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Short communication

Identification and expression profiling of flax (*Linum usitatissimum* L.) polyamine oxidase genes in response to stimuli

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Abstract – Polyamine oxidases (PAOs) are known to be involved in either the terminal catabolism or the back conversion of polyamines, which affect a range of physiological processes, including growth, development, and stress responses. In this study, based on genome-wide analysis, we identified five putative PAO genes (*LuPAO1* to *LuPAO5*) in flax (*Linum usitatissimum* L.) that contain the amino-oxidase domain and FAD-binding-domain. The expression analysis using quantitative real-time PCR revealed spatial variations in the expression of *LuPAOs* in different organs. In addition, the expression level of *LuPAOs* in the flax cell suspension culture was increased by treatment with methyl-jasmonate (MeJA) or pectin, but not with salicylic acid or chitosan. This indicates that LuPAOs might be involved in the MeJA-mediated biological activities. Taken together, our genome-wide analysis of PAO genes and expression profiling of these genes provide the first step toward the functional dissection of LuPAOs.

Keywords: cell suspension culture, flax, methyl-jasmonate, pectin, polyamine oxidase

Introduction

Polyamines (PAs), including spermine (Spm), spermidine (Spd), and putrescine (Put), are low-molecular-mass aliphatic polycations that are ubiquitously distributed in organisms. Due to the cationic nature of PAs, they bind to macromolecules, such as DNA, RNA, and proteins, through electrostatic linkages that can cause either stabilization or destabilization (Kusano et al. 2008). Thus, they have been implicated in a range of fundamental cellular processes, including the regulation of gene expression, translation, cell proliferation, cell growth, differentiation, modulation of cell signaling, membrane stabilization, and modulation of ion-channel function and stability (Kusano et al. 2008, Jiménez-Bremont et al. 2014, Minocha et al. 2014, Tiburcio et al. 2014). Endogenous PA contents depend upon the regulation of biosynthesis, transport, and catabolism in both prokaryotes and eukaryotes, including plants (Kusano et al. 2008, Takahashi et al. 2010). PAs are oxidatively deaminated by two types of amine oxidases: copper-containing amine oxidases (CuAOs, EC 1.4.3.6) and FAD-dependent polyamine oxidases (PAOs, EC 1.5.3.6) (Cona et al. 2006). The extracellular PAOs, such as the PAOs from monocotyledonous plants oxidize the carbon on the endo-side of the N^4-nitrogen of Spd and Spm to produce 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal, respectively, along with 1,3-diaminopropane and H_2O_2, and are thus considered involved in the terminal catabolism of PAs. Differently, intracellular (cytosolic and peroxisomal) PAOs oxidize the carbon at the exo-side of the N^4-nitrogen of Spd and Spm with the production of Spd from Spm and Put from Spd, 3-aminopropanal, and H_2O_2, and are considered involved in a polyamine back-conversion pathway (Planas-Portell et al. 2013, Ahou et al. 2014). Although it has been explained that PAOs in monocotyledonous plants are involved in the terminal catabolism of PAs, four rice PAOs were found to

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be involved in the PA back-conversion pathway (Ono et al. 2012). This suggests that the PA back-conversion pathway also exists in monocotyledonous plants.

Plant PAOs have been suggested to play an important role in physiological processes, including growth, development, and responses to abiotic and biotic stresses (Angelini et al. 2010). The physiological role of PAO proteins is mediated by the regulation of cellular PA levels, but also by H_2O_2 (an important signaling molecule in the promotion of plant cell death and biotic or abiotic stress response) synthesis via the terminal catabolism and back conversion of PAs (Minocha et al. 2014, Tiburcio et al. 2014). Although accumulating evidence has shown that PAOs play roles in modulating a range of physiological processes, most PAO family members in higher plants, except rice and Arabidopsis PAOs, are poorly understood.

Therefore, in this study, we identified genes potentially encoding PAOs in flax (*Linum usitatissimum* L.), which is a medicinally important oil seed crop. Based on *in-silico* analysis, gene structures, sequence homology, intron phase, and *cis*-elements in the promoter regions of five PAO genes were investigated. In addition, the expression patterns of flax PAOs in elicitor-treated flax cell suspensions were examined. Our systematic analysis provides new insights into the understanding of the potential roles of flax PAOs in response to stimuli.

Materials and methods

Identification and sequence analysis of LuPAO genes and promoters

Protein sequences of *Arabidopsis* and rice PAOs were used as queries in a search against the flax genome sequence (Phytozome v9.1; http://www.phytozome.net /search.php?method=Org_Lusitatissimum). The information on LuPAO gene features, including introns and exons, was obtained from Phytozome v9.1. The intron phases of different introns were analyzed using Wise 2.0 (http://www.ebi.ac.uk/Tools/Wise2). In addition, the molecular weight (MW) and the theoretical isoelectric point (pI) were calculated using the Compute pI/Mw tool available on the Expert Protein Analysis System site (http://web.expasy.org/compute_pi/), and the amino acid sequences of putative LuPAO were analyzed to predict subcellular localization using HybridGO-Loc web services (http://bioinfo.eie.polyu.edu.hk/HybridGoServer/) and WoLF PSORT (https://wolfpsort.hgc. jp/). The program MEME (http://meme.sdsc.edu/meme4_6_1/cgi-bin/meme.cgi) was used for the recognition of motifs in LuPAOs. The phylogenetic analysis was performed with the use of the Phylogeny.fr server (http://www.phylogeny.fr) in the "one-click" mode, as described by Hyun et al. (2014).

For the *cis*-element analysis, all 1000-bp upstream sequences of LuPAO genes, except LuPAO1 (437-bp upstream), were compared with known *cis*-regulatory elements in the collection of the PLACE database (http://www.dna.affrc.go.jp/PLACE/).

Plant growth

Flax seeds (golden variety) were obtained from Danong Co. Ltd in South Korea. The seeds were germinated and grown in soil at 22 °C±2 °C /16±2 °C, at a light intensity of 180 μmol m^{-2} s^{-1} and a 16-h-light/8-h-dark cycle. The seeds, cotyledons and young leaves were harvested for tissue specific PAO gene expression analysis.

Cell culture treatment

The cell suspension culture of flax (golden variety), described previously by Hano et al. (2006), was used as the experimental system. For the stress treatment, cell suspension cultures were sub-cultured every two weeks and incubated on a rotary shaker set to 120 rpm in darkness at 25 °C. For elicitor treatment, suspension-cultured cells were treated with 50 μM methyl-jasmonate (MeJA), 1 mM salicylic acid (SA), 50 mg L^{-1} chitosan, or 50 mg L^{-1} pectin. The cells were harvested at different time points (5 h, 24 h, and 48 h after treatment) by centrifugation and stored at –80 °C until analysis.

Quantitative real-time PCR analysis

Total RNA was extracted using the FavorPrep Plant Total RNA Purification Mini Kit (FAVORGEN, Ping-Tung, Taiwan) according to the manufacturer's instructions and was reverse-transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (QIAGEN) in accordance with the manufacturer's recommendations. Quantitative real-time PCR (qRT-PCR) was performed using the AmpiGene qPCR Green Mix (Enzo Life Sciences Inc., Lausen, Switzerland) in the ECO™ Real-time PCR system (Illumina) with default parameters. The expression levels of different genes were normalized to the constitutive expression level of flax actin. Specific primer pairs are listed in On-line Suppl. Tab. 1.

Tab. 1. Gene catalog and nomenclature of polyamine oxidases (PAOs) in *Linum usitatissimum*. The subcellular locations of polyamine oxidases were predicted by HybridGO-Loc web services (a) and WoLF PSORT (b).

Name	Gene ID	Location	CDS (bp)	AA	Intron Nr.	pI	kDa	Subcellular localization
LuPAO1	Lus10020726	scaffold 303:384767 – 390953	1473	490	9	5.43	54.27	Peroxisome [a]
LuPAO2	Lus10005021	scaffold 637:175302 – 177892	1395	464	9	5.67	50.82	Peroxisome [a]
LuPAO3	Lus10039599	scaffold 15:686508 – 691160	1491	496	9	4.95	55.52	Plastid [a]
LuPAO4	Lus10029495	scaffold 55:372940 – 377831	1419	472	9	4.75	52.29	Extracellular [b]
LuPAO5	Lus10019725	scaffold 420:540284 – 543964	1446	481	6	6.78	53.69	Extracellular [b]

Determination of PAO activity

PAO activity was determined according to Han et al. (2014) with slight modifications. Soluble proteins were extracted by grinding cultured cells in 0.1 M sodium phosphate buffer (pH 6.5). After centrifugation (10 min, 10,000 g) at 4°C, the supernatant was used in the assays. Reaction solutions (1.5 mL) contained 0.9 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.45 mL of crude enzyme extracts, 0.05 ml of peroxidase (200 U mL^{-1}), 0.1 mL of 4-aminoantipyrine and N, N'-dimethylaniline solution. The reaction was initiated by the addition of 7.5 μL Spd (20 mM) for the determination of PAO activity. The reaction mixture was incubated at 25 °C for 20 min, and then terminated with the addition of 0.25 mL of 10% trichloroacetic acid. A 0.001 change in the absorbance value at 550 nm was regarded as one enzyme activity unit. Protein concentration was determined according to the method described by Bradford (1976) with bovine serum albumin as the standard.

Statistical analysis

Statistical differences were analyzed using ANOVA based on Duncan's multiple range tests ($p < 0.05$). All experiments were repeated at least three times, and all data were expressed as means ± standard error.

Results and discussion

The availability of the flax genome sequence (Phytozome v9.1) has made it possible to identify the putative PAO family in this plant species for the first time. In order to identify PAO genes, sequences of PAOs from Arabidopsis and rice were analyzed using BLASTp against all scaffold sequences of flax. The redundant sequences were removed according to the self-BLAST of sequences, resulting in a total of five putative PAO genes from flax (Tab. 1). Then, to further support the hypothesis that the five computationally predicted LuPAO proteins belong to the PAO family, the presence of the amino-oxidase domain (PF01593) and FAD-binding- domain, which are conserved in PAOs (Sebela et al. 2001, Gaweska and Fitzpatrick 2011), was analyzed using SMART (http://smart.embl-heidelberg.de/) and Pfam. Based on the phylogenetic analysis of the PAO proteins from different plants, the PAOs were classified into four major classes (I, II, III, and IV). Class I contained LuPAO1 and LuPAO2, whereas LuPAO3 and LuPAO4 were clustered into class III. In addition, LuPAO5 belonged to class IV (On-line Suppl. Fig. 1).It was not clear whether flax lacked class II of the PAO family or whether class II of LuPAOs might be not sequenced.

Multiple-sequence alignments of putative LuPAOs showed that two PAOs (LuPAO 1 and LuPAO2) contained a putative peroxisomal targeting signal (On-line Suppl. Fig. 2), which was defined as a tripeptide of the C-terminus ([SA][RK][LM]) (Reumann 2004). In addition, these LuPAO proteins were predicted to be peroxisomal proteins, whereas LuPAO 4 and 5 localize to the extracellular (Table 1). Arabidopsis (AtPAO2, AtPAO3 and AtPAO4) and rice (OsPAO3, OsPAO4, and OsPAO5) polyamine oxidases, clustered into class I (On-line Suppl. Fig. 1), are known as peroxisomal proteins like LuPAO1 and LuPAO2 (Ono et al. 2012; Planas-Portell et al. 2013). This indicates that class I PAOs are peroxisomal proteins (On-line Suppl. Fig. 1) and are involved in a PA back-conversion pathway. Furthermore, LuPAO3 was predicted as a plastid-associated PAO (Tab. 1). The occurrence of PAs at all stage of plastid development suggested that PAs serve as a nitrogen source for proteins and chlorophyll synthesis, which play a role in plastid differentiation (Sobieszczuk-Nowicka and Legocka 2014). PA content depends not only on biosynthesis, but also on the catabolism (Sobieszczuk-Nowicka and Legocka 2014), suggesting that plastid-associated PAOs including LuPAO3 should be involved in the plastid differentiation via controlling PA catabolism.

Conserved gene structures, including the same number of nucleotides in the exons and the conserved intron phases, indicate the similarities between the studied genes (von Schantz et al. 2006). As shown in Fig. 1, *LuPAO1* and *2* shared eight exons, with the same number of nucleotides and the same intron phase, whereas *LuPAO3* and *4* shared six exons. In addition, we used the MEME program to identify the conserved motifs in LuPAOs. As shown in On-line Suppl. Fig. 3, we found a total of three conserved motifs with low E values. Three motifs were shared by LuPAO3, LuPAO4, and LuPAO5 proteins, and motif 3 was not found in LuPAO1 and LuPAO2. These differences represent the evolutionary and functional relationship between LuPAOs.

To investigate the spatial organization of transcripts for *LuPAOs*, the expression patterns of LuPAO genes in differ-

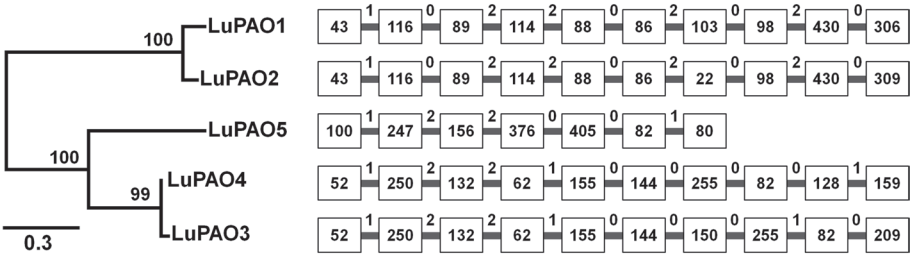

Fig. 1. Phylogenetic analysis and intron-exon structures of PAO gene family in flax. Default values were used except for 100 bootstraps. Numbers in boxes are nucleotide length of each exon, and the connecting thin boxes indicate the positions of the introns. The numbers above the introns indicate the phase of the intron.

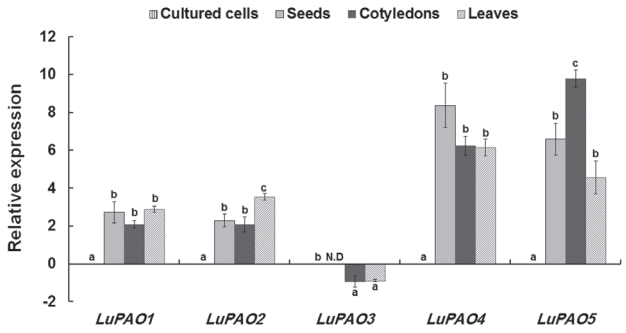

Fig. 2. Tissue-specific expression of *Linum usitatissimum* polyamine oxidase (LuPAO) genes. The expression levels for each gene in different tissue samples were calculated relative to its expression in the cultured cells. The Y-axis represents the normalized relative expression values (Log2). Data represent the means ± SE of three independent experiments. Values with different superscript letters are significantly different ($p < 0.05$). N.D = not detected.

ent tissues and cultured cells were analyzed by qRT-PCR. As shown in Fig. 2, the transcription levels of all *LuPAOs*, except *LuPAO3*, were detected in all the tested tissues with high expression level compared to the cultured cells.

Plant PAOs have been reported to be involved in plant responses to abiotic and biotic stresses (Angelini et al. 2010). Therefore, we analyzed the expression patterns of *LuPAOs* in response to external stimuli by subjecting suspension flax cell cultures to different treatments, including MeJA, SA, chitosan, and pectin. When flax-cultured cells were treated with MeJA or pectin, increased expression levels of all *LuPAOs* were observed, whereas the expression of no *LuPAOs* was significantly affected by SA and chitosan treatments (Fig. 3A). In addition, *LuPAOs* exhibited different expression patterns during response to MeJA or pectin, indicating the divergent functions of LuPAOs in response to stimuli. Although copper amine oxidases are also able to oxidize Put and Spd, with the subsequent release of H_2O_2 (Planas-Portell et al. 2013), the increased transcription level of *LuPAOs* by MeJA or pectin treatment resulted in the induction of enzymatic activity for oxidizing Spd (Fig. 3B). In addition, the enzymatic activity was not changed by treatment with SA or chitosan (Fig. 3B). However, the *cis*-elements like T/GBOX-ATPIN2 for jasmonate signaling were not found in *LuPAOs* (On-line Suppl. Fig. 4), indicating the presence of a novel jasmonate-responsive element in the *LuPAO* promoters. PA accumulation depends on *de novo* synthesis and catabolism under stress conditions (Kusano et al. 2008, Takahashi et al. 2010), suggesting that the expression of *PAOs* under stress conditions is required for the induction of a PA-mediated response. In fact, stress-induced *PAO* expressions have been observed in higher plants (Planas-Portell et al. 2013, Wang and Liu 2015). In addition, several stress-responsive elements were found in the LuPAO gene promoters, including the W box (WBOXNTCHN48), ELRECOREPCRP1 motif (elicitor responsive element), MYB1AT (dehydration-responsive), GT1CONSENSUS (Consensus GT-1 binding site in many light-regulated genes), and BIHD1OS (BELL homeodomain transcription factor in disease resistance responses) (On-line Suppl. Fig. 4). The presence of the aforementioned putative *cis*-elements in *LuPAO* promoters indicates the contribution of PAO to stress defense responses.

In conclusion, based on genome-wide analysis, we identified five flax PAO genes, which belong to three groups. Plant PAOs are known to be responsible for either the terminal catabolism or the back conversion of PAs. Therefore, a further motivating challenge would be to investigate the

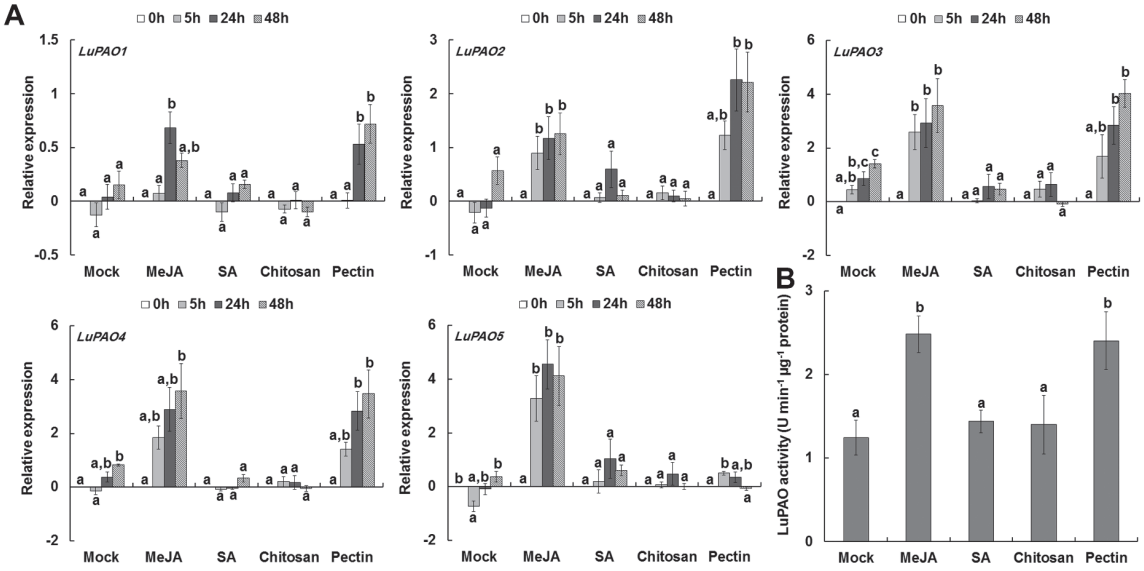

Fig. 3. Effects of elicitation on the expression of *Linum usitatissimum* polyamine oxidase (LuPAO) genes and enzymatic activity in suspension-cultured cells. (A) The expression analysis of LuPAO genes. Transcript levels of *LuPAO1*-5 were normalized to the constitutive expression level of flax actin, and were expressed relative to the values at 0 hour. The Y-axis represents the normalized relative expression values (Log2). (B) The variation of LuPAO enzymatic activity. Flax suspension-cultured cells were treated with methyl-jasmonate (MeJA), salicylic acid (SA), pectin, or chitosan. Mock indicates the treated control (mock-treated control). Data represent the means ± SE of three independent experiments. Values in the same column with different superscripted letters are significantly different ($p < 0.05$).

specific roles of each LuPAO in metabolism. An in-depth analysis of LuPAO gene expression patterns under different stress conditions suggested that LuPAO should be involved in the MeJA-mediated biological activities. Taken together, our genome-wide analysis and expression analysis provide a solid foundation for developing further understanding of the potential function of PAOs.

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