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Research article

A serine carboxypeptidase-like acyltransferase catalyzes synthesis of indole-3-acetic (IAA) ester conjugate in rice (*Oryza sativa*)

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ABSTRACT

Indole-3-acetic acid (IAA) conjugation is one of mechanisms responsible for regulation of free auxin levels in plants. A new member of the serine carboxypeptidase-like (SCPL) acyltransferases family from *Oryza sativa* has been cloned and characterized. 1-*O*-indole-3-acetyl-β-D-glucose (1-*O*-IAGlc): *myo*-inositol acyltransferase (IAInos synthase) is an enzyme of IAA ester conjugates biosynthesis pathway that catalyzes transfer of IAA moiety from 1-*O*-IAGlc to *myo*-inositol forming IA-*myo*-inositol (IAInos). The *OsIAA-At* cDNA has been cloned and expressed using yeast and bacterial expression systems. Proteins produced in *Saccharomyces cerevisiae* and *Escherichia coli* contained 483 and 517 amino acids, respectively. The enzyme functionally expressed in both expression systems exhibits 1-*O*-IAGlc-dependent acyltransferase activity. Analysis of amino acid sequence confirmed that rice IAInos synthase belongs to the SCPL protein family. Recombinant IAInos synthases produced in yeast and bacterial expression systems have been partially characterized and their properties have been compared to those of the native enzyme obtained from 6-days-old rice seedlings by biochemical approach. The oligosaccharide component of the protein enzyme is not necessary for its catalytic activity. The native enzyme showed the lowest specific activity of 5.01 nmol min^{-1} mg^{-1} protein, whereas the recombinant enzymes produced in yeast and bacteria showed specific activity of 18.75 nmol min^{-1} mg^{-1} protein and 18.09 nmol min^{-1} mg^{-1} protein, respectively. The K_M values for *myo*-inositol were similar for all three forms of the enzyme: 1.38, 0.83, 1.0 mM for native, bacterial and yeast protein, respectively. Both recombinant forms of IAInos synthase and the native enzyme also have the same optimal pH of 7.4 and all of them are inhibited by phenylmethylsulfonyl fluoride (PMSF), specific inhibitor of serine carboxypeptidases.

1. Introduction

Auxin, especially indole-3-acetic acid (IAA), regulates numerous growth and developmental processes in plants (Korasick et al., 2013). Plants have developed several mechanisms for maintaining auxin homeostasis. One of them is conjugation of IAA to sugar and alcohol moiety through ester bond or to amino acid, peptide and protein *via* amide bond. IAA ester conjugates are predominant form of the phytohormone in monocotyledonous plants (Ludwig-Müller, 2011). It is estimated that in rice (*Oryza sativa*) kernels, ester conjugates constitute 62–70% of total endogenous auxin pool with 10% of them being indole-3-acetyl-*myo*-inositol (IAInos) (Hall, 1980). IAA conjugates do not exhibit typical auxinic activity, but they are thought to be involved in a variety of hormonally related processes such as transport of IAA, auxin catabolism or protection of the phytohormone from oxidative degradation and can function as auxin temporary storage, (Bajguz and Piotrowska, 2009).

IAA ester conjugates biosynthesis pathway has been studied extensively in the endosperm of maize kernels (Michalczuk and Bandurski, 1980). This pathway is started by formation of 1-*O*-indole-3-acetyl-β-D-glucose (1-*O*-IAGlc) catalyzed by UDPG-dependent IAA glucosyltransferase (1-*O*-IAGlc synthase):

IAA + UDP-glucose ↔ 1-*O*-IAGlc + UDP

In vitro synthesis of 1-*O*-IAGlc is energetically unfavorable due to the thermodynamic constraints of the reactants and products (Kowalczyk and Bandurski, 1990). Therefore this reaction is followed by energetically favourable transacylation of IAA moiety to some acceptors.

Abbreviations: 1-*O*-IAGlc, 1-*O*-indole-3-acetyl-β-D-glucose; GSH, reduced glutathione; GSSH, oxidized glutathione; IAA, indole-3-acetic acid; IAInos, indole-3-acetyl-*myo*-inositol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; OsIAA-At, 1-*O*-indole-3-acetyl-β-D-glucose: *myo*-inositol acyltransferase from rice; PMSF, phenylmethylsulfonyl fluoride; SCPL, serine carboxypeptidase-like; SCT, sinapoylglucose: choline sinapoyltransferase; SMT, sinapoylglucose: malate sinapoyltransferase; SST, sinapoylglucose: sinapoylglucose sinapoyltransferase; UDPG, uridine diphosphate glucose

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In immature maize and rice seeds IAA moiety is transferred to *myo*-inositol:

1-*O*-IAGlc + *myo*-inositol → IAInos + glucose

IAA moiety can also be transferred from 1-*O*-IAGlc to different mono- and oligosaccharides (Starzyńska and Kowalczyk, 2012) and probably to glycoproteins (Ostrowski et al., 2015).

Synthesis of IA-*myo*-inositol (IAInos) was observed for the first time in immature maize kernels (Michalczuk and Bandurski, 1980, 1982) and in consequence the specific IAInos synthase was identified (Kęsy and Bandurski, 1990). Several years later, an electrophoretically homogenous enzyme was obtained and characterized (Kowalczyk et al., 2003). This enzyme is a glycoprotein comprised of two isoforms α and α′ of molecular weight of 56.4 kDa and 53.5 kDa, respectively. Both isoforms have identical amino acid sequence, thus difference in molecular mass is caused by different glycosylation levels. Native enzyme can exist as a monomer of molecular weight of 56.4 kDa or a dimer αα or αα'. Based on analysis of 21-amino acid part of maize IAInos synthase sequence, this enzyme has been classified as a member of serine carboxypeptidase-like (SCPL) acyltransferases family. This classification has been later confirmed by analysis of the complete amino acid sequence of the maize protein (personal communication).

Acyltransferases from the SCPL family catalyze numerous reactions of secondary metabolism pathways in plants using 1-*O*-*β*-glucose esters as an alternative acyl donor instead of coenzyme A thioesters (Bontpart et al., 2015). SCPL enzymes identified so far are involved in biosynthesis of glucose polyesters (Li et al., 1999; Li and Steffens, 2000), sinapate esters (Lehfeldt et al., 2000; Shirley et al., 2001; Stehle et al., 2009), anthocyanins (Fraser et al., 2007; Saito et al., 2013), avenacins (Mugford et al., 2013; Owatworakit et al., 2013), tannins (Niemetz and Gross, 2005; Ikegami et al., 2007; Liu et al., 2012) and IAA ester conjugates (Kowalczyk et al., 2003; Starzyńska and Kowalczyk, 2012). Although SCPL acyltransferases share structural elements with serine carboxypeptidases, i.e. the catalytic triad comprising serine, histidine and aspartate residues as well as the α/β hydrolase fold, they have lost ability to hydrolyze peptide bonds. These enzymes favour transferase activity over hydrolysis using energy-rich 1-*O*-β-glucose esters to acylate a wide range of molecules (Mugford and Milkowski, 2012). To date, only nine SCPL acyltransferases have been cloned and characterized (Bontpart et al., 2015). All of them are from dicotyledones, except of SCPL1 (SAD7) from *Avena strigosa* (Mugford et al., 2009). Despite a number of putative *SCPL* genes have been identified in rice (Feng and Xue, 2006), no enzyme of SCPL acyltransferases family has been characterized on protein level.

In our previous studies we have identified IAInos synthase activity in rice seedlings (Ciarkowska et al., 2013) and observed that this activity is regulated by phytohormones and abiotic stress (Ciarkowska et al., 2016). In this study, we report the cloning and expression of rice IAInos synthase cDNA using yeast and bacterial systems, and we show that the protein encoded by *OsIAA-At* cDNA is a functional SCPL acyltransferase catalyzing formation of IA-*myo*-inositol. We also describe partial purification and characterization of native rice IAInos synthase what makes it the first biochemically characterized enzyme of SCPL acyltransferases family from *Oryza sativa*. Moreover, we compare properties of the native rice IAInos synthase with its recombinant forms produced *S. cerevisiae* and *E. coli*. Such comparison has never been performed on enzymes from IAA ester conjugates biosynthesis pathway.

2. Material and methods

2.1. Plant material

Plant material was black rice whole meal (Bio Planet, Poland). Rice (*Oryza sativa*) seeds were soaked in distilled water at 37 °C for 24 h and planted on Petri dishes. Plants were grown in darkness at 27 °C.

2.2. Cloning of OsIAA-At cDNA

Total RNA was isolated from 6-days-old rice seedlings using GeneMATRIX Universal RNA/miRNA Purification Kit (EUR_x, Poland) according to manufacturer's protocol. RNA was pretreated with RNA-se free DNase I (Thermo Scientific) to remove any contaminating genomic DNA. First-strand cDNA synthesis was performed using 1 μg of RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. cDNA encoding IAInos synthase (*OsIAA-At* cDNA) was synthesized by PCR. Reaction was performed in total volume of 25 μL with 1.5 μL of template cDNA, 0.5 U of Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) in Phusion HF buffer containing 1.5 mM $MgCl_2$, dNTP (0.2 mM each), 0.5 μM primers (F: 5′-AGAATTCCACACAATGTCTATGGTGAGGGCGTC AAC-3'; R: 5′-GCTCTAGACTATAATGAATCAGAACCATCT-3′) and 3% DMSO. Specific primers complementary to *OsIAA-At* cDNA (UniProt EEC73124.1) were generated using Primer3. Restriction site for EcoRI and yeast consensus sequence CACACAATGTCT were added to forward primer and restriction site for XbaI and stop codon were added to reverse primer. Thermo cycling was performed using T100™ Thermal Cycler (BIO-RAD) with the following cycling conditions: 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 59 °C for 30 s; 72 °C for 45 s, followed by final extension of 72 °C for 10 min.

PCR product was cloned into pJET1.2/blunt vector using CloneJET™ PCR Cloning Kit (Thermo Scientific) according to manufacturer's protocol. pJET1.2/blunt-*OsIAA-At* was digested with EcoRI and XhoI (cloning into pET-28a(+)) or XbaI (cloning into pYES2) for 2 h at 37 °C and the reaction was stopped by incubation at 65 °C for 20 min. The restriction mixture of 20 μL contained 10 U of each restriction enzyme, 2x Tango buffer and 1 μg of vector. *OsIAA-At* cDNA was ligated into bacterial (pET-28a(+), Novagen) and yeast (pYES2, Invitrogen) expression vectors digested at the same conditions as pJET1.2/blunt-*OsIAA-At*. The ligation mixture of 20 μL contained 1U of T4 DNA ligase, 1x T4 DNA ligase buffer, 17 ng of linearized pET-28a (+) vector and 13.7 ng of insert or 80 ng of linaerized pYES2 vector and 61 ng of insert. Ligation reaction was performed at 22 °C for 30 min for pET-28a(+) vector and for 1 h for pYES2 vector and then stopped by incubation at 65 °C for 10 min.

2.3. Expression of OsIAA-At cDNA in S. cerevisiae and isolation of the recombinant IAInos synthase

The pYES2-*OsIAA-At* yeast expression construct was transformed into *S. cerevisiae* InvSc1. Yeast cells were grown at 30 °C in SC-U medium containing 2% (w/v) raffinose until OD_{600} = 5.0. IAInos synthase synthesis was induced by dilution of yeast culture to OD_{600} = 0.4 in SC-U medium containing 3% (w/v) galactose. The culture was continued for 8 h at 18 °C. Cells were pelleted by centrifugation (3 500*g*, 3 min, 4 °C, Sigma Sartorius Centrifuge), washed with distilled water and were next lysed with Cell Lytic Y Reagent (Sigma-Aldrich) according to the manufacturer's instruction. Lysate was centrifuged (15 000*g*, 10 min, 4 °C, Sigma Sartorius Centrifuge). Recombinant proteins in supernatant were analyzed by SDS-PAGE on 12% (w/v) running gel and 6% (w/v) stacking gel using Ogita and Markert (1979) method and Western blot analysis.

Supernatant after cell lysis was used for fractionation with poly (ethylene glycol) 6000 (PEG 6000). Solid PEG 6000 was slowly added to the supernatant to final concentration of 15% (w/v). The solution was gently stirred for 1 h and centrifuged (22 000*g*, 1 h, 4 °C, Sigma Sartorius Centrifuge). Supernatant was dialyzed overnight at 4 °C against 25 mM Tris-HCl buffer pH 7.5 containing 2 mM EDTA (buffer A). Supernatant was loaded into ConA-Agarose column (5 cm × 1.5 cm) previously equilibrated with buffer A. Then, the column was washed with buffer A followed by buffer A containing 0.2 M NaCl. IAInos synthase was eluted with buffer A containing 15% (w/v) methyl-α-D-mannopyranoside. The fractions containing IAInos synthase were