BIOLOGIA PLANTARUM 53 (2): 355-359, 2009

BRIEF COMMUNICATION

Expression of *Rhizobium pydA-pydB* fusion gene in *Nicotiana tabacum* confers resistance to the toxic aromatic compound 3-hydroxy-4-pyridone

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Abstract

The toxic aromatic compound 3-hydroxy-4-pyridone (HP) is an intermediate in both synthesis and degradation of mimosine, which is produced by the tree legume *Leucaena leucocephala*. The *L. leucocephala* root-nodule symbiont *Rhizobium* TAL1145 contains a dioxygenase (*pydA*) and a hydrolase (*pydB*) gene that produce enzymes necessary for the degradation of HP. In order to coordinately express both genes in plant tissues under a single promoter, three different *pydA-pydB* fusion constructs (G0, G3, and G7) with varying glycine linkers between the two genes were developed. Prior to transferring the fusion constructs into *L. leucocephala*, which is highly recalcitrant to genetic transformation, we tested the expression and activity of the hybrid proteins in *Nicotiana tabacum*, a model plant system that can be easily transformed and analyzed. Seven independent transgenic tobacco lines were generated by *Agrobacterium*-mediated transformation, and stable integration and expression of *pydA-pydB* in these transgenic lines were confirmed by polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR) and Western analysis. Only one of the fusion constructs, G3, containing a 9-nucleotide linker between *pydA* and *pydB*, provided significant levels of resistance to 3 mM HP, indicating that the hybrid protein produced by this fusion construct could degrade HP.

Additional key words: metabolic pathway engineering, *Leucaena leucocephala*, *Rhizobium* TAL1145, mimosine, hybrid protein.

The foliage of the tropical tree legume *Leucaena leucocephala* is an excellent forage for livestock due to its high protein content, palatability, and digestibility (Jones 1979). Although highly nutritious, *L. leucocephala* foliage can be administered only as a supplement in animal diet since it contains the toxic non-protein amino acid mimosine [β-N-(3-hydroxy-4-pyridone)-α-amino-propionic acid] (Brewbaker and Hylin 1965). Excess consumption of *L. leucocephala* forage can cause hair loss, goiter, decreased fertility and mortality in animals (Jones 1979).

Development of a mimosine-free *L. leucocephala* through the silencing of genes for mimosine biosynthesis is not possible at this time since the genes involved in the biosynthetic pathway for mimosine have not been identified and characterized. An alternative and possibly easier approach for the development of mimosine-free *L. leucocephala* plants is through the expression of enzymes that degrade a precursor for mimosine biosynthesis. 3-hydroxy-4-pyridone (HP) is such a precursor that can be subject to enzymatic degradation to prevent mimosine biosynthesis (Murakoshi *et al.* 1984). We have isolated genes for mimosine and HP degradation from the *Rhizobium* strain TAL1145 that forms nitrogen-fixing symbiotic nodules on the roots of *L. leucocephala* (Soedarjo *et al.* 1994). At least five *mid* (mimosine degrading) genes are involved in the uptake and conversion of mimosine to HP (Borthakur *et al.* 2003). HP is then further converted to ammonia, pyruvate and formate by a dioxygenase encoded by *pydA* and a hydrolase encoded by *pydB* (Awaya *et al.* 2005). Thus both *pydA* and *pydB* are necessary for complete degradation of HP (Fig. 1). In order to coordinately express both genes in transgenic plants, we combined *pydA* and *pydB* into a single *pydA-pydB* fusion gene. We made three such *pydA-pydB* constructs with different

Received 27 August 2007, *accepted* 11 February 2008.

Abbreviations: BA - 6-benzylaminopurine; CaMV - cauliflower mosaic virus; GUS - β-glucuronidase; NAA - naphthaleneacetic acid; NOS - nopaline synthase; PCR - polymerase chain reaction; RT-PCR - reverse transcriptase PCR.

Acknowledgments: This research was supported through USDA-NRI grant 2002-35107-11659 and NSF grant CBET08-27057.

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length of linker sequences between *pydA* and *pydB* (Awaya *et al.* 2007). The goal of this research was to determine which one of these fusion constructs could be expressed in plant tissues to produce a functional dioxygenase-hydrolase hybrid enzyme for degradation of HP. Since no reliable transformation protocol for *L. leucocephala* has yet been established, we transferred the *pydA-pydB* fusion constructs into *Nicotiana tabacum*, and determined the ability of the transgenic plants to withstand toxic levels of HP added to the plant growth medium.

Three different types of *pydA-pydB* fusion gene constructs were previously developed in our laboratory for expression of dioxygenase-hydrolase hybrid enzymes in *Escherichia coli* and *Rhizobium* (Awaya *et al.* 2007). In these *pydA-pydB* fusion constructs, the stop codon and the 38 bp intergenic region between *pydA* and *pydB* were removed so that the two genes could be fused in frame. The genes were linked together by either 0 (G0), 3 (G3) or 7 (G7) glycine (GGN) codons. For the expression of these constructs in tobacco, the genes were amplified with polymerase chain reaction (PCR) from the *E. coli* expression plasmids pNam (Hoang *et al.* 1999) and cloned into the plant binary vector pBI121 (*Clontech*, Palo Alto, CA, USA), which carry an *NPTII* gene for kanamycin resistance as a selectable marker in plants. The PCR primers used for amplification of the fusion genes were designed such that new restriction sites, *Xba*I and *Sac*I, were added to the 5' and 3' ends of the amplified fragments, respectively (Table 1). The pBI121 binary vector was digested with *Xba*I and *Sac*I for the excision of the ß-glucuronidase (GUS) gene, which was replaced with the G0, G3, or G7 fusion gene. The resulting gene constructs were under the control of the constitutive 35S promoter of cauliflower mosaic virus (CaMV) and the nopaline synthase (NOS) terminator (Fig. 2). All the PCR fragments were amplified using 100 ng DNA as templates. Thermocycling was performed at initial 95 °C for 5 min, followed by 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1.5 min for 30 cycles, with a final extension at 72 °C for 15 min, using *Taq*-DNA polymerase from *Promega* (Madison, WI, USA). The PCR fragments were first cloned into TOPO vector (*Invitrogen*, Carlsbad, CA, USA) for DNA amplification and sequence verification, digested with *Xba*I and *Sac*I, and ligated into the binary vector pBI121. The resulting binary plasmids, pBI-G0, pBI-G3 and pBI-G7 were transferred into *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method described by Holsters *et al.* (1978). The transformed LBA4404 were then used to generate transgenic tobacco plants.

Sterilized seeds of tobacco (*Nicotiana tabacum* L. cv. Samsun-NN) were germinated in *Magenta GA7* boxes on hormone-free half-strength Murashige and Skoog (1962; MS) medium containing Gamborg's B5 vitamins (Gamborg *et al.* 1968), 3 % sucrose and solidified with 0.2 % agar (*Sigma-Aldrich*, St. Louis, MO, USA). The plantlets were grown for 4 weeks at 25 °C under a 16-h photoperiod (irradiance of 60 µmol m^{-2} s^{-1}), after which the upper portion of plants, including stems and leaves, were cut and transferred to fresh 1/2 MS medium for further propagation of sterile tobacco tissue. The leaves

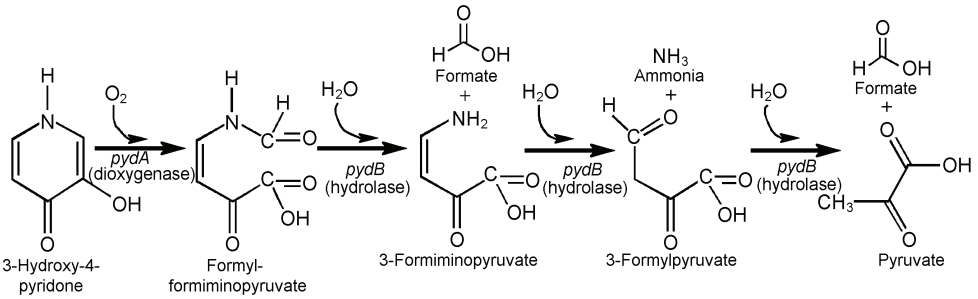

Fig. 1. HP is degraded to ammonia, formate and pyruvate through the concerted enzymatic activity of a dioxygenase and a hydrolase, encoded by *pydA* and *pydB*, respectively.

Table 1. Nucleotide sequences and melting temperatures (Tm) of primers used in the molecular cloning of the *pydA-pydB* fusion fragments. Primer sets Diox-(F1/R1) and Hyd-(F1/R1) were used during RT-PCR analysis.

Primer sets	Nucleotide sequence	Tm [°C]
PydA-F(*Xba*I)	5'-ATA TCT AGA TGA GGA GAA AGT TAA TGG CTG A-3'	59.0
PydA-R(*Sac*I)	5'-ATA GAG CTC TAT GCT GCT GCG TCG TAG G-3'	64.4
PydB-F(*Xba*I)	5'-ATA TCT AGA TGC CTC ATT TTG AAG ACC GAG-3'	60.1
PydB-R(*Sac*I)	5'-ATA GAG CTC TTA TTG TGG TTG CGG GAA GAG C-3'	64.9
Diox-F1	5'-GGC GGC CTG TCG CAT TGG CCT-3'	68.8
Diox-R1	5'-CCA CTC GAC GAC CGG CTC ATA-3'	61.9
Hyd-F1	5'-ATC CAC GGA TCC GGG CCT GGT-3'	66.6
Hyd-R1	5'-GTC CAC TCT CCC GTG CAG CAT-3'	62.9

of young tobacco were excised and used for transformation (Fisher and Guiltinan 1995). The co-cultivation MS medium (CMS) was supplemented with 2 mg dm^{-3} 6-benzylaminopurine (BA), while shoot regeneration MS medium (SMS) contained 2 mg dm^{-3} BA, 500 mg dm^{-3} carbenicillin (for eliminating *Agrobacterium*), and 200 mg dm^{-3} kanamycin for selection of transformed tobacco plants. Rooting MS medium (RMS) was similar to SMS medium but BA was replaced with 0.5 mg dm^{-3} naphthaleneacetic acid (NAA).

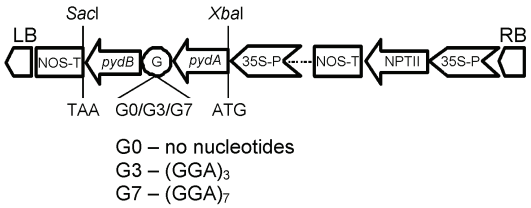

Fig. 2. Three different plasmids (pBI-G0, pBI-G3 and pBI-G7) were constructed using the binary vector pBI121 which contains a *NPTII* gene for kanamycin selection of transgenic plants. A constitutive 35S CaMV promoter and NOS terminator regulate the genes in all T-DNA constructs. The *pydA-pydB* fusion gene constructs differentiate from each other by the size of their glycine linker, varying from 0, 9 and 21 nucleotides.

Cells of transformed *A. tumefaciens* strain LBA4404 carrying the three different plasmids, pBI-G0, pBI-G3 and pBI-G7, were grown for 2 d in 40 cm^3 liquid YEP medium (10 g dm^{-3} yeast extract; 10 g dm^{-3} peptone; 5 g dm^{-3} sodium chloride; pH 7.0) containing 200 μM acetosyringone prior to incubation with tobacco leaf discs. Calluses and shoots were generated from the transformed leaf discs according to the procedure developed by Fisher and Guiltinan (1995). Independent lines of transgenic tobacco plants carrying G0, G3 or G7 fusion gene constructs were assessed for their resistance to toxic levels of HP. The upper parts of aseptically grown transgenic plants containing stem and two leaves, with a fresh mass of approximately 1.2 g, were excised and transferred to *Magenta* boxes containing basic MS medium supplemented with 3 mM HP. Explants from wild-type tobacco were used as a control. Five cuttings from each transgenic line served as replicates. Explants were allowed to remain in the medium for 4 weeks at 25 °C under a 16-h photoperiod (60 μmol m^{-2} s^{-1}), and their capacity to survive at this HP concentration was analyzed by measuring the formation of chlorotic spots on the leaves, and determining the growth inhibition as reduction in fresh mass. Isolation of genomic DNA, total RNA, total protein from the transformed tobacco plants and Western analyses were done according to standard procedures (Sambrook *et al*. 1989). Rabbit polyclonal antibodies against purified PydA and PydB proteins were prepared by *Alpha Diagnostic International* (San Antonio, TX, USA), and used at a dilution of 1:3000.

Successful *Agrobacterium*-mediated transformation resulted in the development of a minimum of four independent transgenic tobacco lines for each of the three fusion constructs. All transgenic plants showed normal phenotype and growth patterns. These plants were propagated in the presence of kanamycin at a concentration of 200 mg dm^{-3} and multiplied for further evaluation. The fusion gene sequences were PCR-amplified with the same set of primers used during the process of molecular cloning into the plant binary vector. PCR amplification followed by gel electrophoresis and ethidium bromide staining revealed the presence of a single band of the expected size of 2.0 kb for all transgenic lines (Fig. 3*A*). RT-PCR products with the size of 1.1 kb, expected for the hybrids, were observed in only half of the 14 transgenic lines that gave positive PCR products (Fig. 3*B*). The expression of the fusion genes in the RT-PCR positive transformed tobacco plants was further confirmed by Western blot analysis using polyclonal antisera raised against PydA (37 kDa) and PydB (30 kDa) proteins. Western analyses revealed that the G0, G3 and G7 proteins were each expressed as a hybrid protein with a molecular mass of 67 kDa (Fig. 3*C*).

After confirming the presence and expression of the fusion genes in the putative transgenic tobacco plants, the enzymatic activity of the dioxygenase-hydrolase hybrid protein was determined by challenging the transgenic plants with toxic amounts of HP in the growth medium. At this concentration of HP at the end of 4 weeks, the growth of wild-type tobacco plants was severely inhibited, showing extreme chlorosis and an 84 % reduction in fresh mass. All 7 transgenic lines expressing one of the hybrid proteins showed higher level of resistance to HP compared to the wild-type plants. The levels of resistance, however, varied greatly among the transgenic lines, from 20 - 79 % reduction in fresh mass. Transgenic line #14, carrying the fusion gene G3, showed the highest resistance to HP, with only a 20 % reduction in fresh mass and no visible chlorosis. Transgenic plants carrying the G0 and G7 fusion genes did not show the same level of resistance as observed with G3 transgenic lines, although they were slightly more resistant to HP than the wild-type.

Successful expression of *pydA-pydB* in tobacco will lead the way for the possible production of a functional PydA-PydB hybrid enzyme in *L. leucocephala*, allowing for degradation of HP and subsequent inhibition of mimosine biosynthesis in this tree legume. After *Agrobacterium* transformation, 14 independent transgenic tobacco lines that underwent multiple passages on kanamycin selection were analyzed by PCR for the presence of the transgenes. Although all of them contained the *pydA-pydB* gene construct, RT-PCR analysis revealed that only 7 transgenic plants produced a *pydA-pydB* mRNA transcript. The absence of the transcript in half of the transgenic plants may be due to the insertion of the *pydA-pydB* fusion gene into inactive regions of the chromosome. Those plants that gave positive results in the RT-PCR analysis also showed expression of the dioxygenase-hydrolase hybrid protein, as indicated by the Western blot analysis. The transgenic lines carrying the G3 fusion construct showed signifi-

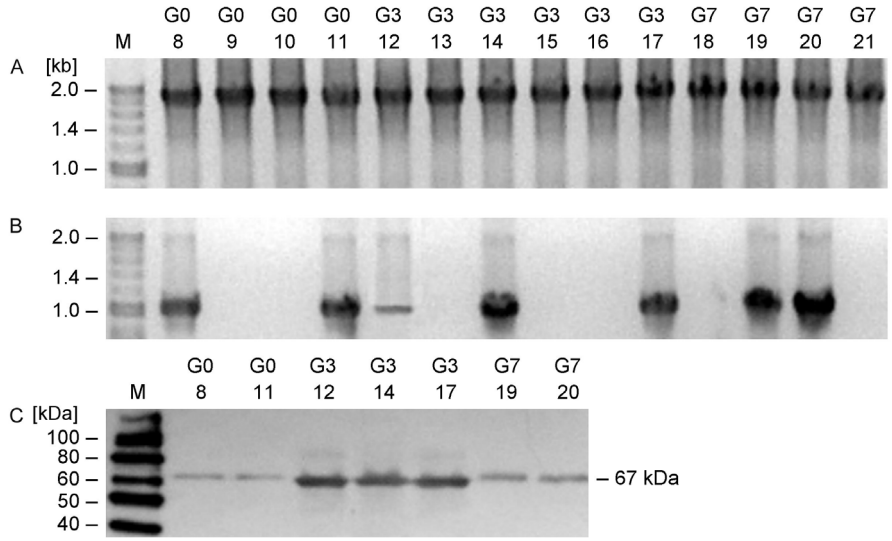

Fig. 3. Molecular analyses of transgenic tobacco containing the *pydA-pydB* hybrid genes: *A* - PCR analysis shows that all kanamycin resistant transformants contained the fusion gene, G0, G3 or G7; *B* - RT-PCR analysis show that the G0, G3 or G7 transgene was transcribed in only 7 out of 14 plants that contained the hybrid gene; *C* - Western analysis shows that the transgenic plants produced a 67 kDa fusion protein; M - molecular mass marker; the numbers above the lanes represent different lines of putative transgenic plants.

cantly higher tolerance to HP than the wild-type tobacco plants. G3 tobacco plants were also more resistant to HP than transgenic plants carrying either G0 or G7 fusion constructs. This result suggests that the G3 fusion gene produced an enzymatically functional PydA-PydB hybrid protein in plants that allowed degradation of HP.

There are numerous examples of expressing fusion genes in tobacco and other plants, but those examples mostly involve expression of a reporter gene such as *gus* or *gfp* that is fused with the 5' segment of a gene to determine transcription levels from a specific promoter (Jube and Borthakur 2007). In these hybrid proteins, the enzymatic properties of only the reporter gene are maintained. In the present study, the fusion gene G3 appeared to maintain the properties of both *pydA* and *pydB* in tobacco. This is consistent with our previous observation that a G3 fusion gene cloned in bacterial expression vectors produced a hybrid enzyme that was functional in both *Rhizobium* and *E. coli* (Awaya *et al.* 2007). Many bacterial genes have been successfully expressed in tobacco and other plants (Guo *et al*. 2007, Zhang *et al*. 2007). Often these genes show only a low level of expression in plants due to differences in codon preferences in bacteria and plants. In the present study, all three fusion gene constructs produced detectable amounts of transcripts and proteins, although only one of them, G3, produced the desired enzymatic effects when expressed in tobacco.

Although tobacco does not produce either HP or mimosine, we have used it as a test organism to express the *Rhizobium* PydA-PydB hybrid proteins because tobacco is readily transformed and allows for prompt analysis of transgene expression. We also wanted to determine which of the fusion gene constructs showed functional enzymatic activity in a higher plant system. We are currently in the process of developing a transformation protocol for *L. leucocephala*, which is highly recalcitrant to *Agrobacterium* transformation. Once the protocol has been established, we will use the best fusion construct obtained from this experiment to transform *L. leucocephala*. Degradation of HP should limit substrate availability for mimosine biosynthesis, resulting in the production of *L. leucocephala* plants with reduced mimosine content. Such a plant will be a valuable asset as a high protein, low maintenance and low toxicity forage legume.

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