Pest and disease resistance enhanced by heterologous suppression of a *Nicotiana plumbaginifolia* cytochrome P450 gene CYP72A2

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Abstract

The functional role of the *Nicotiana plumbaginifolia* cytochrome P450 gene CYP72A2 was investigated in transgenic plants. *N. tabacum* plants transformed with a sense or antisense CYP72A2 construct exhibited diminished heights, branched stems, smaller leaves and deformed flowers. Western blot analysis revealed reduced levels of a 58 kDa protein corresponding to CYP72A2, suggesting that the CYP72A2 homolog was suppressed in the sense and antisense plants. Transgenic plants had increased resistance to *Manduca sexta* larvae that consumed about 35% to 90% less of transgenic versus control leaves. A virulent strain of *Pseudomonas syringae* pv. *tabaci* induced a disease-limiting response followed by a delayed and decreased development of disease symptoms in the transgenics. CYP72A2 gene mediated resistance suggests that the plant-pest or -pathogen interactions may have been modified by changes in bioactive metabolite pools.

Introduction

Plant cytochrome P450s form a large family of heme-containing monoxygenases that are involved in the synthesis of a variety of secondary metabolites that include hormones, sterols, fatty acids, plant allelochemicals and xenobiotics. Specific metabolic functions of cytochrome P450 monoxygenases have been difficult to identify because the enzymes are highly labile and in low abundance making purification difficult. Sequencing of plant genomes has facilitated the classification of some P450s into unique families with predicted functions. Individual P450s have been assigned specific functions using reverse-genetics, knock-out populations and overexpression of P450 genes in transgenic plants (Ito & Meyerowitz 2000, Feldmann 2001).

We cloned a P450 cDNA designated as CYP72A2 (U35226) from *Nicotiana plumbaginifolia* plants that were transformed with an isopentenyl transferase (*ipt*) gene that regulates the synthesis of cytokinins (Mujer & Smigocki 2001). Elevated cytokinin levels in the *ipt* transformed plants correlated with increased insect resistance and induced the accumulation of insecticidal compounds (Smigocki et al. 1997). The deduced amino acid sequence of CYP72A2 has 45% identity to the CYP72A1 gene (L10081) that was cloned from a *Catharanthus roseus* cell culture line selected for high indole alkaloid biosynthesis and shown to code for secologanin synthase, an enzyme that converts loganin to secologanin (Imler et al. 2000). Monoterpenic alkaloids synthesized via the secologanin branch of the pathway include camptothecin and vinblastine that are powerful anticancer drugs and vincristine, a compound with insecticidal activity.

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We demonstrated that CYP72A2 expression is induced by mechanical wounding, insect chewing and exogenously applied cytokinin (Mujer & Smigocki 2001). In addition, the wound response was systemic and occurred more rapidly in response to insect attack, suggesting a possible role for this gene in plant defense responses. We also cloned a highly homologous (75%) Lycopersicon esculentum homologue of CYP72A2 (AF249329) and demonstrated that its expression is regulated by circadian rhythm and wounding as in N. plumbaginifolia, but not by cytokinin (Bartoszewski et al. 2002). It is presumed that the N. plumbaginifolia and L. esculentum P450 clones hydroxylate a different substrate than the C. roseus secologanin synthase enzyme CYP72A1, as they share only 45% identity at the protein level. Therefore, to characterize the functional role of CYP72A2, the gene was reconstructed for constitutive over- and under-expression and introduced into N. tabacum plants. We report on the effects of CYP72A2 gene suppression on plant development and resistance to Manduca sexta and Pseudomonas syringae pv. tabaci.

Materials and methods

**Plant transformation**

Nicotiana tabacum cv. Xanthi leaf disks were co-cultivated with Agrobacterium tumefaciens strain EHA105 carrying the full length CYP72A2 cDNA cloned behind the CaMV 35S promoter in the sense (p35S-p450) or antisense (p35S-ASp450) orientation in pCAMBIA1380-35S binary plasmid that carries the hygromycin phosphotransferase II marker gene for selection of transformed plant cells (Bartoszewski et al. 2002). The 5' end of the CYP72A2 cDNA fragment (corresponding to bp 198–878) was cloned in the antisense orientation (p35S-tASp450). The pCAMBIA1380-35S plasmid was used as a control for transformation. Transformed shoots were selected on 40 mg/l hygromycin B (HgB) and was cloned in the antisense orientation (p35S-tASp450). The pCAMBIA1380-35S plasmid was used as a control for transformation. Transformed shoots were selected on 40 mg/L hygromycin B (HgB) 1−1 (Sigma, 81% pure). Transgenic plants were propagated in the greenhouse and fertilized twice a month with Peters 20N:20P:20K (United Industries Corporation). HgB resistant T2 progeny were selected from seeds of independently derived T1 plants segregating 3:1 for HgB resistance.

**Polymerase chain reaction (PCR)**

Genomic DNA was purified as described by Mujer & Smigocki (2001). PCR was carried out in a reaction mixture with 50–100 ng of genomic DNA as template, 10 mM Tris/HCl (pH 8.3), primers (1 μM each), 200 μM of each dNTP, 1.5 mM MgCl2, 0.001% (w/v) gelatin and 20 units ml−1 AmpliTaq DNA polymerase (Perkin Elmer/Cetus) at 94 °C for 45 s, 55 °C for 45 s followed by 72 °C for 3.5 min for 30 cycles in a 100 μl reaction mixture. The following primer sequences were used: forward (35S promoter region) 5’CCGGATATCCTAGTAGATCGACGACTTACA3’; reverse for the sense 5’GGCAAGCTTTATAATATGGTGGTGGCCAAAGATGATA3’; and reverse for the antisense 5’GGCAAGCTTTATATATGGTGGTGGCCAAAGATGATA3’ constructs.

**Western blot analysis**

Cell-free protein extracts were prepared from 100 mg leaves powdered in liquid N2, homogenized in 1 ml of 0.1 M sodium phosphate (pH 7.6) containing 15 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 15% (v/v) glycerol, and total protein determined (Mujer & Smigocki 2001). Proteins (20 μg per lane) were separated on 9% SDS-PAGE, transferred to PVDF membrane (Bio-Rad) and CYP72A2 proteins detected with Tropix Western Star system (Applied BioSystems). Polyclonal antisera (1:1000 dilution) raised against CYP72A2 synthetic peptides were the primary antibodies (Mujer & Smigocki 2001). Experiments were repeated three times.

**Insect bioassays**

Newly hatched Manduca sexta (tobacco hornworm) larvae were maintained on an artificial diet (Carolina Biological Supply Company). Plant bioassays were conducted as previously described using 3 or 5 excised leaf disks (1.5 cm2) that were placed on water-moistened filter paper with a single second instar hornworm (Smigocki et al. 1997). For extract analysis, leaf disks from untransformed plants were coated with aqueous suspensions of the leaf extracts at 1 and 10 mg ml−1. Non-polar extracts were prepared by briefly