

S. Komarnytsky · A. Gaume · A. Garvey · N. Borisjuk ·
I. Raskin

A quick and efficient system for antibiotic-free expression of heterologous genes in tobacco roots

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Abstract Requirement for antibiotic-resistance selection markers and difficulty in identifying transgenes with the highest expression levels remain the major obstacles for rapid production of recombinant proteins in plants. An alternative approach to producing transgenic plants free of antibiotic-resistance markers is the phenotypic-based selection with root-proliferation genes (*rol* genes) of *Agrobacterium rhizogenes*. By using *Agrobacterium tumefaciens* harboring the pRYG transformation vector with a cluster of *rol* genes linked to a heterologous gene of interest, we have developed a rapid transformation tool using hairy root formation as a selection marker. The expression of β -glucuronidase in newly induced transgenic tobacco roots could be detected as early as 12 days after inoculation. Higher levels of transgene expression in the roots correlated positively with the rates of root elongation on hormone-free medium and thus could be used for positive selection. When tobacco plants were transformed with pRYG harboring the expression cassette for secreted alkaline phosphatase (SEAP), the release of SEAP from roots of the fully regenerated transgenic plants could be quantified at rates as high as 28 $\mu\text{g/g}$ root dry weight per day.

Keywords Plant genetic engineering · Selectable marker · *rol* genes · Hairy roots · Recombinant protein production

Abbreviations *GUS*: β -Glucuronidase · *SEAP*: Secreted alkaline phosphatase · *rolABC*: Cluster of *rolA*, *rolB*, and *rolC* genes

Introduction

Transformation systems usually rely on a selectable marker gene to isolate and propagate a rare transformation event from the non-transformed plant tissue. Traditionally, this is achieved by co-expression of the gene of interest together with the antibiotic or herbicide resistance marker, which remains in the genome of a transgenic plant after the selection and regeneration processes are completed. While the product of the antibiotic-resistance gene does not interfere with plant development directly, the use of appropriate selective agents may significantly decrease the proliferation and regeneration capabilities of the transformed cells. In addition, the potential release of the antibiotic selection markers into the environment creates safety and ethical concerns, further hampering the agricultural use of many transgenic crops. Antibiotic resistance genes can be removed from the genome of the transgenic plant by a number of excision techniques, although each method has limitations that need to be considered (reviewed in Ebinuma et al. 2001). An alternative approach to producing transgenic plants free of antibiotic resistance markers is phenotypic-based selection using regeneration-promoting genes (reviewed in Zuo et al. 2002). This system may improve the overall efficiency of plant transformation by abandoning the need for antibiotic selection (Kunkel et al. 1999). Using this technique, Endo et al. (2002) have developed a single-step transformation system for rice, where marker-free transgenic rice plants were regenerated directly from the *ipt*-induced rice embryogenic tissues.

Similar to the widely used *Agrobacterium tumefaciens*, *A. rhizogenes* stably transfers a portion of its Ri plasmid into a plant cell at the time of infection, thereby inducing the rapid proliferation of hairy roots on stem and leaf sections of many plant species (Chilton 1982). This

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S. Komarnytsky · A. Gaume · A. Garvey · N. Borisjuk ·
I. Raskin (✉)
Biotech Center, Cook College, Rutgers University,
59 Dudley Road, New Brunswick, NJ 08901-8520, USA
e-mail: raskin@aesop.rutgers.edu
Tel.: +1-732-9328165
Fax: +1-732-9326535

Present address:

A. Gaume, ETHZ—Plant Sciences, Group for Plant Nutrition,
Experimental Research Station Eschikon,
8315 Lindau, Switzerland

unique rooting response is caused by the expression of *rol* genes located within the infectious T-DNA region of *A. rhizogenes* (Slightom et al. 1986). When cloned in a cosmid and conjugated into an avirulent strain of *A. rhizogenes*, this region was capable of inducing a root proliferation response comparable to that induced by the wild-type *A. rhizogenes* strain (Vilaine and Casse-Delbart 1987). Since each transformation event can be easily distinguished as an independent hairy root clone developing on the hormone-free medium without any selective agents (Tepfer 1984), the system provides a valuable alternative approach for antibiotic-free plant transformation and regeneration.

Several techniques for the simultaneous introduction of *rol* genes and a gene of interest into the plant genome have been reported over the years. Simpson et al. (1986) used a binary system based on wild-type *A. rhizogenes* culture harboring a binary plant transformation vector to generate a hairy root clone expressing a target gene. However, this system is inefficient and requires an antibiotic selection to propagate the rare co-delivery event (Nicoll et al. 1995; Damiani et al. 1998). Co-transformation using a mixture of *A. rhizogenes* and *A. tumefaciens* cultures harboring the binary expression vector (Torregroza and Bouquet 1997) did not overcome these limitations, while the direct integration of the expression cassette into *A. rhizogenes* T-DNA via homologous recombination (Stougaard et al. 1987) proved cumbersome and inefficient. Finally, a removal MAT vector system combined with a positive rooting marker consisting of four genes—*rolA*, *rolB*, *rolC*, and *rolD* (Ebinuma et al. 1997)—was exploited for the transformation and regeneration of marker-free transgenic snapdragon plants, with only 3% of regenerated shoots reported suitable for the study (Cui et al. 2001).

We report here on a novel, antibiotic-free transformation strategy that results in rapid and efficient expression and secretion of recombinant proteins from the hairy roots only 2 weeks after the initial infection. We show that the hairy root phenotype, which is regarded as being undesirable in standard transformation experiments (Ebinuma et al. 1997; Cui et al. 2001), is beneficial if the goal of the transformation is to rapidly produce and analyze significant quantities of the recombinant protein.

Materials and methods

Vector construction

The *rolABC* gene cluster (harboring the *rolA*, *rolB*, and *rolC* genes) was cut out of the pUC9-*rolABC* plasmid as a 4.3-kb fragment (Schmullig et al. 1988) by *EcoRI* (complete)-*PstI* (partial) digestion and ligated into the *EcoRI*-*PstI* sites of pGreen (Hellens et al. 2000) to construct pRYG, the basic transformation vector for induction of the hairy root proliferation response. The β -glucuronidase (GUS) expression cassette including the *mas2'* promoter, the GUS gene interrupted with an intron sequence, and the *nos* terminator was subcloned as the *PvuII* fragment from pE1226 (provided by Stanton Gelvin, Purdue University) into the *EcoRI*-*PstI* blunted sites of pLit-aps (Borisjuk et al. 2000)

downstream from the *aps* element to produce pLit-aps-mas-GUS. From here, the complete GUS expression cassette including *aps* was ligated as the *KpnI*-*BsiWI* (blunted) fragment into the *KpnI*-*EcoRI* (blunted) sites of pRYG to produce pRYG(GUS). The blunted *HindIII* fragment, containing the SEAP gene with its own secretion signal peptide and the *nos* terminator from pNB-35S-SEAP (Borisjuk et al. 1999), was ligated into the blunted *XbaI*-*XhoI* site of pLit-aps-mas-GUS, replacing the GUS gene with that of SEAP to construct pLit-aps-mas-SEAP. From here, the *KpnI*-*BsiWI* (blunted) fragment harboring the entire SEAP expression cassette was ligated into the *KpnI*-*EcoRI* (blunted) sites of pRYG to produce pRYG(SEAP). *KpnI*-*EcoRI* (blunted) fragment from pLit-aps-mas-GUS was also ligated into the *KpnI*-*HindIII* (blunted) sites of pBin19-aps (Borisjuk et al. 2000), replacing the *aps* element in that construct with a complete GUS expression cassette to produce pBin-mas-GUS.

pRYG-bed plant transformation

Mature tobacco seeds (*Nicotiana tabacum* L. cv. Wisconsin) were surface sterilized in 70% (v/v) ethanol and 10% (v/v) sodium hypochloride and then washed thoroughly in sterilized water. The seeds were germinated and subcultured on half-strength MS medium (Murashige and Skoog 1962) supplemented with 15 g sucrose and 7 g agar (pH 5.8). All reagents were obtained from Sigma-Aldrich (St. Louis, Mo.) unless otherwise stated. Approximately 1-cm² sections of aseptic leaves of 2-month-old plants containing the vascular tissue of the major vein were excised using a sterile surgical blade that had been immersed into a 2-day-old culture of *A. tumefaciens* EHA105 harboring the appropriate transformation vector. The leaf sections were then transferred to half-strength MS medium for infection (25°C, 48 h in the dark). The bacterial culture was eliminated on MSR medium (half-strength MS supplemented with 400 mg/l carbenicillin) under standard conditions [28°C, 16/8-h (light/dark) photoperiod, irradiance of 150 mE/m² per second supplied by a mixture of fluorescent and incandescent lamps]. The leaf sections were subsequently transferred every 4 days to fresh MSR medium with no hormone supplementation. In vivo infection of 3-month-old plants was performed under standard greenhouse conditions. Identically prepared bacterial cultures were inoculated at the mid-section of the tobacco stems using an aseptic syringe and needle (4 ml inoculum/8 cm of plant stem). The infected areas were immediately enclosed within a plastic tube sealed off by Parafilm (Fig. 3C) to provide a high humidity atmosphere favorable for root formation (Gaume et al. 2003). In all experiments, the hairy root proliferation response was observed 9–14 days after bacterial inoculation. Control inoculations with *A. tumefaciens* EHA105 cultures harboring the pGreen vector alone were performed following the identical procedure, and no root development was observed on control plants.

Binary system transformation and root analysis

In vivo infection of 3-month-old plants using the binary transformation system was performed as described above using the *A. rhizogenes* A4 bacterial culture harboring pBin19-mas-GUS or the wild-type A4 culture as a negative control. In these experiments, hairy roots developed 9–12 days post-infection. Three- to five-day-old roots were rinsed thoroughly in sterile water and stained for GUS activity as described below.

Selection and analysis of pRYG(SEAP)-transformed roots

For the experimental data reported in Fig. 5C, 3-day-old hairy roots attached to the leaf section were transferred to half-strength MS liquid medium (no agar) and cultivated on a rotary shaker (80 rpm, 20 ml media in 200-ml Erlenmeyer flasks) in the dark for 7 days. Portions of the fast-growing roots were excised and placed on top

Table 1 Characterization of the root growth and SEAP production in two different populations of pRYG(SEAP)-induced roots. Values represent the means \pm standard deviation of three independent measurements 22 days after inoculation

Root population	Root length (cm)	Total SEAP (% TSP)
Fast growing	4.3 \pm 0.3	0.9 \pm 0.2
Slow growing	1.8 \pm 0.6	0.2 \pm 0.1

of a PVDF membrane with a little pressure applied to ensure the complete contact of the root surface with the membrane. Once the root tissue was removed, the dried membrane was heat-treated and stained for SEAP activity, as described below.

For the experimental data shown in Fig. 5D, E, 3-day-old hairy roots attached to the leaf section were positioned on the surface of the MSR medium and cultivated in a vertical orientation for 3 days. The observed roots were separated into slow- and fast-growing populations and transferred to fresh MSR plates for an additional 4 days of cultivation. At this point, the final root length of both populations was recorded, and 1-cm tip sections of the roots were excised along with the underlining block of medium. Blocks were allowed to air-dry overnight at 25°C, and the resulting gel film with root sections was stained for the SEAP activity as described below. For data presented in Table 1, total soluble protein was extracted from the individual root clones together with the supporting agar block by grinding in PBS buffer and subsequent centrifugation. Determination of protein quantity was done spectrophotometrically according to Bradford (1976), and the quantification of SEAP was performed as described below.

Plant regeneration and SEAP secretion

In all experiments where plant regeneration was performed, fast-growing root clones were cut into 1-cm-long sections and transferred to MSRR medium (MSR supplemented with 2 mg/l benzylaminopurine). Seven weeks after the initial infection, independent regenerated shoots were excised and cultivated on MSR medium. SEAP-transformed shoots with enhanced rooting response were transferred to the greenhouse under hydroponic conditions after 3 weeks, and SEAP secretion was measured as described below during a 24-h period in 100 ml of 1 mM CaCl₂ solution supplied to the plant roots.

GUS detection and SEAP quantification

In all experiments, GUS activity was visualized according to the published procedure of Jefferson et al. (1987). Detection of SEAP activity in plant roots (Fig. 5A, B), on the nitrocellulose membrane (Fig. 5C), and on the surface of the dried agar blocks (Fig. 5D, E) was performed by incubating the samples overnight at 37°C in a thin layer of BCIP-phosphatase substrate (Alkaline Phosphatase Isoenzymes assay; Sigma). Quantification of secreted SEAP in the hydroponic medium was performed using a chemiluminescence assay (Clontech, Palo Alto, Calif.) as described by Borisjuk et al. (1999). In order to minimize the background enzymatic activity associated with the presence of native phosphatases, all samples were treated at 65°C for 30 min prior to visualization and quantification of the SEAP activity. GUS and SEAP visualization was recorded using an Eclipse TE200 inverted microscope (Nikon, Tokyo) or Camedia C3030 camera (Olympus, Tokyo).

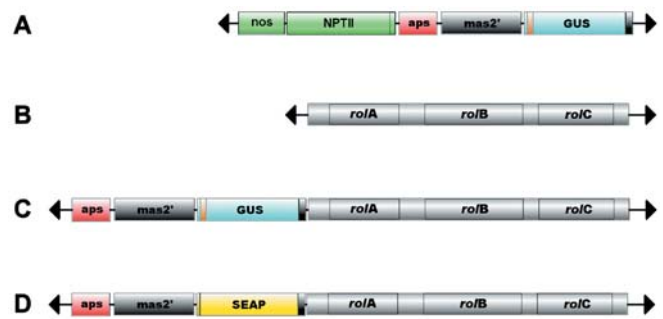


Fig. 1A–D Schematic representation of transformation vectors used in this study. **A** pBin-mas-GUS. *nos*-*NPTII* kanamycin selection marker, *mas2'* mannopine synthase promoter, *aps* amplification-promoting sequence. **B** pRYG. *rolA*, *rolB*, *rolC* are the cluster of *rol* genes responsible for root induction. **C** pRYG(GUS). The GUS gene is interrupted with an intron sequence shown as an orange-shaded box. **D** pRYG(SEAP). The yellow-shaded box depicts the SEAP original secretion signal peptide. Solid black triangles denote the location of the left and right T-DNA borders, respectively

Results and discussion

A. *rhizogenes*-based binary transformation system

The original pBin19 vector carries the neomycin phosphotransferase II (*nptII*) gene under the control of the nopaline synthase (*nos*) promoter and *nos* terminator. For this particular study, a GUS expression cassette was cloned downstream of the *nptII* gene. The resulting vector (pBin-mas-GUS) was electroporated into *A. rhizogenes* strain A4, thus creating a binary transformation system (Fig. 1A). This system contained two independent T-DNA regions—the original *A. rhizogenes* T-DNA harboring the set of *rol* genes, and a binary T-DNA region of the pBin-mas-GUS vector. Upon successful inoculation, either T-DNA region (or both) can be transferred into the plant genome. Since induction of the hairy roots expressing GUS can be observed only as a result of the co-transformation event, this system can be particularly useful for the evaluation of the efficiency of a binary transformation system when no selection agent is applied.

If no exogenous auxins were supplied to the medium, only cells transformed with the *A. rhizogenes* T-DNA developed into auxin-independent root clones 12 days after inoculation. These roots displayed a classic hairy root proliferation response (Fig. 2A)—i.e., a set of phenotypic characteristics typical of the roots expressing *rol* genes (Schmullig et al. 1988). A second T-DNA originating from the pBin-mas-GUS transformation vector and harboring the GUS expression cassette was rarely co-transformed. The efficiency of co-delivery in the absence of selective pressure was low; approximately 5% of induced hairy roots expressed GUS at the level of visual detection (Fig. 2B). Similar co-transformation frequencies (4–7%) were observed in the hairy roots induced on aseptically tobacco leaf sections *in vitro* (data not shown). Although kanamycin selection, not easily appli-

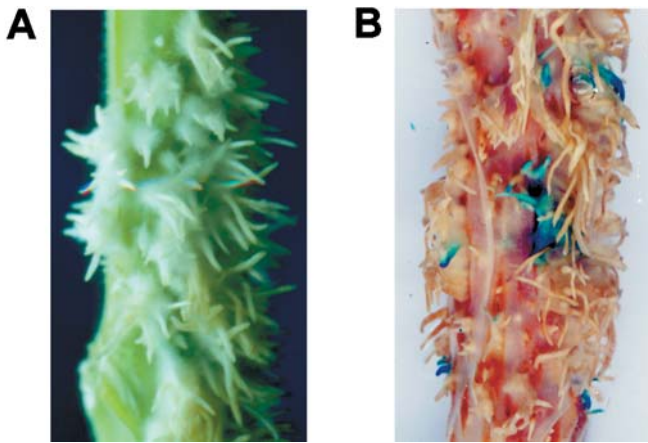


Fig. 2A, B Hairy roots induced on a tobacco stem in vivo using an *Agrobacterium rhizogenes* binary transformation system. **A** A 3-month-old tobacco stem developing a typical hairy root proliferation response 12 days after inoculation with an *A. rhizogenes* culture harboring the pBin-mas-GUS vector. **B** Less than 5% of newly developed roots showed visible GUS staining 15 days after inoculation. No exogenous hormones were added at the time of inoculation, and no kanamycin selection was applied

cable to tobacco stems, would have potentially eliminated the hairy roots that did not carry both *nptII* and GUS transgenes, a significant reduction in the total number of hairy roots would also follow. While not critical for tobacco transformation, this may become a major limitation step in the transformation and regeneration of many recalcitrant species. In addition, the stable introduction of antibiotic resistance genes into the plant genome is cumbersome and increases the risk of horizontal gene transfer (Kay et al. 2002).

Efficiency of *rol* genes as selection markers

Vilaine and Casse-Delbart (1987) and Schumling et al. (1988) reported that a cluster of three *rolA*, *rolB*, and *rolC* genes (*rolABC*) within the 4.3-kb region of the Ri plasmid is sufficient to induce a typical root proliferation response on *A. rhizogenes*-infected plants in vitro when no exogenous auxins are supplied to the cultivation medium. These observations provided the foundation for investigating the use of the *rolABC* cluster as a phenotypic marker for antibiotic resistance-free transformation. We hypothesized that if the *rolABC* cluster is linked to the gene of interest on the same transformation vector, it may become possible to develop a generic, single-step transformation system for the rapid expression and secretion of multiple protein targets. Unlike Cui et al. (2001), we decided to avoid using the *rolD* gene as a part of the selective marker. Earlier, Constantino et al. (1994) reported that *rolD* may encode a transportable factor that induces early flowering in tobacco. Therefore, the presence of *rolD* significantly shortens the life-time potential of a transgenic plant (Mauro et al. 1996) and may become

an obstacle to a long-time secretion of recombinant proteins from plant roots.

The *rolABC* cluster was cloned into the pGreen plant transformation vector (Hellens et al. 2000) to generate a basic backbone vector, pRYG (**R**oot **Y**our **G**ene, Fig. 1B). Using the standard *A. tumefaciens*-mediated transformation protocol, we investigated the ability of this vector to induce hairy roots on the aseptic leaf sections of tobacco plants in vitro (Fig. 3A, B) or on stems of greenhouse-grown plants in vivo (Fig. 3C, D). No exogenous auxins were added throughout the time course of the experiment. Under these conditions no new roots developed on the control leaf sections (Fig. 3A, top) nor on the plant stems (Fig. 3D, top) mock-inoculated with the *A. tumefaciens* culture harboring the pGreen vector alone. To the contrary, plant tissues infected with *A. tumefaciens* containing pRYG showed a remarkable proliferation of roots both in vitro and in vivo 12 days after inoculation (Fig. 3A, D). In all experiments, the development of new roots was observed at the inoculation area only, with the majority of roots emerging from vascular tissue, including the major and secondary veins of the leaf sections. Since the *rolABC* cluster does not participate in auxin biosynthesis directly, but rather increases the sensitivity of plant tissues to the endogenous auxin levels (Cardarelli et al. 1987), a simple hormone-free MS medium was sufficient to select for a transformation event. Considering that no auxin-synthesizing genes are transferred into the plant genome, these newly developed roots should require an external auxin source to support their growth in vitro once the depot of in situ auxin is depleted. To maintain the optimum growth of the transformed roots generated on the leaf sections, we routinely added indole-3-acetic acid to the cultivation medium after the hairy roots were established—usually 3 weeks post-inoculation (Fig. 3B).

Expression of GUS in tobacco hairy roots

In an attempt to estimate the efficiency of the pRYG transformation vector for target protein expression in hairy roots, we cloned GUS under the control of the root-specific *mas2'* promoter (Leung et al. 1991) next to the *rolABC* cluster to produce pRYG(GUS). The *aps* element was subcloned in direct orientation immediately upstream of the *mas2'* promoter region (Fig. 1C). This quantitative enhancer-like element positively influences both the transcription and copy number of nearby genes in either orientation (Borisjuk et al. 2000), putatively decreasing the positional effect often observed after transformation. When introduced into the tobacco genome via *A. tumefaciens*-mediated transformation, pRYG(GUS) induced the hairy root proliferation response similar to that observed with pRYG alone (Fig. 4). The variation in the inoculation technique, physiological status of the inoculated tissue, and/or other environmental conditions might have accounted for a significantly higher number of roots induced by the same amount of inoculum on the aseptic leaf sections.

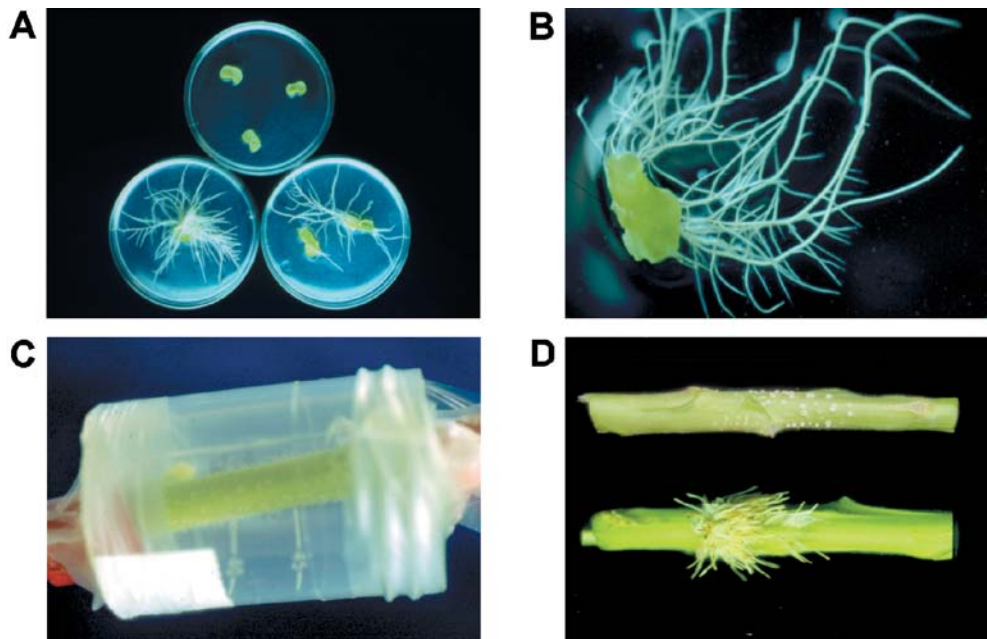
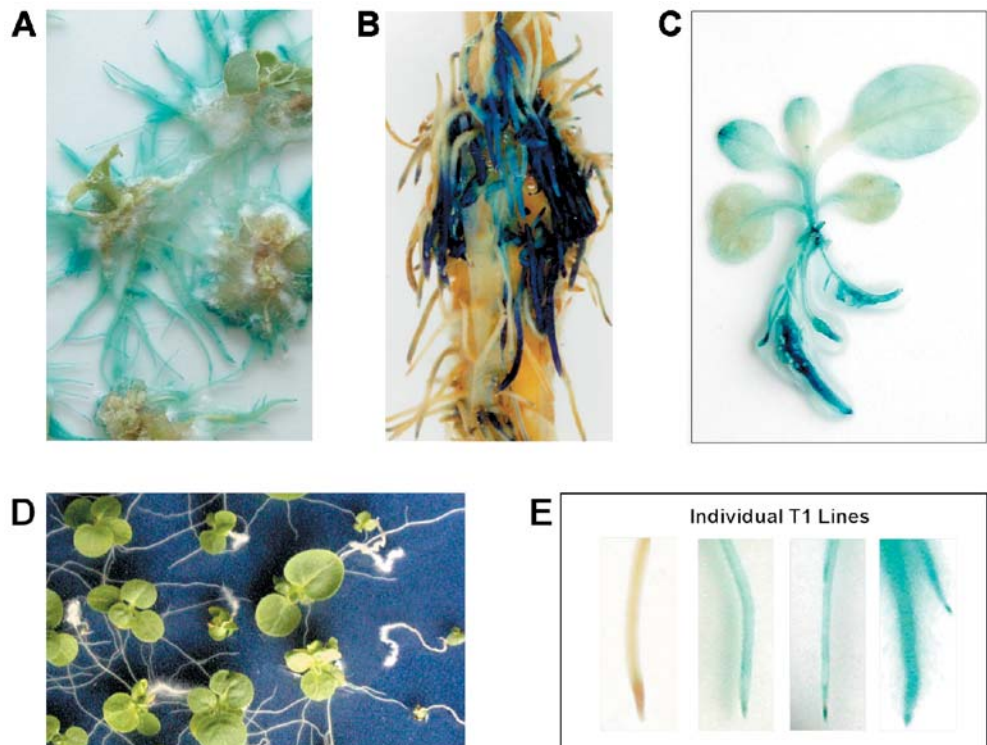


Fig. 3A–D Induction of hairy root proliferation response by *A. tumefaciens* culture harboring pRYG. **A** Aseptic leaf sections developed hairy roots 12 days after inoculation in vitro on the hormone-free MSR medium. Leaf infection was performed using a sterile surgical blade immersed into a 48-h culture of *A. tumefaciens* harboring pGreen alone (negative control, *top*), wild-type *A. rhizogenes* (positive control, *bottom left*), and *A. tumefaciens* harboring pRYG (*bottom right*). **B** pRYG-induced roots retained

the ability to grow in the hormone-free liquid medium 2 weeks after emergence. **C** Plastic tube system used to seal off the inoculation area to maintain the high humidity conditions required for root formation on tobacco stems in the greenhouse. **D** pRYG-based induction caused root proliferation response within 2 weeks post-inoculation (*bottom*); no roots appeared after pGreen-based mock inoculation (*top*)

Fig. 4A–E Expression of GUS in tobacco hairy roots. **A** Uniform GUS expression in the 15-day-old hairy roots induced with pRYG(GUS) on aseptic tobacco leaf sections in vitro. **B** A representative portion of an in vivo-inoculated tobacco stem. The pRYG(GUS) vector induced hairy roots showing significantly higher variation in GUS staining among individual root clones. **C** Juvenile transgenic plant regenerated from a single pRYG(GUS) root clone preferentially expressed GUS in the root tissue. **D, E** Inheritance of the hairy root phenotype (**D**) and GUS gene (**E**) within the population of individual seedlings of the sexually produced T₁ generation of the original transgenic line



GUS activity was visually detected as early as 1–5 days after the initial root appearance in 94% (144/150) and 79% (198/250) of newly developed roots that were induced in vitro (Fig. 4A) and in vivo (Fig. 4B), respectively. Bacterial expression cannot explain the observed GUS activity, since the GUS gene sequence was interrupted with an intron that can be correctly spliced out only in the eukaryotic expression system. Interestingly, while the roots that formed on the leaf sections showed very uniform GUS activity, those that formed on the stems exhibited much more variable spatial patterns and a greater magnitude of GUS expression. (Fig. 4A, B). The former can be at least partially explained by the *aps* effect, which in tobacco enhances transcription and reduces the positional effect (Borisjuk et al. 2000) in the leaf sections but not in the stems. The lack of the 100% transformation/ expression ratio in stem-produced hairy roots could possibly be explained by the positional effect, which is not circumvented by the *aps* effect, or by gene silencing and integration errors more pronounced in the in situ stem tissues.

Seven to eight weeks after inoculation, we obtained multiple regenerated shoots from 81% (71/88) of the root fragments transferred to the regeneration medium. Most of the regenerated shoots showed some degree of Ri symptoms, including the over-proliferation of roots and root hairs. From these, 8% (6/71) of the regenerated plants exhibited very few phenotypic abnormalities in the leaves and flowers and, when grown to maturity, they produced abundant seeds. In most of the regenerants, the strong GUS expression was observed in the roots and localized stem areas, as is typically expected from the *mas2'* promoter (Fig. 4C). Although the *mas2'* promoter is preferentially expressed in roots and wounded leaves (Teeri et al. 1989), a gradual downwards increase in expression beginning from the shoot apex down to the base was observed in the stems, leaves, and petioles (Langridge et al. 1989).

In order to study transgene expression in the next generation, the seeds of the above transgenic plants were surface-sterilized and germinated in vitro. A typical population of 14-day-old seedlings is shown in Fig. 4D. Transgenic seedlings were easily distinguished by their modified hairy root systems, as expected if the activity of the *rol* genes is retained in the next generation. Nearly all of the seedlings (94/121) with hairy roots tested positive for GUS expression, while the seedlings that developed a normal root system failed to show any GUS activity (Fig. 4E).

Extracellular targeting and secretion system based on pRYG

The ability to rapidly introduce a transgene into differentiated plant organs, combined with strong and balanced expression that does not require antibiotic selection, provides a valuable tool for fundamental and applied plant molecular biology. A simple, single-step transfor-

mation system that achieves high transformation frequencies may significantly shorten the gene-to-protein time and make multiple protein evaluations manageable. For example, such a transformation strategy may improve the efficiency of the rhizosecretion-based protein production system (Borisjuk et al. 1999) in terms of speed, multiple target validation, and yield potential. As with many other production systems, including the periplasmic space of *Escherichia coli* (Veiga et al. 1999), the growth medium of mammalian cell cultures (Fouser et al. 1992), or the mammary glands of transgenic animals (Simons et al. 1987), rhizosecretion relies on extracellular targeting and secretion to achieve the highest possible yields.

To test the applicability of the pRYG-based transformation system to rhizosecretion, we cloned the human secreted alkaline phosphatase (SEAP) gene with its secretion signal peptide into pRYG under the control of the same genetic elements as employed for GUS, thereby creating a pRYG(SEAP) transformation vector (Fig. 1D). We used this vector to inoculate aseptically tobacco leaf sections in vitro; no exogenous auxins were added throughout the time course of the experiment. As expected, transgenic roots developed from the inoculation area within 9–12 days, and expression of the SEAP gene was observed at the time of root development. When the emerging root tips were excised from the origin points, heat treated to eliminate the endogenous phosphatase activity, and incubated in the presence of an alkaline phosphatase substrate, the transformed root tissue developed a characteristic blue stain resulting from a substrate breakdown by heat-stable recombinant SEAP (Fig. 5B). The control roots transformed with the pRYG vector alone showed no SEAP activity (Fig. 5A). To further confirm the expression of SEAP, several leaf sections carrying the pRYG(SEAP) roots were transferred to liquid medium and cultivated for 7 days. A portion of the fast-growing root was cut and placed on top of a PVDF membrane with slight pressure applied to ensure complete contact of the root surface with the membrane in a process similar to tissue printing. After the printed root tissue was removed, the membrane was heat-treated and stained for SEAP activity. As expected, SEAP activity was easily detected on the outside surface of the root (Fig. 5C).

Hairy roots generated on the tobacco leaf sections exhibited different rates of growth when kept on the surface of the MSR medium in a vertical orientation. Since root growth-promoting *rol* genes and the SEAP gene were located on the same transformation vector, we wanted to determine whether the rate of root growth could be used for positive visual selection of those root clones with higher levels of transgene expression. Hairy roots from the same transformation experiment were divided into slow- and fast-growing populations based on their elongation rates (Table 1). Slow-growing roots elongated on average 2.4-fold slower than their fast-growing counterparts, and produced four- to five-fold less SEAP.

Both numbers were significant (*t*-test, $P < 0.05$). Some roots developing after the inoculation may have had partial or complete silencing of the entire transgene locus

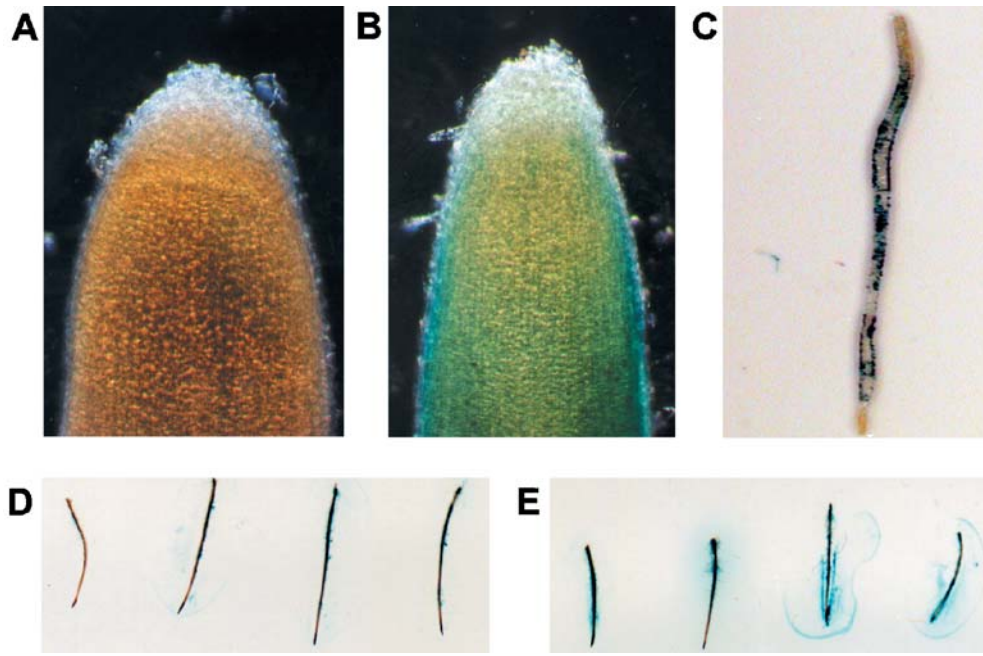


Fig. 5A–E SEAP production and secretion from the pRYG(SEAP)-transformed hairy roots. **A, B** Light microscopy ($\times 100$) of the emerging hairy roots induced by pRYG (**A**) and pRYG(SEAP) (**B**). SEAP activity was visualized as a blue staining of the root tissues producing the recombinant protein. **C** SEAP activity detected on the surface of the 10-day-old pRYG(SEAP) root clone cultivated in the liquid medium ($\times 10$); root tissue was printed onto the PVDF membrane with slight pressure applied and removed prior to

staining. **D, E** Note the visual difference in SEAP secretion from slow- (**D**) and fast-growing (**E**) populations of hairy roots cultivated on the surface of hormone-free MSR medium in a vertical orientation. All stained samples were pre-incubated at 65°C for 30 min to remove background phosphatase activity and then incubated overnight at 37°C in a thin layer of BCIP-phosphatase substrate

due to chromatin confirmation and/or co-suppression and thus did not grow efficiently on the hormone-free medium. Therefore, the slow-growing roots produced much less transgenic protein, and thus can be effectively eliminated after simple visual selection. To further confirm this hypothesis through the visual observations, we excised the tip portions of the roots together with the supporting block of agar medium and stained them for the secreted SEAP. As expected, the fast-growing roots developed a stronger blue-colored zone typical of the greater SEAP enzymatic activity released into the medium (Fig. 5D, E).

The mature plants from independent transgenic lines regenerated from different roots selected within the fast-growing population still showed some variations in SEAP production. Some shoots regenerated spontaneously from the green regions of hairy roots grown on the surface of the MSR medium; other shoots were regenerated by transferring segments of the fast-growing roots onto the regeneration medium after 4 weeks in culture. Fully transgenic regenerants retained the ability to secrete SEAP into hydroponic culture medium. Eighty-seven percent (26/30) of the selected regenerants secreted per day $6\text{--}28\ \mu\text{g/g}$ root DW of biologically active SEAP from their roots (Fig. 6).

Marker-free transformation has been successfully demonstrated by using several plant and non-plant genes that are capable of promoting organ differentiation or

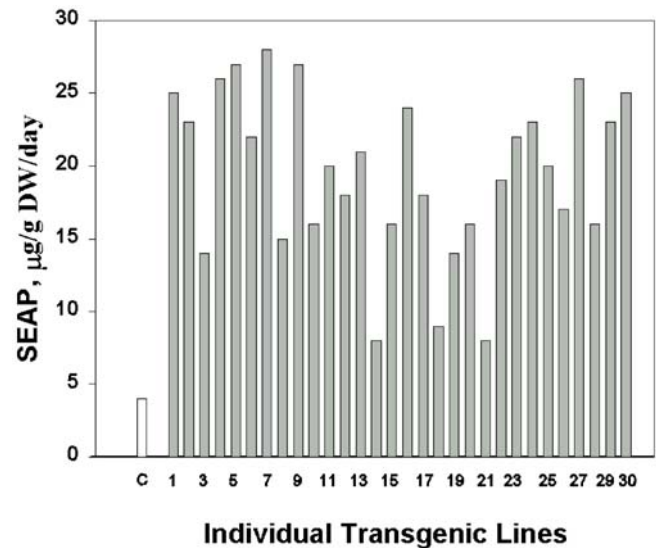


Fig. 6 Quantification of SEAP secretion from the roots of 30 individual transgenic lines (gray columns) regenerated from a fast-growing population of pRYG(SEAP) root clones compared with a control exudate from the wild-type plant (white column). Recombinant SEAP secretion is quantified as micrograms of secreted protein per gram root dry weight per day. Each bar represents the mean values of three replicates. The experiment was repeated with similar results

explant regeneration (Zuo et al. 2002). However, a number of morphological aberrations resulting from the activity of some of these genes (i.e., *rol* cluster) partially limits the use of this strategy as a plant transformation tool aiming at expressing a target protein throughout an entire plant (Cui et al. 2001). On the contrary, the pRYG-based system presented here has not been developed as a merely transformation strategy. Using *rol* genes as a phenotypic selection marker provides an additional advantage of generating a higher ratio of young, metabolically active root tissue with multiple adventitious root growth (Baiza et al. 1998), resulting in higher rates of protein expression. Since a large number of transformed roots can be induced rapidly and every hairy root emerges as the result of an independent transformation event (Tepfer 1984), the described system may provide new means for simultaneous analysis of multiple protein targets and/or cDNA libraries on a large scale. This system also enables the simultaneous subcloning and expression of more than one target gene—for example, a heavy and a light chain of an antibody (Komarnytsky et al. unpublished).

Conclusions

The utilization of the pRYG vector harboring three *rol* genes from *A. rhizogenes* is a fast and effective method for the generation of transgenic hairy roots without the introduction of antibiotic resistance. The system utilizes phenotypic selection in the absence of exogenously supplied hormones and can be visually evaluated for the relative strength of transgene expression. The pRYG-based transformation system can also be used for the rhizosecretion of heterologous proteins, as has been shown for SEAP. Root clones with the highest secretion of the heterologous protein can be readily regenerated into transgenic lines that retain the ability to secrete the target protein over their lifetime. In addition, this approach allows the production of large numbers of independently transformed roots within a small in vitro system or on intact plants. It is likely that future careful selection of genetic elements that regulate target protein expression and secretion will further increase the speed and efficiency of the pRYG-based transformation system. Root clones expressing individual transgenes may be easily propagated as hairy root cultures or regenerated into mature plants, adding to the versatility of the system.

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