# Caleosin/Peroxygenases: multifunctional proteins in plants

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- **Background** Caleosin/peroxygenases (CLO/PXGs) are a family of multifunctional proteins that are ubiquitous in land plants and also found in some fungi and green algae. CLO/PXGs were initially described as a class of plant lipid-associated proteins with some similarities to the oleosins that stabilize lipid droplets (LDs) in storage tissues such as seeds. However, we now know that CLO/PXGs have more complex structure, distribution and functions than oleosins. Structurally, CLO/PXGs share conserved domains that confer specific biochemical features with diverse localizations and functions.
- **Scope** This review surveys the structural properties of CLO/PXGs and their biochemical roles. In addition to their highly conserved structures, CLO/PXGs have peroxygenase activities and are involved in several aspects of oxylipin metabolism in plants. The enzymatic activities and the spatiotemporal expression of CLO/PXGs are described and linked with their wider involvement in plant physiology. Plant CLO/PXGs have many roles in both biotic and abiotic stress responses in plants and in their responses to environmental toxins. Finally, some intriguing developments in the biotechnological uses of CLO/PXGs are addressed.
- **Conclusions** It is now two decades since caleosin/peroxygenases (CLO/PXGs) were first recognized as a new class of lipid-associated proteins, and only 15 years since their additional enzymatic functions as a novel class of peroxygenases was discovered. There are many interesting research questions that remain to be addressed in future physiological studies of plant CLO/PXGs and also their recently discovered roles in the sequestration and possibly detoxification of a wide variety of lipidic xenobiotics that can challenge plant welfare.


**Key words**: caleosin, lipid droplets, peroxygenase, oxylipins, stress responses, plant lipids.

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## INTRODUCTION

The purpose of this review is to examine the nature and roles of a novel family of proteins, the caleosin/peroxygenases (CLO/PXGs). Genes encoding these proteins were initially identified in screening studies in plants in the 1990s (Yamaguchi-Shinozaki et al., 1992, Frandsen et al., 1996). The *Arabidopsis thaliana* gene Responsive to Desiccation 20 (*RD20*) was one of the first proteins that was rapidly upregulated after whole plants were placed on dry filter paper to desiccate. At that time there were no published sequences homologous to *RD20*, but it soon became one of the first stress-induced genes to be identified in plants (Yamaguchi-Shinozaki et al., 1992). The RD20 orthologs in other plants were later characterized and became referred as to 'caleosins' (CLO). Their name was based on the fact that they bound calcium ions plus their similarities with the previously characterized lipid droplet (LD) proteins called oleosins (Chen et al., 1999, Naested et al., 2000, Hernandez-Pinzon et al., 2001).

Most early studies on caleosins took place in plant systems and were largely focused on their apparently ubiquitous presence on the storage LDs, also known as oil bodies, oleosomes, or lipid bodies, that are found abundantly in seeds but which are likely present in most/all other plant tissues (Murphy, 2012). The 'cal' prefix in caleosins denotes the additional presence of a canonical calcium-binding EF-hand motif typically found in all characterized caleosins (Rahman et al., 2018a, Rahman et al., 2018b). EF-hand motifs are present in most organisms and are typically associated with proteins that act as calcium sensors or calcium buffers. While EF-hand motifs are normally found in tandemly arranged pairs, in CLO/PXGs they are present as a single domain, meaning that each protein can only bind one calcium atom, as has been experimentally demonstrated (Chen et al., 1999, Naested et al., 2000). Unlike oleosins, caleosins have additional intracellular locations apart from storage LDs and their presence was detected in most plant tissues. For example, certain CLO/PXG isoforms possess dual localizations into both LDs and other subcellular membrane systems. Hence, some CLO/PXG isoforms can bind to a variety of cellular bilayer membranes, including ER and

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plasmalemma, by a unique transmembrane domain (Naested et al., 2000, Hanano et al., 2006, Purkrtova et al., 2015, Hanano et al., 2015a). CLO/PXG genes and proteins also exhibit complex patterns of induction under different environmental conditions, suggesting addition roles as well as in lipid storage (Hernandez-Pinzon et al., 2001, Partridge and Murphy, 2009, Liu et al., 2022).

Subsequent studies, most notably by the group of Blee et al, revealed that CLO/PXGs are not just non-enzymatic structural proteins like oleosins, but have catalytic functions as peroxygenases (PXGs). Peroxygenases were originally characterized as catalyzing the hydroxylation of aromatic compounds such as aniline (Ishimaru and Yamazaki, 1977), sulfoxidation of methiocarb, a carbamate pesticide (Blee and Durst, 1986) and epoxidation of polyunsaturated fatty acids (Blee and Schuber, 1990b). Such activities were strictly dependent on the presence of hydroperoxides ($H_2O_2$, fatty acid hydroperoxides or cumene hydroperoxide). This function is related to oxylipin metabolism and involves an iron-coordinating heme ligand (Hanano et al., 2006, Blee et al., 2014). In the EBI Enzyme Nomenclature database the enzymatic activity of PXG is classified as an EC type 1.11.2.3 (https://www.ebi.ac.uk/intenz/query?cmd=SearchEC&ec=1.11.2.3). Data from various studies have confirmed that both the PXG activity and lipid binding functions also apply to orthologous caleosin-like proteins in both plants, algae and fungi. For example, it was reported that a fungal CLO/PXG plays a pivotal role in the formation and trafficking of LDs in addition to its peroxygenase activity (Rahman et al., 2018a, Rahman et al., 2018b). For these reasons, the entire family of CLO/PXG proteins, and their encoded genes, are now referred to as caleosin/peroxygenases in order to emphasize their multifunctional nature and physiological roles (Rahman et al., 2018a, Rahman et al., 2018b). Very recently it was reported that OsPXG9 from rice is a caleosin-like peroxygenase that catalyzes lipoxygenase-dependent regiospecific epoxidation of lipid peroxides and is involved in wider responses to abiotic stressors in plants (Tran et al., 2022).

CLO/PXGs have often been annotated under different names and categories, both in protein and nucleic acid sequence databases and in the wider literature, complicating efforts to perform

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comparative analyses of CLO/PXGs, thus researchers should be aware of these different names. Examples of database names for putative and/or experimentally verified *CLO/PXG* genes include: *caleosin, peroxygenase, caleosin-like peroxygenase, calcium-binding protein, EF-hand protein, responsive to desiccation (RD),* and *stress-related protein*. As each of these terms have been used recently in the literature, we have also used them here, for example when describing a particular study where the same gene/protein might be referred to variously as RD20 and/or AtClo3 and/or CLO/PXG despite the fact that all of these terms are synonymous and the proteins/genes describes are identical.

In this review, we first analyze CLO/PXGs in land plants (Embryophytes) where these multifunctional proteins appear to be ubiquitous. We also put this distribution into a wider context by briefly considering current evidence for the presence of characterized or putative CLO/PXGs orthologs in other eukaryotic organisms including algae and fungi. We next examine the structural and biochemical properties of these proteins. This is followed by assessments of their subcellular localizations, the mechanisms of their enzymatic activities as peroxygenases, and their physiological roles in a wide range of plant developmental and stress responses, both biotic and abiotic. Finally, there is an account of some intriguing reports of possible biotechnological roles for CLO/PXGs, including their deployment as nano-carriers for targeted drug delivery in several human disease conditions (Chen et al., 2004, Chen et al., 2005, Chiang et al., 2012, Shih et al., 2017, De et al., 2018, Yuen, 2019).

## OCCURRENCE OF *CLO/PXG* GENES IN PLANTS AND OTHER EUKARYOTES

The presence of *CLO/PXG* genes is actually confirmed in several eukaryotic taxa, but their distribution across these organisms is unusual. Also, *CLO/PXG*-like genes are discovered in the land plants (Embryophyta) and green algae (Chlorophyta) and their biological functions have been well characterized as discussed below and described graphically in Figure 1. *CLO/PXG*-like genes also occur in all major fungal groups, but not in all species of such groups. For example, there are

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numerous reports of the various physiological roles of the encoded CLO/PXG proteins in several important ascomycete and basidiomycete species (Fan et al., 2015, Ortiz-Urquiza et al., 2016, Gao et al., 2013, Wang and St Leger, 2007, Zeng et al., 2017, Rutter et al., 2017, Zhu et al., 2015b). In sharp contrast *CLO/PXG*-like genes are absent, with few exceptions, from other major eukaryotic groups such as protozoans and animals (Rahman et al., 2018a). The restriction of CLO/PXG-like genes to plant and fungal genomes is anomalous because fungi and animals are both members of the monophyletic Opisthokont clade (Cavalier-Smith et al., 2014). Inversely, plants and green algae are clustered in a separate monophyletic eukaryotic lineage refereed as to Viridiplantae. If caleosins were present in the common ancestor of the Opisthokonts and Viridiplantae then their presence would also be expected in at least some metazoan genomes. That is unless these genes were lost prior to the earliest divergence of metazoan groups, about 650 million years ago (Mya) (Rahman et al., 2018a).

In spite of the fact that *CLO/PXG*-like genes are ubiquitous in all plant genomes published to date, their presence was confirmed only in about 30% of sequenced fungal genomes, including species in all of the major fungal taxa. However, fungal CLO/PXGs proteins exhibit similar, but not identical, functions to those in plants. Such roles include stress-related oxylipin signaling, lipid metabolism, reproduction and pathogenesis. While the presence of *CLO/PXG* orthologs in all sequenced plant genomes would suggest that they have essential core housekeeping functions in plants (Rahman et al., 2018b), the absence of *CLO/PXGs* genes in many fungal genomes suggests restricted functions for such fungal *CLO/PXGs* that are not vital for survival. One of the challenges for future research is to identify the core functions of CLO/PXGs that make them essential in plants but optional in fungi.

*CLO/PXG*-like genes are present in the sequenced genomes of all Embryophytes although the isoform content varies in different plant groups. In general, two major CLO/PXG isoforms are typically present, i.e. H (high) and L (low), where H-forms contain an extra C-terminal proximal motif

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of 30–50 residues compared to L-forms. The most primitive Embryophytes are the mosses and liverworts and only one sequenced genome is available for each, *Marchantia polymorpha* and *Physcomitrella patens*, respectively. In both cases their genomes contain only the L isoform, suggesting that the H isoform has been lost in these plants as both forms are present in green algae. The presence of both L and H isoforms was also reported in lycophyte, *Selaginella moellendorfii*, a fern-like seedless vascular plant that has true roots and ABA signaling pathways, with apparent similarity to those reported for AtCLO4 in *A. thaliana* that acts as a negative gene regulator (Zhu et al., 2015a, Kim et al., 2011). Sequence data are available for four species of gymnosperm, which are seed-bearing vascular, but non-flowering, higher plants. In all cases, only the H isoform is present. Therefore, in these non-flowering land plants, most species contain only one of the two major *CLO/PXG* isoforms (Rahman et al., 2018b).

To date *CLO/PXG*-like genes have been found in all of the >300 sequenced angiosperm species. The split between angiosperms and other vascular plants occurred prior to 200 Mya and the former now make up the majority of the terrestrial flora in terms of biomass and species diversity (Murphy and Cardona, 2021). In all of these species our previous analysis suggested that there is at least one copy each of the L and H isoforms of CLO/PXG, although in many species gene duplication has led to relatively large families of *CLO/PXG*-like genes, many of which are differentially expressed in response to developmental and/or environmental conditions (Rahman et al., 2018b). The expansion complexity of *CLO/PXG*-like gene families in terrestrial plants is coordinated with the evolution of a wider framework of functions by the various CLO/PXG protein isoforms. This is in line with the results of comparative phylogenetic analysis previously performed on sixty-seven protein isoforms of CLO/PXG from 34 species across the Viridiplantae as shown Figure 2, adopted from Rahman et al., (Rahman et al., 2018b).

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STRUCTURAL FEATURES OF CLO/PXGS

The first identified plant caleosins were initially described as LD-associated proteins in seeds. This subcellular localization is consistent with the presence of a proline-rich lipid-binding domain with some similarity to that of oleosins, although CLO/PXGs also have important differences from oleosins. It is now known that the structural properties of caleosins are crucial for the involvement of some, but not all, isoforms in stabilizing LDs as well for their catalytic activity. Unlike oleosins, CLO/PXGs have a large N-terminal hydrophilic domain containing an EF-hand calcium-binding motif and a central hydrophobic domain capable of spanning an entire membrane bilayer rather than just a monolayer. CLO/PXGs also contain a highly conserved heme-binding domain with conserved histidine residues that act in concert in the coordination of binding of the binding of one or more heme prosthetic groups. Finally, CLO/PXGs harbor multiple putative phosphorylation sites at the C-terminal hydrophilic domain, although the locations and numbers of these sites are less highly conserved (Hanano et al., 2006, Rahman et al., 2018b).

*The EF-hand calcium binding motif*

The N-terminal CLO/PXG domain contains a highly conserved EF-hand motif of 28 residues in a helix-loop-helix arrangement able to bind a single $Ca^{2+}$ atom. The presence of this motif is often used diagnostically to determine whether an unknown protein is a member of the CLO/PXG family as they are annotated as being putative $Ca^{2+}$-binding proteins. Experimental confirmation of $Ca^{2+}$ binding was determined using an *E. coli*-expressed recombinant rice caleosin, OsCLO (Frandsen et al., 1996), sesame SiCLO (Sopl) and Arabidopsis AtCLO1 (ATS1) caleosins (Chen et al., 1999, Takahashi et al., 2000, Hanano et al., 2006). The presence of a single EF-hand in all CLO/PXG sequences is unusual because this motif is more typically present as a pair. Normally, EF-hand pairs bind two calcium ions within in a hydrophobic pocket that is formed between a pair of EF hands (Ikura, 1996, Kawasaki et al., 1998). In contrast, a single EF-hand binds calcium ions with relatively low affinity. However, it is also possible for two closely adjacent CLOs to form a dimer with two EF

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hand motifs, one on each monomer, in order to bind calcium with higher affinity (Hanano et al., 2006, Naested et al., 2000). Dimerization of two CLOs could happen in two ways. Firstly, a CLO monomer could dimerize by *cis* associations with another monomer integrated into the same membrane or LD to form a pair of EF-hands. Secondly, a CLO monomer could associate with another CLO on a different membrane or LD. The latter possibility is analogous to the v-SNARE/t-SNARE and associating factors involved in membrane fusion in eukaryotic cells where proteins located onto separate membranes can functionally interact (Sanderfoot and Raikhel, 1999, Mayer, 1999).

The catalytic importance of $Ca^{2+}$ binding into CLO/PXGs is demonstrated by the reversible loss of its enzymatic activity upon removal of the $Ca^{2+}$, possibly resulting in a conformational change in the protein (Hanano et al., 2006). Similarly, the recombinant caleosin of Arabidopsis (AtCLO1) reconstituted into LDs *in vitro*, exhibited $Ca^{2+}$-driven aggregation and coalescence, suggesting a role for the $Ca^{2+}$ in the dynamics of LDs (Purkrtova et al., 2008b). The $Ca^{2+}$-mediated fusion of LDs has been proposed as an important aspect of the maturation process where nascent microlipid droplets released from the ER fuse into large LDs in animal cells (Valivullah et al., 1988b, Murphy and Vance, 1999). A similar scenario is found in seeds and lipid-storage plant tissues, where nascent LDs are released from the ER as nano-droplets (100-200 nm) before fusing to produce mature LDs (0.4–2 µm) (Huang, 1996, Sarmiento et al., 1997) (Murphy, 2001a; Murphy, 2012).

*The central hydrophobic domain*

All plant CLO/PXG sequences contain a domain made up of non-polar or hydrophobic motif that typically extends to about 20 residues. This is sometimes referred to as the (putative) transmembrane or lipid-binding domain due to its potential either to span a lipid bilayer or to loop in and out of a lipid monolayer (Rahman et al., 2018b). Frequently, the hydrophobic domain is located close to the N-terminal end of the protein. On its N-terminal side it is flanked by the canonical $Ca^{2+}$ binding domain while on its C-terminal side it is flanked by several conserved phosphorylation sites meaning that it effectively separates these two regions of the protein. For example, if the CLO/PXG is

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located on a membrane such as the ER, the $Ca^{2+}$ binding domain will be located on one side of the membrane while the phosphorylation sites will be located on the other side (Partridge and Murphy, 2009). Due to its substantial role for their association/integration in bilayer membranes or LDs, the hydrophobic domain is a key structural characteristic in CLOs.

The isoelectric point reported for CLO-stabilized LDs was about of pH 4.0 and their aggregation was observed at a pH lower than 4.5, suggesting that their stability and integrity were facilitated by the electronegative repulsion and steric hindrance of C- and N-terminus of CLOs that are exposed onto the surface of LDs (Chen et al., 2004). The hydrophobic domain forms an estimated 15% of total caleosin residues compared to 45% in the case of oleosin residues, exposing about 85% and 55% residues of caleosin and oleosin, respectively, by their N- and C-terminal ends from the surface of LD. When comparable amounts of TAGs and caleosins were mixed for *in vitro* reconstitution of LDs, caleosins covered more surface area of the LDs and encapsulated smaller-sized but larger numbers of LDs than oleosins (Jiang and Tzen, 2010).

Proteinase K digestion of a cytosolic LD-associated CLO/PXG isoform from Arabidopsis, AtCLO3, demonstrated that this caleosin was associated with microsomal and chloroplast envelope fractions (Partridge and Murphy, 2009), where it exhibited a type I membrane orientation (Singer, 1990), with about 2 kDa of its C-terminal facing the cytosol. This suggests that about 9 kDa of the N-terminal region faces the lumen domain of the microsomes (Partridge and Murphy, 2009). A speculative model of the possible associations of CLO/PXG with LDs and bilayer membranes is shown in Figure 3. The orientation of protein is probably mediated by the membrane-spanning helix, which would normally adopt a type I orientation on the ER membrane i.e. N-terminal facing the lumen and the C-terminal facing the cytosol. This would enable the calcium-binding EF hand domain to interact with calcium pools in the ER lumen (Murphy, 2001). Also, in this scenario, the C-terminal domain of CLO/PXG with its kinase phosphorylation sites and disulfide bonds is exposed to the cytosol compartment, suggesting possible roles in stress-induced signaling. In contrast, the N-terminal

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domain is inside the ER lumen with its $Ca^{+2}$-binding domain (residue 75-86) and the essential His70 residue for PXG activity. However, the two essential His residues His70 and His138 are found on opposite sides of the TM domain as predicted in all the algorithms used, raising questions about the structural elements and crucial residues of the active site of PXG that help in better understanding the mechanistic features of this atypical hemoprotein. A possible explanation is that the dimerization of CLOs may be required for PXG activity.

More detailed secondary structural properties were determined using the recombinant seed-specific caleosin, AtCLO1. The protein was found to be well folded in aqueous solution with 16% α-helix and 29% β-sheet structures, with its secondary structure being modulated by increasing the polarity using various aliphatic alcohols and apparently stabilized by a disulfide bridge between cysteine residues C221 and C230 (Purkrtova et al., 2007). From these studies, it was reported that the $Ca^{2+}$-binding domain of CLOs is critically involved in LD-LD fusion processes. This $Ca^{2+}$-mediated fusion has been shown to be a key process of the maturation of micro-LDs released from the ER to form the large cytosolic LDs as discussed above. In a similar way, LDs in plant seeds and other lipid-accumulating tissues are often formed from the ER as micro-nano-droplets and then undergo successive rounds of fusion to shape mature LDs of about 0.4–2 µm diameter typically found in such tissues (Huang, 1996, Sarmiento et al., 1997). Altogether, these data suggest an important role of CLOs in the structural stabilization and conformation of some LDs (Jiang and Tzen, 2010).

*Phosphorylation sites*

Most plant CLOs are typified by the presence of four phosphorylation sites at the C-terminal end, three casein kinase II and one tyrosine kinase and the main putatively phosphorylated residues are threonine, serine and tyrosine (Chen et al., 1999, Rahman et al., 2018a). Experimentally, the native Arabidopsis AtCLO1, CLO/PXG, was partially phosphorylated at S225. However, while the recombinant purified AtCOL1 was phosphorylated by tyrosine kinase this had no significant effect on its peroxygenase activity (Hanano et al., 2006). Comparative genomic analysis for CLO-encoding

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genes identified in 15 plant species demonstrated that the functional divergence of CLO genes was due to a positive selection of codons corresponding to critical amino acids (R70, G74, L88, G89, K100, A106, S107) which located in C-terminal domain (Song et al., 2014). However, the unclear relationship between the phosphorylation of CLOs and their enzymatic activities, raises the possibility of other phosphorylation-mediated physiological roles of plant CLOs. Hence, if phosphorylation is involved in the regulation of CLO/PXG function, it could be related to other functions other than peroxygenase activity.

## LOCALIZATIONS OF CLO/PXGS IN PLANT CELL

Although CLO/PXGs were initially identified in the LDs fraction of plant seeds, their abundance was also confirmed in a range of non-seed plant tissues. Even within the same plant tissue, CLO/PXGs can be differentially expressed at the subcellular level.

### *Tissue localizations of CLO/PXGs*

Several studies have shown that the LD proteome of seeds is dominated by three classes of lipid-binding proteins, named oleosins, caleosins and steroleosins, and their relative abundances were estimated about of 100:5:1 respectively (Jolivet et al., 2011, Umate, 2012, Huang, 2018, Shao et al., 2019, Hamada et al., 2020). Exceptionally, CLOs are also found to be functionally expressed in other plant tissues like roots, leaves, pollen and fruits, suggesting that plants harbor different isoforms of CLO/PXGs with distinct tissue-related functions. For example, Arabidopsis has five well-expressed CLO/PXG isoforms with different tissue-related patterns of expression (Naested et al., 2000, Rahman et al., 2018b). While AtCLO2, AtCLO3 and AtCLO4 were expressed in various vegetative tissues of Arabidopsis seedlings, AtCLO1, was originally characterized as embryo-specific, but later its expression was also detected in other tissues mainly root tips and this mirrors the profile of caleosin expression in plants. It is also now apparent that LDs are not only present in storage tissues such as seeds and pollen grains, but are also found in most, if not all, cell types in eukaryotes

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(Murphy, 2012, Murphy and Cardona, 2021). For example, oleosins are present in LDs that are found in rapidly dividing meristematic tissues in both stems and roots (Murphy et al, 2001). Therefore even if a particular CLO/PXG isoform is expressed in a non-seed tissue, this does not necessarily rule out that it is LD-localized.

The tissue-specific localization of CLO/PXGs was shown in *Brassica napus* by quantifying the transcript levels of two CLO/PXG isoforms in various plant tissues, where two main CLO/PXGs, isoform of 25-kDa and another of 27-kDa, were exclusively detected in embryos. It was shown that the 25-kDa isoform is exclusively localized on lipid bodies of developing embryos and germinating cotyledons while, the 27-kDa is an ER-bound isoform and is expressed in roots, stems and leaves (Hernandez-Pinzon et al., 2001). Similarly, transcripts of two CLO/PXG isoforms, *HvCLO1* and *HvCLO2*, are differentially expressed in barley. While the transcripts of *HvCLO1* are abundant in mature embryos, the transcripts of *HvCLO2* are mainly measured in endosperm tissue, meaning that caleosins, unlike oleosins, can possibly translocate via small vesicles and may be linked to lipid trafficking, membrane expansion and LD biogenesis (Liu et al., 2005, Khalil et al., 2014).

A specific CLO/PXG isoform has been identified in the storage LDs that are abundantly present in pollen grains of lily (Jiang et al., 2008). Another isoform of 30 kDa was identified as a cytosolic LD-specific in olive and pine pollen (Zienkiewicz et al., 2010, Pasaribu et al., 2014, Pasaribu et al., 2017). Pollen grains have some similarity to seeds because they both act as propagules during the reproductive process in higher plants. Similarly, in some species both their seeds and pollen grains can be enriched in storage lipids. Both structures contain large numbers of LDs adapted for storage with specific isoforms of structural and catalytic proteins including oleosins and caleosins (Murphy, 2012). In higher plants, caleosins are encoded by multiple LD-specific isoforms, notably in oilseed species. For example, quantification of *RcCLO1* and *RcClo2* in *Ricinus communis* L. (castor bean) and *LuCLO1* and *LuCLO2* transcripts in *Linum usitatissimum* L. (flax) revealed that *RcCLO2*, *LuCLO1* and *LuCLO2* transcripts were maximal in the seeds of both plants while *RcCLO1* was

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expressed maximally in cotyledons. (Hyun et al., 2013). Similarly, six of eleven CLO genes in *Brassica rapa* were expressed in seed LDs (HU et al., 2013). Such scenario was also found in maize (*Zea mays* L.), where three of twelve CLO/PXG genes were specifically expressed in seed while the other genes were differentially expressed in various tissues (Hu et al., 2014a, Du et al., 2019). The *A. thaliana* AtCLO3 seems to occur in multiple membrane locations in leaf cells, which is consistent with its involvement in responses to several divergent forms of biotic and abiotic stress. Hence, AtCLO3 was especially highly induced in response to salt or dehydration stresses while the expression of AtCLO4 and AtCLO5 was very limited in non-stressed plants. The latter two isoforms may therefore have other, hitherto unrecognized, roles in plants. The role of caleosins in drought stress has been supported by a report indicating that an *AtCLO3*-like gene was upregulated as part of the desiccation tolerance response of the non-seed plant *S. tamariscina* (Liu et al., 2008).

In rice, the first caleosin to be studied, OsCLO, was mainly localized in microsomal fractions of mature seeds, and its expression was effectively induced in abscisic acid (ABA)-treated or osmotic-stressed rice leaves (Frandsen et al., 1996). Also, the expression of the sesame caleosin (SiCLO1) peaked two weeks after flowering and progressively decreased to completely disappear in mature seeds (Chen et al., 1998, Chen et al., 1999). More recently, it was reported that a specific isoform of CLO/PXG, referred to as PdPXG2, was mainly expressed in the apical zone of date palm roots where it specifically reduces 9-hydroperoxide fatty acids. Also in date palm, another isoform, PdPXG4, was mostly expressed in young leaves (Hanano et al., 2016b, Hanano et al., 2018c).

*Subcellular localizations of CLO/PXGs*

Oleosin is well known as an exclusively LD-associated protein, apart from a brief period on the ER where it is co-localized with budding LDs (Murphy, 2012). This meant that oleosin was initially considered as an exclusively seed-specific protein and indeed its gene promoter is still widely used to drive the specific expression of transgenes in seed tissues (for example, US patent 6433252, 2000) (Kriz and Griffor, 2000). Evidence from Arabidopsis suggests that some CLO/PXG isoforms, such as

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AtCLO1, are exclusively localized on LDs but others, like AtCLO3, are localized on other membranes. However, it cannot be ruled out that some CLO/PXG isoforms have dual localizations on both LDs and one or more of the subcellular membranes. There are many examples of other proteins that have such dual localizations, including some of the enzymes involved in the biosynthesis of triacylglycerols (TAGs) and sterol esters (SEs) and other proteins implicated in lipid trafficking (Natter et al., 2005, Ayme et al., 2014, Murphy, 2012, Murphy and Liu, 2022).

The dual localization of CLO/PXG proteins on both LDs and on various cell membrane systems, such as ER, Golgi, plasma membrane and mitochondria, has important implications for the structure of LDs as well as for their mechanisms of interaction and possibly translocation across bilayer membranes (Scholz et al., 2022). In this regard, several models have been proposed for LDs and their wider roles in cellular homeostasis (Chapman et al., 2012). One common theme that has emerged is that there are numerous proteins that, similarly to CLO/PXGs, are able to bind stably to both bilayers and monolayers. Figure 3, represents speculative models for the association of CLO/PXGs in membrane lipids. In other reported cases, LD-associated proteins can possibly form homomeric or heteromeric assembly with other proteins that are either located on the same droplets or elsewhere in the cell. The latter possibility of interaction enable LDs to bind with other cellular organelles. Further, while a particular LD-associated protein still exclusively related to this LD, other proteins might be able to reversibly translocate and bind to different subcellular compartments, such as the ER, mitochondria, vacuoles, or peroxisomes (Pu et al., 2011, Radulovic et al., 2013, Kong et al., 2020, Kory et al., 2016, Walther et al., 2017).

The role of CLO/PXGs in the mobilization of lipid storage reserves has been demonstrated by their involvement in $Ca^{2+}$-mediated membrane-fission and/or fusion events with glyoxysomes to activate the lipolysis and gluconeogenesis, thereby ensuring lipid trafficking between the ER and other storage or transport vesicles (Chapman and Trelease, 1991, Scholz et al., 2022).

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We and others have demonstrated that certain CLO/PXG isoforms in both plants and fungi can bind to ER and plasmalemma, via a putative transmembrane domain (Naested et al., 2000, Hanano et al., 2006, Purkrtova et al., 2015, Hanano et al., 2015a). As pointed out before, the Arabidopsis AtCLO3 isoform was associated with microsomal and chloroplast envelope fractions where it exhibited a type I membrane orientation with the C-terminal region facing the cytosol. But this isoform was also detected in cytosolic LDs of senescent leaves or leaves attacked by pathogens (Shimada et al., 2014). AtCLO3 seems then to occur in multiple membrane locations in leaf cells, which is consistent with its involvement in responses to several divergent forms of biotic and abiotic stress.

Moreover, immunofluorescent labelling of caleosins in pea root tips showed predominant signals in the endoplasmic reticulum, but not in vacuoles of these meristem cells, suggesting that CLOs did not co-localize with antibodies targeting BP-80, a vacuolar receptor found in pre-vacuoles and Golgi (Paris et al., 1996). Co-localization of CLO/PXG was also not observed with two other protein markers for vacuolar functions, the aquaporins $\delta$-TIP and $\gamma$-TIP (Jauh et al. 1999). In contrast, CLOs appeared to co-localize with BiP, an ER marker, and with $\alpha$-TIP, a vacuole marker, indicating that CLOs may be found both outside and inside $\alpha$-TIP-labelled vacuoles. This suggests that these vacuoles may be autophagic rather than protein storage vacuoles (Hoh et al., 1995, Jauh et al., 1999, Paris et al., 1996, Van der Wilden et al., 1980a). The co-localization between CLOs and $\alpha$-TIP-labelled vacuoles could be explained by the fact that LD formation occurs in the ER. The co-localization of CLOs, $\alpha$-TIP-vacuole membranes and the ER marker BiP may hence represent an ER subdomain associated with both vacuoles and LD biogenesis (Yatsu and Jacks, 1972, Slack et al., 1980, Van der Wilden et al., 1980a, Van der Wilden et al., 1980b, Valivullah et al., 1988a, Murphy and Liu, 2022). Within Arabidopsis seeds, the subcellular localization of CLOs varies according to the developmental stage. Early in seed development, before the onset of storage lipid accumulation, only a 27 kDa ER-localized CLO isoform is found while a 25 kDa CLO LD-associated isoform was significantly

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predominated in later stages (Murphy et al., 2001, Murphy et al., 2000, Rozwadowski et al., 1999b). The 27 kDa ER-isoform is then virtually undetectable in dry seeds but reappears during germination, while the 25 kDa LD-isoform disappears during the mobilization of lipid storage. Later, whereas the 27 kDa ER-isoform is also found in roots, stems and young leaves, the 25 kDa LD-isoform isoform is only found in seeds and root tips (Murphy et al., 2000, Naested et al., 2000). In summary, land plants harbor multiple CLO/PXG isoforms that can be localized either on LDs and/or on several bilayer membrane compartments and their expression is regulated by various endo- and /or exogenous stimuli.

## ENZYMATIC ACTIVITIES OF CLO/PXG

The discovery of and characterization of peroxygenase (PXG) as a new enzyme that is distinct from plant peroxidases and cytochromes P450 has been reviewed (Blee and Durst, 1987). In brief, PXG was initially found to catalyze the hydroxylation of aromatic compounds such as aniline or indole (Ishimaru and Yamazaki, 1977), sulfoxidation of pesticides such methiocarb (Blee and Durst, 1986) and epoxidation of *cis*-double bonds of unsaturated fatty acids (Blee and Schuber, 1990b). Such activities were strictly dependent on the presence of hydroperoxides ($H_2O_2$, cumene hydroperoxide or fatty acid hydroperoxides). Further, PXG was identified as a hemoprotein and the presence of a type b protoheme was crucial for its catalytic activity because its destruction totally abolished the enzyme activity (Ishimaru and Yamazaki, 1977, Blee and Schuber, 1990a). Furthermore, the presence of ferric heme liganded with two histidine residues in the purified oat-seed PXG was confirmed by electron paramagnetic resonance (EPR) spectroscopy, predicting therefore that mutation of such histidines might lead to a drastic alteration of the PXG activity (Hanano et al., 2006).

Further detailed studies have clarified the oxygenation reaction catalyzed by PXG that proceeds via a two-step mechanism with linolenic hydroperoxide as oxygen donor as illustrated in Figure 4. In the first step, hydroperoxide fatty acids, e.g. 13-HPOT, are generated from linolenic acid through 13-lipoxygenases activity. The iron of the hemic group in the active site of CLO/PXG attacks the O-O

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bound of the hydroperoxide, fixing therefore one oxygen atom by a heterolytic cleavage and leading to the formation of a reversible oxo-heme complex with an Iron(II) Oxide (Fe-O) polar bond in the active site of the peroxygenase (PXG) and the reduction of the fatty acid hydroperoxide (13-HPOT) to its corresponding hydroxide (13-HOT). This reaction constitutes the fatty acid hydroperoxide-reductase activity of PXG. In the second step, the PXG transfers the oxygen atom via two competing pathways A and B. In pathway A, the PXG transfers the O- to a C=C double bond in the same unsaturated fatty acid hydroxide (13-HOT), resulting from the first step, by an intramolecular transfer, leading to the formation of its corresponding hydroxyl-epoxy fatty acid. Such molecules are directly involved in the deposition of cuticle in the leaves and suberin in the roots of higher plants (Blee et al., 2014, Hanano et al., 2015c, Hanano et al., 2018c). Such oxylipins are known to be strongly induced by biotic and abiotic stresses, suggesting potential roles of these molecule in plant immunity and its responses to environmental stimuli. In pathway B, the PXG transfers O- to another oxidizable substrate via intramolecular transfer, thereby catalyzing the co-oxidation reactions, e.g. the sulfoxidation of sulfur-containing compounds and the epoxidation of unsaturated fatty acids. These activities generate a characteristic signature of mono-, di-, and poly-epoxy fatty acids with major properties as antifungal agents (Blee et al., 1993, Shimada et al., 2014).

Further purification of large amounts of PXG from oat seed microsomal and LD fractions enabled the sequencing of its N-terminus, which revealed that it was a caleosin. The peroxygenases activity of identified proteins was confirmed by biochemical studies of recombinant caleosins of Arabidopsis (AtCLO1 and AtCLO2) and rice (OsCLO10) (Hanano et al., 2006). Site-directed mutagenesis in AtCLO1 identified His70 and His138 as the axial and distal ligands that respectively coordinated the heme of this CLO/PXG. These histidine residues are strictly conserved, not only in plant CLO/PXGs, but also in >230 fungal CLO/PXGs (Rahman et al., 2018a), suggesting that fungal CLOs might also function as peroxygenases. This hypothesis was verified with caleosin AfPXG from *Aspergillus flavus* where the mutagenesis of the His85 residues corresponding to the location of His70 in AtCLO1 from plants totally abolished its peroxygenase activity (Hanano et al., 2018a). This

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raises the possibility that most, and possibly all, fungal CLO/PXGs can function as peroxygenases and this might also be true for algal CLO/PXGs having key histidine in the similar locations (Rahman et al., 2018a, Rahman et al., 2018b). Moreover, it was reported that the enzymatic activity of CLO/PXG is also dependent on $Ca^{2+}$. Thus the removal of the cation resulted in the loss of activity, most likely due to the conversion of CLO-CLO-dimers into monomers in the absence of $Ca^{2+}$ (Murphy and Vance, 1999, Hanano et al., 2006). In contrast, however, protein phosphorylation of CLO/PXGs had no impact on PXG activity (Hanano et al., 2006).

Until now only a few CLOs have been tested for their peroxygenase properties and it is well recognized that the peroxygenases studied to date largely differ in their substrate specificity. This property, likely deriving from differences in the size, the hydrophobicity or/and the sterically hindered entrance of the active site pocket, results in distinct catalytic functionalities. For example, Arabidopsis AtCLO3 possesses an unusual hydroperoxide reductase activity, which is restricted to fatty acid hydroperoxides deriving from linolenic acid. Consequently, Arabidopsis leaves overexpressing AtCLO3 accumulated 13-hydroxy-9,11,15-octadecatrienoic acid which results from the reduction of linolenate hydroperoxide (Blee et al., 2014). In contrast, AtCLO1 and AtCLO2 accept various hydroperoxides as substrates and exhibit elevated hydroxylase and sulfoxidase activities but very poor epoxidase activity (Hanano et al., 2006). Conversely the isoform AtCLO4 is a poor hydroxylase but actively catalyzes regioselective and strictly stereospecific oxygenations of *cis* double bonds of unsaturated fatty acids. Actually AtCLO4 catalyzes the exclusive formation of (*R*),(*S)* epoxide enantiomers which is the stereochemistry of the epoxides found *in planta.* (Hanano et al., 2006). Thus CLO/PXG differ not only by their localizations and their tissular expression patterns in plants but also by their catalytic activities due to the diversity of co-oxidation reactions catalyzed by the peroxygenase.

In rice, the CLO/PXG isoform, OsPXG9, catalyzed a 12-epoxidation that occurred slightly more efficiently with 9(*S*)-hydroperoxyoctadecatrienoic acid rather than with 9(*S*)-

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hydroperoxyoctadecadienoic acid as the preferred substrate (Tran et al, 2023). In this case, the products of 12-epoxidation were labile, and the epoxide ring is hydrolytically cleaved into corresponding trihydroxy compounds. In contrast, the same enzyme OsPXG9 also catalyzed the 15-epoxidation of 13(*S*)-hydro- peroxyoctadecatrienoic acid to generate a relatively stable epoxide product. In this case, expression of the *OsPXG9* gene was upregulated by abiotic stresses such as increased salinity and drought. In contrast, stimuli related to biotic stresses, such as herbivory as elicited by mediators such as salicylic acid and flagellin 2, resulted in downregulation of *OsPXG9* gene expression (Tran et al, 2023).

## PHYSIOLOGICAL ROLES OF CLO/PXGS

### *Roles of CLO/PXG in plant development*

Pivotal roles for CLO/PXGs in different developmental stages of plants have been demonstrated in seed germination, seedling tissues differentiation, leaf senescence, pollen maturation and seed filling (Hanano et al., 2016b, Rejon et al., 2016, Brocard et al., 2017, Pasaribu et al., 2017, Du et al., 2019, Lu et al., 2020, Nuccio and Thomas, 1999, Jiang et al., 2008). The originally described roles for CLO/PXGs in plant development related to their involvement in the formation, maturation and storage functions of seed LDs (Naested et al., 2000). Here, a possible role for CLO/PXGs in calcium-mediated fusion of micro LDs was suggested as analogous to the LD maturation processes in many animal lipid storing cells (Murphy and Vance, 1999, Murphy, 2001, Murphy, 2012). In this context, the role of LDs in interorganellar and possibly in cell-to-cell connection is known to be fundamental for the direct exchange of cellular components. This process is mediated by two newly characterized proteins in *A. thaliana*, LD-localized SEED LD PROTEIN 1 (SLDP1) and PM-localized LD-PLASMA MEMBRANE ADAPTOR (LIPA) (Krawczyk et al., 2022). The connection between LDs and other cellular organelles is essential for overall cellular functioning in terms of cellular metabolic and signaling pathways. The connective LD-organelle network in the plant cell and the roles of LDs in intracellular trafficking was recently reviewed (Scholz et al., 2022).

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The variation of subcellular localizations of LD-associated proteins during different developmental stages of CLO-overexpressing yeast cultures suggests their potential involvement as developmental mediators via their implications in lipid intracellular trafficking and metabolism (Leber et al., 1998). A similar role was proposed for rapeseed and Arabidopsis caleosins during seed germination during which LDs are disassembled to mobilize TAGs (Thompson et al., 1998, Murphy et al., 2000, Naested et al., 2000). A new study has shown that the Arabidopsis four CLO isoforms (CLO1, CLO2, CLO4 and CLO6) were specifically expressed in seeds and knockout plants produced seeds with deformed embryos (Liu et al., 2022).

The involvement of CLOs in the disassembly of LDs was confirmed by two individual insertion mutants of Arabidopsis that were deficient in the substantially LD-associated CLO/PXG isoform, AtCLO1 (Poxleitner et al., 2006). Consequently, although seed germination rates were not seriously affected in these mutants, they were significant delayed in lipid metabolism during germination and seedling development and this was accompanied by a modified pattern in $\alpha$– and $\delta$–tonoplast intrinsic proteins (TIPs), markers for protein storage vacuoles. This strongly suggests that the interaction of LDs with vacuoles is one of the most relevant mechanisms mediating the disassembly of seed LDs (Poxleitner et al., 2006). Additionally, accumulation of both oleosins and CLO/PXGs was detected from early stages of seed development of *Brassica napus* and this was maximal on days 12-17 after pollination, while steroleosins accumulated later (~25 day after pollination) (Jolivet et al., 2011). Lipid oxidation by the CLO/PXG, AtCLO3 in Arabidopsis plants was correlated with reduced ROS levels and delayed floral transition (Blee et al., 2014) and the same isoform appears to play a role in leaf senescence in Arabidopsis (Brocard et al., 2017).

LD-bound CLO/PXGs might be involved in the development of plant reproductive organs, with the Arabidopsis isoform, AtCLO7, being exclusively expressed at the final stage of another development, indicating a potential role in pollen-stigma interactions (Rejon et al., 2016). Also, it was recently reported that AtCLO7 and a CLO-like protein were present in Arabidopsis pollen coat proteome.

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Expression of both *CLO/PXG* genes initially peaked in the tapetum and their proteins were also present in the anther locule. This process was dependent on two tapetum transcription factors, MALE STERILE188 (MS188) and MALE STERILITY 1 (MS1) (Lu et al., 2020). Similarly, it was reported that the number of LDs significantly decreased in olive pollen grains during germination, whereas an inverse scenario was observed in the pollen tube, suggesting that pollen CLO/PXGs might have a role in LD mobilization and in the remodeling of membrane compartments during pollen germination and subsequent tube growth towards the ovary (Zienkiewicz et al., 2010). Furthermore, the CLO/PXG are apparently involved in the architecture of lateral root and elongation. This was demonstrated through the hydroperoxide-reductase activity of RD20 (AtCLO3), where a rd20 null mutant of Arabidopsis possessed extended lateral roots and this was synchronized with exceed level of hydroperoxide FA (Aubert et al., 2010a).

*Roles of CLO/PXG in plant response to abiotic stress*

Several roles for CLO/PXGs in plant responses to abiotic environmental factors have also been found. One of the earliest CLO/PXGs to be reported (although not characterized as such at the time) was the Arabidopsis *RD20* gene product. This was the most rapidly upregulated candidate in a screen for desiccation responsive genes in Arabidopsis (Yamaguchi-Shinozaki et al., 1992). The same gene was also identified in a subsequent screen for cold and salt stress (Yamaguchi-Shinozaki and Shinozaki, 1993). The protein product of this gene is AtCLO3, which was later found to be highly expressed after exposure of Arabidopsis plants to salt and drought (Partridge and Murphy, 2009). A loss-of-function mutant of the related Arabidopsis *CLO/PXG* gene, *AtCLO4*, resulted in plants that were hypersensitive to ABA, salt and mannitol stresses. In contrast, *AtCLO4*-overexpressing plants were hyposensitive to such stresses compared to the control while low levels of water deprivation resulted in an ABA-dependent increase in *AtCLO3* expression in leaves (Kim et al., 2011). This was confirmed in *AtCLO3* knockout Arabidopsis plants that showed a higher transpiration rate correlated with and enhanced stomatal opening and a reduced tolerance to drought as compared with the wild

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type, supporting a vital role for this CLO/PXG isoform in plant response to drought mediating by stomatal control under water deprivation conditions (Aubert et al., 2010b). In addition, *AtCLO3* gene likely assists plant tolerance after exposure to chemicals, heavy metal, and synthetic auxins (Blee et al., 2014).

Further studies on the Arabidopsis *CLO/PXG* gene, *AtCLO3* showed very strong upregulation by ABA and methyl jasmonate (MeJA), and strong upregulation by gibberellin (GA) treatments in both aerial and root tissues after wounding or exposure to cold, osmotic, salt, drought and UV-B (Shen et al., 2014). Also, *AtCLO2* and *AtCLO1* expression were both sensitive to ABA treatments in seeds, while *AtCLO4* was induced in roots as well as in cell culture by the high temperature (Shen et al., 2014). Other data suggested that AtCLO4 acts as a negative regulator of ABA signaling and might have a role in Arabidopsis responses to environmental factors (Kim et al., 2011, Blee et al., 2012). In cereals, *CLO/PXG*s play important roles in plant responses to osmotic stress, with the transcript abundances of *HvCLO3* and *HvCLO5* in barely and *OsCLO5* and *OsCLO11* in rice being greatly attenuated in osmotically stressed seedlings (Liu et al., 2005). The *CLO/PXG* ortholog, *ScCLO9*, was also highly induced in the roots of salt-stressed rye plants, although no induction was detected for such orthologs in the roots of comparably treated triticale plants (Monroy et al., 2007). Microarray analysis of cold acclimation genes in winter and spring wheat revealed a strong induction of the ortholog, *TaCLO3*, in shoots of both types of wheat following cold treatment (Monroy et al., 2007). Although, no changes in *CLO/PXG* expression were detected in wheat plants after exposure to drought (Aprile et al., 2009), some orthologs, notably *TaCLO10* and *TaCLO11*, exhibited increasing levels of gene expression after to ABA treatment (Khalil et al., 2014).

New functions of plant COL/PXGs were recently discovered following the responses of date palm seedlings to dioxin contamination (Hanano et al., 2016b). Two tissue-specific COL/PXGs isoforms were identified, namely PdPXG4 which is a leaf-specific CLO with 13-hydroperoxide reductase activity, and PdPXG2 which is a root-specific with 9-hydroperoxide reductase activity

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(Hanano et al., 2016b). PdPXG2 was highly induced after dioxin treatment and subsequently expressed a characteristic spectrum of dioxin-responsive oxylipin (DROXYL) signature in date palm roots following exposure to dioxin (Hanano et al., 2016b). Interestingly, the inhibition of PdPXG2 activity showed a positive linear relationship between the deposition of suberin in the epidermal cells of the roots and their permeability to environmental dioxins, suggesting a potential involvement of this CLO/PXG in the generalized plant response to dioxin. Consequently, the use of dioxin-responsive oxylipin signature as biomarkers has been suggested for assessing the plant exposure to such environmental contaminants (Pasaribu et al., 2017).

In green algae, the ortholog *CvCLO* from the microalga, *Chlorella vulgaris*, was characterized as a heme-protein with a potential involvement in response to osmotic stress (Charuchinda et al., 2015). This algal protein shared a high similarity with fungal CLO/PXGs, such as the *AfCLO* (*AfPXG*) ortholog found in *Aspergillus flavus*, which was initially characterize as a modulator of aflatoxin B1 secretion as well as of fungal responses to soil organic pollutants (Hanano et al., 2015a, Hanano et al., 2018a, Hanano et al., 2019). Several algal COL/PXGs orthologs are involved in responses to saline and osmotic stresses and it is proposed that these genes were part of the initial movement of some algae from the oceans to freshwater habitats and their subsequent colonization of the land as Embryophyte plants (Rahman et al., 2018b). The other major group of functions of CLO/PXGs in plants is related to LD metabolism and similar functions have been observed in unicellular green algae (Ouyang et al., 2013, Zienkiewicz et al., 2016, Rahman et al., 2018b). These data suggest that plant CLO/PXGs are derived from similar proteins with similar functions that were already present in their algal ancestor

*Roles of CLO/PXG in plant response to biotic stress*

Plant CLO/PXGs play several roles in plant responses to biotic stresses, such as pathogenesis and herbivory. Indeed, a biological role for peroxygenases in plant defense responses was proposed before their molecular characterization as caleosins. Such role are due to their various enzymatic

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activities in the detoxification of xenobiotics, the biosynthesis of antifungal compounds, and in the biosynthesis of cutin monomers (Blee and Schuber, 1992). Regarding the detoxification of organosulfur compounds, plant PXGs oxidizes organosulfur herbicides such as EPTC, and insecticides such as parathion or methiocarb (Blee and Durst, 1987). Concerning antifungal defense, plant PXGs can epoxidize polyunsaturated fatty acids resulting several congeners of epoxy-derivatives, e.g. 9,10-epoxy-12-octadecenoic and 12,13-epoxy-9-octadecenoic acids, that have inhibitory effects against spore germination and germ tube growth of the rice fungal pathogen, *Magnaporthe oryzae* (Kato et al., 1983, Kato et al., 1993, Namai et al., 1993). In addition, PXGs exclusively synthesize 9,10-epoxy-18-hydroxystearate and 9,10,18-trihydroxystearate from oleic acid, both of which are vital structural compounds in plant cuticles, which are the first lines of defense against attack by both pests and pathogens (Blee and Schuber, 1990a).

The discovery that the plant PXG is a caleosin opened up new avenues for research into their involvement in plant defense responses (Hanano et al., 2006). For example the Arabidopsis isoform AtCLO3 (RD20) was highly upregulated following pathogen infection (Partridge and Murphy, 2009). On the other hand, it was reported that the overexpression of AtCLO3 in Arabidopsis plants caused considerable alterations in the composition of their cuticle wax, resulting in greater plant resistance to the fungal pathogen *Alternaria brassicicola*. Silencing of the *AtCLO3* gene enhanced fungal propagation and limited the damage caused by the bacterium *Pseudomonas syringae* pv *tomato* (Hanano et al., 2015c). Finally, the proteome of LDs fractioned from aging Arabidopsis leaves differed from that of seeds and the two most abundant proteins of the leaf LDs were AtCLO3 and the SMALL RUBBER PARTICLE1 (AtSRP1), both of which have proven functions in plant defense (Brocard et al., 2017).

PXGs are now recognized for their roles in one of the pathways involved in the biosynthesis of plant oxylipins. Oxylipins are a large family of oxidized polyunsaturated fatty acids (PUFAs) with proven roles in plant responses to biotic stress. This was demonstrated for four different CLO/PXG isoforms

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(AtCLO1, 2, 3, 4) (Hanano et al., 2006, Partridge and Murphy, 2009, Kim et al., 2011). For example, expression of AtCLO3 was induced by pathogens, suggesting a potential role for this CLO/PXG in biotic stress responses (Shimada et al., 2014). Recombinant AtCLO3 expressed in yeast exhibited a peroxygenase activity through its hydroperoxide reductase activity, catalyzing the formation of 13-hydroxy-9,11,15-octadecatrienoic acid, a linolenate-derived hydroxide. These FA hydroxides enhance plant tolerance to oxidative stress by activation of ROS-neutralizing enzymes, thus reducing cell death (Blee et al., 2014). In line with this, transcriptomic analysis of AtCLO3-overexpressed Arabidopsis plants revealed that AtCLO3 was co-expressed with genes of very long chain fatty acid (VLCFA) biosynthesis, with VLCFAs being key components of cuticular waxes. Interestingly, these plants exhibited an elevated proportion of VLCFAs in their seeds with higher amounts of VLCFA-derived oxylipins in leaf cuticular waxes. Leaves from these plants were also better able to resist infection by the important fungal pathogen *Alternaria brassicicola* (Hanano et al., 2015c).

The Arabidopsis CLO/PXG isoform, AtCLO3 can work together with α-DOX1, a leaf LD protein with α-dioxygenase activity, to catalyze the formation of 2-hydroxyoctadecanoic acid (2-HOT), which shows antifungal activity against *Colletotrichum higginsianum* and mainly accumulates in the zone surrounding the infection site. The functional connection between AtCLO3 and α-DOX1 was confirmed by proteomic analysis of LDs in senescent leaves, suggesting that AtCLO3 and α-DOX1 participate in lipid modification reactions and also, via their LD carriers, in the transport of defense components involved plant responses to pathogens (Shimada et al., 2018, Shimada and Hara-Nishimura, 2015, Fernandez-Santos et al., 2020). Interestingly, some of the fungal pathogens of plants also employ their own CLO/PXGs in roles that include development, reproduction, and interactions with plant hosts. This is the case of the *Aspergillus flavus* CLO/PXG isoform, AfPXG, which mediates fungal development and aflatoxin production via its association to fungal LDs and its peroxygenase activity (Hanano et al., 2018a).

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*Roles of CLO/PXG in plant cell signaling*

The involvement of CLO/PXGs in plant signaling pathways was suggested earlier based on their calcium binding and phosphorylation activities on one side, and on the biological roles of oxylipins produced by COL/PXG pathway, on the other. Concerning the influence of $Ca^{2+}$ binding/unbinding status on CLO/PXG enzymatic activity, it was noted that the removing of $Ca^{2+}$ from CLO/PXGs protein caused a drastic but reversible reduction in its catalytic activity, possibly due to a conformational changes in protein structure (Hanano et al., 2006). It has been suggested that CLO/PXG are involved in abscisic acid (ABA)-mediated responses in rice plants due to its induction following ABA treatment (Frandsen et al., 1996). This could be due to calcium binding and protein phosphorylation, both of which are known to be involved in ABA signaling pathways (Leung et al., 1994). Furthermore, the analysis of ABA-related mutants in Arabidopsis indicated a role of AtCLO3 in the formation of certain oxylipins that act as modulators of stress-related signaling pathways involving both ABA and salicylic acid (Partridge and Murphy, 2009).

In another study, exogenous ABA-treatment of an Arabidopsis AtCLO4 deletion mutant upregulated the expression of key regulatory genes related to ABA pathway, notably ABF3 and ABF4, and inversely, downregulated in AtCLO4-overexpressing lines (Kim et al., 2011). This suggests that AtCLO4 caleosin acts as a negative regulator of ABA-mediated responses in Arabidopsis (Kim et al., 2011). Similarly, overexpression of AtCLO3 (RD20) in Arabidopsis plants led to high levels of reactive oxygen species (ROS), and accelerated flowering in a gibberellin-dependent manner that was related to hypersensitivity to abscisic acid during seed germination (Blee et al., 2014). Interestingly, the latter phenotype, which was characterized by the accumulation of 13-hydroxy-9,11,15-octadecatrienoic acid, a linolenate-derived hydroxide, was strictly dependent on the catalytic activity of the AtCLO3 (RD20) isoform, since it is abolished in the rd20 null mutant and in lines overexpressing RD20, in which PXG activity was inactivated by a single point mutation of a catalytic histidine residue (Blee et al., 2014). More recently, Brunetti et al showed, using yeast two-hybrid

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assays, that the N-terminal regions of AtCLO3 and AtCLO7 interact with GPA1. They also showed that the full length AtCLO3 and AtCLO7 interacted with GPA1 by BiFC in plant cells, *Nicotiana benthamiana* leaf epidermal cells. In the BiFC assays the half YFP fusion protein was fused to the C-terminal end of the caleosins which indicates that the C terminal half of the caleosin is also cytosolic, since the reconstitution of the YFP in the BiFC assay would have to be on the cytosolic side of membrane to be able to interact with the other half of the YFP which was fused to GPA on its C-terminus (Brunetti et al., 2022, Brunetti et al., 2021).

## BIOTECHNOLOGICAL PROSPECTS

Plant LDs and their associated proteins, such as oleosins and CLO/PXGs, are increasingly becoming targets for various biotechnological applications. One of the reasons for this is the exceptional stability of LDs as micro- or nano-level structures both *in vivo* and *in vitro*. For example, in their native environment within a dry seed, the LDs of some species can remain intact and fully capable of regaining biological functions even after prolonged periods of storage under ambient conditions, providing moisture levels remain low. In one remarkable case, a ca. 2000-year old date palm seed, with well-preserved protein and lipid contents, was found to be fully viable (Sallon et al., 2008). There are also numerous reports of seeds that have retained viability for hundreds of years (Porteous et al., 2019). In addition to their exceptional stability, seed LDs are also a highly scalable platform for the production of large quantities of desirable biological materials such as therapeutic or other high value proteins (Murphy, 2007).

A second interesting feature of seeds is that the lipidic core of their LDs can be adapted to serve as vehicles for carrying lipophilic nutraceutical or pharmaceutical molecules. Moreover, LD-associated proteins such as oleosins and CLO/PXGs can be engineered to be carriers of recombinant proteins, such as the blood-clotting factor, hirudin, and the human hormone, insulin (Ling, 2007).

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These recombinant proteins can be expressed on a large scale in seeds as LD-bound fusion proteins with either oleosins or CLO/PXGs and the seeds can be stored almost indefinitely until required. The LDs can then be readily purified from the seeds via floatation and the recombinant proteins cleaved via a pre-engineered proteolytic site to generate the desired end product. So far, in addition to protein carriers, LDs have been used as carriers for antioxidants, steroid hormones, or immunosuppressant drug cyclosporine A, or incorporated into cosmetic formulations (Purkrtova et al., 2008a).

Oleosins were the first class of LD proteins used for such biotechnological applications. For example, plant overexpressed oleosins fused with the insulin peptide (Nykiforuk et al., 2006), β-glucuronidase (Kühnel et al., 1996), xylanase (Liu et al., 1997), or proteins such as hirudin (Parmenter et al., 1995) and cystatin (Peng et al., 2004b) have been successfully expressed and purified and in some cases the products have been tested in clinical trials (Peng et al., 2004a). The potential of CLO/PXGs has been explored for the production of stable Artificial Oil Bodies (AOBs) made from triacylglycerol, phospholipid, and sesame CLO/PXGs (Chen et al., 2004). Interestingly, the AOBs constituted with caleosin were significantly smaller than those formed with oleosins and were smaller than those of the native sesame LDs, suggesting that they might be more suitable to act as carriers for delivery for therapeutically useful amounts of lipid-soluble molecules including drugs. It was also demonstrated that the recombinant version of the sesame CLO/PXG, SiCLO (accession no. AF109921), when expressed in *E. coli* effectively encapsulated AOBs that had physicochemical properties comparable to those encapsulated with native CLO/PXGs. The high stability of AOBs was possibly ensured by recombinant CLO/PXGs that were anchored onto the AOBs via their hydrophobic domain, while their protruding C- and N- terminal regions on the AOB surface conferring anti-aggregation and thermostable properties (Chen et al., 2004).

In terms of pharmaceutical applications of AOBs, the bioavailability of Cyclosporine A was considerably enhanced when encapsulated in CLO/PXG-stabilized AOBs, which also suggests their

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use to encapsulate other hydrophobic drugs for oral administration (Chen et al., 2005). In another study, CLO/PXGs were fused with anti-HER2/neu affibody (ZH2) and then used to assemble AOBs with the aim of the AOBs being selectively internalized by HER2/neu-positive tumor cells involved in breast cancer. The efficiency of this approach was tested in delivering Camptothecin (CPT), a hydrophobic antitumor drug (Chiang et al., 2012, Chiang et al., 2011). After administration of the CPT formulation, CLO/PXG/ZH2-assembled AOBs had a high antitumor activity against HER2/neu-positive cells both *in vitro* and *in vivo* (Chiang et al., 2012, Chiang et al., 2011). In line with this, it was recently demonstrated that cellular LDs and their associated CLO/PXG play important roles in the trafficking and secretion of paclitaxel from *Taxus media* cell suspensions (Hanano et al., 2022).

In a food industry application, artificial LDs were reconstituted by mixing sesame oil and a caleosin fused with histatin 3, a human salivary polypeptide. The artificial LDs were then used to evaluate the astringency of oolong tea infusions. The aggregation of these artificial LDs was induced by mixing them with either a complete oolong tea infusion or its major polyphenolic compound, (-)-epigallocatechin gallate (EGCG). As a result, the aggregated LDs gradually floated and formed a creamy layer whose thickness was proportional to the concentrations of tea infusion. This assay system was applied to test different oolong tea infusions with sensory astringency corresponding to their EGCG contents (Shih et al., 2017).

The *in situ* mechanical properties of both native LDs and AOBs have also been studied by atomic force microscopy in order to investigate the factors behind their unique stability (Yang et al., 2020). In summary, there are numerous potential avenues for the biotechnological use of plant-derived CLO/PXGs, especially in conjunction with either native or artificial LDs.

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## CONCLUSIONS AND PERSPECTIVES

It is only about two decades since caleosin/peroxygenases (CLO/PXGs) were first recognized as a new class of lipid-associated proteins, and only 15 years since their additional enzymatic functions as a novel class of peroxygenases was discovered. During this time CLO/PXGs have been found to be derived from ancient proteins that might have been present in the last eukaryotic common ancestor (LECA) that dates to well over two billion years ago (Rahman et al, 2018b). CLO/PXGs are present in many important groups of unicellular green algae where their emerging functions in LD binding and trafficking and in abiotic stress responses were inherited by the multicellular descendants of those algal groups that went on to colonize the land surface about 500 million years ago. Indeed, CLO/PXGs still play important roles in many contemporary green algal species (de Vries and Ischebeck, 2020, Rahman et al., 2018b, Wang et al., 2019). In the land plants, the CLO/PXG gene family subsequently expanded in terms of the number of different isoforms and in their physiological functions. In particular, they acquired new roles in many biotic stress responses to threats from pathogens and herbivores.

Some plant CLO/PXGs are predominantly bilayer membrane-associated while others appear to be mainly or exclusively LD-associated in which case they are attached to a phospholipid monolayer membrane while a third group can bind to both monolayer and bilayer membranes. The membrane bilayer-associated CLO/PXGs are mainly found in vegetative tissues and might be ubiquitous cellular components. Here, they might be present at low levels for much of the time, but also capable of rapid induction to high levels in response to internal (e.g. hormonal) or external (e.g. biotic or abiotic factors) stimuli. In contrast, the LD-associated CLO/PXGs are largely, but not exclusively, restricted to lipid-storing seeds and pollen grains where they have important functions related to lipid trafficking and reproduction. More recently, the possible roles of CLO/PXGs in plant responses to environmental toxins, such as dioxins, have been explored, as well as their increasingly diverse roles in oxylipin metabolism and other signaling functions. Finally, there are some intriguing

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developments in the biotechnological uses of CLO/PXGs in areas ranging from cancer therapy to the mass production of recombinant pharmaceuticals.

In conclusion, there are many interesting research questions that remain to be addressed in future studies of plant CLO/PXGs. These include elucidating the full range of their physiological roles in plants, e.g. by using genome editing and gene knockout approaches, more detailed characterization of their unusual reaction mechanisms using biophysical methods, and their potential biotechnological uses in areas including human therapeutics.

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AH designed the main body of the manuscript and wrote the first draft. EB contributed in editing the manuscript. DM was a major contributor in editing, expanding and rewriting the manuscript. All authors read and approved the final manuscript. The authors declare that they have no competing interests. The datasets are included in the manuscript.

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## Figures legends

**FIG. 1. Presence of *CLO/PXG* sequences across the Viridiplantae and their estimated evolutionary divergence times.**

The major taxa that contain *CLO/PXG* sequences are shown as green-shaded boxes. Individual species with one or more *CLO/PXG* sequences are shown in blue while other species where *CLO/PXG* sequences are definitely absent from their genomes are shown in red. The estimated evolutionary divergence times of selected key taxa are shown as the number of million years ago (My). Starred major taxa are those with good evidence of monophyletic status while non- starred taxa are probably polyphyletic or paraphyletic. Data from (Rahman et al., 2018b).

**FIG. 2. Phylogenetic analysis of 67 CLO/PXG sequences from 34 species across the Viridiplantae.**

The sequences separate into four clearly distinct clusters that are labelled as follows: **A**. Chlorophyte and Streptophyte green algae; **B**. non-angiosperm Embryophytes; **C**. H-isoform angiosperms; and **D**. L-isoform angiosperms. Data from (Rahman et al., 2018b). For CLO/PXG genes names please refer to Table 1.

**FIG. 3. Speculative models of possible lipid associations of plant CLO/PXGs.**

**A**. Possible association of CLO/PXG to bilayer membranes via its transmembrane domain, which includes a proline-knot motif. In the case of a plasmalemma-bound CLO/PXG, the C-terminal domain of CLO/PXG with its kinase phosphorylation sites and disulfide bonds is exposed outside the cell, suggesting possible roles in stress-induced signaling. In contrast, the N-terminal domain is inside the cell with its $Ca^{+2}$-binding domain (residue 75-86) and the essential His70 residue for PXG activity. However, the two essential His residues His70 and His138 are found on opposite sides of the TM domain as predicted in all the algorithms used, raising questions about the structural elements and crucial residues of the active site of PXG involved in the mechanistic features of this atypical hemoprotein. **B**. Association of CLO/PXG to the monolayer membrane of an LD. In this model, the lipid domain folds back out of the LD, so the both N- and C- terminal of CLO/PXG are found outside

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the LD with the two essential His residues for PXG activity are found in the same side. Although these are speculative models**,** they have provided useful structural predictions that can be tested in the lab.

**FIG. 4. Putative catalytic mechanism of a plant CLO/PXG**.

Hydroperoxide fatty acids, e.g. 13-HPOT, are generated from linolenic acid by 13-lipoxygenases. In the first step, the iron atom attacks the O-O bound of the hydroperoxide group and fixes one oxygen atom by heterolytic cleavage, leading to the formation of a reversible oxo-heme complex with an Iron(II) Oxide (Fe-O) polar bond in the active site of the peroxygenase (PXG). This results in the reduction of the fatty acid hydroperoxide (13-HPOT) to its corresponding hydroxide (13-HOT). This reaction constitutes the fatty acid hydroperoxide-reductase activity of PXG. In the second step, the PXG transfers an oxygen atom via two competing pathways A and B. In A pathway, the PXG possibly transfers the O- to a C=C double bond in the same unsaturated fatty acid hydroxide (13-HOT) molecule, resulting from the first step, by a mechanism known as intramolecular transfer, leading to the formation of its corresponding hydroxyl-epoxy fatty acid, e.g. 13-hydroxy-15,16-epoxy OD. In pathway B, the PXG transfers the O- to another oxidizable substrate by an intramolecular transfer, thereby catalyzing the co-oxidation reactions, e.g. the sulfoxidation of sulfide-containing molecules and the epoxidation of unsaturated fatty acids. These activities generate a characteristic signature of mono-, di-, and poly-epoxy fatty acids. This scheme is adapted from (Blee et al., 1993).

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**Table 1.** Essential information on plant caleosins/peroxygenases identified to date.

| Lineage | Plant species | Gene Name | Gene NCBI Ac. N. | Tissue /role | Reference |
|---|---|---|---|---|---|
| **Bryophytes** | *Physcomitrella patens* | *PpClo1* | *Pp1s56_196V6* | possibly in biotic and abiotic response | (Umate, 2012) |
| | | *PpClo2* | *Pp1s77_124V6* | | |
| | | *PpClo3* | *Pp1s210_68V6* | | |
| | *Marchantia polymorpha* | *MpClo1* | *KZ772698* | LD localization, membrane trafficking | (Huang et al., 2019) |
| | *Selaginella moellendorffii* | *SmClo1* | *Sm167445* | LD localization | (Umate, 2012) |
| **Gymnosperms** | *Cycas revoluta* | *CreClo1* | *FJ455154* | pollen elongate the tube and increasing of triacylglycerol | (Pasaribu et al., 2020, Jiang et al., 2009) |
| | *Pinus massoniana* | *PmClo1* | *AIC74541* | pine LDs | (Pasaribu et al., 2014, Hu et al., 2014b) |
| | *Picea sitchensis* | *PsClo1* | *ABK26654* | lipid accumulation | (Lin et al., 2012) |
| | | *PsCl* | *ABK26* | | |

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|  |  | *o2* | *902* | in tubers from early to late developmental stages |  |
|---|---|---|---|---|---|
|  |  | *PsClo3* | *ABK26466* |  |  |
|  |  | *PsClo4* | *ABK21149* |  |  |
|  |  | *PsClo5* | *ABK24129* |  |  |
| **Angiosperms** | *Amborella trichopoda* | *AtrClo1* | *XM_0068493 13* | desiccation tolerance | (Villegente et al., 2017) |
|  |  | *AtrClo4* | *XM_0068268 36* |  |  |
| **Monocots** | *Brachypodium distachyon* | *BdClo1* | *Bradi1g44200* | LD localization and vegetative tissues, diverse responses to abiotic stresses, | (Khalil et al., 2014, Cao et al., 2016) |
|  |  | *BdClo2* | *Bradi1g44207* |  |  |
|  |  | *BdClo3* | *Bradi1g69571* |  |  |
|  |  | *BdClo4* | *Bradi1g70390* |  |  |
|  |  | *BdClo5* | *Bradi1g70400* |  |  |
|  |  | *BdClo6* | *Bradi3g56810* |  |  |
|  |  | *BdClo7* | *Bradi3g56820* |  |  |
|  |  | *BdClo8* | *Bradi5g15410* |  |  |
|  |  | *BdCl* | *Bradi5g1542* |  |  |

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| | | *o9* | *7* | | |
|---|---|---|---|---|---|
| | | *TaClo1* | *KJ523887* | root/steam leave/anther/pollen /biotic stress/early development | |
| | | *TaClo2* | *BK009956* | | |
| | | *TaClo3* | *BK009957* | | |
| | | *TaClo4* | *KJ523888* | | |
| | | *TaClo5* | *KJ523890* | | |
| | *Triticum aestivum* | *TaClo6* | *BK009963* | | |
| | | *TaClo7* | *KJ523891* | | |
| | | *TaClo8* | *BK009968* | | |
| | | *TaClo9* | *KJ523892* | | |
| | | *TaClo10* | *BK009971* | | |
| | | *TaClo11* | *BK009973* | | (Khalil et al., 2011, Khalil et al., 2014, Feng et al., 2011) |
| | | *HvClo1* | *KAE8800172* | root, steam leave, anther, pollen /biotic stress, early development | |
| | | *HvClo2* | *KAE8782993* | | |
| | *Hordeum vulgare* | *HvClo3* | *KAE8773248* | | |
| | | *HvClo4* | *KAE8775738* | | (Khalil et al., 2014, Sega et al., 2021) |
| | *Secale cereale* | *ScClo1* | *KJ523889* | seedling shoot, ovary, | (Khalil et al., 2015) |

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| | | | | | |
|---|---|---|---|---|---|
| | | | | stigma, pollen | |
| | *Brachypodium distachyon* | *BdClo1* | *XM_0035813 69* | drought and salt stresses | (Zhang et al., 2017, Song et al., 2014) |
| | | *BdClo2* | *XM_0035813 71* | | |
| | | *BdClo3* | *XM_0035584 21* | | |
| | | *BdClo4* | *XM_0035639 47* | | |
| | | *BdClo5* | *XM_0244624 96* | | |
| | *Oryza sativa* | *OsClo1* | *Os02t0734400* | LD localization, embryo-specific, pathogens resistance, fungicide-induced. | (Chen et al., 2012, Wang et al., 2020, Liu and Zhu, 2020) |
| | | *OsClo2* | *Os02t0734500* | | |
| | | *OsClo3* | *Os03t0222600* | | |
| | | *OsClo4* | *Os04t0510900* | | |
| | | *OsClo5* | *Os04t0511200* | | |
| | | *OsClo6* | *Os06t0254300* | | |
| | | *OsClo7* | *Os06t0254600* | | |

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| | | | | | |
|---|---|---|---|---|---|
| | | *OsClo8* | *Os06t0254700* | | |
| | *Setaria italica* | *SiClo1* | *Si007077m* | seed LD localization, vegetative tissues, responses to biotic and abiotic stress | (Horn et al., 2013, Song et al., 2014, Benaragama et al., 2017) |
| | | *SiClo2* | *Si007079m* | | |
| | | *SiClo3* | *Si007300m* | | |
| | | *SiClo4* | *Si010688m* | | |
| | | *SiClo5* | *Si017829m* | | |
| | | *SiClo6* | *Si019785m* | | |
| | | *SiClo7* | *Si036584m* | | |
| | *Zea mays* | *ZmClo1* | *GRMZM2G040121* | LD localization, embryo-specific, seed germination, responses to biotic and abiotic stress in particular drought tolerant. | (Schlueter et al., 2004, Qu et al., 1986, Chen et al., 1999, Mishra et al., 2020, Gonzalez-Morales et al., 2021) |
| | | *ZmClo2* | *GRMZM2G062717* | | |
| | | *ZmClo3* | *GRMZM2G063220* | | |
| | | *ZmClo4* | *GRMZM2G063693* | | |
| | | *ZmClo5* | *GRMZM2G075456* | | |
| | | *ZmClo6* | *GRMZM2G090505* | | |
| | | *ZmClo7* | *GRMZM2G090609* | | |

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|---|---|---|---|---|---|
| | | *ZmClo8* | *GRMZM2G139797* | | |
| | | *ZmClo9* | *GRMZM2G164308* | | |
| | | *ZmClo10* | *GRMZM2G342685* | | |
| | *Sorghum bicolor* | *SbClo1* | *Sb01g042490* | LD localization, structural stabilization, increase lipid content in leaf | (Song et al., 2014, Schlueter et al., 2004, Gidda et al., 2016) |
| | | *SbClo2* | *Sb01g042500* | | |
| | | *SbClo3* | *Sb04g028730* | | |
| | | *SbClo4* | *Sb04g028740* | | |
| | | *SbClo5* | *Sb06g022280* | | |
| | | *SbClo6* | *Sb10g009140* | | |
| | | *SbClo7* | *Sb10g009150* | | |
| | | *SbClo8* | *Sb10g009160* | | |
| | | *SbClo9* | *Sb10g009170* | | |
| | *Avena sativa* | *ASClo1* | *JN390966* | seed LD localization, epoxy-hydroxy oxylipins biosynthesis, respon | (Benaragama et al., 2017, Hanano et al., 2006, Meesapyodsuk and Qiu, 2011) |

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| | | | | | |
|---|---|---|---|---|---|
| | | | | se to abiotic stress | |
| | *Phoenix dactylifera* | *PdClo1* | *XP_008803896* | seed LD localization, tissues - specific isoforms in leaf and root, induced by dioxins | (Hanano et al., 2016b, Hanano et al., 2018c, Hanano et al., 2016a) |
| | | *PdClo2* | *XP_008775946* | | |
| | | *PdClo3* | *XP_008801250* | | |
| | | *PdClo4* | *XP_008775947* | | |
| | | *PdClo5* | *XP_008796441* | | |
| | *Elaeis guineensis* | *EgClo1* | *XP_010920301* | | |
| | | *EgClo2* | *XP_010932570* | identified by multiple alignments of CLOs sequences | (Rahman et al., 2018b, Xiao et al., 2019) |
| | | *EgClo3* | *XP_010909571* | | |
| | | *EgClo4* | *XP_010917508* | | |
| | | *EgClo5* | *XP_010917507* | | |
| | | *EgClo6* | *XP_010917509* | | |
| | *Musa acuminata* | *MaClo1* | *XP_009412834* | identified by bioinfo | (Rahman et al., 2018b) |

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| | | MaClo1 | XP_0094191 19 | rmatics analyses | |
|---|---|---|---|---|---|
| | *Ananas comosus* | *AcClo1* | *XM_0202229752* | identified by bioinformatics analyses | (Rahman et al., 2018b) |
| | | *AcClo2* | *XM_0202228395* | | |
| | | *AcClo3* | *XM_0202249522* | | |
| | | *AcClo4* | *XM_0202234319* | | |
| | *Zostera marina* | *ZmClo1* | *LFYR01000773* | identified by bioinformatics analyses | (Rahman et al., 2018b) |
| | | *ZmClo2* | *LFYR01000981* | | |
| **Eudicots** | *Arabidopsis thaliana* | *AtClo1* | *At4g26740* | embryo-specific, tissue-differential expression, oxylipins biosynthesis, response to abiotic and abiotic stress and environment | (Blee et al., 2014, Blee et al., 2012, Fernandez-Santos et al., 2020, Frandsen et al., 1996, Hanano et al., 2015b, Hanano et al., 2018b, Hanano et al., 2006, Partridge and Murphy, 2009) |
| | | *AtClo2* | *At5g55240* | | |
| | | *AtClo3* | *At2g33380* | | |
| | | *AtClo4* | *At1g70670* | | |
| | | *AtClo5* | *At1g70680* | | |
| | | *AtClo6* | *At5g29560* | | |
| | | *AtClo7* | *At1g23250* | | |
| | | *AtClo8* | *At1g23240* | | |

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| | | | | | |
|---|---|---|---|---|---|
| | | | | al toxins | |
| | *Citrus x sinensis* | *CxsClo1* | *orange1.1g032767m* | identified by bioinformatics analyses. | (Rahman et al., 2018b) |
| | | *CxsClo2* | *orange1.1g042070m* | | |
| | | *CxsClo3* | *orange1.1g043673m* | | |
| | *Cucumis sativus* | *CsClo1* | *Cucsa.029890* | Leaf-specific LDs, antifungal oxylipins, phytoalexins. | (Shimada and Hara-Nishimura, 2015) |
| | | *CsClo2* | *Cucsa.178330* | | |
| | *Brassica rapa* | *BrClo1* | *Bra002921* | LD degradation storage lipid during seed germination, seed development, responses to biotic and abiotic stress | (HU et al., 2013, Rozwadowski et al., 1999a, Poxleitner et al., 2006, Shen et al., 2016) |
| | | *BrClo2* | *Bra003948* | | |
| | | *BrClo3* | *Bra005501* | | |
| | | *BrClo4* | *Bra007934* | | |
| | | *BrClo5* | *Bra012369* | | |
| | | *BrClo6* | *Bra016195* | | |
| | | *BrClo7* | *Bra020623* | | |
| | | *BrClo8* | *Bra021847* | | |
| | | *BrClo9* | *Bra022936* | | |
| | | *BrCl* | *Bra026* | | |

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|---|---|---|---|---|---|
| | | *o10* | *407* | | |
| | *Glycine max* | *Gm Clo1* | *Glyma 03g41 030* | tissue-differential expression, oil seed content, response to abiotic stress | (Zhang et al., 2018, Fisk and Gray, 2011) |
| | | *Gm Clo2* | *Glyma 09g22 310* | | |
| | | *Gm Clo3* | *Glyma 09g22 330* | | |
| | | *Gm Clo4* | *Glyma 09g22 580* | | |
| | | *Gm Clo5* | *Glyma 10g33 350* | | |
| | | *Gm Clo6* | *Glyma 19g43 680* | | |
| | | *Gm Clo7* | *Glyma 20g34 300* | | |
| | *Populus trichocarpa* | *PtClo1* | *POPTR_0008S 13330* | tissue-differential expression, response to drought | (Umate, 2012, Song et al., 2014) |
| | | *PtClo2* | *POPTR_0010S 07710* | | |
| | | *PtClo3* | *POPTR_0010S 11820* | | |
| | *Ricinus communis* | *RcClo1* | *NW_0 02994 594* | LDs localization, oil seed content, | (Hyun et al., 2013, Nogueira et al., 2013) |
| | | *RcClo4* | *NW_0 02994 482* | | |
| | *Jatropha curcas* | *JcClo1* | *XM_01 22145* | seed-specifi | (Liu et al., 2015, Yang et al., 2009, Liu et al., 2013) |

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|---|---|---|---|---|---|
| | | | *14* | c LDs, carbon flux and lipid accumulation | |
| | | *JcClo2* | *XM_0206785 61* | | |
| | | *JcClo4* | *XM_0122169 01* | | |
| | *Solanum tuberosum* | *StClo1* | *XM_0063426 21* | storage oil hydrolysis, germination, seedling growth, drought tolerance | (Quettier and Eastmond, 2009, Schafleitner et al., 2007) |
| | | *StClo2* | *XM_0063544 72* | | |
| | | *StClo5* | *XM_0063675 46* | | |
| | *Morus notabilis* | *MnClo1* | *XM_0241713 06* | identified by bioinformatics analyses | (Kong and Yang, 2017) |
| | | *MnClo2* | *XM_0100909 09* | | |
| | | *MnClo3* | *XM_0100909 07* | | |
| | | *MnClo4* | *XM_0241696 26* | | |
| | *Nicotiana tabacum* | *NtClo1* | *XM_0165813 89* | seed LDs-specific, pollen tube, leaves LDs, drought | (De Domenico et al., 2011, Muller et al., 2017) |
| | | *NtClo5* | *XM_0166131 95* | | |
| | | *NtClo5-2* | *XM_0166334* | | |

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| | | | *80* | tolerance | |
|---|---|---|---|---|---|
| | *Pyrus x bretschneideri* | *PxbClo1* | *XM_0093455 03* | identified by bioinformatics analyses | (Li et al., 2019) |
| | | *PxbClo2* | *XM_0093359 35* | | |
| | | *PxbClo4* | *XM_0093532 57* | | |

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Figure 1

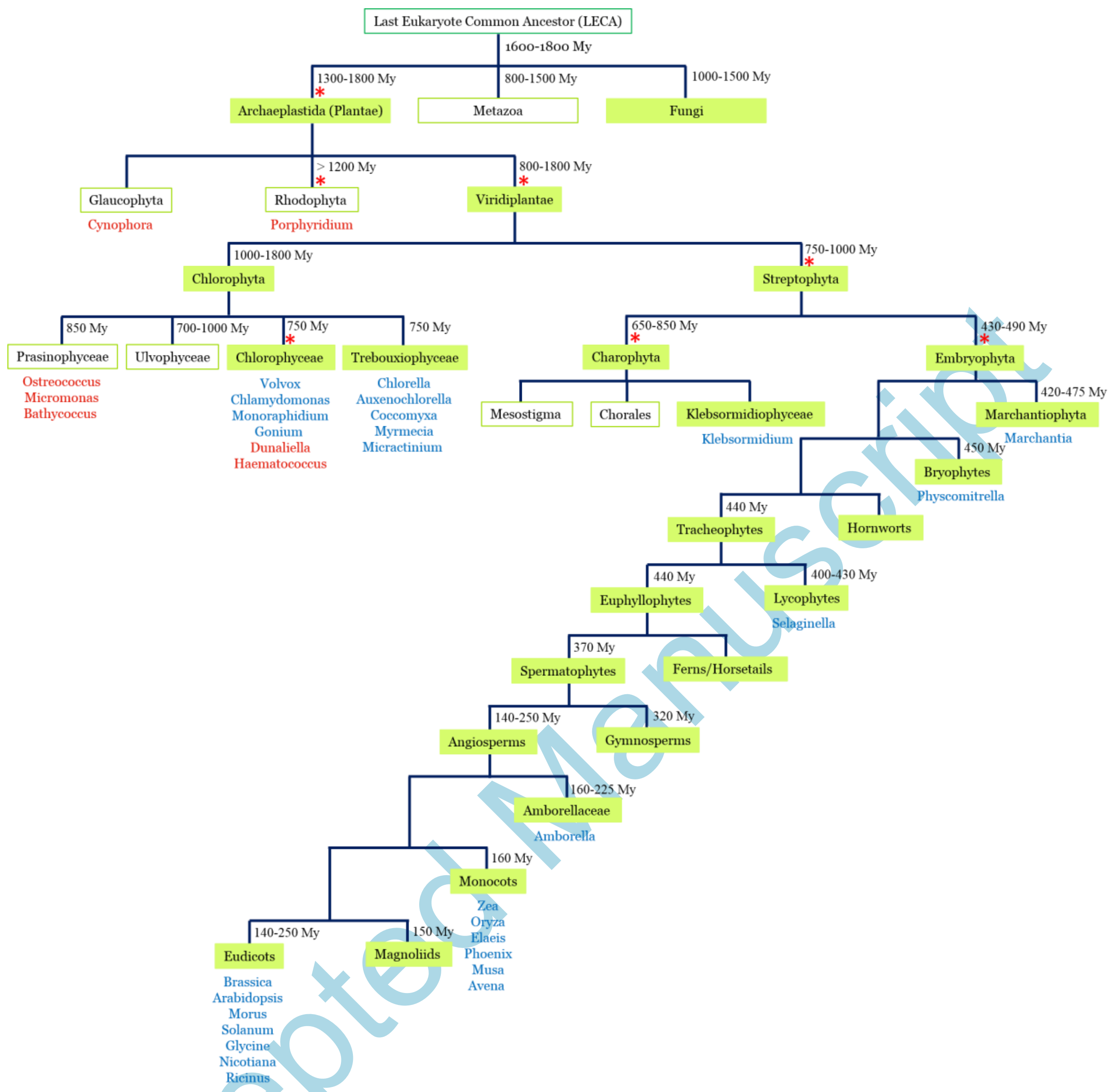


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Figure 2

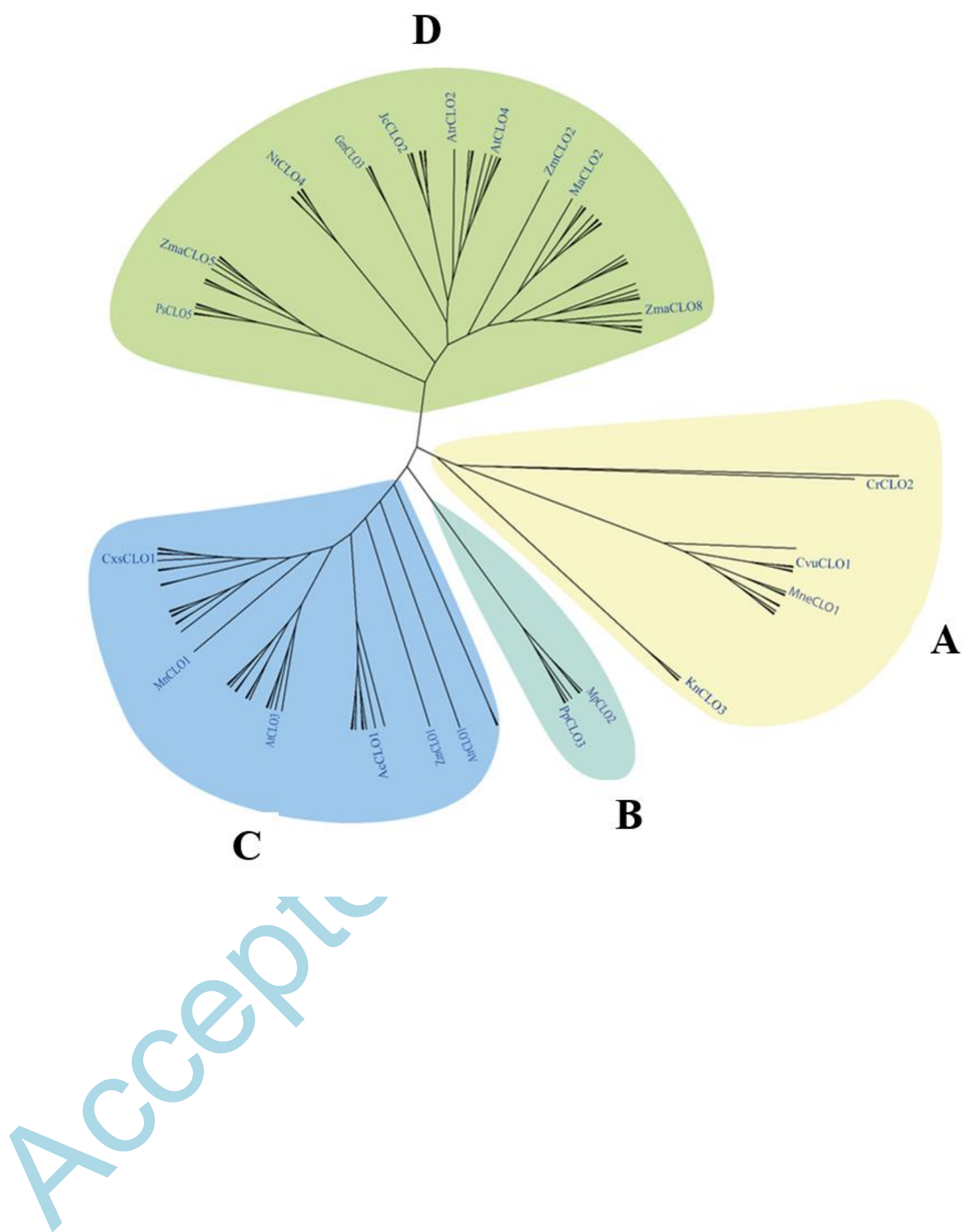


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Figure 3

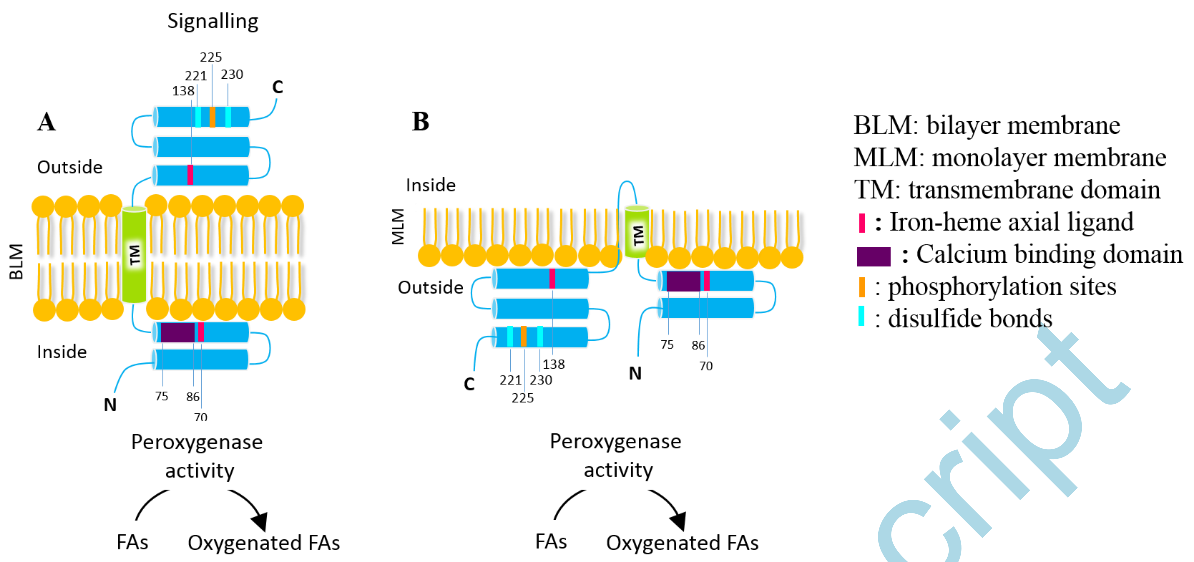


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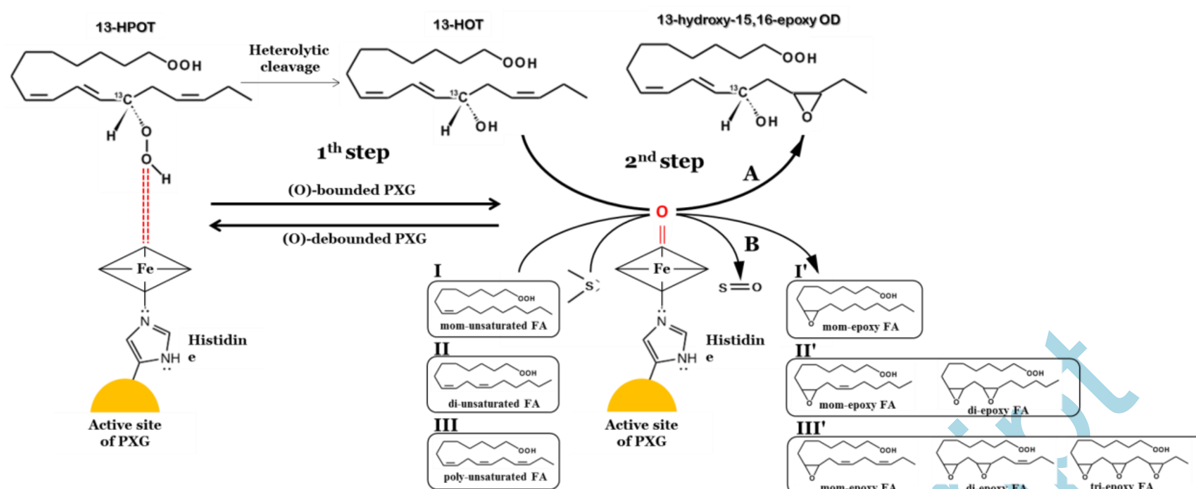
Figure 4

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