Australian Journal of Crop Science AJCS
AJCS 6(5):904-911(2012) ISSN:1835-2707

Transform of an ectopically expressed bulb lectin gene from *Pinellia pedatisecta* into tobacco plants conferring resistance to aphids (*Myzus nicotianae*)

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Abstract

Plant lectins are widely distributed in the plant kingdom, and a number of cDNAs have been isolated from many plants. Here we reported the isolation an expression analysis of a cDNA from *Pinellia pedatisecta* named PPAb. The cDNA clone was obtained from the bulb using a reverse transcriptase-polymerase chain reaction (RT-PCR). The coding region of the gene is 777 bp encoding 258 amino acids of a predicted 28.4 kDa molecular mass and with a pI of 8.32, and containing a 24 amino acid signal peptide. The gene shares 85.5 % and 98.8 % homology on the protein level with the lectin cDNA from *P. pedatisecta and Arisaema heterophyllum*, respectively. The deduced amino acid sequence contains the conserved features of mannose-binding lectins, including three mannose-binding sites (QXDXNXVXY). Southern blot analysis indicated that *PPAb* belongs to a multi-copy gene family, and Northern blot analysis revealed that the *PPAb* is preferentially expressed in the tuber. The insecticidal activity of PPAb against the tobacco aphids (*Myzus nicotianae*) was studied using transgenic tobacco plants expressing *PPAb* gene under the control of the constitutive CaMV 35S promoter. Northern blot assays revealed that lectin gene was expressed at various levels in the transgenic tobacco lines. Insect bioassays demonstrated that the ectopically expressing *PPAb* had significantly increased mortality, to tobacco aphids (*Myzus nicotianae*) fed on the transgenic lines when compared to wild type. These findings suggest that the PPAb is a suitable candidate protein for insect resistance in the control of various sap-sucking insects through a transgenic approach.

Keywords: *Pinellia pedatisecta*; bulb lectin (PPAb); transgenic tobacco; *Myzus nicotianae*; insect bioassay.

Introduction

Aphids have devastating effects on crops as a result of their feeding activities: sucking plant sap, transmitting plant viruses and excreting honeydew, which induces mold (Blackman and Eastop, 1984). Aphid populations are usually controlled with chemical insecticides, however the extensive use of insecticides can result in detrimental consequences for the environment (Boatman et al., 2007), and aphid species are becoming resistant to chemical attack (Devonshire et al., 1998; Hill et al., 2006; Margaritopoulos et al., 2007). Therefore, there is a need to reduce the use of these insecticides by introducing safer alternatives with control the aphids. Genetic engineering technology with a potent control agent has been thought to be an alternative solution, which necessitates the identification of an appropriate control agent against the target pests. Therefore, many researches have concentrated on finding the effective insect-resistance genes which could express toxic agents to aphids. The toxicity of plant lectins towards insects, especially sap-feeding hemipterans, is particularly important as potential control agents for hemipteran pests because these insects are not susceptible to any known Bt toxins, and so cannot be controlled by existing plant genetic engineering technologies using Bt toxin genes. The potential of plant lectins in pest control has been demonstrated by the expression of snowdrop lectin (GNA) in transgenic rice which conferred partial resistance to planthoppers, leafhoppers, the pea aphid *Acyrthosiphon pisum* (Harris), the peach potato aphid *Myzus persicae* (Sulzer), the glasshouse potato aphid *Aulacorthum solani* (Fabricius) and the grain aphid *Sitobion aenae* (Fabricius), etc (Foissac et al., 2000; Rahbé et al., 1995; Gatehouse et al., 1996; Stoger et al., 1999). Also, the discovery that GNA can successfully be exploited as a carrier for toxins across the insect gut that are normally only toxic when injected into the insect hemolymph (Powell et al., 1998), offers the opportunity to use GNA as a carrier molecule to deliver other peptides to the circulatory system (Fitches et al., 2002; Fitches et al., 2004; Down et al., 2006; Peumans et al., 2007). Insecticidal activity was also recently shown for some Araceae lectins. The *Arum maculatum* lectin (AMA) was tested in an artificial diet against the aphids *Lipaphis erysimi* and *Aphis craccivora* (Majumder et al., 2005). When incorporated in an artificial diet *Arisaema jacquemontii* lectin (AJA) affected the development of *Bactrocera cucurbitae* larvae (Kaur et al.,

2006). The *Pinellia* spp. lectin had more or less insecticidal activities towards cotton aphids *Aphis gossypii* (Glover) and *M. persicae* (Sulzer) when incorporated into artificial diets (Huang et al., 1997; Pan et al., 1998). Transgenic tobacco expressing *P. ternata* lectin (PTA) and *Arisaema heterophyllum* lectin (AHA) significantly inhibited the growth of *M. persicae* Sulzer (Yao et al., 2003a; Yao et al., 2004). *P. pedatisecta*, a traditional Chinese medicinal herb belonging to family Araceae, may also have the potential for the control of sap-sucking or homoptera insect pests in genetically engineered crops. Potential application for *P. pedatisecta* lectins are not limited to control of hemipterans, even in insects where the lectin itself has limited toxicity such as lepidopteran larvae (Sadhegi et al., 2008), the possibility exists of using a *P. pedatisecta* lectin as a "carrier" domain in a fusion protein in corporating a toxic protein or peptide, in a similar manner to the use of GNA in this role (Fitches et al., 2002) and the fusion protein is resistant to gut proteolysis and is insecticidal after oral ingestion. Though a mannose-binding lectin from *P. pedatisecta* has been cloned, its anti-insect property is still unkown (Lin et al., 2007). In this study, we reported the over-expression of *P. pedatisecta* bulb lectin (*PPAb*) gene in transgenic tobacco plants, and evaluation of increasing plant resistance to aphids by genetic engineering technologies for the control of aphids.

Results

Cloning of full-length cDNA of PPAb gene and sequence analysis

Based on the reported full-length cDNA sequence of *PPA* (AY451853), the full-length cDNA sequence of PPAb was amplified through RT-PCR using the gene-specific primers 1 and 2, and confirmed by sequencingAccession numbers. no. HM593586. The cDNA of PPAb contained a 777 bp ORF encoding a precursor of 258 amino acids with a calculated molecular weight of 28.4 kDa and pI of 8.32. Sequence analysis by BLASTX program in NCBI showed that PPAb was a member of the monocot mannose-binding lectin gene superfamily. Sequence alignment revealed that the putative amino acid sequence of *PPAb* has higher identity with those of other Arareae species (from 98.8% to 71.5%) than with those of Alliaceae, Amaryllidaceae, Orchidaceae, Bromeliaceae, Liliaceae, Araceae, Iridaceae and Zingiberaceae (from 23.1% to 40.2 %). The PPAb has only 85.9% identity with the reported PPA but 98.8% identity with the reported AHA . Like most of the mannose-binding lectins from Araceae species, the deduced amino acid sequence of the *PPAb* gene contained three mannose-binding sites (QXDXNXVXY). The amino acid sequences of sites I and II were the same as those of GNA. However, the amino acid sequence of site III was different from that of GNA, in which Asn (N) were substituted by Phe (F), which was similar to AHA (Zhao et al., 2003), but it was substituted by hydrophobic amino acid Leu and Tyr in the third site of AMA and PTA (Yao et al., 2003b; Van Damme et al., 1995). According to the NCBI conserved domain search and the plant lectin database Lectin DB (Chandra et al., 2006), the predicted PPAb precursor possessed two domains, called PPAb-DOM_1 and PPAb-DOM_2. PPAb-DOM_1 was between T_{27} and P_{133} amino acids, and PPAb-DOM_2 was between D_{148} and P_{255}. The amino acid sequences of PPAb-DOM_1 showed 40.6% (43/108) identity to PPAb-DOM_2. Comparison of the sequences of PPAb-DOM_1 and PPAb-DOM_2 with one-domain lectins such as GNA and AKA showed a higher identity. A 24-amino acid signal peptide was found in PPAb via iPSORT, the most likely cleavage site between A_{24} and V_{25} based on the rules of predicting signal peptides (Gatehouse et al., 1999), which was in agreement with reports of most mannose-binding lectins from the Araceae family (Fig. 2) (Zhao et al., 2003; Van Damme et al., 1995).

Southern blotting analysis

To investigate if *PPAb* belonged to a multi-copy gene family in *P. pedatisecta*, southern blotting analysis was carried out with genomic DNA extracted from young leaves and digested with *Eco* RI, *Hind* III and *Bam* HI that had no cleavage sites in the probe region (*PPAb*). Hybridization under high stringency conditions demonstrated that the three enzyme-digested products generated multiple hybridization bands in each lane, indicating that *PPAb* belonged to a multicopy gene family in the genome (Fig. 3a), which was in agreement with reports of most mannose-binding lectins from the Araceae family previously (Zhao et al., 2003; Van Damme et al., 1995).

Tissue-specific expression of the gene

Expression patterns of *PPAb* in different tissues, stems, leaves, spadixs and tubers, were examined with the same probe as that for Southern blotting. *PPAb* was expressed at the highest level in the tubers, followed by leaves, whereas no signal could be detected in the spadixs and stems (Fig. 3b).

Selection, plant regeneration and molecular analysis of transgenic plants

Tobacco leaf discs were transformed with *A. tumefaciens* EHA 105 harboring pBIPPAb. After 3 weeks of selection with 100 mg l^{-1} kanamycin, a total of 16 independent kanamycin-resistant plants were recovered. PCR analysis revealed that 15 out of 16 plants were positive for the *PPAb* gene. For further analyses described below, seven independent PPAb-PCR positive plants were analyzed by Northern blot analysis, and demonstrated that *PPAb* expression could be detected in transgenic plants at various levels, and it could be detected in plant nos. 1, 2, 5, 6 and 7, with plant nos.1, 2, 5 and 6 showing much higher expression than other plants, while there was hardly any PPAb expression in plant nos.3 and 4 (Fig. 3c). This demonstrated that *PPAb* was expressed at different levels in different transgenic plants.

Aphid bioassays

The transgenic tobacco T_0 lines (plant nos. 1-7), together with untransformed control tobacco plants, were challenged by tobacco aphids (*M. nicotianae*) and their effects on aphid survival and growth were investigated. About twenty late instar aphid nymphs were introduced onto each plant and insect survival and growth was measured at 3-day intervals for 15 days. The results suggested that the three independent lines (nos.1,2and 6), which had strong expression of the *PPAb* gene, showed significant levels of resistance against aphids and the growth of the aphid population was significantly reduced, when compared to untransformed control plants (Fig 4 Cand Fig 5). The mean number of aphids on these plants was significantly lower than that on the control plants constantly throughout the assay period with significant differences at $P < 0.05$ after day 3. Aphid number increased from 21 insects per plant (initial inoculum) to an average of 288 insects (13 times increase) per plant over a 15-day assay period on the control plants. However, the number of nymphs produced per transgenic plant was decreased by 100% (no.2), 92.01% (no. 1), 78.8% (no. 6) and 58.84% (no.5), respectively, when compared to controls. Especially in no.2 transgenic plant lines, the highest reduction

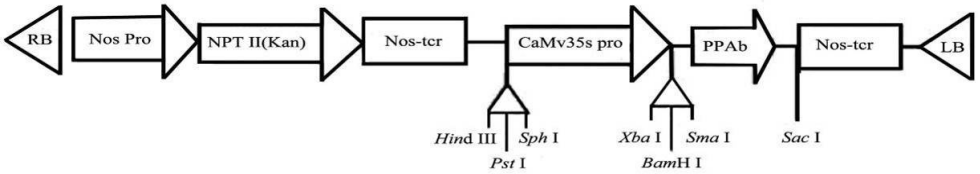

Fig 1. Map of T-DNA plasmid pBIPPAb used for tobacco transformation. LB, RB: Left and right borders of Agrobacterium T-DNA. The *PPAb* gene was placed between the enzyme *Sma* I.and *Sac* I and and was controlled by 35S promoter

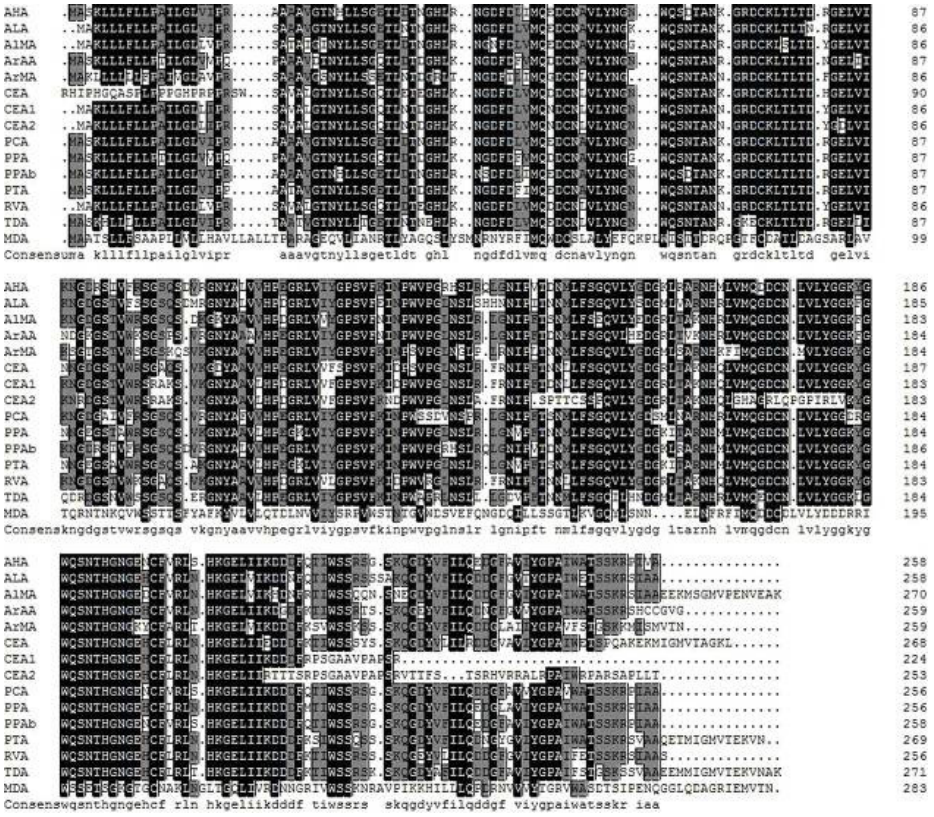

Fig 2. Multiple alignments of the deduced amino acid sequence of PPAb and those of *Arisaema heterophyllum* agglutinin (AHA, AAP50524); *Arisaema lobatum* agglutinin (ALA, AAS66304); *Alocasia macrorrhizos* agglutinin (AlMA,ABC69036); *Arisaema amurense* agglutinin (ArAA,ABY91323);*Arum maculatum* agglutinin (ArMA, AAC48998); *Colocasia esculenta* agglutinin (CEA,BAA03722); *Monstera deliciosa* agglutinin (MDA,ABM74186); *Pinellia cordata* agglutinin (PCA,ABK88277); *Pinellia ternata* agglutinin (PTA, AAP20876); *Remusatia vivipara* agglutinin (RVA,ACH41914); *Typhonium divaricatum* agglutinin (TDA ,AAQ55289). Black and gray shaded backgrounds indicated that the amino acids were identical or similar to PPAb, respectively. Mannose-binding sites (QXDXNXVXY) are underlined.

rate of aphid population, all the aphids were dead 7 days after inaculation, while the growth of aphid population on the other transgenic lines (nos. 3, 4 and 7), which had very low expression of the *PPAb* gene, was not significantly different from that on the untransformed control plants. This indicated the low expression levels of the PPAb gene in the transgenic plants.

Discussion

Notable efforts have been made towards development of transgenic plants with control of aphids. The monocot mannose-binding lectins from Araceae have drawn much attention for their remarkable insecticidal activities towards aphids. Insect bioassays showed significant insecticidal activity against *A. gossypii* (Glover) and *M. persicae* (Sulzer) with the lectins from Araceae (Huang et al., 1997; Pan et al., 1998). Transgenic tobacco expressing *PTA* and *AHA* gene significantly inhibited the growth of *M. persicae* Sulzer (Yao et al., 2003a; Yao et al., 2004). However, to the best of our knowledge, there is no report about expressing *PPA* gene with resistance to aphids in transgenic plants. We have over-expressed *PPAb* gene which isolated from the bulb of *P. pedatisecta* under a constitutive promoter, CaMV *35S*. The transgenic lines exhibited high resistance to aphids, making PPAb a potential candidate for the control of aphids by genetic engineering. Southern blot analysis showed that multiple hybridization bands were present in each lane, indicating that PPAb belonged to a multicopy gene family (Fig. 3a). The presence of multiple copies of the mannose-binding lectin gene in the genome has been reported in many plant species, particularly those

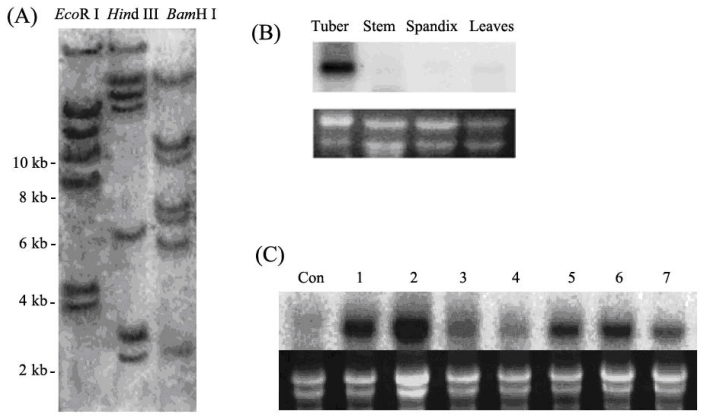

Fig 3A. Southern blotting analysis showing distribution of PPAb in *P. pedatisecta* genome. Thirty micrograms of genomic DNA from young leaves digested with *Eco*R I, *Hin*d III and *Bam*H I were fractionated on 0.8% agarose gel, blotted to nylon Hybond N membrane, followed by hybridization to the probe labeled from PPAb. Molecular size of the hybridized signals is indicated on the left. **B**. RNA gel blotting analysis of PPAb expression in different tissues of. *P. pedatisecta*. Expression of PPAb in tubers, leves, stems, and spadixs, respectively. Equal loading of the total RNA was confirmed by EtdBr staining, shown as rRNA below the signal panel. Lane1: tubers; Lane2: stems; Lane3:spandixs; Lane 4: leaves. **C**. Northern analysis for transgenic plants with pBIPPAb. Total RNA isolated from pBIPPAb -transformed tobacco plants. Formaldehyde–agarose gel separated and blotted RNA was hybridized with the PPAb cDNA probe. Lane 1, non-transgenic tobacco (control); Lanes 2-8, transgenic tobacco plants (nos.1,2,3,4,5,6,7)

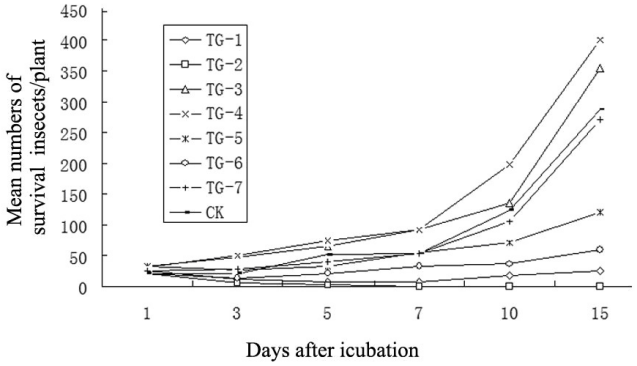

Fig 4. Aphids bioassay tests on transgenic tobacco line no.1-7.Average about twenty one aphid nymphs were incoulated on each plants on day 1 and the insect survival and growth of aphid population were measured at 3 days intervals for a 15-day period. Points and bars showed the means ± SE.

belonging to the Amaryllidaceae and Araceae families (Zhao et al., 2003; Van Damme et al., 1995). In most species, mannose-binding lectins occur in almost all vegetative tissues, such as leaves, flowers, ovaries, bulbs, tubers, roots and nectar (Van Damme et al., 1998). However, lectins from different tissues may differ in their isolectin composition. Our research indicated that the PPAb was expressed only in the tubers, with almost no expression in the leaves, stems and spadixs (Fig.3b). The expression pattern of PPAb was different from other lectin genes such as AHA, PCA, and PPA from Araceae species, in which these genes expressed in various tissues such as leaves, stems, roots and spadixs, while PPAb gene expression is tuber-specific lectin. This expression pattern was similar to ASA, the lectin from *Allium sativum*, containing two different bulb specific lectins, one leaf-specific lectin, and one root-specific lectin (Van Damme et al., 1992). The PPAb had only 85.9% identity with the reported PPA which was expressed in leaves (Lin et al., 2007), suggesting that lectins from different tissues might differ in amino acid composition and the PPA lectin family might contain some isoforms in different tissues. This was similar to the antifungal protein from *Gastrodia elata* (GAFP-1) which contains four mannose-binding lectins among which the amino acid sequence identity was 98.2% (Wang et al., 2001). In our study, the levels of transgenic plants resistance to aphids activity was correlated with mRNA expression of PPAb. Our results showed that there was a significant reduction of the survival rate of the aphids on the transgenic T0 plants expressing PPAb when compared to the untransformed controls, and especially, some transgenic plants are lethal to aphids (Fig. 4). The growth of aphid populations was reduced on three tested transgenic lines when compared to the control line, the average reduction of aphid population in T0 plants was 90.28%. The highest reduction rate of aphid population is 100% in the 2 transgenic plant lines in

which all the aphids were dead 7 days after inoculation. While the inhibition rates of the 1 and 6 transgenic plant lines were 92.01% and 78.84%, respectively. Furthermore, northern blot analysis of the transgenic lines demonstrated variable expression of PPAb among events, similar to transgenic plants containing other lectin genes such as the *GNA* and *PTA* (Gatehouse et al., 1996; Yao et al., 2004). Those data were also consistent with previous assays showing toxicity of purified mannose-binding lectins to aphids (Huang et al., 1997), and deleterious effects on aphids development in transgenic plants expressing mannose-binding lectins (Chakraborti et al., 2009; Wu et al., 2006). The expression levels of PPAb in plant 2 lines were the highest when compared to other lines, and their inhibitory effects on *M. persicae* were also higher than that of transgenic plants with lower levels of expression. The effects of plant lectins on insects in transgenic plants might be relevant with the formation of active forms of lectin in transgenic plants, which included the processing of lectin precursor and the expression level of the lectin gene in transgenic plants (Yu and Wei, 2008; Gatehouse et al., 1999). The results of this study also showed that the expression of *PPAb* in transgenic plant was dose-dependently toxic to the aphid *M. persicae*, and the trangenic tabacco plant line 2 was lethal to aphids, consistent with an interference with normal growth and development. This lethal toxicity is different from the PTA, which is only moderately toxic (Huang et al., 1997; Yu and Wei, 2008), and the resistance level might be in the third mannose-binding sites III between the PPAb and PTA, where it has a site difference in amino acids sequence. Presently, there is no clear correlation between lectins specificity and toxicity, but binding to glycoproteins in the midgut of the insect, which appears to be a prerequisite for any toxic effect of lectins (Czapla, 1997). The mannose-binding lectins have been shown to interact with glycosylated receptors in the insect midgut, one of the major receptors for GNA in *N. lugens* was identified as a subunit of ferritin, suggesting that GNA may interfere with the insects' iron metabolism (Du et al., 2000). The garlic bulb lectins were also bound to two membrane-associated aphid midgut protein, alanyl aminopeptidase N and sucrose (Fitches et al., 2008). In addition to binding certain carbohydrate structures in the insect, a second prerequisite for insecticidal activity is the resistance of the lectins to proteolytic degradation. It has been shown that the mannose-binding lectins e.g. GNA and ASAL remain stable and survive the insect gut (Fitches et al., 2002; Christou et al., 2006). In the previous study, fusion proteins were made with allostatin to deliver this insect neuropeptide hormone to the hemolymph of *L. oleracea* (L) (Fitches et al., 2002), and also it was used to deliver a venom protein of the spider *Segestria florentina* to the hemolymph of *L. oleracea* (Fitches et al., 2004), the latter fusion protein was shown to affect survival of *N. lugens* and *M. persicae* when incorporated into artificial diets (Down et al., 2006). Finally, a fusion protein consisting of a toxin from the red scorpion (*Mesobuthus tamulus*) N-terminally fused to GNA was acutely toxic when fed to *L. oleracea* larvae (Peumans et al., 2007). Whether or not, PPAb as a carrier molecule to deliver other peptides to the circulatory system toward aphids still needs to be studied. The lethal toward *M. persicae* with transgenic plants expressing PPAb in this study indicated that PPAb was a novel candidate for plant transgenic engineering against homopteran insect pests, and the PPAb gene might be used in combination with other insecticidal proteins like Bt and protease inhibitors (Christou et al., 2006). It has been reported that transgenic rice plants expressing two Cry Bt proteins in combination with GNA were resistant to the rice leaf folder (*Cnaphalocrocis medinalis*), the yellow stemborer (*Scirpophaga incertulas*) which are the Bt gene targets and the leafhopper (*N. lugens*) where the GNA gene targets (Bano-Maqbool, et al., 2001). Although the usefulness of PPAb might be constrained by the relatively high dose necessary for the control of hemipteran, it is viable to find a way to operate PPAb in combination with one or more additional insecticidal factors, either separately or as components of fusion proteins by genetic engineering for the control of insects. Work to evaluate insecticidal fusion proteins based on PPAb is currently in progress in our laboratory.

Materials and methods

Plant material and tissue sampling

The bulbs of *P. pedatisecta* were collected from Lingshou county of Hebei Province, China. The bulbs were grown in pots in the greenhouse under standard conditions. Roots, leaves, spadixs and tubers were collected and quickly frozen in liquid nitrogen and stored at –80 °C for subsequent utility.

Extraction and purification of RNA

Total RNA was isolated using the guanidine isothiocyanate phenol-chloroform method as described by Sambrook *et al* with modification (Sambrook et al., 1989). Quality of the extracted RNA was checked by agarose gel electrophoresis and quantified spectrophotomerically (UV 2450, Shimadzu Corp., Kyoto, Japan).

Cloning of PPAb complete cDNA by RT-PCR and sequence analysis

Single-strand cDNA was synthesized using a BD SMART™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol. Based on the cDNA sequence of *PPA* (GenBank accession. no. AY451853) (http://www.ncbi.nlm.nih.gov/), a pair of primers was designed to amplify *PPAb* cDNA including the complete ORF and with *Sma* I and *Sac* I site, respectively (Primer 1: 5'- ATCCCGGG-ATGGCCTCCAAGCTCCTCCTC -3′ and Primer 2: 5'-ATG-AGCTCTACGCGGCAATTGGGCGCTT -3′). The PCR amplification was 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 s; final extension at 72 °C for 10 min. PCR products of expected size were recovered and purified by DNA Gel Extraction Kit (TaKaRa, Japan), and the purified fragments were cloned into pMD18-T vector (TaKaRa, Japan), labeled as pMDPPAb, and then sequenced by TaKaRa Bio Inc.

The full-length cDNA sequence was used to search homologous sequences via BLASTX in National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Multiple alignments of amino acid sequences were performed between *P. pedatisecta* and other plants using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/). A phylogenetic relationship tree was then constructed by the neighbor-joining (NJ) method. Typical motifs of *PPAb* that are conserved among *P. pedatisecta* and other plants were analyzed via ScanProsite (http://www.expasy.ch/tools/scanprosite/), while signal peptide was analyzed by iPSORT (http://www.hc.ims.u-tokyo.ac.jp/iPSORT/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/).

Genomic Southern blot analysis

Genomic DNA was isolated from young fresh leaves of *P. pedatisecta* plants followed by CTAB extraction method (Saghai-Maroof et al., 1984). For southern blotting, 20 µg of genomic DNA was digested with 3 enzymes (*EcoR* I, *Hind* III

or *BamH* I) without restriction site in *PPAb* that was labeled with [α-^{32}P]-dCTP by PCR. After digestion the DNA was separated on 0.8 % agarose gel overnight and transferred to a Hybond-N + nylon membrane (Amersham Pharmacia Biotech, UK) via capillary transfer, fixed by incubation for 2 h at 80 °C and cross-linked with a UV-Stratalinker Model 1800 (Stratagene). Prehybridization was performed at 60 °C for 1-2 h in 6× SSPE, 0.5% SDS, 5× Denhardt's solution, and 100 µg /l salmon sperm DNA. The blots were hybridized overnight at 60 °C in a buffer (pH 7.4) containing 1mM EDTA, 250mM $Na_2HPO_4 \cdot 7H_2O$, 1% hydrolyzed casein, and 7% SDS with random-primed ^{32}P-labeled probes made from *PPAb* coding sequences. The membrane was washed twice for 20 min at 60 °C with 2× SSC plus 0.1% SDS, and twice for 20 min at 60 °C with 2×SSC plus 1% SDS. The hybridization signals were detected by autoradiography using X-ray film (Kodak) with an intensifying screen at –80 °C, and the film was developed according to manufacturers' recommendation.

Tissue expression profile analysis

Total RNA of leaves, tubers, stems and spadix was extracted and each RNA sample (20 µg) was analyzed by electrophoresis in a 1.2 % (w/v) agarose and 1 % (v/v) formaldehyde denaturing gel. To ensure that equal amounts of RNA per lane were loaded, gels were stained with ethidium bromide and individual lanes evaluated for comparable fluorescence levels upon exposure to a short UV light source. After running, RNA was transferred to a Hybond-N+ nylon membrane. Prehybridization, hybridization, membrane washing and detection followed the same procedure as Southern blotting.

Construction of plant binary vectors and transformation

The recombinant plasmid pMD18-PPAb was completely digested with *Sma* I and *Sac* I to release a fragment containing the coding sequence of the *PPAb* and the latter was inserted into *Agrobacterium* binary vector pBI121 predigested with *Sma* I and *Sac* I to generate pBIPPAb. The recombinant plasmid, pBIPPAb, contained the selectable marker neomycin phosphotransferase gene (*nptII*) conferring kanamycin resistance and the *PPAb*, both driven by *CaMV* 35S (Fig. 1). The recombinant vector pBIPPAb was transferred from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* strain EHA 105 by triparentalmating and was used to transform tobacco (*Nicotiana tabacum* cv. Xanthi) (Holsters et al., 1978). The tobacco (*N. tabacum* cv. Xanthi) was used for transformation and was performed essentially as described by a previous study (Horsch et al., 1988). *A. tumefaciens* strain EHA 105 containing pBIPPAb was grown for 2 days at 28 °C in YEB supplemented with 100 mg l^{-1} kanamycin and 50 mg l^{-1} rifampycin. The bacteria (OD_{600}=0.8) were collected and suspended in hormone-free MS liquid medium before use. The leaf discs of approximately 0.5–1.0 cm^2 were immersed in the bacterial suspension for 8 min, transferred onto MS solid medium supplemented with 1.0 mg l^{-1} 6-benzylaminopurine (6-BA), and incubated at 25 °C in the dark for 2 days. After the cocultivation, the discs were placed on selection medium (MS medium supplemented with 1.0 mg l^{-1} 6-BA, 0.1 mg l^{-1} 1-naphthaleneacetic acid (NAA), 100 mg l^{-1} kanamycin, 500 mg l^{-1} cefotaxime) and cultured for 2 weeks at 25 °C under a light/dark cycle of 16 h/8 h until shoots developed. The regenerated green healthy shoots were separated from the explants and transferred to hormone-free MS medium containing 100 mg l^{-1} kanamycin, 500 mg l^{-1} carbenicillin for rooting. The well rooted plants were eventually transferred to soil in pots in the greenhouse.

PCR and Northern blotting analyses

Putative transformants and the control (wild type) plants were used for PCR analysis. Genomic DNA was isolated from young green leaves of putatively transformed and untransformed control tobacco plants followed the same procedure as Southern blotting. PCR analysis for the detection of the *PPAb* gene followed the same procedure as the cloning of *PPAb*. Seven independently transgenic plants were identified and used for detection of mRNA expression. The RNA extraction and northern blotting analyses of transformed and untransformed control tobacco plants were performed by the same procedures as the *PPAb* northern blotting in different tissues of *P. pedatisecta* as described above.

Aphid bioassay

The seven independently derived transgenic tobacco T_0 lines which were identified by northern blot analysis (plant nos. 1-7, three cloned replicate plants per line), together with untransformed tobacco controls, were challenged by *M. nicotianae* and investigated for their effects on aphid survival and the development of aphid population using the protocol described by Hilder et al (Hilder et al., 1995). *M. nicotianae* nymphs were obtained from mature tobacco plants which were cultured under laboratory conditions at 25 °C in an insect-culturing room and aphid bioassays were carried out at the Crop Protection Institute of Hebei Academy of Agricultural Sciences in China. Each plant (10–15cm tall) was confined to an insect-proof fine mesh nylon cage, and about 20 late instar aphid nymphs were introduced with a brush to tobacco leaves of each plant on the first day and the insect survival and growth of the insect populations were measured at 2-3-day intervals for a 15-day period. The experiment was repeated three times (each independently derived transgenic line was micropropagated into three cloned plants).

Acknowledgements

This study was supported by grants from: (i) China National Novel Transgenic Organisms Breeding Project (No. 2009ZX08002-005B), (ii) Hebei Provincial Natural Science Foundation (No. C2009001301) and (iii) foudation for young scientists from Hebei Academy of Agricultural and Forestry Sciences (A06030101).

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