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Research article

Characterisation of recombinant *Hevea brasiliensis* allene oxide synthase: Effects of cycloxygenase inhibitors, lipoxygenase inhibitors and salicylates on enzyme activity

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Abstract

Mechanical wounding and jasmonic acid (JA) treatment have been shown to be important factors in controlling laticifer differentiation in *Hevea brasiliensis* (rubber tree). With the long-term aim of potentially modifying the endogenous levels of JA in *H. brasiliensis* by gene transfer, we describe in this paper the molecular cloning of a *H. brasiliensis* allene oxide synthase (*AOS*) cDNA and biochemical characterisation of the recombinant AOS (His_6-HbAOS) enzyme. The *AOS* cDNA encodes a protein with the expected motifs present in CYP74A sub-group of the cytochrome P450 super-family of enzymes that metabolise 13-hydroperoxylinolenic acid (13-HPOT), the intermediate involved in JA synthesis. The recombinant *H. brasiliensis* AOS enzyme was estimated to have a high binding affinity for 13-HPOT with a K_m value of 4.02 ± 0.64 μM. Consistent with previous studies, mammalian cycloxygenase (COX) and lipoxygenase (LOX) inhibitors were shown to significantly reduce His_6-HbAOS enzyme activity. Although JA had no effect on His_6-HbAOS, salicylic acid (SA) was shown to significantly inhibit the recombinant AOS enzyme activity in a dose dependent manner. Moreover, it was demonstrated that SA, and various analogues of SA, acted as competitive inhibitors of His_6-HbAOS when 13-HPOT was used as substrate. We speculate that this effect of salicylates on AOS activity may be important in cross-talking between the SA and JA signalling pathways in plants during biotic/abiotic stress.

Keywords: Allene oxide synthase; *Hevea brasiliensis*; Jasmonic acid; Laticifers

1. Introduction

Hevea brasiliensis (Willd. Ex Adr. Juss), a tropical perennial Euphorbiaceae originating from the Amazon, is an important source of natural rubber and timber. In the bark of the tree, latex vessels are formed from reticulated chains of anastomosed cells that are dedicated to the synthesis of rubber particles which are made up of cis-1,4-polyisoprene chains surrounded by a single layer membrane. Upon tapping (excision of a thin layer of the trunk bark, about 1 mm thick) the latex vessels are severed and the latex flows out until the latex coagulates and plugs the wound [6,10].

The economic importance of natural rubber has prompted biochemical investigations into rubber biosynthesis (reviewed in ref. [24]). By comparison, less effort has been devoted to

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studies on laticifer differentiation, despite the fact that it has long been established that the number of laticifers is one of the most important factors influencing rubber yield in *H. brasiliensis* [10]. It seems that wounding, which inevitably occurs during tapping, is an important parameter which influences laticifer differentiation, but more recently, and related, it has been demonstrated that application of exogenous jasmonic acid (JA) to the bark of young *H. brasiliensis* trees can also lead to increases in latex vessel numbers [13]. Moreover, treatment with linolenic acid (LA), a precursor molecule in JA synthesis, caused an increase in secondary laticifer formation, whereas the lipoxygenase inhibitors diethyl dithiocarbamic acid (DIECA) and nor-dihydroguaiaretic acid (NDGA), which both inhibit the formation of JA from LA, in conjunction with wounding, caused a dramatic decrease in the number of secondary laticifers formed compared to those control plants that were only wounded [40].

These results clearly show that JA induces laticifer differentiation in *H. brasiliensis* and it seems likely that increases in endogenous JA levels, in response to wounding, might be important in controlling laticifer differentiation. This is consistent with the postulated role of JA in plant defence [27,31] and in various aspects of plant development [5,38].

JA and its methyl ester, methyl jasmonate (MeJA), are derived from LA via a series of reactions that occur in a biochemical pathway referred to as the octadecanoid pathway [5]. The octadecanoid pathway has three specific enzymes; allene oxide synthase (AOS), allene oxide cyclase (AOC) and 12-oxophytodienoate reductase (OPR). These enzymes convert 13-hydroperoxylinolenic acid (13-HPOT) into 3-oxo-2(2′(z) pentenyl)-cyclopentane-1 octanoic acid (OPC-8:0) which is subsequently converted to JA following three rounds of β-oxidation.

The substrate for AOS, the first committed octadecanoid enzyme, is 13-HPOT, a hydroperoxy derivative of LA which is formed by the action of 13-lipoxygenase (13-LOX) on LA. AOS is a CYP74 family member of the cytochrome P450 super-family of enzymes [16,17,19]. The CYP74A sub-family of enzymes include all of the AOSs that have specificity for 13-HPOT and are the only ones involved in JA synthesis. They catalyse the dehydration of 13-HPOT to the unstable epoxide, 12,13-epoxy-linolenic acid, which is then cyclised to form 12-oxo-phytodieonoic acid (OPDA) by the enzyme allene oxide cyclase (AOC). Due to the relative instability of the 13-HPOT epoxide, it is thought that AOS and the AOC enzyme are both physically and functionally linked [37].

Some CYP74 members are involved in processes other than JA synthesis. These include the enzymes hydroperoxide lyases (HPLs) and divinyl ether synthases (DESs) which produce essential signals for plant defence against pest attack [8,15,33]. These enzymes, including certain isoforms of AOS, can metabolise 9-hydroperoxides as well as 13-hydroperoxides and the preferred substrate reflects the sub-group they belong to [15]. The CYP74 enzyme types and their substrate specificity can to a large extent be computationally distinguished because they cluster – during phylogenetic analysis – into separate sub-groups CYP74A (13-AOSs), CYP74B (13-HPLs), CYP74C (9/13-HPLs) and CYP74D (9-DESs) [8,15]. However, the recent revelation that the recombinant AOS from *Arabidopsis* has dual specificity, despite its identification as 13-AOS based on sequence relatedness, has led to the suggestion that CYP74 assignation should be based on both substrate and product specificity of the purified enzyme *in vitro* [16,17].

The next step in the octadecanoid pathway leading to JA synthesis is the reduction of OPDA to OPC-8:0 by the enzyme 12-oxo-phytodienoate reductase (OPR). To date three OPRs have been identified in *A. thaliana*, with only the OPR3 being involved in the metabolism of the correct entaniomeric form of OPDA (cis-(+)-12-oxo-phytodienoic acid) that leads to the production of biologically active JA [28]. The intermediate product (OPC-8:0) is then converted into JA by three rounds of β-oxidation. Both the AOS and AOC enzymes are located within chloroplasts [9,39], whereas OPR is located in the peroxisomes [32]. In *Arabidopsis*, OPDA is transported into the peroxisome by the ATP-binding cassette (ABC) transporter COMATOSE (CTS) [36]. The final step in the pathway of JA synthesis is β-oxidation [4,22,36]. JA can also undergo a fourth round of β-oxidation to form jasmone [37].

The primary abiotic/biotic stress signalling mechanisms which induce JA synthesis are, at present, poorly understood. Once synthesised, JA and other as yet unidentified downstream components comprising the JA signal transduction pathway, eventually lead to detectable changes in gene expression and the accompanying adaptive responses. These responses must be integrated with outputs from the other major stress signalling pathways including the salicyclic (SA) and the ethylene (ET) signalling pathways, as well as the abscisic acid (ABA) general stress signalling pathway [1,14,20,34].

In this paper we report the molecular cloning and characterisation of a recombinant *H. brasiliensis* AOS enzyme, a new CYP74 family member. The K_m value for the enzyme was established using the substrate 13-hydroperoxylinolenic acid (13-HPOT) and inhibition studies were carried out using a range of mammalian lipoxygenase and cycloxygenase inhibitors, as well as investigating the effects of the JA and salicylates on enzyme activity. Although JA had no effect on AOS enzyme activity, SA was shown to significantly inhibit the *H. brasiliensis* AOS enzyme. It was further demonstrated that SA, including various chemical analogues of SA, acted as competitive inhibitors of the AOS enzyme.

2. Results

2.1. H. brasiliensis AOS is a new member of the CYP74A sub-family

The final assembled *AOS* cDNA sequence was 1843 bp in length (accession number DQ004684) and contained an open reading frame of 1572 bp encoding a 524 amino acid protein with a calculated molecular weight of 58.7-kDa (Fig. 1A). At the N-terminus of the protein, a 35 amino acids chloroplast transit peptide was predicted using the ChloroP chloroplast transit peptide computer program [7]. Analysis of the deduced amino acid sequence showed that the AOS from *H. brasiliensis*