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Polyketide synthesis in tobacco plants transformed with a *Plumbago zeylanica* type III hexaketide synthase

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ABSTRACT

A type III polyketide synthase from *Plumbago zeylanica* (*PzPKS*) was cloned and expressed in tobacco plants to study whether the transgenic tobacco plants expressing *PzPKS* synthesize the pharmacologically important polyketide, plumbagin. High resolution mass spectrometry based metabolite profiling of two transgenic events and wild type tobacco plants was carried out to investigate changes in polyketides, including plumbagin. Ten polyketides, which included six pyrones and four naphthalene derivatives, were identified in *PzPKS* transgenic plants. While one pyrone, styryl-2-pyranone, was detected in both, wild type and transgenic tobacco plants, three pyrones were expressed only in the leaves of transgenic tobacco plants. The transgenic tobacco plants did not accumulate plumbagin, but showed accumulation of isoshinanolone in the roots, which is postulated to be the reduction product of plumbagin. In addition, leaves of transgenic tobacco plants accumulated 3-methyl-1,8-naphthalenediol, a postulated precursor of plumbagin. The results indicated the requirement of additional Plumbago-specific components in the biosynthetic pathway of this polyketide.

Introduction

Polyketides are a group of secondary metabolites derived from the reiterative condensation of acetate units. They show large structural and functional diversity and include a number of pharmacologically important compounds (Staunton and Weissman, 2001). In plants, polyketides are synthesised by type III polyketide synthases (PKSs), which catalyze decarboxylative condensation reactions followed by cyclization of the intermediate polyketide. The type III PKSs are small homodimeric proteins unlike type I and II PKSs, which consist of large multidomain protein complexes (Weng and Noel, 2012). Type III polyketide synthases are an important group of enzymes that are able to carry out reiterative malonyl-CoA condensations on different starter molecules to form a wide array of polyketides. Chalcone synthase (CHS) is a type III PKS found ubiquitously in plants and it catalyses the synthesis of chalcone, a precursor of phenylpropanoids, by three malonyl-CoA condensations on a coumaroyl starter molecule (Ferrer et al., 1999). Other type III PKSs, which show a more restricted distribution in plants, include pentaketide chromone synthase and octaketide synthase from *Aloe arborescens* (Abe and Morita, 2010), benzalacetone synthase and heptaketide synthase from *Rheum palmatum* (Abe et al., 2005), stilbene synthase (STS) from *Polygonum cuspidatum* (Liu et al., 2011) and *Arachis hypogeal* (Hain et al., 1990) and a hexaketide synthase from *Drosophyllum lusitanicum* and *Plumbago indica* (Jindaprasert et al., 2008; Springob et al., 2007). They differ from CHS in the starter molecule on which malonyl-CoA condensations occur, the number of condensations and cyclization of the intermediate polyketides.

Naphthaquinones of polyketide origin are found in several families of the order Caryophyllales, which include Droseraceae, Nepenthaceae, Polygonaceae and Plumbaginaceae (Heubl et al., 2006). *Plumbago zeylanica* L. is an important medicinal plant of the family Plumbaginaceae and is traditionally used in treatment of skin diseases, infections and inflammation (Kishore et al., 2013). The active ingredient is plumbagin, a naphthaquinone, which has been shown to have anticancer properties (Nair et al., 2008). Plumbagin is synthesised by the acetopolymalonate pathway, in which Type III PKS plays a role in bringing about six condensations of malonyl-CoA to form the basic hexaketide skeleton (Durand and Zenk, 1971).

The hexaketide synthases from *P. indica* as well as *D. lusitanicum* have been cloned and expressed in *Escherichia coli* (Jindaprasert et al., 2008; Springob et al., 2007). *In vitro* assays using the purified recombinant PKS from *P. indica* showed synthesis of several pyrones. However napthaquinones, which are natural products of PKS in the respective plants, were not observed in the *in vitro*

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assays. In the present paper we describe cloning of a hexaketide synthase from *P. zeylanica* (*PzPKS*) and its expression in tobacco plants (*Nicotiana tabacum* L.). The objective was to study whether polyketides, especially plumbagin, are synthesised by the transgenic tobacco plants expressing *PzPKS*. Metabolite profiling of the transgenic and wild type tobacco plants using ultra high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) revealed changes in polyketides accumulated in tobacco plants due to heterologous *PzPKS* expression.

Results

Cloning and expression of PzPKS in tobacco

A 1185 bp long coding sequence of *PzPKS* was obtained (JQ015381) by PCR amplification, using primers designed from the coding sequence of *PiPKS* and RNA as the template. The sequence of this cDNA showed 94% similarity to the *PiPKS* sequence. Phylogenetic analysis carried out with the protein sequence of PzPKS and of several CHS and non-CHS types of PKSs reported from other plants, showed that the PzPKS belonged to non-CHS type of PKS, which formed a cluster with non CHS type PKSs from *R. palmatum*, *P. cuspidatum* and *D. lusitanicum* (Fig. 1).

The *PzPKS* cDNA was cloned into a modified pCAMBIA1300 vector, where its expression was driven by the constitutive CaMV35S promoter. On introducing this construct into tobacco plants using *Agrobacterium*-mediated transformation of leaf discs, two transgenic events were obtained on the selection medium from a total of 200 explants used for transformation, giving a transformation frequency of 0.01%. The transgenic plants corresponding to event 1 (E1) and event 2 (E2) showed amplification of *hpt* (hygromycin phosphotransferase) and *PzPKS* sequences in PCR analysis using DNA as the template (Fig. 2A). Leaf and root tissues of E1 and E2 plants (T_0) showed the presence of *PzPKS* transcripts, while they were not expressed in the wild type tobacco plants (Fig. 2B). The wild type and transgenic tobacco plants showed similar levels of expression of *NtCHS*, which is the native CHS of tobacco. Quantification of *PzPKS* transcript abundance, using quantitative real time PCR, showed that leaves of the two transgenic events had similar levels of expression, but the expression level in roots of E2 was over 6-fold higher than that in roots of E1 (Fig. 3).

Metabolite profiling of transgenic tobacco plants expressing PzPKS

The total ion chromatogram profiles obtained for the transgenic tobacco plants differed from that obtained for wild type plants (Supplementary Figs. 1A and B). The chromatogram for *P. zeylanica* leaf and root tissues showed different profiles from that of tobacco (Supplementary Fig. 2). An explicit difference in the grouping pattern for total ion metabolite profiles of E1 and E2 transgenics and wild type samples was observed in PCA analysis along PC1 and PC 2, which together accounted for over 81% variation in the metabolite profiles (Fig. 4).

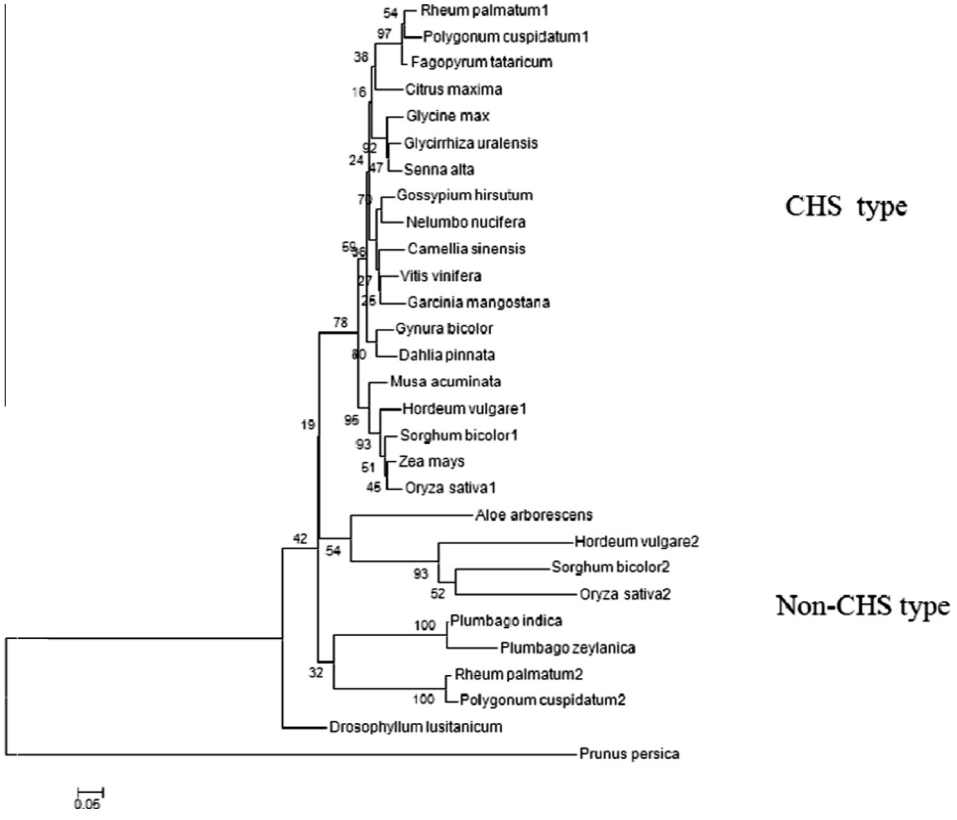

Fig. 1. Genetic relatedness of *P. zeylanica* polyketide synthase (PzPKS) to chalcone synthase (CHS) type and non-CHS type of PKS proteins of other monocotyledonous and dicotyledonous plants. Phenylammonia lyase (PAL) sequence from *Prunus persica* was used as an outgroup. Alignment of sequences was carried out using CLUSTALW. The tree was constructed with MEGA4 software, using Neighbour Joining algorithm. Numbers at the nodes indicates percent bootstrap values. The bar at the bottom indicates 0.05 amino acid substitutions. *Rheum palmatum1*(ABB13608), *Polygonum cuspidatum1*(ACC76754), *Fagopyrum tataricum* (ACH70135), *Citrus maxima* (ACX37403), *Glycine max* (CAA46590), *Glycirrhiza uralensis* (ADZ45296), *Senna alta* (AAM00232), *Gossypium hirsutum* (ABS52573), *Nelumbo nucifera* (ADD74167),*Camellia sinensis* (AAT75302),*Vitis vinifera* (BAA31259), *Garcinia mangostana* (ACM62742), *Gynura bicolor* (BAJ17656),*Dahlia pinnata* (BAK08888), *Musa acuminate* (ADE59487), *Hordeum vulgare*1(BAJ88598), *Sorghum bicolor*1 (XP002450871), *Zea mays* (NP001149022), *Oryza sativa*1 (NP001068009), *Aloe arborescens* (ACR19997), *Hordeum vulgare* 2 (BAJ93669), *Sorghum bicolor*2 (XP002450871), *Oryza sativa* 2 (BAD31062), *Plumbago indica* (BAF44539), *Plumbago zeylanica* (AEX86944), *Rheum palmatum*2 (AAS87170), *Polygonum cuspidatum* 2 (ACC76752), *Drosophyllum lusitanicum* (ABQ59603), *Prunus persica* (AAF17247).