



***Lupinus luteus* Pathogenesis-Related Protein as a Reservoir for Cytokinin**

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Received 20 January 2008;
received in revised form
7 March 2008;
accepted 12 March 2008
Available online
19 March 2008

Edited by R. Huber

Plant pathogenesis-related (PR) proteins of class 10 (PR-10) are small and cytosolic. The main feature of their three-dimensional structure is a large cavity between a seven-stranded antiparallel β -sheet and a long C-terminal α -helix. Although PR-10 proteins are abundant in plants, their physiological role remains unknown. Recent data have indicated ligand binding as their possible biological function. The article describes the structure of a complex between a classic PR-10 protein (yellow lupine LIPR-10.2B) and the plant hormone, *trans*-zeatin. Previously, *trans*-zeatin binding has been reported in a structurally related cytokinin-specific binding protein, which has a distant sequence relation with classic PR-10 proteins. In the present 1.35 Å resolution crystallographic model, three perfectly ordered zeatin molecules are found in the binding cavity of the protein. The fact that three zeatin molecules are bound by the protein when only a fourfold molar excess of the ligand was used indicates an unusual type of affinity for this ligand and suggests that LIPR-10.2B, and perhaps other PR-10 proteins as well, acts as a reservoir of cytokinin molecules in the aqueous environment of the cell.

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Keywords: plant hormones; cytokinin; *trans*-zeatin; plant PR-10 protein; yellow lupine

Introduction

Plants forced to grow in harsh conditions and exposed to pathogens have developed several means of defense. Besides establishing a physical barrier by strengthening their cell walls, plants also produce antibiotic compounds called phytoalexins and accelerate cell death to suppress the

spread of infectious pathogens. The expression of a number of genes is induced by various types of pathogens or by chemicals such as ethylene and salicylic acid, which mimic the effect of pathogen infection and thus induce stress.¹ These genes code the so-called pathogenesis-related (PR) proteins.²

PR proteins are grouped into 17 classes according to their biological activity or physicochemical properties and sequence homology.^{2,3} According to accepted definition, they are synthesized *de novo* under stress conditions. Among them are chitinases, glucanases, enzymes of phenylpropanoid pathway, thionins, osmotins, proteases, proline-rich glycoproteins, and PR class 10 (PR-10) proteins of unknown function, also known as intracellular pathogenesis-related (IPR) proteins. PR-10 proteins have been identified in more than 70 species belonging to both monocotyledonous and dicotyledonous plants.⁴ They are small (155–163 residues), slightly acidic, resistant to proteases and are usually encoded by multigene families (for instance, 10 genes in yellow lupine, divided into two subclasses),⁵ suggesting

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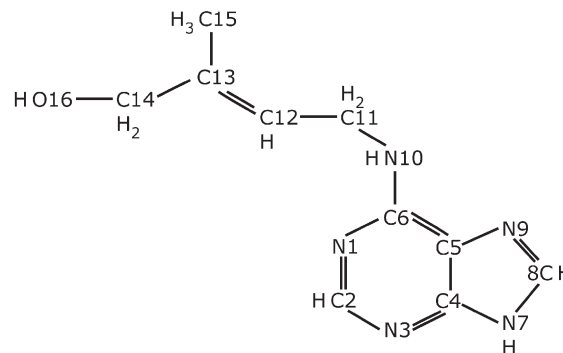
Abbreviations used: CSBP, cytokinin-specific binding protein; IPR, intracellular pathogenesis-related protein; LIPR-10.2B, *Lupinus luteus* (yellow lupine) PR-10 protein of subclass 2B; MLP, major latex protein; PR, pathogenesis-related; RMS, root-mean-square; STAR, steroidogenic acute regulatory protein; START, star-related lipid transfer.

that a number of different protein homologs can be expressed in various plant organs at different conditions. This makes the study of the function of PR-10 proteins very complex.

Based on sequence similarity (~50%), common allergens present in birch pollen (Betv1),⁶ celery,⁷ apple⁸ and in other fruits are also included in the PR-10 group. Proteins from another PR-10 subfamily, called major latex proteins (MLPs), are found in the latex of some plants, including opium poppy⁹ and bell pepper.¹⁰ Interestingly, the classic PR-10 proteins and the MLPs have never been found to coexist in the same plant species.¹¹ Furthermore, based on distant sequence identity (<20%) and secondary-structure prediction, the group of cytokinin-specific binding proteins (CSBPs) were also postulated to be related to the PR-10 family.¹² This tentative classification has been confirmed recently by X-ray crystallography,¹³ which has demonstrated close structural similarity between CSBP and classic PR-10 proteins.

The sequence similarity and wide distribution of PR-10 proteins throughout the plant kingdom indicates their indispensable function. However, it is difficult to assign a unique function to all PR-10 members because there is no coordinated expression pattern. They are involved in general defense mechanisms, with some of the homologs accumulating around sites of pathogen invasion.^{14,15} It is also believed that PR-10 proteins play a role in plant development, since they have been found in seeds^{16,17} and constitutive expression has been observed in roots¹⁸ and other developing organs.^{6,17} Moreover, senescent leaves often have increased levels of PR-10 protein expression¹⁸ and some homologs appear to be induced by phytohormones, such as cytokinins,¹⁹ abscisic acid²⁰ or ethephon, an ethylene-releasing compound.²¹

Crystal and solution structures are known for several PR-10 homologs.^{13,22–28} All these structures have the same general fold consisting of a seven-stranded antiparallel β -sheet wrapped around a long C-terminal α -helix. These two elements, together with two short helices, form a hydrophobic cavity whose volume is unusually large for proteins of this size. The abnormal size of the cavity suggests a physiological function, the obvious possibility being the binding of hydrophobic ligands. Additional evidence for this likely function is provided (i) by the structural similarity between the PR-10 members and the START (steroidogenic acute regulatory protein-related lipid transfer) domain of human MLN64 protein, which is a steroid-binding domain related to steroidogenic acute regulatory protein involved in cholesterol translocation in human placenta and brain,²⁹ (ii) by the structural similarity with CSBP proteins,¹³ and (iii) by the crystal structure of Betv1 in complex with deoxycholate.²⁶ Deoxycholate is not a plant metabolite and cannot be a natural PR-10 ligand, but it is structurally similar to brassinosteroids, which are plant steroid hormones involved in plant development and growth.³⁰ Finally, several studies have been reported demon-



Scheme 1. The structural formula of trans-zeatin, with atom numbering used in the PDB entry 2qim and in this article.

strating the ability of PR-10 proteins to bind steroids, cytokinins, fatty acids, and flavonoids.^{26,31,32}

Here, we report the crystal structure of a classic PR-10 protein from yellow lupine (LIPR-10.2B) in complex with a plant hormone from the cytokinin group. LIPR-10.2B was co-crystallized with *trans*-zeatin (Scheme 1) and the structure reveals the presence of three zeatin molecules within the hydrophobic cavity of the protein.

Results

Model quality and overall fold

The refined 1.35 Å resolution crystallographic model of LIPR-10.2B has good overall geometry and Ramachandran statistics (Table 1). The quality of the electron density maps is high and there are only a few less clear areas in the loop regions. The recombinant protein lacks the N-terminal methionine, excised by *Escherichia coli* methionylamino-peptidase, whose catalytic efficiency is inversely proportional to the size of the side chain of the amino acid in the penultimate position.³³ In the LIPR-10.2B sequence this amino acid residue (corresponding to position 1 of the native sequence) is a glycine, therefore the cleavage is almost complete.

The crystallographic asymmetric unit contains one LIPR-10.2B molecule with an overall fold consisting, as in other PR-10 members, of a seven-stranded antiparallel β -sheet wrapped around a long C-terminal helix α 3. Two additional short helices (α 1, α 2), in a right-handed crossover, separate the strands β 1 and β 2, which form the two edges of the β -sheet. The consecutive strands β 2– β 7 are connected by five hairpin loops bringing strand β 7 back into hydrogen-bond contact with β 1. Six β -bulges distort the regularity of the β -sheet endowing it with a highly curved shape (Fig. 1). The β -bulges are structurally conserved in all PR-10 structures as well as in the birch pollen protein, confirming their importance for maintaining the overall structure of the β -sheet (Fig. 2a). A large hydrophobic cavity is present within the protein core, formed between the

Table 1. Data collection and refinement statistics

<i>Data collection</i>	
Space group	$P6_5$
Cell parameters (Å)	$a=73.8, c=67.2$
Resolution limits (last shell) (Å)	30.0–1.35 (1.40–1.35)
Radiation source	DESY, X13 (EMBL)
Wavelength (Å)	0.8030
Temperature (K)	100
No. of measured reflections	442,110
No. of unique reflection	45,644
R_{int} (last shell)	0.042 (0.670)
Completeness (last shell) (%)	100 (100)
Redundancy	9.7
$\langle I/\sigma(I) \rangle$ (last shell)	56.7 (2.5)
<i>Refinement</i>	
Programs used	Refmac5
Resolution limits (Å)	15.0–1.35
No. of reflections	45,644
No. of reflections in test set	1163
No. of atoms	
Protein	1211
Ligand	64
Metal	1 (Ca)
Glycerol molecules	1
Water molecules	233
R/R_{free}	0.147/0.194
$\langle B \rangle$ (Å ²)	
Protein atoms	24.9
Ligand atoms	21.9
Water molecules	41.5
rmsd from ideal	
Bond lengths (Å)	0.020
Bond angles (°)	2.04
Ramachandran φ/ψ angles (%)	
Most favored	93.9
Additionally allowed	6.1

residues lining the interior face of the β -sheet and the three α -helices (Fig. 1).

The C-terminal α -helix of PR-10 proteins is structurally and sequentially the most divergent element of their structure (Figs. 2a and 3). This divergence is higher in the N-terminal half of the helix. The present protein, LIPR-10.2B, is a close homolog of LIPR-10.2A, described earlier.²⁷ Although the two sequences are nearly identical (91% sequence identity) and there are no differences in the N-terminal part of helix $\alpha 3$ (Fig. 3), the strong inward kink in the middle of helix $\alpha 3$ reported for LIPR-10.2A is not observed in the present structure. This makes the volume of the internal cavity much larger (Fig. 2a, Supplementary Table 1). It is possible that the structural change of helix $\alpha 3$ is a result of the zeatin cargo present in the internal cavity (*vide infra*).

One of the loops (L4) shows extraordinary rigidity despite the presence of four glycine residues. This glycine-rich loop is characterized by excellent electron density, low B -factors and the highest degree of PR-10 sequence conservation (Figs. 3 and 4). The sequence and structural conservation is observed even in distant homologs, such as CSBP. The rigidity of the loop is maintained by a pattern of three hydrogen bonds between the O ^{γ} 1 atom of Thr51 and the main-chain N–H groups of Asn46, Gly47 and Gly48. The sequence motif of the glycine-rich loop, xEGNGGPGTxKKx, is simi-

lar to the nucleoside phosphate binding motif (P-loop) present in other protein families.³⁷ However, the PR-10 proteins have no affinity for ATP.³¹ The loop appears to be shifted when the homologous PR-10 structures are aligned, but if only the loop regions are superposed, the agreement is excellent. The glycine-rich loop has been postulated as a putative ligand-binding site for cytokinins,^{25,31} but the present structure shows no zeatin molecules close to it.

Asymmetric unit contents

Apart from the protein–zeatin complex, the asymmetric unit contains 233 modeled water molecules, one molecule of glycerol, and one calcium cation. The metal cation is octahedrally coordinated close to loop L3 by three carbonyl oxygen atoms (Pro31, Val34, Ile37) and three water molecules. The correctness of the interpretation of the metal site as calcium is confirmed by the satisfactory refinement of the B -factor (29.0 Å²), by the final Ca²⁺...O distances (2.2–2.7 Å) and by the bond-valence test.³⁸ Calcium cations have not been found in PR-10 structures before. Previously, sodium cations were found in the crystal structures of LIPR-10.2A (coordinated close to L3) and CSBP (coordinated close to L9). Interestingly, in both those structures, the sodium cation was found only in 50% of the protein molecules present in the respective asymmetric units, indicating rather weak affinity for the metal. On the other hand, calcium was not present in the crystallization buffer of the LIPR-10.2B/zeatin complex. Therefore, it must have been coordinated already at the protein

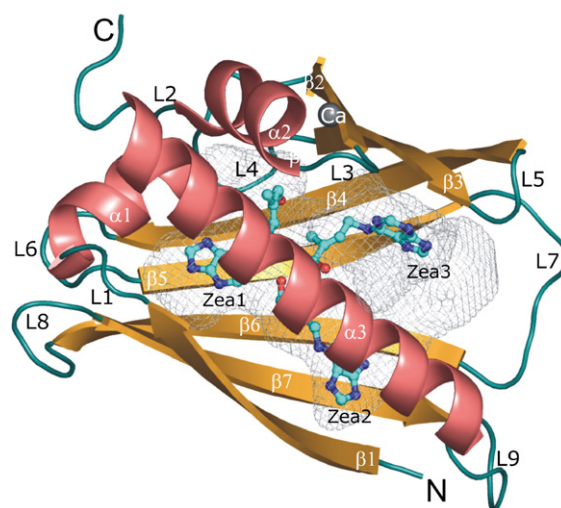
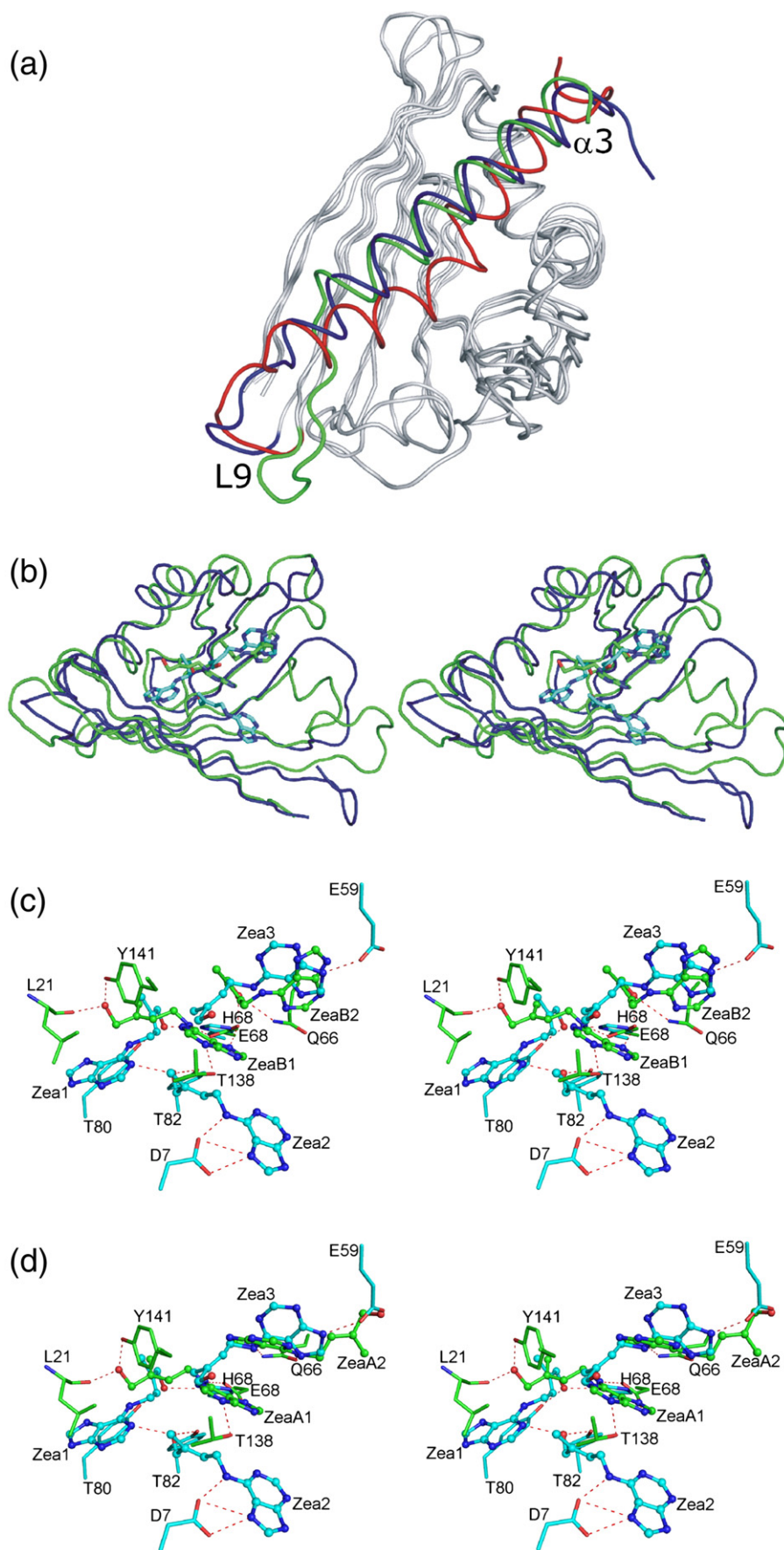


Fig. 1. Overall fold of the LIPR-10.2B molecule with annotation of secondary-structure elements. The three *trans*-zeatin molecules found inside the binding cavity are shown in ball-and-stick representation. The binding cavity is represented as a gray cast, calculated in VOIDOO.³⁴ A calcium cation is represented as a sphere. The orientation has been adjusted to visualize the O16 atoms of the zeatin molecules. This and all other structural illustrations have been prepared using PyMol [<http://pymol.sourceforge.net/>].

**Fig. 2** (legend on next page)

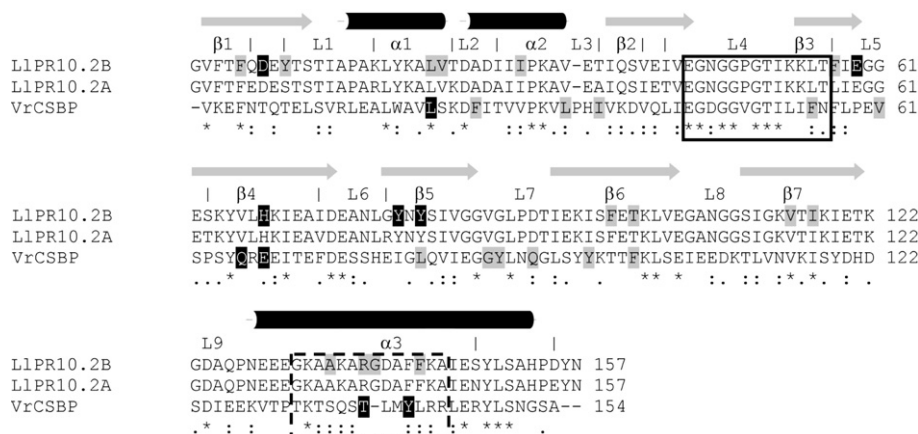


Fig. 3. Sequence alignment of representative PR-10 proteins, calculated in ClustalW.³⁶ LIPR-10.2A²⁷ and LIPR-10.2B (this work) are yellow lupine proteins and CSBP is cytokinin-specific binding protein from mung bean.¹³ Residues identical in all sequences are marked with *. The symbols ":" and "." below the sequences indicate conservative and semiconservative substitutions, respectively. The differences between the two members of the yellow lupine PR-10.2 subfamily are marked with |. The boxes outline regions that are most conserved (continuous line) or most divergent (dash line). The residues marked in gray interact with the ligands via van der Waals contacts, and those in black via hydrogen bonds. The secondary-structure elements correspond to the LIPR-10.2B structure.

expression stage, which indicates strong binding, perhaps specific for this cation.

The glycerol molecule is located on the outer face of helix $\alpha 3$ at its C-terminus but it forms no direct hydrogen bonds with the protein. Instead, its two hydroxyl groups are connected via one or two water molecules to a common hydrogen-bonding center in the side chain of Ser148.

The LIPR-10.2B/zeatin complex is formed by one protein molecule and three *trans*-zeatin molecules. Additionally, one zeatin molecule has an interstitial position between symmetry-related protein molecules. This fourth zeatin molecule is most probably a crystallographic artifact but it is interesting to note that the LIPR-10.2B:(3+1) zeatin complex strictly corresponds to the 1:4 protein/ligand molar ratio used for co-crystallization.

The binding cavity

The cavity enclosed within the LIPR-10.2B core has an unusually large volume, calculated as 4500 Å³ by the SURFNET program.³⁹ The residues shaping the cavity endow it with hydrophobic character. Nevertheless, a few polar residues are also pointing into its volume. Both the hydrophobic and polar residues are responsible for the binding of the ligand molecules, as described below. The cavity is not closed and can be accessed by a number of openings. The largest

opening, located between $\alpha 3$, L3, L5 and L7, is gated by a salt bridge between Arg138 and Glu59. Close to and connected with the first opening is another one, between $\beta 5$, L7 and $\beta 6$. This opening is clearly enlarged by a disruption of the antiparallel β -sheet by a water molecule, Wat195, two hydrogen bonds away from the end of the β -sheet. An additional opening is found between $\beta 1$ and $\alpha 3$. A small opening is also present in the opposite wall of the cavity, between L2 and L4. The binding cavity is filled with 3 zeatin molecules and 25 water molecules (Fig. 5). Most of the water molecules function as hydrogen-bonding interface between the zeatin ligands and the protein wall of the cavity, or simply satisfy the hydrogen-bonding centers of the ligands. There are also water molecules that are only linked with other water molecules and/or with protein partners. In general, the present cavity-enclosed water molecules have no conserved counterparts in other PR-10 structures.

The LIPR-10.2B/zeatin complex

The full occupancy and excellent electron density of the zeatin molecules (Zea1–Zea4), including the one outside of the binding cavity (Zea4), leaves no doubt about the identity and conformation of the ligands (Fig. 6). All the zeatin molecules have evident *trans* configuration (Table 2). Each of them

Fig. 2. Superposition of PR-10 structures, calculated with ALIGN.³⁵ (a) Superposition of three proteins with PR-10 fold. Color code: blue, LIPR-10.2B (this work); red, LIPR-10.2A molecule A (PDB code 1xdf); green, CSBP molecule B (2flh). The orientation emphasizes the differences in loop L9 and the C-terminal helix $\alpha 3$. (b) Stereo view of the superposition of the LIPR-10.2B complex (blue) with three zeatin molecules in the binding cavity (this work), onto CSBP (molecule B, green) with two zeatin molecules in the binding cavity (PDB code 2flh). The C-terminal helix $\alpha 3$ has been omitted for clarity. (c) Zoom-in stereo view of the zeatin molecules (ball-and-stick models) from (b) with their interacting partners contributed by the protein environment (stick models). Hydrogen bonds are shown by red dash lines. Molecule coloring scheme as in (b). (d) An analogous illustration as in (c), but showing the superposition of the LIPR-10.2B complex onto molecule A of the CSBP complex. Note that ZeaA2 is flipped with respect to ZeaB2 in (c).

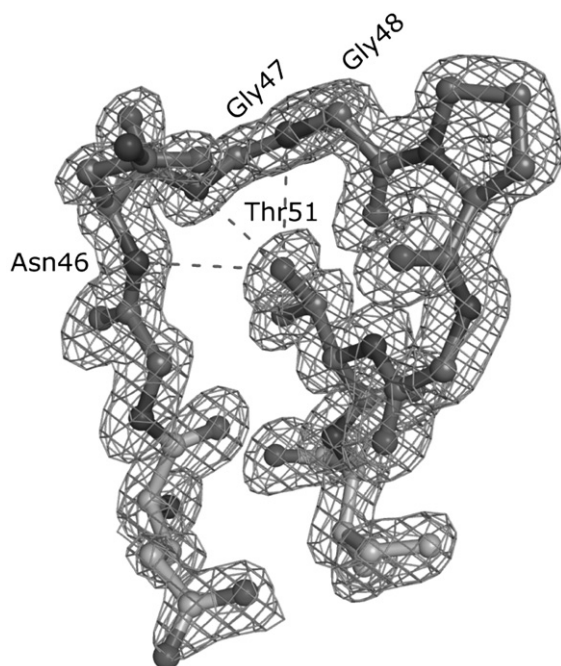


Fig. 4. The glycine-rich loop (L4) with the three hydrogen bonds formed by the side chain of Thr51 and the main-chain amides of residues Asn46-Gly47-Gly48. The $2F_o - F_c$ electron density map is contoured at the 1.5σ level.

has at least one direct hydrogen-bond contact with the protein. Additionally, the ligands are stabilized by numerous van der Waals interactions (Figs. 3, 5).

Zea1 is bound deep in the cavity close to the two short helices, with the adenine moiety closer to $\alpha 1$ and the aliphatic tail closer to $\alpha 2$ (Fig. 1). It is anchored to the protein only by two hydrogen bonds at the tip of the aliphatic tail (His68, Tyr80). The hydrogen bonds of the adenine moiety are with Zea2 (at N1) and with several water molecules. Zea1 also forms several van der Waals contacts (Fig. 5).

Zea2 is aligned with helix $\alpha 3$ near the $\beta 1/\alpha 3$ opening, with the adenine moiety closer to the N-terminal half and the aliphatic tail pointing in the C-terminal direction of the helix (Fig. 1). The terminal hydroxyl group of the aliphatic tail of Zea2 forms a hydrogen bond with Zea1 and with Tyr82. N9 and N10 donate three hydrogen bonds to the carboxylate group of Asp7 ($N9 \cdots O^{61}$; $N9 \cdots O^{62}$; $N10 \cdots O^{62}$). Zea2 is also stabilized by several van der Waals contacts (Fig. 5), and the remaining hydrogen-bonding centers of the adenine ring are satisfied by water molecules.

Zea3 is placed near the L3/L5/L7/ $\alpha 3$ opening of the cavity with the aliphatic tail pointing into the cavity (Fig. 1). The molecule is surrounded by six water molecules and forms only one hydrogen bond with the protein [$N7 \cdots O^{62}$ (Glu59)]. Zea3 also participates in van der Waals contacts with Phe57, Arg138, Gly139 and zeatin molecules 1 and 2 (Fig. 5).

The terminal hydroxyl group of the aliphatic tail of Zea4, which is located between symmetry-related

protein molecules, forms three hydrogen bonds, two with protein residues (Glu129 and Lys54') and one with a water molecule. Additional two hydrogen bonds of Zea4 are formed between two water molecules and the adenine moiety.

Although this study presents a high-resolution structure of the LIPR-10.2B/zeatin complex and the definition of the electron density, especially for the ligand molecules, is excellent, the current 1.35 Å resolution X-ray diffraction data are not sufficient to locate the hydrogen atoms. Location of hydrogen atoms would be particularly important in the case of the zeatin molecules because their tautomeric state (N7 or N9 protonation, Scheme 1) or even the degree of protonation (neutral, or protonated at N1, N3, N7 or N9) is not known. However, some deduction based on the pattern of hydrogen bonds in the binding cavity is possible (Fig. 5). First, it should be noted that although zeatin was added to the crystallization buffer as a cation (see Experimental Procedures), the crystallization solution was buffered near neutral pH (6.5). Consequently, either protonation state of zeatin would be possible. The fact that among the residues surrounding the cavity there is a strong negative net charge (positive Arg138 *versus* negative Asp7, Glu59, Glu131) indicates that the contents of the cavity have a positive net charge and that at least some of the zeatin ligands are protonated. With respect to Zea1, it is not possible to speculate about the tautomeric state of the adenine ring as it interacts exclusively with O-H groups. Zea2, which forms a salt bridge with Asp7 through its N9/N10 atoms, must be protonated at N9. The situation is opposite at Zea3, where the proton must reside at N7 in order to form a hydrogen bond with Glu59.

All four zeatin molecules have evident trans form of the C12=C13 double bond (Scheme 1). However, the isoprenoid tail has diversified conformation and assumes three different forms (Fig. 6, Table 2). The terminal hydroxyl group assumes two rotamers. There seems to be some correlation between the torsion angles around N10-C11 and C11-C12. Namely, one of them is always close to 90° (absolute value), while the other defines a very open angle (>120°) between the two non-H substituents. Interestingly, each of the zeatin molecules in the binding cavity has a different rotamer of the aliphatic tail. The isoprenoid tail of the interstitial zeatin molecule Zea4 is very similar to that of Zea2, despite the completely different environment of these two molecules. The rotation of the *exo*-amino substituent at N10 is generally the same, with the aliphatic tail distal to the imidazole ring.

In the CSBP complex, all the cavity-bound zeatin molecules had the same conformation as the present Zea3 molecule, except one "outer" ligand, which has no counterpart in the LIPR-10.2B complex. Taking into account the interstitial ligands in the CSBP structure, the zeatin molecule has been observed in five different conformational forms in just two complexes with PR-10-type molecules. We can

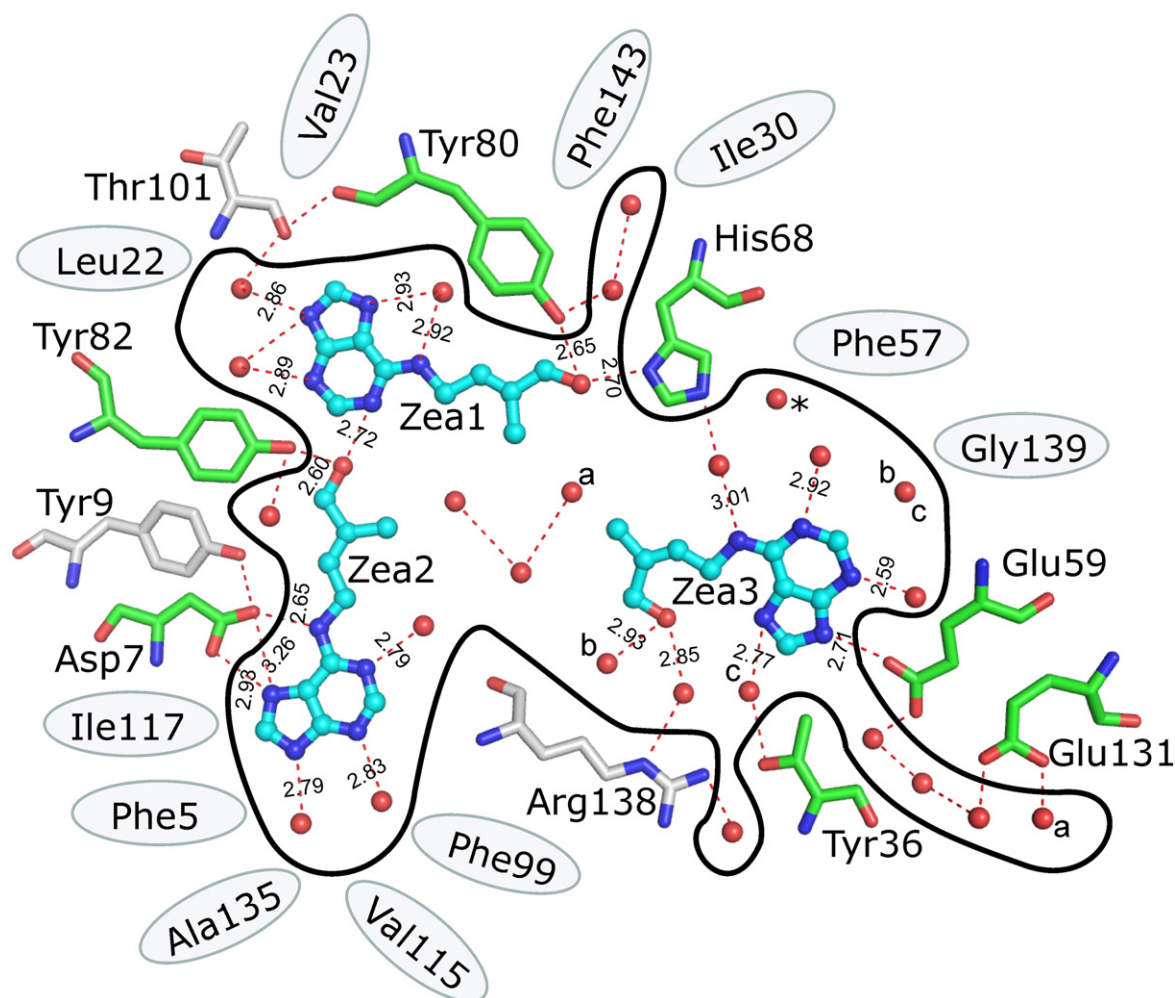


Fig. 5. Representation of the three zeatin molecules found in the protein cavity (black outline) with their environment. Hydrogen bonds are represented by dash lines. Those formed by the zeatin molecules are annotated with the donor-acceptor distance in angstroms. The letters a–c mark water molecules that are linked by hydrogen bonds but could not be connected because of the distortion of this two-dimensional representation. The residues involved in hydrogen bonding are represented in stick mode (gray, if they participate in van der Waals interactions with the ligands). The ellipses indicate amino acid residues involved only in van der Waals contacts with the zeatin molecules. The asterisk * indicates a water molecule that has hydrogen-bonding interactions with bulk solvent only.

speculate that it is the flexibility of the aliphatic tail and the plasticity of the adenine ring in hydrogen-bonding interactions (protonation state and tautomerism) that make the zeatin molecule a versatile ligand, capable of adjusting itself to different molecular environments. This aspect of the zeatin molecule may explain the ease with which three ligands could be accommodated in the binding cavity of LIPR-10.2B.

Comparison with other PR-10 homologs

The amount of crystallographic information about PR-10 proteins has been accumulating rather quickly in the recent years. In addition to the present structure, three other yellow lupine homologs have been described, as well as several structures of the Betv1 protein and one structure of the distant CSBP homolog from mung bean. Taking into account that in several cases more than one protein molecule was

found in the asymmetric unit (e.g., LIPR-10.1B, 2; LIPR-10.2A, 2; CSBP, 4), the amount of structural information is quite significant. However, no report is available about structural characterization of the same protein with and without a natural ligand. The nearest such case is available for the Betv1 protein, but its complex with deoxycholate does not represent a physiological possibility.

Although all the crystallographic models of PR-10 proteins share the same canonical fold, their superpositions reveal very significant structural differences (Supplementary Table 1). When analyzed in detail, the differences are located mostly at the C-terminal helix $\alpha 3$, which displays different axial shifts as well as a variable degree of deformation in the central part and in its N-terminal connection with loop L9. In addition, the volume of the internal cavity, formed with the participation of helix $\alpha 3$, shows remarkable variability. The cavity volume is determined not only by the variability of the $\alpha 3$ helix

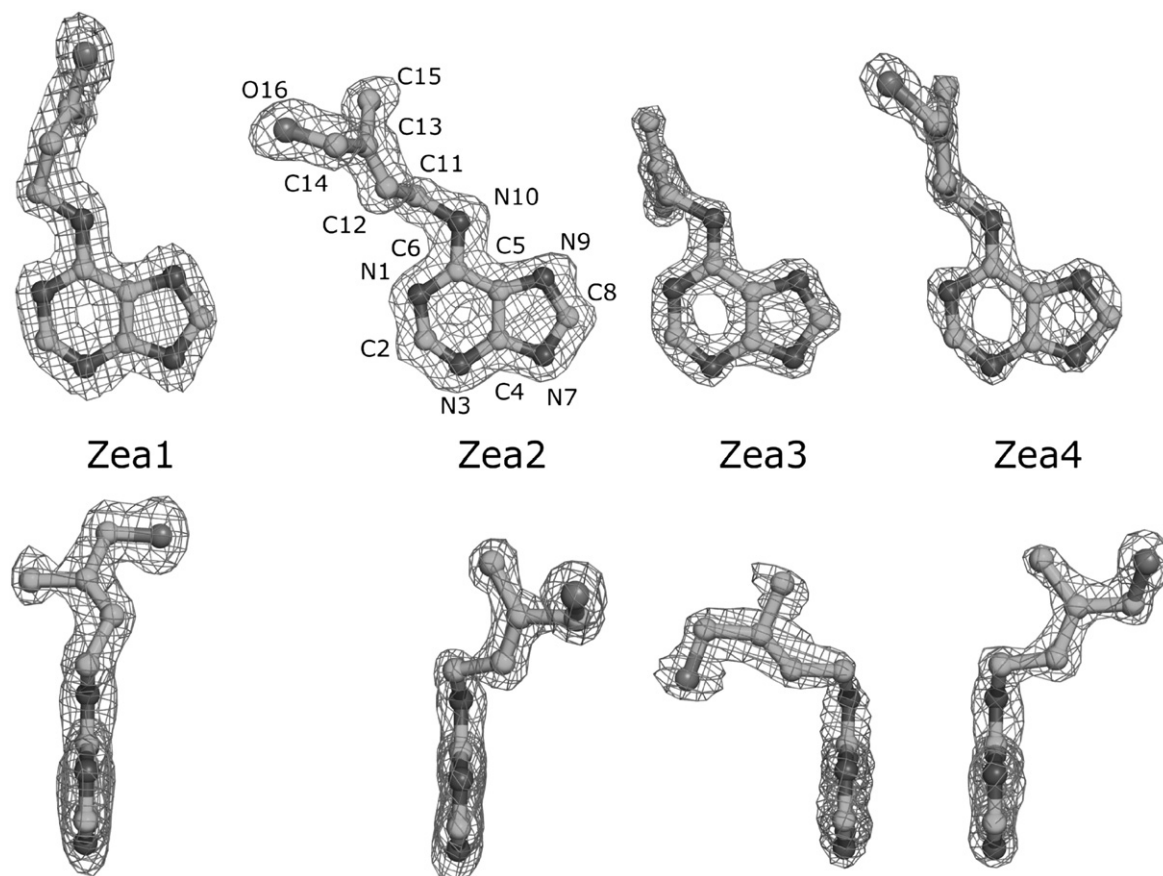


Fig. 6. The four zeatin molecules present in the crystal structure of the LIPR-10.2B/zeatin complex, shown in two different views (rotated by 90°). The $2F_o - F_c$ electron density maps are contoured at the 1.5σ level. The zeatin atom numbering scheme used here (as defined in the PDB entry 2qim) is shown at Zea2.

sequence but also by its geometrical deformation, such as the strong inward kink reported for the LIPR-10.2A protein. Another important factor is the occupation of the cavity by small-molecule ligands. It is not immediately obvious if the shape of the cavity and its volume are adjusted to the ligands on complex formation, or conversely, if the cavities of different PR-10 homologs are preformed and rigid, i.e., designed for high-affinity binding of specific ligands. In this context, it is instructive to consider the following comparisons: (i) the structures of the two homologous yellow lupine LIPR-10.1 proteins 1A [Protein Data Bank (PDB) code 1icx] and 1B (1ifv), which share 77%/90% sequence identity/similarity, (ii) apo Betv1a (1bv1) and its 94%/97% identical/similar variant *l* in complex with deoxycholate

(1fm4), (iii) the four crystallographic copies of the CSBP/zeatin complex (2flh), and (iv) the structures of the two homologous yellow lupine LIPR-10.2 proteins (91%/96% sequence identity/similarity), one (2A) in apo form (1xdf) and one (2B) in complex with zeatin (this work). Comparison (i) indicates a PR-10 cavity with a rigid shape and volume, although, surprisingly, the C^α superposition reveals structural differences that are not insignificant (Supplementary Table 1). Comparison (ii) suggests that the PR-10 cavity with and without a ligand has similar volume and that, indeed, there are no structural changes on deoxycholate binding. The four CSBP molecules in (iii) are practically identical and have the same cavity volume (although significantly smaller than in the remaining, classic PR-10 molecules) despite the fact that only two of them bind their tandem zeatin molecules in the same way (in one case the “outer” zeatin ligand has a flipped orientation and in one complex it is absent altogether). The most surprising, however, is case (iv), where an almost identical protein sequence encloses a very different cavity, depending on the presence of the zeatin ligands. In addition, the C^α deviation between these two nearly identical homologs is the highest (2.1 Å). This large C^α deviation results not only from the kink in helix α_3 in LIPR-10.2A but also from a rearrangement of the α_2 , β_2 , β_3 , β_4 , L3, L5,

Table 2. Conformation of the isoprenoid chain of the zeatin molecules

	Zea1	Zea2	Zea3	Zea4
N1-C6-N10-C11	-1.3	-6.8	-8.9	5.7
C6-N10-C11-C12	154.8	83.2	-82.1	90.4
N10-C11-C12-C13	87.7	134.7	-131.1	126.5
C11-C12-C13-C14	-177.5	-177.5	178.6	-178.2
C12-C13-C14-O16	8.0	-124.2	3.8	-131.0
Torsion angles in degrees.				

L7, L8 and L9 elements. In consequence, the volume of the cavity has increased from 2000 Å³ in LIPR-10.2A to 4500 Å³ in LIPR-10.2B.

Discussion

Although the biological function of PR-10 proteins has not been determined yet, their gene expression patterns, accumulation in different organs and physicochemical properties are well characterized. Many genes encoding PR-10 proteins show organ or tissue-specific expression and appear to be developmentally regulated. Some of them are expressed constitutively,^{11,14,18} while others are induced in aerial parts by various stress factors, e.g., wounding,⁴⁰ UV radiation,¹⁵ chemical pollutants,⁴¹ or in response to pathogen invasion.^{14,15} Presumably the PR-10 proteins constitutively expressed in roots are involved in the plant developmental program, while the inducible ones can participate in defense reactions.

Based on structural homology with a human steroid carrier²⁹ and because of the characteristic large cavity of the PR-10 protein family, a role in ligand binding has been postulated as their possible physiological function. This hypothesis has been supported recently by experimental evidence from the crystallographic study of a CSBP.¹³ CSBP is a structural PR-10 homolog despite low sequence similarity. In addition, several assays showed PR-10 proteins to be able to bind a number of important physiological ligands, including cytokinins, fatty acids, flavonoids and brassinosteroids.^{26,31,32}

The present structure shows the atomic detail of a classic PR-10 protein in complex with *trans*-zeatin. Three ligand molecules are buried in the internal cavity (Fig. 1). The fourth zeatin molecule is located near the β 1/ α 3 cavity entrance of one protein molecule and on the back of the β -sheet of a symmetry-related protein molecule. It is conceivable that the position of the fourth zeatin molecule is showing a way to enter/exit the cavity, but it is also possible that it is simply trapped between two protein molecules, being only a crystallization artifact. The remaining discussion is, therefore, focused on the "internal" zeatin molecules.

In the CSBP/zeatin complex, a maximum of two zeatin molecules ("inner" and "outer") were found in the protein cavity,¹³ in contrast to the three ligand molecules found in the present structure. In addition to the surprising higher cytokinin-binding ability of the LIPR-10.2B protein, the crystal structures show that the ligands are located differently in the cavity (Fig. 2b–d). When the structure of LIPR-10.2B is aligned with the four protein molecules found in the asymmetric unit of the CSBP structure, it is evident that the inner zeatin in the CSBP complexes, which is conserved in all four copies, does not coincide with any ligand molecule in the LIPR-10.2B structure. In particular, while the inner zeatin ligand in the CSBP complex is aligned along the α 3-helix, the innermost zeatin molecule in the LIPR-10.2B complex (Zea1) is

aligned with helices α 1 and α 2 (Fig. 2b–d). Considering the position of the outer zeatin molecule in the CSBP structure, present only in three of the four copies of the protein molecule, it is noticeable that it corresponds to Zea3 in the LIPR-10.2B structure. However, a poor overlap is observed only with one of the outer CSBP ligands, while the other two are rotated by 180°, pointing the aliphatic tail in the opposite direction.

Due to the different stoichiometry and position of the zeatin molecules in the two complexes, the patterns of binding residues are also different (Fig. 3). Although both proteins employ five residues for hydrogen bonding with the ligands, only one is positionally conserved. Nevertheless, this amino acid identity is not conserved, as it is His68 in LIPR-10.2B and Glu69 in CSBP. It is interesting to note that the same position, but once again occupied by a different amino acid residue (Asp), is used by Betv1l to form a hydrogen bond with a deoxycholate ligand.²⁶ The remaining hydrogen bonds in the LIPR-10.2B and CSBP complexes are present at different spatial positions. In the lupine protein they are formed by Asp7, Glu59, His68, Tyr80 and Tyr82, and in CSBP by Leu21, Gln66, Glu68, Thr138 and Thy141 (Figs. 3, 5). The main forces responsible for the stabilization of the zeatin molecules in the LIPR-10.2B cavity are weak hydrogen bonds formed with mediation of water molecules, and van der Waals interactions. Again, the amino acid residues responsible for these interactions are generally different in the LIPR-10.2B and CSBP proteins. The number of residues used for van der Waals contacts is also different, 14 in LIPR-10.2B versus 11 in CSBP (Fig. 3).

Since ligand binding is a dynamic process and the side chains pointing toward the vast volume of the cavity must have some conformational freedom, one can expect the PR-10 proteins to be rather general than specific ligand binders. This view is supported by the studies carried out to demonstrate the binding capability of the PR-10 proteins^{13,26,31,32} and by the crystal structure of Betv1l in complex with deoxycholate, a non-plant metabolite.²⁶ The observation that the residues involved in ligand binding are not conserved in the PR-10 family suggests that the proteins could be involved in storage of water-insoluble hydrophobic ligands in an unspecific way.

Structural superposition of the LIPR-10.2B and CSBP molecules reveals that their C α traces differ by 1.7–1.8 Å. They also have different volumes of the internal cavity, 4500 Å³ in LIPR-10.2B and 1100–1600 Å³ in CSBP.

The accumulating knowledge about the PR-10 protein structure allows some speculations. One of them concerns the influence of the ligand on the protein shape. Unfortunately, no published data are available for the same protein in the apo form and in complex with a natural ligand. Nevertheless, one can use the ligand-free crystal structure of LIPR-10.2A,²⁷ which belongs to the same subclass as the protein described here. Structural superposition and sequence alignment of the lupine LIPR-10.2A and LIPR-10.2B proteins suggest that the ligand has a

strong influence on the structure of the molecule. The two proteins show 96% sequence similarity with only 14 residues different. However, their structural superposition shows a large rmsd between their C α atoms. In LIPR-10.2A, the volume of the cavity is less than one-half of the 4500 Å³ found in LIPR-10.2B. This large difference is mainly due to the 60° inward kink of helix α 3 around Phe142, present in both copies of the LIPR-10.2A molecule,²⁷ and to a rearrangement of the remaining secondary-structure elements shaping the internal cavity. The residues involved in hydrogen bonds with zeatin in the LIPR-10.2B complex are all conserved in LIPR-10.2A. Among the 14 residues involved in van der Waals contacts, only two are dissimilar, namely, at position 10 (Tyr in LIPR-10.2B *versus* Ser in LIPR-10.2A) and at position 57 (Phe *versus* Leu). This strongly supports the hypothesis of helix α 3 remodeling by the presence, number and identity of the ligands in the binding cavity. Additional support comes from multiple alignment of the PR-10 sequences, which shows that the most divergent element is located in helix α 3, especially in its N-terminal half. However, in the sequences of LIPR-10.2A and LIPR-10.2B this segment is perfectly conserved (Fig. 3), with only two conservative substitutions at the C-terminus of helix α 3, namely, at positions 149 (Ser in LIPR-10.2B *versus* Asn in LIPR-10.2A) and 156 (Asp *versus* Glu). It is important to note that the residues at these positions are not involved in ligand binding in the LIPR-10.2B/zeatin complex. The only substitution that could possibly disturb zeatin binding is at position 57, where a phenylalanine that (together with Arg138) forms stacking interactions with Zea3 is replaced in LIPR-10.2A by leucine. A substitution that could indirectly affect ligand binding because of different side-chain electrostatics and size is Gly79→Arg, but the Arg residue is not pointing into the cavity. Since these substitutions are obviously not responsible for the difference in the cavity volume, and since the sequences around Phe142, where the α 3-helix is kinked in the LIPR-10.2A structure, are exactly the same, it is logical to conclude that it is the presence and identity of the ligands in the binding cavity that modulate the conformation of the C-terminal helix α 3.

The residues of LIPR-10.2B that are involved in *trans*-zeatin binding are not strictly conserved in all yellow lupine PR-10 homologs. Specifically, Asp7 is conserved in subclass 2 proteins but not in subclass 1, where it can be replaced by Asn or Glu. Similarly, Glu59 is conserved among subclass 2 homologs but in subclass 1 this position is occupied by His. The remaining amino acids involved in hydrogen bonding with the ligands, His68, Tyr80 and Tyr82, are strictly conserved in the two subclasses. It is interesting to note that Zea1 interacts only with strictly conserved residues (His68, Tyr80), Zea2 with a conserved (Tyr82) and a variable (Asp7) residue, while Zea3 is attached at a variable site (Glu59).

Although a single crystal structure represents only a snapshot of a (possibly) dynamic situation, collectively the currently available structural infor-

mation allows a more accurate view of the hormone-binding protein–ligand interaction. In particular, it becomes clear that the PR-10 molecules are adaptable ligand binders, capable of binding a variety of ligands with the same amino acid sequence. The small-molecule partners of these interactions, such as cytokinins, are also plastic because of their unique adaptability to hydrogen-bonding situations and relative conformational freedom of the aliphatic fragment. Thus, the accumulating structural data on PR-10 proteins and their complexes may be revealing an exciting new mode discovered by nature for interactions between partners that are both capable of mutual adaptation. These interactions may not be very strong but are unusually versatile and, indeed, when characterized by relatively low binding constants, could guarantee efficient release of the small-molecule signals to their final receptors.

Considering the sequence similarity, wide distribution and lack of coordinated expression, one can speculate that the PR-10 family might have evolved to participate in biologically important processes, e.g., in the development program, through binding of physiologically relevant small molecules, such as cytokinins or brassinosteroids. Cytokinins, together with other plant hormones, are involved in differentiation of the shoot meristem and the tissues of the root, mitosis, leaf formation and senescence, and chloroplast development.⁴² Brassinosteroids contribute to the regulation of cell division and differentiation, and participate in the control of the overall development program, leading to morphogenesis.³⁰

In conclusion, a possible role of the PR-10 proteins could be to sequester water-insoluble hydrophobic ligands from the cytosol and to deliver them to their receptors. In this sense, they can be viewed as broad-specificity carriers and/or reservoirs for plant hormones and possibly other small-molecule ligands.^{26,31,32}

Experimental Procedures

Purification and crystallization

Recombinant LIPR-10.2B was expressed and purified from *Escherichia coli* cells as described.⁴³ The LIPR-10.2B/zeatin complex was formed by incubating 6 mg/ml protein (in 3 mM sodium citrate buffer, pH 6.3) with *trans*-zeatin (100 mM, dissolved in 50 mM HCl) in 1:4 molar ratio. Crystals were grown by the hanging-drop vapor-diffusion method at room temperature by equilibrating a mixture of 2 μ l protein solution with 2 μ l reservoir solution containing 1.2 M sodium citrate in 0.1 M Mes, pH 6.5, against 0.5 ml of the reservoir solution. Crystals grew within 2 weeks to dimensions suitable for X-ray analysis (0.25 mm $\times$ 0.25 mm $\times$ 1.00 mm).

Data collection, structure solution and refinement

X-ray diffraction data extending to 1.35 Å resolution were collected at the EMBL X13 beamline at the DORIS ring of the DESY synchrotron. The crystal was harvested

into a cryoprotectant solution composed of the reservoir solution containing 30% (v/v) glycerol, and was flash-frozen in a stream of N₂ gas at 100 K. The images were indexed, integrated and scaled using the HKL program package.⁴⁴ The crystals are hexagonal (Table 1) with one protein molecule in the asymmetric unit, corresponding to a solvent content of 60.6% (V_m 3.13 Å³/Da).⁴⁵ The structure was solved by molecular replacement with the program AMoRe⁴⁶ using the crystal structure of LIPR-10.2F (R. Dolot, personal communication) as search model (sequence similarity/identity 94%/89%). Most of the residues were clearly visible in the electron density map phased by molecular replacement. The remaining side chains, zeatin and water molecules and other components of the solvent region (Ca²⁺, glycerol) were built during interactive cycles of model building in XtalView,⁴⁷ followed by structure refinement in REFMAC5.⁴⁸ When the isotropic refinement had converged, full anisotropic model was introduced resulting in a drop of R and R_{free} by 0.032 and 0.027, respectively. In the last stages of the refinement, the model was supplemented with geometrically positioned riding hydrogen atoms. The occupancies of residues in double conformation were manually adjusted based on electron density maps and temperature factors. The final model includes 157 protein residues, four *trans*-zeatin ligands, 233 water molecules, one glycerol molecule and a calcium ion (Table 1).

Protein Data Bank accession numbers

Coordinates and structure factors of the LIPR-10.2B/zeatin complex have been deposited in the PDB, accession code 2qim.

Acknowledgements

We thank Luiza Handschuh for a batch of recombinant LIPR-10.2B protein and Alina Kasperska for help with protein purification. This work was supported by grants from the State Committee for Scientific Research to M.M.S. (grants 6 P04B 004 21 and 2 P04A 053 27) and to M.J. (grant N N204 2584 33) and by a subsidy from the Foundation for Polish Science to M.J. H.F. was supported by a Marie Curie Fellowship from the European Union.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2008.03.027](https://doi.org/10.1016/j.jmb.2008.03.027)

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