Polyamine Oxidase 7 is a Terminal Catabolism-Type Enzyme in Oryza sativa and is Specifically Expressed in Anthers

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Introduction

Polyamines (PAs) are low molecular mass, aliphatic amines that play a role in various biological processes (Tabor and Tabor 1984, Cohen 1998). In plants, the major PAs include (i) the diamines putrescine (Put) and cadaverine (Cad), the latter being abundant in legumes; (ii) the triamine spermidine (Spd); and (iii) the tetraamines spermine (Spm) and thermospermine (T-Spm) (Kusano et al. 2008, Alcazer et al. 2010, Handa and Mattoo 2010, Takano et al. 2012). The concentrations of PAs are controlled by a dynamic balance between biosynthesis and catabolism. PA biosynthetic pathways are well established in plants (Knott et al. 2007, Kakehi et al. 2010, Alcazer et al. 2010, Mattoo et al. 2010). Two types of enzymes, copper-dependent diamine oxidases and FAD-associated polyamine oxidases (PAOs), are primarily involved in PA catabolism (Kusano et al. 2008, Moschou et al. 2012). Mammalian PAOs require acetyl modification at the N1 position of Spm (or Spd) prior to back-conversion (BC) to Spd (or Put) (Casero and Pegg 1993). In addition, mammalian Spm oxidase and its yeast ortholog (encoded by Fms1) back-convert Spm to Spd without acetyl modification (Wang et al. 2001, Vujcic et al. 2002, Cervelli et al. 2003). These PAOs are categorized as BC-type PAOs. Maize ZmPAO1, the best characterized plant PAO (Federico et al. 1990, Federico et al. 1996, Tavladoraki et al. 1998) and two barley PAOs, HvPAO1 and HvPAO2 (Cervelli et al. 2001, Radova et al. 2001, Cervelli et al. 2004, Cervelli et al. 2006), catalyze the terminal catabolism (TC) of Spm and Spd and produce N-(3-aminopropyl)-4-aminobutanal from Spm and 4-aminobutanal from Spd, along with 1,3-diaminopropane (DAP) and H2O2 (Cona et al. 2006, Angelini et al. 2010).
The dicotyledonous model plant *Arabidopsis thaliana* has five PAO genes, termed sequentially AtPAO1–AtPAO5; all of the products of these genes except AtPAO5 have been homogeneously purified and characterized (Tavladoraki et al. 2006, Lim et al. 2006, Moschou et al. 2008, Kamada-Nobusada et al. 2008, Takahashi et al. 2010, Fincato et al. 2011, Fincato et al. 2012). All of these AtPAOs have BC-type activities that either convert Spm (or T-Spm) to Spd and then to Put, or convert Spm (or T-Spm) to Spd but not subsequently to Put (Moschou et al. 2008, Kamada-Nobusada et al. 2008, Takahashi et al. 2010, Fincato et al. 2011, Fincato et al. 2012).

*Oryza sativa*, a representative monocotyledonous model plant, contains seven PAO-encoding genes, sequentially termed OsPAO1–OsPAO7, beginning with the gene with the smallest chromosome number and gene ID number (Ono et al. 2012). Of these, four OsPAO gene products, OsPAO1, OsPAO3, OsPAO4 and OsPAO5, catalyze the BC reaction of Spm (or T-Spm) to Spd and/or to Put (Ono et al. 2012, Liu et al. 2014).

In this study, we examined OsPAO7 and its gene product. As expected (due to its high identity with ZmPAO1, a representative TC-type enzyme), OsPAO7 is a TC-type PAO, and it is the first such PAO identified in *O. sativa*. The predominant site of OsPAO7 expression is the anthers. Based on the results, we discuss the functional role of OsPAO7 in anthers and further explore PA catabolic pathways in rice plants.

**Results**

OsPAO7 belongs to clade II, which includes maize and barley PAOs, and localizes to the peripheral edge of the plant cell

Plant PAOs are classified into five clades (I–V; Fig. 1). OsPAO7 shares high identity with OsPAO6 (93%), OsPAO2 (73%), ZmPAO1 (83%), HvPAO1 (82%), HvPAO2 (74%), VvPAO1 (57%) and VvPAO2 (63%), all of which were grouped into clade II (Fig. 1; Supplementary Fig. S1). Furthermore, the tertiary structure of OsPAO7 predicted by the Protein Structure Prediction Server program (http://140.113.239.111/~ps2/) resembles those of the other clade members (Supplementary Fig. S2), all of which (except OsPAO2) have N-terminal extensions in common, which are predicted to be signal peptides (SPs) and transmembrane domains (TDs).

Amino acid positions 1–19 and 20–29 of OsPAO7 were predicted to be an SP and TD, respectively, by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0/). The results suggest that OsPAO7 is an apoplastic protein. To examine this possibility, we employed a green fluorescent protein (GFP) fusion approach. Constructs encoding the full-length (FL) form of OsPAO7 fused to GFP and its SP–TD portion fused to GFP (SP + TD) were transiently expressed in onion cells (Fig. 2A) by particle bombardment. Both GFP signals derived from the OsPAO7 FL construct and its SP + TD construct were detected in anthers and further localized to the peripheral edge of the anther (Fig. 2B). These results strongly suggest that OsPAO7 is an apoplastic protein and that the peripheral localization is due to the presence of the signal peptide.

**Discussion**

OsPAO7 encodes an apoplastic protein. The predominant site of OsPAO7 expression is the anthers. Based on the results, we discuss the functional role of OsPAO7 in anthers and further explore PA catabolic pathways in rice plants.
in the peripheral zones of onion cells, which overlapped with the red fluorescent signals from the fusion construct comprising DsRED–rice plasma membrane intrinsic protein 2 (OsPIP2;1), a plasma membrane localization marker (Sakurai et al. 2005) (Fig. 2B). These results suggest that OsPAO7 is situated in the apoplastic space with the aid of its SP–TD region.

**Purification and characterization of OsPAO7**

We cloned the OsPAO7 cDNA coding fragment from rice flower organs (see below) into the pCold-I vector (TAKARA) and introduced the resulting plasmid into the *Escherichia coli* Rosetta-gami B (DE3) pLysS strain (TAKARA). Following a procedure described previously (Chen et al. 2002, Liu et al. 2014), the isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *E. coli* cells were harvested, disrupted by sonication and centrifuged again to remove cell debris. The supernatant fraction was applied to an Ni-charged column, washed, and eluted with 250 mM imidazole solution. Using gel filtration chromatography, we determined that the molecular masses of the non-denatured OsPAO7 proteins were approximately 64 kDa (data not shown) using bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) as standards. After the above purification procedures, the recombinant (His)$_6$-tagged OsPAO7 protein was homogeneously purified (Fig. 3A). The calculated molecular masses of nascent OsPAO7 and recombinant (His)$_6$-tagged OsPAO7 were 53.2 and 56.7 kDa, respectively. The latter value matches that of purified OsPAO7 protein determined by SDS–PAGE (~55 kDa; Fig. 3A). Thus, it is likely that OsPAO7 is present in monomeric form.

The OsPAO7 preparation showed absorption maxima at 380 and 460 nm, indicating that FAD was associated with the purified enzyme (Fig. 3B). Using the two PAs, Spd and Spm, the optimal pH values for the highest activities of recombinant OsPAO7 were determined to be pH 7.0 for Spd (Fig. 3C) and pH 6.5 for Spm (Fig. 3D). The optimal temperatures for OsPAO7
activity were 37°C for Spd and 30°C for Spm, respectively (Fig. 3E, F). We then examined the PA substrate specificity of OsPAO7 at pH 7.0, 37°C and pH 6.5, 30°C. We tested the substrates Put, Cad, Spd, norspermidine (NorSpd), Spm, T-Spm, norspermine (NorSpm), N1-acetyl Spm and N1-acetyl Spd. At pH 7.0, 37°C, Spm and Spd were the preferred substrates for OsPAO7, followed by N1-acetyl Spm, N1-acetyl Spd, then NorSpm, T-Spm and NorSpd (Fig. 4A). At pH 6.5, 30°C, OsPAO7 showed a similar PA preference; it favored Spm and Spd, followed by N1-acetyl Spm, NorSpm, T-Spm, N1-acetyl Spd and NorSpd (Fig. 4B). The diamines Put and Cad were not recognized by OsPAO7 under either of these conditions (Fig. 4A, B).

**OsPAO7 is a TC-type PAO enzyme**

As OsPAO7 belongs to clade II (Fig. 1) and shares high identities with ZmPAO1, HvPAO1 and HvPAO2, which are characterized as ‘TC-type enzymes’ (Cona et al. 1991, Federico et al. 1990, Cervelli et al. 2001, Cervelli et al. 2004), we hypothesized that OsPAO7 would catalyze a reaction of the TC type. Previously, Flores and Galston (1982) used a methanol (MeOH)–water solvent system to separate Put, DAP and other PAs on a reversed-phase HPLC column. Therefore, we tested various MeOH–water systems (45–64% MeOH; Supplementary Fig. S3) and decided to use a 60% MeOH–40% water solution (Supplementary Fig. S4). Under these conditions, Put was first eluted [retention time (RT) = 7.25 min], followed by DAP...
(RT = 7.66 min), Spd (RT = 10.33 min), T-Spm (RT = 12.87 min) and Spm (RT = 13.30 min; Supplementary Fig. S4). When OsPAO7 was incubated with Spd (or Spm), we detected DAP from both of these substrates in a time-dependent manner (Fig. 4C, D). These results clearly indicate that OsPAO7 catalyzes the TC-type reaction.

**Kinetic parameters of OsPAO7**

We determined the kinetic parameters of the OsPAO7 protein under two different pH and temperature conditions: pH 7.0 at 37°C and pH 6.5 at 30°C. The $K_m$ values with Spm and Spd were 38.3 and 42.2 μM, respectively, at their optimum conditions.
Table 1 Kinetic parameters of OsPAO7

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}/K_m$ ($\times 10^5$ M$^{-1}$s$^{-1}$)</th>
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</thead>
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<tr>
<td>pH 7.0, 37°C</td>
<td></td>
<td></td>
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<tr>
<td>Spm</td>
<td>30.6 ± 0.5</td>
<td>40.7 ± 1.2</td>
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<td>T-Spm</td>
<td>4.5 ± 0.2</td>
<td>81.4 ± 1.0</td>
<td>553.2 ± 2.9</td>
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<tr>
<td>Spd</td>
<td>27.9 ± 0.6</td>
<td>42.2 ± 0.6</td>
<td>660.9 ± 22.9</td>
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<tr>
<td>Put</td>
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<td>0</td>
<td>--</td>
</tr>
<tr>
<td>Cad</td>
<td>0</td>
<td>0</td>
<td>--</td>
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<tr>
<td>N$^1$-Acetyl Spm</td>
<td>18.2 ± 0.3</td>
<td>70.1 ± 2.7</td>
<td>259.7 ± 10.8</td>
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<td>NorSpm</td>
<td>8.3 ± 0.5</td>
<td>82.9 ± 2.8</td>
<td>969.8 ± 8.4</td>
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<td>N$^1$-Acetyl Spd</td>
<td>9.8 ± 1.2</td>
<td>80.3 ± 5.2</td>
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<td>NorSpd</td>
<td>2.3 ± 0.2</td>
<td>108.8 ± 9.4</td>
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<td>pH 6.5, 30°C</td>
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<tr>
<td>Spm</td>
<td>32.6 ± 1.2</td>
<td>38.3 ± 2.5</td>
<td>853.9 ± 71.4</td>
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<td>T-Spm</td>
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<td>75.0 ± 3.5</td>
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<td>Spd</td>
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<td>0</td>
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<tr>
<td>N$^1$-Acetyl Spm</td>
<td>20.5 ± 1.7</td>
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<td>NorSpm</td>
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<td>78.8 ± 3.4</td>
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<td>NorSpd</td>
<td>1.1 ± 0.1</td>
<td>157.8 ± 11.6</td>
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Enzymatic reactions were performed at pH 7.0 and 37°C or at pH 6.5 and 30°C, and product (H$_2$O$_2$) formation was quantified using a multiplate reader.

Table 1. The $k_{\text{cat}}/K_m$ values for Spm and Spd were 853 and 660 x 10$^{-5}$ M$^{-1}$s$^{-1}$, respectively. These values are quite similar to those obtained for TC-type barley and maize PAOs (Federico et al. 1996, Cervelli et al. 2001). OsPAO7 showed rather high catalytic efficiency ($k_{\text{cat}}/K_m$) values for T-Spm, N$^1$-acetyl Spm, N$^1$-acetyl Spd, and NorSpm (Table 1). NorSpd did not appear to be a good substrate for OsPAO7 in vitro (Table 1).

OsPAO7 is specifically expressed during anther development

In silico data (RiceXPro; http://ricexpro.dna.affrc.go.jp/) indicate that the expression of OsPAO7 is modulated by ABA and jasmonic acid (JA; Supplementary Fig. S5A) and that OsPAO7 is specifically expressed during a late stage of anther development (Supplementary Fig. S5B). Although reverse transcription–PCR (RT–PCR) data indicate that OsPAO7 is not highly responsive to ABA and JA during the first 4 h of anther development (Supplementary Fig. S5B), OsPAO7 transcripts were detected at a late stage of anther development (Fig. S5A).

Initially, we separated rice flowers into two stages, encompassing early and late anther development; the anthers were distinguished by size and color. Early-stage anthers are smaller and pale yellow and late-stage anthers are larger and darker yellow. No expression of OsPAO1, OsPAO2, or OsPAO6 was observed in anthers (Fig. S5A), whereas three members, OsPAO3, OsPAO4, and OsPAO5, were abundantly expressed in the early and late anther developmental stages (Fig. S5A). We then prepared cDNA from four different stages of rice anthers, i.e. the tetrad, unicellular pollen, bicallecular pollen, and tricellular pollen stages (Fujioka et al. 2008, Suwabe et al. 2008, Ariizumi and Toriyama 2011), and we examined the transcript levels of seven OsPAO genes in these anthers (including OsPAO7) using RT–PCR. OsPAO7 was expressed in all anther stages, with an expression peak at the bicallecular stage (Fig. S5B). OsPAO3 was consistently expressed at high levels except in the tricellular mature anther stage. OsPAO4 had a similar expression profile to that of OsPAO3, while its expression peak was observed at the unicellular stage. OsPAO5 was also consistently expressed at high levels, with a mild peak in the bicallecular stage (Fig. S5B).

Next, we generated transgenic rice plants carrying OsPAO7 promoter–GFP as a transgene. In agreement with the RT–PCR results, weak GFP signals were detected in anthers of early-stage rice flowers (Fig. 6A, a–h), and the signals became intense in anthers of the later stage flowers (Fig. 6A, e, f, g, and h). In both stages, weak signals were detected in filaments but no signals were detected in pistils (Fig. 6A, e, f, g, and h). We further confirmed the above results by observing single detached anthers at two different stages (Fig. 6B, a–h). The inner regions of pollen grains of late-stage anthers, in addition to the anther walls, emitted GFP signals (Fig. 6C, a–h).
PA contents in seedlings and flower organs of O. sativa

We also analyzed the PA contents of young seedlings, leaves (leaf blades), stems (leaf sheaths), and early- and late-stage rice flowers. Small amounts of DAP were detected in seedlings and in early-stage flowers (Table 2), suggesting that DAP is rapidly metabolized. This DAP pattern is likely to have been derived from the action of OsPAO7 or OsPAO6. In 2-week-old seedlings, Put, Spd, T-Spm and Spm were detected...
(Table 2; Supplementary Fig. S8A). In mature leaves, high levels of Put, Spd and T-Spm were detected at an approximately 2:1:1 ratio, while in stems Put levels dropped to one-eighth of those of mature leaves, and the levels of Spd and T-Spm were approximately 50% of those of mature leaves (Table 2; Supplementary Fig. S8B, C). In early-stage rice flowers, the levels of Put and Spd increased relative to those of seedlings, but the Spm levels decreased slightly (Table 2; Supplementary Fig. S8D). In late-stage rice flowers, Put levels increased to approximately 3-fold that of early-stage flowers, while Spd levels increased to approximately 1.3-fold early-stage levels (Table 2; Supplementary Fig. S8E). The Spm content was comparable with that of seedlings and early-stage flowers. No T-Spm was detected in reproductive organs (Table 2; Supplementary Figs. S8D, E).

**Discussion**

Phylogenetic analysis has suggested the existence of five clades of plant PAOs (Agudelo-Romero et al. 2013, Liu et al. 2014). Seven PAO genes, OsPAO1–OsPAO7, are present in *O. sativa*. Of these, three PAOs, namely OsPAO2, OsPAO6 and OsPAO7, are categorized into clade II. One PAO, OsPAO1, belongs to clade III and the remaining three PAOs, OsPAO3, OsPAO4 and OsPAO5, are classified into clade IV (Fig. 1; Table 2; Agudelo-Romero et al. 2013, Liu et al. 2014). All biochemically characterized clade IV members, including OsPAO3, OsPAO4 and OsPAO5, localize to peroxisomes and catalyze BC-type reactions, albeit that they differ in terms of PA specificity (Moschou et al. 2008, Kamada-Nobusada et al. 2008, Takahashi et al. 2010, Fincato et al. 2011, Fincato et al. 2012, Ono et al. 2012). To date, only one clade III member, OsPAO1, has been biochemically and molecular biologically characterized (Liu et al. 2014). OsPAO1 remains in the cytoplasm and back-converts tetraamines, Spm and T-Spm, to Spd but not to Put (Liu et al. 2014). Thus, the above four rice PAOs catalyze BC-type reactions (Table 3).

Here, we successfully cloned OsPAO7 cDNA from rice flower organs. The deduced N-terminal sequence of OsPAO7 is assumed to contain an SP (amino acid positions 1–19) and a TD (amino acid positions 20–29), as determined with the SignalP program (Supplementary Fig. S1). In fact, OsPAO7 localizes to the periphery of plant cells (Fig. 2), suggesting that it is secreted into the apoplastic space, which is similar to ZmPAO1 and HvPAO1 (Cervelli et al. 2000, Cervelli et al. 2001). Recombinant OsPAO7 functions at neutral pH (Fig. 3C, D) and prefers Spm and Spd as substrates (Fig. 4A, B). This enzyme also recognizes T-Spm less efficiently. However, T-Spm is not present in rice flower organs (Table 2; Supplementary Fig. S8D, E); therefore,
the physiological substrates of OsPAO7 are likely to be Spm and Spd. When these substrates were incubated with OsPAO7, DAP was produced in a time-dependent manner (Fig. 4C, D), strongly indicating that OsPAO7 is a TC-type PAO. The kinetic parameters of OsPAO7 for Spm and Spd are quite comparable with those of ZmPAO1 and HvPAOs (Federico et al. 1996, Radová et al. 2001, Cervelli et al. 2001, Moschou et al. 2012) (Table 1). Clade II contains two other OsPAOs, OsPAO2 and OsPAO6. While almost all clade II member proteins consist of 474–500 amino acids (Supplementary Figs. S1, S2), OsPAO2 is a 351 amino acid protein and lacks the N-terminal portion that harbors catalytically essential residues (Cervelli et al. 2001). Therefore, OsPAO2 may not exhibit PAO enzyme activity. Meanwhile, the entire OsPAO6 protein shows high identity to other clade II members (Supplementary Figs. S1, S2). Thus, we predict that OsPAO6 is another TC-type PAO in O. sativa. In summary, O. sativa has two alternative catabolic PA pathways; one is the BC-type pathway, in which OsPAO1, OsPAO3, OsPAO4 and OsPAO5 are involved, whereas the other is the TC-type pathway, in which OsPAO7 (Fig. 4, Table 3) and possibly OsPAO6 are involved. BC-type rice PAO genes such as OsPAO3, OsPAO4 and OsPAO5 are expressed constitutively and ubiquitously (Ono et al. 2012; Supplementary Fig. S7C, D). In contrast, TC-type rice PAO genes are expressed in specific tissues. In particular, OsPAO7 showed anther-specific expression (Figs. 5, 6; Supplementary Fig. S6). Expression of OsPAO7 was high in roots at the vegetative stage, in ripening stems and at 28 d after flowering (Supplementary Fig. S7B). Expression of OsPAO2 was specifically high in anthers, roots at the vegetative stage and ripening stems (Supplementary Fig. S7A), although we assume that its gene product is not a functional enzyme (see above). TC-type PAOs are thought to function in wound healing and defense against pathogens (Angelini et al. 2010, Moschou et al. 2012).

As discussed above, OsPAO7 exhibited anther-specific expression (Figs. 5, 6). What role does OsPAO7 play in anthers? Hu et al. (2011) reported that the rice floral homeotic C-class gene MADS3 regulates reactive oxygen species (ROS) homeostasis during late anther development. The authors analyzed changes in expression of genes involved in ROS homeostasis in anthers of mads3-4, a loss-of-function mutant of MADS3. Two OsPAO genes, OsPAO7 (Os09g0368500) and OsPAO5 (Os04g0671300), are down-regulated and up-regulated, respectively, >2-fold in mads3-4 mutants compared with the wild type. The observation that MADS3 is differentially expressed, with expression only at the later anther stage, while OsPAO7 is constitutively expressed, with peak expression at the bicellular stage, suggests that OsPAO7 is not a direct target of the MADS3 transcriptional regulator. Furthermore, the authors noted that changes in the levels of H2O2, which is produced by the action of PAO, do not differ between the wild type and mads3-4, suggesting that there is no direct link between MADS3 and PAO.

Genome-scale transcriptomic analysis revealed that genes encoding signal transduction components, cell structure components, transporters, transcription factors and defense/stress-related pathways are up-regulated during anther development in O. sativa (Wang et al. 2010, Aya et al. 2011, Deveshwar et al. 2011). One of the reaction products catalyzed by plant PAO is H2O2, albeit that PAO catalyzes BC or TC reactions. The catalytic efficiency (kcat/Km) values of OsPAO7 are >100 times higher than those of OsPAO3, OsPAO4 and OsPAO5 (Table 1; unpublished data); thus, OsPAO7 produces H2O2 efficiently. As membrane-permeable H2O2 is assumed to be a second messenger for defense reactions (Cona et al. 2006), it is possible that OsPAO7 functions in defense reactions against abiotic/biotic stresses via H2O2 production. Another possible function of OsPAO7 is its involvement in lignin formation. Lignin is a major component of secondary wall thickening in anthers, and in late-stage anthers lignin formation occurs to thicken the anther wall (Boerjan et al. 2003). Lignin polymerization in the anther endothecium is dependent on H2O2 levels (Kawasaki et al. 2006, Jung et al. 2013). Cinnamoyl-CoA reductase is a key enzyme in lignin biosynthesis (Kawasaki et al. 2006), and the oxidative polymerization of monolignols is catalyzed by either laccase or peroxidase in the presence of H2O2 (Boerjan et al. 2003). In rice plants with suppressed OsPAO7 expression due to a mutation in MADS3, three genes encoding cinnamoyl-CoA reductase are down-regulated. In particular, the expression of Os08g0277200 is down-regulated by a factor of 37.39. Furthermore, seven peroxidase precursor genes (including Os01g0327100 and Os05g0499300) and three laccase genes (including Os01g0850800 and Os07g0101000) are also down-regulated >3-fold, respectively (Hu et al. 2011). Thus, a likely role for OsPAO7 during anther development is to deliver H2O2 for secondary wall thickening through lignin formation. To substantiate this hypothesis, extensive analysis of anther development in O. sativa plants harboring silenced or down-regulated OsPAO7 is required.

In rice anthers, three peroxisomal OsPAO genes in addition to OsPAO7 were expressed rather constitutively (Fig. 5). An examination of PA content among 2-week-old seedlings and leaves, stems, early flowers and late flowers derived from the same maturing rice plants revealed the following: (i) T-Spm is missing in leaves, stems and reproductive organs of mature plants; (ii) total PA contents increased in mature leaves and flowers compared with seedlings; (iii) Put and Spd contents increased more in later stage flowers while Spm content remained constant; and (iv) a small amount of DAP was detected in seedlings and early-stage flower organs but not in late-stage flower organs. High amounts of Spd conjugated to hydroxycinnamic acids are present in the trypine of A. thaliana pollen grains (Grienberger et al. 2009, Aripzuimu and Torijama 2011, Fellenberg et al. 2012). These observations indicate that dynamic PA metabolism occurs during anther development (Falasca et al. 2010, Fellenberg et al. 2012). The increase in Put and Spd contents in later stage flowers may be partially explained by the presence of BC-type OsPAOs. To reveal the physiological significance of OsPAO7 during anther development, further study is required.
**Materials and Methods**

**Plant materials and growth conditions**

Rice plants (*Oryza sativa* cv. Nipponbare) were grown hydroponically in 2/5 strength Hoagland’s solution #2 (2 mM Ca(NO₃)₂·4H₂O, 2 mM KH₂PO₄, 0.8 mM MgSO₄·7H₂O and 0.0002% FeSO₄·EDTA) in a plant incubator (NK system, Nippon Medical & Chemical Instruments Co. Ltd.). To obtain mature rice plant samples, rice seeds were germinated in distilled water in a Petri dish placed in a plant incubator set at 30°C for 3–5 d. Each plantlet was then transferred to the center of a plastic beaker filled with 2/5 strength Hoagland’s solution #2 and cultured for 2 weeks before being transferred to soil. Rice plants were further grown in a growth chamber at 28°C, 80% humidity under a 12 h light/12 h dark photocycle (15,000 lux).

**Chemicals**

Put, Cad, Spd and Spm were purchased from Nacalai-Tesque Ltd. DAP was purchased from Sigma-Aldrich. T-Spm, NorSpm, NorSpd, N₁-acetyl Spm and N₁-acetyl Spd were chemically synthesized as described (Samejima et al. 1984, Niitsu et al. 1986). All other analytical grade chemicals were obtained from Wako Pure Chemical Industries Ltd., Sigma-Aldrich and Nacalai-Tesque Ltd.

**RT–PCR analysis**

Total RNA was extracted from whole aerial parts of 2-week-old rice seedlings and rice flowers using Sepasol-RNA I Super (Nacalai-Tesque). Stage-specific RNA samples from tetrad-, uni-, bicellular- and tricellular-stage rice anthers were prepared as described (Fujioka et al. 2008, Suwabe et al. 2008). First-strand cDNA was synthesized with ReverTra Ace (Toyobo Co. Ltd.) and oligo(dT) primers (Endo et al. 2004, Oda et al. 2008). First-strand cDNA was synthesized with ReverTra Ace (Toyobo Co. Ltd.) and oligo(dT) primers (Endo et al. 2004, Oda et al. 2010). RT–PCR analysis was performed as described (Zhu et al. 2012) using the above cDNAs and the primers listed in Supplementary Table S1. Constitutively expressed OsActin (accession No. NC_008396.2) was used as an internal control for the analyses.

**Generation of transgenic rice plants expressing OsPAO7 promoter–GFP**

The OsPAO7 promoter–GFP construct was prepared as follows: the OsPAO7 promoter fragment, covering the region from −2,245 to −1 (with the ‘A’ of the ATG start codon of OsPAO7 defined as +1), and the GFP fragment were amplified using the primer pairs (OsPAO7_Pro_F and OsPAO7_Pro_R) and (OsPAO7_GFP_F and OsPAO7_GFP_R), respectively. Aliquots of the resulting fragments were mixed and amplified again using the primers OsPAO7_Pro_F and OsPAO7_GFP_R. The resulting amplification products were digested with KpnI and XhoI, and cloned into the respective sites of pPZP2H-Iac (Fuse et al. 2001), yielding pPZP2H-IacOsPAO7 promoter–GFP. This binary construct was introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986), which was then co-cultured with *O. sativa* cv. Nipponbare seeds. The resulting regenerated transformed rice lines were selected on hygromycin medium as described (Toki et al. 2006).

**Preparation of recombinant OsPAO7 protein in *E. coli***

Total RNA was extracted from whole flowers of *O. sativa*. The coding region of the OsPAO7 cDNA was amplified by RT–PCR using gene-specific primers (see Supplementary Table S1). Amplified PCR products were digested with the respective restriction enzymes and cloned in-frame with the 6×His tag of the pCold-I vector (TAKARA BIO INC.), resulting in pCold-OsPAO7. After confirming the cloned fragment by DNA sequencing, pCold-OsPAO7 was transformed into *E. coli* Rosetta-gami B (DE3) pLySs cells and recombinant OsPAO7 protein tagged with 6×His at its N-terminus was produced according to the manufacturer’s instructions (TAKARA BIO INC.). In brief, an *E. coli* cell culture at an optical density at 600 nm of approximately 1.2 was treated with 2% ethanol, heat shocked at 60°C for 1.5 min as described (Chen et al. 2002) and cooled to 15°C for 45 min. IPTG was then added to a final concentration of 0.5 mM and the culture was further incubated at 15°C for 20 h. The *E. coli* cells were collected by centrifugation, resuspended in 50 mM Na-phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole and 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication. After centrifugation at 15,000 r.p.m. for 25 min at 4°C, the cleared supernatant was collected and applied to an Ni-NTA agarose column (Qiagen). The column was washed with washing buffer [50 mM Na-phosphate buffer (pH 8.0) containing 300 mM NaCl and 5 mM imidazole] and the bound proteins were eluted with elution buffer [50 mM Na-phosphate buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole]. The eluate was further separated by gel filtration chromatography on a GILSON HPLC system using a G3000SW column (TSK gel, TOSOH). The molecular mass of the enzymatic active fraction was calculated using the molecular weight markers for gel filtration chromatography (Sigma-Aldrich). The active fractions were concentrated with an Amicon Ultra device (NMWL 30,000, Millipore).

**PAO activity assay**

The catalytic activities of recombinant OsPAO7 protein for the oxidation of Spm, T-Spm, Spd and NorSpm, NorSpd, N₁-acetyl Spm and N₁-acetyl Spd were determined spectrophotometrically by examining the formation of a pink adduct, which resulted from the oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid catalyzed by horse-radish peroxidase (Tavladoraki et al. 2006). To determine the optimum pH, 100 mM MES buffer for the pH 4.0–5.5 range, 100 mM Na-phosphate buffer for the pH 5.5–8.5 range and 100 mM HEPES buffer for the pH 8.5–10.0 range were used. In a typical experiment, 1 µg of protein was added to a buffered solution containing 500 µM of each substrate, 100 µM
4-aminooantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid and 10 U ml⁻¹ horseradish peroxidase, and the increase in absorbance at 515 nm was measured using a multplate reader (Tecan-M200) or spectrophotometer (Hitachi U-2900). Kinetic analysis was performed as follows: 0.5 μg of purified enzyme protein was added to a buffered solution (100 mM Na-phosphate buffer; pH 7.0 or pH 6.5) containing the substrate (0, 10, 20, 40, 80, 160, 320 and 640 μM each), 4-aminooantipyrine (100 μM), 3,5-dichloro-2-hydroxybenzenesulfonic acid (1 mM) and horseradish peroxidase (10 U ml⁻¹), and the increase in absorbance at 515 nm was measured using a multplate reader (Tecan-M200).

Analysis of enzyme reaction products by HPLC

To determine the reaction products of PA oxidation, purified recombinant OsPAO7 protein was incubated with 150 μM Spd in 100 mM phosphate buffer (pH 7.0) at 37°C or with 150 μM Spm in 100 mM phosphate buffer (pH 6.5) at 30°C for various periods of time. The reactions were stopped by adding 9-fold volumes of 5% (v/v) perchloric acid (PCA) as described previously (Naka et al. 2010). To each 1 ml of reaction products, 1 ml of 2 N NaOH was added, followed by 10 μl of benzoyl chloride, and the mixture was incubated at room temperature for 20 min. After the addition of 2 ml of saturated sodium chloride and 2 ml of diethyl ether followed by vigorous mixing, the phases were separated by centrifugation for 5 min at 1,500 × g at 4°C. A 1.5 ml aliquot of each organic solvent phase was evaporated, and the residue was resuspended in 50 μl of methanol. The benzoylated PAs were analyzed with a programmable Hewlett Packard series 1100 liquid chromatograph using a reverse-phase column (4.6 × 250 mm, TSK-GEL ODS-80Ts, TOSOH), eluted isocratically with an MeOH–water solvent (see the Results) at a flow rate of 1 ml min⁻¹ for 20 min and detected at 254 nm (Flores and Galston 1982).

Fluorescence microscope observations of OsPAO7–GFP in onion cells

Fragments encoding FL OsPAO7 and the hypothetical SP and TD portions of OsPAO7 were amplified using the respective primer pairs (OsPAO7_FL_Smal_F and OsPAO7_FL_SacI_R for ‘FL’ and OsPAO7_TD_Smal_F and OsPAO7_TD_SacI_R for ‘TD’), digested with Smal and SacI and cloned into the respective sites of pBI221:GFP (Ono et al. 2012), resulting in pBI221:OsPAO7–GFP (FL) and pBI221:OsPAO7–GFP (TD). A plasmid, pBI221:OsSPD–OsPSP2:1 (a positive plasma membrane-localized marker, see also Sakurai et al. 2005), was constructed by two-step fusion PCR using the respective primer pairs (OsSPD_XbaI_F and OsPSP2:1_SacI_R for ‘OsSPD’ and OsPSP2:1_F and OsPSP2:1_SacI_R for ‘OsPSP2:1’) and then OsSPD_XbaI_F and OsPSP2:1_SacI_R for ‘OsSPD’ and OsPSP2:1_SacI_R for ‘OsSPD’. After bombardment with gold particles coated with either control pBI221:GFP or pBI221:OsPAO7–GFP and pBI221:OsPSP2:1–DsRED, the onion bulbs were incubated at 23°C overnight in the dark. Onion epidermal layers were peeled off and observed with a fluorescence microscope (BX61; Olympus).

PA analysis of plant samples

Plant samples (0.3–0.5 g per sample) were pulverized with a mortar and pestle under liquid nitrogen. Five volumes (2.5 ml per 0.5 g plant sample) of 5% (v/v) cold PCA were added to the resulting fine powders. Then, as described previously (Naka et al. 2010), PAs were derivatized by benzoyl chloride and analyzed by HPLC with a 60% MeOH–40% water solvent system as described above.

Statistical analysis

Data analysis was performed using Microsoft Excel statistical tools (Student’s t test).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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