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Hairy root-activation tagging: a high-throughput system for activation tagging in transformed hairy roots

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Abstract

Activation tagging is a powerful technique for generating gain-of-function mutants in plants. We developed a new vector system for activation tagging of genes in ''transformed hairy roots''. The binary vector pHR-AT (Hairy Root-Activation Tagging) and its derivative pHR-AT-GFP contain a cluster of *rol* (rooting locus) genes together with the right border facing four tandem repeats of the cauliflower mosaic virus (CaMV) 35S enhancer element on the same T-DNA. Transformation experiments using *Arabidopsis*, potato, and tobacco as model plants revealed that upon inoculating plants with *Agrobacterium tumefaciens* harboring these vectors, a large number of independently transformed roots could be induced from explants within a short period of time, and root culture lines were subsequently established. Molecular analyses of the pHR-AT-GFP-transformed *Arabidopsis* lines showed that expression of the genes adjacent to the T-DNA insertion site was significantly increased. This system may facilitate application of the activation-tagging approach to plant species that are recalcitrant to the regeneration of transgenic plants. High-throughput metabolic profiling of activation-tagged root culture lines will offer opportunities for identifying regulatory or biosynthetic genes for the production of valuable secondary metabolites of interest.

Introduction

Activation tagging is a powerful tool for identifying novel genes involved in a variety of biological pathways. Owing to the ease of generating large numbers of independently transformed plant lines, this approach has been especially successful in *Arabidopsis* (for a review, see Weigel *et al.*, 2000; Tani *et al.*, 2004). High-throughput activation-tagging program were recently extended to rice (Jeong *et al.*, 2002) and tomato (Mathews *et al.*, 2003), and a flanking sequence database is currently being generated to enable the ''reverse activation tagging'' approach.

Since activation tagging creates gain-of-function mutations, it is not necessary to generate a large collection of fertile transgenic plant lines. Instead, transformed explants or cultured cells can be screened for desired phenotypes. This approach was used with *Arabidopsis* tissue cultures to identify CKI1, a histidine kinase whose overexpression bypasses the requirement for cytokinin in shoot regeneration (Kakimoto, 1996). van der Fits and Memelink (2000) used this

approach with *Catharanthus roseus* cell cultures to identify ORCA3, an AP2/ERF class of transcription factor that regulates indole alkaloid production.

Like *A. tumefaciens*, which is widely used, *A. rhizogenes* also has the ability to transfer its T-DNA from the root-inducing (Ri) plasmid to the host genome, thereby inducing the formation of hairy roots (Chilton *et al.*, 1982; Nillson and Olsson, 1997). In addition, *A. rhizogenes* can transfer the T-DNA of binary vector plasmids in trans, enabling the production of "transgenic" hairy roots containing other foreign genes carried on a binary vector (Simpson *et al.*, 1986).

A. rhizogenes-mediated transformation has several desirable features making it suitable for activation tagging: (1) the rapid, efficient induction of hairy roots from explant tissues of a wide variety of plant species has been reported (Tepfer, 1990); (2) it is easy to identify and separate individual transgenic clones, since each transformation event can be readily distinguished as an independent hairy root; and (3) hairy root cultures, unlike undifferentiated cell cultures, are normally thought to have the ability to synthesize the same compounds as the roots of the intact plant (Shanks and Morgan, 1999; Uozumi, 2004), providing attractive materials for investigating the biosynthesis of a variety of secondary metabolites.

Due to these desirable features, we initially attempted to generate activation-tagged hairy root lines via *A. rhizogenes*-mediated delivery of binary T-DNA for activation tagging. However, our initial attempts were not very successful because (1) the frequency of co-delivery of Ri plasmid-derived and binary vector-derived T-DNA was low without antibiotic selection pressure, whereas a significant reduction in the total number of hairy roots occurred under antibiotic selection, as reported by several researchers (Komarnytsky *et al.*, 2004, and references therein); (2) the integration patterns of T-DNAs were highly complexed and included the formation of repeats between Ri plasmid-derived and binary vector-derived T-DNA, with *A. rhizogenes*-mediated transformation, which resulted in a lower frequency of genome tagging and complicated characterization of the tagged genes. Karimi *et al.* (1999) and Visser *et al.* (1996) both reported more complex T-DNA arrays when *A. rhizogenes* was used instead of *A. tumefaciens*. To circumvent these problems, we designed new binary vectors specialized for hairy root-activation tagging. Here, we show that these vectors can be used to generate large numbers of transformed root culture lines and activate the expression of genes adjacent to T-DNA. The potential of the hairy root-activation tagging system is discussed.

Materials and methods

Construction of pHR-AT and pHR-AT-GFP

Standard gene cloning methods (Sambrook *et al.*, 1989) were used to create the vector constructs. *Pfu*-turbo DNA polymerase was used for PCR according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The pHR-AT and pHR-AT-GFP vectors were made from pPCVICEn4HTP (Walden *et al.*, 1994).

To yield the intermediate construct pBCR−15, the *Asc*I-*Avr*II linker (5′-GGCGCGCCGTCAACCCTAGG-3') was added to one of the *Cla*I sites in the T-DNA of pPCVICEn4HTP. The DNA fragment harboring the cluster *rolA*, *rolB*, *rolC*, ORF13, and ORF13a, derived from mikimopine-type pRi1724 in *A. rhizogenes* strain MAFF03–01724, was amplified from the pRi1724-derived plasmid clone pRTE7.6 (Tanaka *et al.*, 1994) with the primers 5′-CTTGGCGCGCCAAGGAATTC-CAGCGGATATCTGGAGG-3′ and 5′-CGAT-CCTAGGGATACGCTAGAAAAGGAAGCA TTC-3′. A 7.6-kb PCR fragment was digested with *Asc*I/*Avr*II and cloned into *Asc*I/*Avr*II-digested pBCR−15, resulting in pHR-AT. A fragment containing the *sGFP*(S65T) gene flanked by the CaMV 35S promoter and nopaline synthase polyadenylation signal was amplified from the CaMV 35S promoter-*sGFP*(S65T) plasmid (Chiu *et al.*, 1996) with the primers 5′-CTT GGCGCGCCCCCCTCAGAAGACCAGAGGG CT-3′ and 5′-CGAGGAATTCTCATGTTTGAC AGCTTATCATC-3′. A 1.4-kb PCR fragment was digested with *Asc*I/*Eco*RI and cloned into *Asc*I/*Eco*RI-digested pHR-AT, resulting in pHR-AT-GFP. The underlined sequences in each of the primers correspond to the restriction enzyme sites used for cloning purposes.

Plant transformation and establishing root cultures

The binary vector construct was introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) using chemically based transformation (An *et al*., 1988). In all plant transformation experiments described here, hormone-free half-strength (0.5×) MS medium was used. 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 hygromycin (25 mg l^{-1}). A root cluster was separated from the seedling and transferred to liquid medium, or the seedling was transplanted to soil and allowed to continue growing.

Potato (*Solanum tuberosum* L. cv. May Queen) stem segments (approximately 10 mm in length) were dipped in *Agrobacterium* culture suspended in 3% sucrose ($OD_{600} = 0.05$) for 2 min, and then co-cultivated on agar medium for 2 days at 26 °C under a 16/8 h (day/night) photoperiod. After co-cultivation, the explants were transferred onto agar medium supplemented with 200 mg l^{-1} cefotaxime to eliminate bacteria and maintained at 23° C under a 16/8 h (day/night) photoperiod.

Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) leaf segments (15×15 mm) were dipped in *Agrobacterium* culture suspended in 3% sucrose (OD_{600} = 0.15) for 2 min, and then co-cultivated on agar medium for 2 days at 26° C in the dark. Control explants were dipped in 3% sucrose solution. After co-cultivation, the explants were transferred onto agar medium supplemented with 200 mg l^{-1} cefotaxime to eliminate the bacteria and maintained at 26° C in the dark. Putative transformed roots were excised and transferred to selective medium supplemented with hygromycin (25 mg l^{-1}). PCR analyses were performed using the primers 5′-GAGAAACTACTATACCACGGGTG−3′ and 5′-ACGGACGAGCCCATCCGTCCAGA−3′.

GFP-expressing *Arabidopsis* and potato roots, as well as hygromycin-resistant tobacco roots were transferred to liquid medium after several subcultures on agar medium at 2-week intervals. Liquid cultures were maintained at 26 °C on a rotary shaker (100 rpm) under a 16/8 h (day/night) photoperiod.

Southern blot analysis

Genomic DNA was isolated using a Nucleon Phytopure plant DNA extraction kit (Amersham Biosciences, Little Chalfont, UK). Genomic DNA (10 μg) was digested with *Nhe*I or *Spe*I, separated on 0.8% agarose gels, and transferred onto Hybond N^+ nylon membranes (Amersham), as described by Chomczynski (1992). Hybridization was performed in DIG Easy Hyb solution (Roche Diagnostics, Basel, Switzerland) for 12 h at 42° C. The membrane was then washed twice for 5 min with 2× SSC and 0.1% SDS at room temperature, and twice for 20 min with 0.1× SSC and 0.1% SDS at 68 °C. Detection was performed with anti-DIG antibody conjugated with alkaline phosphatase and its chemiluminescent substrate, CDP-*Star*™ (Roche). A probe corresponding to the ampicillin-resistance gene was PCR-amplified from pHR-AT vector using the primers 5′-ATGAGTATTCAACATTTCCGTG−3′ and 5′-TTACCAATGCTTAATCAGTGA-3′, and labeled with digoxigenin−11-dUTP using a PCR DIG Probe Synthesis Kit (Roche).

Plasmid rescue

Genomic DNA (1 μg) was digested overnight with *Nhe*I in a volume of 200 μl. The digested DNA was ethanol precipitated and dissolved in 10 μl of water. The DNA was self-ligated using T4 DNA ligase (New England Biolabs, Beverly, MA, USA) in a volume of 400 μl at 16 °C overnight, and then ethanol precipitated and dissolved in 10 μl of water. Twenty-five μl of ElectroMax™ DH10B cells (Invitrogen, Carlsbad, CA, USA) were transformed by electroporation with 2 μl of self-ligated DNA. The rescued genomic DNA adjacent to the right T-DNA border was sequenced using the primer 5′-ACGTCGCGGTGAGTTCAGGC−3′ (from inside the *Nhe*I site), following the primer-walking strategy. The sequence results were analyzed using the BLASTN program at NCBI.

Northern blot and semi-quantitative RT-PCR analysis

Total RNA was prepared using RNAwiz™ (Ambion Inc., Austin, TX, USA) according to the manufacturer's specifications. For Northern blot analysis, total RNA (15 μg) was separated on 1% agarose-formaldehyde gels and transferred onto Hybond N^+ nylon membranes (Amersham), as described by Chomczynski (1992). For semi-quantitative RT-PCR analysis, first-strand cDNA was prepared from 1 μg of total RNA with the TaKaRa RNA PCR kit (Takara Shuzo, Shiga, Japan). A 1-μl aliquot of the total RT reaction volume (20 μl) was used as the template in a PCR amplification, ensuring that the amount of amplified product remained linearly proportional to the initial template present in the reaction. Five μl from the PCR were separated on 0.8% agarose gels and transferred onto Hybond N^+ nylon membranes (Amersham). The membranes were hybridized with digoxigenin (DIG)-labeled antisense RNA probes specific for each of the genes analyzed. Hybridization was performed in DIG Easy Hyb solution (Roche) for 16 h at 68 °C. To obtain gene-specific probes, a cDNA fragment was amplified by RT-PCR, and the products were cloned into pCR II-TOPO cloning vector (Invitrogen). The resulting plasmids were digested with the appropriate enzyme to yield template for antisense *in vitro* transcription. Antisense RNA probes were made using an RNA transcription kit (Stratagene), and labeled with digoxigenin (DIG)-UTP using DIG RNA labeling Mix (Roche).

Results and discussion

Design of pHR-AT and pHR-AT-GFP

The vectors described here, designated pHR-AT and pHR-AT-GFP, are derivatives of the widely used activation-tagging vector, pPCVICEn4HTP (Walden *et al*., 1994). The vectors were designed as "*all-in-one*" constructs with (1) the *rol* gene cluster for inducing transformed roots from explant tissues, (2) a hygromycin-resistance gene for antibiotic selection of the transformed roots, (3) a unit of the ampicillin-resistance gene and replication origin working in *Escherichia coli*, which allows isolation of the genomic sequences adjacent to T-DNA via plasmid rescue, (4) four tandem repeats of the CaMV 35S enhancer element for activating adjacent plant genes, and (5) the green fluorescent protein (GFP) expression cassette in pHR-AT-GFP as a visible, 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 *rolC* gene products are particularly important in the induction and development of these transformed roots (Spena *et al*., 1987; Schmulling *et al*., 1988). To circumvent the problems involved in *A. rhizogenes*-mediated transformation, the 7.6-kb DNA fragment harboring the *rol* gene cluster (*rolA, B, C*), derived from mikimopine-type pRi1724 in *A. rhizogenes* strain MAFF03–01724 (Tanaka *et al*., 1994), was

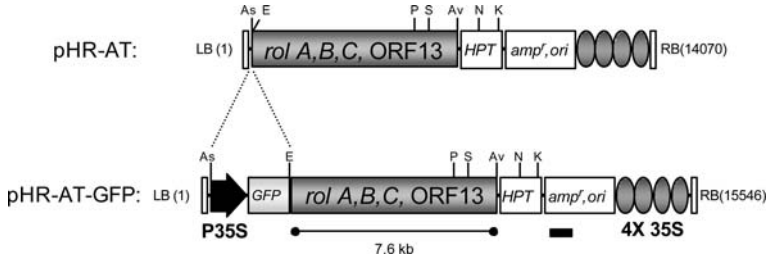


Figure 1. Map of pHR-AT and pHR-AT-GFP. Only the region between the left and right borders is shown (not to scale). pHR-AT and pHR-AT-GFP are identical except for the *Asc*I/*Eco*RI fragment in pHR-AT-GFP, which contains the *sGFP*(S65T) coding sequence flanked by the CaMV 35S promoter (P35S) and nopaline synthase polyadenylation signal. *rolA, B, C*, ORF13, 7.6-kb fragment derived from pRi1724 in *A. rhizogenes* strain MAFF03-01724; *HPT*, hygromycin phosphotransferase gene; *amp*^{r}, ampicillin-resistance gene; *ori*, origin of replication in *E. coli*; 4× 35S, four copies of the CaMV 35S enhancer sequence; LB and RB, T-DNA left and right border, respectively. Numbers in parenthesis indicate the positions relative to the left T-DNA border. The restriction enzyme sites that can be used for rescue of sequences adjacent to the right T-DNA border are shown; *Asc*I (As), *Eco*RI (E), *Pme*I (P), *Spe*I (S), *Avr*II (Av), *Nhe*I (N), and *Kpn*I (K). The thick line represents the region corresponding to the ampicillin-resistance gene sequence used as the probe in Southern analyses (Figure 4).

packaged in a binary vector T-DNA together with CaMV 35S enhancer repeats. This 7.6-kb DNA fragment contains, in addition to the *rol* genes (*rolA, B, C*), two open reading frames (ORFs), ORF13 and ORF13a. The ORF13 was reported to promote the *rol*-mediated rooting in tobacco (Aoki and Syono, 1999), while the function of the ORF13a in root induction remains unknown. The vector should be introduced into *A. tumefaciens*, but not into *A. rhizogenes*, and the binary vector T-DNA containing *rol* gene cluster will be subsequently integrated into the plant genome via typical *A. tumefaciens*-mediated transformation, resulting in the induction of transformed roots and activation tagging of the plant genes therein.

The vector T-DNAs contain several restriction enzyme sites that can be used for rescue of sequences adjacent to the right T-DNA border, whereas no appropriate restriction enzyme sites for left border rescue (see Figure 1). Therefore, the left border adjacent sequences will be recovered using other techniques such as an inverse PCR (Spertini *et al*., 1999) or adapter-PCR method (Yamamoto *et al*., 2003).

Antibiotic selection of *Agrobacterium*-inoculated explants sometimes causes a significant reduction in the total number of hairy roots (Komarnytsky *et al*., 2004, and references therein); therefore, the engineered *sGFP*(S65T) expression cassette was included in pHR-AT-GFP due to the utility of *sGFP*(S65T) as a visible, non-invasive selectable marker in a number of plant species (Niwa *et al*., 1999; Stewart, 2001; Niwa, 2003).

Rapid and efficient induction of transformed roots

To evaluate the root-inducing ability of vector T-DNAs, transformation experiments were performed using three different types of explant tissue as model systems. In all of the transformation experiments described here, hormone-free half-strength (0.5×) Murashige and Skoog (MS) medium was used (Murashige and Skoog, 1962).

Arabidopsis hypocotyl-derived calli and potato stem segments were inoculated with *A. tumefaciens* harboring pHR-AT-GFP. After 2 days of co-cultivation, the explants were transferred to non-selective (without hygromycin) agar medium. After 2–3 weeks, GFP-expressing roots appeared on the surface of the *Arabidopsis* calli and the cut edge of the potato stem segments (Figure 2A and B). Since no appreciable background fluorescence is observed in *Arabidopsis* or potato adventitious roots (non-transformed roots), the pHR-AT-GFP-transformed roots were readily identified with GFP fluorescence under non-destructive, sterile conditions. Five weeks after inoculation, the induction of GFP-expressing roots was observed on 43% (84/194) and 60% (132/219) of the *Arabidopsis* and potato explants, respectively (Table 1). The number of independent GFP-expressing roots per explant averaged 0.73 and 2.1 on *Arabidopsis* calli and potato stem segments, respectively (Table 1).

Tobacco leaf segments were inoculated with *A. tumefaciens* harboring pHR-AT or pHR-AT-GFP, to compare the root-inducing ability of these two vectors. After 2 days of co-cultivation, the leaf segments were subsequently cultured on non-selective agar medium. Within 2 weeks, putative transformed roots were induced at wound sites on leaf segments inoculated with pHR-AT or pHR-AT-GFP. Figure 2C shows the leaf segments 18 days after inoculation with pHR-AT-GFP (right) compared to mock-inoculated leaf segments (left). Three weeks after inoculation, the induction of putative transformed roots was observed on

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[a] (%)	Total number of GFP-expressing roots[b]	No. of GFP-expressing roots[b]/explant[a]
Arabidopsis hypocotyl-derived calli	84/194[a] (43)	142[b]	0.73[b/a]
Potato stem segments	132/219 (60)	454	2.1

[a] Number of explants inoculated.
[b] Total number of GFP-expressing roots emerged.
Explants were inoculated with *A. tumefaciens* harboring pHR-AT-GFP. The number of GFP-expressing roots was counted 5 weeks after inoculation.

more than 60% of the leaf segments with both pHR-AT and pHR-AT-GFP (Table 2), and the numbers of roots per leaf segment averaged 2.3 and 5.7 with pHR-AT and pHR-AT-GFP, respectively (Table 2). We observed 2.5-fold more roots per leaf segment with pHR-AT-GFP inoculation than with pHR-AT, perhaps because the CaMV 35S promoter, which drives GFP expression in pHR-AT-GFP, also affects expression of the *rol* genes.

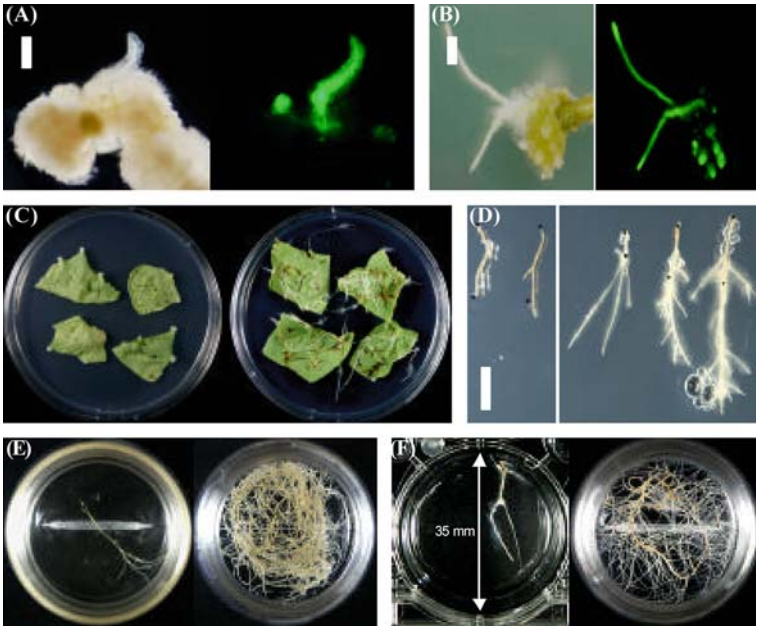


Figure 2. Induction of transformed roots from explants. (A) GFP-expressing roots emerging on the surface of an *Arabidopsis* callus 3 weeks after inoculation with *A. tumefacience* harboring pHR-AT-GFP. A reference image under reflected light (left) and GFP fluorescence of induced roots in the same field of view (right) are shown. Scale bar = 1 mm. (B) Induction of GFP-expressing roots from a potato stem segment 3 weeks after inoculation with *A. tumefaciens* harboring pHR-AT-GFP. A reference image under reflected light (left) and GFP fluorescence of induced roots in the same field of view (right) are shown. Scale bar = 1 mm. (C) Induction of putative transformed roots from tobacco leaf segments. Tobacco leaf segments 18 days after mock-treatment (left) or inoculation with *A. tumefaciens* harboring pHR-AT-GFP (right). The Petri dish is 10 cm in diameter. (D) Hygromycin resistance of transformed tobacco roots. Apical tips from non-transformed roots (left) and pHR-AT-GFP-transformed roots (right) were transferred onto selective medium containing 25 mg l^{-1} hygromycin and held vertical for 2 weeks. Dots indicate the length of the root before the 2-week culture. Scale bar = 1 cm. (E) Liquid culture of transformed potato roots 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. (F) Liquid culture of transformed tobacco roots at the onset (left) and 4 weeks after the onset (right) of liquid culture in hormone-free 0.5× MS medium. The culture vessels are 3.5 (left) and 9 (right) cm in diameter.

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

Bacteria strain (plasmid)	No. of explants yielding roots/No. of explants[a] (%)	Total number of roots[b]	No. of roots[b]/explant[a]	PCR-positive roots/roots analyzed (%)
GV3101 (pHR-AT)	51/80[a] (64)	184[b]	2.3[b/a]	9/10 (90)
GV3101 (pHR-AT-GFP)	83/100 (83)	569	5.7	8/10 (80)
Control	9/30 (30)	15	0.5	Not tested

[a] Number of explants inoculated.
[b] Total number of roots emerged.
Leaf segments were inoculated with *A. tumefaciens* harboring pHR-AT or pHR-AT-GFP. As a negative control, leaf segments were dipped in 3% sucrose solution instead of bacteria suspension. Leaf segments were cultured on nonselective (without hygromycin) agar medium. The number of putative transformed roots was counted 3 weeks after inoculation.

Since visual GFP selection of transformed roots was difficult due to the strong background fluorescence in tobacco roots (non-transformed roots), mock-inoculated leaf segments were prepared as a negative control for transformation. Leaf segments were dipped in 3% sucrose, instead of the bacteria suspension, and then cultured on non-selective agar medium. Although adventitious roots appeared on 30% of the mock-inoculated leaf segments within 3 weeks, the average number of roots per leaf segment was 0.5, suggesting that the majority of roots that appeared on the pHR-AT- or pHR-AT-GFP-inoculated leaf segments were induced by the transformation events. This assumption was supported by further analyses. Ten independent, randomly selected roots were excised from both pHR-AT- and pHR-AT-GFP-inoculated leaf segments, and the integration of T-DNA was analyzed using PCR. Nine of the 10 (90%) root clones from pHR-AT, and 8 of 10 (80%) root clones from pHR-AT-GFP inoculation were positive for T-DNA integration (Table 2). The difficulties with visual GFP selection prompted us to evaluate the effectiveness of antibiotic selection of transformed tobacco roots. In contrast to the non-transformed root clones (PCR negative; left), pHR-AT-GFP-transformed root clones (PCR positive; right) grew on the selective medium containing 25 mg l^{-1} hygromycin at an average elongation rate of 16.1 ± 4.2 mm per week ($n = 20$) (Figure 2D). However, cultivation of inoculated leaf segments on the same selective medium resulted in a drastic reduction in the number of putative transformed roots that emerged (data not shown).

These results show that our vectors can be used to generate a large number of transformed root clones in *Arabidopsis*, potato, and tobacco, and probably in other plant species as well, using an extremely simple transformation protocol. However, to obtain the most efficient root initiation and root growth, the transformation protocol should be improved further. Karimi *et al.* (1999) reported 2.6-fold more hairy roots per *Arabidopsis* leaf segment inoculated with wild-type *A. rhizogenes*, when Phytagel (Sigma, St. Louis, MO, USA) was used to solidify medium instead of agar. We obtained 3-fold more GFP-expressing roots per pHR-AT-GFP-inoculated *Arabidopsis* callus cultured on agar medium containing 0.1 mg l^{-1} indole−3-acetic acid (IAA) than on hormone-free agar medium (data not shown), possibly, at least partly, due to the transcriptional activation of the integrated *rolB* gene by exogenous auxin as reported (Baumann *et al.*, 1999, and references therein). More intense root induction, accompanying the transcriptional activation of the *rolB* gene, by exogenous application of auxin was observed on *A. rhizogenes*-inoculated tobacco leaf segments (Handayani *et al.*, 2005).

Establishing liquid cultures

To propagate the transformed *Arabidopsis* and potato roots, GFP-expressing roots were excised from explant tissues 4–5 weeks after inoculation and transferred to non-selective agar medium. After one or two passages on fresh agar medium at 2-week intervals, the roots were transferred to hormone-free 0.5× MS liquid medium. Figure 2E 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, the apical tip of the putative transformed roots (10–15 mm in length) were excised from leaf segments 3–4 weeks after inoculation and transferred to selective medium containing 25 mg l^{-1} hygromycin. After 2 weeks, the hygromycin-resistant clones were transferred to liquid medium. The transformed tobacco roots could also be cultured in hormone-free 0.5× MS liquid medium for several months (Figure 2F), although the older parts of the roots became dark brown with increasing incubation time, and the growth rate decreased (data not shown), which is often the case in tobacco hairy root liquid cultures generated by infection with wild-type *A. rhizogenes* (T. Hashimoto, pers. comm.). Therefore, the basal medium, phytohormone concentrations, and culture conditions should be optimized for each plant species.

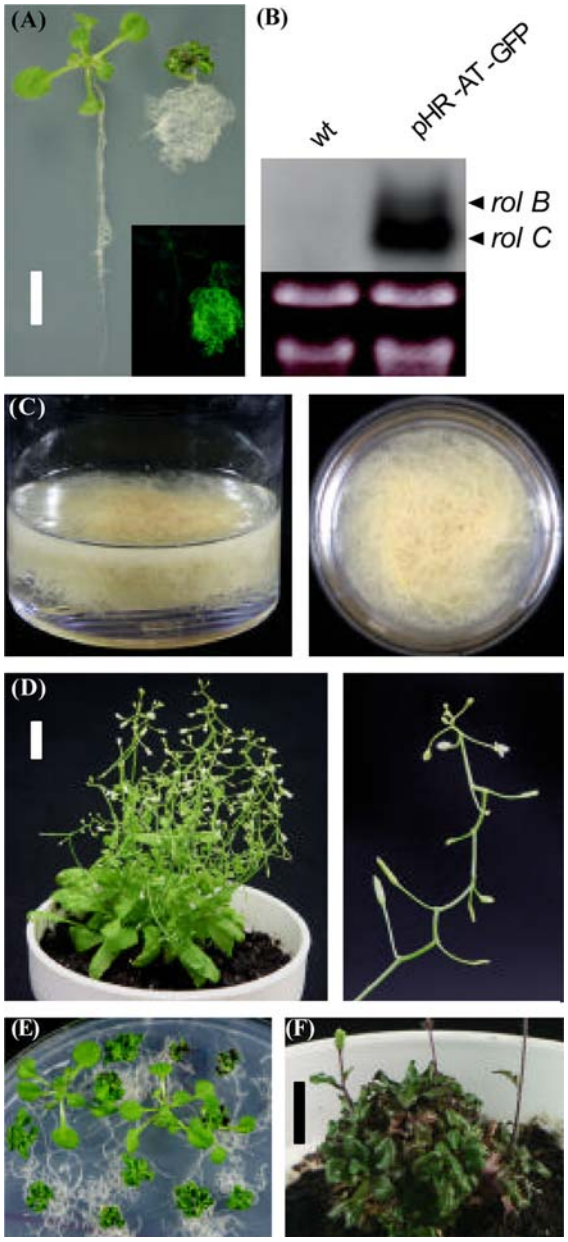
Generating Arabidopsis *root culture lines using floral dip transformation*

The "floral dip" transformation method has been developed and widely used as an easy, high-throughput method for generating transgenic *Arabidopsis* plants (Clough and Bent, 1998). We tested whether this method works for the *rol*-type pHR-AT-GFP vector. A transformation experiment

revealed that transgenic plants could be obtained with an efficiency comparable to that of typical non-*rol*-type binary vectors. Moreover, we were able to establish root culture lines from transgenic seedlings using an extremely simple protocol. The pHR-AT-GFP-transformed seedlings displayed typical morphological phenotypes represented by an overabundance of roots and curled leaves, accompanied by GFP fluorescence (Figure 3A). The expression of the *rolB* and *rolC* genes was confirmed in the transgenics (Figure 3B).

To establish root cultures, a cluster of roots was separated from an individual 3-week-old seedling and transferred to hormone-free 0.5× MS liquid medium. Figure 3C shows the established root culture 3 months after the onset of culturing in liquid medium, with the fresh weight of root tissues exceeding 10 g. We confirmed that established root cultures can be maintained for at least 10 months up to this stage, without the loss of GFP fluorescence, by subculturing every 2 weeks. A comparison of two different transformation protocols, i.e., transformation of hypocotyl-derived calli (Figure 2A) and floral dip transformation, revealed that the latter process is better for establishing large numbers of *Arabidopsis* root culture lines, because there is no need to prepare callus tissues for transformation, and less labor is required to separate independent transformed clones, which can be simply cut from the roots of each seedling.

For a phenotypic evaluation of pHR-AT-GFP-transformed *Arabidopsis*, approximately 300 seedlings were transferred to soil, where they continued to grow. Almost all of these plants showed the morphological phenotypes of ''hairy root syndrome'' (Tepfer, 1984) such as dwarfism, wrinkled leaves, decreased apical dominance, and unusual stem elongation in the adult stage (Figure 3D). Although the transformants had significantly reduced fertility, more than 80% of the primary transformants set seed. T_2 heterozygous seeds from one of the primary transformants were germinated on medium without the selective antibiotic hygromycin. Twelve of 15 seedlings displayed the phenotypes of the parent plant that co-segregated with GFP fluorescence, confirming the heritability of the ''*rol*'' phenotypes (Figure 3E). This is important from a technical perspective, because it indicates that a large collection of activation-tagged root culture lines can be maintained as seeds, without regular



subculturing. Furthermore, this also means that the loss-of-function mutations potentially can be recovered from these lines. We confirmed that the pHR-AT-GFP-transformed seeds were stable for at least 2 years under room temperature conditions.

From approximately 300 independent primary pHR-AT-GFP transformants, we identified a single mutant plant with an aberrant phenotype that differed from the other transformants. This plant

Figure 3. Generation of pHR-AT-GFP-transformed *Arabidopsis* lines using the floral dip method. (A) Phenotype of a 2-week-old pHR-AT-GFP transgenic plant (right) compared to a wild-type plant (left). Scale bar = 1 cm. The inset shows the GFP fluorescence from the transgenic plant. (B) Expression of *rol* genes in a pHR-AT-GFP transgenic plant. RNA isolated from 2-week-old wild-type (WT) and transgenic plants (pHR-AT-GFP) was hybridized with probes specific to each of the *rolB* and *rolC* gene on the same blot. Ethidium bromide staining of rRNA is shown to demonstrate equal loading of RNA in each lane. (C) Established root culture of a pHR-AT-GFP transgenic line generated through the seedling stage. The root organ of a 3-week-old transgenic plant was excised, transferred to hormone-free 0.5× MS liquid medium, and photographed 3 months after the onset of liquid culture. Topical view of the culture vessel (right), which is 9 cm in diameter. (D) Phenotype of an 80-day-old pHR-AT-GFP transgenic plant. Scale bar = 1 cm. Closer view of stem (right). (E) Inheritance of the *"rol"* phenotype. Seeds of a pHR-AT-GFP transgenic line (T_2 heterozygous seeds) were germinated on non-selective medium and photographed 2 weeks after germination. The "*rol*" phenotype co-segregated with GFP fluorescence (data not shown). (F) Dome-shaped phenotype of transgenic line #12 (T_1 generation), photographed 7 months after germination. Scale bar = 1 cm.

had a dome-like appearance caused by aerial rosettes, and it did not flower until approximately 6 months after germination (Figure 3F). We obtained T_2 seeds from this plant (hereafter, transgenic plant line #12), and root cultures were subsequently established from each of the six T_2 seedlings. None of these root culture lines showed obvious morphological differences when compared to the other pHR-AT-GFP-transformed root culture lines. However, one of the #12 (T_2) root culture lines was subjected to further analysis for mapping of T-DNA insertion sites (Figure 5), due to the aberrant phenotype of its parent plant (Figure 3F).

Genomic Southern analysis

To get an idea of the number of T-DNA insertion sites (loci), in pHR-AT-GFP-transformed lines, we performed genomic Southern analysis of (a) five *Arabidopsis* root culture lines generated from hypocotyl-derived calli (lines #1 to #5), (b) six *Arabidopsis* root culture lines (lines #6 to #11) and transgenic plant line #12 (T_1) generated using floral dip transformation (Figure 3F), and (c) 10 tobacco root culture lines (lines #N1 to #N10). The genomic DNA was digested with *Nhe*I or *Spe*I at a unique site within the vector T-DNA (see Figure 1) and probed with the ampicillin-resistance gene fragment, which hybridized only with restriction fragment(s) including the right T-DNA border.

Of the 22 lines examined, three *Arabidopsis* lines (#7, #11 and #12) contained a single T-DNA insertion, while the other 19 lines had multiple T-DNA insertions. The number of bands does not simply represent the number of insertion sites because they could be derived from concatemeric insertion of the T-DNAs. However, these data suggest that the number of T-DNA insertion sites ranges from one (lines #7, #11 and #12) to a maximum of five (line #N9).

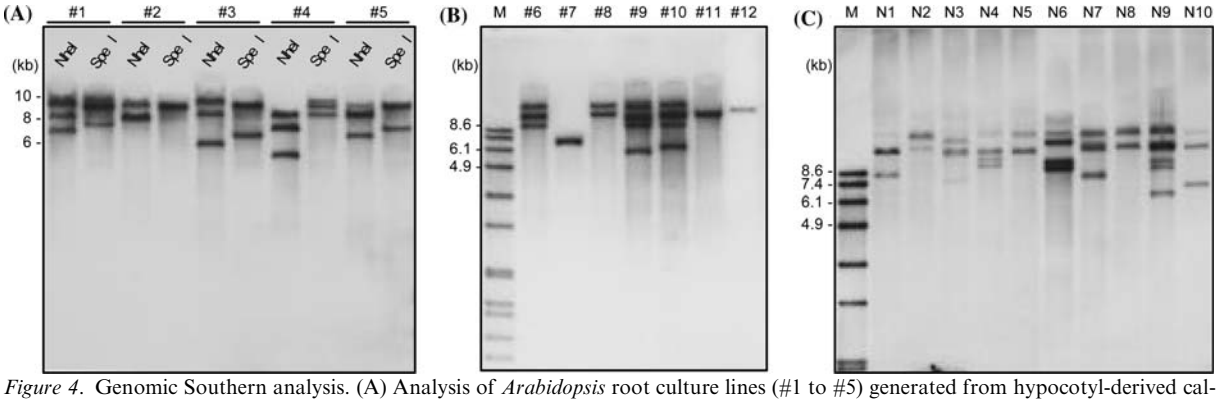


Figure 4. Genomic Southern analysis. (A) Analysis of *Arabidopsis* root culture lines (#1 to #5) generated from hypocotyl-derived calli. Genomic DNA was digested with *Nhe*I and *Spe*I. (B) Analysis of *Arabidopsis* root culture lines (#6 to #11) and a transgenic plant line (#12, see Figure 3F) generated using floral dip transformation. Genomic DNA was digested with *Nhe*I. (C) Analysis of tobacco root culture lines (#N1 to #N10). Genomic DNA was digested with *Nhe*I. All digested DNA was probed with a DIG-labeled ampicilin-resistance gene fragment (for the location of the probe region, see Figure 1). M, molecular maker lane.

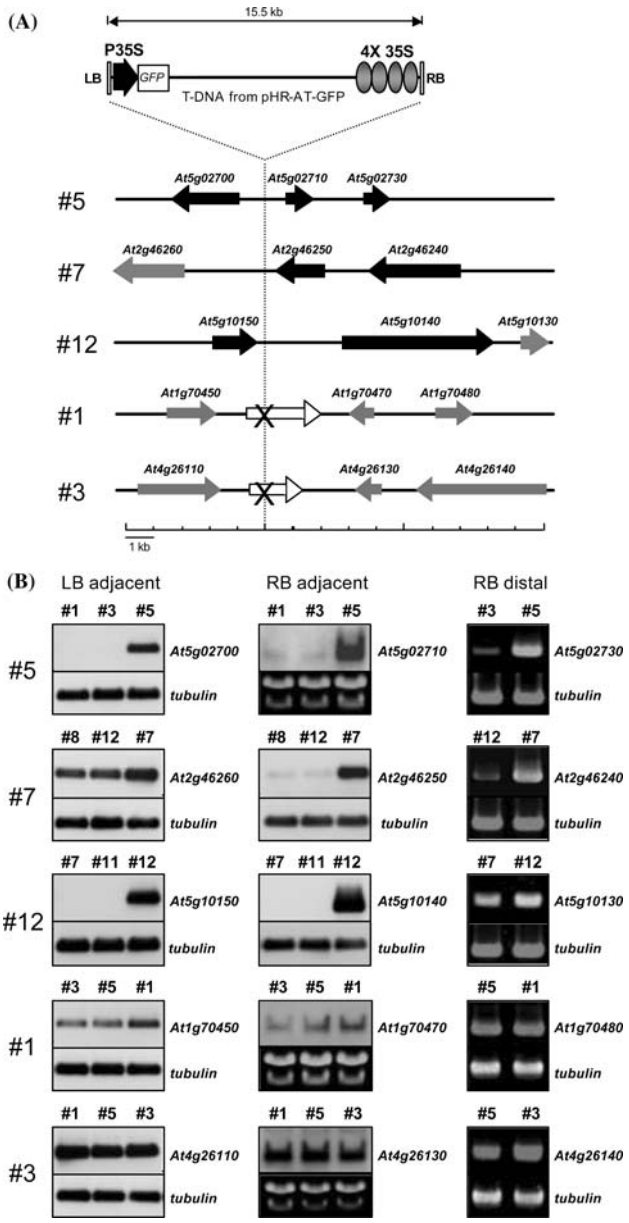


Figure 5. Analysis of *Arabidopsis* lines transformed with pHR-AT-GFP. (A) Schematic representations of the adjacent genes relative to the integrated T-DNA. Five different insertion sites recovered from five root culture lines (#1, #3, #5, #7 and #12) are shown. The gene adjacent to the T-DNA is indicated with a black (activated), gray (not activated) or open (disrupted) arrow pointing in the direction of transcription. P35S, CaMV 35S promoter which drives the expression of the *sGFP*(S65T) coding sequence; 4× 35S, four copies of the CaMV 35S enhancer sequence; LB and RB, T-DNA left and right border, respectively. (B) Expression analysis of the genes adjacent to the T-DNA (left panel, genes adjacent to the left border; center panel, genes adjacent to the right border; right panel, genes more distal to the right border). The far right bands are the results of the corresponding lines. Total RNA was prepared from root tissues cultured for at least four months in 0.5× MS liquid medium. Northern analyses were performed with *At5g02710* (line #5, center), *At1 g70470* (line #1, center) and *At4g26130* (line #3, center). Ethidium bromide staining of rRNA is shown to demonstrate equal loading of RNA in each lane. Semi-quantitative RT-PCR analyses were performed with the remaining genes. The genes on the left panel and the two genes (*At2g46250* and *At5g10140*) on the center panel were detected by DNA blot analysis using the corresponding cDNA as probes. The genes on the right panel were detected by ethidium bromide staining. The lower bands correspond to beta-tubulin fragments amplified as a template adjustment control.

All of the *Arabidopsis* lines that had a single T-DNA insertion site were generated by floral dip transformation. Therefore, the floral dip transformation method (Figure 4B) may generate more frequent single insertion sites than does the transformation of hypocotyl-derived calli (Figure 4A). However, a much greater number of lines would have to be analyzed to provide statistical data.

Expression of T-DNA adjacent genes

To examine the efficiency of activation tagging using pHR-AT-GFP T-DNA, five *Arabidopsis* root culture lines were subjected to further analysis because the entire genome sequence (Arabidopsis Genome Initiative, 2000) is available, which facilitated the mapping of the T-DNA insertion sites in the genome. Three root culture lines (lines #1, #3 and #5) with multiple T-DNA insertions (Figure 4A) and line #7 with a single insertion (Figure 4B) were chosen, along with the root culture line #12 (T_2, single insertion). None of these lines showed an obvious root phenotype compared with the other transformed root culture lines.

Genomic sequences adjacent to the T-DNA right border were recovered by plasmid rescue. Despite the multiple T-DNA insertions in lines #1, #3 and #5 (Figure 4A), only the single genomic region flanking the T-DNA right border was recovered from each of these lines, probably because of concatemeric insertion of multiple T-DNAs, in particular, the presence of the inverted repeat of the T-DNA right border. Database searches revealed that three of the T-DNA insertions occurred in the intergenic regions (lines #5, #7 and #12), whereas the remaining two occurred within the transcriptional units (lines #1 and #3). The structures of five different insertion sites are shown schematically in Figure 5A; in these, the position and direction of the adjacent genes relative to the inserted T-DNA are depicted. The distance between the enhancer repeats and the ATGs of these nearest genes, except for the disrupted genes (open arrows), ranged from 0.6 to 4.1 kb.

The expression of the genes adjacent to the T-DNA was examined by Northern or reverse transcription-polymerase chain reaction (RT-PCR) analysis using RNA prepared from root tissues cultured for at least four months in 0.5× MS liquid medium. Of the five root culture lines examined, three lines (#5, #7 and #12) showed a significant increase in the expression level of the corresponding gene adjacent to the right border (AGI accession numbers *At5g02710*, *At2g46250* and *At5g10140* in lines #5, #7 and #12, respectively) compared to those in the other root culture lines (Figure 5B, center panel). Among these three root culture lines, lines #5 and #7 also showed increased expression of corresponding genes that were more distal to the right border, *At5g02730* (ATG locates 3.6 kb away from the right border) and *At2g46240* (ATG locates 7 kb away from the right border), respectively (Figure 5B, right panel).

Furthermore, we investigated whether the gene adjacent to the left border could be activated by the pHR-AT-GFP T-DNA that is approximately 15.5 kb in size. Of the five root culture lines examined, lines #5 and #12 showed a significant increase in the expression level of corresponding genes adjacent to the left border, whose ATG locates more than 16 kb away from the right border enhancer repeats (Figure 5B, left panel). The activation of the gene adjacent to the left border by the right border enhancer repeats was not surprising because a previous report had suggested this possibility (Ichikawa *et al*., 2003). However, it should be noted that the pHR-AT-GFP T-DNA contains a 35S promoter next to the left border to drive the expression of the *GFP* sequence. Therefore, we cannot rule out the possibility that the 35S promoter, rather than the right border enhancer repeats, was the main factor affecting the expression of the left border adjacent gene. A comparison of data on the activated genes in pHR-AT (see Figure 1) and pHR-AT-GFP transformed lines will help to answer this point.

Based on previous reports, T-DNA activation tagging is most likely to activate the nearest gene to the enhancer repeats (Kakimoto, 1996; Kardailsky *et al*., 1999; Weigel *et al*., 2000; Huang *et al*., 2001; Ichikawa *et al*., 2003). However, lines #1 and #3 did not show elevated expression of the gene adjacent to the right border, the gene distal to the right border, or the gene adjacent to the left border (Figure 5B) in the cultured roots. At this stage, we have no convincing explanation for these results. However, this may be explained by the presence of an insulator sequence or the promoter preference of the 35S enhancers (Weigel *et al*., 2000; Jeong *et al*., 2002) or the activation of gene(s) more

distal from either left- or right border cannot be excluded. In addition, it should be noted that in these cases, the T-DNA was inserted within a transcriptional unit (Figure 5A); this may lower the efficiency of activation. Ichikawa *et al.* (2003) reported that the rate of T-DNA insertion within an ORF was slightly lower (28%) in 1262 *Arabidopsis* plants that showed visible phenotypes in the T_1 generation than that in other random knockout populations (30–35%). It is not surprising that the 35S enhancer repeats did not always increase the expression of the nearest gene. Jeong *et al.* (2002) showed that in rice, only four of the 10 genes for which the enhancers were inserted within 1.5–4.5 kb of the genes displayed elevated expression.

Our results clearly showed that the pHR-AT-GFP T-DNA can activate its adjacent gene(s) in cultured roots (Figure 5). However, the 35S enhancer is relatively less active in roots than in shoots (Benfey and Chua, 1989). Moreover, as shown by recent activation tagging experiments, the 35S enhancer is likely to increase the expression of nearby genes without altering the original expression pattern, rather than causing ectopic overexpression (Neff *et al.*, 1999; Weigel *et al.*, 2000; Jeong *et al.*, 2002). Therefore, to fully exploit the potential of the hairy root-activation tagging system, further improvement of the vectors is needed, for example, using an enhancer or promoter that confers a high level of expression in hairy roots, such as the *parAt* gene promoter (Muranaka *et al.*, 1994). An additional limitation of the current version of pHR-AT-GFP vector is creation of double-headed activation tagging, possibly affected by 35S promoter locating next to the left border (see Figure 1), which will make it time-consuming process to find out which gene is responsible for the phenotypes. Therefore, alteration of the placement and/or the orientation of GFP expression cassette within T-DNA will be desired.

We observed a significant increase in the expression of *At5g10140* and *At5g10150* genes in root culture line #12. The parent T_1 plant showed a severe late-flowering phenotype (Figure 3F). In contrast to the *At5g10150* gene whose biological function remains unknown, the *At5g10140* gene has been identified as the *FLOWERING LOCUS F* (*FLF*) gene encoding a member of the MADS box class of transcription factors that repress floral initiation (Sheldon *et al.*, 1999). The defined biological function of *FLF* as a repressor of flowering, combined with the facts that (1) the #12 plant (T_1) has a single T-DNA insertion (Figure 4B), (2) the #12 plant (T_1) showed increased expression of *FLF* in rosettes (data not shown), and (3) the *flf–1* mutant plant (with a high level of *FLF* gene expression in rosettes) shows a dome shape (Sheldon *et al.*, 1999) extremely similar to that of the #12 plant (Figure 3F), implies that the elevated expression of *FLF* caused the late-flowering phenotype of the #12 (T_1) plant (Figure 3F). Because it had no obvious root phenotype, we did not try to recapitulate the late-flowering phenotype of the #12 (T_1) plant with a construct overexpressing the *FLF* gene. Recently, we developed another vector set for overexpression and RNA interference (RNAi) of the target gene in cultured roots (Seki *et al.*, unpublished results) using the same *rol* gene cluster used for the vectors described here. Once a candidate gene that yields the desired phenotype is isolated using hairy root-activation tagging, its function can be defined using these vectors. However, it should be noted that possible involvement of the combinatorial effects from the activation of candidate gene and the function of *rol* gene product on the desired phenotype cannot be eliminated.

Perspective

Potential of the hairy root-activation tagging system

Activation tagging has been applied primarily to plant species for which a high-efficiency transformation procedure has been established. However, the production of transgenic plants remains difficult and slow for most species. We developed the vectors pHR-AT and pHR-AT-GFP for hairy root-activation tagging, and these can be used to generate a large number of activation-tagged root clones in *Arabidopsis*, potato, tobacco, and probably other plant species as well. The hairy root-activation tagging system may facilitate the application of the activation-tagging approach to plant species that are recalcitrant to the regeneration of transgenic plants. If successful, this approach could be extended to tree species in which the induction of hairy roots by inoculation of *A. rhizogenes* has been reported (Damiano and Monticelli, 1998, and references therein).

Plant root systems are exposed to various biotic and abiotic environmental stresses. However, the mechanisms by which plant root systems tolerate these stresses are poorly understood, compared with our knowledge of aboveground organs. To dissect the mechanisms of stress tolerance and isolate underlying genes, pHR-AT- or pHR-AT-GFP-inoculated explants or induced/excised roots could be exposed to various stress conditions of interest, and screened for "resistant" root clones. As another strategy, the hairy root-activation tagging system could be applied to a targeted activation-tagging approach. Grant *et al.* (2003) conducted activation tagging in a transgenic *Arabidopsis* line containing a *PR−1 promoter::luciferase* (*LUC*) reporter construct in order to isolate dominant mutants with constitutively enhanced pathogenesis-related−1 (*PR−1*) gene expression. The expression of *PR−1* is a reliable marker for the establishment of systemic acquired resistance (SAR) in *Arabidopsis*. Hairy root-activation tagging could be undertaken in a transgenic background, pre-transformed with the reporter gene under the control of certain gene promoters involved in the stress signaling pathway of interest, to isolate root clones exhibiting altered reporter gene expression.

High-throughput metabolic profiling will be promising approach for screening activation-tagged root culture lines. Targeted metabolite analysis can be performed in plant species that produce specific valuable metabolites. Hairy root cultures have served as a useful model system in many so-called "exotic" plants to investigate the production of a variety of secondary metabolites, including compounds of pharmaceutical value (for a review, see Shanks and Morgan, 1999). Recently, Woo *et al.* (2004) used liquid chromatography/mass spectrometry (LC/MS) and high-performance liquid chromatography with ultraviolet (HPLC-UV) analyses to screen 993 independent *Panax ginseng* hairy root lines transformed with Ri T-DNA for ginsenoside-overproducing lines. A similar approach could be applied for activation-tagged root culture lines to identify the regulatory or biosynthetic genes for the production of secondary metabolites of interest.

Activation-tagged *Arabidopsis* root culture lines will provide a useful model system for studying other aspects of root biology. Plant roots produce and secrete secondary metabolites, potentially acting in plant–microbe or plant–plant interactions, plant defense, or in changing the chemical and physical properties of the soil to shape their immediate environment, i.e., the rhizosphere. To facilitate our understanding of the molecular basis of these biological processes, several researchers have used *Arabidopsis* as a model system. Using HPLC analysis, Walker *et al.* (2003) detected 289 possible secondary metabolites in root exudates of *Arabidopsis* with various elicitor treatments, versus 68 with no elicitation. Bouwmeester *et al.* (2003) reported that exudates from *Arabidopsis* hairy roots contain germination stimulant(s) for *Orobanche ramosa*, a parasitic weed that infects agricultural crops. However, little is known about the biosynthesis pathway of secreted metabolites and how this pathway is regulated, or about the mechanisms that drive and regulate root secretion. We developed an easy, high-throughput system for establishing activation-tagged *Arabidopsis* root culture lines (Figure 3C). This system has two advantages: (1) root biomass under tightly controlled sterile conditions can be readily obtained from each activation-tagged clone, and (2) root culture lines can be maintained for a long period, which is attractive for the non-targeted metabolic analysis of root exudates. This approach will identify genes involved in root-specific secondary metabolism or secretion, or in regulating these processes in response to various environmental stimuli.

The vectors described in this paper are available to academic researchers for non-commercial projects.

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References

Akama, K., Shiraishi, H., Ohta, S., Nakamura, K., Okada, K. and Shimura, Y. 1992. Efficient transformation of

Arabidopsis thaliana: comparison of the efficiencies with various organs, plant ecotype and *Agrobacterium* strains. Plant Cell Rep. 12: 7–11.

An, G., Ebert, P., Mitra, A. and Ha, S. 1988. Binary vectors. In: S.B. Gelvin and R.A. Schilperoort (Eds.), Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, pp. 1–19.

Aoki, S. and Syono, K. 1999. Synergistic function of *rolB*, *rolC*, ORF13 and ORF14 of TL-DNA of *Agrobacterium rhizogenes* in hairy root induction in *Nicotiana tabacum*. Plant Cell Physiol. 40: 252–256.

Arabidopsis Genome Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 0: 796–815.

Baumann, K., De Paolis, A., Costantino, P. and Gualberti, G. 1999. The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the *rolB* oncogene in plants. Plant Cell 11: 323–333.

Benfey, P.N. and Chua, N-H. 1989. The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. 8: 2195–2202.

Bouwmeester, H.J., Matusova, R., Zhongkui, S. and Beale, M.H. 2003. Secondary metabolite signalling in host–parasitic plant interactions. Curr. Opin. Plant Biol. 6: 358–364.

Chilton, M.D., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F. and Tempe, J. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature 295: 432–434.

Chiu, W.-I., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J. 1996. Engineered GFP as a vital reporter in plants. Curr. Biol. 6: 325–330.

Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal. Biochem. 201: 134–139.

Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735–743.

Damiano, C. and Monticelli, S. 1998. In vitro fruit trees rooting by *Agrobacterium rhizogenes* wild type infection. Electron. J. Biotechnol. [online]. 15 December 1998, vol. 1, no. 3. Available from: http://www.ejbiotechnology.info/content/vol1/issue3/full/4/index.html. ISSN: 0717–3458.

Grant, J.J., Chini, A., Basu, D. and Loake, G.J. 2003. Targeted activation tagging of the *Arabidopsis* NBS-LRR gene, *ADR1*, conveys resistance to virulent pathogens. Mol. Plant Microbe Interact. 16: 669–680.

Handayani, N.S.N., Moriuchi, H., Yamakawa, M., Yamashita, I., Yoshida, K. and Tanaka, N. 2005. Characterization of the *rolB* promoter on mikimopine-type pRi1724 T-DNA. Plant Sci. 168: 1353–1364.

Huang, S., Cerny, R.E., Bhat, D.S. and Brown, S.M. 2001. Cloning of an *Arabidopsis* patatin-like gene, *STURDY*, by activation T-DNA tagging. Plant Physiol. 125: 573–584.

Ichikawa, T., Nakazawa, M., Kawashima, M., Muto, S., Gohda, K., Suzuki, K., Ishikawa, A., Kobayashi, H., Yoshizumi, T., Tsumoto, Y., Tsuhara, Y., Iizumi, H., Goto, Y. and Matsui, M. 2003. Sequence database of 1172 T-DNA insertion sites in *Arabidopsis* activation-tagging lines that showed phenotypes in T1 generation. Plant J. 36: 421–429.

Jeong, D.H., An, S., Kang, H.G., Moon, S., Han, J.J., Park, S., Lee, H.S., An, K. and An, G. 2002. T-DNA insertional mutagenesis for activation tagging in rice. Plant Physiol. 130: 1636–1644.

Kakimoto, T. 1996. CKI1, A histidine kinase homolog implicated in cytokinin signal transduction. Science 274: 982–985.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. 1999. Activation tagging of the floral inducer *FT*. Science 286: 1962–1965.

Karimi, M., Van Montagu, M. and Gheysen, G. 1999. Hairy root production in *Arabidopsis thaliana*: cotransformation with a promoter-trap vector results in complex T-DNA integration patterns. Plant Cell Rep. 19: 133–142.

Komarnytsky, S., Gaume, A., Garvey, A., Borisjuk, N. and Raskin, I. 2004. A quick and efficient system for antibiotic-free expression of heterologous genes in tobacco roots. Plant Cell Rep. 22: 765–773.

Mathews, H., Clendennen, S.K., Caldwell, C.G., Liu, X.L., Connors, K., Matheis, N., Schuster, D.K., Menasco, D.J., Wagoner, W., Lightner, J. and Wagner, D.R. 2003. Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15: 1689–1703.

Muranaka, T., Niwa, Y. and Machida, Y. 1994. A model for a bioconversion system with the promoter of the *parAt* gene, which confers a high level of expression of a transgene in hairy roots. Appl. Microbiol. Biotechnol. 40: 481–485.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco culture. Physiol. Plant 15: 473–497.

Neff, M.M., Nguyen, S.M., Malancharuvil, E.J., Fujioka, S., Noguchi, T., Seto, H., Tsubuki, M., Honda, T., Takatsuto, S., Yoshida, S. and Chory, J. 1999. *BAS1*: a gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 83: 735–739.

Nillson, O. and Olsson, O. 1997. Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. Physiol. Plant. 100: 463–473.

Niwa, Y. 2003. A synthetic green fluorescent protein gene for plant biotechnology. Plant Biotechnol. 20: 1–11.

Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. and Kobayashi, H. 1999. Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. Plant J. 18: 455–463.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Schmulling, T., Schell, J. and Spena, A. 1988. Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J. 7: 2621–2629.

Shanks, J.V. and Morgan, J. 1999. Plant 'hairy root' culture. Curr. Opin. Biotechnol. 10: 151–155.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J. and Dennis, E.S. 1999. The *FLF* MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. Plant Cell 11: 445–458.

Simpson, R.B., Spielmann, A., Margossian, L. and McKnight, T.D. 1986. A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes*. Plant Mol. Biol. 6: 403–415.

Spena, A., Schmulling, T., Koncz, C. and Schell, J. 1987. Independent and synergistic activities of the *rolA*, *B*, and *C* loci in stimulating abnormal growth in plants. EMBO J. 6: 3891–3899.

Spertini, D., Beliveau, C. and Bellemare, G. 1999. Screening of transgenic plants by amplification of unknown genomic DNA flanking T-DNA. Biotechniques 27: 308–314.

Stewart Jr, C.N. 2001. The utility of green fluorescent protein in transgenic plants. Plant Cell Rep. 20: 376–382.

Tanaka, N., Ikeda, T. and Oka, A. 1994. Nucleotide sequence of the *rol* region of the mikimopine-type root-inducing plasmid pRi1724. Biosci. Biotechnol. Biochem. 58: 548–551.

Tani, H., Chen, X., Nurmberg, P., Grant, J.J., SantaMaria, M., Chini, A., Gilroy, E., Birch, P.R.J. and Loake, G.J. 2004. Activation tagging in plants: a tool for gene discovery. Funct. Integr. Genomics 4: 258–266.

Tepfer, D. 1984. Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. Cell 37: 959–967.

Tepfer, D. 1990. Genetic transformation using *Agrobacterium rhizogenes*. Physiol. Plant 79: 140–146.

Uozumi, N. 2004. Large-scale production of hairy root. Adv. Biochem. Eng. Biotechnol. 91: 75–103.

van der Fits, L. and Memelink, J. 2000. ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295–297.

Visser, R.G.H., Kuipers, A.G.J. and Jacobsen, E. 1996. Antisense granulebound starch synthase effects in potato. In: D. Grierson, G.W. Lycett and G.A. Tucker (Eds.), Mechanisms and Applications of Gene Silencing, Nottingham University Press, Nottingham, pp. 97–104.

Walden, R., Fritze, K., Hayashi, H., Miklashevichs, E., Harling, H. and Schell, J. 1994. Activation tagging: a means of isolating genes implicated as playing a role in plant growth and development. Plant Mol. Biol. 26: 1521–1528.

Walker, T.S., Bais, H.P., Halligan, K.M., Stermitz, F.R. and Vivanco, J.M. 2003. Metabolic profiling of root exudates of *Arabidopsis thaliana*. J. Agric. Food Chem. 51: 2548–2554.

Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Frankhauser, C., Lamb, C.J., Yanofsky, M.F. and Chory, J. 2000. Activation tagging in *Arabidopsis*. Plant Physiol. 122: 1003–1013.

Woo, S.S., Song, J.S., Lee, J.Y., In, D.S., Chung, H.J., Liu, J.R. and Choi, D.W. 2004. Selection of high ginsenoside producing ginseng hairy root lines using targeted metabolic analysis. Phytochemistry 65: 2751–2761.

Yamamoto, Y.Y., Tsuhara, Y., Gohda, K., Suzuki, K. and Matsui, M. 2003. Gene trapping of the *Arabidopsis* genome with a firefly luciferase reporter. Plant J. 35: 273–283.