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Genetic Transformation of *Ipomoea purpurea* Mediated by *Agrobacterium rhizogenes*

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ABSTRACT

The transformation of *Ipomoea purpurea* mediated by *Agrobacterium rhizogenes* was studied. Almost all roots induced by four bacterial strains, R1000, A4, R1601 and R1025 were putative hairy roots. Hairy roots were formed on wounds in cotyledons or hypocotyls infected for 10-15 days by *A. rhizogenes*. The frequency of hairy root formation in hypocotyls infected by A4, R1025, R1000 and R1601 was 7.6%, 16.5%, 20.0 and 28.7%, respectively while that for cotyledons infected by R1000, R1601 and R1025 ranged between 30.5%, 44.6% and 19.80%, so that R1000 was the optimal bacterial strain to infect cotyledons. Almost all roots which could survive on selection medium (MS containing 500 mg L^{-1} carbenicillin and 200 mg L^{-1} kanamycin) were putative hairy roots. The effects of silver nitrate, infection time, aeration and light treatment on the transformation were also described in subsequent experiments used with R1000. The transformation frequency increased to 42.6% when the infection time was increased to 10 minutes as previously described, or to 34.61% when 2 mg L^{-1} silver nitrate was used for selection of transformed *I. purpurea* plants. The experiments also showed that aerating cultures were better than airproof cultures, and that light was better than darkness. After selecting on 200 mg L^{-1} kanamycin in solid medium, the results of PCR and Southern blot analysis of the *rolB* gene for the hairy roots indicated that the Ri plasmid had integrated into the genome of *I. purpurea*.

Keywords: acetosyingone, hairy root formation, silver nitrate

INTRODUCTION

Most *Agrobacterium rhizogenes* strains carrying agropine type Ri-plasmids, transfer two independent T-DNAs, denoted T_L-DNA and T_R-DNA. Both T_L-DNA and T_R-DNA are transferred and integrated independently into the host plant genome, but the transfer of T_L-DNA is essential for induction of the hairy root syndrome, and transfer of T_R-DNA does not provoke formation of roots from transformed cultures (Sevon and Oksman-Caldentey 2002). T_R-DNA contains two genes, *iaaM* and *iaaH*, responsible for the biosynthesis of auxins and the genes responsible for the synthesis of the opines mannopine and agropine (ags). T_L-DNA carries 18 open reading frames, four of which are essential for hairy root formation; ORF10, ORF11, ORF12 and ORF15 corresponding to the *rolA*, *rolB*, *rolC* and *rolD* genes, respectively. Genes of T_L-DNA, *rolA*, *rolB*, *rolC* and *rolD* directly infect the synthesis of a substance that recruits the cells to differentiate into roots under the influence of endogenous auxin synthesis. The *rolB* gene is absolutely essential for the induction of hairy roots. Even when expressed alone, the *rolB* gene can induce significant hairy root production (Gelvin 2000; Tzfira *et al*. 2004).

A. rhizogenes causes production of transformed hairy roots at the infection site in plants. Hairy roots are characterized by high growth rate and genetic stability. Since the early stage of hairy-root research it has been known that *in vitro* cultures of hairy roots are able to regenerate whole plants in *Panax ginseng* (Yang and Choi 2000), spinach (Ishizaki *et al*. 2002) and *Catharanthus roseus* (Choi *et al*. 2004).

Most have altered phenotypes including the hairy root syndrome, dwarfing, altered flowering, wrinkled leaves and increased branching, which have proven useful in ornamental plant breeding programs (Giovanni *et al*. 1997). Many plants have been transformed by making use of the Ri plasmid, and some of them had been regenerated into whole viable transgenic plant with high genetic stability (Choi *et al*. 2004; Crane *et al*. 2006).

Common Morning Glory (*Ipomoea purpurea*), an annual herbaceous vine, is a popular garden flower and a medicinal plant belonging to the family Convolvulaceae (Bhate 2001; Mori *et al*. 2005). The development of new transformation methods is important for functional genomic studies in ornamental plant species. The genetic transformation of ornamentals has potential for model cultivar breeding, as demonstrated by Satou *et al*. (2004) in a study in which the transgenic plants were characterized by modified flowers with various longevities and colors. However, the transformation of *I. purpurea* mediated by *A. rhizogenes* has not yet been reported. In this study, we described an effective system for the Ri plasmid-mediated transformation of *I. purpurea* and investigate the effects of several major physical and chemical factors on hairy root formation.

MATERIALS AND METHODS

Plant materials

Seeds of *I. purpurea* were soaked in 70% ethyl alcohol for 1 minute followed by 4 successive rinses with sterilized distilled water, then surface-sterilized with 0.1% $HgCl_2$ for 10 minutes, followed by 4 successive rinses with sterilized distilled water. Seeds were germinated on half-strength Murashige and Skoog (1962; MS) medium solidified with 0.8% (w/v) agar. Unless mentioned otherwise, the pH of all media was adjusted to 6.0 before autoclaving. All cultures were maintained under light (approximately 35 μmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent lamps with a 16-h photoperiod) at 25°C. For transformation experiments, cotyledons and hypocotyls were excised from *I. purpurea* which were grown *in vitro* for about 15 days after germination.

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Original Research Paper

Agrobacterium rhizogenes strains

A. rhizogenes strains R1000, R1601, R1025, and A4 belong to the agropine type and harbor the Ri plasmid with a two-part T-DNA (left T-DNA and right T-DNA) (**Table 1**). For the transformation experiments, each strain was inoculated into liquid YEB medium (5 g L^{-1} sucrose, 1 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 5 g L^{-1} peptone) with 100 mg L^{-1} kanamycin and shaken for 25 hours at 28°C. The cultures were resuspended in liquid YEB medium containing 30 μmol L^{-1} acetosyingone before dilution for infection.

Table 1 Bacterial strains and plasmids used in this work and their relevant characteristics.

Strains	Ri plasmid	Type of plasmid	References
A4	pRiA4 and pB1121	Agropine	White and Nester 1980
R1000	pRiA4b and pB1121	Agropine	White *et al.* 1985
R1601	pRi1500 and pTVK291	Agropine	Pythoud *et al.* 1987
R1025	Ri (*tms-1* and *tms-2* deletion)	Agropine	White *et al.* 1985

Transformation

Cotyledons or hypocotyls were precultured on half-strength MS medium solidified with 0.8% (w/v) agar containing silver nitrate (0, 1, 2, 4, 6 mg L^{-1}) and 30 μmol L^{-1} acetosyringone for 2 days (Yu *et al.* 2002). The explants were then transferred to the *Agrobacterium* suspensions containing 30 μmol L^{-1} acetosyingone and incubated for a particular period of time (8, 10, 12, 14 min), then blotted dry on sterilized paper. As control explants, cotyledons incubated with water. The explants were returned to the same precultivation medium for co-cultivation. After 2 days, these explants were transferred to half-strength MS medium containing 500 mg L^{-1} carbenicillin and 200 mg L^{-1} kanamycin to inhibit bacterial growth. Every five days, the explants were transferred to selection medium (half-strength MS containing 250 mg L^{-1} carbenicillin and 200 mg L^{-1} kanamycin) until hairy roots formed. The number of explants with roots, which was about 13 days after infection, was recorded.

The following conditions were considered: 0, 1, 2, 4, 6 mg L^{-1} silver nitrate; 0, 8, 10, 12, 14 minutes infection time; two days pre- and co-cultivation. In all cases the pH was 6.5 with a bacterial concentration of 1.0 (OD_{600}). To determine the optimum conditions for the transformation (such as silver nitrate, infection time, ventilation and light treatment), one parameter of the considered conditions was varied at a time and the effect on hairy root formation was measured. Explants are grown in a growth chamber with 16 h of light and 8 h of darkness or 24 h of darkness, respectively. In the aerating cultures, cultural flasks were covered with a semi-permeable membrane to let air diffusing into the flasks, while in the airproof cultures, cultural flasks were tightly covered with plastic sheets without air diffusion through the sheets.

The transformation frequency was percentage of explants number formed hairy roots/all explants number infected by *Agrobacterium* suspensions.

PCR amplification of the *rolB* gene

To determine the presence of Ri T-DNA in the hairy-root lines, total DNA was extracted from a wild-type plant root as a control and the four independent hairy-root lines using the procedure of Edwards *et al*. (1991). Primers for detecting the *rolB* gene (5′-CG CAAGCTACAACATCATAG-3′ and 5′-CAGTAGATCTCACTCC AGCA-3′), were used to amplify a 583 bp fragment of the *rolB* gene. Total DNA from wild-type roots, hairy-root lines, and *A. rhizogenes* cells, in addition to primers and the reaction buffers, were incubated in a DNA thermal cycler [GeneAmp PCR system 9700 (Applied Biosystems)] at 94°C for 5 min followed by 30 cycles of 92°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 min, and a final 7-min extension at 72°C.The DNA size marker was DL 2000 (TaKaRa Biotechnology (Dalian) Co., Ltd.).

Southern blot analysis

Genomic DNA was digested with *Hind*III and resolved by electrophoresis on a 0.8% agarose gel before transferring to a nylon membrane according to the method of Southern (Sambrook and Russell 2001). The membrane was pre-hybridized at 65°C in 7% SDS and 0.25 mol L^{-1} $NaHPO_4$ and then hybridized with the *rolB* gene fragment, which was isolated from the restricted Ri plasmid with *nar*I and *nde*I, labeled with ^{32}P-dCTP. The hybridized blot was subjected to three washes in 20 mmol L^{-1} $NaHPO_4$ and 1% SDS. The blot was exposed to X-ray film (Kodak) at -70°C for 3 days.

Statistical analysis

All data on hairy root frequency were the mean values of three independent experiments (±SD) with a minimum of 35-54 explants per treatment. All roots that survived on the selection medium for 20 days were designated as hairy roots. All data among different treatment were tested by SSR testing (Duncan 1955).

RESULTS

Four strains of *A. rhizogenes*, A4, R1000, R1601 and R1025 were tested for their transformation efficiency, after infecting *I. purpurea* hypocotyl or cotyledon explants for 8 mins (**Fig. 1**). After 10 days culture, hairy roots formed on the wound of the explants (**Fig. 2A**). The transformation frequency cotyledons infected by R1000 (44.6%) or R1025 (30.5%) was higher than that of hypocotyls (20.0% and 16.5%, respectively) so cotyledons were used in the following experiments. R1000-infected cotyledons with a transformation frequency of 44.6% had the highest transforming ability and A4, transformed cotyledons at a 3.0% transformation frequency, had the lowest (**Fig. 1**).

A. rhizogenes R1000 was used in subsequent experiments. **Fig. 3** shows the effect of infection time on the frequency of hairy root formation. The transformation frequency increased as the infection time increased from 8 to 10 minutes, rising to 42.6% at 10 minutes. Thereafter the transformation frequency began to decrease when the infection time was increased to 12 minutes. The transformation frequency was lowest at 14 minutes, at just 20.0%. As expected, bacterial growth in the selection medium increased significantly when the infection period exceeded 12 minutes, causing problems in subsequent work, especially the inability of inhibiting bacterial growth on medium. Consequently the infection time was limited to 10 minutes in subsequent experiments.

The experiments showed that aerating cultures or light

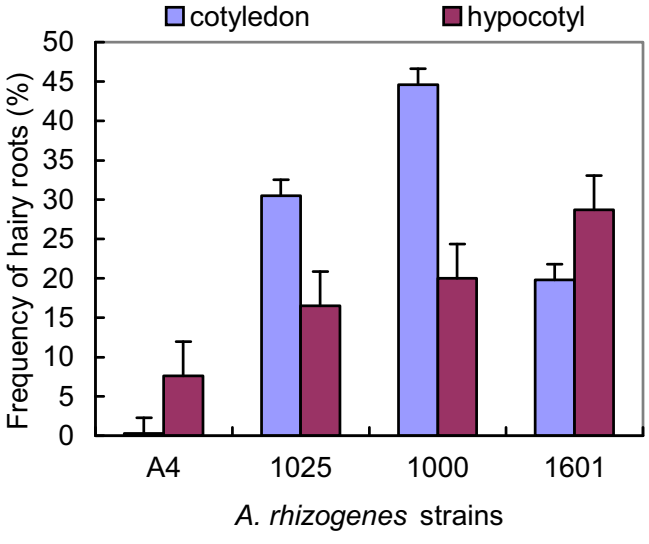


Fig. 1 Efficiency of transformation to hairy roots infected by four *A. rhizogenes* strains for 10 days. Each value represents the mean of three different experiments.

Fig. 2 Hairy root formation in cotyledons infected by R1000 for: 10 days (**A**) and 16 days (**B**). Hairy roots sub-cultured on hormone-free MS medium for 10 days (**C**).

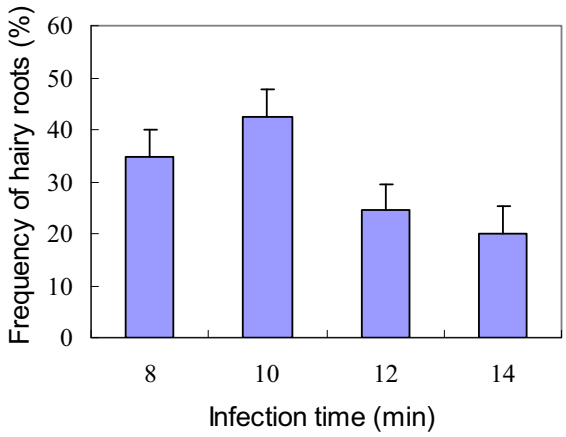
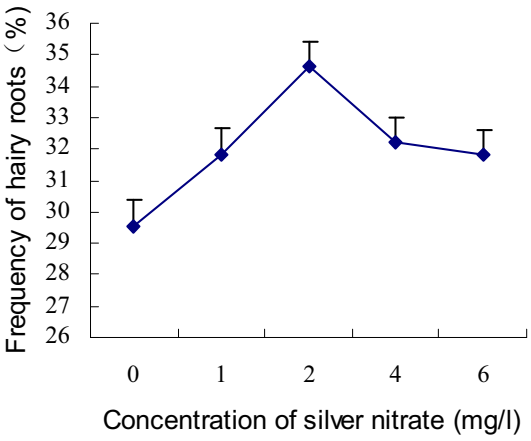


Fig. 3 Effect of infection period on hairy root formation of 200 mg L^{-1} kanamycin-resistant roots.



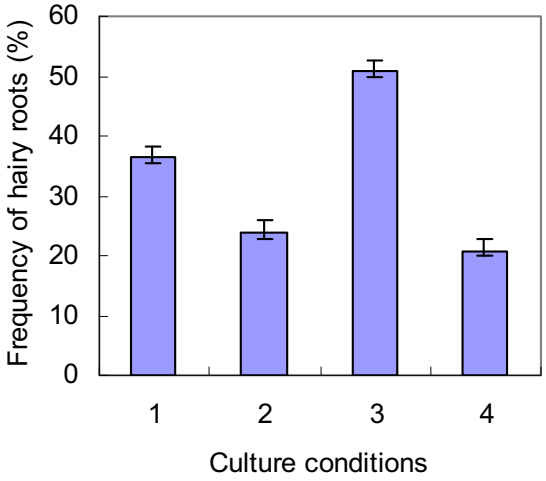


Fig. 4 Frequency of hairy roots under different cultivation conditions. 1. Aerated cultures; 2. Airproof cultures; 3. Light (16 $h.day^{-1}$) 4. Darkness.

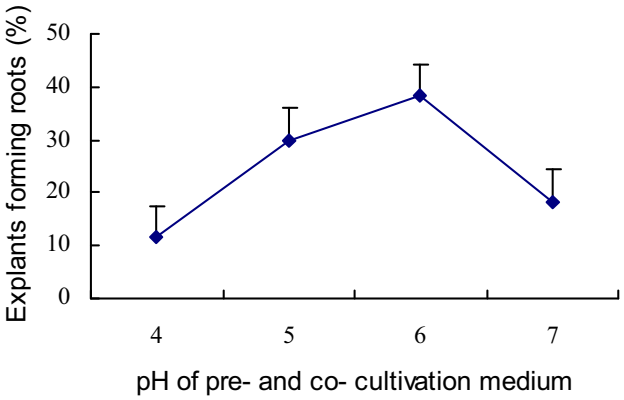


Fig. 5 Effects of silver nitrate (A) and pH (B) in the pre- and co-cultivation medium. (**A**) Different concentrations of silver nitrate were added to the standard composition. (**B**) All variables were standardized except pH of pre- and co-cultivation medium and 4 mg L^{-1} silver nitrate. Each value represents the mean of three different experiments.

treatment (16 h d^{-1}) induced hairy roots better than darkness (**Fig. 4**). The frequency of hairy root formation in aerated cultures during explant culture was 36.4%, while that of airproof cultures was 23.8%. The frequency of hairy root induction in the light (16h of light and 8h of darkness) was higher than in the darkness.

Fig. 5A shows the effect of silver nitrate on the frequency of hairy root formation in *I. purpurea*. The transformation frequency was to 34.61%, when the silver nitrate concentration at 2 mg L^{-1}. The frequency decrease at above 2 mg L^{-1}. Silver nitrate at 6 mg L^{-1} decreased the induction of hairy roots compared with the control medium (no silver nitrate).

The pH of the pre- and co-cultivation medium affected hairy root induction. Increasing pH from 4.0 to 6.0 strongly stimulated hairy root formation, with an optimum at about pH 6.0, but a higher pH (7.0) inhibited growth (**Fig. 5B**).

Finally, hairy root production under the cumulative optimal conditions determined above was performed: use of bacterial strain R1000; co-cultivation for 3 days; 30 μmol L^{-1} acetosyringone, 2 mg L^{-1} silver nitrate and pH 6.0 in the co-cultivation medium. Under this condition, hairy root production frequency was 44.6%.

For amplification of the *rolB* gene, roots were excised from hairy roots that had been infected for 16 days (**Fig. 2B**) and cultivated on the selection medium. Four individual root clones that had survived on the selection medium after 16 days culture were randomly selected. All roots, induced by R1000, were found to harbor the *rolB* gene (**Fig. 6A**). This showed that almost all roots that could grow on selection medium containing 200 mg L^{-1} kanamycin and were putative hairy roots. We selected two root clones for Sou-

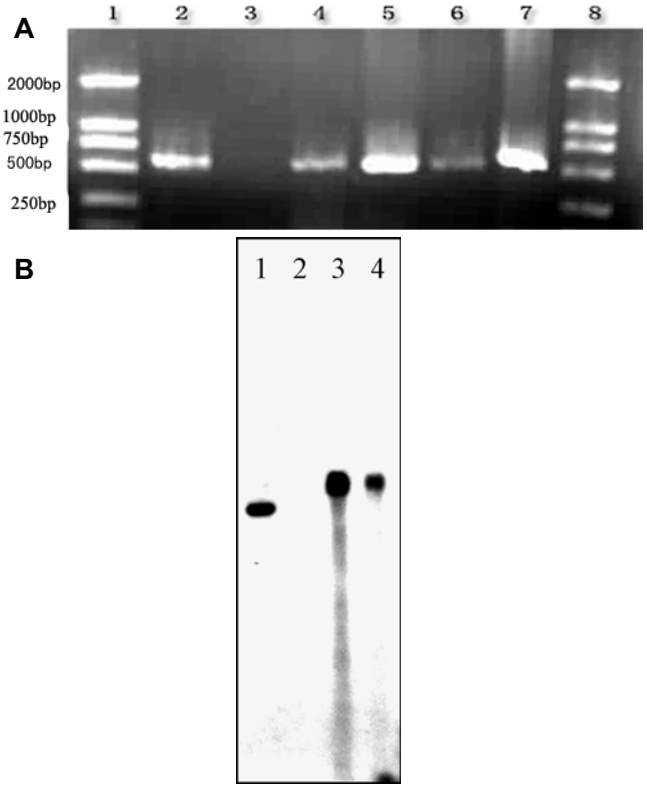


Fig. 6 PCR of DNA templates (A) and Southern blot analysis (B) from hairy root induced from infected *Ipomoea purpurea* cotyledon 16 days. (**A**) Line 1, DL 2000 Marker; line 2, Ri plasmid; line 3, control; lines 4-7, roots induced by R1000; Line 8, DL 2000 Marker. (**B**) Line 1, plasmid DNA; line 2, control; lines 3-4, roots induced by R1000.

thern blot analysis out of four clones that were putative hairy roots. Two roots analysed by Southern blot analysis were putative hairy roots, which indicated that the roots were transformed (**Fig. 6B**).

To induce callus formation from hairy-root explants for plant generation in the further study, we cultured hairy–root in MS medium hormone-free, the result shown the percentage frequency (%) of callus formation was more than 75% (**Table 2**).

Table 2 Frequency (%) of callus formation on hairy root explants of *I. purpurea*.

Root line	Number of hairy roots	Number of hairy roots forming callus	Frequency (%)
1	80	65	81.2
2	75	62	82.6
3	70	54	77.1
4	68	51	75.0

DISCUSSION

Hairy roots have been induced in many dicotyledonous plants following transformation with the *Agrobacterium rhizogenes* Ri T-DNA. The procedure used to induce hairy roots includes cultivation of wounded plant parts (called explants) with suspensions of *A. rhizogenes* in aseptic conditions (Sevon and Oksman-Caldentey 2002). Wounded plant explants can be infected with *Agrobacterium* strains either by direct inoculation with bacterial suspensions and incubation on a solid medium or by co-cultivation in liquid media. In either case, the infected explants have to be subsequently transferred to a solid medium with antibiotics (claforan or penicillin derivates) to eliminate the bacteria, which can survive on medium without the presence of any hormone (Teixeira da Silva 2006). Successful genetic transformation can be demonstrated by detecting T-DNA or opines, respectively (Sevon and Oksman-Caldentey 2002). To detect T-DNA, either polymerase chain reactions or Southern blot hybridizations can be used (Xie *et al*. 2001).

The genetic transformation mediated by *Agrobacterium* is affected by explant genotype and structure, chemical and physical factors, bacterial strains, signal molecules. Different strains of *A. rhizogenes* vary in their transforming ability (Giri *et al*. 1997; Choi *et al*. 2004). This was confirmed in this study which showed that the R1000 strains had greater hairy root generating capacity than A4, R1025 and R1601 in *I. purpurea* transformation. This result could possibly be explained by the different plasmids contained by these strains, in which genes of Ri T_L-DNA directly infect the synthesis of a substance that recruits the cell to differentiate into root formation under the influence of endogenous auxin synthesis and/or Ri TR-DNA containing tms loci which directly synthesize auxin (Capone *et al*. 1989; Zhao *et al*. 2004) and which then induces hairy root formation.

Bacterial concentration plays an important role in the production of transformed roots. Suboptimal concentration resulted in a lower availability of bacteria to transform plant cells, while a high concentration decreased their potentiality by competitive inhibition in soybean transformation mediated by *A. rhizogenes* (Kumar *et al*. 1991). Our results showed an optimum bacterial concentration of 1.0 (OD_{600}) on hairy root formation, which is similar to the effect of bacterial concentration in *Torenia* transformation (Tao and Li 2006), therefore possibly sharing the same mechanism.

Some recalcitrant plant species can be transformed by inducing the *vir* genes of the bacteria by signal molecules or it can be achieved *in vitro* by co-cultivating *Agrobacterium* with wounded tissue or in media that contains signal molecules (Rahman *et al*. 2004). Most studies indicate that the application of silver nitrate in the co-cultivation medium strongly promotes transformation mediated by *Agrobacterium* (Bu *et al*. 2000). Liu suggested that was because silver nitrate can induce the activation of the *Agrobacterium VIR* gene (Liu *et al*. 2003). Moreover, other scientists agree that because silver nitrate can affect the activity of the receptor cell protoplast, which competitively binds to ethylene receptor protein of *in vitro* cells to inhibit or reduce the contamination effects of ethylene, which influences the ability of *in vitro* cells to grow. Some studies indicate that silver can stimulate organogenesis *in vitro* by inhibiting ethylene signaling (Zhang *et al*. 1997). Different concentrations of silver nitrate in the medium were used in leaf tissue culture of tobacco (*Nicotiana tabacum* L.) *in vitro*, the results showed that 1-5 mg L^{-1} silver nitrate increased bud differentiation percentages of tobacco callus, especially the concentration of 5 mg L^{-1} (Wang *et al*. 2006). Our results in this study showed that when the concentration of silver reached 1-4 mg L^{-1}, the frequency of genetic transformation in *I. purpurea* increase compared with the control (without silver nitrate treatment), which is consistent with these findings (**Fig. 5A**). It suggests that the promotive effect of silver at a low dose (2 mg L^{-1}) on hairy root formation is due to the inhibition of ethylene signaling affecting *Agrobacterium* growth and stimulating to some degree *vir* gene expression. There were many preliminary experiments which show that Ag^{2+} ions interact with the ethylene binding sites located in cell membranes (Yang and Hoffman 1984) and blocks ethylene binding *in vivo* (Goren *et al*. 1984) and usage of $AgNO_3$ can possibly counteract this ethylene-caused recalcitrance. Silver nitrate can inhibit the growth of *Agrobacterium*, which may be caused by different silver nitrate concentrations and different types of explants used (Lin *et al*. 2003).

The genetic transformation frequency of aerating cultures is higher than that of airproof cultures, and we supposed that is because under airproof cultures ethylene accumulates so much that it inhibits the transport of auxin, and determined that EIR (ethylene-insensitive root) proteins as probable auxin-efflux carriers to effect the polar

auxin transport in vascular (Galweiler *et al*. 1998).

Studies indicate that YEB medium pH for activating bacteria promotes activation of some *vir* genes. VirD2, for example, was effectively activated under pH 5.4-5.6, peaking at pH 5.1-5.2 (Yu *et al*. 2002), when pH in the medium decreased from 7.2 to 5.8, the frequency hairy root formation strongly increased. However, the effects of pre- and co-cultivation medium pH on hairy root formation have not been previously reported. **Fig. 5B** shows that hairy root formation frequency increased when pre- and co-cultivation medium pH increased from 4.0 to 6.0, but decreased when pH was over 6.0. The underlying mechanism of this is not clear.

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