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REVIEW

Lipid transfer proteins: structure, classification and prospects of genetic engineering for improved disease resistance in plants

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Abstract

Plant non-specific lipid transfer proteins (nsLTPs) are small, basic, and cysteine-rich proteins found abundantly in higher plants. Apart from main processes like the membrane stabilization, cell wall organization, cuticle synthesis, plant growth and development, and signal transduction, nsLTPs have an active role in abiotic and biotic stress tolerance. Their structure consists of a conserved motif with eight-cysteine residues, stabilized by four disulfide bonds that make an inner hydrophobic cavity for ligand binding. This structural conformation renders stability and means for the transport of a variety of hydrophobic molecules. The nsLTPs possess significant inhibitory activity against the pathogenic microorganisms and thus make a part of the immunity in the plant's defense system. Due to their small size, LTPs penetrate the fungal and bacterial membrane, creating pores that cause the efflux of the intracellular ions and eventually the cell death. Several genes encoding LTPs with antimicrobial potential have been integrated and overexpressed in plants either alone or in combination with other peptides for improved disease resistance. This review summarizes nsLTPs, their structural characteristics, and expression in various plant species to combat phytopathogens with enhanced disease resistance.

Key message

The development of classification system for nsLTPs, isolated from different plant species with their identified role.

Keywords Nonspecific-lipid transfer proteins (ns-LTPs) · AMPs · Genetic engineering · Disease resistance

Abbreviations

ns-LTPs	Nonspecific-lipid transfer proteins
AMPs	Antimicrobial peptides
8CM	Eight cysteine motif
PR	Pathogenesis-related
3D	Three dimensional
GPI	Glycosylphosphatidylinositol-anchor

Introduction

In plants, lipids are actively involved in regulating cellular activities and mediating responses to different stresses during growth and developmental stages. Lipids build and maintain membrane structure and store energy for regulating metabolic pathway machinery as well as build a surface cuticle layer, thus protecting plants from aridity during drought conditions. The membrane lipids are responsible for mediating cell signaling associated with environmental responses. The non-specific lipid transfer proteins (nsLTPs) in plants make a large protein family, and constitute up to 4% of the total soluble plant proteins (Liu et al. 2015). They are the most functional proteins in plants that can bind and transport lipids across the membrane and comprised of various plant lipids with broad specificity thus referred to as non-specific

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LTPs (Yeats and Rose 2008; Salminen et al. 2016). Initially discovered in 1970 (Abdelkader and Mazliak 1970), they had been termed phospholipid exchange proteins, but later in the 1990s, these proteins have been renamed as lipid transfer proteins (Vergnolle et al. 1992).

Although the first LTP was determined approximately 50 years ago, remarkable progress has been made in terms of elucidating the functions of the LTPs in the recent past. Since then, several other nsLTPs from bacteria, yeast, plants, and mammals have been purified, and thoroughly studied in free cells and intact cells (Peretti et al. 2020). LTPs have high expression in plant tissues (below and above the ground) and are usually present in extracellular spaces. A large gene family in seed plants encodes LTPs; however, small gene families of up to 50 members encode LTPs in the non-flowering plants like bryophytes and ferns (Edstam et al. 2011, 2014; Wei and Zhong 2014). The family of *nsLTP* genes exhibits different expression patterns during growth and developmental stages, and in different physiological stress conditions (Wei and Zhong 2014). However, all nsLTPs share few biochemical similarities. For example, they are all small proteins with low molecular weight, with a basic isoelectric point from 9 to 11. Their structure is comprised of highly conserved residues, and is highly stable against heat, proteases and denaturant agents. Moreover, all the nsLTPs are generated in the plant cells as pre-proteins, usually comprising of an N-terminal signal peptide as a pre-requisite for their secretion into sub-cellular localization (Wang et al. 2012). Other reports also show updates on this interesting and important feature of nsLTPs (Salcedo et al. 2007; Ng et al. 2012; Edqvist et al. 2018; D'Agostino et al. 2019; Missaoui et al. 2022).

The plant nsLTPs are soluble, secreted, abundant, and cysteine (Cys)-rich proteins of 6 to 12 kDa (Li et al. 2022a, b). These Cys-rich proteins comprised of 8 cysteine residues, are arranged in an 8CM pattern, stabilizing the folding of 4 or 5 α-helices. The formation of 4 disulfide bridges results in an interior hydrophobic cavity that have a ligand-binding site for fatty acids such as fatty acyl-CoA, and other lipids such as glycolipids, phospholipids, and cutin monomers (Kader 1996; Carvalho and Gomes 2007; Salminen et al. 2016). Based on primary structure similarity and molecular size, initially, LTPs were grouped into two sub-families, nsLTP1 and nsLTP2, until new proteins of intermediate characteristics have been discovered. Thus considering other features like the position of evolutionarily conserved introns, amino acids sequence resemblance, post-translational modification, and the cysteine pattern, comprehensive systems of classification have been proposed, according to which LTPs were classified into ten sub-families (Boutrot et al. 2008; Liu et al. 2010; Wang et al. 2012; Edstam et al. 2011, 2014; Edqvist et al. 2018).

LTPs, initially, were thought to be responsible for transfer of the fatty acids and lipids across the cell membrane (Kader et al. 1984; Kader 1975). However, subsequent studies recommended other functions, including cell-wall loosening activities, the synthesis of protective cuticle layer, cell signaling and mediating cellular responses, cell proliferation, somatic embryogenesis, wax deposition, seed storage, flower development, and fiber elongation. They also perform different functions during the biosynthesis and assemblage of lipid-based polymers like suberin and sporopollenin (Nieuwland et al. 2005; Debono et al. 2009; Lee et al. 2009; Zhang et al. 2010; Kim et al. 2012; Liu et al. 2014; Deeken et al. 2016; Jacq et al. 2017; Meng et al. 2018). In the past decades, several reports investigated the role of nsLTPs in abiotic stress resistance (Guo et al. 2013; Yu et al. 2014; Gonzalez et al. 2017; König et al. 2018; Xu et al. 2018a, b; Akhiyarova et al. 2019). In the context of biological activity, being small in size, the LTPs are believed to penetrate cell membrane of the pathogens leading to flowing out of the intracellular ions and cell death (Selitrennikoff 2001). As the first in vivo functions related to some nsLTPs was their role in plant protection by inhibiting the growth of phytopathogens, several reports described their involvement in biotic stress tolerance (García-Olmedo et al. 1995; Molina et al. 1996; Jung et al. 2005; Sun et al. 2008; Zhu et al. 2012; Gangadhar et al. 2016; Nawrot et al. 2014; Iqbal et al. 2019). For this reason, the nsLTPs have been categorized in the pathogenesis-related proteins (PR-14) (Blein et al. 2002). Antimicrobial potential of the nsLTPs and their overexpression in several plant species for enhanced disease resistance against pathogens will be the primary focus of this review.

Classification of ns-LTPs

As mentioned earlier, LTPs were initially classified based on their molecular size into two types, LTP1 and LTP2. The LTP1 were basic proteins of molecular mass 9-kDa and found abundantly in higher plants (Ng et al. 2012), whereas type 2 LTPs of 7-kDa were isolated from cereal kernels (Kalla et al. 1994). Noticeably distinct in molecular masses, several reports indicated that both the families of nsLTPs are also dissimilar by the characteristic of the residue present between Cys5 and Cys6 that is hydrophilic in the LTP1 while apolar in the LTP2 family (Douliez et al. 2001). Later, the study on the three-dimensional (3-D) structures of proteins led to the confirmation of the hypothesis that only two main structural types of proteins could be classified. Hereafter, they were called as "Type-onefold" and "Type-twofold". The fold of type I nsLTPs was called as "Type-onefold" while the alternative fold of the Types II to XI was referred to as Type-twofold. As in the types III, IV, V, VI, VIII, IX, and XI ns-LTP, the C5 and C6 residues follow the exact spatial conformation as of type II proteins, thus so-called "Type-twofold" (Fleury et al. 2019).

Another system of classification for nsLTPs has been suggested with the investigation of 200 sequences of nsLTPS in rice, wheat, and Arabidopsis. This system is based on the sequence homology and the spacing pattern among 8 Cys residues. All these identified sequences were grouped into 9 types (type I–IX) of nsLTPs (Boutrot et al. 2008) (Table 1). Thereafter, Liu et al. (2010) identified 135 nsLTPs from Solanaceae and characterized them into 5 types (type I, II, IV, IX, and X). Type X was almost a new group that accounts for more than 50% of the Solanaceae nsLTPs (Liu et al. 2010). Boutrot et al. (2008) method of classification was modified with the identification of 63 putative ns-LTPs, grouped as type I–XI including a novel type XI (Li et al 2014). However, with the characterization of LTPs in the non-flowering plants (liverworts and mosses), these LTPs could not readily be classified within Boutrot et al. (2008) system of classification because of limited sequence similarity among nsLTPs from those of flowering plants (Wang et al. 2012). Therefore, Edstam et al. (2011) introduced a well-modified and expanded system of classification for nsLTPs which is comprised of major types including LTP1, 2, c, d, and g, and five minor types like LTP e, f, h, j, and k. Rather than their molecular sizes, this system of classification was based on the sequence homology, the location of a conserved intron, and the intervals among the eight cysteine residues. This system has also considered the post-translational modifications, for example, LTPs with a C-terminal sequence motif of a glycosylphosphatidylinositol (GPI)-anchor, are classified in the type LTP g. As this system covered various aspects of the ns-LTPs, it is more novel and robust than the previous systems of classification. However, the conventional system of classification (type LTP1 and LTP2) is also preserved.

Despite this novel system of classification described, naming the LTPs has been ambiguous for years. Salminen et al. (2016) suggested a well-defined and informative system for naming the LTPs like AtLTP1.3, OsLTP2.4, HvLTPc6, PpLTPd5, and TaLTPg7. In these, the first two letters represent the plant species (At = *Arabidopsis thaliana,* Os = *Oryza sativa*, Hv = *Hordeum vulgare* etc.). Here LTP1, LTP2, LTPc, LTPd and LTPg show the type, and the last digit (3, 4, 6, 5 and 7) indicates the specific number assigned to each gene or protein within a particular LTP type.

Zhang et al. (2019) identified 70 *nsLTP* genes in barley in a genome-wide analysis and placed these into five types, types 1, 2, C, D, and G, according to Edstam et al. (2011) system of classification. Each type of barley nsLTPs shares a similar type of exon and intron gene structures. These barley nsLTPs were found with diverse expression patterns, indicating their various roles. Similarly, a total of 89 *nsLTP* genes have been characterized from the cabbage genome and classified into six groups (type 1, 2, C, D, E, and type G). The identified *nsLTPs* genes exhibited differential and tissue-specific expression in response to the biotic and abiotic stresses and many of the genes were found to be linked to stress resistance (Ji et al. 2018). Odintsova et al. (2019) identified 243 putative nsLTPs sequences and characterized them into 6 structural types (Type 1, 2, D, G, and X). In this classification, nsLTPs with GPI-anchor were isolated and grouped in type G nsLTPs. While the other sequences (without GPI-anchor site) have been characterized as type 1, 2, D, and X, based on their specific cysteine spacing patterns (Edstam et al. 2011, 2014). Type 1 and 2 were found the same as types I and II of the Boutrot et al. (2008). Type D was then comprised of the IV, V, VI, VIII, and XI types of the Boutrot et al. (2008) and of the Li et al. (2014). Type X ns-LTPs included the sequences with unusual spacing patterns among cysteine residues.

Wang et al. (2020) identified and characterized a total of 39 *CsLTP_2* genes using databases for the cucumber-specific LTP_2. This family was differed from the typical ns-LTPs in having 5-cys motif (5CM) with the basic form CC-Xn-CXC-Xn-C. Depending on their structure and the phylogenetic relationship, the members of CsLTP_2 were categorized into six families in which nine were grouped as type I, three as type II, eight as type III, three as type IV, four as type V and 12 members were grouped as type VI of Boutrot et al. (2008) system of classification.

Li et al. (2019) carried out genome-wide characterization and identified a total of 83 *StnsLTP* genes. Based on Boutrot et al. (2008) method of classification, the identified genes were classified into eight types, keeping in view the characteristics like gene structure and protein domains, conserved motifs, phylogenetic relationship, and chromosome location. Moreover, the expression pattern of the genes was checked in various tissues and found to be expressed mainly in younger tissues of the plant, indicating their role in the development and growth of plant tissues.

Fleury et al. (2019) presented an updated and comprehensive structure-based classification of the ns-LTP superfamily using 3D structure modeling, phylogenetic analysis, and functional annotation of proteins. They investigated 797 nsLTP protein sequences and classified these proteins based on structural characteristics like sequence length and composition. Recently Fang et al. (2020) identified 330 nsLTPs in wheat and classified them into five types (type1, 2, c, d, and g). The type nsLTPs have three sub-types (d1d3) and the type g has seven sub-types (g1–g7). It was found for the first time that the GPI-anchors were also present in the non-g type nsLTPs. All the five types of nsLTPs were found unequally and unevenly distributed on the 21 chromosomes as indicated by chromosome mapping.

Table 1 Tjcbsdjvbsjdvgjsdngjdsng

Type	Classification standard	Total no. of nsLTPs	Species	Identified role	References
I, II	Molecular weight	–	Monocotyledonous and dicotyledonous plants	Cutin formation, embryogenesis, symbiosis, defense reactions	Kader (1996)
III	Sequence similarity	9	Wheat	Germination and seedling growth	Boutrot et al. (2005)
I to IX	Sequence similarity, intervals of eight cysteine residues	200	Wheat, Rice and Arabidopsis	Antimicrobial properties, developing anther and pollen wall	Boutrot et al. (2008)
I to X	Sequence similarity, intervals of eight cysteine residues	135	Solanaceae, Gramineae and Arabidopsis	Biotic and abiotic stress tolerance	Liu et al. (2010)
I, II, C, D, E, F G, H, K, J, K	Sequence similarity, GPI modification site, and spacing between the cysteine residues	146	Green and red algae, liverworts, moss, lycopods, ferns and conifers	Cuticular lipid accumulation, adaptation to stressful condition	Edstam et al. (2011)
I-V	Sequence similarity matrix, properties of 8-cysteine motifs	–	Nine plant species	Defence signalling, or vascular tissue differentiation	Wang et al. (2012)
I-XI(excluding VII)	Sequence similarity, intervals of eight cysteine residues	63	*Brassica rapa*	Antimicrobial activity	Li et al. (2014)
1,2,C,D,E and G	Homology, conserved motifs, and tertiary structure	89	cabbage	Biotic and abiotic stress resistance	Ji et al. (2018)
Eight types based on Boutrot's method		83	*Solanum tuberosum*	Growth and development of different tissues	Li et al. (2019)
1, 2, C, D, and G	Edstam's system of classification	70	Barley	Plant reproduction, biotic and abiotic stress resistance	Zhang et al. (2019)
1, 2, D, G and X	Cysteine spacing patterns	243	*Triticum kiharae*	Stress tolerance	Odintsova et al. (2019)
I to IX	Sequence length and composition	797	120 plant species	Pathogen resistance Fleury et al. (2019)	
1, 2, c, d, and g	Phylogenetic analysis	330	Wheat	Abiotic stress tolerance	Fang et al. (2020)
1, 2, D and G	Homology alignment, conserved motifs, gene structure and evolution	40, 35	Barley and Qingke	Abiotic stress tolerance	Duo et al. (2021)
I,II,IV,V and VI	Boutrot's method of classification	45	*Setaria italica*	Abiotic stress tolerance	Li et al. (2022a, b)
I, II, IV, V, VII, and VIII	Gene structures, motifs, tertiary structures, gene duplications and expression patterns	50, 51 and 100	*Nicotiana. sylvestris, N. tomentosiformis, N. tabacum*	Biotic and abiotic stress tolerance	Yang et al (2022)
I, II, III, IV, V, VI and VII	Phylogenetic analysis, gene structure, conserved motifs	283	*Brassica napus*	Plant growth, development, biotic and abiotic stress tolerance	Xue et al. (2022)

Structure function relationship

The protein tertiary structure is a basic prerequisite to finding the secrets and describing the biological roles of proteins. However, the first tertiary structure (3-D) of LTPs was shown in the early 1990s. LTPs, as reported, are generated as precursor proteins bearing an N-terminal signal peptide, generally of 21 to 27 amino acids (Edstam et al. 2011). The 3-D structures of various plants nsLTPs have been investigated using the nuclear magnetic resonance and X-ray crystallography, both in bound and in an unbound state (Lin et al. 2005; Salminen et al. 2016; Jain and Salunke 2017). All these findings recommended that the tertiary structure of LTPs is characterized by an 8 Cys motif in a sequence pattern like C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C, stabilizing the folding of 4 α-helices into a compact 3-D structure and a non-structured long C-terminal tail. The α-helix domain is stabilized by disulfide bridges forming an interior hydrophobic cavity (Cuevas-Zuviría et al. 2019) (Fig. 1). This conformation renders protein resistant to heat and proteolytic activity through intermolecular hydrogen bonds (H-bonds) and a disulfide bond linkage. Likewise, in the structural conformation of wheat LTP, the 4 amphipathic α-helices (from H1–H4) are separated by a total of three loops (L1 to L3), whereas a C-terminal component with a β-turn is located at Asn84–Cys87 position (Tassin-Moindrot et al. 2000).

Embedding of aliphatic chains of the ligands into the hydrophobic cavity of the protein is a common feature of the maize, wheat and barley nsLTPs. However, various binding modes have been discovered in various protein complexes depending on the structure of the protein and/or the ligand. Such as in the wheat and maize nsLTPs, the ligands orient linearly within the tunnel, with their polar heads pointing closer to the primary opening of the tunnel-like cavity located in loop L2 and the β-turn of the C-terminus. These complexes get stability by electrostatic interactions with the Tyr79 side-chain leading to alterations in orientation to allow the introduction of lipids within the tunnel. Within the structure of the palmitate/barley nsLTPs, ligand orientation is almost upside-down compared to the maize or wheat nsLTPs. While the barley nsLTP has a complex with palmitoyl CoA, orientation of the lipid is comparable; however, its aliphatic chain is surprisingly curved and its polar head is directed closer to the second opening of the cavity located in between the ends of helices H1 and H3. Also, in some nsLTPs, Tyr79 can form H-bonds with the polar head of lipid ligands (Melnikova et al. 2018). Furthermore, in the case of peach nsLTP1, the residues Leu 10, Ile 14, Val 17, Leu 51, Leu 54, Ser 55, and Ala 66 in peach nsLTPs have been reported to interact with the joint of the polar head and the aliphatic chain of its native ligand (Cuevas-Zuviría et al. 2019).

Together with molecular, the biophysical and crystallographic approaches have been extensively used to explore

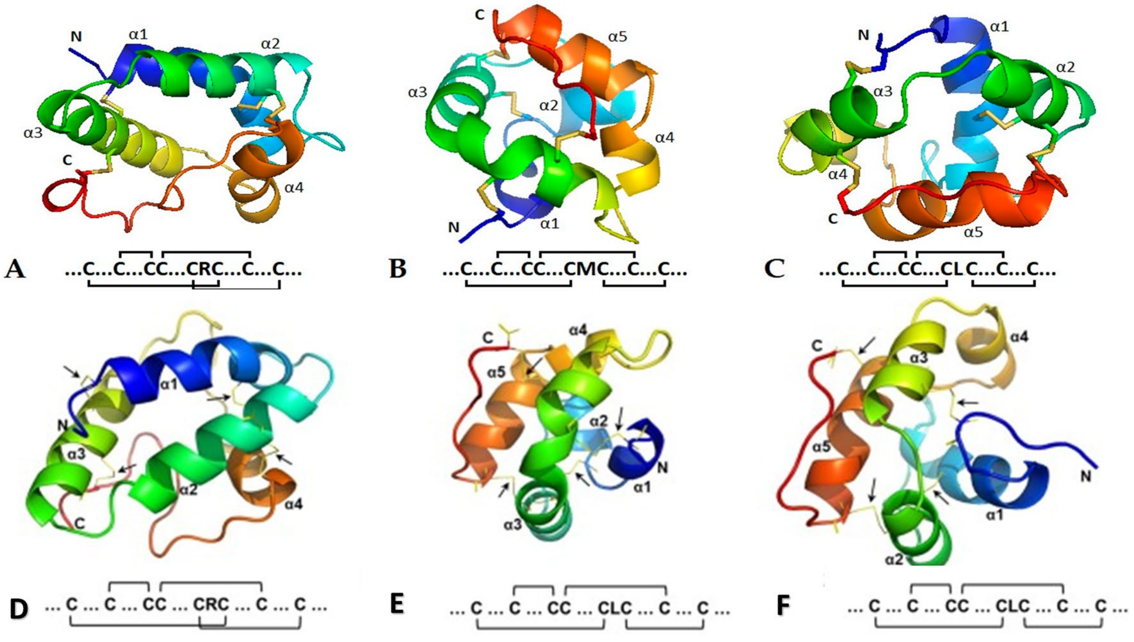

Fig. 1. 3-D structures and Cys-pairing pattern of TkLTP1.36 (**A**), TkLTP2.21 (**B**), TkLTPd7.1 (**C**), BoLTP1.5 (**D**), BoLTP2.1 (**E**) and BoLTPd15 (**F**). The disulfide bonds in the given structures are shown in yellow color. (The figure is reproduced from Ji et al. 2018; Odintsova et al. 2019)

the mechanism of action of different plants' nsLTPs (Amador et al. 2021). Studies have shown that disrupting the disulfide bonds, the key components for structure stability of plant LTPs, the inhibitory effect of the proteins towards the pathogenic microorganism as well as the ability of binding the lipid can be lost. The 3-D fold of the rice nsLTP2 consists of a triangular hydrophobic cavity formed with the aid of 3 prominent helices (Finkina et al. 2016). The four disulfide bonds, required for the structural stabilization of nsLTP2, display an exceptional pattern of cysteine pairing as compared to nsLTP1. The flexible C-terminus of the protein forms a cap over the hydrophobic cavity. Studies on molecular modeling also suggested that the hydrophobic cavity must accommodate larger molecules with an inflexible structure like sterols (Samuel et al. 2002).

The positively charged residues located on the surface of nsLTP2 have been reported to be similar in structure to other plant defense proteins. Employing site-directed mutagenesis, Ge et al. (2003) investigated the structure–function relationship of the rice LTP110. The results showed that some of the conserved residues like Tyr17, Arg46, and Pro72 have key roles in maintaining the resistance function of the LTP110. In addition, they determined that the Cys50–Cys89 disulfide bridge was important for the resistance features, in contrast to preceding reports and expectations that the disulfide bridges are crucial for antimicrobial activity.

Another study on the wheat nsLTP isoforms indicated that the replacement of only one residue i.e. Pro3Ser in TaLt10B6 and TaLt710H24 and Asn24Ser in TaLt10F9 and TaBs116G9 isoforms significantly affected the resistance function of the proteins against pathogens. It is thus deduced that the difference in only one amino acid residue results in changes in LTPs structural conformation that also affects its antimicrobial activity (Sun et al. 2008).

The real structural composition of nsLTPs and their role in biological activity was recently suggested by Fleury et al. (2019). Many residues have been found conserved in type 1 nsLTPs that corresponded to vital folding distinctions among type1 and other types of nsLTPs. As an example, Gly37, Arginine, and lysine residues at the position 51 and hydrophobic residues at the positions 87 and 89 are the conserved residues among type I nsLTPs. The residue Asparagine (Asp) at the position 259 and the isoleucine (Ile) at the position 402 were as strongly conserved as the 8 Cys residues. Three different residues placed at the positions 137, 154, and 266 of the structural alignment have been otherwise conserved inside the 3 defense clusters.

Using structural trace analysis, the specific potential residues were recognized that are liable for plant protection and pathogen resistance. The nsLTPs involved in defense have been normally found in the type I family (28 proteins), with only 3 defense nsLTPs within the type II family. Within the defense cluster trace, either valine or alanine is located at position 137, while position 154 was occupied either by leucine or by a valine residue. Similarly, position 266 was occupied by positively charged residues i.e. arginine or a lysine residue in defense proteins (Fig. 2). Site-directed mutagenesis showed that replacement of this intermediate residue at the position 63 by an alanine residue disturbed folding, ligand binding, and lipid transfer activity in the type II nsLTPs (Cheng et al. 2008). It seems interesting that alanine residues have been found at position 54 in the type I nsLTPs, while the larger hydrophobic residues mostly occupied this buried position in another nsLTP type. In the recent past, various reports have explored the structure-functions relationships of LTPs (Salminen et al. 2016; Edqvist et al. 2018).

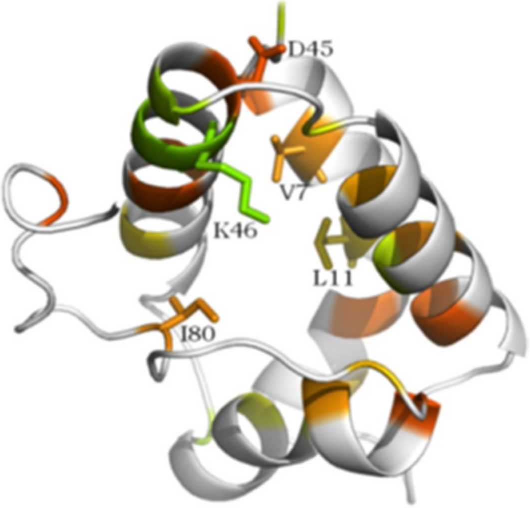

Fig. 2 3-D structure of nsLTP 525, showing conserved amino acid residues among the so-called defense cluster (Fleury et al. 2019)

LTPs as antimicrobial peptides

Plants have a natural and important mechanism to combat certain pathogens by producing natural antimicrobial peptides that can contribute to the natural defense system in plants (Owayss et al. 2020). These naturally produced antimicrobial peptides, also called as plant antimicrobial peptides (PAMPs), have been characterized based on their structure, molecular size, the charge on molecules, number of disulfide bonds, activity against different microbes, and mechanism of action. Plant AMPs inhibit the pathogen in several ways including membrane permeabilization, interfering DNA, RNA, and protein synthesis that render them stop from developing resistance (Iqbal et al. 2019; Khan et al. 2019). LTPs belong to a functional proteins family of AMPs that exhibit antimicrobial activities and are classified as PR14 class of the pathogenesis-related (PR) proteins.

Buhot et al. (2004) showed that a recombinant tobacco LTP1 had the potential to load fatty acids and jasmonic acid, and binds to specific plasma membrane sites, and was reported to be involved in activation of plant defense. Chanda et al. (2011) reported that an LTP, DIR1 has a role in translocation of Glycerol-3-phosphate (G3P), an important metabolite which induces systemic acquired resistance (SAR) in plants. Various members of the LTP class exhibit biotic stress resistance including antibacterial, antifungal, antiproliferative, and enzyme-inhibitory activities (Carvalho and Gomes 2007). Compelling pieces of evidence show that nsLTPs can play a crucial role in imparting resistance to phytopathogens.

Terras et al. (1992) isolated the first antifungal nsLTP of 9-kDa from the radish seeds. It was a dimeric, basic protein with $Pi > 10.5$, and was found highly active against a number of fungal pathogens by restricting hyphal growth instead of inhibiting spore germination. Extracts of LTPs from leaves of barley and maize exhibited strong antifungal activity against *Clavibacter michiganensis* subsp. sepedonicus, *Fusarium solani,* and antibacterial activity against *Pseudomonas solanacearum* (Molina et al. 1993). Plant ns-LTPs possess highly specific antimicrobial activity against specific pathogens. The LTPs isolated from onion seed, exhibited strong antifungal activity against a number of fungal pathogens, while a radish seed LTP showed only a moderate effect towards fungi but the LTPs from maize and wheat seeds were reported to be inactive against fungal infection (Cammue et al. 1995). Like other classes of plant AMPs, the antimicrobial activity of plant LTPs was also found to decrease in the presence of ionic solutions and their level of pathogenicity varied with the type of LTPs. The LTP from onion seeds showed the same antimicrobial activity in a low ionic containing medium and a medium containing 1.0 mM Ca^{2+} and 50.0 mM K^{+}, while in the presence of such cations, the radish LTP was found inactive (Cammue et al. 1995).

A number of nsLTPs isoforms have been described to be differentially expressed during pathogenic infections. Of the two barley genes, *HvLtp4.2* and *HvLtp4.3*, expression of the *HvLtp4.3* was found up-regulated during *Xanthomonas campestris* infection, and down-regulated with the infection by *P. syringae* pv. Japonica (Molina et al. 1996). In another study, involving promoter analysis, expression of the rice *LTP1* gene (involved in organ development) was induced by the fungal pathogen, *Magnaporthe grisea* (Guiderdoni et al. 2002). The nsLTP, Ha-AP10, from the seeds of sunflower, is reported to be the first ns-LTP, that caused membrane permeability of intact spores of the *F. solani* and affected its viability and caused even lethal effects at an elevated concentration (Regente et al. 2005). Arabidopsis DIR1 encodes nsLTP, which can provide immunity against pathogens (Maldonado et al. 2002). *CsLTP_2* genes have a tissue-specific expression in cucumber tissues. During nematode infection, the two genes showed substantial expression alteration in the roots of the susceptible and resistant lines, depicting their role in response to *Meloidogyne incognita* (Wang et al. 2020).

Some plant nsLTPs are only fungistatic while some possess fungicidal activity with the ability to cause membrane permeabilization of the pathogens like other PAMPs (Regente et al. 2005; Sun et al. 2008). As an example, LTPs isolated from barley (Caaveiro et al. 1997), onion (Tassin et al. 1998), and sunflower (Regente et al. 2005) were found to induce permeabilization of liposomes and caused its leakage. Some reports have shown temperature dependence on the activity of nsLTPs, for example, the ns-LTP (9-kDa) from a wild grass *Echinochloa crusgalli,* was reported to be more potent at lower temperature than at room temperature against zoosporangia of two major pathogenic fungi, causing late blight in tomato and potato and root rot in herbs. It was found to be more effective relatively at lower temperature than other nsLTPs (Rogozhin et al. 2009).

Ge et al. (2003) tested the purified protein extract of rice LTP110 against rice pathogens, and *Pyricularia oryzae* and *Xanthomonas oryzae* under in vitro conditions. The LTP110 strongly inhibited spore germination of the *P. oryzae* but was found slightly effective against the *X. oryzae*. LTPs-like protein with antifungal potential was isolated from seeds of chili pepper that showed a strong inhibitory effect towards *F. oxysporum, Candida tropicali, Colletotrium lindemunthianum, Candida albicans, Pichia membranifaciens,* and *Saccharomyces cerevisiae* (Cruz et al. 2010). In another experiment, *TdLTP4* of wheat, encoding an antifungal protein was isolated and overexpressed in *E. coli* and its antimicrobial activity was checked against several food-borne and spoilage bacterial and fungal pathogens. The inhibition zones and the minimal inhibitory concentration (MIC) values of the bacterial strains were found 14–26 mm and 62.5–250 µg/mL, respectively. Moreover, a significant inhibitory role against several fungal pathogens revealed the potential of *TdLTP4* to be used as an antimicrobial agent in food preservation (Hsouna et al. 2021). Many other ns-LTPs with antimicrobial activity have been reported so far, which are summarized in Table 2.

Transgenic plants overexpressing *LTPs* for enhanced disease resistance

As an effective and modern strategy regarding protection against a number of phytopathogens, transgenic approaches have been employed to modify the natural defense system of plants (Dong and Ronald 2019). Genetic engineering approaches can be employed to overcome the hazardous effects of pesticides on the environment and to reduce the cost of crop protection (Khan et al. 2006, 2010, 2011a, b). Similarly, transgenic strategies have confirmed the strong

Table 2 The identification and characterization of ns-LTPs with antimicrobial activity against targeted organism

Protein	Size (kDa)	Species	Activity	Targeted organism	References
Hv- LTP Cw-18,	6.5	*Hordeum vulgare*	Antifungal	*Fusarium solani*	Molina et al. (1993)
(PKG2316)		Antibacterial	*Pseudomonas solanacearum*		
Hv-LTP-1			*Clavibacter michiganensis*		
LTP-a1 LTP-a2	6.5	*Arabidopsis thaliana*	Antifungal	*F. solani*,	Segura et al. (1993)
LTP-s1 LTP-s2	6.5	*Spinacea oleracea*	Antibacterial	*Sepedonicus P. solanacearum*	
				C. michiganensis subsp.	
Ace-AMP1	10	*Allium cepa*	Antibacterial	*Bacillus megaterium*, *Sarcina lutea*	Cammue et al. (1995)
			Antifungal	*Alternaria brassicola*, *Ascockyta pisi*, *Botrytis cinerea*, *Colletotrickum lindemutkianum*,	
IWF1 (Bv-LTP), JWF2 (Bv-LTP2)	9	*Beta vulgaris*	Antifungal	*Cercospora beticola F.culmorum, Pyricularia oryzae*	Nielsen et al. (1996)
IWF5	9	*B. vulgaris*	Antifungal	*C. beticola*	Kristensen et al. (2000)
nsLTP	9	*Phaseolus mungo*	Antifungal	*F. solani, F. oxysporum*, *Pythium aphanidermatum*	Wang et al. (2004)
			Antibacterial	*Staphylococcus aureus*	
La-LTP (LJAFP)	6.2	*Leonurus Artemisia*	Antibacterial	*B. subtilis, P. solanacearum Ralstonia solanacearum*	Yang et al. (2005)
LJAMP2	6.2	*L. japonicas*	Antifungal	*A. brassicae, B. maydis, Rhizoctonia cerealis Aspergillus niger*, *B. maydis, F. oxysporum,*	Yang et al. (2006)
			Antibacterial	*B. subtilis, Penicillium digitatum,*	
			Antiyeast	*Saccharomyces cerevisiae*	
LJAMP1	7.8	*L. japonicas*	Antifungal	*A. alternata, C. personata, A. niger*	Yang et al. (2007)
nsLTP1	9.4	*Brassica campestris*	Antifungal	*F.oxysporum*, *Helminthosporium staivum, Mycosphaerella arachidicola, Sclerotinia sclerotiorum, Verticivium albotarum*	Lin et al. (2007)
Lc-LTP2	9.26	*Lens culinaris*	Antibacterial	*A.tumefaciens*	Finkina et al. (2007)
Lc-LTP4,	9.3				
Lc-LTP7,	9.1				
Lc-LTP8	9.1				
Cy-AMP3	9.2	*Cycas revolute*	Antifungal	*F.oxysporum*, *Geotrichum candidum*	Yokoyama et al. (2008)
			Antibacterial	*Clavibacterium michiganensis Curtobacterium flaccumfaciens*, *A. radiobacter, A. rhizogenes*, *Erwinia carobora*	
Ec-LTP	9.1	*Echinochloa crusgalli*	Antifungal	*Phytophthora infestans*, *Helminthosporium sativum*	Rogozhin et al. (2009)
LTP	9	*Capsicum annuum*	Antifungal	*F. oxysporum*, *C. lindemunthianum*, *S. cerevisiae*, *Pichia membranifaciens*, *Candida tropicalis, C. albicans*	Cruz et al. (2010)
Ca-LTP(1)	9	*C. annuum*	Antifungal	*C. lindemuthianum, C. tropicalis*	Diz et al. (2011)
Cc-LTP-1	9	*Coffea canephora*	Antifungal	*C. albicans, C. tropicalis*	Zottich et al. (2011)
Cc-LTP2	9	*C. canephora*	Antibacterial	*Xanthomonas euvesicatoria*	Bard et al. (2016)
Ps-LTP1	9.5	*Pisum sativum,*	Antifungal	*F. solani, F. oxysporum*	Bogdanov et al. (2016)

Table 2 (continued)

Protein	Size (kDa)	Species	Activity	Targeted organism	References
BrLTP2.1	7.3	*B. rapa*	Antifungal	*A. solani, F. oxysporum F. graminearum, C. trifolii Bipolaris oryzae*	Schmitt et al. (2018)
			Antibacterial	*P. syringae*	

potential of ns-LTPs to be used for enhanced pathogen resistance (Table 3). For example, Molina et al. (1993) reported that transgenic Arabidopsis and tobacco expressing the barley LTP2 showed reduced necrotic effects caused by infection by *Pseudomonas.* Their results also confirmed the biotechnological roles of plant LTPs and reinforced the idea that LTPs are potent inhibitors of phytopathogens and are involved in defense mechanisms. Similarly, the transformation of the barley *LTP* gene in the tobacco and Arabidopsis genome resulted in a decreased level of damage caused by fungal pathogens (Molina and García-Olmedo 1997).

Roy-Barman et al. (2006) produced transgenic wheat expressing Ace-AMP1 from *Allium cepa* harboring fungal disease resistance. The inhibition percentage of engineered wheat was checked through leaf assay and found to be more resistant to *Blumeria graminis* f. sp. Tritici. In addition, Ace-AMP1 increased the expression of *PR* genes when inoculated with *Neovossia indica*. Hence, transgenic plants showed a high level of salicylic acid (SA), phenylalanine ammonia lyase (PAL), glucanase, and chitinase transcripts, indicating systemic acquired resistance (SAR) during pathogen infection. This gene was also transformed in rice plants and the protein extract from leaves of transgenic lines showed a strong inhibitory effect on three rice pathogens, *Rhizoctonia solani, M. grisea,* and *X. oryzae*, in vitro. Importantly, with stable transformation and expression of the *Ace-AMP1*, the transgenic rice plants retained their agronomic properties as well as acquired strong resistance towards fungal and bacterial pathogens (Patkar and Chattoo 2006).

Zhu et al. (2012) produced transgenic *Triticum aestivum* plants overexpressing the *TaLTP5* gene that exhibited strong resistance towards *Cochliobolus sativus* and *F. graminearum***.** Fan et al. (2013) transformed *Brassica napus* with the LTP gene and reported that the disease resistance is related to lower malondialdehyde (MDA) and higher content level of SOD (superoxide dismutase) and POD (peroxidase). As the transgenic plants showed low MDA contents and high SOD and POD activities, it was concluded that the transgenic plants were more resistant to disease compared to non-transgenic. The over-expression of the *TdLTP4* in Arabidopsis plants resulted in increased resistance of the plants to abiotic stresses as the plant growth was promoted under different treatments of salt, abscisic acid (ABA), jasmonic acid (JA), and hydrogen peroxide (H_2O_2). Furthermore, the transgenic Arabidopsis expressing *TdLTP4* have been found effective against the fungal pathogens, *A. solani* and *B. cinerea*. These findings suggested the role of the *TdLTP4* gene in enhanced resistance to biotic as well as abiotic stresses in crop plants (Safi et al. 2015).

For a functional approach, Jülke and Ludwig-Müller (2015) analyzed *Arabidopsis* plants with altered expression of *LTPs* genes concerning their phenotypic expression during clubroot development including abiotic stress resistance and lipid composition in galls. Their findings showed that overexpression of the *LTP1*, *3*, *4* and *8,* caused little susceptibility to clubroot while downregulation of the genes caused higher susceptibility. In addition, the plants overexpressing *LTP1* & *3* tolerated NaCl containing media indicating its role in the adaptation to the abiotic stresses. Additionally, NaCl treatment caused growth reduction in the two lines with decreased *LTP* expression. It was suggested that *LTP1* and *LTP3* can regulate different pathways in a cross manner and their overexpression results in highly resistant plants to the clubroot pathogen as well as abiotic stress. Fahlberg et al. (2019) used reverse genetics to evaluate the role of GPI-anchored LTPs (LTPGs) in harboring resistance against non-host mildews in *Arabidopsis*. According to their findings, loss of either LTPG1, 2, 5 or 6 might increase the permeability of the *B. graminis* f. sp. *hordei* to epidermal cell wall. LTPG1 was found to be localized to papillae at the sites of *B. graminis* penetration. The study showed that LTPGs can also play role in pre-invasive defense against certain non-host pathogens causing powdery mildew. Similarly, LTPg5 was overexpressed in leaves of *Arabidopsis* upon infection with *P. syringae* and *B. cinerea*. The transgenic lines over-expressing the *LTPg5* were found more resistant to *Meloidogyne incognita*, *P. syringae,* and *B. cinerea*, whereas, a knocked-out mutant was found more susceptible to these pathogens. Although LTPg5 might have no antimicrobial activity directly but could mediate responses by associating with a receptor-like kinase, leading to the higher expression of the defense-related genes (Ali et al. 2020).

A study on the expression pattern of potato StLTP10 showed a positive role of the StLTP10 during infection by *P. infestans.* In addition, over-expression of the ROS scavenging- and defense-related genes for ABA, SA, and JA was also indicated (Wang et al. 2021). These findings provided insight into the role of StLTP10 in resistance to *P.*

Table 3 Transgenic plant overexpressing *LTPs* genes for enhanced disease resistance

Plant peptide	Origin species	Transgenic plants	Targeted pathogens	Type of transformation/method	References
LTP2	*Hordeum vulgare*	Arabidopsis, tobacco	*Pseudomonas syringae*	Nuclear/*A. tumefacien*	Molina and García-olmedo (1997)
LTPS	*Oryza sativa*	*Oryza sativa*	*Glomus mosseae*	Protoplast/electroporation	Blilou et al. (2000)
CALTP1	Pepper	Arabidopsis	*P. syringae, Botrytis. Cinerea,*	Nuclear/*A. tumefacien*	Jung et al. (2005)
Ace-AMP1	*Allium cepa*	*Oryza sativa*	*Magnaporthe grisea, Rhizoctonia solani Xanthomonas oryzae*	Nuclear/*A. tumefacien*	Patkar and Chattoo (2006)
Ace-AMP1	*Allium cepa*	Wheat	*Blumeria graminis*	Nuclear/microprojectile	Roy-Barman et al. (2006)
LjAMP1&LjAMP2	*Leonurus japonicas*	Tomato	*Verticillium dahlia, A. solani,*	Nuclear/A. tumefaciens	Li et al. (2007)
Ltp	*Triticum aestivum*	*Daucus carota*	*Alternaria. radicicola,*	Nuclear/*A. tumefaciens*	Jayaraj and punja (2007)
LJAMP1	*Leonurus japonicas*	Tobacco	*A. alternata, Aspergillus niger, Cercospora personata,*	Nuclear/*A. tumefacien*	Yang et al. (2007)
LJAMP2	*Leonurus japonicas*	Tobacco	*A. alternate, Ralstonia solanacearum*	Nuclear/*A. tumefacien*	Yang et al. (2008)
LTP 3F1	*Triticum aestivum*	Tobacco	*Rhizoctonia solani, Curvularia lunata, Alternaria sp. Cylindrocladium scoparium Bipolaris oryzae, B. cinerea Sarocladium oryzae*	Nuclear/*A. tumefacien*	Kirubakaren et al. (2008)
CALTPI&II	Pepper plant	Tobacco	*Phytophthora nicotianae, Pseudomonas syringae pv. Tabaci*	Nuclear/*A. tumefacien*	Sarowar et al. (2009)
NsLTP	*Leonurus japonicus*	*Populus tomentosa*	*A. alternate, Colletotrichum gloeosporioides*	Nuclear/*A. tumefacien*	Jia et al. (2010)
GtLTPs	*Gentiana trifora*	Tobacco	*B. cinerea*	Nuclear/*A. tumefacien*	Kiba et al. (2012)
(Bbchit1, LJAMP2)	*Beauveria bassiana, Leonurus japonicas*	*Populus tomentosa*	*A. alternate*	Nuclear/*A. tumefacien*	Huang et al. (2012)
TaLTP5	*Triticum aestivum*	*Triticum aestivum*	*Cochliobolus sativus, F. graminearum*	Nuclear/biolistic bombardment	Zhu et al. (2012)
LTP	*Oryza sativa*	*Brassica napus*	*Sclerotinia sclerotiorum*	Nuclear/*A. tumefaciens*	Fan et al. (2013)
LJAMP2	*Leonurus japonicas*	*Brassica napus*	*Sclerotinia sclerotiorum*	Nuclear/*A. tumefaciens*	Jiang et al. (2013)
Lc-LTP2	*Lens culinaris*	*Escherichia coli*	*A. tumefaciens, P. syringae, Clavibacter michiganens A.alternate, A. niger, B. cinerea Neurospora crassa, F. solani*	Ecoli transformation	Gizatullina et al. (2013)
TdLTP4	Durum wheat	*Arabidopsis thaliana*	*A.solani, B. cinerea*	Nuclear/*A. tumefaciens*	Safi et al. (2015)
Ace-AMP1	*Allium cepa*	*Triticum aestivum*	*Blumeria graminis* f. sp. *tritici*		Fleury et al. (2019)
StLTP10	*Solanum tuberosum*	*Solanum tuberosum*	*Phytophthora infestans*	Nuclear/*A. tumefaciens*	Wang et al. (2020)
LTPg5	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Heterodera schachtii, *P. syringae, B. cinerea*	Nuclear/*A. tumefaciens*	Ali et al. (2020)
TdLTP4	Durum whea	*Escherichia coli*	Foodborne pathogenic bacteria		Hsouna et al. (2021)
AtLTP4.4	*Arabidopsis thaliana*	*Triticum aestivum*	*Fusarium graminearum*		McLaughlin et al. (2021)

infestans and suggested StLTP10 as a candidate for greater and enhanced resistance to pathogens in potato.

Recently, McLaughlin et al. (2021) produced transgenic wheat plants expressing the *AtLTP4.4.* As a result, the production of the Trichothecene mycotoxins such as deoxynivalenol (DON; virulence factors of *F. graminearum* causing FHB) was found to decrease and less accumulation was reported in the field. This reduced expression of DON-induced reactive oxygen species by *AtLTP4.4* was believed to be the possible mechanism by which the spread of fungal spores and mycotoxin accumulation have been inhibited in transgenic wheat plants. NsLTPs with antifungal and antimicrobial activity, as BrLTP2.1 from *Brassica rapa,* have been found in the nectar of various species (Schmitt et al. 2021).

Multi-transgene stacking or pyramiding of genes is one of the advantageous methods in the production of genetically modified (GM) crop plants. Researchers have been elucidating ways to co-transform multiple genes for more desirable, improved, and effective disease resistance in transgenic plants. Multiple transformation or transgene stacking has been successfully employed in rice (Jha and Chattoo 2009), tobacco (Ntui et al. 2011), potato (Khan et al. 2014) and others (Shehryar et al. 2020). A chitinase (*chit-2*) from barley and LTP from wheat were transformed into carrot singly and in combination through *Agrobacterium*-mediated transformation. The percent resistance in the transgenic plants co-expressing both the genes was found as 95% and 90% against *Botrytis* and *Alternaria,* respectively, while that of single gene-expressing lines was between 40 and 50%. These findings suggested that transforming more than one PR protein gene could be a good strategy for improving resistance to fungal pathogens (Jayaraj and Punja 2007). In another study, transgenic poplar carrying the *LJAMP2* (motherwort lipid-transfer protein) gene was transformed with a chitinase gene, *Bbchit1* (*Beauveria bassiana chitinase*) to generate double-transgenic lines which were found with significantly higher resistance to the *A. alternata* compared to the lines carrying only *LJAMP2* gene and the wild-type plants (Huang et al. 2012).

The *CALTPI* and *CALTPII* genes (isolated from *Capsicum annum*) constitutively expressed in the tobacco plants exhibited broad-spectrum resistance against oomycete pathogen, *Phytophthora nicotianae* as well as a bacterial pathogen*, syringae pv. Tabaci* (Sarowar et al. 2009).

Conclusion and future prospects

Ns-LTPs are widely expressed in the whole plant kingdom, in nearly all investigated land plants tissues and organs with either intracellular or extracellular localization, performing various significant physiological roles. Several nsLTPs and LTPs-like proteins, possessing different structures and functional role in plant's body, have been investigated during evolution which is believed to be resulted from the need to enlarge the range of their physiological and biological functions.

Plant LTPs could be used as possible alternatives to pesticides to manipulate pathogenic fungi. Genetic engineering as an advanced and more powerful protection strategy and genome targeting tools have made it possible to over-express the nsLTPs in the targeted plant species for imparting enhanced biotic and abiotic stress resistance. For higher and more effective disease resistance, multiple transformations or transgene stacking can be employed for the co-expression of nsLTP genes with the other PR-genes in transgenic plants.

The cysteine-rich AMPs are broad-spectrum antimicrobial agents that can mediate immune responses in different life forms, such as, protozoans, bacteria, fungi, insects, plants, and animals. Plants ns-LTPs are also cysteine-rich peptides that express in several tissues to counteract the effects of invading pathogens. They can also play a crucial role in regulating plant growth and development while as a potent antimicrobial agent, the nsLTPs can supplement or replace conventional antibiotics. As cysteine-rich AMPs, the nsLTPs can open up new avenues for the plants to be used as bio-factories for the production of antimicrobials and can be considered as promising antimicrobial drugs in bio-therapeutics.

Most of the recent studies included in this review focused on the antifungal and antibacterial properties of plants nsLTPs while the antiviral effect of these proteins is still under-explored and further investigation in tackling this issue remains to be determined.

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