) with poly(dT) and adaptor primers (Frohman et al. 1988) at 42ºC for 1 hr. One-tenth volume of the cDNA solution was used for PCR. Primers used to amplify *GUS* transcripts were 5'-GGAATTTCGCCGATTTTGCG-3' and the adaptor

primer used for cDNA synthesis. For an RT-PCR positive control, a 0.3-kb portion of the cysteinyl tRNA synthase transcript was amplified using the primers 5'-TGACAAGATAATTGCCAGAG-3' and 5'-CTTTCTCGAATCAATAGCTACC-3' (Kubo et al., personal communication).

5

Results

Differences in the regeneration frequency from shoot bases among *B. maritima* and *B. vulgaris* accessions

10 Based on the report that shoot bases are amenable to both *Agrobacterium*-mediated transformation and regeneration (Lindsey and Gallois 1990), we attempted to optimize a sugarbeet transformation system using these tissues. We prepared shoot base tissue using a simple method suitable for the production of a large number of transformed plants (for protocol details, see Materials and Methods; Fig. 1).

15 We first examined the frequency of regeneration from our shoot base preparations in seven accessions of sugarbeet and two accessions of *B. maritima* to choose the most suitable materials for genetic transformation. Six explants of leaf blades, on which shoot-base tissues were exposed, from each individual plant, were used. The number of individual plants in which shoot formation was observed in at least one explant 20 was counted to determine regeneration frequency (data not shown). Regeneration frequency was very high in the *B. maritima* “acc. France” (80%) and was higher in the “NK150” (48%) and “TK-80” (48%) lines than in the other *B. vulgaris* accessions. The frequency distribution of the number of explants that generated shoots among six explants was shown in Table 2, which shows that two accessions of *B. maritima* (3.7 and 25 4.1) and *B. vulgaris* line “T2n-24-115-24” (4.5) showed the highest average regeneration frequencies. Based on these results we used the *B. maritima* “acc. France” and *B. vulgaris* “NK150”, “TK80”, and “T2n-24-115-24” for the following transformation procedures.

Transformation using *Agrobacterium* strain EHA101 and hygromycin selection

Transformation was first carried out using *Agrobacterium* strain EHA101 harboring the plasmid pGM221, which contains a hygromycin resistance gene and a *GUS* reporter (for gene construct, see Fig. 2A). To determine the amount of hygromycin suitable for selection of transformed cells, 10-15 mg/l of hygromycin was added to the shoot-formation medium, and regeneration from non-transformed explants was tested prior to the screening of transformed cells. In the *B. maritima* “acc. France”, two of 16 individuals generated emerging shoots on a medium that contained 10 mg/l hygromycin, whereas zero of nine individuals generated shoots on a medium that contained 12 mg/l hygromycin (Table 3).

Using these selection conditions, we analyzed the formation of shoots after infection of shoot bases with *Agrobacterium* containing a vector with the hygromycin resistance gene in the T-DNA region. Shoots were obtained under both selection conditions (see Fig. 1E). The presence of the transgenes was analyzed by PCR (for typical PCR results, see Fig. 3) of the *GUS* gene, which was linked to the hygromycin resistance gene (*HPH*) in the T-DNA region. The number of resistant *B. maritima* “acc. France” shoots that also contained the transgenes was 6 out of 8 using 12 mg/l hygromycin and 3 out of 8 using 10 mg/l hygromycin (Table 4). This result is consistent with the observation that regeneration from non-transformed cells could occur in the presence of hygromycin (see Table 3). We subsequently obtained regenerated plants from the resistant shoots by transferring them to root-formation medium (Fig. 1F-H).

Similar experiments were done using accessions “NK150”, “TK80”, and “T2n-24-115-24” of *B. vulgaris*. Shoots were also generated in *B. vulgaris* “NK150” on a medium that contained 10 mg/ml hygromycin (see Table 3), further suggesting that the concentration of hygromycin suitable for screening transformed cells should be higher than 10 mg/ml. However, no transformants were obtained under these conditions (Table 4). It is possible that either this hygromycin selection or the *Agrobacterium* strain

EHA101 is not suitable for transformation in these accessions. Further examination is necessary to clarify these results and to find the optimal conditions for hygromycin selection.

5 Transformation using *Agrobacterium* strain LBA4404 and kanamycin selection

We also transformed beet plants using kanamycin selection and different transgene constructs (see Figs. 2B-D). We tested the resistance of explants of *B. maritima* "acc. France" and "NK150" of *B. vulgaris* to various concentrations (50–150 mg/l) of
10 kanamycin as described above and found that regeneration from non-transformed cells could be efficiently suppressed on medium containing 150 mg/l of kanamycin (data not shown). Transformation was carried out using *Agrobacterium* strain LBA4404 harboring plasmids with the neomycin phosphotransferase II gene (*NPTII*), which imparts kanamycin resistance. The plasmid also contained a gene [*cryIA(b)*, *cryIC*, or chitinase]
15 that conveys an agronomically important trait. The effect of the expression of these genes in sugarbeet will be described elsewhere. Successful transformation was confirmed by PCR amplification of the genes linked to *NPTII* in the T-DNA region (see Fig. 3).

Transformants were obtained for all plasmids and all materials tested. The rate of transformation is summarized in Table 5. When we introduced *cryIA(b)* to *B. maritima*
20 "acc. France", 142 explants were infected with *Agrobacterium*. As a result, 88 explants were selected on kanamycin-containing medium, and plants were regenerated. We isolated DNA from 21 plants, and the *cryIA(b)* sequence was PCR-amplified from 12 of these plants. In other experiments using different combinations of genes and accessions, the results were similar: about 2 of 3 explants were resistant to drug selection, and about
25 half of the regenerated plants that we examined contained the transgene. The exceptions were the *cryIA(b)* gene in "NK150", in which only 4/14 regenerated plants contained the transgene, and chitinase in "TK80", in which all 21 drug-resistant plants contained transgene (see Table 5).

Analysis of subsequent generations of primary transformants using the *GUS* reporter

To analyze the heritability of the transgene, progenies of three GUS-containing T0 plants of *B. maritima* "acc. France" (AC4-1, AC4-2, and AC5) were obtained as follows. Progeny of AC4-1 and AC4-2 were obtained by selfing, while that of AC5 was obtained by crossing AC5 with a cytoplasmic male sterile (CMS) line, "S22-CMS". The presence of the transgenes in the progeny was analyzed by PCR. 8/16, 9/16, and 5/16 of the progeny of AC4-1, AC4-2, and AC5, respectively, contained a transgene (data not shown). This suggests that the transgene was meiotically heritable. For unknown reasons, the frequency of transmission was slightly lower than expected for the transmission of a single copy gene (75% for selfing and 50% for crossing with CMS line), although the number of individuals examined was small. GUS activity in T1 plants was assayed (Fig. 4). Leaf extracts from transformants showed between 3- and 231-fold higher GUS activity than did extracts from non-transgenic control plants, with the majority showing 10-20 fold higher activity.

The presence of transgenes in subsequent generations (T2) was also confirmed by gel blot analysis of genomic DNA (Fig. 5), which showed that the transgene integrated into the genome and was transmitted through meiosis. In addition, RNA was isolated from transformant leaves, and *GUS* expression was analyzed by RT-PCR (Fig. 6). The results indicate that the transgene is expressed in the T2 generation and that *GUS* mRNA levels vary among individual transformants.

Discussion

We have established a genetic transformation system for plants in the genus *Beta* using shoot bases prepared by a procedure that is simpler than previously published procedures. One of the advantages of our method is the simplicity with which materials suitable for transformation are prepared. In our protocol, shoot bases, to which *Agrobacterium* was

inoculated, were prepared by simply cutting shoots that had formed on leaf blades. This procedure may save a considerable amount of time in material preparation, as compared to the method of Lindsey and Gallois (1990), in which shoot-base slices (1 cm x 1 cm x 2 mm) are used, or the method requiring preparation of, and regeneration from, protoplasts (Hall et al. 1996). Our method also differs from previously reported methods in its media supplements, including phytohormones for tissue culture and antibiotic concentrations for the selection of transformed cells. PCR analysis showed that, in three out of the five combinations of genes and accessions that we tried, more than 50% of plants that regenerated from drug resistant explants contained transgenes (Table 5). Plants that were found by PCR to contain transgenes also showed higher GUS activity levels than did non-transformed plants (see Fig. 5). Given these results, the transformation frequency with our method is significantly higher than with that of Lindsey and Gallois (1990), in which approximately 30% of kanamycin-resistant regenerated shoots showed reporter gene activity.

The high transformation rate could be due in part to the selection medium kanamycin concentration (150 mg/l), which is higher than that used in Lindsey and Gallois (1990) (100 mg/l). In addition, our media were supplemented with phytohormones, 0.25 mg/l of benzyladenine (BA) and 1.0 mg/l of indolebutyric acid (IBA), to induce shoot and root formation, respectively, as BA and IBA were found to be suitable for sugarbeet morphogenesis from leaf explants (Mikami et al. 1989). IBA was also added at a lower concentration (0.1 mg/l) to other media besides the rooting medium. We used 0.5 × MS rather than 1 × for all culturing steps. Our results show that these modifications improve the selection efficiency of transformed cells without disturbing the morphogenic ability of the beet cells. Our results also show that a lack of drug selection in the rooting medium does not significantly increase the rate of escapes from drug selection, although the possibility of chimaeric tissues in terms of the presence or absence of transgenes cannot be excluded.

In this study, we examined the selection of transformed cells using hygromycin and kanamycin. For *B. maritima* accessions, we obtained transformants using both hygromycin (in combination with infection by *Agrobacterium* strain EHA101) and kanamycin (with *Agrobacterium* strain LBA4404). For *B. vulgaris*, we obtained transformants only with the latter combination. This probably indicates that transformation of plants in the genus *Beta* is controlled more easily by the latter combination. In any case, this is, to our knowledge, the first report of transformation of *B. maritima*. Transformation of *B. maritima* may contribute to expanding its genetic diversity for improving *B. vulgaris*. In addition, this is also the first report of transformation of plants in the genus *Beta* using hygromycin selection.

Multiple shoots were generated from shoot-base tissue in this study, allowing regeneration of multiple transformed plants from a single leaf blade segment. A high proportion of multiple shoots generated from a single explant, selected for drug resistance, were found to have a transgene, suggesting that selection occurred equally from cell to cell. In one outstanding case in our study, 21 plants (derived from 7 explants of "TK80") all contained the chitinase gene (see Table 5). Thus, this method provides a suitable system for practical transformation purposes, as it allows for the production of sugarbeet transformants on a large scale. This allows subsequent screening of the lines that express the transgenes at a high level. We found that the tissues used in this method are also useful for bombardment-mediated gene transfer; we routinely bombarded plasmid DNA on shoot bases prepared by this method and obtained transgenic plants in sugarbeet lines "NK150" and "TK80" (unpublished results).

In this study, we analyzed the transmission and expression of transgenes using *GUS* as a reporter in *B. maritima*. In our transformants containing the *GUS* gene, variation in expression level among transgenic lines was observed by both RT-PCR and GUS activity assays. The observed variations could be due to variation in transgene expression caused by epigenetic effects involving copy number, the insertion position in

the genome, or methylation of the transgenes (for reviews, see Matzke and Matzke 1995; Kooter et al. 1999).

The transformation method using shoot bases does not involve a detectable callus phase prior to regeneration, suggesting that the possibility of somaclonal variation is minimized (Lindsey and Gallois 1990). In fact, no morphological abnormality was observed in any of the transformed plants in this study. In addition, the preparation of materials for *Agrobacterium* inoculation in our method requires simply cutting shoots and exposing the shoot bases on leaf blades; it does not require any special cutting skills, and thus does not produce differences in transformation efficiency among individuals. This simplicity may contribute to the reduced variation in transformation and to maintaining the regeneration ability of the cells. These features would contribute, presumably, to the production of uniform transgenic plants at a high frequency.

Acknowledgements We would like to thank Dr. T. Mikami (Hokkaido University, Japan) for valuable suggestions for tissue culture of plants in the genus *Beta*, Dr. M. Murase (Plantech Reseach Institute, Japan) for providing us the pGA221 vector, Dr. R. Akashi (Miyazaki University, Japan) for pumpkin chitinase gene clone, Natl. Agr. Res. Cent. for Hokkaido Region (Japan) for accessions in the genus *Beta*, Dr. T. Kubo (Hokkaido University, Japan) for sequence information of PCR primers for sugarbeet cysteinyl tRNA synthase gene, and Dr. T. Iizuka (Hokkaido University, Japan) for his advices on the use of BT toxin genes. We also thank Mr. Y. Yamashita, Ms. K. Kuwahara, Ms. Y. Iida, Ms. Y. Matsumura, and Ms. H. Nonokawa (Hokkaido University, Japan) for their technical supports.

References

Bevan MW (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 22:8711-8721

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001)

Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. Mol Breeding 7:25-33

D'Halluin K, Bossut M, Bonne E, Mazur B, Leemans J, Botterman J (1992) Tranaformation of sugarbeet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Bio/Technology 10:309-314

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11-15

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998-9002

Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev 67:16-37

Hall RD, Riksen-Bruinsma T, Weyens GJ, Rosquin IJ, Denys PN, Evans IJ, Lathouwers JE, Lefebvre MP, Dunwell JM, van Tunen A, Krens FA (1996) A high efficiency technique for the generation of transgenic sugar beets from stomatal guard cells. Nature Biotech 14:1133-1138

Hamill JD, Prescott A, Martin C (1987) Assessment of the efficiency of cotransformation of the T-DNA of disarmed binary vectors derived from *Agrobacterium tumefaciens* and the T-DNA of *A. rhizogenes*. Plant Mol Biol 9:573-584

Harpster MH, Townsend JA, Jones JDG, Bedbrook J, Dunsmuir P (1988) Relative strengths of the 35S cauliflower mosaic virus, 1', 2' and nopaline synthase promoters in transformed tobacco, sugar beet and oilseed rape callus tissue. Mol Gen Genet 212:182-190

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907

Kondo S, Tamura N, Kunitate A, Hattori M, Akashi A, Ohmori I (1987) Cloning and nucleotide sequencing of two insecticidal delta-endotoxin genes from *Bacillus thuringiensis* var. kurstaki HD-1 DNA. Agric Biol Chem 51:455-463

Konwar BK (1994) *Agrobacterium tumefaciens*-mediated genetic transformation of sugar beet (*Beta vulgaris* L.). J Plant Biochem Biotech 3:37-41

Kooter JM, Matzke MA, Meyer P (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends Plant Sci 4:341-347

Krens FA, Zijlstra C, van der Molen W, Jamar D, Huizing HJ (1988) Transformation and

regeneration in sugarbeet (*Beta vulgaris* L.) induced by 'shooter' mutants of *Agrobacterium tumefaciens*. Euphytica 5:185-194

Lindsey K, Gallois P (1990) Transformation of sugarbeet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. J Exp Bot 41:529-536

5 Lindsey K, Jones MGK (1987) Transient gene expression in electroporated protoplasts and intact cells of sugar beet. Plant Mol Biol 10:43-52

Mannerlof M, Tuvesson S, Steen P, Tenning P (1997) Transgenic sugar beet tolerant to glyphosate. Euphytica 94:83-91

Matzke MA, Matzke AJM (1995) How and why do plants inactivate homologous
10 (trans)genes? Plant Physiol 107:679-685

Mikami T, Sudoh R, Nagao E, Kinoshita T (1989) Genotypic variation in the in vitro morphogenesis from leaf explants of *Beta vulgaris* L. and *B. maritima* L. Euphytica 40:271-273

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with
15 tobacco tissue cultures. Physiol Plant 15:473-497

Sanchis V, Lereclus D, Menou G, Chaufaux J, Guo S, Lecadet MM (1989) Nucleotide sequence and analysis of the N-terminal coding region of the Spodoptera-active delta-endotoxin gene of *Bacillus thuringiensis* aizawai 7.29. Mol Microbiol 3:229-238

20 Sevenier R, Hall RD, van der Meer IM, Hakkert HJC, van Tunen AJ, Koops AJ (1998) High level fructan accumulation in a transgenic sugar beet. Nature Biotech 16:843-846

Figure legends

Fig. 1 The process of transformation and regeneration of plants in the genus *Beta*. A Germination from seeds and formation of cotyledon by *in vitro* culture. B Young plants transplanted onto shoot formation medium by cutting in the bottom part of hypocotyls after cotyledons are generated. C Generation of leaves. D Leaf blades placed on the medium. Note that shoots are generated from the leaf blades. These shoots were removed by cutting at the base in order to expose shoot-base tissues on the leaf explants prior to the infection with *Agrobacterium*. E Generation of shoots from shoot-base tissues on selection medium after inoculation of *Agrobacterium* to the leaf explants. F Regeneration and induction of roots after the selection. G Plants transferred to soil and acclimated to non-aseptic environment. H A mature plant grown on a pot.

Fig. 2 Gene constructs used for transformation. A The GUS gene construst. B The *cryIA(b)* gene construct. C The *cryIC* gene construct. D The chitinase gene construct. HPH, hygromycin phosphotransferase gene; NPTII, neomycin phosphotransferase II gene; NOS Pro, nopaline synthase promoter; NOS Ter, nopaline synthase terminator. DNA regions amplified by PCR are indicated by lines with arrowhead on both sides.

Fig. 3 Detection of transgene by PCR. A representative result of transformation experiment with the *cryIC* gene is shown. Portion of *cryIC* gene was amplified by PCR from the DNA isolated from transformed individual plants of T0 generation (lanes 1-10; lanes 1, 3, 4 and 5 are transformants of “NK150”, while others are transformants of “TK80”), non-transformed plant of “NK150” (lane 11), and non-transformed plant of “TK80” (lane 12), as well as from a plasmid vector in which *cryIC* had been cloned for a positive control. The PCR products were separated by agarose gel electrophoresis, and subsequently, hybridized with the *cryIC* probe.

Fig. 4 Analysis of GUS activity in T1 generation. The GUS activities of individual plants in T1 generation relative to that of non-transformed control plants are shown. Lane 1, non-transformed plants; lanes 2-9, T1 individuals of AC4-1; lanes 10-18, T1 individuals of AC4-2; lanes 19-23, T1 individuals of AC5. The data of non-transformed plants is given a value of 1. All the transformed plants used in this analysis were found to contain transgene(s) by PCR analysis.

Fig. 5 Gel-blot analysis of genomic DNA of transformed plants using GUS gene as a probe. Total DNA from six individuals of T2 generation (lanes 1–6) was digested with *Hin*dIII, and hybridized with the GUS gene probe. In this example, the hybridization profile indicates that multiple copies of transgene are integrated in the genome because there is only one *Hin*dIII site within the T-DNA region.

Fig. 6 Analysis of transgene expression by RT-PCR. A Gel-blot analysis of RT-PCR products using GUS gene specific primers. The cDNA prepared from leaves of individual transformants (numbered 1-6) were used as templates for PCR using a combination of GUS gene specific primer and the adaptor sequence primer that was used for cDNA synthesis. To confirm the specificity of amplification, the PCR products were hybridized with a GUS gene probe. B Ethidium bromide-staining of RT-PCR products of cycteinyl tRNA synthase gene after separation by agarose gel electrophoresis for positive control of RT-PCR. In this case, a combination of gene-specific primer was used. This resulted in amplification of transcripts (0.3 kb) from cDNA template as well as amplification of intron-containing fragment (0.5 kb) from DNA template that co-existed in the cDNA sample. Specific amplification of fragments from cDNA (see lane RT+) rather than from DNA was confirmed by that these fragments were not amplified when reverse transcriptase was not included in the reaction mixture for cDNA synthesis (see lane RT−). Note that there were differences in the level of GUS transcript between individual transformants.

Fig. 1

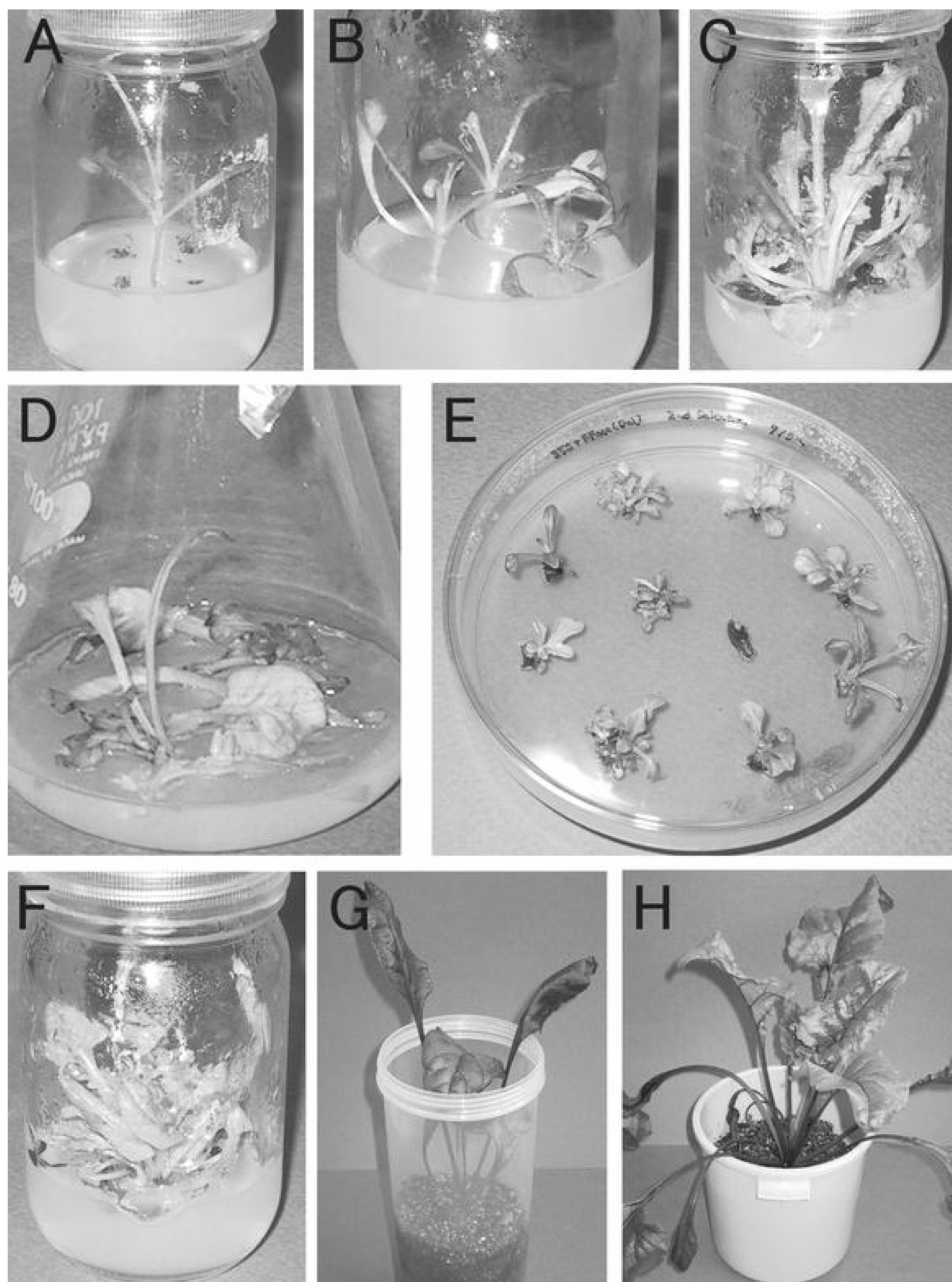


Fig. 2

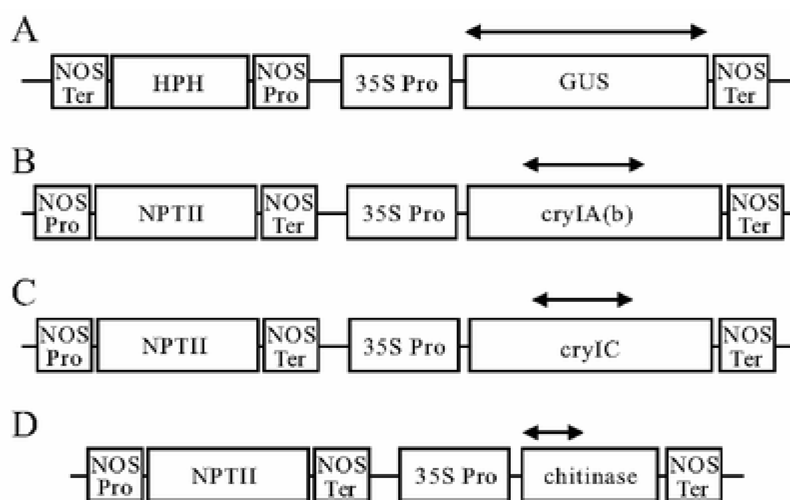
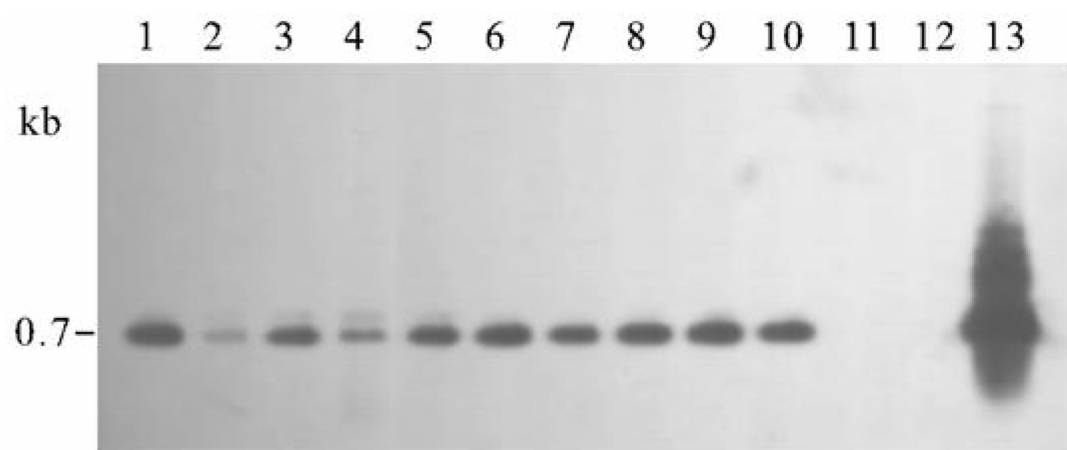


Fig. 3



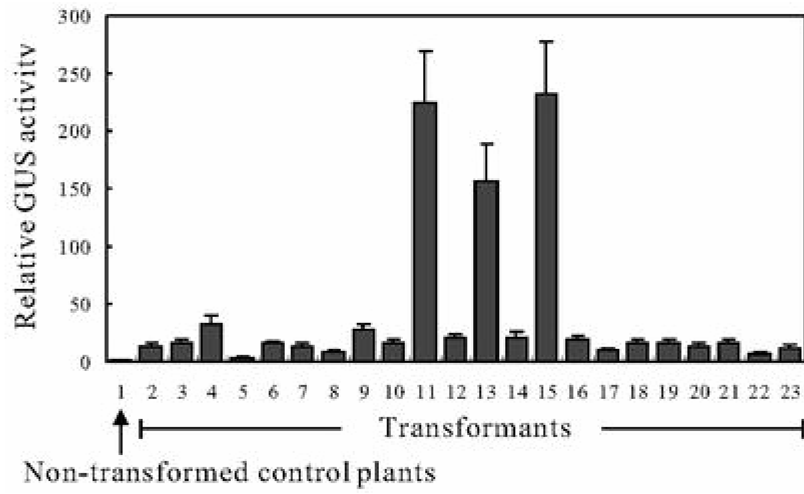

Relative GUS activity
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Transformants
Non-transformed control plants

Fig. 5

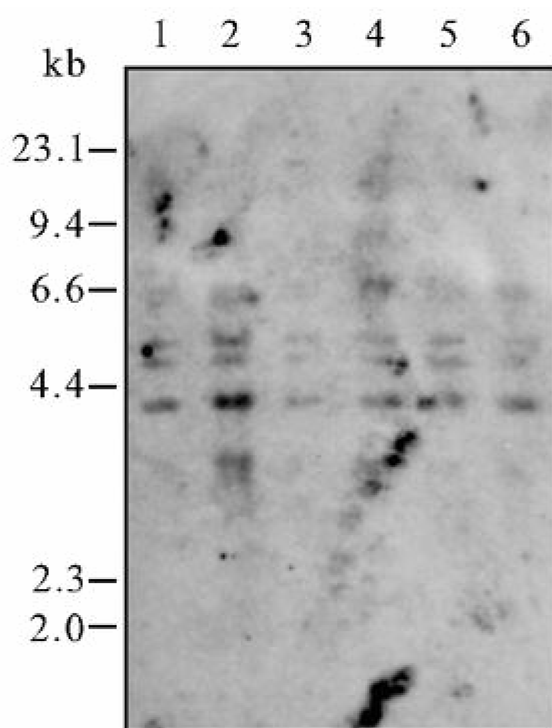
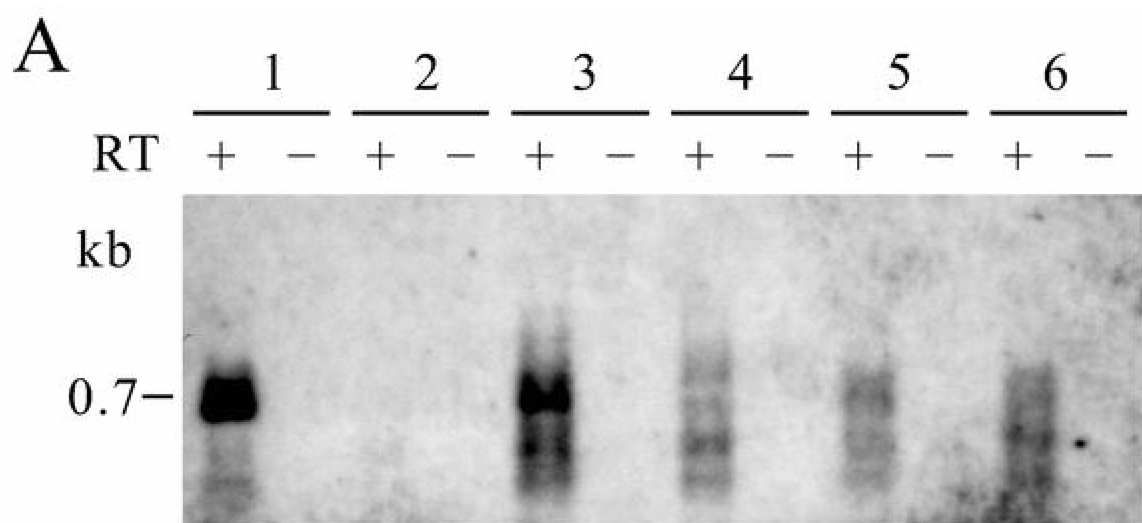


Fig. 6



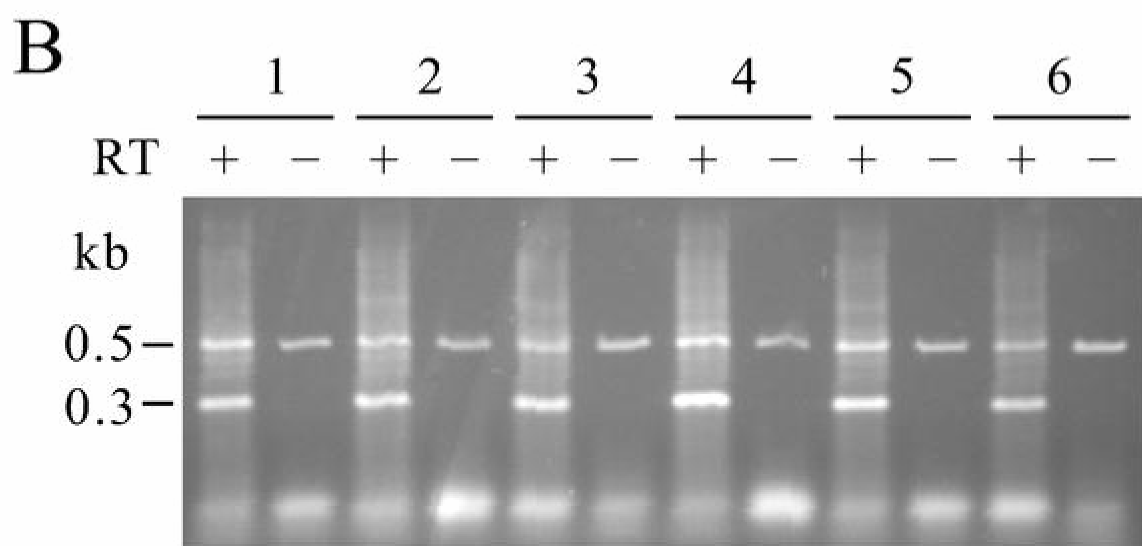


Table 1 Supplements in medium used for transformation

Medium	Plant hormone (mg/l)		Antibiotics (mg/l) hygromycin (kanamycin)	Carbenicillin (mg/l)	Asetosyringone (μM)	Sucrose (g/l)	Agar (g/l)
	IBA	BA					
Germination medium						30	9
Shoot-formaiton medium	0.1	0.25				30	9
Co-cultivation medium					20	30	9
Washing medium	0.1	0.25		1000			
Selection medium	0.1	0.25	10-15 (50-150)	500		30	9
Growth medium	0.1	0.25		500		30	9
Rooting medium	1.0			500		30	9

1/ 2 MS medium was used as basic medium for all the medium

Table 2 Frequency distribution of the number of explants from which regeneration occurred in *B. maritima* and *B. vulgaris* accessions

Accession	Number of individuals	Frequency distribution of the number of explants (1 - 6) from which regeneration occurred						Average [a]
		1	2	3	4	5	6	
B. maritima								
acc. France	66	1	10	18	21	14	2	3.7
SP673000	15	0	2	2	5	5	1	4.1
B. vulgaris								
NK150	47	25	16	1	4	1	0	1.7
TK80	13	2	4	5	1	1	0	2.6
NK183	3	0	2	1	0	0	0	2.3
T2n-24-115-24	8	0	0	1	3	3	1	4.5
NK185	5	4	1	0	0	0	0	1.2
NK172	14	11	2	1	0	0	0	1.3
TA33	20	11	6	1	2	0	0	1.7

[a] Average number of explants from which regeneration occurred

Table 3 Regeneration frequency of non-transformed cells under the presence of hygromycin

Accession	Amount of antibiotic (hygromycin mg/l)	Number of individuals	Number of explants	Number of individuals that allowed regeneration	Number of regenerated shoots
B. maritima					
acc. France	10	16	59	2	11
	12	9	23	0	0
B. vulgaris					
NK150	10	5	15	4	20
	15	11	36	1	1
TK80	10	2	6	0	0
	12	1	3	0	0
T2n-24-115-24	10	2	12	0	0
	12	1	5	0	0

Table 4 Frequency of transformation using *Agrobacterium* strain EHA101 and hygromycin selection

Accession	Amount of antibiotic (hygromycin mg/l)	Number of individuals	Number of explants	Number of explants resistant to hygromycin	Number of regenerated plants [a]	Number of plants from which transgene was detected
B. maritima						
acc. France	10	29	44	5	8	3
	12	11	68	3	8	6
B. vulgaris						
NK150	15	13	41	0	0	0
TK80	10	10	48	1	4	0
	12	1	3	0	0	0
T2n-24-115-24	10	6	48	0	0	0
	12	2	6	0	0	0

[a] All the regenerated plants were analyzed by PCR for presence or absence of transgene

Table 5 Frequency of transformation using *Agrobacterium* strain LBA4404 and kanamycin selection

Gene	Accession	Number of explants	Number of explants resistant to kanamycin	Number of regenerated plants examined by PCR	Number of plants from which transgene was detected
cryIA(b)	acc. France	142	88	21	12
	NK150	42	32	14	4
cryIC	NK150	32	21	10	5
	TK80	79	43	23	11
chitinase	TK80	13	11	21	21