Journal of Experimental Botany, Vol. 66, No. 19 pp. 5663–5681, 2015
doi:10.1093/jxb/erv313 Advance Access publication 2 July 2015

REVIEW PAPER

Non-specific lipid transfer proteins in plants: presenting new advances and an integrated functional analysis

Fang Liu[1], Xiaobo Zhang[2], Changming Lu[1], Xinhua Zeng[1], Yunjing Li[1], Donghui Fu[3] and Gang Wu[1,*]

[1] Key Laboratory of Oil Crop Biology of the Ministry of Agriculture, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, China
[2] Life Science and Technology Center, China National Seed Group Co. Ltd., Wuhan 430206, China
[3] The Key Laboratory of Crop Physiology, Ecology and Genetic Breeding, Ministry of Education, Agronomy College, Jiangxi Agricultural University, Nanchang, China

* To whom correspondence should be addressed. E-mail: wugang@caas.cn

Received 1 April 2015; Revised 26 May 2015; Accepted 28 May 2015

Editor: Christine Raines

Abstract

Plant non-specific lipid-transfer proteins (nsLTPs) are small, basic proteins present in abundance in higher plants. They are involved in key processes of plant cytology, such as the stablization of membranes, cell wall organization, and signal transduction. nsLTPs are also known to play important roles in resistance to biotic and abiotic stress, and in plant growth and development, such as sexual reproduction, seed development and germination. The structures of plant nsLTPs contain an eight-cysteine residue conserved motif, linked by four disulfide bonds, and an internal hydrophobic cavity, which comprises the lipid-binding site. This structure endows stability and increases the ability to bind and/or carry hydrophobic molecules. There is growing interest in nsLTPs, due to their critical roles, resulting in the need for a comprehensive review of their form and function. Relevant topics include: nsLTP structure and biochemical features, their classification, identification, and characterization across species, sub-cellular localization, lipid binding and transfer ability, expression profiling, functionality, and evolution. We present advances, as well as limitations and trends, relating to the different topics of the *nsLTP* gene family. This review collates a large body of research pertaining to the role of nsLTPs across the plant kingdom, which has been integrated as an in depth functional analysis of this group of proteins as a whole, and their activities across multiple biochemical pathways, based on a large number of reports. This review will enhance our understanding of nsLTP activity *in planta*, prompting further work and insights into the roles of this multifaceted protein family in plants.

Key words: Classification, evolution, gene expression, nsLTP, protein function, sub-cellular localization.

Introduction

Lipids play a vital role in maintaining cell function and mediating responses to stress during plant growth and development. They build and maintain energy stores and membranes for the compartmentalization of metabolic pathway machinery, and construct the surface cuticle layer, protecting plants from desiccation under water stress. Membrane lipids also mediate cell signalling associated with responses to the environment. Plant non-specific lipid transfer proteins (nsLTPs) have the ability to bind or transfer various types of hydrophobic molecules *in vitro*, such as fatty acids, fatty acyl-CoA, phospholipids, glycolipids and cutin monomers (Carvalho and Gomes, 2007). Information pertaining to their

structures, classification, sub-cellular localization, expression patterns and evolution help us to understand the function of these proteins at different stages of development, as well as the nature of the mechanisms they are involved in during stress responses. An understanding of the regulatory features controlling the specific expression and activity of nsLTPs support further work involving the manipulation of nsLTP expression, through transgenic technologies and molecular breeding, for the enhancement of crop quality and resistance to stress.

Plant *nsLTP*s belong to a multigene family, and have been isolated from numerous plant species. Members of the *nsLTP* gene family display variable expression patterns at different stages of development, in different tissues, and under varying levels of physiological stress. The proteins fulfil different roles across protein structures, nsLTP family types and plant species. nsLTPs were first characterized nearly 40 years ago, however, their specific biological function and the relationship between structure and lipid transfer mechanism is still not clear. Existing functional reports on this gene family are highly varied and disjointed. To provide an integrated review here, a wide body of nsLTP-related topics are covered, demonstrating the progress made towards further characterization of the nsLTP family in recent years, as well as exploring the limitations and trends of nsLTP research across different disciplines. New perspectives are highlighted and are related to existing functional reports for the nsLTP family, providing an integrated and comprehensive investigation of nsLTP function in plants.

Structure and biochemistry of nsLTPs

nsLTPs are widely distributed in the plant kingdom and are present in abundance, representing as much as 4% of total soluble protein. They are small, basic proteins, ranging in size from 6.5–10.5 kDa, which are synthesized as precursors with an N-terminal secretory signal peptide, generally varying from 21–27 amino acids in size. Their structure confers stability through disulfide bonding, rendering the protein resistant to heat and proteolysis. The tertiary structure is characterized by an eight-cysteine motif (8 CM) forming a backbone, with the following sequence: C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C (José-Estanyol *et al.*, 2004). The cysteine residues are linked by four disulfide bonds that stabilize a hydrophobic cavity, which has been shown to bind various lipids and hydrophobic compounds *in vitro* (Douliez *et al.*, 2000) (Fig. 1A, B). The nsLTP fold represents a large internal tunnel-like cavity along the axis of the molecule, which accommodates a range of different lipid types, with broad specificity, and exhibits a high plasticity upon binding (Lerche and Poulsen, 1998; Charvolin *et al.*, 1999; Douliez *et al.*, 2000; Han *et al.*, 2001; Sy *et al.*, 2003).

The 3-D structure of plant nsLTPs, in both an unbound state and in complex formation with various lipid ligands, has been determined based on X-ray crystallography and nuclear magnetic resonance (NMR) in various species (Gomar *et al.*, 1998; Lee *et al.*, 1998; Lerche and Poulsen, 1998; Tassin-Moindrot *et al.*, 2000; Han *et al.*, 2001; Cheng *et al.*, 2004; Da Silva *et al.*, 2005; Lin *et al.*, 2005; Pasquato *et al.*, 2006). These analyses show that nsLTPs possess a typical tertiary

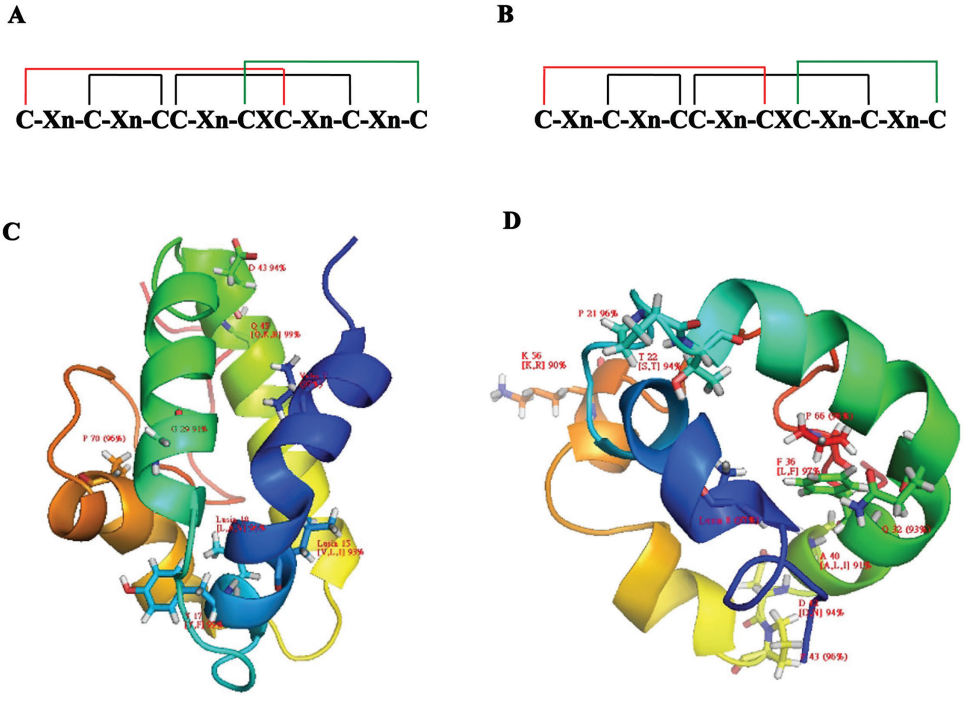

Fig. 1. Structural features of plant nsLTPs: the 8-Cys patterns (primary structure), four disulfide bridges (secondary structure) (A, B), and graphic mode (tertiary structure) (C and D) for type I (A, C), and type II (B, D) nsLTPs. 'C' indicates cysteine residue at highly conserved positions, 'X' indicates other amino acid residues, and 'n' indicates numbers. The four linkages between cysteine residues indicate disulfide bridges: black links indicate the common linkage mode between type I and type II nsLTPs, and red and green links indicate different linkage modes between type I and type II nsLTPs. The figure is reproduced from Carvalho and Gomes (2007), and Wang *et al.* (2012).

fold, characterized by four α-helices, connected by flexible loops, and a non-structured C-terminal tail. The α-helix compact domain is further stabilized by disulphide bonds linking the cysteine residues (Sy *et al.*, 2003) (Fig. 1C, D). Additionally, a large number of intramolecular H-bonds contribute to the stabilization of the 3-D protein structure. These characteristics contribute to the thermal stability and proteolytic resistance of nsLTPs (Scheurer, *et al.* 2004; Gaier *et al.*, 2008). The structures of type I and II nsLTPs have been relatively well studied. The difference between the two types is defined in the nature of the disulphide bonding and the resulting effects on tertiary structure. The disulfide bond linkages of type I at C_1–C_6 and C_5–C_8 differ from those of type II at C_1–C_5 and C_6–C_8 (Fig. 1A, B). Type I nsLTPs are characterized by a long tunnel-like cavity, while type II nsLTPs have two adjacent hydrophobic cavities (Fig. 1C, D).

nsLTP systems of classification

Categorization of nsLTPs based on sequence similarity-derived phylogenetic clustering has provided comprehensive information into the protein family and has facilitated further functional analysis. nsLTPs were first classified into two types based on molecular weight, which include nsLTP1 (type I, ~9 kDa) and nsLTP2 (type II, ~7 kDa) (Kader, 1996). However, this method excludes classification of several newly identified anther-specific proteins, displaying substantial homology to plant nsLTPs. These proteins have been excluded from the original groupings and could form a new split (Lauga *et al.*, 2000; Boutrot *et al.*, 2005), termed type III nsLTPs, which differ from type I and II by the number of amino acid residues present in the intervals of 8 CM structure (Boutrot *et al.*, 2005).

Recently, a new classification system was proposed by Boutrot *et al.* (2008), where nsLTPs are grouped according to sequence similarity and intervals of eight cysteine amino acid residues. This system categorized nsLTPs into nine types (type I–IX) based on a genome-wide analysis of rice, wheat and *Arabidopsis thaliana* (Arabidopsis) (Boutrot *et al.*, 2008). Additional studies have applied this classification system to other species with slight modification in some cases (Liu *et al.*, 2010; Wang *et al.*, 2012; Tapia *et al.*, 2013; Li *et al.*, 2014) (Table 1). Liu *et al.* (2010) clustered 135 Solanaceae nsLTPs into five types (I, II, IV, IX and X) within Boutrot's system. It is worth noting that type X is a new group, which had not yet been reported in any other plant, and accounts for >50% of Solanaceae nsLTPs (Liu *et al.*, 2010). In *Lotus japonicus,* 25 nsLTPs-encoding sequences were also classified into seven types (I, II, III, IV, V, VIII and IX) (Tapia *et al.*, 2013), and in *Brassica rapa*, Li *et al.* (2014) identified 63 putative nsLTPs, which were classified into nine types (I, II, III, IV, V, VI, VIII, IX and XI) according to Boutrot's method (2008), including a novel XI grouping (Li *et al.*, 2014). A database has now been established containing 595 nsLTPs from 121 different species, which have been divided into five types (I, II, III, IV, V) by feature of intervals between eight cysteine residues, overlapping with type I, II, IV, V, VI separately in Boutrot's system (Wang *et al.*, 2012) (Table 1).

However, the above classification scheme largely excludes non-flowering plants due to limited sequence homology between nsLTPs from flowering and non-flowering plants in this system. Thus, Edstam *et al.* (2011) developed a new classification system for nsLTPs, based on sequence similarity, glycosylphosphatidylinositol (GPI) modification site, intron position and spacing between the cysteine residues. The long-established type I and II groupings are retained, while the other *nsLTP* genes are classified in the subfamilies types C, D, E, F, G, H, J and K. Types D and G are expressed in liverwort, mosses and vascular plants, while other types may be restricted to a single species (Edstam *et al.*, 2011). Type C is overlapping with Boutrot's type III, type D with type V and VIII, type E with type IX, and type G with type VII and VIII, while type F, H, J and K are new types which comprise non-flowering plants (Table 1). This expanded classification system contributes significantly to the general development of methods for classification

Table 1. *The development of a classification system for nsLTPs*

Year	Type	Classification standard	Type	Remark	Species	Reference
1996	2	Molecular weight	I, II	/	Most monocotyledonous and dicotyledonous plants	Kader, 1996
2005	/	Sequence similarity	III	III is a new type	Wheat	Boutrot *et al.*, 2005
2008	9	Sequence similarity, intervals of eight cysteine residues	I to IX	/	Rice, *Arabidopsis* and wheat	Boutrot *et al.*, 2008
2010	10	Sequence similarity, intervals of eight cysteine residues	I to X	X is a new type	Solanaceae	Liu *et al.*, 2010
2012	5	Sequence similarity matrix, properties of 8-cysteine motifs	I, II, III, IV, V	Corresponds to types I, II, IV, V, VI in Boutrot's nine-type system	121 species	Wang *et al.*, 2012
2014	9	Sequence similarity, intervals of eight cysteine residues	I to XI	XI is a new type	*Brassica rapa*	Li *et al.*, 2014
2011	10	Sequence similarity, GPI modification site, intron position and spacing between the cysteine residues	I, II, C, D, E, F, G, H, J, K	Types I and II are the same as above	Green and red algae, liverworts, moss, lycopods, ferns and conifers	Edstam *et al.*, 2011

of genes and proteins across plant species and allows for a greater understanding of the function and evolutionary history of the many forms of nsLTP in plants. In maize (*Zea mays*) 63 nsLTP genes were divided into five types (I, II, C, D and G) (Wei and Zhong, 2014), according to this Edstam's method (2011).

Boutrot's classification system and its successors (Boutrot *et al.*, 2008, Liu *et al.*, 2010, Li *et al.*, 2014) focused on a comprehensive classification of *nsLTP* genes with adequate coverage in only a few species, while the nsLTPs database (Wang *et al.*, 2012) is based on a wider range of incomplete *nsLTP* gene sequences in a number of species. It is noteworthy that nsLTP-like proteins that contain the GPI-anchored domain were not included in the above-mentioned systems of classification, with the exception of Edstam's, where GPI-anchored nsLTPs are grouped in type G. These are common in liverworts and are present in many important terrestrial plant species including *A. thaliana* and rice (Edstam *et al.*, 2011). Regardless of the classification system employed, type I and II nsLTPs are present across all species that express nsLTPs. The current nsLTP classification schemes provide a valuable resource for researchers, however, further improvements can be made to increase both the comprehensiveness and robustness of the system.

The identification and characterization of nsLTPs across species

The first plant lipid transfer protein fraction was isolated from potato tuber in 1975 (Kader, 1975). Later, complete nsLTPs were purified and characterized from spinach leaves, and named for their ability to mediate the *in vitro* transfer of phospholipids between membranes (Kader *et al.*, 1984). nsLTPs are widely distributed in the plant kingdom and belong to a complex multigene family. Over the past 40 years, many more nsLTPs have been identified in both flowering and non-flowering plants (Kader, 1996; Jang *et al.*, 2007; Boutrot *et al.*, 2008; Edstam *et al.*, 2011; Wang *et al.*, 2014). Originally, *nsLTP* genes were isolated and characterized in the flowering plants (angiosperms), predominantly in major crops such as wheat (*Triticum aestivum*) (Boutrot *et al.*, 2008), rice (*Oryza sativa*) (Boutrot *et al.*, 2008), soybean (*Glycine max*) (Wang *et al.*, 2014), Chinese cabbage (*Brassica rapa*) (Li *et al.*, 2014) and maize (Wei and Zhong, 2014) (Table 2). *nsLTP* genes are present across a wide variety of plant families, including Poaceae, Liliaceae, Musaceae in the monocots, Cruciferae, Leguminosae, and Vitaceae in the dicots (Table 2) and in non-flowering land plants such as liverworts, mosses, lycopods, ferns and gymnosperms. However, nsLTPs have not been found in algae (Edstam *et al.*, 2011) (Table 2, Fig. 2).

The availability of fully sequenced plant genomes has facilitated the identification of nsLTPs through genome-wide analysis of the putative *nsLTP* genes (Boutrot *et al.*, 2008, Liu *et al.*, 2010, Edstam *et al.*, 2011, Tapia *et al.*, 2013, Li *et al.*, 2014, Wang *et al.*, 2014, Wei and Zhong, 2014). To species not sequenced, BLAST analysis has often been employed to match unigenes against an EST database in order to find additional genes, not yet annotated as *nsLTP* genes (Jang *et al.*, 2007, 2008, Boutrot *et al.*, 2008, Edstam *et al.*, 2011, Wang *et al.*, 2012). The number of nsLTPs contained in the database established by Wang *et al.* (2014) will grow with the increasing numbers of genomes becoming available, thus facilitating a more extensive categorization of the *nsLTP* gene family. Expansion of the database will support the advancement of genomics-based investigations into the structure and biological function of nsLTPs, such as through reverse genetics.

Targeting and localization of nsLTPs

nsLTP must be targeted to their proper sub-cellular domains before they become fully functional. Therefore, information pertaining to the localization of nsLTP proteins is of major importance in functional studies. nsLTPs were originally named for their ability to transfer phospholipids across membranes *in vitro* (Kader, 1975). They were originally proposed to play a major role in the intracellular movement of lipids in general, via membrane biogenesis, trans-membrane transfer of phospholipids and through altering membrane lipid composition (Wirtz, 1991; Wu *et al.*, 2004; Kirubakaran *et al.*, 2008). nsLTPs were also thought to play similar roles *in vivo* as were found *in vitro*. However, later researchers showed that the intracellular transfer of lipids by nsLTPs is unlikely *in vivo* (Kader, 1996) because several nsLTPs were detected extracellularly and their secretion was inferred by the presence of a signal peptide in the deduced protein sequence (Kader, 1997). Recent studies again support the point of view of intracellular transfer of lipids through several observations in nsLTPs' dynamic distributions (Pagnussat *et al.*, 2012; Ambrose *et al.*, 2013; Edstam *et al.*, 2013).

Numerous studies performed across diverse species have demonstrated the extracellular localization of nsLTPs, including work done in barley (Mundy and Rogers, 1986), carrot (Sterk *et al.*, 1991), grape (Coutos-Thevenot *et al.*, 1993), Arabidopsis (Maldonado *et al.*, 2002), tobacco (Dani *et al.*, 2005), soybean (Djordjevic *et al.*, 2007) and *Medicago* (Kusumawati *et al.*, 2008). Although nsLTPs are recognized as apoplastic proteins, some experimental evidence has shown that certain family members are localized to cell walls (Thoma *et al.*, 1993; Pyee *et al.*, 1994; Park *et al.*, 2002), plasma membranes (Debono *et al.*, 2009; Lee *et al.*, 2009; Edstam *et al.*, 2014), and to the intracellular matrix (Tsuboi *et al.*, 1992; Dubreil *et al.*, 1998; Carvalho *et al.*, 2001, 2004; Kielbowicz-Matuk *et al.*, 2008; Pagnussat *et al.*, 2009, 2012; Diz *et al.*, 2011) (Table 3). Some seed nsLTPs have been detected within multiple sub-cellular localizations: intracellularly, in the cell wall, plasma membrane and in the extracellular space (Tsuboi *et al.*, 1992; Carvalho *et al.*, 2004; Pagnussat *et al.* 2009; Diz *et al.*, 2011) (Table 3), and this prompts new considerations into the varied physiological role of nsLTPs.

Recently, studies in germinating sunflower (*Helianthus annuus*) seeds have demonstrated that HaAP10 (a nsLTP protein) is apoplastic in dry seeds but, upon imbibition, is rapidly endocytosed and relocalized to intracellular organelles

Table 2. *nsLTP genes identified in various plant species*

Classification	Family	Common name	Species	Gene numbers	Reference
Red algae	Bangiaceae	Porphyra	*Porphyra yezoensis*	0	Edstam *et al.*, 2011
Green algae	Prasinophyceae	Mesostigma	*Mesostigma viride*	0	Edstam *et al.*, 2011
Liverwort	Marchantiaceae	Marchantia	*Marchantia polymorpha*	14	Edstam *et al.*, 2011
Moss	Funariaceae	Physcomitrella	*Physcomitrella patens*	40	Edstam *et al.*, 2011
Lycopod	Selaginellaceae	Herba Selaginellae	*Selaginella moellendorffii*	43	Edstam *et al.*, 2011
Fern	Adiantaceae	Adiantum	*Adiantum capillus-veneris*	6	Edstam *et al.*, 2011
Gymnosperms	Pinaceae	Loblolly pine	*Pinus taeda*	43	Edstam *et al.*, 2011
Angiosperm (monocot)	Liliaceae	Onion	*Allium cepa*	4	Jang *et al.*, 2008
Angiosperm (monocot)	Musaceae	Banana	*Musa nana*	5	Jang *et al.*, 2008
Angiosperm (monocot)	Poaceae	Rice	*Oryza sativa*	52	Boutrot *et al.*, 2008
Angiosperm (monocot)	Poaceae	Wheat	*Triticum aestivum*	156	Boutrot *et al.*, 2008
Angiosperm (monocot)	Poaceae	Sorghum	*Sorghum vulgare*	5	Pelèse-Siebenbourg *et al.*, 1994
Angiosperm (monocot)	Poaceae	Sorghum	*Sorghum bicolor*	16	Wang *et al.*, 2012
Angiosperm (monocot)	Poaceae	Purple false brome	*Brachypodium distachyon*	14	Wang *et al.*, 2012
Angiosperm (monocot)	Poaceae	Maize	*Zea mays*	63	Wei and Zhong, 2014
Angiosperm (monocot)	Poaceae	Barley	*Hordeum vulgare*	14	Jang *et al.*, 2007
Angiosperm (dicot)	Cruciferae	Arabidopsis	*Arabidopsis thaliana*	49	Boutrot *et al.*, 2008
Angiosperm (dicot)	Cruciferae	Chinese cabbage	*Brassica rapa*	63	Li *et al.*, 2014
Angiosperm (dicot)	Leguminosae	Crowtoe	*Lotus japonicus*	25	Tapia *et al.*, 2013
Angiosperm (dicot)	Leguminosae	Peanut	*Arachis hypogaea*	5	Zhao *et al.*, 2009
Angiosperm (dicot)	Leguminosae	Soybean	*Glycine max*	25	Wang *et al.*, 2014
Angiosperm (dicot)	Malvaceae	Cotton	*Gossypium hirsutum*	11	Feng *et al.*, 2004
Angiosperm (dicot)	Pedaliaceae	Sesame	*Sesamum indicum*	34	Wang *et al.*, 2014
Angiosperm (dicot)	Ranunculaceae	Colorado blue columbine	*Aquilegia coerulea*	10	Wang *et al.*, 2012
Angiosperm (dicot)	Rubiaceae	Coffee	*Coffea arabica*	6	Cotta *et al.*, 2014
Angiosperm (dicot)	Rubiaceae	Coffee	*Coffea canephora*	3	Cotta *et al.*, 2014
Angiosperm (dicot)	Solanaceae	Potato	*Solanum tuberosum*	28	Liu *et al.*, 2010
Angiosperm (dicot)	Solanaceae	Tomato	*Solanum lycopersicum*	28	Liu *et al.*, 2010
Angiosperm (dicot)	Solanaceae	Tobacco	*Nicotiana glauca*	5	Cameron *et al.*, 2006*a*
Angiosperm (dicot)	Solanaceae	Tobacco	*Nicotiana tabacum*	33	Liu *et al.*, 2010
Angiosperm (dicot)	Solanaceae	Tobacco	*Nicotiana benthamiana*	17	Liu *et al.*, 2010
Angiosperm (dicot)	Solanaceae	Pepper	*Capsicum annuum*	19	Liu *et al.*, 2010
Angiosperm (dicot)	Solanaceae	Garden petunia	*Petunia hybrida*	10	Liu *et al.*, 2010
Angiosperm (dicot)	Vitaceae	Grapevine	*Vitis vinifera*	6	Wang *et al.*, 2014

involved in lipid metabolism, thus playing the role originally suggested in intracellular function (Pagnussat *et al.*, 2012). LTPG distribution is also dynamic in *A. thaliana* apoplast or cell wall, responding to changes in cell shape and cell wall curvature during cell growth and differentiation (Ambrose *et al.*, 2013). Alternative splicing of several type G nsLTPs (LTPGs) results in the generation of one transcript encoding the GPI-anchor signal and another transcript lacking the signal, hence playing a role in regulating the cellular localization of LTPGs depending on tissue type and environment (Edstam *et al.*, 2013).

Until recently, several methods have been employed to investigate the sub-cellular localization of nsLTPs, including cell culture (Mundy and Rogers, 1986; Sterk *et al.*, 1991; Coutos-Thevenot *et al.*, 1993; Kusumawati *et al.*, 2008), proteomics analysis of apoplastic fluid (Dani *et al.*, 2005; Djordjevic *et al.*, 2007), immunochemical studies (Pyee *et al.*, 1994; Thoma *et al.*, 1993; Pagnussat *et al.*, 2012) and the expression of fusion proteins in protoplasts or in plant tissue cells by transformation (Park *et al.*, 2002; Debono *et al.*, 2009; Lee *et al.*, 2009; Edstam *et al.*, 2014; Guo *et al.*, 2013*b*). It seems that a relationship may exist between research methods and the specific sub-cellular localization of nsLTPs identified (Table 3). For example, most of the nsLTPs identified to be extracellularly located were isolated from cell culture or by proteomic studies in apoplastic fluid, while immunochemical studies were more likely to locate nsLTPs in cell walls. Studies using fusion proteins expressed in protoplasts or in plant tissue cells by transformation showed the majority of nsLTPs identified to be located in the plasma membrane and/or cytoplasm. Protoplast transformation is, in fact, not the most reliable method as it is fails to detect proteins in cell walls and/or extracellularly (Guo *et al.*, 2013*b*). While different detection methods and conditions often give varied results with respect to sub-cellular localization profiles (Tsuboi *et al.*, 1992; Carvalho *et al.*, 2004; Pagnussat *et al.* 2009; Diz *et al.*, 2011), employment of a combination of experimental methods, under varied conditions, allows for the more accurate localization of nsLTPs.

Within the nsLTPs multigenic family, proteins with varied localizations may perform the corresponding specific functions (Clark and Bohnert 1999). LTPGs were primarily localized to the plasma membrane, on the surface of stem epidermal cells, where wax is actively secreted (Debono *et al.*,

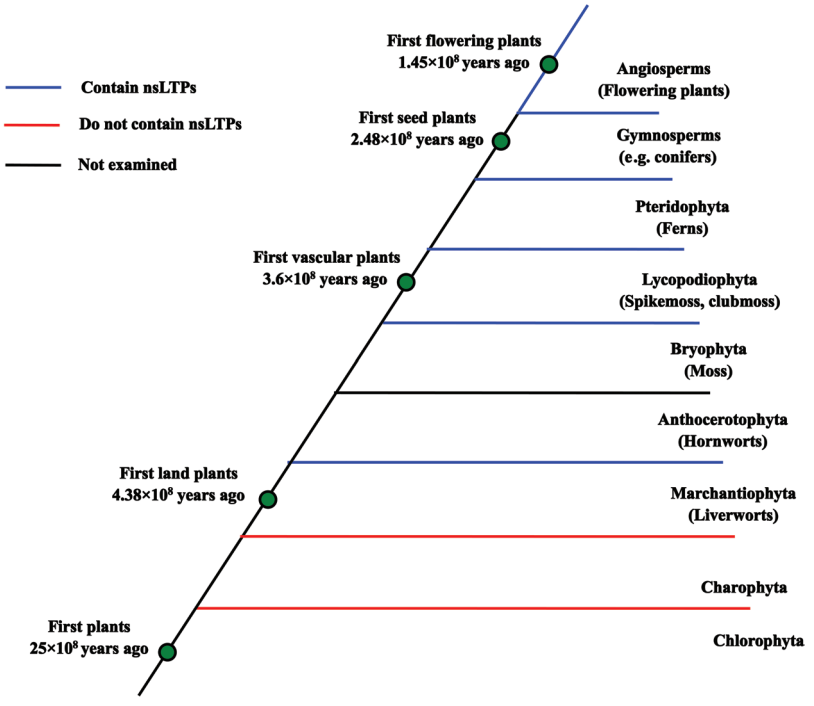

Fig. 2. The presence and distribution of *nsLTP*s across plant species. Red branches in the cladogram indicate that no *nsLTP*s have been identified, and blue branches indicate the presence of *nsLTP*s. Green dots mark major divergence events in plant evolution (Edstam *et al.*, 2011).

2009; Lee *et al.*, 2009; Edstam *et al.*, 2014). As LTP3 has been shown to bind lipids *in vitro*, and is localized in cytosol, it may act as a co-signal for binding and transferring lipids from the cytosol to cell membranes or cell walls, to form cuticular wax (Guo *et al.*, 2013*b*). DIR1 in Arabidopsis is located extracellularly, which may play a role in production or transmission from the inoculated leaf of an essential mobile long distance signal (Maldonado *et al.*, 2002). In addition, two soybean nsLTP protein XPs identified to be located extracellularly, have known roles in plant signalling (Djordjevic *et al.*, 2007). The extracellular localization of nsLTPs is thought to contribute to the generation of a defensive shield over plant surfaces, which are susceptible to pathogen attack, thus strengthening resistance to various forms of stress (García-Olmesdo *et al.*, 1995). HaAP10 is located in the apoplast in dry sunflower seeds, and is then relocated to the intracellular matrix in imbibed seeds, which may have relevant consequences on the function of the protein when targeting oil bodies to participate in the degradation of major seed storage reserves of triacylglycerides triggered during seed germination (Pagnussat *et al.*, 2012). These findings have generated new perspectives in the functionality of nsLTP family members, based on a greater understanding of their tissue-specific localization.

Lipid binding and transfer ability of nsLTPs

Plant nsLTPs show a broad lipid-binding specificity *in vitro*, including to fatty acids, fatty acyl-CoA, phospholipids, glycolipids, hydroxylated fatty acid and prostaglandin B2 (Kader, 1996; Sodano *et al.*, 1997; Zachowski *et al.*, 1998; Douliez *et al.*, 2000, 2001; Tassin-Moindrot *et al.*, 2000; Carvalho and Gomes, 2007). However, such capacity varies among different nsLTP members, depending on the specific characteristics of the nsLTP tertiary fold. Some nsLTPs can bind one or two lipid molecules at a time (Sodano *et al.*, 1997; Charvolin *et al.*, 1999; Da Silva *et al.*, 2005; Cheng *et al.*, 2004), and some nsLTPs with a hydrophobic cavity obstructed by bulk side chains of aromatic amino acids could not bind and transport free lipids (Cammue *et al.*, 1995; Tassin *et al.*, 1998), while some are completely lacking any internal lipid-binding cavity (Baud *et al.*, 1993; Salcedo *et al.*, 2004; Monsalve *et al.*, 2007). Studies in *Z. mays* showed that saturated molecules containing 16–18 carbons, rather than 12–14 or 20–22 carbons, best interact with this type of nsLTP (Zachowski *et al.*, 1998). A computational study on nsLTP2 in *O. sativa* identified the structural determinant controlling the affinity of nsLTPs for fatty acids, which is facilitated by longer carbon chains, the presence of a hydroxyl group, an increased number of double bonds in the acyl chain, as well as a trans configuration (Tousheh *et al.*, 2013). Purified moss LTPGs in *Physcomitrella patens* showed a preference for binding unsaturated fatty acids, and displayed a lipid profile rich in cutin monomers, such as C16 and C18 mono- and di-hydroxylated fatty acids (Edstam *et al.*, 2013).

Although nsLTPs were originally discovered and named for their *in vitro* lipid binding or transfer ability between membranes (Kader, 1996; Douliez *et al.*, 2000), increasing lines of evidence suggest that nsLTPs do not mediate a simple vectorial lipid transport from one membrane to another. Instead, they facilitate lipid transport between membranes in response to their membrane environment. nsLTPs can therefore locally modulate lipid composition and/or fluidity of membranes, and consequently regulate various cellular processes, including vesicular trafficking, signal transduction, and lipid transfer and metabolism (Cockcroft, 1999; De Matteis *et al.*, 2007; Fairn and McMaster, 2008). *In vitro* studies suggest that

Table 3. *Summary of nsLTP sub-cellular localization studies*

Protein	Species	Experimental method	Distribution	Reference
PAPI	Barley (*Hordeum vulgare*)	Detected in cell culture medium	Extracellular	Mundy and Rogers, 1986
EP2	Carrot (*Daucus carota*)	Detected among extracellular proteins in embryogenic cell cultures	Extracellular	Sterk *et al.*, 1991
Four LTPs	Grape (*Vitis vinifera*)	Purification from extracellular medium of somatic embryo cultures	Extracellular	Coutos-Thevenot *et al.*, 1993
DIR1	Arabidopsis (*Arabidopsis thaliana*)	Detected in petiole exudate	Extracellular	Maldonado *et al.*, 2002
Two LTPs	Tobacco (*Nicotiana tabacum*)	Proteomic analysis of apoplastic fluid	Extracellular	Dani *et al.*, 2005
Two LTPs	Soybean (*Glycine max*)	Proteomic analysis of xylem and apoplast fluid	Extracellular	Djordjevic *et al.*, 2007
One LTP	Medicago (*Medicago truncatula*)	Secreted proteins isolated and identified in suspension cultures	Extracellular	Kusumawati *et al.*, 2008
LTP1	Arabidopsis (*Arabidopsis thaliana*)	Immunocytochemical studies using a polyclonal antibody against a fusion protein	Cell wall	Thoma *et al.*, 1993
WAX9	Broccoli (*Brassica oleracea*)	Immunogold labelling studies	Cell wall	Pyee *et al.*, 1994
CaLTP1	Pepper (*Capsicum annuum*)	35S::CaLTP1:smGFP introduced into detached pepper leaves through biolistic gene bombardment	Cell wall	Park *et al.*, 2002
LTPG	Arabidopsis (*Arabidopsis thaliana*)	Gene promoter::YFP-LTPG transgenic experiment	Plasma membrane	Debono *et al.*, 2009
LTPG1	Arabidopsis (*Arabidopsis thaliana*)	Tranformation of Arabidopsis protoplast and tobacco epidermal cell with P35S::LTPG1:EYFP	Plasma membrane	Lee *et al.*, 2009
PpLTPG2	Physcomitrella (*Physcomitrella patens*)	35S::YFP-PpLTPG2 fusion protein expressed in *P. patens* protoplasts and in tobacco leaves	Plasma membrane	Edstam *et al.*, 2014
LTP3	Arabidopsis (*Arabidopsis thaliana*)	P35S::LTP3-GFP Arabidopsis protoplast transformation	Cytoplasm	Guo *et al.*, 2013*b*
SsLTP1	Eggplant (*Solanum sogarandinum*)	Western analysis of soluble, apoplastic and membrane fractions	Intracellular	Kielbowicz-Matuk *et al.*, 2008
nsLTP	Castor bean (*Ricinus communis*)	Immunolocalization experiments, cell fractionation analysis	Glyoxysome matrix and cell wall	Tsuboi *et al.*, 1992
ns-LTP1e$_1$	Wheat (*Triticum aestivum*)	Immunolocalization experiments	Aleurone granules	Dubreil *et al.*, 1998
LTP	Cowpea (*Vigna unguiculata*)	Immunolocalization of tissue sections and sub-cellular fractionation analysis	Extracellular space, cell wall, intracellular localization in protein storage vacuoles and in lipid-containing vesicles	Carvalho *et al.*, 2004
HaAP10	Sunflower (*Helianthus annuus*)	Sub-cellular fractionation analysis, fluorimmunolocalizationstudies	Apoplastic, plasma membrane, intracellular	Pagnussat *et al.*, 2009
Ca-LTP1	Pepper (*Capsicum annuum*)	Immunolocalization experiments, western blotting of seed exudate proteins	Intracellular vesicles, and extracellular space	Diz *et al.*, 2011
HaAP10	Sunflower (*Helianthus annuus*)	Fluorimmunolocalization experiments	Apoplastic (in dry seeds), intracellular organelles of oil bodies and glyoxysomes (upon imbibition)	Pagnussat *et al.*, 2012

nsLTPs could also be involved in mediating lipid trafficking in intact cells, however *in vivo* studies provide more evidence for an extracellular role (Maldonado *et al.*, 2002; Dani *et al.*, 2005; Djordjevic *et al.*, 2007; Kusumawati *et al.*, 2008). nsLTPs appear to be involved in secretion of extracellular lipophillic material, including cutin monomers (Sterk *et al.*, 1991; Debono *et al.*, 2009; Lee *et al.*, 2009). Until recently, an apoplastic localized HaAP10 from sunflower dry seeds relocalizes to intracellular organelles involved in lipid metabolism during seed germination, and may carry out an intracellular function (Pagnussat *et al.*, 2012). Similar to many proteins, it appears that nsLTP localization and function varies according to developmental stage and in response to certain environmental conditions. Although nsLTPs have been extensively studied, their modes of action in intact cells have not yet been fully elucidated and there has been some debate as to their true *in vivo* activity. Further studies of the precise mechanisms involved in lipid metabolism could deepen our understanding of nsLTPs' function.

Expression profiling and functional roles of nsLTPs

As *nsLTP* genes were first cloned and characterized, it became possible to determine their tissue-specific and

temporal expression patterns, as well as changes in expression in response to certain environmental conditions and molecular signals. Transcriptional studies have greatly enhanced investigations into the roles played by nsLTPs *in vivo*. Northern blot analysis, real-time PCR, and *in situ* hybridization are commonly employed to investigate the expression of nsLTPs. Reporter gene constructs, such as β-glucuronidase fused with the nsLTP promoter, demonstrate the complex temporal and spatial expression patterns of nsLTPs in response to a wide variety of environmental conditions and signalling events.

Plant nsLTP involvement in a range of biological processes has been indicated, although the precise mechanisms employed by the protein are not yet clearly understood. Several potential functions have been proposed thus far, including in resistance to biotic and abiotic stress (Jung *et al.*, 2005; Guo *et al.*, 2013*a*), cutin and wax metabolisms (Debono *et al.*, 2009), seed development and germination (Pagnussat *et al.*, 2012), sexual reproduction (Chae *et al.*, 2009, 2010), and as components in food allergens (Sawano *et al.*, 2008). Moreover, of these, the role of nsLTPs in plant defence is now relatively well established (Maldonado *et al.*, 2002).

nsLTPs and biotic stress

It is well verified that nsLTPs play a key role in the protective mechanisms developed by plants against attack by bacteria and fungi. It has also been well established that nsLTPs protect against viruses (Sohal *et al.*, 1999; Park *et al.*, 2002), as well as insect pests (Jang *et al.*, 2005). In some cases, nsLTPs have been classified as pathogenesis-related (PR) proteins, such as the PR-14 family (Van Loon and Van Strien, 1999; Sels *et al.*, 2008). Ample evidence exists supporting the role of nsLTPs in plant disease resistance: (i) Firstly, many *nsLTP* genes exhibit differential expression patterns in response to bacterial, fungal and viral infection, or upon treatment with various defence-related signalling molecules, such as abscisic acid, salicylic acid, ethylene and methyl jasmonate (Molina and García-Olmedo, 1993; Park *et al.*, 2002; Guiderdoni *et al.*, 2002; Gomès *et al.*, 2003; Jung *et al.*, 2003; Jang *et al.*, 2005; Lu *et al.*, 2005). (ii) nsLTP proteins isolated from diverse plant species exhibit strong antimicrobial activity *in vitro* (Terras *et al.*, 1992; Molina *et al.*, 1993; Segura *et al.*, 1993; Cammue *et al.*, 1995; Nielsen *et al.*, 1996; García-Garrido *et al.*, 1998; Kristensen *et al.*, 2000; Regente and De La Canal, 2000; Ge *et al.*, 2003; Wang *et al.*, 2004; Patkar and Chattoo, 2006; Lin *et al.*, 2007; Yang *et al.*, 2007; Kirubakaran *et al.*, 2008; Jia *et al.*, 2010; Zottich *et al.*, 2011; Gizatullina *et al.*, 2013). (iii) nsLTPs with antifungal activity also have the ability to infiltrate artificial membranes and liposomes, as shown by permeabilization assay, indicating that their antifungal activity could be achieved through interference with the biological membranes of target organisms, leading to loss of membrane integrity (Kader, 1996; Caaveiro *et al.*, 1997; Regente and De La Canal, 2000; Regente *et al.*, 2005; Diz *et al.*, 2006). (iv) Over-expression of *nsLTP* genes, including *LTP* in barley, *CALTP*1 and *CALTP2* in pepper, *Ace-AMP1* in onion, and *LJAMP1* or *LJAMP2* in motherwort, have been found to significantly enhance resistance to fungal and bacterial pathogens (Molina and García-Olmedo, 1997; Jung *et al.*, 2005; Roy-Barman *et al.*, 2006; Patkar *et al.*, 2006; Yang *et al.*, 2007, 2008; Sarowar *et al.*, 2009; Jia *et al.*, 2010), and *ltpg1* mutant in Arabidopsis were found to be more susceptible to infection by the fungus *Alternaria brassicicola* than wild type (Lee *et al.*, 2009). (v) Recent studies have lead to breakthroughs in our understanding of the nsLTP defence mechanism controlling these responses. nsLTPs may be involved in long distance signalling associated with systemic acquired resistance (SAR), probably through interaction with a lipid-derived molecule, e.g. jasmonic acid or lysophosphatidylcholines, then forming a complex which competitively binds receptors of fungal elicitins. The elicitins include small cysteine-rich proteins secreted on plasma membranes, for example by *Phytophthora*, with structural similarities to nsLTPs (Buhot *et al.*, 2001, 2004; Blein *et al.*, 2002; Maldonado *et al.*, 2002; Suzuki *et al.*, 2004; Lascombe *et al.*, 2008; Sarowar *et al.*, 2009; Pii *et al.*, 2010; Yu *et al.*, 2013).

Further questions remain as to whether the signaling function is linked to a specific lipid transport system, or whether the nsLTP lipid molecule complex is in fact the mobile signal, and whether or not the binding of the complex to receptors on the plasma membrane is specifically required for production of the mobile signal, as well as how the protein is recognized by the receptor, and subsequently transmitted. Additional work is needed to further characterize the role of nsLTPs in signal transduction.

nsLTPs and abiotic stress

The roles played by nsLTP in responses to abiotic stress may help plants adapt to changes in environmental conditions, namely drought (Jang *et al.*, 2002, 2004; Jung *et al.*, 2005; Giordani *et al.*, 2011; Guo *et al.*, 2013*a*, *b*), freezing stress (Hincha *et al.*, 2001; Wu *et al.*, 2004; Kielbowicz-Matuk *et al.*, 2008) and salinization (Jang *et al.*, 2004; Jung *et al.*, 2005; Pitzschke *et al.*, 2014). These roles of nsLTPs have been investigated mainly by four aspects: (i) Members of the nsLTP family are responsive to one or multiple abiotic stressors, including drought (Treviño and MA, 1998; Jang *et al.*, 2002, 2004; Jung *et al.*, 2003; Wu *et al.*, 2004; Cameron *et al.*, 2006*a*, *b*; Kielbowicz-Matuk *et al.*, 2008; Guo *et al.*, 2013*a*, *b*; Edstam *et al.*, 2014), low temperature (Yubero-Serrano *et al.*, 2003; Jung *et al.*, 2003; Wu *et al.*, 2004; Carvalho *et al.*, 2006; Kielbowicz-Matuk *et al.*, 2008; Maghuly *et al.*, 2009; Guo *et al.*, 2013*a*, *b*; Edstam *et al.*, 2014), high temperature (Wu *et al.*, 2004), salt (Jung *et al.*, 2003; Wu *et al.*, 2004; Jang *et al.*, 2004; Kielbowicz-Matuk *et al.*, 2008; Choi *et al.*, 2008; Wang *et al.*, 2009; Guo *et al.*, 2013*a*), alkali (Wang *et al.*, 2009), osmotic stress (Jang *et al.*, 2004; Choi *et al.*, 2008; Wang *et al.*, 2009), hydrogen peroxide (Jang *et al.*, 2004; Tapia *et al.*, 2013), heavy metal (Wang *et al.*, 2009), light (Sohal *et al.*, 1999) and wounding (Yubero-Serrano *et al.*, 2003; Jung *et al.*, 2003; Jang *et al.*, 2004; Cameron *et al.*, 2006*a*; Maghuly *et al.*, 2009). (ii) nsLTPS also show responses to the abiotic stress-related plant hormones, including abscisic acid (ABA) (Treviño and MA, 1998; Yubero-Serrano *et al.*, 2003; Jung *et al.*, 2003; Wu *et al.*, 2004; Choi *et al.*, 2008; Wang *et al.*, 2009; Guo *et al.*, 2013*a*;

Tapia *et al.*, 2013), salicylic acid (Yubero-Serrano *et al.*, 2003; Jang *et al.*, 2004; Jung *et al.*, 2005; Maghuly *et al.*, 2009), methyl jasmonate (Jung *et al.*, 2003; Tapia *et al.*, 2013) and ethephon (Jung *et al.*, 2003, 2005; Jang *et al.*, 2004; Tapia *et al.*, 2013). (iii) Transgenic overexpression of *nsLTP* genes has been shown to significantly enhance tolerance to drought (*OsDIL* in rice, *CALTP1* in pepper and *LTP3* in Arabidopsis) (Jung *et al.*, 2005; Guo *et al.*, 2013*a*, *b*), cold stress (*LTP3*) (Guo *et al.*, 2013*b*), and salinization (*AZI1* in Arabidopsis) (Jung *et al.*, 2005; Pitzschke *et al.*, 2014). The loss-of-function mutant *ltp3* in Arabidopsis exhibits increased sensitivity to drought stress (Guo *et al.*, 2013*b*), and the null mutant *azi1* is hypersensitive to salt stress (Pitzschke *et al.*, 2014). (iv) Recent investigations have focused on the regulatory association between *nsLTP* genes and their upstream regulatory genes. For example, *LTP3* is positively regulated by the transcription factor MYB96 to mediate freezing and drought stress in Arabidopsis (Guo *et al.*, 2013*b*). Also, the lipid transfer protein AZI1 interacts with the protein kinase MPK3 to form complexes, and is up-regulated by MPK3 to mediate salt stress in Arabidopsis (Pitzschke *et al.*, 2014). The presence of various cis-regulatory sequences within the promoter of *nsLTP* genes, which are a response to abscisic acid, cold or wounding stress, provides further evidence of the regulatory role of nsLTPs during plant stress (Treviño and MA, 1998; García-Garrido *et al.*, 1998; Yubero-Serrano *et al.*, 2003; Jung *et al.*, 2005, 2006; Cameron *et al.*, 2006*a*).

nsLTPs in cutin and wax metabolism

The cuticle layer is made up of cutin polymers, containing hydroxy fatty acids (C16 and C18), glycerolmonomers, and waxes composed of long chain fatty acids (C20–34) and their derivatives. It is a hydrophobic structure, which insulates plant surfaces to prevent non-stomatal water loss, and to protect against pathogen attack. Plant epidermal cells dedicate most of their lipid metabolism to the synthesis of cuticular lipids (Riederer and Muller, 2006). In *Brassica oleracea* and Arabidopsis, the expression pattern of some nsLTPs demonstrates that they are expressed at high levels in young developing tissues, which are actively synthesizing surface wax. Expression diminishes in fully expanded tissues, suggesting a role for nsLTPs in the deposition of cuticular material during the expansion of leaf tissues (Pyee *et al.*, 1994; Thoma *et al.*, 1994). This concept is reinforced by the observation elevation in *nsLTP* gene expression in epidermal cells, in the protoderm cells of embryos, and in the petal and sepal abscission zone, where lipophilic substances are deposited to form the protective layer (Sterk *et al.*, 1991; Thoma *et al.* 1993, 1994; García-Olmedo *et al.*, 1995; Vroemen *et al.* 1996; Sohal *et al.*, 1999; Yubero-Serrano *et al.*, 2003; Wu and Burns, 2003; Jang *et al.*, 2005; Debono *et al.*, 2009; Lee *et al.*, 2009; Tapia *et al.*, 2013). nsLTPs have also been located extracellularly, mainly in cell walls or in the plasma membrane of epidermal cells and in certain secretory tissues, where they play a role in the secretion and/or deposition of cutin monomers (Thoma *et al.*, 1993, 1994; Debono *et al.*, 2009; Lee *et al.*, 2009; Potocka *et al.*, 2012). Increased synthesis of the wax, stimulated by drought, heat stress, exposure to heavy metals, and other environmental factors, is synchronous with the increased expression of *nsLTP*s (Pyee *et al.*, 1994; Hendriks *et al.*, 1994; Sohal *et al.*, 1999; Kunst and Samuels, 2003; Cameron *et al.*, 2006*b*; Tapia *et al.*, 2013).

Reverse genetic studies show that in *nsLTP* mutants, decreased expression of LTPG reduces wax load on the stem surface and may cause alterations in cuticular lipid composition (Debono *et al.*, 2009; Lee *et al.*, 2009). Systematic investigation of type G nsLTPs by Tapia *et al.* (2013), using microarray data from Arabidopsis and rice, combined with gene ontology analyses, has lead to the identification of three independent modes of expression for nsLTPs: primary involvement of the AtI/OsI-module in cuticular wax production, the AtII/OsII-module in the synthesis of suberin, and the AtIII/OsIII-module in the synthesis of sporopollenin. However, the actual mechanism of transport for lipid components to form the cuticle and the precise role that nsLTPs play in cuticle deposition are still unclear.

nsLTPs in seed development and germination

The specific expression of nsLTP genes and their protein distribution profiles indicate that they are present in the endosperm, embryo and/or surrounding regions, during seed development (Eklund and Edqvist, 2003; Boutrot *et al.*, 2005; Carvalho *et al.*, 2006; Kovalchuk *et al.*, 2012; Cotta *et al.*, 2014), as well as in the cotyledon or hypocotyl during seed germination (Soufleri *et al.*, 1996; Edqvist and Farbos, 2002; Gonorazky *et al.*, 2005). Evidence for their specific presence in seed is presented in the promoter regions of nsLTPs in coffee (*Coffea arabica* and *C. canephora*), which contain several DNA boxes essential for seed-specific expression in plants (Cotta *et al.*, 2014). The biochemical and physiological role of nsLTPs in seed germination has been further elucidated in several species. In castor bean, nsLTP1 regulates fatty acid beta-oxidation through the enhancement of acyl-CoA oxidase activity in glyoxysomes, in order to facilitate mobilization of seed storage lipid during seed germination (Tsuboi *et al.*, 1992). Gb-nsLTP1 in *Euphorbia lagascae* functions as a protease inhibitor, protecting the cotyledons from proteases released during programmed cell death (Eklund and Edqvist, 2003). Recently, it was found that HaAP10 in sunflower was rapidly relocalized upon seed imbibition to organelles involved in lipid metabolism (oil bodies and glyoxysomes) (Pagnussat, *et al.*, 2012). However, the precise mechanism by which nsLTPs mobilize lipids during seed development/germination is still unclear.

nsLTPs in plant sexual reproduction

nsLTP expression has been detected in the flowers of a variety of plant species and may be associated with reproduction (Sterk *et al.*, 1991; Pyee *et al.*, 1994; Soufleri *et al.*, 1996; Suelves and Puigdomènech, 1997; Clark and Bohnert, 1999; Sohal *et al.*, 1999; Arondel *et al.*, 2000; Botton *et al.*, 2002; Yubero-Serrano *et al.*, 2003; Jung *et al.*, 2003; Nieuwland *et al.*, 2005; Kim *et al.*, 2008; Choi *et al.*, 2008; Kielbowicz-Matuk *et al.*,

2008; Lee *et al.*, 2009). This is demonstrated by the identification of nsLTPs that are specifically expressed in the anthers, such as *t42*, *Wda1* and *OsC6* in *O. sativa* (Imin *et al.*, 2006; Jung *et al.*, 2006; Zhang *et al.*, 2010), protein 108 in *Solanum lycopersicum* (Chen and Smith, 1993), *FIL1* in *Antirrhinum majus* (Nacken *et al.*, 1991), *A9* and *AtLtp12* in *A. thaliana* (Nakamura *et al.*, 1998; Ariizumi *et al.*, 2002), *MZm3-3* in *Zea mays* (Lauga *et al.*, 2000), and *CaLTP* and *CaMF2* in pepper (*Capsicum annuum*) (Hong *et al.*, 2001; Chen *et al.*, 2011), and most of them are preferentially detected in the tapetum at the early stage of anther development. Stigma- and style-abundant nsLTPs are also identified, including *SCA* (stigma/style Cys-rich adhesin) in *Lilium longiflorum* (Park *et al.*, 2000, 2003), and *LTP5* and *LTP1* in *A. thaliana* (Chae *et al.*, 2009, 2010; Chae and Lord, 2011). Studies in reverse genetics, biochemistry and cytology suggest a role for nsLTPs in pollen and/or anther development, such as in pollen formation and germination (Chen *et al.*, 2011), pollen exine generation (Zhang *et al.*, 2010; Huang *et al.*, 2013), anther epidermal cell formation (Jung *et al.*, 2006), adhesion of pollen tubes to the stigma/stylar transmitting tract epidermis during pollen elongation (Park *et al.*, 2000; Park *et al.*, 2003; Chae *et al.*, 2010), pollen tube adhesion-mediated guidance and growth (Kim *et al.*, 2006; Chae *et al.*, 2007, 2009; Chae and Lord, 2011), and in the protection of reproductive tissues from environmental stress (Hong *et al.*, 2001; Guo *et al.*, 2013*a*).

nsLTP activity in cell wall growth, nodulation and CaM binding

A number of studies have found that nsLTPs play an integral role in cell development and organogenesis. *TobLTP2* was shown to facilitate cell wall loosening and extension *in vitro* in tobacco (Nieuwland *et al.*, 2005). Arabidopsis LTPGs are involved in responses to changes in cell shape and wall curvature, and in promoting proper cell geometry during cell growth and differentiation (Ambrose *et al.*, 2013).

In cowpea (*Vigna unguiculata*), *nsLTP* mRNA levels increased transiently in root hairs following inoculation with *Rhizobium*, indicating a role for nsLTPs in nodulation (Krause *et al.*, 1994). In Chinese milk vetch (*Astragalus sinicus*), *AsE246* has been shown to participate in the transport of plant lipids to symbiosome membranes and in nodule organogenesis associated with infection thread formation (Lei *et al.*, 2014). *MtN5* expression was induced in *Medicago trunculata* during the early phases of symbiosis in root hairs and nodule primordia, and also appears to be involved in the regulation of root tissue invasion, probably linking the progression of bacterial invasion with restricting the competence of root hairs for infection (Pii *et al.*, 2009, 2010, 2013).

The ubiquitous Ca^{2+}-binding protein, calmodulin (CaM), regulates the activity of many Ca^{2+}-dependent cellular processes and targets molecules involved in plant stress response. Some nsLTPs contain a putative CaM-binding site consisting of ~12 highly conserved amino acid residues at the C terminus (Wang *et al.*, 2005, 2008; Gao *et al.*, 2009). In Arabidopsis, nsLTP1 has been identified as a Ca^{2+}-independent CaM-binding protein (Wang *et al.*, 2005), and in *Brassica chinensis,* BcLTP exhibits both Ca^{+} dependent and independent binding to CaM, in turn facilitating BcLTP lipid binding capability via Ca^{2+} mediated signalling (Wang *et al.*, 2008). In potato (*Solanum tuberosum*), *StLTPa7* has been identified as a possible Ca^{2+}-responsive plant defence gene, due to an increase in *StLTPa7* transcripts as Ca^{2+} accumulates in response to interactions with the bacterium *Ralstonia solanacearum* (Gao *et al.*, 2009). However, the different expression profiling of *CaM* and *Ltp* genes in Tibetan cherry trees (*Prunus incisa*×*serrula*) suggests they play an important, but independent, role in the adaptation of plants to environmental stresses (Maghuly *et al.*, 2009).

nsLTPs responses to plant allergens

Subsequent to studies of adult patients with an allergy to Rosaceae fruits (Sánchez-Monge *et al.*, 1999; Pastorello *et al.*, 1999), several members of the nsLTP family have been identified as IgE-mediated food allergens in plant foods and pollens. These members have been identified and characterized predominantly in fruits (especially from those in Rosaceae), but also in vegetables, nuts, cereals and pollens (Sánchez-Monge 1999; Hoffmann-Sommergruber, 2000; Salcedo *et al.*, 2004; Breiteneder and Mills, 2005; Hartz *et al.*, 2007; Lauer *et al.*, 2009; Ciardiello *et al.*, 2010; Zoccatelli *et al.*, 2010). Active allergen forms of nsLTPs may also be present in processed plant-based products, including beverages, juice, jam and in heat-treated foodstuffs (Pastorello *et al.*, 2003; Scheurer *et al.*, 2004; Schad *et al.*, 2005).

nsLTPs are highly stable proteins, resisting heat treatments of up to 100°C, and the presence of glucose, for example in fruits, is contributive to the thermostability (Brenna *et al.* 2000; Lindorff-Larsen *et al.*, 2001; Asero *et al.*, 2003; Pastorello *et al.*, 2003; Scheurer *et al.*, 2004; Sancho *et al.*, 2005). nsLTPs are also resistant to proteolytic digestion *in planta*, and in simulated gastric fluid (Asero *et al.*, 2000; Lindorff-Larsen *et al.*, 2001; Duffort *et al.*, 2002; Enrique *et al.*, 2004; Vassilopoulou *et al.*, 2006; Sawano *et al.*, 2008). This explains the presence of active allergen forms of nsLTPs in processed and heat-treated plant products, and their ability to induce both sensitization and systemic response symptoms after passing through the gastrointestinal tract.

The evolution of nsLTPs

The plant *nsLTP* gene family evolved with the colonization of land by terrestrial plants, as they are present in all land plants but not in green alga. New *nsLTPs* may have evolved during land plant evolution as the diversity of nsLTP subfamilies or types in non-seed plants is more limited compared to seeded plants. The adoption of novel nsLTPs types likely assisted plants in adjusting to the harsh new environment on land (Edstam *et al.*, 2011) (Fig. 2).

The evolution of *nsLTP* genes within the Poaceae family has been characterized in a comprehensive survey of *nsLTP* genes in rice, wheat and sorghum (Jang *et al.*, 2007, 2008; Wang *et al.*, 2010, 2012). *nsLTPs* in rice and wheat show evidence of a varied genomic distribution, exhibiting somewhat

disproportionate shares of EST clones among the cereal *nsLTP* genes. This suggests the occurrence of independent duplication event(s), followed by increasing functional diversity in each species, which likely occurred during speciation (Jang *et al.*, 2007). The theory is supported to some extent by the observed differential expression profiles of *nsLTP* genes in rice and wheat, although the genes of both species in the same group were processed via a similar selection mode (Jang *et al.*, 2008). Microarray-based transcriptional profiling of *nsLTP*s indicates that rice *nsLTP* genes may have been subjected to a complex evolutionary selection mechanism, involving processing subfunctionalization, where pairs of genes originating from a duplication event take on independent functions, in concert with other mechanisms. As inferred by the constructed *nsLTP* gene-coexpression networks, increased functional diversity of *nsLTP* genes appears not to have occurred in a random fashion, but instead originated within specific biological processes over the course of evolutionary time (Jang *et al.*, 2008). Additional analysis of *nsLTP*s gene expression regulations in wheat has contributed towards further elucidation of evolutionary mechanisms governing the diversifying roles of nsLTPs. Analyses again indicate that their distinct physiological function appears to result predominantly from subfunctionalization involving degenerative mutations in the regulatory regions of the genome (Wang *et al.*, 2010). In a comparative analysis carried out on a cluster of *nsLTP* genes from rice and sorghum (Wang *et al.*, 2012), a highly redundant tissue-specific expression pattern displayed by members of the rice *nsLTP* family, compared with sorghum, suggested that a concerted evolution via gene conversion had occurred, favouring the preservation of crucial expression motifs through the homogenization of proximal promoter sequences under high selection constraints. However, extensive regulatory subfunctionalization might have also occurred under relatively low selection constraints, resulting in functional divergence at the expression level.

Clear evolutionary stories with regard to the *nsLTP* gene family on a wider range of species are expected to aid in future studies of nsLTP functions and the mechanisms relevant to their evolutionary fate.

Biological function of nsLTPs

The current literature describing the functionality of *nsLTP*s is extensive and wide ranging, and has revealed a variety of roles for nsLTPs based on a diversity of research. Our understanding of nsLTP biological function is based on the comprehensive amalgamation of the wide body of information available, pertaining to the structure, activity, expression, localization and function of these proteins. Here, an integral notion as to the precise activities of nsLTPs is provided based on the interrelated information generated from previous investigations into the functional roles of the protein at different biological levels (Fig. 3).

nsLTP expression is regulated through interaction between cis-elements within the promoter and upstream regulatory elements, such as transcription factors or protein kinases, which alter expression at different development stages, in specific organs and tissues, as well as in reaction to complex biotic and abiotic stress pressures, thus determining the spatiotemporal expression patterns of nsLTPs (Jung *et al.*, 2003; Jang *et al.*, 2005; Lu *et al.*, 2005; Kielbowicz-Matuk *et al.*, 2008; Guo *et al.*, 2013*a*, *b*; Edstam *et al.*, 2014). The presence of a signal peptide in the N-terminus region is predicted to be required for protein secretion, which determines subcellular localization or relocalization, as shown microscopically (Kader, 1997; Maldonado *et al.*, 2002; Dani *et al.*, 2005; Pagnussat *et al.*, 2012; Ambrose *et al.*, 2013; Edstam *et al.*, 2013) (Fig. 3).

Like most proteins, structural characteristics impart biochemical features to nsLTPs. The hydrophobic cavity determines the capability of binding and transporting of lipid materials, the CaM-binding sites modulate $Ca2^{+}$ interactions, and the disulfide bonding confers heat stability and proteolytic resistance to nsLTPs (Fig. 3). These characteristics are clearly reflected at the cytological level, where nsLTPs function in stabilization of membranes, permeabilization of pathogenic membranes, cell wall organization, cuticle formation, signal translocation, and as plant allergens. From a resistance physiological point of view, nsLTPs play critical and multifaceted roles in biotic stress, like disease defence, abiotic stress, drought, cold and high salinity resistance. Regarding plant growth and development, nsLTPs function pivotally in embryogenesis, sexual reproduction, seed development and germination, and in nodule organogenesis (Fig. 3).

nsLTP stabilization of membranes under stress has been explicitly demonstrated, particularly under cold stress (Hincha *et al.*, 2001; Bubier and Schläppi, 2004). The mechanism involves the ability of nsLTPs to participate in hydrophobic interactions and stable binding with membrane lipids, thereby reducing lipid fluidity and decreasing the solute permeability of the membrane (Hincha *et al.*, 1997). This, in turn, reduces the diffusion rates of solutes across the membrane during cooling and freezing, therefore preventing osmotic membrane rupture during thawing. This adaptive process is associated with numerous biochemical processes involving changes to membrane lipid composition, in the maintenance of cellular integrity under stress (Örvar *et al.*, 2000).

Plant nsLTPs are capable of forming short-lived, unstable complexes with membrane lipids, which is a prerequisite for transferability, as stable binding would preclude rapid transfer (Hincha *et al.*, 2001). The role of nsLTPs in membrane stabilization under stress is associated with a loss in lipid transfer activity (Hincha *et al.* 2001). The same loss of transfer activity was also observed with antimicrobial nsLTP from onion seeds (Cammue *et al.*, 1995), which, however, showed membrane-destabilizing effects with artificial liposomes (Tassin *et al.* 1998). Likewise, studies on the inhibitory effects of nsLTPs on phytopathogens indicate nsLTPs interference with the membranes of target organisms, leading to a loss in membrane integrity (Kader, 1996; Caaveiro *et al.*, 1997; Regente and De La Canal, 2000; Regente *et al.*, 2005; Diz *et al.*, 2006). Two possible mechanisms may explain how nsLTPs members exert their functions to stabilize membrane of plants or destroy pathogen membranes. Firstly, limited structural variety among members of the nsLTP family may underlie the

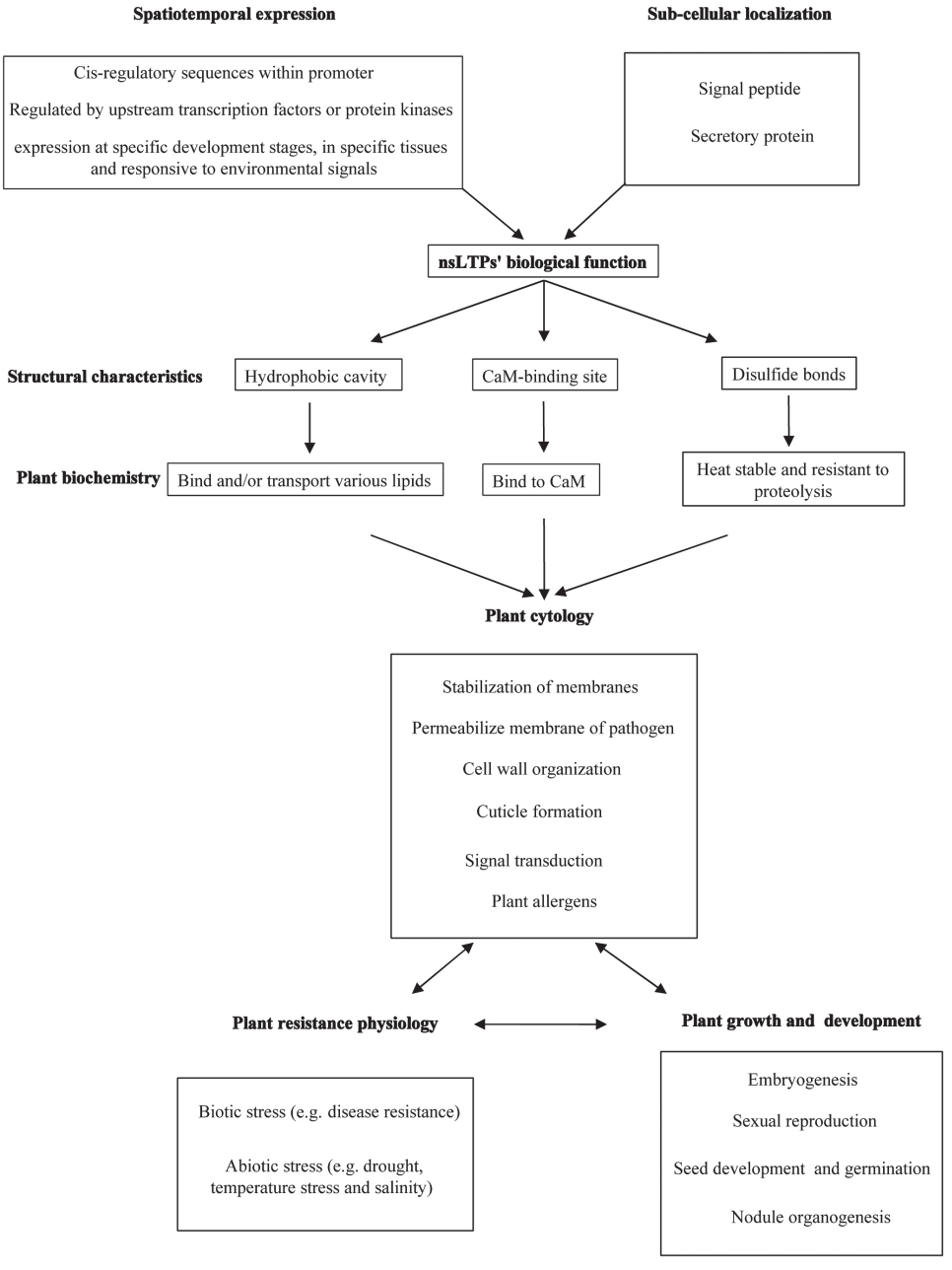

Fig. 3. Diagram of nsLTPs' functions associated with biochemical and structural features and their resulting roles at different levels including plant cytology, plant resistance physiology, and plant growth and development.

dramatic variety in biochemical activities displayed by these proteins. Secondly, modulated signalling may allow nsLTPs to switch between modulating the membranes of the plant, and those of the invading pathogens.

Localization of nsLTPs to the epidermal cell walls is generally consistent with a role in assembly or deposition of cell wall or cuticular structural material (Sterk *et al.*, 1991). In tobacco, *TobLTP2* facilitates cell wall loosening and extension through interaction of the binding cavity with hydrophobic molecules in the cellulose/xyloglucan network of the cell wall following TobLTP2 secretion. Cell wall loosening by nsLTPs may be instrumental in the initiation of cell expansion, or local and directional growth leading to cell specialization (Nieuwland *et al.*, 2005). *A. thaliana* LTPG plays an instrumental role in guiding cell geometry. It is hypothesized that wax-laden LTPG is targeted to functional sites, sealing the vulnerable border surrounding cell-cell junctions, and assisting in cell wall fortification and cuticular wax deposition. During cellular morphogenesis, changes in cell shape and cell junction topology are fundamental to normal tissue and organ development (Ambrose *et al.*, 2013). The external pollen cell wall, or exine, protects the pollen grain from dehydration and other environmental damage to maintain the reproductivity of the microspore, facilitates pollen-stigma interactions, and releases the pollen tube to effect fertilization (Blackmore *et al.*, 2007). Synthesis of lipidic components in anthers, including the pollen exine, is essential for plant reproductive development. nsLTPs play a central role in the assembly of pollen exine

during anther development, as the constituents are secreted from the tapetum in the synthesis microspore exine (Jung *et al.*, 2006; Zhang *et al.*, 2010; Huang *et al.*, 2013).

Plant nsLTPs are thought to be traffickers of cutin and wax to the plant surface for assembly and deposition of the cuticle. Evidence for this role is based on their abundant expression in epidermal cells (Thoma *et al.*, 1993), their secretion to the extracellular matrix (Sterk *et al.*, 1991) and specific structural features such as their small size allowing infiltration of pores in the plant cell wall (Baron-Epel *et al.*, 1988). The cuticle is made up of hydrophobic protective layers, which seal and protect the plant shoot, and can adapt to various biotic and abiotic stresses. The cutin monomer 16-hydroxypalmitic acid has been identified as the signal mediator *OsLTP5* in the response of rice plants to pathogen invasion (Kim *et al.*, 2008). The *ltpg1* mutant displays an increased susceptibility to infection by fungi, providing further evidence supporting a role for nsLTPs in cutin deposition (Lee *et al.*, 2009). In addition, nsLTP involvement in the formation of a protective layer of cutin in the cell wall, surrounding the young embryo, is necessary for initiation of the somatic embryogenesis pathway (Pedroso and Pais 1995; Potocka *et al.*, 2012).

Lipids, and their derivatives, are involved in many important cell-signalling pathways. A relatively large number of investigations have been carried out into nsLTP involvement in defence signalling, such as the aforementioned long distance signalling association with SAR through interaction with a lipid molecule. Moreover, the control of bacterial infection by antimicrobial peptides, such as nsLTPs, seems to be a common phenomenon during symbiosis, so there is possibly a similar role of nsLTPs involved in signalling between rhizobia and host cells during nodule organogenesis, through interactions with the rhizobia plasma membrane (Pii *et al.*, 2009, 2010, 2013; Lei *et al.*, 2014). During compatible pollination, adhesion between the pollen tube and the stigma and/or stylar transmitting tract is an essential aspect of the process (Sanders and Lord, 1989). In the pistil, SCA and SCA-like nsLTPs are secreted and endocytosed into the pollen tube tip, where they function in establishing or maintaining cell polarity at the tip of pollen tubes and forming an adhesive matrix in the pollen tube cell wall with pectin that guides pollen tubes to the ovules. The process is involved in a signal transducer with hierarchy (Mollet *et al.*, 2000; Park *et al.*, 2000; Lord and Russell, 2002; Kim *et al.*, 2006; Chae *et al.*, 2007, 2009). This highly orchestrated pollen-pistil interaction, and the associated signalling events, enables the plant species to avoid inbreeding and outcrossing, thus providing a species-specific barrier in sexual reproduction.

Calcium ions act as intracellular second messengers, which relay extracellular signals from the cell membrane, including various phytohormones, lipids and their derivatives, into intracellular signalling pathways. Calmodulin is a multifunctional Ca^{2+} sensor, which acts as a mediator of intracellular Ca^{2+} signal transduction pathways, and represents a critical component of the inducible repertoire of biotic and abiotic stress in plants. nsLTPs participate in CaM-mediated plant signal transduction through their conserved CaM-binding site (Wang *et al.*, 2005, 2008; Gao *et al.*, 2009).

The structural resilience of nsLTPs, as relayed in their thermal stability and proteolytic resistance displayed by nsLTPs, underlies their role as active allergen forms in plant foods. Increased understanding of the structure and function of nsLTPs may help to develop novel therapies for allergies. The robustness of these proteins underlies their resilience during abiotic stress response and defence activities towards blight or pathogens (Lindorff-Larsen and Winther, 2001; Sancho *et al.*, 2005). Supporting evidence for this link between structure and function is provided in food allergens often showing homology to PR-14 type proteins, such as nsLTPs.

A number of nsLTPs isolated from the seeds of different species have been characterized as antimicrobial peptides, due to their strong antifungal and/or antibacterial activity *in vitro*, including onion (Cammue *et al.*, 1995), radish (Terras *et al.*, 1992), maize (Sossountzov *et al.*, 1991), sunflower (Regente and De La Canal, 2000), mung bean (Wang *et al.*, 2004; Lin *et al.*, 2007), wheat (Boutrot *et al.*, 2005), *Brassica campestris* (Lin *et al.*, 2007), chilli pepper (Diz *et al.*, 2011), cumin (Zaman and Abbasi, 2009), coffee (Zottich *et al.*, 2011) and lentil (Gizatullina *et al.*, 2013), participating in seed defence against microorganisms. Studies on HaAP10 from sunflower seeds have helped us to understand the links between seed germination and pathogen defence. HaAP10 displays antifungal activity *in vitro*, and the ability to disturb phospholipid layers leading to fungal membrane permeabilization (Regente *et al.*, 2005). HaAP10 localizes extracellularly in dry seeds, but upon imbibition, is rapidly targeted to intracellular oil mobilization-related structures (Pagnussat *et al.*, 2012), indicating a shift in the role of HaAP10 from seed protection to mobilization of seed storage lipids in order to sustain seedling growth during germination.

The overall complexity of the nsLTP family can be observed in the multitude of functions carried out by nsLTPs, often encoded by the same gene. *AtLTP1* in Arabidopsis carries out roles in cuticle deposition (Thoma *et al.*, 1994), CaM binding (Wang *et al.*, 2005), and stigma and pollen adhesion (Chae *et al.*, 2010). *LTPG* in Arabidopsis is also involved in cuticle deposition (Debono *et al.*, 2009; Lee *et al.*, 2009), as well as in pathogen resistance (Lee *et al.*, 2009), and cell wall organization (Ambrose *et al.*, 2013). *CaLTP1* in pepper (*C. annuum*) is involved in protecting anther tissues (Hong *et al.*, 2001), pathogen resistance (Jung *et al.*, 2003), tolerance to NaCl and drought stresses (Jung *et al.*, 2005). *BraLTP1* in *Brassica rapa* is involved in wax deposition, with additional effects on cell division and flower development (Liu *et al.*, 2014). A translated nsLTP gene may not fulfill all functions ascribed to the protein, but instead, may produce one or several isoforms, which cooperatively accomplish specific functions in specialized tissues, during certain developmental stages, or depending upon environmental conditions.

Outlook

nsLTPs play multifaceted and key roles in plant architecture and in the adaptation of plants to their environment. Most efforts in the functional characterization of nsLTPs have so far been focused on type I, II and III nsLTPs; further studies are needed into the functional roles of the other types

(Liu *et al.*, 2014). It will also become necessary to establish the precise relationship between nsLTP groupings and functions. Systematic analyses based on gene expression profiles using microarray or RNA-seq data will help to develop a more informative and comprehensive description of the different categories of *nsLTP*s (Suh *et al.*, 2005, Edstam *et al.*, 2013). In addition, the precise mechanisms whereby nsLTPs interact with lipids, and the specific relation between their structures and the activities during the binding and transport process, is not fully understood. Further elucidation of nsLTP activity at the biochemical and cytological level will facilitate a better understanding of nsLTP functionality. Spatiotemporal analysis of nsLTP expression and activity at different stages of development can be further employed in determining the *in vivo* activity of the protein. A focus of future work should be on using reverse genetics to answer some of these questions. In recent years, the omic (transcriptomics, proteomics and metabolomics) technologies have formed a pillar for methods in research into gene function and regulation of the expression and activity of many proteins. The integration of large sequence and transcriptional datasets with further downstream biochemical analyses and transgenic studies will be a catalyst for discoveries into nsLTP function and evolution, and will provide clarification of the precise activities of the protein *in planta*. This review links a comprehensive body of information on nsLTP form and function, presenting the inherent connections among nsLTPs at different biological levels, thus facilitating our understanding of this very complex family of plant proteins, and accelerating the application of our current knowledge into various plant improvement initiatives for increased quality and stress resistance.

Acknowledgements

This work was supported by a Major Research Project of CAAS Science and the Technology Innovation Program at the National Natural Science Foundation of China (grant no: 31400243 and 31201152), and by the Natural Science Foundation of Hubei Province (grant no: 2013CFB423).

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