Agrobacterium tumefaciens and Agrobacterium rhizogenes transformed roots of the parasitic plant Triphysaria versicolor retain parasitic competence

Alexey Tomilov · Natalya Tomilova · John I. Yoder

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Abstract Parasitic plants in the Orobanchaceae invade roots of neighboring plants to rob them of water and nutrients. Triphysaria is facultative parasite that parasitizes a broad range of plant species including maize and Arabidopsis. In this paper we describe transient and stable transformation systems for Triphysaria versicolor Fischer and C. Meyer. Agrobacterium tumefaciens and Agrobacterium rhizogenes were both able to transiently express a GUS reporter in Triphysaria seedlings following vacuum infiltration. There was a correlation between the length of time seedlings were conditioned in the dark prior to infiltration and the tissue type transformed. In optimized experiments, nearly all of the vacuum infiltrated seedlings transiently expressed GUS activity in some tissue. Calluses that developed from transformed tissues were selected using non-destructive GUS staining and after several rounds of in vivo GUS selection, we recovered uniformly staining GUS calluses from which roots were subsequently induced. The presence and expression of the transgene in Triphysaria was verified using genomic PCR, RT PCR and Southern hybridizations. Transgenic roots were also obtained by inoculating A. rhizogenes into wounded Triphysaria seedlings. Stable transformed roots were identified using GUS staining or fluorescent microscopy following transformation with vectors containing GFP, dsRED or EYFP. Transgenic roots derived from both A. tumefaciens and A. rhizogenes transformations were morphologically normal and developed haustoria that attached to and invaded lettuce roots. Transgenic roots also remained competent to form haustoria in response to purified inducing factors. These transformation systems will allow an in planta assessment of genes predicted to function in plant parasitism.

Keywords Parasitic plants · Gene transformation · Orobanchaceae · Triphysaria

Abbreviations
- DMBQ 2,4-Dimethoxybenzoquinone
- IAA Indole acetic acid
- 6-BAP 6-Benzylaminopurine

Introduction

Parasitism is a lifestyle that has been repeatedly adapted throughout evolution. Several thousand-plant species are parasitic and able to obtain at least some of their nutrients by directly invading other plants (Kuijt 1969). Phylogenetic analyses of extant species suggest that parasitic plants originated at least 12 times independently in angiosperm evolution (Nickrent 2005). Consequently, parasitic plant species display extensive variations in growth habits, host and tissues parasitized and heterotrophic dependency. Plants in the Orobanchaceae, a family recently revised to include parasitic Scrophulariaceae, parasitize neighboring plants through their roots (Musselman 1980; Olmstead et al. 2001). The host range of Orobanchaceae includes many important crop plants making root parasites amongst the world’s most devastating agricultural pests (Parker and Riches 1993).

Alexey Tomilov and Natalya Tomilova made an equal contribution in the paper.

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Orobanchaceae import water, minerals and fixed carbon resources directly from host roots, but the degree to which they are dependent on these resources varies considerably. At one extreme are obligate parasites like *Striga* and *Orobanche* that have inefficient or absent photosynthesis systems and require host resources from germination through flowering (Boone et al. 1995; Wigchert and Zwanenburg 1999). At the other extreme are facultative parasites like *Triphysaria* and *Rhinanthus* that are able to survive as free-living plants but will parasitize a host plant if one is available (Atsatt and Strong 1970; Gibson and Watkinson 1989). Typical of facultative parasites, *Triphysaria* has a broad host range that includes at least 20 monocotyledon and eudicot families, including maize, rice and *Arabidopsis* (Atsatt and Strong 1970; Goldwasser et al. 2002).

Parasitic plants invade host roots through a specialized structure called the haustorium (Riopel and Timko 1995). Haustoria are initiated to develop in Orobanchaceae roots through a combination of allelochemical and tactile effectors that ensure the parasite is close to a host root before initiating haustorium development (Lynn and Chang 1990; Yoder 2001). The attachment and invasion of host roots by *Triphysaria* can be monitored in vitro by overlaying aseptic *Triphysaria* and host roots. Haustorium development is induced in the absence of host roots by applying host root exudates or purified haustoria inducing phenolics directly to *Triphysaria* roots (Albrecht et al. 1999; Jamison and Yoder 2001). Under these conditions haustoria develop within 12 h near most of the *Triphysaria* root tips. Attachment is mediated through haustorial hairs which firmly connect the two roots and make it possible for intrusive cells within the haustorial body to invade the host root cortex (Baird and Riopel 1985). Once the host stele has been contacted, xylem elements differentiate within the haustorium and provide an obvious conduit for robbing water, amino acids, organic acids, and ions from the host xylem sap (Dörr 1997). The nature of parasite–host phloem connections is less obvious and seems to vary among species (Hibberd and Dieter Jeschke 2001). Sieve elements were identified in *Orobanche* and *Striga asiatica* but no obvious phloem–phloem linkages (Kuijt and Toth 1976). In contrast, interspecific plasmodiogonia were observed in *Striga gesneriodes–Pisum* associations and between adjacent sieve tubes in *Orobanche crenata* associations (Dörr 1996; Dörr and Kollman 1995). Extensively invaginated transfer cells have been described that apparently link the host and parasite vasculature in *Triphysaria* haustoria (Heide-Jorgensen and Kuijt 1993, 1995).

Cloning genes for haustorium development will provide insights into how plants originate parasitism during evolution. Also, genes responsible for haustorium development are potential intervention targets for engineering genetic resistance to parasitic weeds. To begin the identification of genes responsible for haustorium development, EST databases have been developed representing transcripts regulated in *Triphysaria* roots before and after contact with host roots and factors (Torres et al. 2005). A gene transformation system is critical to determine the function of these genes in parasitic plants. We describe here transient and stable transformation systems for monitoring in planta functions of parasite genes in *Triphysaria* roots.

Both *Agrobacterium tumefaciens* and *A. rhizogenes* mediate gene transfer into plants through a conserved type IV secretion–conjugation mechanism (Chen et al. 2005). Elimination of the tumor inducing genes on the *A. tumefaciens* Ti plasmid yields disarmed T-DNA vectors that can be used to introduce foreign genes into plants (Fraley et al. 1986; Lichtenstein and Fuller 1987). Infections with *A. rhizogenes* stimulate hairy root development because of the root locus genes (rol) on the Ri plasmid (Chilton et al. 1982; Tepfer 1984). Co-transformation with the Ri plasmid and a second T-DNA carrying a gene of interest can result in transgenic roots emerging from non-transgenic plants (Christey 2001; Limpens et al. 2004; Tepfer et al. 1989; Visser et al. 1989). In many cases the transgenic roots on these composite plants appear normal in morphology and function. *Agrobacterium rhizogenes* transformed legume roots develop nitrogen fixing nodules and support endomycorrhizal symbioses (Boisson-Dernier et al. 2001; Hansen et al. 1989; Jensen et al. 1986). Similarly, *A. rhizogenes* root cultures support the growth of soybean cyst nematodes and have been used to study the function of nematode resistance genes in tomato (Cho et al. 2000; Hwang et al. 2000). *Agrobacterium rhizogenes* transformation has also been used to deliver inhibitor RNA constructions into *Medicago* and *Lotus* roots where their inhibitory phenotypes can be assessed. (Kumagai and Kouchi 2003; Limpens et al. 2004). For some species *Agrobacterium rhizogenes* transformed roots can be regenerated into fertile plants (Crane et al. 2006).

An alternative approach to investigate transgene function is to transiently express the gene or gene product by infiltrating plant tissues with *Agrobacterium* cultures. The approach is considered transient when the transgenic tissue does not undergo cell division and the phenotype is directly assayed in Agro-infiltrated tissue (Bendahmane et al. 2000; Janssen and Gardner 1990; Johansen and Carrington 2001; Schob et al. 1997; Wroblewski et al. 2005). The T-DNA carried by the infiltrating *Agrobacterium* is integrated into the genomes of some cells and when these are selected for
subsequent to mitosis, stable lines of transformed cells can be obtained.

We examined transient and stable transformation systems for expressing transgenes in *Triphysaria* roots. *Agrobacterium tumefaciens* and *A. rhizogenes* strains were evaluated for their ability to introduce a GUS reporter construct into *Triphysaria* following vacuum infiltration, co-inoculation, wound inoculation, or hypocotyl dips. Three fluorescence proteins were compared to GUS to determine their effectiveness in marking transgenic roots. Transgenic calluses were selected by non-destructive GUS staining which proved more effective than selection with antibiotics. Uniformly stained calluses were regenerated into transgenic root cultures that retained their ability to develop haustoria and infect host roots. The GUS reporter construct allows unambiguous distinction between host and parasite cells in haustorial connections.

**Materials and methods**

Plants, plasmids and bacteria

*Triphysaria versicolor* seeds were collected from hundreds of open pollinated plants growing in a cow pasture south of Napa California. Seeds from the lettuce cultivar Monishe were obtained from Dr. R. Michelmore (UC Davis, CA, USA).

The plasmid pCAMBIA 1305.2 is a T-DNA based vector bearing a GUSPlus reporter gene under the control of a CaMV 35S promoter. A castor bean catalase intron interrupts the GUS reading frame and eliminates protein expression in bacteria. The effectiveness of this intron was confirmed by staining the bacteria with X-Glu and not detecting any GUS activity. The GUSPlus gene contains a glycine rich protein secretion signal sequence that facilitates movement of the protein to the apoplastic space. The plasmid has genes for selecting kanamycin resistance in bacteria and hygromycin resistance in plants and was CAMBIA (CAMBIA 2006).

pTFS40 is a T-DNA based vector containing a GUS reporter gene under the control of CaMV 35S promoter. This GUS gene is also interrupted by a plant intron (Chang et al. 2002). pTFS40 carries a kanamycin resistance gene for selection in bacteria and a hygromycin resistance gene for selection in plants. The plasmid was obtained from Dr. Richard Michelmore (UC Davis, CA, USA).

The plasmid pBIN-mgf5-ER contains a GFP gene containing an N-terminal signal peptide from a vacuolar chitinase and a C-terminal HDEL motif. Together these motifs direct the secretion and retention of the GFP protein to endoplasmic reticulum (Haseloff et al. 1997). The plasmid carries two kanamycin resistance genes, one for selection in bacteria and one for selection in plants.

The plasmid pBIN-YFP contains the *Aequorea victoria* enhanced yellow fluorescence protein (EYFP) gene (Clonetech, Mountain View, CA, USA). It was cloned into the CaMV 35S promoter of pBIN for expression in plants and obtained from Dr. Albrecht Von–Arnim (University of Tennessee, TN, USA) (Subramanian et al. 2006).

The plasmid pRedRoot is a binary vector containing the red fluorescence marker DsRED under the control of an AtUBQ10 promoter (Limpens et al. 2004). pRedRoot contains kanamycin resistance for selection in bacteria and DsRED for selection in plants. pRedRoot was obtained from Dr. Rene Geurts (Wageningen University, Netherlands).

The *Agrobacterium* strains used in this study are shown in Table 1. Growth media for the bacteria are described in the references. The T-DNA plasmids were introduced into *Agrobacterium* by electroporation (Sambrook and Russell 2001).

DMBO was purchased from Pfaltz and Bauer, Inc. (Waterbury, CT, USA). Indole acetic acid (IAA) and cytokinin (6-BAP) were purchased from Sigma (St Louis, MO, USA) and X-Glu from Gold Bio Technology, Inc. (St Louis, MO, USA).

**Transformation protocols**

*Triphysaria* seeds were surface sterilized in 50% (v/v) bleach [sodium hypochlorite 2.13% (w/v) final] and 0.1% (v/v) Triton X-100 (Sigma, St Louis, MO, USA), rinsed in several volumes of sterile de-ionized water and germinated at 16°C as described (Jamison and Yoder 2001). *Triphysaria* seedlings and cultures were typically maintained in 0.25× Hoagland’s medium [1.25 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1.25 mM KNO<sub>3</sub>, 0.25 mM KH<sub>2</sub>P O<sub>4</sub>, 0.50 mM MgSO<sub>4</sub>, and micronutrients (50 mM H<sub>3</sub>BO<sub>3</sub>, 9.0 mM MnCl<sub>2</sub>·4H<sub>2</sub>O, 70 nM ZnSO<sub>4</sub>·7H<sub>2</sub>O, 30 nM CuSO<sub>4</sub>·5H<sub>2</sub>O, and 10 nM Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O)] supplemented with 1% (w/v) sucrose and 0.5% (w/v)

<table>
<thead>
<tr>
<th>Table 1</th>
<th><em>Agrobacterium</em> strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>A281</td>
<td><em>A. tumefaciens</em></td>
</tr>
<tr>
<td>C58</td>
<td><em>A. tumefaciens</em></td>
</tr>
<tr>
<td>GV3101</td>
<td><em>A. tumefaciens</em></td>
</tr>
<tr>
<td>ATCC 15834</td>
<td><em>A. rhizogenes</em></td>
</tr>
<tr>
<td>VIGG 15834</td>
<td><em>A. rhizogenes</em></td>
</tr>
<tr>
<td>A4RS</td>
<td><em>A. rhizogenes</em></td>
</tr>
<tr>
<td>MSU440</td>
<td><em>A. rhizogenes</em></td>
</tr>
</tbody>
</table>
Phytagar (Gibco-BRL Life Technologies, Rockville, MD, USA) (Hoagland and Arnon 1938).

Transformation efficiencies were estimated from the number of GUS positive tissues after staining with 500 μg/ml of X-Glu (Gold Bio Technology, Inc) and destaining in 70% ethanol (Jefferson et al. 1987). Stained materials were examined under a Carl Zeiss Stemi SV 11 stereomicroscope (Microimaging, Inc., Thornwood, NY, USA). Inoculations with medium without Agrobacterium or with Agrobacterium but without vector were used as negative controls.

Vacuum infiltration

Vacuum infiltration of Triphysaria seedlings with Agrobacterium was previously described (Tomilov et al. 2005). After germination, Triphysaria seedlings were placed into Petri dishes containing Hoagland’s medium, covered with aluminum foil, and placed into a 4°C cold room for several months. Prior to transformation, Agrobacterium were grown overnight, harvested by centrifugation, and resuspended in MS medium at an OD_{600} of 0.5. Triphysaria seedlings were removed from cold storage, mixed with the Agrobacterium suspension, placed into a plastic syringe and subjected to a gentle vacuum that was applied by hand pulling on the syringe plunger. After 10 min the explants were removed from the Agrobacterium solution and placed into fresh MS medium for 2 days with shaking at 40 rpm. The explants were then stained non-destructively with X-Glu by transferring onto solid medium supplemented with 50 μg/ml X-Glu. The GUSPlus protein is secreted and stains the agar around the tissue blue (Tuan Nguyen, CAMBIA, personal communication). Alternatively, plant material was immersed in MS medium containing 100–200 μg/ml X-Glu and stained at room temperature while shaking at 40 rpm. Positive stained tissues were blotted dry and placed on medium without X-Glu for 2 days and then into callus inducing medium (1× Hoagland’s, 2% glucose, 5.4 μM NAA, 0.44 μM 6-BAP, 1% Gelrite (Sigma)). Agrobacterium strains were inhibited using 120 mg/l of timentin (Glaxo Smith Kline, NC, USA) except A 281 and MSU 440, which were selected against with 500 mg/l of cefotaxime.

Co-cultivation

Triphysaria explants were obtained by cutting seedlings and root cultures with a razor into approximately 5–10 mm pieces and co-cultivated 10 min at room temperature with mid-log phase Agrobacterium cultures diluted 1–5 with MS medium (Minlong et al. 2000). Explants were blotted dry, transferred onto square Petri dishes containing solidified Hoagland’s medium, wrapped with parafilm and incubated at 25°C. After 2 days the explants were rinsed with sterile water and transferred onto fresh medium supplemented with 250 mg/l cefotaxime or timentin. After approximately 1 month calluses were stained for GUS expression or visually monitored for GFP as appropriate.

Table 2 Efficiency of primary transformation using different Agrobacterium strains and infection methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>C58 (Ti)</th>
<th>A281 (Ti)</th>
<th>GV3101 (Ti)</th>
<th>A4RS (Ri)</th>
<th>ATCC 15834 (Ri)</th>
<th>VIGG 15834 (Ri)</th>
<th>MSU440 (Ri)</th>
<th>Labor^1 (h/1,000)</th>
<th>K(re)^2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vacuum infiltration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seedlings</td>
<td>2.00 ± 1.73^{a,b}</td>
<td>0.00</td>
<td>0.00</td>
<td>2.80 ± 1.48^{a}</td>
<td>0.00</td>
<td>0.13 ± 0.25^{b}</td>
<td>1.60 ± 1.95^{a,b}</td>
<td>0.20</td>
<td>14.00</td>
</tr>
<tr>
<td>Germinating seeds</td>
<td>0.33 ± 0.58{a}</td>
<td>–</td>
<td>–</td>
<td>0.33 ± 0.58^{a}</td>
<td>0.11 ± 0.19^{a}</td>
<td>0.00</td>
<td>–</td>
<td>0.20</td>
<td>1.65</td>
</tr>
<tr>
<td>Root cultures</td>
<td>0.55 ± 0.51^{a,b}</td>
<td>0.00</td>
<td>0.00</td>
<td>0.67 ± 0.58^{a}</td>
<td>0.11 ± 0.19^{b}</td>
<td>0.00</td>
<td>–</td>
<td>0.20</td>
<td>3.35</td>
</tr>
<tr>
<td><strong>Co-cultivation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seedlings</td>
<td>1.00 ± 1.00^{a}</td>
<td>0.00</td>
<td>0.33 ± 0.58^{a}</td>
<td>0.33 ± 0.58^{a}</td>
<td>0.00</td>
<td>0.00</td>
<td>–</td>
<td>5.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Root cultures</td>
<td>0.40 ± 0.55^{a}</td>
<td>0.40 ± 0.89^{a}</td>
<td>0.00</td>
<td>0.40 ± 0.55^{a}</td>
<td>0.00</td>
<td>0.20 ± 0.45^{b}</td>
<td>–</td>
<td>5.00</td>
<td>0.08</td>
</tr>
<tr>
<td>Cell cultures</td>
<td>0.00</td>
<td>0.00</td>
<td>2.00 ± 2.65</td>
<td>0.00</td>
<td>0.00</td>
<td>–</td>
<td>–</td>
<td>5.00</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>Microinjection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seedlings</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.11 ± 0.19^{b}</td>
<td>0.55 ± 0.51^{a,b}</td>
<td>1.44 ± 1.71^{a}</td>
<td>–</td>
<td>50.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Hypocotyl dip</td>
<td>8.33 ± 3.81^{a}</td>
<td>–</td>
<td>–</td>
<td>0.00</td>
<td>8.06 ± 2.41^{a}</td>
<td>10.00</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each bacteria/transformation combination, the frequency of transformation is determined as the percentage of explants having at least one sector of tissue staining for GUS. Values with similar letters in each row indicate no significant difference using the Student-Newman–Keuls test at P ≤ 0.05 (SAS Version 9.1, SAS Institute Inc., Cary, NC, USA)

a Labor is approximately the number of hours one person needs to transform 1,000 explants

b K (re) is a coefficient of reliability calculated as best transient transformation efficiency divided by the labor cost. More robust transient transformation methods have higher K (re) values.
Microinjection

Suspensions of *A. rhizogenes* at an OD$_{600}$ of 0.5 in MS medium were inoculated into *Triphysaria* hypocotyls using a glass capillary. Inoculated plantlets were placed on 0.25× Hoagland’s medium and after a few weeks roots emerged from the site of inoculation. Reporter gene expression was monitored in the roots after 1 month of growth.

Hypocotyl dip

Reporter plasmids were introduced into *Triphysaria* roots using *A. rhizogenes* mediated transformation of hypocotyls as described for *Medicago* (Limpens et al. 2004). Roots were excised from 10-day-old *Triphysaria* seedlings and the cut ends dipped into colonies of *Agrobacterium* growing on a plate. The rootless, inoculated seedlings were then transferred onto filter paper placed on the surface of an agar plate containing 0.25× Hoagland’s medium. The plates were vertically oriented and placed into a 25°C growth room for 2–6 weeks over which time transgenic and non-transgenic roots emerged from the cut ends. Transformed root were identified by GUS staining or by visualization of fluorescent markers. Throughout this period *Agrobacterium* growth was minimized by growing the inoculated seedlings on filter paper and wrapping the plates with Micropore tape to allow good air circulation.

Callus development and selection

Callus formation was induced by plating on callus inducing medium [1× Hoagland’s containing 5.4 µM...

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**Fig. 1** GUS staining of primary transformed tissues and derived calluses. GUS activity in (a) roots, (b) hypocotyls and cotyledons and (e) shoot meristem 4 days after vacuum infiltration with *A. tumefaciens*. d GUS activity in flower bud that developed on a transgenic shoot. Non-destructive GUS staining of (e) transgenic roots, (f) transgenic callus and (g) non-transformed callus. Transgenic calluses (identified by non-destructive GUS staining) on medium (h) without antibiotics, (i) with 100 µg/ml kanamycin, or (j) with 12.5 µg/ml hygromycin. (k) *Triphysaria* cell cultures co-incubated with *A. tumefaciens* GV3101. GFP transgenic and control calluses, respectively, under (l, m) white light or (n, o) fluorescence. Uniformly GUS staining calluses obtained from (p) vacuum infiltration with *A. tumefaciens* CS8 or (q) hypocotyl dip into *A. rhizogenes* MSU440. r Differentiation of leaf-like structures after about 1 year in culture. (s, t) Haustorium development in transgenic *Triphysaria* roots treated with 30 µM DMBQ and stained with X-Glu GUS 24 h later...
NAA, 0.44 μM 6-BAP, 2% (w/v) glucose and 1% of Gelrite (Sigma). Root cultures were maintained in Hoagland’s medium and 2% (w/v) glucose with monthly transplants onto 23 μM IAA to induce secondary root development (Tomilov et al. 2004). Cell cultures were obtained by shaking finely divided, non-transgenic
calluses in liquid Hoagland’s medium supplemented with 2% glucose, 5.4 μM NAA and 0.44 μM 6-BAP. Cell cultures were shaken for 1 month at 25°C at which time they were co-cultivated with Agrobacterium as described above.

For antibiotic selection, transformed calluses were subdivided and aliquots plated in medium containing 100 μg/ml of kanamycin or 12.5 μg/ml of hygromycin, depending on the vector. Other pieces of callus were monitored by GFP fluorescence or GUS staining.

Southern hybridization and PCR analyses

Genomic DNA for Southern blots was isolated from calluses as described (Rogers and Bendish 1988). Genomic DNA was digested with Hind III restriction enzyme; fragments were separated on 1% agarose gels and blotted onto Zeta-Probe GT blotting membrane nylon membrane according to the recommendations of the manufacturer (Bio–Rad Laboratories, Hercules, CA, USA). The blots were hybridized in 7.0% SDS, 6.7 SSPE at 65°C (Sambrook et al. 2001).

The hybridization probe was a GUSPlus fragment PCR amplified from pCAMBIA 1305.2 and radiolabeled by random priming using Ready-To-Go DNA Labeling Beads (Amersham Biosciences, England).

### Table 3 Optimum time for in vivo GUS selection of seedlings and calluses

<table>
<thead>
<tr>
<th>Time of in vivo staining (h)</th>
<th>Percent viability (%)</th>
<th>Seedlingsa</th>
<th>Callusesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>89.9 ± 9.2</td>
<td>100 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>17.9 ± 9.0</td>
<td>87.4 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>0.0</td>
<td>15.6 ± 8.8</td>
<td></td>
</tr>
</tbody>
</table>

a Stained in liquid medium with 200 μg/ml X-Glu

b Stained on solid medium with 50 μg/ml X-Glu

Values are means with standard deviations from three to four independent experiments with 15–100 explants each

The GUSPlus fragment was amplified using the following primers: Pr1: 5’-CTCTTGCCATCCCTTGTC TCCTTAGC-3’ and Pr2: 5’-CTCTTCAGTCTCTT CCCGTAGTCCA-3’. The primers for the 18S control amplifications were 18SF: 5’-AAATACGTGAAACA AC CCC -3’ and 18SR: 5’-CACCACCCATAGAATC AAGAAAG-3’.

The PCR conditions were an initial hot start at 94°C for 1 m, then 45 cycles of denaturation (94°C, 15 s), annealing (56°C, 30 s) and extension (72°C, 30 s), followed by a final extension of 10 m at 72°C. The volume of reaction mixtures was 50 μl which contained 100 ng genomic DNA, 2.0 μl of each primer (10 μM), 4 μl of dNTP mix (2.5 mM each), 5 μl PCR buffer and 1 μl Taq DNA polymerase. PCR amplification products were analyzed by electrophoresis in 2% agarose gels with ethidium bromide.

For RT-PCR analysis, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA synthesis was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) with random primers. The PCR reactions conditions were the same as above.

### Microscopy

Plant tissues were fixed in FAA, embedded in plastic using JB-4 Embedding kit (Polysciences Inc., Warington, PA, USA), and sectioned at 4 μm (Ruzin 1999). The sections were observed using an Axioskop Routine microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA) with an attached Microcolor tunable RGB filter (Cambridge Research and Instrumentation, Inc.).

GFP was monitored using a filter set with excitation HQ470/40×, dichroic beamsplitter Q495LP, emission HQ525/50m; dsRED was monitored with a filter EX-HQ545/12× DM-Q560DCLP, EM-HQ605/75 and EYFP with EX-HQ500/20, DM-Q515LP, EM-HQ535/30. Digital photographs were taken with a Sensys air-cooled CCD camera (Photometrics Ltd, Tucson, AZ, USA) using SimplePC1.1 software (Compix Inc, Sewickley, PA, USA).

### Haustorium assays

Haustorium development was monitored in aseptic Triphysaria seedlings following contact with lettuce roots. Lettuce seedlings were grown vertically for 4 weeks on solid Hoagland’s medium in 225 mm2 Petri plates (Q-Trays from Genetix Inc.). Triphysaria root cultures were placed on top of the lettuce roots and liquid medium was added to ensure good root–root

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Fig. 3 Agrobacterium rhizogenes transformations. a–g Formation of new roots from Triphysaria hypocotyls 0, 1, 2, 3, 4, 5, 6 weeks after dipping in MSU440. h Thick, abnormal roots that sometimes formed along with more normal transgenic roots. i–l are fluorescence and (m–p) white light photos of Triphysaria seedlings after inoculation with A. rhizogenes. i, m Seedlings 2 weeks after glass needle inoculations with medium (left panel), ATCC15834 without reporter plasmid (middle panel), or ATCC15834 harboring pBIN-mgfp5-ER (right panel). j–l and n–p Seedlings inoculated by hypocotyl dips with MSU440 harboring j GFP, k DsRed or l EYFP. q–r GUS staining of A. rhizogenes transformed roots. u, w Haustorium development in transgenic roots following exposure to 30 μM DMBQ. s–x Are closer magnifications of (q–w)
Selection of transgenic callus using antibiotic resistance or in vivo GUS staining

Selection of primary transformed tissue
- No of infiltration experiments: 12
- No of seedlings infiltrated: 200,000
- No of transformed calluses\( ^b \): 63

Selection of uniformly transformed callus from primary transformed tissue
- Viability\( ^c \): 100 (100)
- Increase in weight over 4 months (g): (2.97 ± 0.30)
- Uniformly staining calluses\( ^d \): 5 (20)
- Chimeric calluses (%): 40 (80)
- Non-transgenic escapes (%): 55 (0)

<table>
<thead>
<tr>
<th>Step</th>
<th>Parameter</th>
<th>In vivo GUS selection(^a)</th>
<th>Antibiotic selection(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection of primary transformed tissue</td>
<td>No of infiltration experiments</td>
<td>12</td>
<td>Hygromycin: 6</td>
</tr>
<tr>
<td></td>
<td>No of seedlings infiltrated</td>
<td>200,000</td>
<td>Kanamycin: 20</td>
</tr>
<tr>
<td></td>
<td>No of transformed calluses( ^b )</td>
<td>63</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\( ^a \) The first number in each cell is the frequency after 2 months of cultivation, the number in parenthesis is the frequency after 4 months
\( ^b \) Calluses with at least some cells staining GUS positive
\( ^c \) Calluses selected as GUS positive were plated in medium with and without hygromycin or kanamycin and the viability determined after 2 and 4 months. The 4-month value is in parentheses
\( ^d \) Calluses were stained with GUS and the percentage of uniformly staining, chimeric or non-transgenic calluses determined

Contact. About thirty Triphysaria root cultures were added to approximately 200 mm of lettuce root. Infection was allowed to proceed for 2 weeks.

Haustorium initiation was examined by treating Triphysaria roots with 30 μM of the haustorium inducing factor 2,4-dimethoxybenzoquinone (DMBQ). In these experiments, the plates were kept horizontal for 24 h before scoring. Haustoria were identified as swollen, hairy knobs near the Triphysaria root tips.

Results and discussion

Agrobacterium mediate transient transformation

Agroinfection was used to deliver a GUS reporter to Triphysaria seedlings. First, the plasmid pCAMBIA 1305.2 was electroporated into the different A. tumefaciens and A. rhizogenes strains shown in Table 2. Bacterial suspensions were mixed with aseptic Triphysaria seedlings that had undergone an extensive post-germination conditioning regime described below. A vacuum was applied with a plastic syringe and the seedlings stained with X-Glu 3 days later. The degree of GUS staining ranged from a few cells to staining across most of the seedling (Fig. 1). The best strains were A. tumefaciens C58 and A. rhizogenes A4RS and MSU440 and resulted in 1–3% of seedlings staining GUS positive (Table 2). Newly germinated seeds and root cultures also showed GUS staining after Agroinfection with either Ti or Ri containing strains.

Significant enhancement of expression was obtained when seedlings were conditioned post germination for several months in the dark at 4°C. The highest transformation efficiencies were obtained after a 3–6 month conditioning period although seedlings that had been incubated up to 1 year, still expressed GUS activity when vacuum infiltrated. Interestingly, the time of conditioning affected the types of tissues transformed (Fig. 2e). With a month or less of conditioning the overall number of transformants was low but a high proportion expressed GUS in the shoot meristem (13 out of 17 transformed seedlings) (Fig. 1c). Developmentally these seedlings were just opening their apical hooks. After 2–3 months the cotyledons and hypocotyls were expanding and vacuum infiltration at this period gave staining distributed throughout different parts of the seedlings (Fig. 2b). These seedlings provided the best friable callus. Only after 3 months or more of dark conditioning did a large proportion of roots became susceptible to Agroinfection (Fig. 1a). These experiments suggest that conditioning for different periods of time provides a means for enriching transformation events for particular plant tissues.

The overall effectiveness of plant tissue to be transiently express transgenes is a balance between the susceptibility of that tissue to Agrobacterium infection and its subsequent viability. There is a clear negative correlation between the transformation efficiency and the ultimate viability of the tissue as seen in Fig. 2d where the transformation efficiencies of 20 independent experiments is plotted as a function of viability.

Agrobacterium rhizogenes hairy root transformations

Triphysaria plants were inoculated with A. rhizogenes either by puncturing the seedlings with a glass needle carrying bacteria or by dipping the freshly cut ends of hypocotyls into bacterial cultures (Limpens et al. 2004; Petit et al. 1987). Wound inoculation resulted in transgenic hairy roots developing at the point of inoculation in approximately 1–2% of the
Plants inoculated with VIGG 15834. Hairy roots formed with A4RS and ATCC 15834 strains as well but about an order of magnitude less frequently (Fig. 3; Table 2).

The wound inoculation method was time consuming and the hypocotyl dip adaptation proved an effective way to generate transgenic roots. De-rooted Triphysaria seedlings regenerated roots within about 2 weeks of inoculation (Fig. 3a–h). Some were normal in appearance and others were thick and stunted but both types could be transformed. Up to 8% of seedlings dipped generated at least one morphologically normal transgenic root (Table 2). Agrobacterium rhizogenes transformed roots form pre-attachment haustoria when exposed to DMBQ and are suitable for early haustorium development assays (Fig. 3u–x).

In order to develop an effective visual marker for A. rhizogenes mediated transformation, we examined the effectiveness of GFP, dsRED, or EYFP as fluorescent reporters for Triphysaria roots. The three reporter constructions were introduced into MSU440 and transformed into Triphysaria seedlings using the hypocotyl dip. After 3 weeks transgenic roots were monitored using the recommended filter sets (Materials and methods). There was considerable autofluorescence in Triphysaria roots when using the filter sets suggested for GFP and dsRED fluorescence (Fig. 3i, j). The most robust fluorescent marker for Triphysaria roots was EYFP since it gave the lowest background of auto-fluorescence (Fig. 3i).

**Fig. 4** Detection of transgene by Southern hybridization and PCR. a Schematic of GUSPlus gene from pCAMBIA 1305.2 showing the glycine rich protein secretion signal (GRP), catalase intron, and GUS coding sequences (CAMBIA 2006). Arrows Pr1 and Pr2 show the positions and orientations of primers used to amplify the GUSPlus gene. Presence of the transgene results in a 363 bp PCR fragment when genomic DNA is used as a template and a 174 bp fragment when cDNA was used. b Restriction map of pCAMBIA 1305.2. A HindIII asymmetrically digests the T-DNA near its middle. c Genomic PCR and RT-PCR using GUSPlus primers on transgenic and non-transgenic root cultures. The top photo results from using GUSPlus specific primers, the bottom photo is the same substrates amplified with 18s primers for loading control. Lane 1, genomic DNA from Triphysaria root culture number 1; lane 2, cDNA from root culture number 1; lane 3, genomic DNA from non-transgenic Triphysaria root culture; lane 4, cDNA from non-transgenic root culture; lane 5, amplification of pCAMBIA1305.2 plasmid for control. d Southern blot of transgenic roots. Lane 1: Triphysaria callus line number 1 obtained by A. rhizogenes inoculation; lane 2: Triphysaria callus line no. 13 obtained by A. tumefaciens vacuum infiltration; lane 3: non-transgenic Triphysaria calluses; lane 4: no sample loaded; lane 5: pCAMBIA 1305.2 plasmid DNA digested with HindIII. The size markers included a 1 kb Plus DNA ladder and DNA of the phage Lambda.
Selection of stable transformed roots

Transformed cell lines were obtained by co-cultivating seedlings, roots or cell cultures with *Agrobacterium* and plating onto callus inducing medium containing cefotaxim or timentin to inhibit *Agrobacterium* growth. We also generated callus by plating vacuum infiltrated *Triphysaria* onto medium containing auxin and cytokinin.

The efficiency of GUSPlus staining together with its secretion into the apoplastic space allows GUS to be stained without destroying the transgenic cells (CAMBIA 2006). The Cambia protocol for non-destructive staining includes low concentrations of X-Glu, staining at room temperature, omission of Triton from the staining buffer and omission of the vacuum infiltration. *Triphysaria* seedlings and calluses were stained under these conditions for increasing times of GUS staining to maximize visualization of transgenic material. We determined that *Triphysaria* tissues could be stained for 24 h without compromising viability, however, longer incubation periods were toxic (Table 3).

Ultimately, 63 transgenic calluses were obtained from about 200,000 vacuum infiltrated seedlings using in vivo staining for selection (Table 4). Selection for antibiotic resistance carried on the T-DNAs was considerably less successful and resulted in a single transgenic callus from about one million seedlings in vivo. Antibiotic selection again proved ineffective for obtaining uniformly transgenic callus from primary transformed cells. Even calluses known to be transgenic by GUS staining grew poorly or were killed by the antibiotics (Fig. 1h–j; Table 3).

The transgenic nature of GUS-positive calluses was confirmed by PCR and Southern hybridization analyses (Fig. 4). We conclude that in vivo GUS selection is superior to antibiotic selections for *Triphysaria*.

Transgenic root cultures form functional haustoria

Roots developed from *A. tumefaciens* and *A. rhizogenes* transformed calluses after 1 or 2 months in callus inducing medium. Roots were excised and placed into hormone free medium with monthly treatment with auxin to induce secondary root development (Tomilov et al. 2004). Thirty micro molar DMBQ was applied to several root tips and haustorium development visualized 24 h later. Essentially all of the root tips formed pre-attachment haustoria that were indistinguishable from those of non-transgenic plants (Fig. 1t). Roots were subsequently stained with X-Glu confirming that the haustoria forming roots were transgenic.
Transgenic Triphysaria roots were applied to lettuce roots in vitro and within few days, haustoria had developed, attached to and invaded the host. Haustoria connecting Triphysaria and lettuce roots were stained with X-Glu and cytological sections prepared to examine the parasite–host interface (Fig. 5). As seen in Fig. 5b, the GUS staining Triphysaria haustorium surrounds about one half of the lettuce root circumference. Closer examination shows compression of the lettuce root and penetration of parasite intrusive cells into the host vasculature (Fig. 5c, d). Xylem tissues were observed internal to the haustorium body, presumably to eventually connect the host and parasite vascular systems. We take this as evidence that potentially functional haustoria are made by transgenic roots.

Conclusions

We have generated transgenic roots of the parasitic plant Triphysaria that retain their ability to invade and parasitize host plants. The transgenic roots also developed haustorium upon contact the haustorium-inducing factor DMBQ. The ability to make functional transgenic roots from parasitic plants provides the technology to investigate the role of selected genes in mediating the host–parasite association.

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References


