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Original Paper

The "all-in-one" *rol*-type binary vectors as a tool for functional genomic studies using hairy roots

Hikaru Seki[1,2], Kiyoshi Ohyama[2], Tomoko Nishizawa[1], Shigeo Yoshida[2] and Toshiya Muranaka[1,2,*]

[1] Kihara Institute for Biological Research, Yokohama City University, Totsuka-ku, Yokohama, Kanagawa 244-0813, Japan; [2] RIKEN, Plant Science Center, Yokohama, Kanagawa 230-0045, Japan
* E-mail: muranaka@yokohama-cu.ac.jp Tel: +81-45-820-2445 Fax: +81-45-820-1901

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Abstract The hairy root culture system has several desirable features as an experimental model for functional analyses of the genes involved in root metabolism and/or physiology. We developed a binary vector set for efficient target gene overexpression and RNA interference (RNAi) in transformed (hairy) roots. The vectors, pHR-OX and pHR-RNAi, contain a cluster of *rol* (rooting locus) genes, together with the single GATEWAY™ conversion cassette (in pHR-OX vectors) or inverted repeats of the GATEWAY™ conversion cassette separated by an intron sequence (in pHR-RNAi vectors), flanked by a dual CaMV 35S promoter and nopaline synthase polyadenylation signal, on the same T-DNA. Transformation experiments with pHR-OX vectors using *Arabidopsis*, potato, and tobacco as model plants revealed that by inoculating *Agrobacterium tumefaciens* harboring these vectors, a large number of independently transformed roots could be induced from explants in a short period of time, and subsequent establishment of root culture lines was possible. A model experiment that focused on the sterol biosynthetic pathway in *Arabidopsis* validated the utility of pHR-RNAi vectors for gene functional analysis in cultured roots. Use of our vector system may facilitate identification of regulatory or biosynthetic genes for the production of valuable secondary metabolites in plant roots.

Key words: *Agrobacterium*, hairy root culture, *rol* genes, sterol methyltransferase, binary vector.

Hairy root cultures have several desirable features, including rapid growth and maintenance under tightly controlled sterile conditions, which make them suitable as an experimental model for functional analyses of the genes involved in root metabolism using a metabolic profiling approach. In contrast to cell suspension cultures, hairy root cultures generally have the ability to synthesize the same compounds as the roots of the intact plant (Shanks and Morgan 1999; Uozumi 2004); therefore, they offer attractive material to investigate the biosynthesis of a variety of secondary metabolites, including compounds of pharmaceutical value (for review, see Shanks and Morgan 1999).

Hairy root formation is induced as a consequence of the transfer of T-DNA from the root-inducing (Ri) plasmid of *Agrobacterium rhizogenes* to the host plant genome (Nillson and Olsson 1997). Like the widely used *A. tumefaciens*, *A. rhizogenes* can also transfer the T-DNA of binary vector plasmids in trans, thereby enabling the production of "transgenic" hairy roots containing other foreign genes carried on a binary vector (Simpson et al. 1986). However, *A. rhizogenes*-mediated delivery of binary T-DNA has several disadvantages when applied to gene functional analyses at larger scales: 1) the frequency of co-delivery of Ri plasmid-derived and binary vector-derived T-DNA is often very low without antibiotic selection pressure, but antibiotic selection media lead to a significant reduction in the total number of hairy roots (Komarnytsky et al. 1999, and references therein); 2) apart from the well studied *rol* genes (*rol A*, *rol B*, and *rol C*) that are particularly important in the induction of hairy roots, at least 16 more open-reading frames (ORFs), whose functions are not yet well characterized, are present on the Ri T-DNA (TL-DNA in the agropine-type Ri plasmid; Slightom et al. 1986), and may increase the bias from normal root metabolism and/or physiology; and 3) the Ri plasmids of the *A. rhizogenes* agropine-type strains, which are most widely used, contain two independently transferable T-DNAs, TL-DNA and TR-DNA, which are not always transferred together into the plant genome would thus increase inter-line genetic variation (Batra et al. 2004). To avoid the need to perform less efficient co-delivery of several different T-DNAs, and to minimize bias from normal root metabolism/physiology and inter-line genetic/biochemical variation, we used the cluster of *rol* genes

Abbreviations: HR, hairy root; RNAi, RNA interference; *rol*, rooting locus; SMTs, sterol-C24-methyltransferases.
This article can be found at http://www.jspcmb.jp/

packaged in a binary vector T-DNA, instead of the original Ri plasmid, for hairy root induction. Here, we show that *rol*-type binary vectors can be used to generate large numbers of transformed roots from several different types of explant tissue.

We also developed an extremely simple protocol using the widely used "floral dip" transformation protocol (Clough and Bent 1998), which works in the *rol*-type vectors to generate *Arabidopsis* hairy root culture lines. The potential use of our new vector system for functional genomic analysis of root biology is discussed.

Materials and Methods

Binary vector construction

Standard gene cloning methods (Sambrook et al. 1989) were used to make the vector constructs. *Pfu*-turbo DNA polymerase was used in PCRs according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The vectors were constructed with pBCR35 (Seki et al. unpublished) as a vector backbone. pBCR35 is a derivative of pBI121 (Clontech, Palo Alto, CA, USA), in which the *Asc*I site is added into the *Hin*dIII site upstream of the CaMV 35S promoter, and the *uidA* gene (GUS) is removed (*Sma*I/*Sac*I digestion) and replaced with the *Kpn*I site linker. A fragment containing the CaMV 35S promoter with a duplicate enhancer region (P35SS) was amplified from pAM-PAT-GW with the primers 5′- CGGTTTGGGCGCGCC-CGGCCTAGGTCTCAGAAGA-3′ and 5′-CGATCTAGACGA-CCTCGAGGTCCTCTCCAAATG-3′. The underlined sequences in each of the two primers correspond to the restriction enzyme sites for cloning purposes. A PCR fragment was digested with *Asc*I/*Xba*I and cloned into *Asc*I/*Xba*I-digested pBCR35, resulting in pBCR35.2. The fragment containing the single GATEWAY™ conversion cassette was excised from pAM-PAT-GW (a kind gift from Dr. Bekir Ülker, Max Planck Institute for Plant Breeding, Cologne, Germany) as the *Xho*I/*Spe*I fragment and cloned into *Xho*I/*Xba*I-digested (*Xba*I and *Spe*I are compatible) pBCR35.2, to obtain the intermediate construct, pBCR-79. Similarly, a fragment containing inverted repeats of the GATEWAY™ conversion cassette separated by a 250-bp intron sequence was excised from pJawohl17-RNAi (a kind gift from Dr. Bekir Ülker) as an *Xho*I/*Spe*I fragment and cloned into *Xho*I/*Xba*I-digested pBCR35.2, to obtain the intermediate construct pBCR-80. The 7.6-kb fragment containing the *rol* gene cluster was excised from the pHR-AT vector (Seki et al. 2005) as the *Asc*I/*Avr*II fragment and cloned into *Asc*I/*Avr*II-digested pBCR-79 and pBCR-80, resulting in pHR-OX and pHR-RNAi, respectively. A 9.0-kb fragment containing the *sGFP* (S65T) expression cassette (P35S-*GFP*) and the *rol* gene cluster together was excised from the pHR-AT-GFP vector (Seki et al. 2005) as an *Asc*I/*Avr*II fragment and cloned into *Asc*I/*Avr*II-digested pBCR-79 and pBCR-80, resulting in pHR-OX(*gfp*) and pHR-RNAi(*gfp*), respectively. The GATEWAY™-compatible vectors were maintained in *E. coli* strain DB3.1 (Invitrogen, Carlsbad, CA, USA) in which the *ccdB* gene is not lethal.

Generating entry and expression clones

To create pHR-*GUS* and pHR-*GUS*(*gfp*), the entire coding region of the *GUS* gene was amplified from pBI121 with the primers 5′-CACCATGTTACGTCCTGTAGAAACC-3′ and 5′-TCATTGTTTGCCTCCCTGCTGC-3′, and cloned into pHR-OX and pHR-OX(*gfp*) via the pENTR™/D-TOPO® vector (Invitrogen) using the Gateway LR Clonase Enzyme Mix (Invitrogen).

To create the negative control vector, the pHR-emp(*gfp*) pENTR™1A vector was digested with *Eco*RI and subsequently self-ligated to remove the *ccdB* gene. The resulting entry clone was used for the LR recombination reaction for pHR-OX(*gfp*).

For RNAi constructs, an 800-bp fragment within the coding sequence of each of the *SMT2* (*At1g20330*) and *SMT3* (*At1g76090*) genes was RT-PCR-amplified with the primers 5′-ACCCTCGAGTACGAGTGGGGATGGGGACAATC-3′ and 5′-GCTGGATCCCAGAGAATCATATGCATCGGAG-3′. The underlined sequences in each of the primers correspond to the restriction enzyme sites for cloning purposes. The amplified region included 253–1052 bp of the *SMT2* and *SMT3* cDNAs. These numbers indicate the nucleotide positions from the translation initiation site. PCR products were digested with *Xho*I/*Bam*HI and cloned into the *Xho*I/*Bam*HI-digested pENTR™1A vector (Invitrogen). The resulting entry clones were linearized with the appropriate restriction enzyme and used for LR recombination reactions for pHR-RNAi(*gfp*).

Plant transformation and establishment of root cultures

The binary vector construct was introduced into *A. tumefaciens* strain GV3101 (pMP90) by chemically-based transformation (An et al. 1988). In all transformation experiments described here, half-strength (0.5×) Murashige and Skoog (MS; Murashige and Skoog 1962) medium was used as the basal medium. Transformation of *Arabidopsis* (ecotype Wassilewskija) hypocotyl-derived calli was essentially performed as described by Akama et al. (1992), with minor modifications. Transformation of *Arabidopsis* (ecotype Columbia) using the floral dip method was performed as described by Clough and Bent (1998). Seeds collected from dipped plants were selected on agar medium containing kanamycin (50 mg l^{-1}). A root cluster was separated from a seedling and transferred to liquid medium, or the seedling was transplanted to soil and allowed to continue growing.

Transformation and establishment of root cultures of potato (*Solanum tuberosum* L. cv. May Queen) stem segments and tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) leaf explants (15×15 mm) were essentially performed as described by Seki et al. (2005). After co-cultivation, explants were transferred to agar medium supplemented with 200 mg l^{-1} cefotaxime to eliminate bacteria. Potato stem segments were maintained at 23°C under a 16/8 h (day/night) photoperiod. Induced *GFP*-expressing roots were excised and transferred to agar medium supplemented with 200 mg l^{-1} cefotaxime. Tobacco leaf explants were maintained at 26°C in the dark. Putative transformed roots were excised and transferred onto the selection medium supplemented with kanamycin (50 mg l^{-1}) and cefotaxime (200 mg l^{-1}). After several subcultures

at 2-week intervals, isolated roots were transferred to liquid medium. Liquid cultures were maintained at 26°C on a rotary shaker (100 rpm) under a 16/8 h (day/night) photoperiod.

Northern blot analysis

Total RNAs were prepared from root tissues cultured for at least 3 months in hormone-free 0.5× MS liquid medium. Northern blot analysis was performed as described (Seki et al. 2005). To obtain gene-specific probes, a cDNA fragment was amplified by RT-PCR, and products were cloned into the pCR II-TOPO cloning vector (Invitrogen). The primers used for RT-PCR were, for *rolB*; 5′-ATGGCACTGAACTTGCCGTT-3′ and 5′-TCAAGTCGCCGAGGTTTCTTTC-3′, for *rol*C; 5′-ATGGCGGAATTTGACCTATG-3′ and 5′-TGGGCACCGTCAGAACAGCT-3′, and for *SMT2* and *SMT3*; 5′-TACGAGTGGGGATGGGGACAATC-3′ and 5′-CAGAGAATCATATGCATCGGAG-3′.

Sterol analysis

Root tissues were sampled at the same time as for Northern analyses. Sterol analyses were performed as described (Suzuki et al. 2004; Ohyama et al. 2007).

Results

Design of vectors

The vectors described here were designed as an "*all-in-one*" construct with: 1) the *rol* gene cluster for induction of transformed roots from explant tissues; 2) the kanamycin resistance gene (*nptII*) for antibiotic selection of the transformed roots; 3) the single GATEWAY™ conversion cassette [in pHR-OX and pHR-OX(*gfp*) vectors] or inverted repeats of the GATEWAY™ conversion cassette separated by a 250-bp intron sequence [in pHR-RNAi and pHR-RNAi(*gfp*) vectors], flanked by a dual cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase polyadenylation signal, to facilitate the rapid construction of target gene overexpression or RNAi constructs, respectively; and 4) the green fluorescent protein (GFP) expression cassette was also included in pHR-OX(*gfp*) and pHR-RNAi(*gfp*) as a visual and non-invasive selectable marker for successful transformation within a single T-DNA fragment (Figure 1).

Hairy roots result from the integration of genes located on the Ri plasmid of *A. rhizogenes* into the host genome. The synergistic activities of the *rolA*, *rolB*, and *rol*C gene products are particularly important in the induction and development of these transformed roots (Schmulling et al. 1988; Spena et al. 1987). The 7.6-kb DNA fragment harboring the *rol* gene cluster was packaged in a binary vector T-DNA. This DNA fragment contains, in addition to the *rol* genes (*rolA*, *B*, *C*), two ORFs, ORF13 and ORF13a. ORF13 promotes *rol*-mediated rooting in tobacco (Aoki and Syono 1999), while the function of ORF13a in root induction remains unknown.

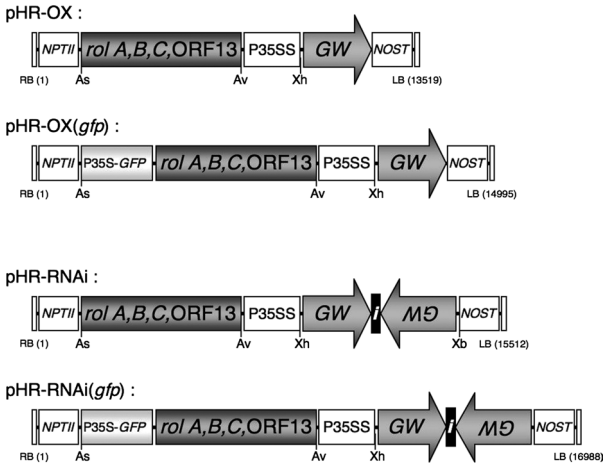

Figure 1. Vector structure. In this figure, only the region between the right and left borders is shown (not to scale). *NPTII*, neomycin phosphotransferase II gene; P35S-*GFP*, *sGFP*(S65T) coding sequence flanked by the CaMV 35S promoter and nopaline synthase polyadenylation signal; *rol A*, *B*, *C*, ORF13, 7.6-kb fragment derived from pRi1724 in *A. rhizogenes* strain MAFF03-01724 (Tanaka et al. 1994); P35SS, CaMV 35S promoter with duplicated enhancer region; *GW*, GATEWAY™ conversion cassette (*attR1*, Cm^R, *ccdB*, *attR2* orientation); *i*, a 250-bp intron sequence derived from the *Arabidopsis WRKY33* gene (*At2g38470*); *NOST*, nopaline synthase polyadenylation signal; RB and LB, T-DNA right and left border, respectively. Numbers in parentheses indicate the positions relative to the right T-DNA border. Several of the unique sites on T-DNA are shown: *Asc*I (As), *Avr*II (Av), *Xho*I (Xh), *Xba*I (Xb).

The vectors were constructed as GATEWAY™-compatible destination vectors to facilitate easy and reliable DNA cloning. Inverted repeats of the GATEWAY™ conversion cassette, separated by an intron, in the pHR-RNAi and pHR-RNAi(*gfp*) vectors enables the single target gene fragment flanked by the attL1 and attL2 sites in an entry clone to be recombined into it simultaneously to form the inverted repeats in a single-step LR clonase recombination reaction.

The expression cassette of the engineered *sGFP*(S65T) was included in pHR-OX(*gfp*) and pHR-RNAi(*gfp*) because the utility of *sGFP*(S65T) as a visual and non-invasive selectable marker has been demonstrated in a number of plant species (Niwa et al. 1999; Stewart 2001).

The binary vector T-DNA will be integrated into the plant genome via the usual *A. tumefaciens*-mediated transformation procedure for the induction of transformed roots and overexpression or RNAi silencing of the target gene therein.

Rapid and efficient induction of transformed roots

To evaluate the root-inducing ability of the vector T-DNAs, transformation experiments were performed using three different types of explant tissue. In all transformation experiments described here, hormone-

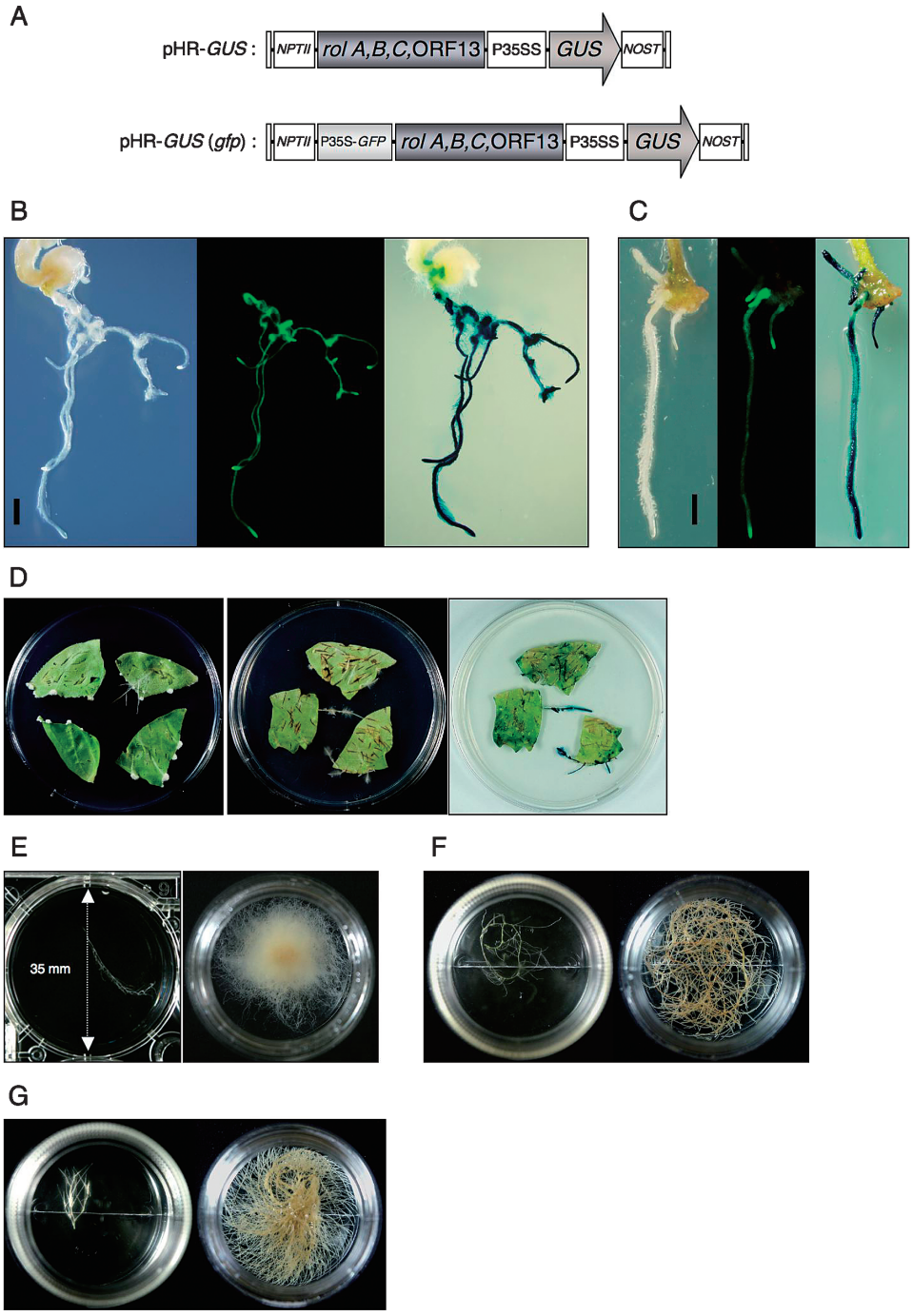

Figure 2. Induction and establishment of transformed root cultures. (A) Constructs pHR-*GUS* and pHR-*GUS*(*gfp*), used for transformation experiments. (B) *GFP*-expressing roots induced from *Arabidopsis* callus 4 weeks after inoculation with *A. tumefaciens* harboring pHR-*GUS*(*gfp*). Images under reflected light (left), under a fluorescence microscope (center), and after histochemical GUS staining (right). Scale bar=1 mm. (C) Induction of GFP-expressing roots from potato stem segments 3 weeks after inoculation with *A. tumefaciens* harboring pHR-*GUS*(*gfp*). Images under reflected light (left), under a fluorescence microscope (center), and after histochemical GUS staining (right). Scale ba=2 mm. (D) Induction of putative transformed roots from tobacco leaf explants. Tobacco leaf explants 18 days after mock inoculation (left) or after inoculation with *A. tumefaciens* harboring pHR-*GUS* (center) with its corresponding image after histochemical GUS staining (right). The Petri dish is 9 cm in diameter. (E) Liquid culture of pHR-*GUS*(*gfp*)-transformed *Arabidopsis* roots, photographed at the onset (left) and 4 weeks after the onset (right) of liquid culture in hormone-free 0.5× MS medium. The culture vessel (right) is 9 cm in diameter. (F) Liquid culture of pHR-*GUS*(*gfp*)-transformed potato roots. Photographed at the onset (left) and 2 weeks after the onset (right) of liquid culture in hormone-free 0.5× MS medium. The culture vessel is 9 cm in diameter. (G) Liquid culture of pHR-*GUS*(*gfp*)-transformed tobacco roots, photographed at the onset (left) and 4 weeks after the onset (right) of liquid culture in hormone-free 0.5× MS medium. The culture vessel is 9 cm in diameter.

free half-strength (0.5×) MS medium was used.

Arabidopsis hypocotyl-derived callus and potato stem segments were inoculated with *A. tumefaciens* harboring pHR-*GUS*(*gfp*) (Figure 2A). After 2 days of co-cultivation, explants were transferred to non-selective (without kanamycin) agar medium. After 2 to 3 weeks, GFP-expressing root(s) appeared on the surface of *Arabidopsis* calli and on the cut edge of potato stem segments (Figure 2B, C). Five weeks after inoculation, induction of GFP-expressing root(s) was observed in 56% (137/245) and 86% (83/96) of *Arabidopsis* and potato explants, respectively (Table 1). The average number of independent GFP-expressing roots per explant was 0.8 and 3.1 on *Arabidopsis* calli and potato stem segments, respectively (Table 1). Because no appreciable background fluorescence was observed in *Arabidopsis* and potato adventitious roots (non-transformed roots), pHR-*GUS*(*gfp*) transformed roots were readily identified with *GFP* fluorescence under non-destructive, sterile conditions. All GFP-expressing roots examined showed GUS staining, indicating the utility of GFP as a visual selection marker for target gene expression.

Tobacco leaf explants were inoculated with *A. tumefaciens* harboring pHR-*GUS* or pHR-*GUS*(*gfp*) (Figure 2A) to compare the root-inducing ability of these two vectors. After 2 days of co-cultivation, explants were subsequently cultured on non-selective agar medium. Within 2 weeks, putative transformed roots were induced at wound sites on pHR-*GUS*- or pHR-*GUS*(*gfp*)-inoculated leaf explants. Figure 2D shows the leaf explants 18 days after inoculation with pHR-*GUS* before (center) and after GUS staining (right), compared to the mock inoculation (left). Three weeks after inoculation, induction of putative transformed root(s) was observed on more than 60% of leaf explants for both pHR-*GUS* and pHR-*GUS*(*gfp*) (Table 2), with average numbers of roots per leaf segment of 2.0 and 1.9 with pHR-*GUS* and pHR-*GUS*(*gfp*), respectively (Table 2). There was no significant difference in root-inducing ability between these two vectors. Because GFP visual selection of transformed tobacco roots was difficult due to strong background fluorescence, even in non-transformed roots (adventitious roots), mock-inoculated leaf explants were prepared as a negative control for transformation. Leaf explants were dipped in 3% sucrose, instead of the bacterial suspension, and subsequently cultured on non-selective agar medium. Adventitious roots appeared on 28% (11/40) of mock-inoculated leaf explants within 3 weeks; however, the average number of roots per leaf segment was 0.4, suggesting that the majority of roots on the pHR-*GUS*- and pHR-*GUS*(*gfp*)-inoculated leaf explants had been induced by the transformation events. This was confirmed by the fact that 93% (96/103) of root clones from pHR-*GUS* and 83% (96/115) of root clones from pHR-*GUS*(*gfp*) inoculation were positive for GUS staining (Table 2). These results indicate that the vectors can be used to create a large number of transformed root clones in *Arabidopsis*, potato, tobacco, and probably in other plant species as well, using an extremely simple transformation protocol.

Establishment of liquid cultures

To propagate the transformed *Arabidopsis* and potato roots, *GFP*-expressing roots were excised from the explant tissues 4 to 5 weeks after inoculation, and transferred to fresh non-selective agar medium. After one or two passages on fresh agar medium at 2-week intervals, roots were transferred to liquid medium. Figure

Table 1. Frequency of transformed root induction on *Arabidopsis* hypocotyl-derived calli and potato stem segments

Explant tissues	No. of explants yielding GFP-expressing roots/No. of explants inoculated (%)	Total number of GFP-expressing roots	No. of GFP-expressing roots/explant
Arabidopsis hypocotyl-derived calli	137/245 (56)	189	0.8
Potato stem segments	83/96 (86)	297	3.1

Explants were inoculated with *A. tumefaciens* harboring the pHR-*GUS*(*gfp*). The number of GFP-expressing roots was counted 5 weeks after inoculation.

Table 2. Frequency of transformed root induction on tobacco leaf explants

Bacteria strain (plasmid)	No. of explants yielding roots/No. of explants inoculated (%)	Total number of roots	No. of roots/explant	GUS positive roots/ roots analyzed (%)
GV3101: pHR-*GUS*	35/56 (68)	113	2.0	96/103 (93)
GV3101: pHR-*GUS*(*gfp*)	37/60 (62)	116	1.9	96/115 (83)
Control	11/40 (28)	15	0.4	Not tested

Leaf explants were inoculated with *A. tumefaciens* harboring pHR-*GUS* or pHR-*GUS*(*gfp*). As a negative control, leaf explants were dipped in 3% sucrose solution instead of bacterial suspension. Inoculated leaf explants were cultured on hormone-free non-selective (without kanamycin) agar medium. The number of putative transformed roots was counted 3 weeks after inoculation. The numbers of GUS-positive roots were counted 24 h after staining.

2E shows transformed *Arabidopsis* roots at the onset (left) and 4 weeks after the onset (right; culture vessel is 9 cm in diameter) of liquid culture. Figure 2F shows transformed potato roots at the onset (left) and 2 weeks after the onset (right) of liquid culture.

To establish liquid cultures of transformed tobacco roots, putative transformed roots were excised from leaf explants 2 to 3 weeks after inoculation and transferred to selective medium containing 50 mgl^{-1} kanamycin. After 2 weeks, the kanamycin-resistant clone was transferred to liquid medium. Figure 2G shows transformed tobacco roots at the onset (left) and 4 weeks after the onset (right) of liquid culture. Transformed tobacco roots could also be cultured in hormone-free 0.5× MS liquid medium for several months, but older parts of roots became dark brown with increasing incubation time and the growth rate decreased (data not shown). Therefore, the basal medium, phytohormone concentrations, and culture conditions should be optimized for each plant species.

Generation of Arabidopsis *root culture lines by floral dip transformation*

The "floral dip" transformation method has been developed and widely used as an easy and high-throughput method to generate transgenic *Arabidopsis* plants (Clough and Bent 1998). We tested if this method is suitable for the *rol*-type vectors. Transformation experiments using pHR-*emp*(*gfp*) (Figure 3A) revealed that transgenic plants could be obtained at a comparable efficiency to the usual non-*rol*-type binary vectors. Moreover, we found that root culture lines can be established from transgenic plants using this extremely simple protocol. The pHR-*emp*(*gfp*)-transformed seedlings showed typical morphological phenotypes, represented as an overabundance of roots and curled leaves, accompanied by GFP fluorescence (Figure 3B). Expression of *rolB* and *rolC* genes was confirmed in transgenic lines (Figure 3C).

To establish root cultures, a cluster of roots was separated from individual seedlings (2 weeks old) and transferred to hormone-free liquid Gamborg B5 medium (Gamborg et al. 1986). The established root culture 3 months after the onset of culturing in liquid medium had more than 17 g (fresh weight) of root tissue (Figure 3D). We subcultured the established root cultures every 2–3 weeks and confirmed that they could be maintained for at least 2 years without loss of GFP fluorescence. A comparison of hypocotyl-derived callus transformation (Figure 2B) and floral-dip transformation (Figure 3) of *Arabidopsis* revealed that the latter process is preferable for the establishment of large numbers of root culture lines, because it does not require the preparation of callus tissues for transformation and it is less laborious to separate independently-transformed clones because the roots can simply be cut off from each seedling.

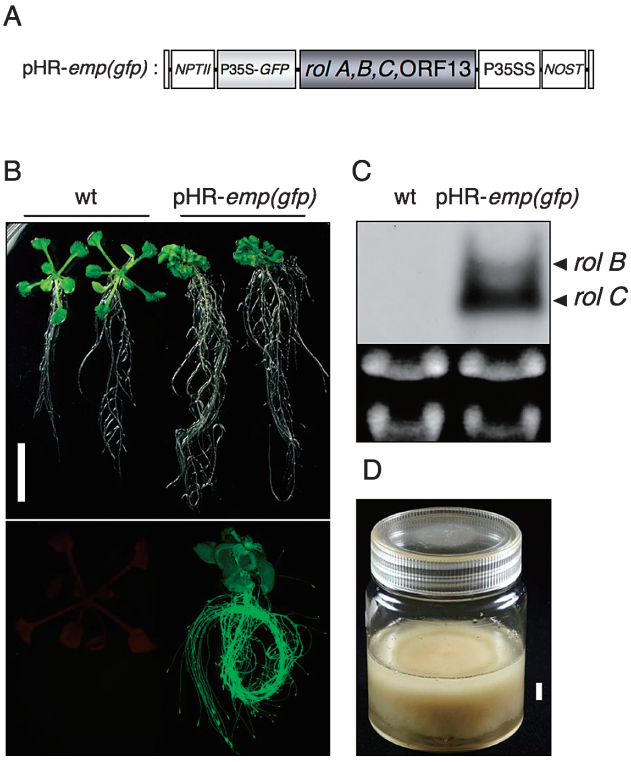

Figure 3. Generation of transformed *Arabidopsis* root culture lines by the floral dip method. (A) The construct pHR-*emp(gfp)* used for floral dip transformation. (B) Phenotype of 2-week-old pHR-*emp*(*gfp*) transgenic plants [pHR-*emp*(*gfp*), right] compared to wild-type plants (wt, left). Scale bar = 1 cm. The lower panel shows a closer view of the wt (left) and pHR-*emp*(*gfp*) transgenic plants (right) under a fluorescence microscope. (C) Expression of *rol* genes in a pHR-*emp*(*gfp*) transgenic plant. RNAs isolated from 2-week-old wt and transgenic plants [pHR-*emp*(*gfp*)] were hybridized with probes specific to the *rol* B and *rolC* gene on the same blot. Ethidium bromide staining of rRNAs is shown to demonstrate equal loading of RNA in each lane. (D) Established root culture of a pHR-*emp*(*gfp*) transgenic line generated through the seedling stage. The roots of a 2-week-old transgenic plant were excised, transferred to hormone-free liquid Gamborg B5 medium, and cultivated, and photographed 3 months after the onset of liquid culture. Scale bar = 1 cm.

For a phenotypic evaluation, pHR-*emp(gfp)*-transformed seedlings were transferred to soil, where they continued to grow. Although the transformants had significantly reduced fertility, more than 80% of the primary transformants set seed. Moreover, T2 seedlings displayed the phenotype of the parent plant that co-segregated with GFP fluorescence, confirming the heritability of the "*rol*" phenotypes (data not shown). This is important from a technical perspective, because it indicates that a large collection of transformed root culture lines can be maintained as seeds, without the need for regular subculturing.

Generating Arabidopsis root culture lines with reduced expression of SMT genes

The utility of our vector system for gene functional analyses was evaluated in a model experiment focused

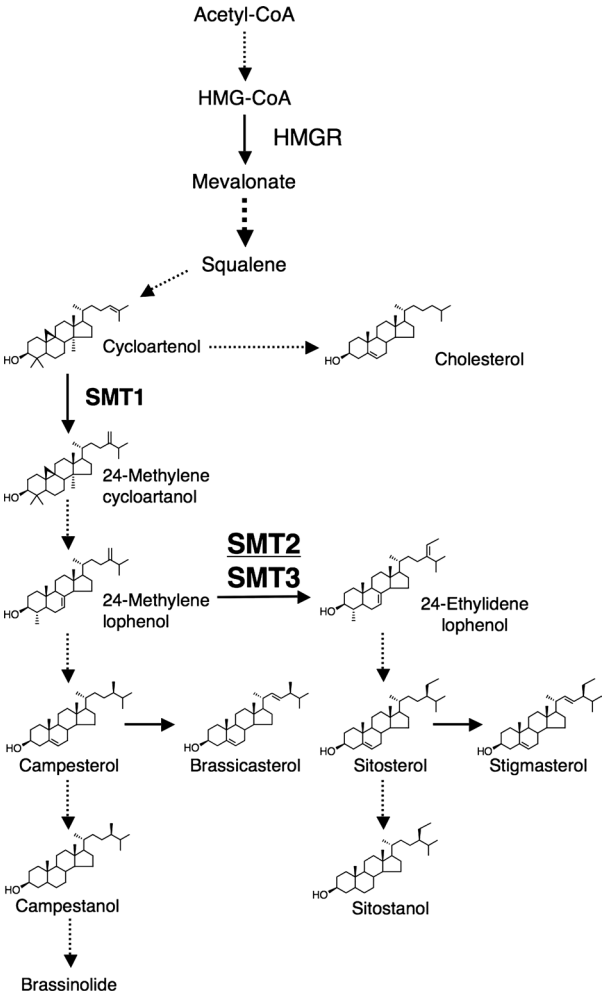

Figure 4. Simplified biosynthetic pathway for plant sterols. Solid arrows indicate a single enzymatic step, and dashed arrows indicate more than one enzymatic step. HMGR: 3-hydroxy-3-methylglutaryl CoA reductase, FPS: farnesyl diphosphate synthase, SMT: sterol-C24-methyltransferase.

on the sterol biosynthetic pathway in *Arabidopsis*. The *Arabidopsis* genome contains three distinct genes encoding sterol-C24-methyltransferases (SMTs) involved in sterol biosynthesis (Figure 4). SMT2 and the functionally redundant SMT3 mainly catalyze a second methyl transfer, as a branch point distinguishing the sterols from brassinosteroid precursors (Bouvier-Navé et al. 1997; Carland et al. 2002).

We made constructs for RNAi of *Arabidopsis SMT2* and *SMT3* genes using pHR-RNAi(*gfp*). The 800-bp fragment corresponding to the 253–1052-bp region (these numbers indicate the nucleotide positions from the translation initiation site) within the coding sequence of *SMT2* and *SMT3* was included in the RNAi construct. These constructs, along with an empty vector, were used in *A. tumefaciens*-mediated transformation of *Arabidopsis* hypocotyl-derived callus, and subsequently transformed root culture lines were established by the method described in Figure 2.

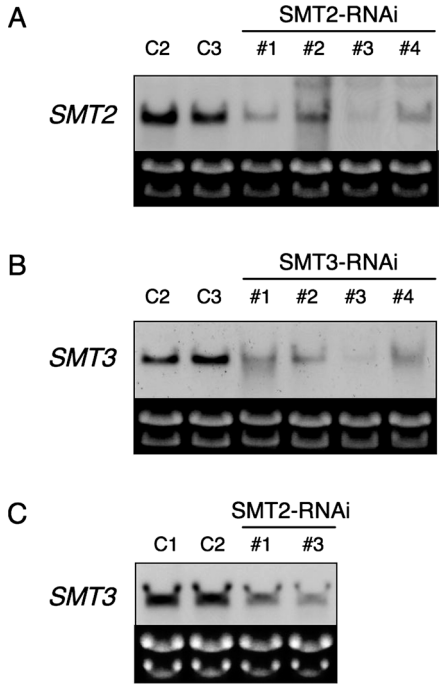

Figure 5. Northern analysis of *SMT* RNAi lines. (A) Expression of *SMT2* in the empty vector lines (C2 and C3) and four independent *SMT2* RNAi lines (SMT2-RNAi). (B) Expression of *SMT3* in the empty vector lines (C2 and C3) and in four independent *SMT3* RNAi lines (SMT3-RNAi). (C) Expression of *SMT3* in the empty vector lines (C1 and C2) and in *SMT2* RNAi lines (#1 and #3). Ethidium bromide staining of rRNAs is shown to demonstrate equal loading of RNA in each lane.

Total RNAs were prepared from root tissues cultured in hormone-free 0.5× MS liquid medium. Northern analyses of root culture lines transformed with SMT-RNAi constructs confirmed that although the transcript levels varied among the lines, all lines analyzed showed decreased transcript levels of the corresponding gene compared to those in the empty vector lines (Figure 5A, B). Because cDNA fragments included in *SMT2*- and SMT3-RNAi constructs show 78.6% nucleotide sequence identity (including a 37-bp perfect match), we also analyzed the transcript levels of *SMT3* in SMT2-RNAi lines (#1 and #3). Figure 5C shows that the expression of *SMT3* is also mildly affected in SMT2-RNAi lines.

Altered sterol profile in SMT-RNAi transformed hairy roots

The sterol profile of root tissue from three representative lines for each construct was analyzed. Average values of independent lines are given in Table 3. Aside from minor variations, major changes to the total sterol content and composition were common among the independent lines of each construct, except that 24-methylenecycloartanol was not detectable in one of the three SMT2-RNAi lines analyzed.

SMT2-RNAi lines (#1, #3, and #4) exhibited

Table 3. Sterol composition in *Arabidopsis* root culture lines with decreased expression of *SMT2* and *SMT3* genes

Sterol	Empty vector control ($n=3$)	SMT2-RNAi ($n=3$)	SMT3-RNAi ($n=3$)
Total	4568 (279)	5266 (594)	5296 (216)
Cycloartenol	40 (6)	26 (15)	33 (5)
24-Methylenecycloartanol	n.d.	10 (6)[a]	n.d.
Cholesterol	256 (58)	370 (120)	229 (54)
Campesterol	625 (103)	1570 (666)	1140 (370)
Campestanol	27 (1)	75 (22)	52 (15)
Brassicasterol	28 (15)	68 (18)	57 (21)
Sitosterol	1599 (305)	1344 (446)	1297 (133)
Sitostanol	63 (8)	80 (12)	71 (12)
Stigmasterol	1930 (168)	1631 (593)	2418 (257)

Average values (mg g^{-1} DW) of three independent lines for each construct are shown. The standard deviation is given in parentheses. n.d., not detected.

[a] Not detected in one of the three independent lines analyzed.

increased levels of the 24-methylsterols campesterol (2.5-fold), campestanol (2.8-fold), and brassicasterol (2.4-fold), when compared to empty vector lines. This alteration of sterol profile is consistent with the loss or reduction of SMT2 function and is similar to the situation described for co-suppressed 35S::*SMT2 Arabidopsis* plants (Schaeffer et al. 2001), and *cvp1* (Carland et al. 2002) and *frl1* (Hase et al. 2005) mutants that have a mutation in *SMT2*. Similar alterations were observed in SMT3-RNAi lines (#1, #2, and #3), with increased levels of campesterol (1.8-fold), campestanol (1.9-fold), and brassicasterol (2.0-fold). This is consistent with the overlapping function of SMT2 and SMT3 (Carland et al. 2002), and thus validated the utility of pHR-RNAi vectors for gene function analyses using hairy roots.

However, in contrast to the co-suppressed 35S::*SMT2 Arabidopsis* plant, where 5-week-old plants were analyzed for sterol profiles (Schaeffer et al. 2001), and *cvp1* (1-week-old seedlings were analyzed, Carland et al. 2002) and *frl1* (inflorescence samples were analyzed, Hase et al. 2005) mutants, SMT2- and SMT3-RNAi hairy roots did not show decreased levels of 24-ethylsterols such as sitosterol and stigmasterol. This could indicate that in hairy roots compared to leaves or shoots of 35S::*SMT2*, cvp1, and *frl1* mutants, the pool of substrate of SMT2 (and 3), namely, 24-methylene lophenol, which is located at the branching point in the pathways leading to 24-methyl- or 24-ethyl sterols, is not limiting. To verify this speculation, more detailed biochemical analyses of different tissues and organs have to be carried out.

Discussion

We developed a binary vector set for efficient target gene overexpression and RNAi in transformed hairy roots. Transformation experiments revealed that the vectors could be used to create a large number of root culture lines in *Arabidopsis*, potato, tobacco, and probably also in other plant species. As a proof-of-concept, we have shown in this study that engineered deficiencies in the expression of known phytosterol biosynthetic genes in hairy roots result in similar biochemical modifications as in classical plant lines. Use of our vectors will facilitate *in vivo* gene function analyses in plant species that are recalcitrant to regeneration of transgenic plants.

Since a large number of transformed roots can be induced from explants, and every hairy root emerges as the result of an independent transformation event (Tepfer 1984), the described system may provide a new means for the screening of genes involved in the production of useful metabolites. The presence of GATEWAY™ recombination site(s) allows cDNAs to be easily moved into our vectors, rendering the method suitable for high-throughput functional genomic approaches. Our transformation system described here could easily be scaled up for large-scale functional analysis of cDNA libraries. High-throughput targeted metabolite analysis will be a promising approach for screening of root culture lines overproducing useful metabolites, and could lead to the identification of a direct functional link between integrated genes and the production of target metabolites.

Historically, hairy root cultures have served as a useful model system in many so-called "exotic" plants, but the hairy root culture system has seldom been applied to the model plant *Arabidopsis*. However, *Arabidopsis* hairy root cultures, coupled with the tools available for *Arabidopsis*, such as the large collection of full-length cDNAs, DNA arrays, and knock-out mutants, will make it an ideal system for the systematic dissection of certain aspects of root biology. We have developed a simple system for the establishment of transformed *Arabidopsis* root culture lines (Figure 3). This system has several advantages: 1) adequate quantities of root biomass under tightly controlled sterile conditions can be readily obtained from each transformed root clone, and 2) root culture lines can be maintained for a long period of time and are thus attractive for metabolic analyses of root exudates. Use of our vectors and *Arabidopsis* hairy root cultures may accelerate functional analyses and/or discovery of the genes involved in root-specific secondary metabolism, secretion, or regulation of these processes in response to various environmental stimuli.

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