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ORIGINAL ARTICLE

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Sweet pepper ferredoxin-like protein (*pflp*) gene as a novel selection marker for orchid transformation

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Abstract A novel method for selection of transgenic plants utilizing the sweet pepper (*Capsicum annuum* L.) ferredoxin-like protein (*pflp*) gene as selection marker and *Erwinia carotovora* as the selection agent has been developed. An expression vector containing a *pflp* cDNA driven by a cauliflower mosaic virus 35S promoter was successfully transformed into protocorm-like bodies of *Oncidium* orchid by *Agrobacterium tumefaciens* and particle bombardment, respectively. *Erwinia carotovora* was used as a selection agent to screen transformants, thereby obtaining transgenic plants without the use of an antibiotic selection agent. A total of 32 independent transgenic orchid lines were obtained, out of which 9 transgenic lines (β-glucuronidase positive) were randomly selected and confirmed by Southern and northern blot analyses. The transgenic orchid plants showed enhanced resistance to *E. carotovora*, even when the entire plant was challenged with the pathogen. Our results suggest the novel use of the *pflp* gene as a resistance selection marker in plant genetic engineering strategies. In the future, the use of the *pflp* gene as a selection marker may facilitate the use of smaller gene constructs due to removal of bulky antibiotic selection and reporter genes. These constructs can then be used to incorporate additional genes of choice.

Keywords *Agrobacterium* · *Erwinia* · Ferredoxin-like amphipathic protein · *Oncidium* orchid · Particle bombardment · Selection marker

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Abbreviations CaMV: cauliflower mosaic virus · GUS: β-glucuronidase · PLB: protocorm-like body · WT: wild type

Introduction

The selection of transformants has been accomplished routinely by the introduction of an antibiotic- or herbicide-resistance gene, enabling the transformants to survive on medium containing the selective agent(s). However, there are several disadvantages in using antibiotics and/or herbicides as selective agents, for example possible toxicity or allergenicity of the gene product to humans (Haldrup et al. 1998a, 1998b). In some cases, the presence of such resistance genes may be undesirable in transformants (Flavell et al. 1992).

The currently available methods for selecting transgenic orchid are based on antibiotic (Kuehnle and Sugii 1992; Anzai et al. 1996; Belarmino and Mii 2000; Yu et al. 2001), herbicide (Knapp et al. 2000), or visual selection using firefly luciferase as a screenable marker gene (Chia et al. 1994). However, these selection systems are costly, time consuming and labor intensive during the selection process. To avoid the use of antibiotic or herbicide selection agents, alternative selection systems of non-plant origin have been established and applied to crop plants (Haldrup et al. 1998a, 1998b; Joersbo et al. 1998; Wang et al. 2000; Zhang et al. 2000). However, the use of these selection systems is a matter of concern regarding their acceptability by society. Recently, a sweet pepper ferredoxin-like protein (*pflp*, formerly called *AP1*) was isolated from sweet pepper. This peptide has been reported to have anti-microbial activity against *Pseudomonas syringae*, by delaying the hypersensitive response (HR) in non-host plants through the release of harpin proteinaceous elicitor (PSS), thus suppressing bacterial growth. It is hypothesized that a membrane-bound signal transduction pathway allows the harpin to induce an HR. However, the molecular mechanisms underlying harpin PSS perception by the

plant cell remain uncharacterized (Lin et al. 1997). Similarly, over-expression of *pflp* in transgenic rice has been known to confer resistance to *Xanthomonas oryzae* (Tang et al. 2001). Orchids are one class of ornamentals that are in constant demand worldwide for their exotic flowers. Genetic engineering has been successfully used to generate insect- and disease-resistant cereals and pulses, but not orchids. One of the most devastating diseases encountered in orchids is the soft-rot disease caused by *E. carotovora*. The *Oncidium* genus, known for its high commercial value, is highly susceptible to the pathogen. Thus we reasoned that introduction of this antimicrobial peptide (*pflp*) into orchid might confer resistance to attack by *E. carotovora* and hence be useful as a selection marker. To test this hypothesis, we generated transgenic orchid plants expressing the *pflp* gene and used *E. carotovora* as the selection agent. The protocorm-like bodies (PLBs) of transgenic *Oncidium* showed enhanced resistance to *E. carotovora*; moreover, the non-transgenic PLBs did not survive under similar conditions. Therefore, this evidence shows that the *pflp* gene could be a useful selection candidate and bacterial resistance gene for genetic engineering strategies.

Materials and methods

Plasmid construction

A DNA fragment containing a cauliflower mosaic virus (CaMV) 35S promoter, a *pflp* coding sequence (including the signal peptide) and a nopaline synthase (*nos*) poly(A), was excised from pBIAP1 by digestion with *Eco*RI and *Hin*dIII (Tang et al. 2001) and cloned into the *Eco*RI and *Hin*dIII site of pCAMBIA 1304 (Center for the Application of Molecular Biology of International Agriculture, Black Mountain, Australia) to form pSPFLP (Fig. 1). The pCAMBIA 1304 vector contains two selection markers: the green fluorescent protein (GFP) and β-glucuronidase (*GUS*) fusion gene and the hygromycin phosphotransferase (*hph*) gene driven by two separate 35S promoters. A schematic map of the plasmid is shown in Fig. 1.

Plant material and culture conditions

Protocorm-like bodies (PLBs) of the commercial variety of *Oncidium* orchid 'Sherry Baby cultivar OM8′ were used as explants. They were maintained by sub-culturing chopped PLBs onto G10 medium containing MS salts, 1 g/l tryptone, 20 g/l sucrose, 1 g/l charcoal, 65 g/l potato tuber and 3 g/l phytagel at pH 5.4, and incubating for a 16-h photoperiod at 25 °C.

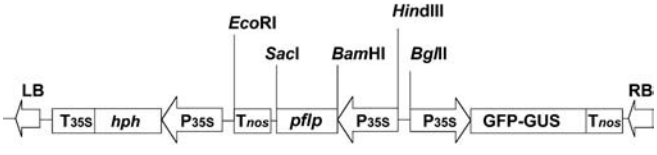

Fig. 1 Schematic representation of pSPFLP. *pflp* Sweet pepper ferredoxin-like protein cDNA, *Hph* hygromycin phosphotransferase cDNA sequence, *GFP–GUS* green fluorescent protein (GFP) and β-glucuronidase fusion protein, *P35S* CaMV 35S promoter, *nos* nopaline synthase terminator sequence, *T35S* CaMV 35S terminator sequence, *LB* left border, *RB* right border

Erwinia carotovora subsp. *carotovora*, kindly provided by The Asian Vegetable Research and Development Center (AVRDC), Tainan, Taiwan, was propagated in NB medium (5 g/l peptic digest of animal tissue, 5 g/l yeast extract, 1.5 g/l beef extract, 1.5 g/l and sodium chloride) at 28 °C. The culture was allowed to grow to $A_{600} = 1.0$ and cells were pelleted by centrifugation and re-suspended in fresh NB medium.

Agrobacterium tumefaciens-mediated transformation

The pSPFLP plasmid was transformed into *A. tumefaciens* strain EHA105 cells by electroporation. *Agrobacterium tumefaciens* containing pSPFLP was cultured on YEB medium (Chan et al. 1993) containing 100 mg/l kanamycin at 28 °C for 2 days. A single colony was transferred into 5 ml AB medium (7.25 g/l KH_2PO_4, 10.25 g/l K_2HPO_4, 0.15 g/l NaCl, 0.15 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $CaCl_2$, 5 g/l glucose, 2.5 mg/l $FeSO_4$, and 8 g/l agar) containing 100 mg/l kanamycin and incubated at 28 °C for 2 days. The bacterial culture was then transferred into 50 ml AB medium containing 100 mg/l kanamycin and incubated at 28 °C overnight. Four hours before transformation, 200 μM acetosyringone (AS) was added to the bacterial culture. The *A. tumefaciens* culture ($A_{600} = 0.8–1.0$) was centrifuged at 3,000 rpm for 10 min and the pellet was suspended in 20 ml MS medium.

Newly formed PLBs of 45-day-old cultures were chopped, pretreated with 0.5 M sucrose for 2 h, cultured in G10 medium and then incubated at 25 °C in the dark for 7 days. These pre-cultured PLBs were infected with *A. tumefaciens* for 30 min, then co-cultured on G10 medium plates, without charcoal but containing 200 μM AS along with a nurse culture of tobacco suspension cells (Fillatti et al. 1987), and incubated at 25 °C in darkness for 3 days. The nurse culture was used as an additional supplement along with AS since the amount of phenolics exuded by the cut PLBs was not sufficient to obtain a high transformation efficiency (Belarmino and Mii 2000). The infected PLBs were washed with MS medium containing 200 mg/l timentin to prevent *A. tumefaciens* overgrowth and then transferred to G10 medium supplemented with 100 mg/l timentin and 50 mg/l cefatoxime. The PLBs were cultured for a 16-h photoperiod (1,000 lux) at 25 °C and sub-cultured every week for 1 month. After this step, the traditional selection procedure was as follows: Transformed PLBs were transferred to PR medium containing Knudson C orchid medium salts (Knudson 1946; Duchefa BioChemie BV, Netherlands), 20 g/l sucrose, MS vitamins and 3 g/l phytagel at pH 5.4, supplemented with 200 mg/l timentin, 200 mg/l cefatoxime and 5 mg/l hygromycin for the selection of putative transformants. The hygromycin-resistant green PLBs were further sub-cultured to fresh G10 medium every month for 3 months. At this stage, shoots would regenerate from PLBs. After 3 months the shoots were sub-cultured in G10 medium without hygromycin for root formation. The alternative selection approach was as follows: Transformed PLBs were transferred to PR medium without any antibiotic agents but an overnight culture of *E. carotovora* (10^6 colony-forming units) was added to the medium for 1 week. This procedure was repeated twice. After 2 weeks, the resistant PLBs were transferred into PR medium supplemented with 200 mg/l timentin and 200 mg/l cefatoxime for 1 week. After eliminating bacteria, the PLBs were transferred to G10 medium for shoot regeneration. The juvenile shoots emerging from resistant PLBs were formed within 1 month. Regenerated plants about 3–4 cm in height, with five to six leaves and three to four roots, were eventually transferred to pots containing sphagnum moss and acclimatized under greenhouse conditions.

Transformation by particle bombardment

The transformation procedure was according to the method of Chan and Yu (1998). The bombarded PLBs were incubated in darkness for 7 days and then cultured for 16 h under light (1,000 lux) at 25 °C. After 30–40 days, the proliferating green

PLBs were sub-cultured onto G10 medium for regeneration. At this stage, an overnight culture of *E. carotovora* (10^6 colony-forming units) was added to new G10 medium and incubated for 1 week. This procedure was repeated twice. The surviving PLBs or shoots were transferred to PR medium, supplemented with 200 mg/l timentin and 200 mg/l cefatoxime, for 1 week. The following regeneration procedure was the same as for *A. tumefaciens*-mediated transformation.

Identification of transgenic orchid plants

All putative transgenic PLBs or rooted shoots on G10 medium were tested by GUS histochemical staining [0.1 M Na-phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc), 0.5 mM K-ferrocyanide (pH 7.0), 0.5 mM K-ferricyanide (pH 7.0)].

To confirm transgene integration, Southern blot analyses were performed in GUS-positive orchid plants. Nine independent transgenic lines were randomly selected for Southern blots. Total genomic DNA was extracted from leaves of rooted shoots on G10 medium, as described previously (Chan et al. 1993). The genomic DNA was digested with *Bam*HI and probed with *pflp*.

The transgenic plants subjected to Southern blot analysis were analyzed for *pflp* transcripts by northern blot. Total RNA was isolated using Triazole (Gibco-BRL) solution. RNA blots were prepared according to the method of Chan et al. (1994). The *GUS* gene excised from the *Bam*HI–*Sac*I restriction fragment of plasmid pBI221 (Clontech) and *pflp* cDNA isolated from pSPFLP were used as probes. To amplify an internal control rRNA fragment, the orchid 5.8 rRNA gene (accession number PMA237597) was used as a template for primer design. The 5′ primer (5′-ATGACTCTCG-ACAATGGATTT-3′) and 3′ primer (GCTTGAAGCCCAGGC-AGACG) were chosen to amplify the 161-bp 5.8 rRNA cDNA fragments. The PCR fragments were cloned into pT7Blue(R) and an ABI PRISM 373 automatic DNA sequencing system determined the DNA sequences. The rRNA cDNA was also used as a probe; however, low-stringency hybridization conditions were used when using this probe. These fragments were labeled with α-[^{32}P]dCTP, using the random-primer method (Feinberg and Vogelstein 1983).

Results

Transformation of *Oncidium* PLBs by *A. tumefaciens* and particle bombardment

The *pflp* gene was first transformed into *Oncidium* orchid PLBs via *A. tumefaciens*-mediated transformation. After 1 month, the newly formed PLBs rapidly grew in the G10 medium, devoid of selection agent. After the proliferation stage, *E. carotovora* was used to select the putative transformants. After two repeated infections, the untransformed PLBs turned brown and died within the *E. carotovora* infection period (Fig. 2A), whilst resistant PLBs were green (Fig. 2A). After the surviving PLBs had been sub-cultured, shoots developed rapidly, with roots forming spontaneously after 1 month. From 500 transformed PLBs, 60 PLBs survived *Erwinia* selection and 18 putative transgenic plants were successfully regenerated from culture (Table 1). The 18 putative transgenic plants were derived from independent transformation lines and designated as AG1 to AG18. These transgenic plants exhibited a normal phenotype. We randomly selected five independent *Erwinia*-resistant transgenic orchid plants for molecular analyses.

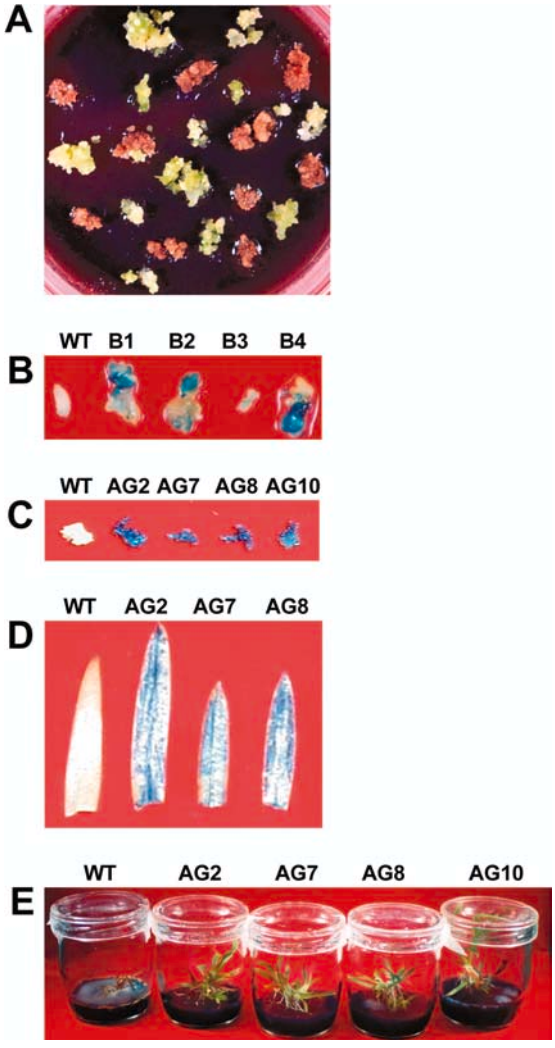

Fig. 2A–E Selection of transgenic orchid (*Oncidium*) by *Erwinia carotovora* and confirmation of foreign GUS gene expression in transgenic orchid. **A** Non-transgenic orchid PLBs (*brown*) did not survive on medium containing *E. carotovora*, whilst transgenic orchid PLBs (*green*) grew on the same medium. **B** Expression of GUS (*blue*) in PLBs of WT and transgenic orchids AG2, AG7, AG8 and AG10 derived from *A. tumefaciens*-mediated transformation. **C** Expression of GUS (*blue*) in PLBs of WT and transgenic orchids B1, B2, B3 and B4 derived from particle bombardment-mediated transformation. **D** Expression of GUS (*blue*) in leaves of WT and transgenic orchid plants AG2, AG7 and AG8. **E** The leaves of WT and transgenic orchid plants (AG2, AG7, AG8 and AG10) were injected with 3 μl of *E. carotovora* and incubated at 25 °C. The disease symptoms were assessed visually after 1 week

To establish whether the various transformation methods had any effect on transgene selection, we also used particle bombardment to transfer the *pflp* gene into *Oncidium* PLBs. After 1 month of the proliferation stage, we used *E. carotovora* to select transformed PLBs. We obtained results similar to those for *Agrobacterium*-mediated transformation. From 500 bombarded PLBs, 54 PLBs survived *Erwinia* infection and 14 putative transgenic plants were successfully regenerated from culture (Table 1). The 14 putative transgenic plants were derived from independent transformation lines and designated as

Table 1 Comparison of the selection efficiency of transformed PLBs by hygromycin and *Erwinia carotovora*. *ES*, *Erwinia* selection; *HS*, hygromycin selection; *SM*, selection medium

Transformation event	No. of PLBs transformed		No. of PLBs surviving on SM		Transformation efficiency (%)		No. of plants transferred to pots	
	ES	HS	ES	HS	ES	HS	ES	HS
Agrobacteriumtumefaciens	500	500	60	52	12	10.4	18	17
Particle bombardment	500	500	59	45	11.8	9	14	10

B1 to B14. We randomly selected four independent *Erwinia*-resistant transgenic orchid plants for molecular analyses. These results indicate that the *pflp* gene can be used as a novel selection gene in plant transformation.

Identification of transgenic *Oncidium* orchid plants

As a preliminary screening procedure the transformed PLBs were assayed by GUS histochemical staining. As shown in Fig. 2, independent *E. carotovora*-resistant PLBs derived from particle bombardment-mediated (Fig. 2B) and *A. tumefaciens*-mediated (Fig. 2C) transformation methods, exhibited intense blue color while non-transformed PLBs did not show blue coloration. After selection on *E. carotovora*-containing medium, the leaves of the putative transgenic orchid plants were further assayed by GUS histochemical staining. Leaves of three putative transgenic plants (AG2, AG7 and AG8) showed intense blue coloration compared with the leaf of a wild-type (WT) plant (Fig. 2D). These putative transgenic plants were selected for molecular analyses.

Transgenic *Oncidium* plants exhibited enhanced resistance to soft-rot disease

To clarify whether transgenic plants expressing the *pflp* gene had enhanced resistance to bacterial infection, we then injected shoots of four transgenic orchid and WT plants with suspensions of *E. carotovora* subsp. *carotovora* and incubated at 25 °C. One week later, WT plants exhibited water-soak symptoms, maceration and rotting caused by bacterial infection, whilst transgenic orchid plants showed resistance to bacterial infection and remained green (Fig. 2E). Initial maceration was also observed in transgenic orchid plants after injection; however, further invasion by the pathogen was significantly inhibited. These data, shown in Fig. 2E, clearly demonstrate that transgenic *Oncidium* orchid plants expressing the *pflp* gene confer enhanced resistance to soft-rot disease.

Molecular analyses of transgenic plants

The genomes of the five independent GUS-positive, *E. carotovora* resistant transgenic lines (AG2, AG7, AG8, AG10, and AG12) were analyzed for transgene integration by Southern hybridization. Total genomic DNA, isolated from the leaves of the transformed and WT plants, was digested with *Bam*HI and probed with the *pflp* gene. As *pflp* is known to be a sweet pepper ferredoxin-like protein gene (Lin et al. 1997), we used high-stringency hybridization conditions to identify transgene integration. All of the determined transgenic plants had a single copy of the integrated gene. The fragment size integrated ranged from around 9 kb for AG2, AG10 and AG12 (lanes 2, 5 and 6 in Fig. 3A) to 6.7 kb and 5 kb in AG7 and AG8 respectively (lanes 3 and 4 in Fig. 3A). Transgenic plants B1 to B4 derived from particle bombardment-mediated transformation, however, showed multiple integration of the transgene (Fig. 3B). These results confirmed the integration of the *pflp* transgene in the *Oncidium* genome. These results clearly demonstrate that *pflp* combined with the selection agent *E. carotovora*, can be used efficiently to select transgenic plants.

High expression of *pflp* mRNA in transgenic *Oncidium* orchid plants

Northern blot analyses were performed to evaluate transgene expression in nine transgenic orchid plants (AG2, AG7, AG8, AG10, AG12, B1, B2, B3 and B4) (Fig. 4A, B). *pflp* and *GUS* mRNA transcripts were highly expressed in all transgenic orchid plants (lanes 2–6 in Fig. 4A; lanes 2–5 in Fig. 4B), whereas *pflp* and *GUS* mRNA transcripts were not detected in WT plants (lanes 1 in Fig. 4A, B). rRNA transcripts were used as an internal control in these experiments. The level of *rRNA* was similar in both transgenic and WT orchid plants (Fig. 4).

Comparison of the selection efficiency between the antibiotic and *pflp* systems

The selection efficiency of the *pflp* system was now compared to that of a traditional antibiotic selection system by using hygromycin to select transformants in a parallel experiment. From 500 *Agrobacterium*-mediated transformed PLBs, 52 survived hygromycin selection and 17 putative transgenic plants were successfully regenerated from culture. These transgenic plants were confirmed by Southern and northern blot analyses (data not shown).

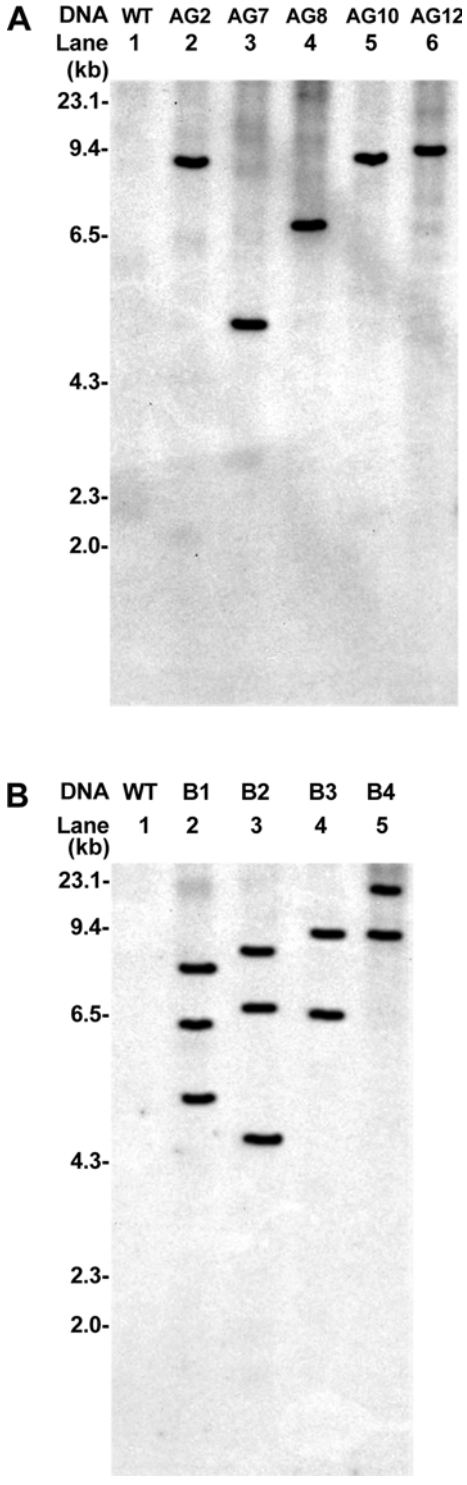

Fig. 3A, B Foreign genes integrated into transgenic orchid plants. **A** Transgenic orchid plants driven from *Agrobacterium*-mediated transformation. *Lane 1* DNA from a WT plant, *lanes 2–6* DNA from transgenic plants (AG2, AG7, AG8, AG10, AG12). **B** Transgenic orchid plants derived from particle bombardment-mediated transformation. *Lane 1* DNA from a WT plant, *lanes 2–5* DNA from transgenic plants (B1, B2, B3 and B4). Genomic DNA (10 μg) was digested with *Bam*HI and hybridized with the *AP1* probe. The probe used was ^{32}P-labeled *pflp* cDNA from pSPFLP

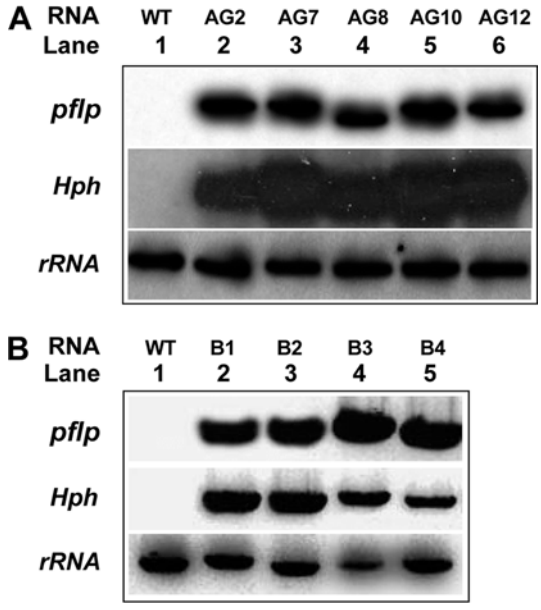

Fig. 4A, B Transcripts of the *pflp* gene expressed in transgenic orchid plants but not in WT plants. **A** Total RNA (10 μg) was extracted from WT (*lane 1*) and transgenic plants (AG2, AG7, AG8, AG10, AG12). **B** Total RNA (10 μg) was extracted from WT (*lane 1*) and transgenic plants (B1, B2, B3 and B4). Probes used were ^{32}P-labeled *pflp*, *GUS* and orchid rRNA cDNA fragments

Furthermore, using the particle-bombardment method we transformed 500 PLBs and obtained 45 transformed PLBs and 10 transgenic plants, which survived hygromycin selection. These results indicate that the transgene selection efficiency of the *pflp* gene is similar to those using antibiotics. The comparison of selection efficiency of transformed PLBs by hygromycin and *E. carotovora* is given in Table 1. The data were collected from 5 separate experiments for each transformation event; each event contained a minimum of 100 PLBs, including controls (transformed PLBs on hygromycin selection).

Discussion

In this study, we demonstrate a novel method for transgenic plant selection utilizing the sweet pepper ferredoxin-like protein (*pflp*) gene and *E. carotovora* as the selection agent. Transformed PLBs survived the *E. carotovora* selection whereas the non-transformed PLBs died on the medium containing the pathogen (Fig. 2A). The transgenic nature of the putative orchid plants was confirmed by Southern and northern blotting (Fig. 4). In this study, the transgenic orchid plants showed a normal phenotype and we did not observe any growth retardation compared with WT orchid plants. The use of *pflp* and *E. carotovora* as selection systems reduces the selection time for obtaining transgenic plants as compared to the routinely followed selection procedure. Putative transgenic *pflp* plants were obtained by *E. carotovora* selection after 2 weeks, whilst putative transgenic plants were obtained by hygromycin selection

only after 2–3 months. This is an advantage of the *pflp* system because long-term tissue culture selection may cause somaclonal variation of orchid PLBs. In addition, *pflp* selection is less labor intensive, avoiding frequent sub-culturing of putative transformants. Furthermore, selection by hygromycin always inhibits the root formation of transgenic plants, especially in orchid (our unpublished data). Using the *pflp* selection system, we were able to overcome this phenomenon because transgenic plants grew well in medium devoid of antibiotic.

Our results suggest that the *pflp* gene may be extremely useful for transgenic selection in plant genetic engineering strategies. Transgenic orchid plants, created in this manner, as well as conferring resistance against soft-rot disease, could also be used to carry additional genes of choice, due to the removal of the large fragment containing the promoter, and sequences of the antibiotic gene and terminator. This *pflp* selection system makes it possible to design strategies for introducing economically important genes into any orchid genus and testing the efficiency of transformation more rapidly than currently existing protocols. This study also suggests that this *pflp* selection system can be applied to improve other crop plants, such as potato and maize, which are natural hosts of *E. carotovora*. However, using plants that are not natural hosts of *E. carotovora* as transformation materials might be the limitation of the *pflp* selection system, as the antimicrobial properties of the *pflp* gene against other microbes remains unproven. From the published evidence and our recent results, it is suggested that this selection system can be applied for rice and tobacco transformation. Our studies have demonstrated that transgenic *pflp* tobacco plants are resistant to *Pseudomonas* (unpublished data) and that transgenic *pflp* rice plants are resistant to *Xanthomonas* (Tang et al. 2001), respectively.

So far the *pflp* system has not been optimized by comprehensive selection experiments, but is already more efficient than traditional hygromycin-based systems. Although the selection efficiency does not exceed that of the hygromycin selection, the reduced time period required for obtaining transgenic orchid plants is an advantageous factor for potential plant transformation, especially orchid. Moreover, we believe that the employment of this selection system may diminish the possibility of toxicity or allergenicity of the gene product to humans, a concern raised in relation to the use of antibiotic or herbicide resistance marker genes and their presence in transgenic plants. The disease resistance mechanism of the *pflp* gene in plants is presently unclear; however, we have demonstrated in this study that the *pflp* gene can be used successfully as part of a novel selection system in plant transformation experiments.

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