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Introduction of pathogen defense genes and a cytokinin biosynthesis gene into sugarbeet (*Beta vulgaris* L.) by *Agrobacterium* or particle bombardment

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Abstract Two different methods for sugarbeet (*Beta vulgaris* L.) transformation were developed, one using *Agrobacterium* with excised cotyledons, the other, particle bombardment of embryogenic hypocotyl callus. Transformation efficiencies averaged 0.7% for the *Agrobacterium* method (number of transgenic plants obtained per treated cotyledon) and about 8% for the bombardment method (number of transgenic plants obtained per plate of embryogenic callus treated). Transgenic sugarbeet plants were produced carrying genes encoding either pathogen-defense-related proteins or the reporter enzyme β-glucuronidase (GUS) under transcriptional control of stress- or wound-inducible promoters. In addition, two plants were regenerated carrying a gene associated with enhanced insect resistance, the cytokinin biosynthesis gene, fused to a patatin gene promoter from potato. Expression of the GUS gene (*gusA*) under the control of the tobacco osmotin promoter was wound inducible with detectable activity at 8 h and maximal activity at 72 h post-wounding.

Key words Sugarbeet · Transformation · Disease resistance · Insect resistance · Particle bombardment

Abbreviations *As* Acetosyringone · *BAP* 6-Benzylaminopurine · *CaMV* Cauliflower mosaic virus · *GUS* β-glucuronidase · *MS* Murashige and Skoog · *4-MU* 4-Methylumbelliferone · *NAA* α-Naphthaleneacetic acid · *PR-S proteins* Pathogenesis-related S proteins

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Introduction

Sugarbeet (*Beta vulgaris*) supplies approximately 35% of the world's sugar, but for the past 20 years, sugar yields have been steadily declining due to disease and insect attack. Effective control measures for microbial pathogens and major insect pests are lacking. Biotechnological improvement of sugarbeet has been hampered by the lack of reproducible, high-frequency, gene transfer methods. Initially, success in sugarbeet transformation was reported using *Agrobacterium*-mediated transformation of shoot-base explant material (Lindsey and Gallois 1990), cotyledonary node explants (Krens et al. 1996), or embryogenic callus from either leaf disks (Ben-Tahar et al. 1991) or seedlings (D'Halluin et al. 1992). Recently, a new method employing polyethyleneglycol-mediated transformation of guard cell protoplasts has been described (Hall et al. 1996).

We have been unable to employ successfully the methods using sugarbeet shoot bases (Lindsey and Gallois 1990), cotyledonary node explants (Krens et al. 1996), or leaf protoplasts (Hall et al. 1996), and the seedling method of D'Halluin et al. (1992) was impractical due to overgrowth of *Agrobacterium*. Since the patented method of Ben-Tahar et al. (1991) lacked peer review, it was included in this study as a comparison.

The goal in the present research was to develop an efficient and reproducible method of sugarbeet transformation for introduction of pathogen and insect defense genes for control of major sugarbeet diseases and pests. Transgenic sugarbeets were produced that carry genes coding for osmotin (Osm) (Nelson et al. 1992; Zhu et al. 1995) and the pathogenesis-related S (PR-S) proteins (Vigers et al. 1992) from tobacco, a barley leaf α-thionin polypeptide (Bohlman et al. 1988), a modified cecropin polypeptide MB39 (Owens and Huette 1997), or a bacterial cytokinin biosynthesis gene (*ipt*) that has been associated with enhanced insect resistance in transgenic plants (Smigocki et al. 1993). Transgenic plants were derived using both *Agrobacterium* inoculation of excised cotyledons and particle bombardment of embryogenic hypocotyl callus.

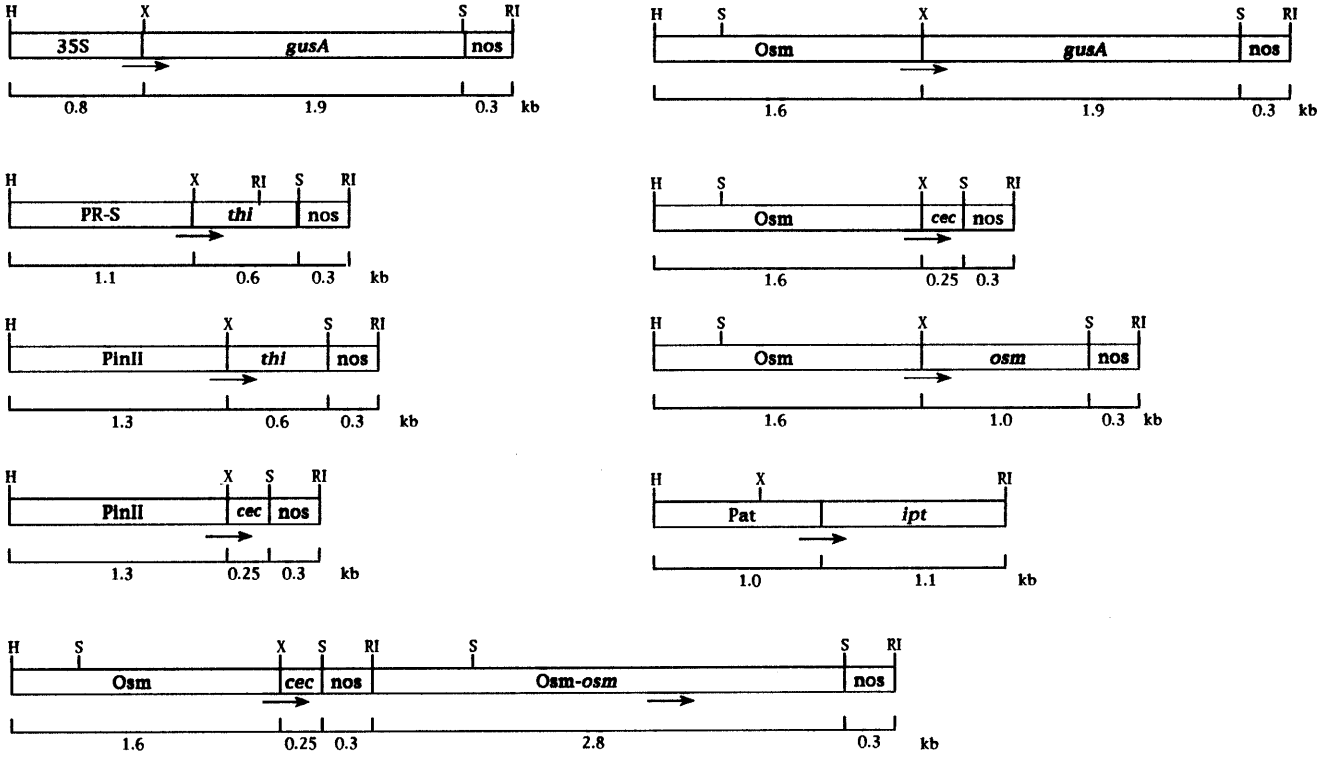


Fig. 1 Schematic representation of gene constructs used to produce transgenic plants. Promoters (*roman*) and coding regions (*italics*) were derived from: 35S (from CaMV) and *gusA* (from *Escherichia coli*) from pBI121 (Jefferson et al. 1987); Osm and *osm* from tobacco osmotin (Nelson et al. 1992); PR-S from tobacco pathogenesis-related S (van Kan et al. 1989); PinII from proteinase inhibitor II (Keil et al. 1986); *thi* from barley leaf α-thionin DB4 (Bohlmann et al. 1988); *cec* from the MB39 modification of cecropin B (Huang et al. 1997); Pat from potato patatin (Mignery et al. 1988); *ipt* from *Agrobacterium tumefaciens* isopentenyl transferase (Smigocki et al. 1993) genes

Materials and methods

Gene constructs

The plant promoters used in this study were described previously by Ingersoll et al. (1996) and consisted of the 35S promoter from cauliflower mosaic virus (CaMV), the tobacco Osm (Nelson et al. 1992) and PR-S (van Kan et al. 1989) promoters, and the potato proteinase inhibitor II (PinII) (Keil et al. 1986) promoter. Coding regions from *Escherichia coli* β-glucuronidase (GUS) *uidA* (*gusA*) (Jefferson et al. 1987), tobacco Osm (*osm*) (Nelson et al. 1992) and PR-S (Kauffmann et al. 1990), barley leaf α-thionin DB4 (*thi*) (Bohlmann et al. 1988), and cecropin MB39 (*cec*) (Huang et al. 1997) genes were cloned downstream from the aforementioned promoters (Fig. 1). Additional constructs included the Osm-*osm* gene fused in tandem with Osm-*cec*, and the coding region from a bacterial isopentenyl transferase (*ipt*) gene (Smigocki et al. 1993) fused to a tuber-specific, class I patatin promoter (Pat) from potato (Mignery et al. 1988). The chimeric defense genes were ligated into the pGPTV binary vector (Becker et al. 1992), and the 35S-*gusA* segment from pBI121 (Jefferson et al. 1987) was ligated into the pKYLX71 vector (Schardl et al. 1987).

Tissue culture media

Tissue culture was performed using variations of a basal medium (MSB) composed of Murashige and Skoog (1962) (MS) salts, B5 vitamins (Gamborg et al. 1970), 1.0 mg/l pantothenic acid, 0.01 mg/l biotin, 0.5 g/l MES, 30.0 g/l sucrose, and 5.0 g/l agargel (Sigma, St. Louis, Mo.) (pH 5.8).

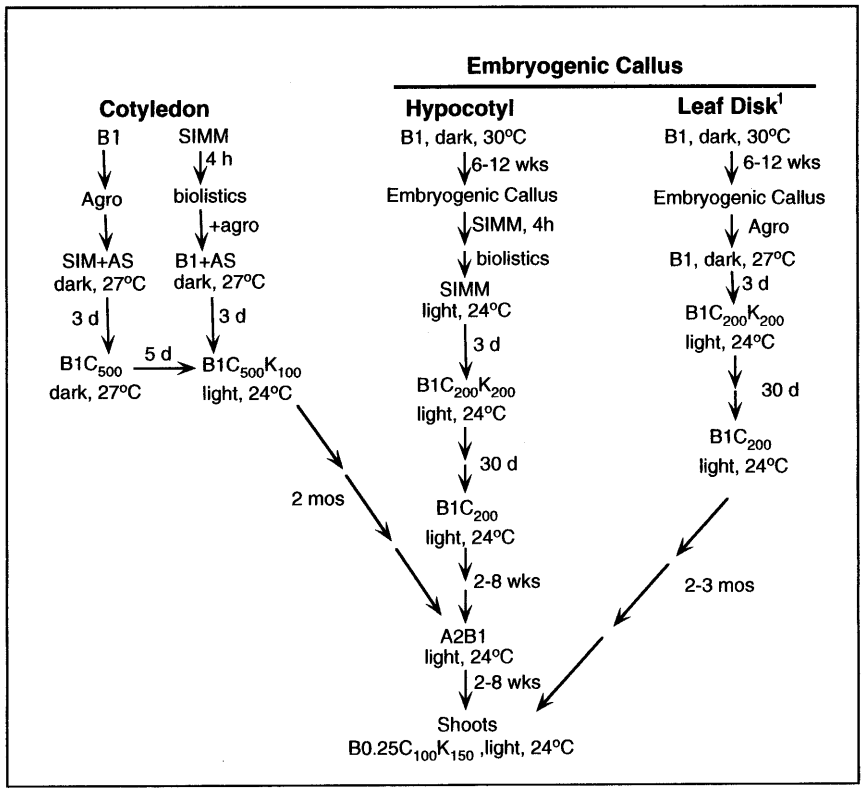
Plant material

The sugarbeet genotype Rel-1 (Saunders et al. 1992) was used in all experiments. Clones of this cultivar and subsequent transgenic R0 plants were maintained in tissue culture on B0.25 medium [MSB with 0.25 mg/l 6-benzylaminopurine (BAP)], rooted on RM medium [MSB with 1.0 mg/l α-naphthaleneacetic acid (NAA)], and transferred to soil for growth in the greenhouse. Transgenic R0 plants were induced to flower (during the first or second year) under a 16-h photoperiod, and R1 seed was produced following self-pollination.

Rel-1 S1 seeds were surface-sterilized in a 15% commercial Clorox, 0.01% SDS solution for 40 min and washed five times with sterile deionized water. Eight seeds were placed on GM medium (MSB with 1.0 mg/l BAP, 0.5 mg/l 2,3,5-triiodobenzoic acid) in a disposable petri dish (100×25 mm) and incubated at 27°C in the dark for 21 days. Seedlings were either transferred to 30°C in the dark, or hypocotyl segments (1–2 cm) and cotyledons were excised. Hypocotyl segments were placed on B1 (MSB with 1.0 mg/l BAP) medium and incubated at 30°C in the dark until callus appeared (1.5–3 months). Excised cotyledons were treated as described below.

Bacterial strains

Agrobacterium supervirulent strains EHA101 (Hood et al. 1986) and EHA105 (Hood et al. 1993) harboring one of the modified binary vector plasmids described above were grown overnight at 28°C in YEB (5.0 g/l beef extract, 5.0 g/l peptone, 5.0 g/l sucrose, 1.0 g/l yeast extract, 2.0 mM $MgSO_4$, pH 7.2) or YOB (YEB without $MgSO_4$) medium. The overnight culture (in stationary phase) was diluted 1:2 in 10 ml of an induction medium composed of AB salts and buffer (An 1987), 5% glucose, and 100 μM acetosyringone (AS), pH 5.6, and grown for 24 h at 28°C.



Agro=*Agrobacterium* AS=acetosyringone

[1] The procedure of Ben-Tahar et al. (1991)

Fig. 2 Flow chart of the transformation methods used employing excised cotyledons and embryogenic callus from cultured hypocotyls or leaf disks

Transformation

Four different approaches were used to generate transgenic sugarbeet (Fig. 2). Two utilized cotyledons as the starting material, and two utilized embryogenic callus.

When cotyledons were used, they were removed from dark-grown seedlings (described above) with forceps or scalpel, placed abaxial side down on B1 or SIMM medium (MSB with 0.3 mg/l BAP, 0.1 mg/l NAA, 50 mg/l adenine sulfate, 44.6 g/l mannitol, 44.6 g/l sorbitol) for 4 h (Fig 2). The explants were then either left untreated or bombarded (BIOLISTIC Particle Delivery System PDS-1000He, Bio-Rad, Hercules, Calif.) three times at 1300 psi with 500 μg of gold microcarriers (Ingersoll et al. 1996) coated with 0.83 μg of plasmid DNA from one of the gene constructs described above. Bombarded or untreated cotyledons were immersed in a suspension of *Agrobacterium* in induction medium diluted 1:1 with liquid B1 medium, blotted dry on sterile Whatman 3MM paper and placed abaxial side up on B1 or SIM (SIMM, without sorbitol and mannitol) medium supplemented with 100 μM AS and incubated for 3 days in the dark. After cocultivation, the cotyledons were washed in liquid B1 medium containing 500 μg cefotaxime/ml (C_{500}) and placed either on selective B1 medium supplemented with 100 μg kanamycin/ml (K_{100}) and C_{500} in the light at 24°C or placed on non-selective $B1C_{500}$ in the dark at 27°C for 5 days before transfer to $B1C_{500}K_{100}$ in the light. After three transfers, over a 2-month period, embryogenic callus arising from the cotyledons was cultured on A2B1 medium (MSB, 2.0 mg abscisic acid, 1.0 mg BAP/l). Shoots that appeared were transferred to $B0.25C_{100}K_{150}$ medium to select for transgenics (Fig. 2).

Two sources of embryogenic callus were utilized (Fig. 2). For biolistic experiments, embryogenic callus (3.0–5.0 g fresh weight) from cultured hypocotyl segments was harvested and spread as a thin layer on a sterile filter paper disk overlaying solidified SIMM (Fig. 2). The callus was incubated for 4 h and subjected to biolistic treatment with DNA-coated particles, as described by Ingersoll et al. (1996). Following biolistic treatment, the filter disk was moved to a fresh plate of SIMM medium and incubated for 3 days in the light at 24°C, then transferred to $B1C_{200}K_{200}$. C_{200} was added to control any endophytic or other bacterial contaminants. After 14 days, the filter disk was transferred to fresh $B1C_{200}K_{200}$ medium and, after 30 days, to $B1C_{200}$ medium. Viable callus was harvested and cultured on A2B1 medium until shoots formed. Shoots were grown on $B0.25C_{100}K_{150}$ medium to select for transgenics (Fig. 2). Leaves were assayed for GUS activity as described by Ingersoll et al. (1996).

For *Agrobacterium*-mediated transformation, embryogenic callus was produced from leaf disks of young sugarbeet leaves and treated according to the method of Ben-Tahar et al. (1991) (Fig. 2).

Isolation of genomic DNA and Southern hybridization

Genomic DNA was isolated from leaves using the method of Dellaporta et al. (1983), digested with restriction enzymes and subjected to agarose gel electrophoresis. DNA was transferred to a nylon membrane and hybridized according to the manufacturer's suggestions (Boehringer Mannheim or BioRad) with probes prepared by the Genius nonisotopic method or ^{32}P random-primed labeling (Boehringer Mannheim).

Results and discussion

Agrobacterium-mediated transformation

Embryogenic leaf disk callus cocultivated with *Agrobacterium* according to the patented method of Ben-Tahar

Table 1 Transgenic sugarbeet plants produced by treatment of excised cotyledons and embryogenic hypocotyl callus

Transformation method/construct	Number of independent transformants	Efficiency (%)[a]
Agrobacterium excised cotyledons		
Pat-*ipt* (unwounded)	2/57	3.5
Osm-*gusA* (unwounded)	1/55	1.8
Osm-*gusA* (wounded)[b]	1/40	2.5
Agrobacterium success rate	4/572[c]	0.7
Biolistics embryogenic hypocotyl callus		
Osm-*cec*	1/6	16.7
Osm-*osm*/Osm-*cec*	1/6	16.7
Osm-*osm*	1/5	20.0
PinII-*cec*	1/3	33.3
PinII-*thi*	1/3	33.3
PR-S-*thi*	1/4	25.0
Biolistic success rate	6/78[d]	7.7

[a] Number of transgenic plants obtained per treated cotyledon explant or plates of embryogenic callus (3–5 g fresh weight/plate)
[b] Wounded biolistically with DNA-coated gold particles (see Materials and methods)
[c] Includes data from nine additional experiments comprising 420 cotyledons treated with *Agrobacterium* carrying various constructs from which no transformants were obtained. Additional constructs used were Osm-*osm*, Osm-*thi*, Osm-*thi*/Osm-*gusA*, PinII-*osm*, PinII-*thi*, PR-S-*osm*, PR-S-*thi*, 35S-*prs*, and 35S-*gusA* [*prs* is the coding region from the pathogenesis-related S gene (van Kan et al. 1989); see legend to Fig. 1 for other abbreviations]
[d] Includes data from nine additional experiments comprising 51 plates from which no transformants were recovered. Additional constructs used were 35S-*thi*, 35S-*cec*, 35S-*osm*, 35S-*prs*, PR-S-*gusA*, PR-S-*cec*, PR-S-*osm*, PR-S-*prs*, Osm-*thi*, Osm-*prs*, PinII-*gusA*, PinII-*osm* and PinII-*prs*

et al. (1991) produced only a single transgenic plant carrying the 35S-*gusA* construct (data not shown). A major problem experienced using this method was the frequent loss of callus due to overgrowth by an endophytic microorganism from the greenhouse-grown explant and/or agrobacteria. This problem led us to devise other methods that employ cotyledons or hypocotyls derived from axenic tissue-cultured seedlings.

Cocultivation of cotyledons with *Agrobacterium* carrying 11 different gene constructs yielded four transgenic plants at an overall efficiency of 0.7% as defined by the number of independent transgenic plants obtained per treated cotyledon (Table 1).

Biolistic wounding of cotyledons prior to cocultivation with *Agrobacterium* carrying the Osm-*gusA* construct appeared to slightly enhance the transformation efficiency. The efficiency was 2.5% for the microprojectile-wounded cotyledons compared to 1.8% for the unwounded ones (Table 1). These results are in accordance with the stimulation reported by Bidney et al. (1992) for microprojectile-wounded sunflower tissue. The presence of plasmid DNA on the gold particles used for sugarbeet cotyledon wounding had no apparent effect on the transformation efficiency. Southern analysis of the Osm-*gusA* transgenic plant derived from wounded cotyledons established that it was transformed by *Agrobacterium*-vector DNA and not by the DNA on the microprojectiles (data not shown).

Interestingly, a higher transformation efficiency was observed with the Pat-*ipt* gene construct (3.5%) than with the Osm-*gus* construct (1.8%). This may possibly reflect an enhancing effect of cytokinin on regeneration of plants due to expression of the introduced *ipt* gene.

Biolistic transformation

As has been observed by others (D'Halliun et al. 1992; Jacq et al. 1992), in-vitro-germinated sugarbeet seedlings proved to be an excellent source of embryogenic callus. Particle bombardment of embryogenic hypocotyl callus yielded transformation efficiencies that ranged from 0 to 33% and averaged 7.7%, as defined by the proportion of treated plates that produced transgenic plants (Table 1). Embryogenic callus was initiated using the method of Jacq et al. (1992) except that the callus was induced on B1 rather than SIM medium (Fig. 2). Preliminary experiments had shown that B1 medium, as opposed to SIM, resulted in higher callus survival during the long period between transformation and regeneration (data not shown), thereby increasing the chances of producing transgenics.

During incubation on $B1C_{200}K_{200}$, most of the treated callus became brown and necrotic, but a few small areas of white callus appeared. After transferring the filter disk to nonselective medium, the white callus proliferated. This callus was subcultured onto A2B1 medium (Fig. 2) to stimulate shoot production. After two to three monthly subcultures on $B0.25C_{100}K_{150}$ medium, the transgenic shoots flourished while escapes deteriorated and became chlorotic.

Molecular analysis

Southern blot analyses confirmed transformation and revealed the number of genes inserted. Fig. 3A, B shows analysis of transgenic plants produced from cotyledons (Fig. 3A) or leaf disk callus (Fig. 3B) cocultivated with *Agrobacterium*, and Fig. 3C–E from embryogenic callus bombarded with DNA-coated microparticles. The two Pat-*ipt* plants had four and two copies of the introduced gene (Fig. 3A, plants 01 and 02), the Osm-*gusA* plant had two copies, and the 35S-*gusA* plant one copy (Fig. 2B) of their respective transgene.

Generally, transformation via *Agrobacterium* inserts into plant genomes only the genes between the T-DNA borders of the vector, whereas particle bombardment may introduce the whole binary vector, often resulting in DNA rearrangements. Of all the plants transformed solely by microprojectile bombardment of embryogenic callus, only the PR-S-*thi* transgenic plant contained a single inserted gene (Fig. 3E). All other lanes showed evidence of multiple-copy DNA insertions and molecular rearrangements, as noted by bands of unexpected sizes, in addition to a band of the expected size, appearing in lanes where genomic DNA was cut within the insert. The consequences of these rearrangements are unknown, but all the

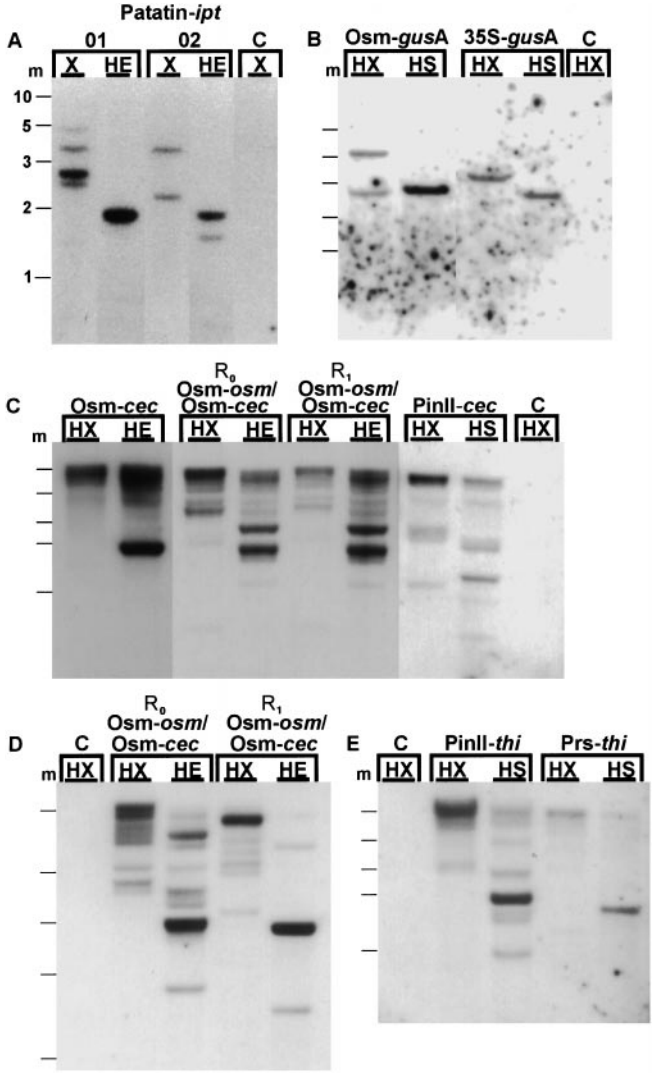


Fig. 3A–D Southern blot analysis of transgenic sugarbeets. **A** was probed with a ^{32}P-labeled *ipt* gene fragment; all other blots were hybridized with a digoxygenin-labeled DNA probe from the coding regions of gusA (*B*); *cec* (*C*); *osm* (*D*); and *thi* (*E*). Notations for the plants from which the DNA was isolated appear above the boxes enclosing the restriction enzymes used (*E Eco*RI, *H Hin*dIII, *S Sst*I, *X Xba*I). Except for the Rel-1 untransformed control (*C*), genomic DNA was cut in two ways: one restriction digest (cut once within the insert and probed with coding-region DNA) was used to show the number of inserts (*left lane*) and the other digest (cut at both ends of the promoter-gene insert) to show a fragment of a predicted size (*right lane*). Predicted sizes for promoter gene constructs: Pat-*ipt* 2.1 kbp, Osm-*gusA* 2.8 kbp, 35S-*gusA* 2.7 kbp, Osm-*cec* 2.2 kbp, PinII-*cec* 1.7 kbp, Osm-*osm* 3.0 kbp, PinII-*thi* 1.8 kbp, Pr-S-*thi* 1.6 kbp (*m* molecular weight markers at 1, 2, 3, 5, and 10 kbp from bottom to top). **C** Composite of three hybridizations

plants appeared to have at least one intact copy of the inserted gene.

To characterize the phenotypes, more plants must be generated either by selfing or crossing with wild types. A banding pattern identical to the R0 was noted in an R1 plant produced after selfing the sugarbeet transformed with the Osm-*osm*/Osm-*cec* dual construct (Fig. 3C).

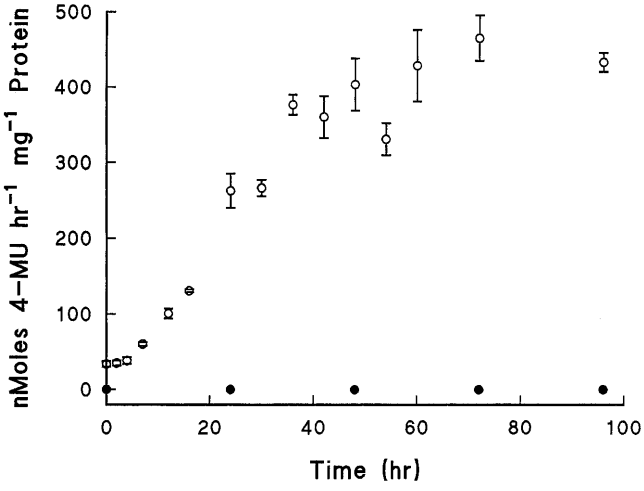


Fig. 4 Wound-inducible GUS activity in Osm-*gusA* transgenic sugarbeet leaves from greenhouse-grown plants (n=3). Leaves were removed, surfaced sterilized, wounded by cutting into 1-cm^2 pieces and cultured on moist filter paper until assayed. Each time point for the Osm-*gusA* (○) is the average of three leaf pieces and for the untransformed control (●), one leaf piece

Osmotin-*gusA* expression

The regeneration of sugarbeet plants with the *gusA* gene driven by the tobacco osmotin promoter provided the opportunity to examine the pattern of in vivo expression. Ingersoll et al. (1996) have shown that the Osm-*gusA* construct exhibited inducible GUS activity in sugarbeet suspension cells. In the present study, leaves were removed from tissue-cultured plants transformed with either 35S-*gusA* or Osm-*gusA*, wounded by cutting the leaf into three to four pieces and assayed for GUS activity at 0, 8, and 24 h. Both transgenic plants showed constitutive GUS expression based on observations that GUS activity was not significantly different over three time points (data not shown). However, the GUS activity of the Osm-*gusA* plant [2070±470.0 nmol 4-methylumbelliferone (4-MU) h^{-1} mg^{-1} protein] averaged almost ten-fold higher than that of the 35S-*gusA* plant (246±16.6 nmol 4-MU h^{-1} mg^{-1} protein).

In contrast to tissue-cultured plants, the pattern of GUS expression in the leaves of the Osm-*gusA* plant grown in the greenhouse was different and inducible (Fig. 4). In this experiment, leaves were removed, surface sterilized, placed in petri dishes on moist filter paper, and assayed at various time points from 0 to 96 h. At time zero, GUS activity in the leaf was only slightly higher than background. Induction occurred by 8 h, and activity peaked at 72 h and remained high through to 96 h. The temporal nature of this response is similar to that reported by Zhu et al. (1995) who found that GUS activity in *Solanum commersonii* transformed with an Osm-*gusA* construct and infected with *Phytophthora infestans* was detectable at 1 day post-infection and continued to increase up to 4 days. The osmotin promoter is known to be induced by wounding and abiotic factors such as abscisic acid, ethylene, and NaCl (Nelson et al. 1992).

Histochemical staining revealed that GUS activity in leaves from tissue-cultured plants appeared to be uniform throughout the tissue, whereas in the wounded soil-grown plant, staining of the leaves was limited to the cut edges and vascular tissue (data not shown). This observation may explain the reduction in GUS activity, as measured by hydrolysis of 4-MUG, in soil-grown plants (Fig. 4) relative to tissue-cultured ones, and indicate the presence of a tissue culture factor, or factors, possibly sucrose and/or ethylene, which uniformly induced the osmotin promoter in vitro.

Two new methods for the transformation of sugarbeet have been developed: in one, the excised cotyledons were cocultivated with *Agrobacterium*, in the other, the procedure employed particle bombardment of hypocotyl callus. A greater diversity of genes was introduced with the particle bombardment method, and the transformation efficiency was higher. However, the higher efficiency was achieved at a cost of 2–3 months additional time and effort required to produce the callus as compared to cotyledon explants. The *Agrobacterium*-cotyledon method has the additional advantages of not requiring a particle-delivery system, and of producing transgenic plants with fewer molecular rearrangements of the inserted DNA.

The methods were used to introduce eight different defense gene constructs into sugarbeet. Plants are currently being selfed and subjected to genetic analysis to produce seed for subsequent testing for expression of resistance to the respective pathogens.

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