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Isoform-specific subcellular localization of *Zea mays* lipoxygenases and oxo-phytodienoate reductase 2

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ABSTRACT

Plant 9- and 13-lipoxygenases (9- and 13-LOXs) function in an early step of the oxylipin biosynthesis pathway. Here we report diverse subcellular localization profiles of four maize LOX family proteins and one oxo-phytodienoate reductase 2, subcellular localization of which could not be predicted by bioinformatics tools. Fluorescent protein-tagged LOX proteins were transiently expressed in maize B73 protoplasts as well as in stably transformed *Arabidopsis thaliana*. Localization profiles of maize 9-LOXs were more diverse than that of reported 13-LOX isoforms and spanned cytoplasm, plastids and tonoplasts, suggesting compartmentation of different oxylipin production inside of the cells. Localization profiles of maize 9-LOX were mostly consistent between maize and Arabidopsis, suggesting subcellular targeting mechanisms of these isoforms are conserved between monocots and dicots.

1. Introduction

Oxylipins are a group of diverse lipid hydroperoxide compounds produced from polyunsaturated fatty acids, namely, linoleic (18:2) and linolenic acids (18:3) (Feussner and Wasternack, 2002). Plants collectively produce an estimated 650 molecular species of oxylipins with many exhibiting hormone-like signaling or direct antimicrobial activities (Borrego and Kolomiets, 2016). Some of plant oxylipins including jasmonic acid (JA), conjugate dienoic acids, and volatile aldehydes like green leaf volatiles (GLV) are well characterized (Sofo et al., 2004). Certain oxylipins are highly reactive and toxic to the host cells, others function as signal molecules in plant defense reaction against biotic and abiotic stresses (Wang and Yang, 2005; Xue et al., 2008; Mueller and Berger, 2009; Hou et al., 2016; Wasternack and Strnad, 2016; Lim et al., 2017). The prototypical plant oxylipin signal molecules are JA and its derivatives produced via octadecanoid pathway, collectively called jasmonates (Farmer and Ryan, 1992; Koiwa et al., 1997). Production of oxylipins starts with a release of polyunsaturated fatty acids from membrane lipids. In the case of JA biosynthesis, linolenic acid from plastidial membrane galactolipids are released by the function of conserved plastidial galactolipases (Ishiguro et al., 2001; Hyun et al., 2008; Ellinger et al., 2010). Linolenic acid released to the stroma undergoes a 7-step conversion in plastid (lipoxygenase [LOX], allene oxide synthase, allene oxide cyclase) and peroxisome (oxo-phytodienoate reductase [OPR]) and three rounds of beta-oxidations to produce JA (Schaller, 2001). Resulting JA could be esterified to produce a volatile methyl-jasmonate, which can function in interplant signal communications (Farmer and Ryan, 1990; Tamogami et al., 2008), or conjugated with amino acids including biologically active jasmonate, JA-Ile (Staswick and Tiryaki, 2004).

Lipoxygenases (LOXs) (linoleate: oxygen oxidoreductase, EC 1.13.11.12) are a family of nonheme, iron-containing enzymes that catalyze the first step of converting polyunsaturated fatty acids derived from membrane lipids to oxylipins (Feussner and Wasternack, 2002). Plant lipoxygenase consists of 2 domains, a C-terminal catalytic domain (lipoxygenase domain) and an N-terminal PLAT/LH2 (Polycystin-1, Lipoxygenase, Alpha-Toxin/lipoxygenase homology 2) domain found in many lipid associated proteins (Bateman and Sandford, 1999). Two different plant LOX subgroups exist, 9-LOX and 13-LOX, based on the position specificity for the incorporation of the molecular oxygen into these fatty acids at C9 or C13 (Feussner and Wasternack, 2002). Functions of plant 13-LOX have been studied extensively in both monocots and dicots (Feussner and Wasternack, 2002; Lim et al., 2017). All plant 13-LOXs characterized to date accumulated in plastids, and some of them were shown to function in the production of jasmonate (Acosta et al., 2009; Chauvin et al., 2013) and GLV (Christensen et al.,

Abbreviations: OPR, oxo-phytodienoate reductase; LOX, lipoxygenase; JA, jasmonic acid

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2013). By contrast, little is known about the pathways mediated by each 9-LOX isoform. Most of 9-LOX proteins reported lack plastid targeting signals and likely function outside of the plastids, suggesting that different subcellular compartments other than plastids are responsible for the production of distinct sets of oxylipins (Wardale and Lambert, 1980; Vernooy-Gerritsen et al., 1984; Tranbarger et al., 1991; Matsui et al., 1992; Nalam et al., 2012).

The genome of monocot *Zea mays* cultivar B73 contains 12 isoforms of LOX genes, which represent six 13-LOXs and six 9-LOXs (Borrego and Kolomiets, 2016). In addition, maize, sorghum, and few other grasses harbor a unique monocot-specific LOX isoform, LOX6. LOX6 lacks an activity to oxidize fatty acids but functions as a hydroperoxide lyase that cleaves 13-LOX-derived hydroperoxide of linolenic acid into 13C- and 5C- containing oxylipins (Gao et al., 2008). Previous studies determined functions of maize 13-LOX paralogues in the production of JA (TS1/LOX8), and GLV (LOX10) (Acosta et al., 2009; Christensen et al., 2013). As for maize 9-LOX homologs, genetic analysis suggested that oxylipins produced via different 9-LOX isoforms differentially impact plant-microbe and nematode interactions (Gao et al., 2009; Constantino et al., 2013; Christensen et al., 2014; Wang et al., 2017). By contrast, our understanding is still obscure concerning the mechanism of how 9-LOX functions are integrated into different branches of the oxylipin pathway (Christensen et al., 2015), and how plants differentially activate different 9-LOX pathways and subsequent signaling. As a first step to obtain molecular insight of maize 9-LOX homologs, we conducted a systematic survey of subcellular localization using fluorescent protein (XFP) tagging approach. Four 9-LOX/LOX6 and one OPR proteins were fused to XFP in different configurations, and their subcellular localization patterns were determined using maize and Arabidopsis hosts. The data obtained from the analysis revealed surprisingly diverse localization profiles of individual 9-LOX isoforms suggesting that compartmentation of the different oxylipin biosynthesis branches extends beyond the plastidial 13-LOX/non-plastidial 9-LOX paradigm.

2. Materials and methods

2.1. Preparation of expression cassettes for maize lipoxygenases

Primers used for the PCR reactions were listed in Table S1. PCR fragments for LOX/OPR coding region were prepared by PCR using primer pairs (LOX2, 1444/1445/; LOX4,1428/1429; LOX6, 1430/1431; LOX12, T1 /T2; OPR2,1432/1433) and cDNA clones encoding LOX2 (ZM_BFc0165N03) LOX4 (ZM_BFc0171G14), LOX6 (Gao et al., 2008), LOX12 (Christensen et al., 2014), and OPR2 (p0010.cbpbi09, provided by Pioneer- a DuPont Company) as templates.

The PCR products were then introduced into Gateway entry vectors (pEnSOTG) (Bang et al., 2006) by SLiCE (Seamless Ligation Cloning Extract) homologous recombination cloning (Zhang et al., 2012). For internally tagged TP-mCherry-LOX6, cDNA fragments were prepared using primer pairs (1435/1436, 1434/1437, 1438/1437), and SLiCE reaction was prepared using three fragments and pEnSOTG digested with *Nco*I/*Not*I. After completion of the SLiCE reaction, each mixture was treated with Proteinase K at 37 °C for 10 min and used for *E.coli* transformation by electroporation. To prepare fusion proteins using monomeric GFP, pEnSOTG was modified to pEnSOmG by ligating a PCR fragment for mGFP (primer pair 922/923) and NcoI/NotI-digested pEnSOTG. LOX-mGFP expression cassettes were prepared as described above for LOX-GFP.

2.2. PLAT/LH2 domain deletion constructs

Deletion constructs were generated using pEnSO-LOX4-mGFP. LOX4Δ2 and Δ6 were prepared by digesting pEnSO-LOX4-mGFP with *Xcm*I/NcoI (Δ2) and *Rsr*II/XcmI (Δ6), and subsequent blunting and self-ligation. For LOX4 Δ9 and Δ11, cDNA fragments containing deletions were prepared by PCR using primer pairs (LOX4Δ9, T32/T33; LOX4Δ11, T34/T35) and ligated to pEnSO-LOX4-mGFP digested with RsrII/XcmI for Δ9, or RsrII/*Xho*I for Δ11.

2.3. Protoplast transfection assay

Protoplast transfection of maize cultivar B73 was performed using the polyethylene glycol (PEG)-mediated method (Jeong et al., 2013) with modifications. Maize seedlings were germinated under 16 h light/8 h dark condition then were moved under the dim light and kept for three days before the harvest. The middle part of the leaf blades was harvested and used for the protoplast preparations. All protoplasts were transfected using 10 μg of purified plasmid and cultured for 12 h at 25 °C. Confocal microscopy images were collected using Nikon FN1 C1si.

2.4. Arabidopsis transformation and selection

LOX/OPR-GFP/mCherry entry plasmids were recombined with pMDC99 (Curtis and Grossniklaus, 2003) using Gateway LR clonase (Thermo Fisher) according to the manufacturer's protocol. Agrobacterium-mediated transformation and selection of *Arabidopsis thaliana* Col-0 were performed as described previously (Rips et al., 2014).

2.5. Tonoplast isolation

The 35-day-old leaf tissues of stable transgenic Arabidopsis lines overexpressing GFP-LOX4/5 were used to isolate vacuoles as described (Robert et al., 2007). The isolated intact vacuoles were observed using the confocal microscope.

3. Results

3.1. Preparation of GFP/mCherry reporter gene for LOX/OPR subcellular localization

In contrast to the observations that enzymes related to the 13-LOX-derived oxylipin biosynthesis possess a clear plastid- or peroxisome-targeting (as in OPR3) signal, many 9-LOXs lack obvious targeting sequence making bioinformatics prediction of their subcellular localization difficult. To empirically determine the localization of maize 9-LOX family proteins in live cells, expression plasmids containing LOX-XFP fusion cassettes were prepared. In addition to 9-LOXs, OPR2, another unusual oxylipin pathway enzyme lacking peroxisome targeting signal (Zhang et al., 2005) was included in the analysis. Expression plasmids containing LOX/OPR with C-terminally fused GFP for protoplast transfection were prepared using the pEnSOTG vector (Fig. 1). To ensure expression in maize protoplasts, expression of reporter genes was driven by the superpromoter (Ni et al., 1995). LOX and OPR2 were

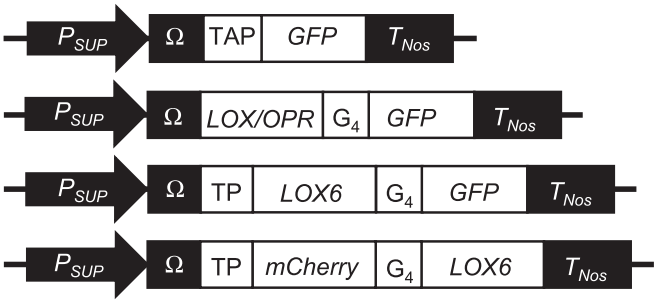

Fig. 1. Schematic drawing of expression cassettes for GFP/mCherry-tagged LOXs and OPR. P_{SUP}, superpromoter; Ω, tobacco mosaic virus omega sequence; G4, Glycine Linker; GFP, green fluorescence protein; TAP, TAP-tag; TP, transit peptide of LOX6; Tnos, NOS terminator.