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

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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 stabilization 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 nsLTPs 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 fulfill 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-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
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 disulphide bond linkages of type I at C₁-C₄ and C₂-C₆ differ from those of type II at C₁-C₅ and C₃-C₆ (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, ~9kDa) and nsLTP2 (type II, ~7kDa) (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**

<table>
<thead>
<tr>
<th>Year</th>
<th>Type</th>
<th>Classification standard</th>
<th>Type</th>
<th>Remark</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996</td>
<td>2</td>
<td>Molecular weight</td>
<td>I, II</td>
<td>/</td>
<td>Most monocotyledonous and dicotyledonous plants</td>
<td>Kader, 1996</td>
</tr>
<tr>
<td>2005</td>
<td>/</td>
<td>Sequence similarity</td>
<td>III</td>
<td>III is a new type</td>
<td>Wheat</td>
<td>Boutrot et al., 2005</td>
</tr>
<tr>
<td>2008</td>
<td>9</td>
<td>Sequence similarity, intervals of eight cysteine residues</td>
<td>I to IX</td>
<td>/</td>
<td>Rice, Arabidopsis and wheat</td>
<td>Boutrot et al., 2008</td>
</tr>
<tr>
<td>2010</td>
<td>10</td>
<td>Sequence similarity, intervals of eight cysteine residues</td>
<td>I to X</td>
<td>X is a new type</td>
<td>Solanaceae</td>
<td>Liu et al., 2010</td>
</tr>
<tr>
<td>2012</td>
<td>5</td>
<td>Sequence similarity matrix, properties of 8-cysteine motifs</td>
<td>I, II, III, IV, V</td>
<td>Corresponds to types I, II, IV, V, VI in Boutrot’s nine-type system</td>
<td>121 species</td>
<td>Wang et al., 2012</td>
</tr>
<tr>
<td>2014</td>
<td>9</td>
<td>Sequence similarity, intervals of eight cysteine residues</td>
<td>I to XI</td>
<td>XI is a new type</td>
<td>Brassica rapa</td>
<td>Li et al., 2014</td>
</tr>
<tr>
<td>2011</td>
<td>10</td>
<td>Sequence similarity, GPI modification site, intron position and spacing between the cysteine residues</td>
<td>I, II, C, D, E, F, G, H, J, K</td>
<td>Types I and II are the same as above</td>
<td>Green and red algae, liverworts, moss, lycopsids, ferns and conifers</td>
<td>Edstam et al., 2011</td>
</tr>
</tbody>
</table>
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 Arabidopsis, 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 relocated to intracellular organelles.
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; Coutsos-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., 2013b). 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., 2013b). 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 various 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.,

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

<table>
<thead>
<tr>
<th>Classification</th>
<th>Family</th>
<th>Common name</th>
<th>Species</th>
<th>Gene numbers</th>
<th>Reference</th>
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<td>Red algae</td>
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<td>Porphyra yezoensis</td>
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<td>Edstam et al., 2011</td>
</tr>
<tr>
<td>Green algae</td>
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<td>Mesostigma</td>
<td>Mesostigma viride</td>
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<td>Marchantiaceseae</td>
<td>Marchantia</td>
<td>Marchantia polymorpha</td>
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</tr>
<tr>
<td>Moss</td>
<td>Funariaceae</td>
<td>Physcomitrella</td>
<td>Physcomitrella patens</td>
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<td>Lycopod</td>
<td>Selaginellaceae</td>
<td>Selaginella moellendorffii</td>
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<td>Adiantaceae</td>
<td>Adiantum</td>
<td>Adiantum capillus-veneris</td>
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</tr>
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<td>Gymnosperms</td>
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<td>Loblolly pine</td>
<td>Pinus taeda</td>
<td>43</td>
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<td>Lilaceae</td>
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<td>Allium cepa</td>
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<td>Musaceae</td>
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<td>Musa nana</td>
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<td>52</td>
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<td>Brassica rapa</td>
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</tr>
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<td>Coffea arabica</td>
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<td>Cotta et al., 2014</td>
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<tr>
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<td>Coffee</td>
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<td>Cotta et al., 2014</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>Capsicum annuum</td>
<td>19</td>
<td>Liu et al., 2010</td>
</tr>
<tr>
<td>Angiosperm (dicot)</td>
<td>Solanaceae</td>
<td>Garden petunia</td>
<td>Petunia hybrida</td>
<td>10</td>
<td>Liu et al., 2010</td>
</tr>
<tr>
<td>Angiosperm (dicot)</td>
<td>Vitaceae</td>
<td>Grapevine</td>
<td>Vitis vinifera</td>
<td>6</td>
<td>Wang et al., 2014</td>
</tr>
</tbody>
</table>
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 lipid binding or transfer ability between membranes (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
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 lipophilic 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 relocated 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

Table 3. Summary of nsLTP sub-cellular localization studies

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Experimental method</th>
<th>Distribution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP1</td>
<td>Barley (Hordeum vulgare)</td>
<td>Detected in cell culture medium</td>
<td>Extracellular</td>
<td>Mundy and Rogers, 1986</td>
</tr>
<tr>
<td>EP2</td>
<td>Carrot (Daucus carota)</td>
<td>Detected among extracellular proteins in embryogenic cell cultures</td>
<td>Extracellular</td>
<td>Sterk et al., 1991</td>
</tr>
<tr>
<td>Four LTPs</td>
<td>Grape (Vitis vinifera)</td>
<td>Purification from extracellular medium of somatic embryo cultures</td>
<td>Extracellular</td>
<td>Coutsos-Thevenot et al., 1993</td>
</tr>
<tr>
<td>DIR1</td>
<td>Arabidopsis (Arabidopsis thaliana)</td>
<td>Detected in petiole exudate</td>
<td>Extracellular</td>
<td>Maldonado et al., 2002</td>
</tr>
<tr>
<td>Two LTPs</td>
<td>Tobacco (Nicotiana tabacum)</td>
<td>Proteomic analysis of apoplastic fluid</td>
<td>Extracellular</td>
<td>Dani et al., 2005</td>
</tr>
<tr>
<td>Two LTPs</td>
<td>Soybean (Glycine max)</td>
<td>Proteomic analysis of xylem and apoplastic fluid</td>
<td>Extracellular</td>
<td>Djordjevic et al., 2007</td>
</tr>
<tr>
<td>One LTP</td>
<td>Medicago (Medicago truncatula)</td>
<td>Secreted proteins isolated and identified in suspension cultures</td>
<td>Extracellular</td>
<td>Kusumawati et al., 2008</td>
</tr>
<tr>
<td>LTP1</td>
<td>Arabidopsis (Arabidopsis thaliana)</td>
<td>Immunocytochemical studies using a polyclonal antibody against a fusion protein</td>
<td>Cell wall</td>
<td>Thoma et al., 1993</td>
</tr>
<tr>
<td>WAX9</td>
<td>Broccoli (Brassica oleracea)</td>
<td>Immunogold labelling studies</td>
<td>Cell wall</td>
<td>Pyee et al., 1994</td>
</tr>
<tr>
<td>CaLTP1</td>
<td>Pepper (Capsicum annuum)</td>
<td>35S::CaLTP1:smGFP introduced into detached pepper leaves through biolistic gene bombardment</td>
<td>Cell wall</td>
<td>Park et al., 2002</td>
</tr>
<tr>
<td>LTPG</td>
<td>Arabidopsis (Arabidopsis thaliana)</td>
<td>Gene promoter::YFP-LTPG transgenic experiment</td>
<td>Plasma membrane</td>
<td>Debono et al., 2009</td>
</tr>
<tr>
<td>LTPG1</td>
<td>Arabidopsis (Arabidopsis thaliana)</td>
<td>Tranferanation of Arabidopsis protoplast and tobacco epidermal cell with P3SS::LTPG1:GFYP</td>
<td>Plasma membrane</td>
<td>Lee et al., 2009</td>
</tr>
<tr>
<td>PpLTP2</td>
<td>Physcomitrella (Physcomitrella patens)</td>
<td>35S::YFP-PpLTP2 fusion protein expressed in P. patens protoplasts and in tobacco leaves</td>
<td>Plasma membrane</td>
<td>Edstam et al., 2014</td>
</tr>
<tr>
<td>LTP3</td>
<td>Arabidopsis (Arabidopsis thaliana)</td>
<td>P3SS::LTP3-GFP Arabidopsis protoplast transformation</td>
<td>Cytoplasm</td>
<td>Guo et al., 2013b</td>
</tr>
<tr>
<td>SsLTP1</td>
<td>Eggplant (Solanum gosogardinum)</td>
<td>Western analysis of soluble, apoplastic and membrane fractions</td>
<td>Intracellular</td>
<td>Kielbowicz-Matuk et al., 2008</td>
</tr>
<tr>
<td>nsLTP</td>
<td>Castor bean (Ricinus communis)</td>
<td>Immunolocalization experiments, cell fractionation analysis</td>
<td>Glyoxysome matrix and cell wall</td>
<td>Tsuboi et al., 1992</td>
</tr>
<tr>
<td>ns-LTP1e</td>
<td>Wheat (Triticum aestivum)</td>
<td>Immunolocalization experiments</td>
<td>Aleurone granules</td>
<td>Dubreil et al., 1998</td>
</tr>
<tr>
<td>LTP</td>
<td>Cowpea (Vigna unguiculata)</td>
<td>Immunolocalization of tissue sections and sub-cellular fractionation analysis</td>
<td>Extracellular space, cell wall, intracellular localization in protein storage vacuoles and in lipid-containing vesicles</td>
<td>Carvalho et al., 2004</td>
</tr>
<tr>
<td>HaAP10</td>
<td>Sunflower (Helianthus annuus)</td>
<td>Sub-cellular fractionation analysis, fluorimmunolocalization studies</td>
<td>Apoplastic, plasma membrane, intracellular</td>
<td>Pagnussat et al., 2009</td>
</tr>
<tr>
<td>Ca-LTP1</td>
<td>Pepper (Capsicum annuum)</td>
<td>Immunolocalization experiments, western blotting of seed exudate proteins</td>
<td>Intracellular vesicles, and extracellular space</td>
<td>Diz et al., 2011</td>
</tr>
<tr>
<td>HaAP10</td>
<td>Sunflower (Helianthus annuus)</td>
<td>Fluorimmunolocalization experiments</td>
<td>Apoplastic (in dry seeds), intracellular organelles of oil bodies and glyoxysomes (upon imbibition)</td>
<td>Pagnussat et al., 2012</td>
</tr>
</tbody>
</table>
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., 2013a), cutin and wax metabolisms (Debonyo 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, 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; Zöttich 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, CALTP1 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 elicitors. The elicitors 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; Pi 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., 2013a, 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 (Trevisio and MA, 1998; Jang et al., 2002, 2004; Jung et al., 2003; Wu et al., 2004; Cameron et al., 2006a, b; Kielbowicz-Matuk et al., 2008; Guo et al., 2013a, 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., 2013a, b; Edstam et al., 2014), high temperature (Wu et al., 2004), salt (Jung et al., 2003; Wu et al., 2004; Jung et al., 2004; Kielbowicz-Matuk et al., 2008; Choi et al., 2008; Wang et al., 2009; Guo et al., 2013a), 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., 2006a; Maghuly et al., 2009). (ii) nsLTPs also show responses to the abiotic stress-related plant hormones, including abscisic acid (ABA) (Trevisio 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., 2013a;
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., 2013a, b), cold stress (LTP3) (Guo et al., 2013b), and salinization (AZII 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., 2013b), and the null mutant azil 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., 2013b). Also, the lipid transfer protein AZII 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., 2006a).

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; Garcia-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 nsLTPs (Pyee et al., 1994; Hendriks et al., 1994; Sohal et al., 1999; Kunst and Samuels, 2003; Cameron et al., 2006b; 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 clytendron or hypocotyl during seed germination (Souleri 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 clytendrons 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; Souleri et al., 1996; Suvelles and Puigdoménech, 1997; Clark and Bohnert, 1999; Botton 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.,
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 (Nieuwdant et al., 2005). Arabidopsis LTPs 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 symbiotic membranes and in nodule organogenesis associated with infection thread formation (Lei et al., 2014). MtN5 expression was induced in Medicago truncatula 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 (Pit et al., 2009, 2010, 2013).

The ubiquitous Ca\textsuperscript{2+}-binding protein, calmodulin (CaM), regulates the activity of many Ca\textsuperscript{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\textsuperscript{2+}-independent CaM-binding protein (Wang et al., 2005), and in Brassica chinensis, BeLTP exhibits both Ca\textsuperscript{2+} dependent and independent binding to CaM, in turn facilitating BeLTP lipid binding capability via Ca\textsuperscript{2+} mediated signalling (Wang et al., 2008). In potato (Solanum tuberosum), StLTPa7 has been identified as a possible Ca\textsuperscript{2+}-responsive plant defence gene, due to an increase in StLTPa7 transcripts as Ca\textsuperscript{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\times 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 those from 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; Ciardelli 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 algae. 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 seceded plants. The adoption of novel nsLTP 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 (Jung 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 nsLTPs 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 nsLTPs 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 nsLTPs 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., 2013a, 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 (Orvar 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-stabilizing effects with artificial liposomes (Tassin et al., 1998). Likewise, studies on the inhibitory effects of nsLTPs on phytopathogens indicate nsLTP interference with the membranes of target organisms, leading to a loss in membrane integrity (Kader, 1996; Cavaire 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
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-hydroxy palmitic 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 (Lindorf-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). BratLTP1 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 nsLTPs (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 planta 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 clarifying the precise activities 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.

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