DIANTHIN, a negative selection marker in tobacco, is non-toxic in transgenic rice and confers sheath blight resistance

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Abstract

The *DIANTHIN* gene encoding a ribosome-inactivating protein (RIP) from *Dianthus caryophyllus* L. was tested for negative selection in tobacco and rice. Tobacco leaf discs and scutellum-derived callus of rice were transformed with *Agrobacterium tumefaciens* strain LBA4404 (pSB1, pJAS1). pJAS1 harbors the *DIANTHIN* gene under the control of the CaMV 35S promoter. Tobacco transformation efficiency, in comparison to pCAMBIA1301, was reduced by 87 % in pJAS1-transformed leaf discs. The *DIANTHIN* gene proved to be completely toxic to tobacco as all the recovered hygromycin-resistant transgenic plants harbored truncated T-DNAs with deletions of the *DIANTHIN* gene. Transformation of the *DIANTHIN* gene under a *Mungbean yellow mosaic virus* (MYMV)-inducible promoter did not cause any toxicity in tobacco as reflected by the recovery of transgenic tobacco plants with the complete *DIANTHIN* gene. Transformation efficiency of pJAS1 did not decline in rice. Interestingly, all transgenic rice plants harbored the complete *DIANTHIN* gene and expressed the gene. The T_1 transgenic lines showed reduction of sheath blight symptom in the range of 29 to 42 %. The difference in the sensitivity to *DIANTHIN* between tobacco and rice provides a new direction to study the mechanisms underlying RIP toxicity in plants.

Additional key words: *Nicotiana tabacum*, *Oryza sativa*, *Rhizoctonia solani*, ribosome-inactivating protein, truncated T-DNA.

Introduction

Negative selection marker (NSM) genes, which allow selection of cells or organisms that lack these genes, are categorized into conditional (substrate-dependent) and non-conditional (substrate-independent) types (reviewed in Miki and McHugh 2004). Conditional NSMs like *codA*, indoleacetamide hydrolase (*tms2*), dehalogenase (*dhlA*), cytochrome P450 monooxygenase (*P450*), alcohol dehydrogenase (*cue*), D-amino acid oxidase (*dao1*) (Erikson *et al*. 2004) and herpes simplex virus thymidine kinase (*HSVtk*) (Czako and Marton 1994) require the substrates 5-fluorocytosine, naphthalene acetamide, dihaloalkanes, sulfonylurea R7402, allyl alcohol, D-isoleucine and ganciclovir, respectively, which get converted to toxic products (reviewed in Miki and McHugh 2004). Non-conditional NSMs are toxic and hence do not require any additional steps. The genes encoding barnase (Mariani *et al*. 1992), *Pseudomonas aeruginosa* exotoxin A (Koning *et al*. 1992), yeast RAS2 (Hilson *et al*. 1990) and diphtheria toxin A fragment (DT-A) (Terada *et al*. 2002) are examples of non-conditional NSM genes. NSMs have been deployed for gene targeting in higher plants through homologous recombination (Terada *et al*. 2002, Thykjaer *et al*. 1997), to engineer male sterility by anther specific expression

Received 27 October 2008, *accepted* 19 May 2009.

Abbreviations: *int-gus* - β-glucuronidase gene with an intron; *hph* - hygromycin phosphotransferase gene; Hyg^r - hygromycin-resistant; Hyg^s - hygromycin-sensitive; MS - Murashige and Skoog; NSM - negative selection marker; RIP - ribosome-inactivating protein.

Acknowledgements: We gratefully acknowledge Dr. John Stanley, UK, for providing the *DIANTHIN* gene. We thank Dr. Toshihiko Komari, Japan Tobacco Inc., Japan, for providing pSB1 and Dr. M. Samiappan, Tamil Nadu Agricultural University, Coimbatore, India for providing the *Rhizoctonia solani* strain RS7. We thank Dr. K. Dharmalingam for permitting us to use his instrumentation facilities. R. Rajeswaran is acknowledged for constructing pRAJ18 and for performing tobacco transformation with pRAJ18. This research was supported by funds from the Department of Biotechnology (DBT) and University Grants Commission (UGC), Government of India. JMS is thankful to Council of Scientific and Industrial Research (CSIR), New Delhi for Fellowship.

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(Mariani *et al*. 1992, Cho *et al*. 2001), as tools in transposon tagging (Sundaresan *et al*. 1995), to generate selectable marker-free transgenic plants (Gleave *et al*. 1999) and to eliminate transfer of vector backbone in *Agrobacterium*-mediated plant transformation (Hanson *et al*. 1999).

Ribosome-inactivating proteins (RIPs) are toxic proteins that enzymatically damage ribosomes in an irreversible manner (reviewed in Stirpe and Battelli 2006). RIPs cleave the glycosidic bond of the first adenine residue A_{4324} in the tetranucleotide GAGA of the highly conserved α-sarcin loop of 26-28 S rRNA (Endo *et al*. 1987). RIPs are proposed to have a defensive role in nature in view of their antiviral and antifungal activity and toxicity against predators (reviewed in Nielsen and Boston 2001).

Transgenic tobacco plants expressing a barley RIP gene (Logemann *et al*. 1992, Jach *et al*. 1995) and a maize RIP gene (b-32 protein) (Maddaloni *et al*. 1997) showed resistance against *Rhizoctonia solani*. The *PhRIP1* gene from *Phytolacca heterotepala*, expressed in transgenic tobacco under the control of a wound-inducible promoter of the bean polygalacturonase-inhibiting protein I gene, exhibited resistance against *Alternaria alternata* and *Botrytis cinerea* (Corrado *et al*. 2005). Transgenic rice expressing trichosanthin, an RIP from *Trichosanthes kirilowi*, exhibited resistance against *Magnaporthe grisea* (Yuan *et al*. 2002). Transgenic rice plants expressing a modified maize RIP b-32 in combination with rice chitinase (Kim *et al*. 2003) and transgenic tobacco plants expressing barley RIP, barley chitinase and barley β-1,3-glucanase (Jach *et al*. 1995) genes were resistant to *R. solani*. Expression of pokeweed antiviral protein II (PAPII) and curcin2, RIPs from *Phytolacca americana* (Wang *et al*. 1998) and *Jatropha curcas* (Huang *et al*. 2008), respectively, generated fungal (*R. solani*) and viral (*Tobacco mosaic virus*) resistance in transgenic tobacco.

The aim of this research was to evaluate the toxicity of the *DIANTHIN* gene encoding a type 1 RIP from *Dianthus caryophyllus* L. (carnation) (Stirpe *et al*. 1981) in tobacco and rice using the transgenic approach reported by Terada *et al*. (2004) and highlight the possible difference in the toxicity of RIP in monocotyledonous and dicotyledonous plants.

Materials and methods

Binary vector and *Agrobacterium* strains: pJAS1 is a pCAMBIA1301-derived binary vector harboring the *DIANTHIN* gene (Hong *et al*. 1996) under the control of CaMV 35S promoter. pCAMBIA1301 and pJAS1 (Fig. 1*A*) harbor the hygromycin phosphotransferase (*hph*) gene as the plant positive selection marker and the β-glucuronidase (*int-gus*) gene with an intron as the reporter. pRAJ18 (Fig. 1*B*) is a pGA472-derived binary vector harboring the *DIANTHIN* gene under the control of *Mungbean yellow mosaic virus* (MYMV)-induced BV1 promoter (Rajeswaran *et al*. 2007). The binary plasmids pJAS1 and pCAMBIA1301 were mobilized by triparental mating into the *Agrobacterium tumefaciens vir* helper strain LBA4404 (pSB1) (Komari *et al*. 1996). PRAJ18 was mobilized into the *A. tumefaciens* strain EHA105.

Transformation of tobacco and rice: Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) leaf discs were transformed using *Agrobacterium* as described by Sunilkumar *et al*. (1999). The regenerated transgenic shoots were established on the shoot-induction medium [Murashige and Skoog (MS) medium + 4 μM benzylaminopurine + 0.5 μM naphthaleneacetic acid + 0.9 % agar] supplemented with 250 mg dm^{-3} cefotaxime and 50 mg dm^{-3} hygromycin (for pJAS1 and pCAMBIA1301) or 250 mg dm^{-3} cefotaxime and 100 mg dm^{-3} kanamycin (for pRAJ18). Scutellum-derived calli from mature seeds of *Oryza sativa* L. subsp. *indica* cv. Pusa Basmati1 (PB1) were used for infection with *A. tumefaciens* strain LBA4404 (pSB1, pJAS1) and LBA4404 (pSB1, pCAMBIA1301). Callus induction, *Agrobacterium*-mediated transformation and regeneration of transgenic rice plants were done as described earlier (Sridevi *et al*. 2003).

DNA analysis: DNA was extracted from tobacco and rice plants (Rogers and Bendich 1988). The primers used to amplify the 1.0-kb *hph* gene were 5'-AAAGCCTGAACTCACCCGC-3' and 5'-GGTTTCCACTATCGGCGAC-3'. A 627-bp *DIANTHIN* fragment was amplified using the primers 5'-TCGAGGAACGGTCTCTCTTG-3' and 5'-ATGAGCTGTGTCGTCAGTGG-3'. For Southern hybridization analysis, 2.5 μg of rice DNA or 10 μg of tobacco DNA were digested with *Hin*dIII, electrophoresed in 0.8 % agarose gels and transferred onto *Zeta-probe* nylon membrane (*Bio-Rad*, Hercules, CA). The *hph* and *DIANTHIN* gene fragments were labeled with [α-^{32}P]dCTP (*Board of Radiation and Isotope Technology*, Hyderabad, India) using the *Megaprime*^{TM} DNA labeling system (*GE Healthcare*, Little Chalfont, UK) and used as probes.

Expression analysis: Accumulation of the *DIANTHIN* transcript in transgenic plants was studied by Northern blot analysis. Total RNA was extracted (Pawlowski *et al*. 1994) and 10 μg RNA was electrophoresed in a denaturating 1.2 % agarose gel containing 1 % formaldehyde, transferred onto a positively charged nylon membrane (*Roche Diagnostics*, Mannheim, Germany) and hybridized to the [α-^{32}P]dCTP-labelled *DIANTHIN* gene probe.

Resistance analysis against *Rhizoctonia solani*: Infection assay of the transgenic and control rice plants for sheath blight resistance was done as described previously (Sridevi *et al*. 2003). Seven days after inoculation with *R. solani*, the sheath blight symptom was graded in a scale of 0 to 5 and disease index was determined using the formula: disease index = (mean grade point per plant/5) × 100.

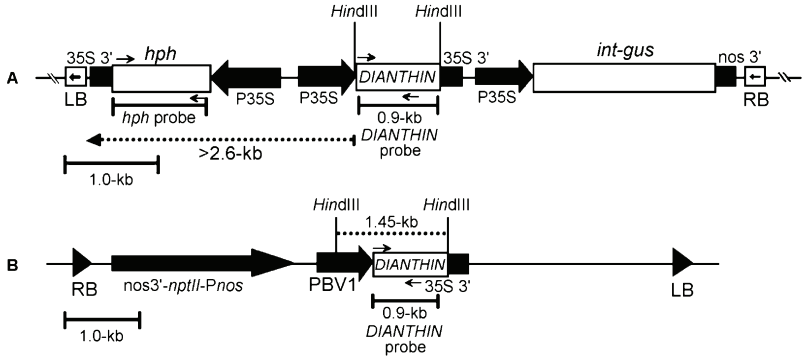

Fig. 1. The T-DNA regions of binary plasmids. The T-DNA of pJAS1 (*A*), which harbors P35S-*DIANTHIN*, P35S-*hph* and P35S-*int-gus* genes. The left border junction fragment (>2.6 kb, the distance between the *Hin*dIII site and LB), marked with a *dotted arrow*, will hybridize to the *hph* probe (marked with a *full line*). The 0.9-kb *DIANTHIN* gene (marked with a *full line*), flanked by *Hin*dIII sites on either side, will hybridize to a 0.9-kb *Hin*dIII fragment. P35S, *Cauliflower mosaic virus* 35S promoter; 35S3', *Cauliflower mosaic virus* 3' region; LB, left T-DNA border; RB, right T-DNA border; *hph*, hygromycin phosphotransferase gene; *int-gus*, β-glucuronidase gene with an intron; nos3', nopaline synthase gene 3' region. The T-DNA of pRAJ18 (*B*), which harbors the PBV1-*DIANTHIN* and P*nos*-*npt*II genes. The 1.45-kb internal T-DNA fragment, flanked by two *Hin*dIII sites (marked with a dotted line) will hybridize to the *DIANTHIN* probe (marked with a *full line*). P*nos*, nopaline synthase gene promoter; *nptII*, neomycin phosphotransferaseII. Scale (1.0 kb) is marked. *Arrows* mark the locations of forward and reverse primers of *hph* and *DIANTHIN* genes.

Results

***DIANTHIN* expression contributes to negative-selection in tobacco:** Tobacco transformation with pCAMBIA1301, a binary vector without the *DIANTHIN* gene, yielded 100 % transformation of tobacco leaf discs. However, transformation with pJAS1, which harbored the *DIANTHIN* gene, resulted in transformation of only 13 %

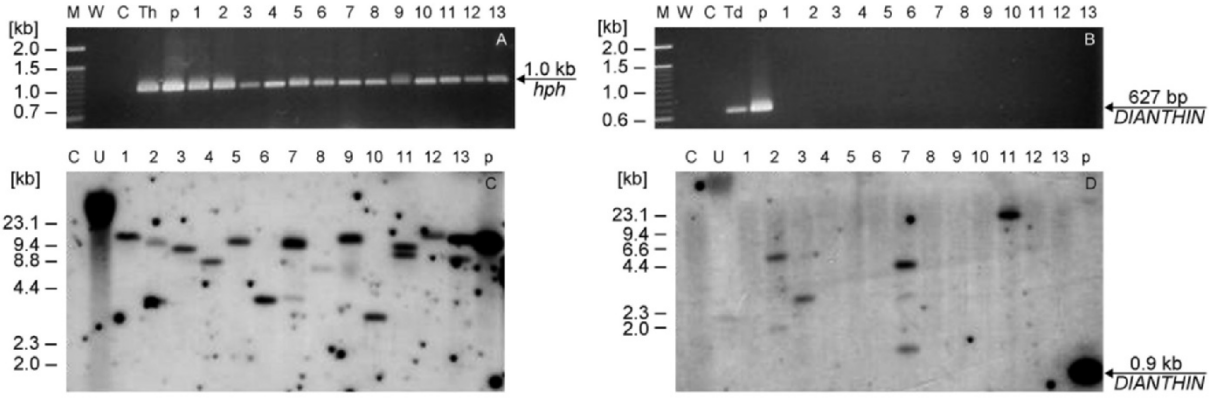

Fig. 2. PCR and Southern blot analysis of tobacco plants transformed with pJAS1. PCR analysis with *hph* primers (*A*) and *DIANTHIN* primers (*B*). Analysis of left border junction fragments with the [α-^{32}P]dCTP-labelled *hph* probe (*C*). Analysis of integration of the complete *DIANTHIN* gene with the [α-^{32}P]dCTP-labelled *DIANTHIN* probe (*D*). C - untransformed tobacco plant DNA (100 ng undigested DNA in *A* and *B* and 10 µg *Hin*dIII-digested DNA in *C* and *D*); U - undigested DNA of the plant 1; 1 to 13 - DNA samples of pJAS1-transformed tobacco plants (100 ng undigested DNA in *A* and *B* and 10 µg *Hin*dIII-digested DNA in *C* and *D*); p - pJAS1 (50 pg undigested DNA in *A* and *B* and 100 pg *Hin*dIII-digested DNA in *C* and *D*); W - water control; Th - Southern blot analysis-confirmed tobacco plant DNA harboring the *hph* gene; Td - BV1 promoter-driven *DIANTHIN* gene-harboring tobacco DNA; numbers on the left of *A* and *B* indicate the sizes of 100-bp ladder; of *C* and *D* indicate the sizes of λ-*Hin*dIII marker.

of the leaf discs. Thirteen hygromycin-resistant (Hyg^r) tobacco plants that regenerated following pJAS1 transformation were analyzed by PCR with *hph* and *DIANTHIN* primers. DNA from all 13 Hyg^r plants showed the expected amplification of 1.0 kb *hph* gene (Fig. 2*A*). Though the plasmid pJAS1 (positive control for PCR) amplified the expected 627 bp *DIANTHIN* fragment, DNA from all 13 Hyg^r plants did not amplify the *DIANTHIN* gene (Fig. 2*B*).

To study the T-DNA integration, the pJAS1-transformed tobacco plant DNA was digested with *Hin*dIII and subjected to Southern hybridization analysis with *hph* and *DIANTHIN* probes. Upon integration of the complete T-DNA, one or more *Hin*dIII-released junction fragments longer than 2.6 kb are expected to hybridize with the *hph* probe (Fig. 1*A*). *Hin*dIII sites are present on both sides of the *DIANTHIN* gene in the T-DNA. Hence, if complete T-DNA is inserted, the *Hin*dIII-digested DNA of transgenic plants is expected to display hybridization of a 0.9 kb band with the *DIANTHIN* probe (Fig. 1*A*). DNA from all 13 Hyg^r plants hybridized to the *hph* probe (Fig. 2*C*), indicating that they harbored the *hph* part of the T-DNA. *Hin*dIII-digested pJAS1, the plasmid positive control, displayed hybridization of 0.9 kb fragment to the *DIANTHIN* probe (Fig. 2*D*). However, DNA from none of the plants showed hybridization of the *DIANTHIN* probe to the expected 0.9 kb internal T-DNA fragment. In four plants (2, 3, 7 and 11), the *DIANTHIN* probe hybridized to fragments longer than 0.9 kb (Fig. 2*D*), suggesting that integration of T-DNA with the truncated *DIANTHIN* gene generated junction fragments of varying lengths. Plants 1, 4, 5, 6, 8, 9, 10, 12 and 13 did not exhibit signal with the *DIANTHIN* probe, indicating that they harbored truncated T-DNAs, devoid of the *DIANTHIN* gene. Toxicity of the expressed dianthin protein may have contributed to the elimination of the events comprising complete T-DNAs with intact *DIANTHIN* gene (Terada *et al*. 2004). Consequently, only transgenic events with incomplete T-DNAs either without the *DIANTHIN* gene or with truncated *DIANTHIN* gene were recovered.

The dominant negative selection of the *DIANTHIN* gene can be avoided if it is placed under an inducible promoter. The promoter of the *Mungbean yellow mosaic virus* (MYMV) *BV1* gene, induced by the MYMV AC2 protein (Rajeswaran *et al*. 2007), was fused to the *DIANTHIN* gene and placed in a binary vector pGA472 to yield pRAJ18. Since the *DIANTHIN* gene will not express constitutively, we expected to recover transgenic plants with the whole T-DNA harboring the complete *DIANTHIN* gene. Eight BV1- *DIANTHIN* plants were subjected to Southern blotting. In case of complete T-DNA transfer, *Hin*dIII digestion of pRAJ18-T-DNA will result in the hybridization of a 1.4 kb internal T-DNA fragment with the *DIANTHIN* probe (Fig. 1*B*). The *Hin*dIII-digested DNA of all 8 transgenic tobacco plants showed hybridization of the 1.4 kb fragment (Fig. 3). Thus, the whole T-DNA with complete *DIANTHIN* gene was integrated in all 8 transgenic plants obtained by PBV1- *DIANTHIN* gene transformation.

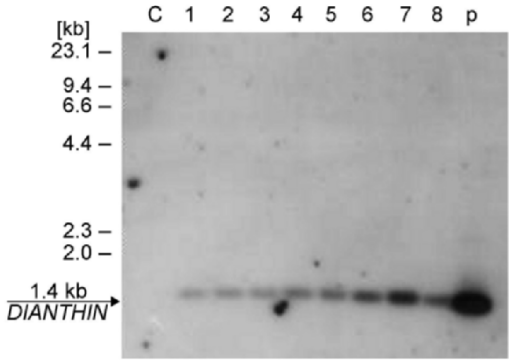

Fig. 3. Southern blot analysis of pRAJ18-transformed tobacco plants using the [α-^{32}P]dCTP-labelled *DIANTHIN* gene probe. C - untransformed tobacco plant DNA (10 μg) digested with *Hin*dIII; 1 to 8 - *Hin*dIII-digested DNA (10 μg) of pRAJ18-transformed tobacco plants; p - 250 pg of pRAJ18 digested with *Hin*dIII; numbers on left indicate the sizes of λ-*Hin*dIII marker.

Recovery of transgenic rice plants expressing the *DIANTHIN* gene: Transformation of scutellum-derived calli (150 for each) with LBA4404 (pSB1, pJAS1) (with the *DIANTHIN* gene) and LBA4404 (pSB1, pCAMBIA1301) yielded 11 and 9 Hyg^r plants, respectively. Unlike in tobacco, the numbers of Hyg^r rice plants recovered following transformation with pCAMBIA1301 (without *DIANTHIN*) and pJAS1 (with *DIANTHIN*) were not much different. PCR analysis with *hph* primers and *DIANTHIN* primers on 11 Hyg^r plants from pJAS1 transformation resulted in the amplification of the expected 1.0 kb and 627 bp fragments, respectively (Fig. 4*A,B*). Upon integration of the complete T-DNA, *Hin*dIII-released junction fragments longer than 2.6 kb and an internal T-DNA fragment of 0.9 kb are expected to hybridize to the *hph* and *DIANTHIN* probes, respectively (Fig. 1*A*). DNA from all 11 Hyg^r plants hybridized to the *hph* probe and displayed junction fragments longer than 2.6 kb (Fig. 4*C*). As expected for the integration of the whole T-DNA with complete *DIANTHIN* gene, all 11 transgenic rice plants exhibited hybridization of the 0.9 kb internal T-DNA fragment to the *DIANTHIN* probe (Fig. 4*D*). Northern blot analysis of six single-copy T_0 plants (PBDIA-2, PBDIA-4, PBDIA-7, PBDIA-8, PBDIA-10 and PBDIA-11) revealed hybridization of a 1.1 kb *DIANTHIN* transcript (Fig. 5).

Six single-copy transgenic rice plants (PBDIA-2, PBDIA-4, PBDIA-7, PBDIA-8, PBDIA-10 and PBDIA-11) were selfed and 40 seeds from each line were germinated on half-strength MS medium containing 50 mg dm^{-3} hygromycin to study segregation of the *hph* gene in the T_1 plants. Five lines showed 3:1 (Hyg^r/Hyg^s) segregation ratio of the *hph* gene, as expected for single-

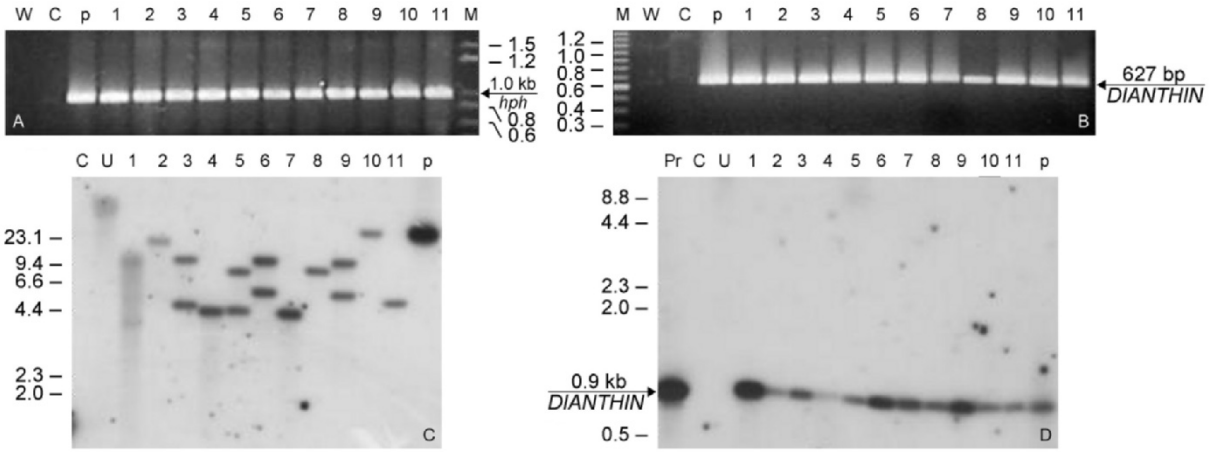

Fig. 4. PCR and Southern blot analysis of rice plants transformed with pJAS1. PCR analysis with *hph* primers (*A*) and *DIANTHIN* primers (*B*). Analysis of left border junction fragments with the [α-^{32}P]dCTP-labelled *hph* probe (*C*). Analysis of integration of complete *DIANTHIN* gene with the [α-^{32}P]dCTP-labelled *DIANTHIN* probe (*D*). C - untransformed rice plant DNA (100 ng undigested DNA in *A* and *B* and 2.5 μg *Hin*dIII-digested DNA in *C* and *D*); U - undigested DNA of the plant 1. Numbers 1 to 11 are DNA of pJAS1-transformed rice plants, PBDIA-1, PBDIA-2, PBDIA-3, PBDIA-4, PBDIA-5, PBDIA-6, PBDIA-7, PBDIA-8, PBDIA-9, PBDIA-10 and PBDIA-11 (100 ng undigested DNA in *A* and *B* and 2.5 μg *Hin*dIII-digested DNA in *C* and *D*). p - the plasmid pJAS1 (50 pg undigested DNA in *A* and *B* and 100 pg *Hin*dIII-digested DNA in *C* and *D*), Pr - 50 pg of *DIANTHIN* gene, W - water control, numbers on the left of *A* and *B* indicate the sizes of 100-bp ladder, numerals on the left of *C* and *D* indicate the sizes of λ-*Hin*dIII marker.

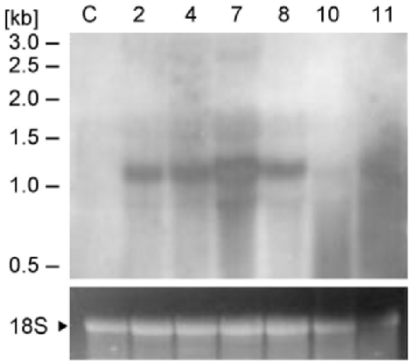

Fig. 5. Expression of the *DIANTHIN* gene in the single-copy transgenic rice plants transformed with pJAS1. Total RNA (10 μg) from the untransformed control rice plant (C) and six transgenic rice plants (2, 4, 7, 8, 10 and 11, corresponding to PBDIA-2, PBDIA-4, PBDIA-7, PBDIA-8, PBDIA-10 and PBDIA-11, respectively) was probed with the [α-^{32}P]dCTP-labelled *DIANTHIN* gene. Bottom panel is the portion of ethidium bromide stained gel indicating equal levels of 18S-rRNA in all lanes. The sizes of marker RNA are shown *on the left*.

copy integration events (results not shown). Sufficient seeds of the line PBDIA-8 were not available for reliable statistical analysis of segregation.

Analysis of resistance against *Rhizoctonia solani*: An infection assay with *R. solani* was performed to test whether constitutive expression of the *DIANTHIN* gene reduced the sheath blight disease in transgenic rice. Five T_1 transgenic rice lines (PBDIA-2, PBDIA-4, PBDIA-7, PBDIA-10 and PBDIA-11) and untransformed control plants were inoculated with *R. solani*, and the disease index was scored 7 d after infection. The disease index, which was 62.6 in the control plants, reduced to the range of 44.4 to 36.3 in the transgenic plants (Table 1). The T_1 plants from the line PBDIA-11 did not show resistance (disease index of 62.2). The maximum reduction (42 %) of sheath blight disease was exhibited by T_1 plants of the line PBDIA-7.

Table 1. *Rhizoctonia solani* infection assay on transgenic T_1 rice lines harboring the *DIANTHIN* gene. Means ± SE, n = 27. Means marked by different letters are significantly different at 1 % level as determined by Student's t-test.

Plant	Disease index	Plant	Disease index
Control	$62.6^{a} \pm 4.2$	PBDIA-7	$36.3^{b} \pm 5.8$
PBDIA-2	$42.9^{b} \pm 4.3$	PBDIA-10	$37.0^{b} \pm 4.0$
PBDIA-4	$44.4^{b} \pm 4.3$	PBDIA-11	$62.2^{a} \pm 5.5$

Discussion

Tobacco transformation efficiency markedly reduced from 100 % with pCAMBIA1301 to 13 % with pJAS1 (pCAMBIA1301 harboring the *DIANTHIN* gene), suggesting a negative impact of *DIANTHIN* on tobacco

transformation. Terada *et al*. (2004) reported that transgenic rice plants recovered following transformation with the non-conditional NSM gene *DT-A*, harbored truncated T-DNAs that lacked the *DT-A* part of the T-DNA. PCR with *hph* and *DIANTHIN* primers and Southern blot analysis with *hph* and *DIANTHIN* probes revealed that all 13 pJAS1-transformed Hyg^r tobacco plants harbored truncated T-DNAs, which had the intact *hph* gene, but the *DIANTHIN* gene was either absent or was incomplete. We did not recover even a single transgenic tobacco plant harboring the whole T-DNA with the complete *DIANTHIN* gene. As in the case of the *DT-A* gene in rice (Terada *et al*. 2004), the non-conditional NSM gene *DIANTHIN* exerted a total negative selection in tobacco and no escape was observed. In contrast, conditional NSM genes like *codA*, *P450* and *HSVtk* yielded a low frequency of escapes in plants transformed with them (Czako and Marton 1994, Koprek *et al*. 1999).

In accordance with the hypothesis linking toxicity of constitutively expressed *DIANTHIN* and T-DNA truncation, we found that transformation of tobacco with the *DIANTHIN* gene under the control of the MYMV-inducible BV1 promoter (pRAJ18) resulted in the recovery of transgenic tobacco plants harboring the whole T-DNA with the complete *DIANTHIN* gene. In an earlier report (Hong *et al*. 1996), viable transgenic *Nicotiana benthamiana* plants were obtained with the *DIANTHIN* gene placed under the control of the *AV1* promoter of *African cassava mosaic virus* (ACMV). Transactivation of the PAV1-*DIANTHIN* gene brought about by ACMV infection, caused hypersensitive cell death and conferred protection against ACMV.

Since *DIANTHIN* is a non-conditional NSM, it directly exhibits toxicity without requiring the treatment with a substrate. This is a desirable feature that will help in eliminating ectopic integrations in gene targeting experiments (Terada *et al*. 2002). The substrates for conditional NSMs like *P450* (Korpek *et al*. 1999), *codA* (Schlaman and Hooykaas 1997, Korpek *et al*. 1999), *tms2* (Upadhyaya *et al*. 2000), nitrate reductase (*NR*; Nussaume *et al*. 1991) and *HSVtk* (Czako and Marton 1994), though considered non-toxic, did cause inhibitory effect on non-transgenic plants also. Since the expressed dianthin protein is localized only in the cells expressing it, the action of *DIANTHIN* is cell autonomous, an important factor required for an NSM (Schlaman and Hooykaas 1997). Conditional NSMs including *dao1* (Erikson *et al.* 2004), nitrite reductase (Nussaume *et al*. 1991), *P450* (Korpek *et al*. 1999), *codA* (Korpek *et al*. 1999) and *tms2* (Upadhyaya *et al*. 2000) have been tested on seedlings. Exertion of negative selection in the tissue culture phase is advantageous since it enriches the selection of only desirable transgenic events in gene targeting (Terada *et al.* 2002). *DIANTHIN* exhibited this advantage in tobacco.

Interestingly, *DIANTHIN*, which was toxic to tobacco, did not exhibit toxicity in rice. RIPs are known to show different substrate specificities (reviewed in Nielsen and Boston 2001, Stirpe and Battelli 2006). Proteins that interact with RIPs and arrest translation may exhibit differences in different plants. In contrast to tobacco transformation, PCR and Southern blotting analysis of the pJAS1-transformed rice plants showed that all Hyg^r plants harbored the whole T-DNA with the complete *DIANTHIN* gene. Six single-copy rice plants taken for analysis expressed the *DIANTHIN* gene as revealed by Northern blot analysis. Resistance against *R. solani* was higher in *DIANTHIN*-harboring T_1 rice plants (disease index in the range of 42.9, 44.4, 36.3 and 37), when compared to the untransformed control plants (disease index of 62.6). This report describes the effectiveness of *DIANTHIN* as an antifungal protein gene in transgenic rice.

This work serves as the basis for developing vectors comprising *DIANTHIN* as an NSM for gene targeting in tobacco and other dicotyledonous plants in which it is toxic. An RIP derived from barley (a monocotyledonous plant) was not toxic to the dicotyledonous plants tobacco (Logemann *et al*. 1992) and soybean (Li *et al*. 2004), but was toxic to the monocotyledonous plant wheat (Bliffeld *et al*. 1999). Constitutively expressed dicotyledonous plant-derived RIPs, ricin A chain (Frigerio *et al*. 1998) and PhRIP I (Corrado *et al*. 2005) were toxic to tobacco and PAP was toxic to potato (Lodge *et al*. 1993). We report that toxicity of dianthin (derived from a dicotyledonous plant) is markedly different between a dicotyledonous plant, tobacco and a monocotyledonous plant, rice. These studies offer new directions to understand the mechanisms underlying RIP toxicity in plants.

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