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Crystallographic and CD probing of ligand-induced conformational changes in a plant PR-10 protein

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ABSTRACT

Plant pathogenesis-related class 10 (PR-10) proteins are a family of abundant proteins initially identified as elements of the plant defense system. The key structural feature suggesting PR-10 functionality is a huge hydrophobic cavity created in the protein interior by a scaffold composed of an extended β-sheet wrapped around a long and flexible C-terminal α-helix. Several crystallographic and NMR studies have shown that the cavity can accommodate a variety of small molecule ligands, including phytohormones. The article describes ~1.3 Å resolution crystal structures of a *Lupinus luteus* PR-10 isoform LlPR-10.1A, in its free form and in complex with *trans*-zeatin, a naturally occurring plant hormone belonging to the cytokinin group. Moreover we present the structure of the same protein where the saturation with zeatin is not complete. This set of three crystal structures allows us to track the structural adaptation of the protein upon *trans*-zeatin docking, as well as the sequence of the ligand-binding events, step-by-step. In addition, titration of LlPR-10.1A with *trans*-zeatin monitored in solution by CD spectra, confirmed the pattern of structural adaptations deduced from the crystallographic studies. The ligand-biding mode shows no similarity to other zeatin complexes of PR-10 proteins. The present work, which describes the first atomic models of the same PR-10 protein with and without a physiological ligand, reveals that the conformation of LlPR-10.1A undergoes a significant structural rearrangement upon *trans*-zeatin binding.

1. Introduction

Within the huge superfamily of plant pathogenesis-related (PR) proteins, the members of class 10 (PR-10) are defined as small, slightly acidic and mainly cytosolic proteins. In variance with other PR classes, PR-10 proteins do not have a clearly defined biological function and their classification is often made according to structural similarity, even though it is quite apparent now that not all plant proteins with PR-10 fold and physicochemical properties are linked to pathogenesis mechanisms (van Loon et al., 2006). The key and common feature of the PR-10 fold is the presence of a hydrophobic cavity formed at the interface of the principal secondary structure elements, which are a long and flexible C-terminal α-helix α3, and a seven-stranded, antiparallel β-sheet. This folding scheme, resembling the thumb in a clenched fist, is completed by a number of loops (L1–L9) and by two additional short α-helices, α1 and α2, which form a V-shaped support for the C-terminal end of helix α3. The cavity has two entrances: E1 formed by helix α3 and loops L3, L5 and L7, and E2 situated between helix α3 and strand β1 (Fig. 1a).

Numerous structural and biophysical studies have demonstrated that the PR-10 cavity has the ability to bind diversified small molecules, leading to the suggestion that the PR-10 fold could be a generic solution to ligand binding utilized in different physiological processes in plants. Indeed, the PR-10 fold is exhibited by enzymes such as S-norcoclaurine synthase (Berkner et al.,

Abbreviations: 2iP, 6-(γ,γ-dimethylallylamino)purine; ABA, abscisic acid; ANS, 8-anilinonaphthalene-1-sulfonate; CD, circular dichroism; CPPU, *N*-(3-chloropyridyl)-*N*′-phenylurea; CSBP, Cytokinin Specific Binding Protein; DPU, diphenylurea; GA3, gibberellic acid; IAA, 3-indoleaceticacid; LlPR, *Lupinus luteus* pathogenesis-related protein; MPD, 2-methyl-2,4-pentanediol; PDB, Protein Data Bank; PEG, polyethylene glycol; PhBP, Phytohormone Binding Protein; PR, pathogenesis-related; R.M.S.D., root mean square deviation; ZEA, *trans*-zeatin.

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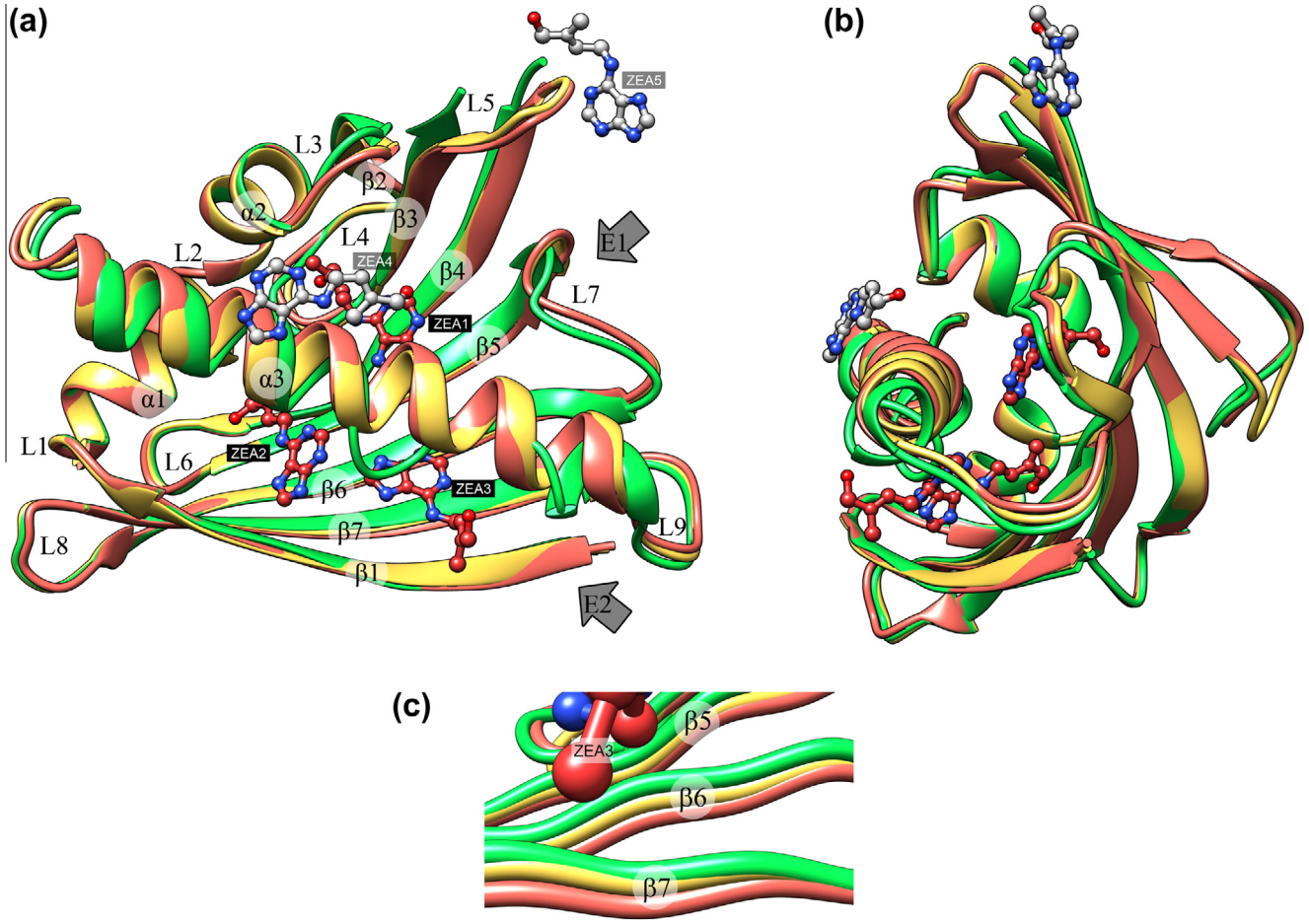

Fig. 1. (a) Superposition of the three LlPR-10.1A structures determined in this work, with annotation of the canonical structural elements of the PR-10 fold. The free form **F** is shown in green, the unsaturated form **U** in yellow, and the saturated form **Z** in red (with the external *trans*-zeatin molecules in gray). (b) A different view of the same superposition. (c) A zoom-in view illustrating the variable degree of caving of the β5–β7 fragment of the β-sheet.

2008) or TcmN aromatase/cyclase (Ames et al., 2008), hormone receptors such as Pyl1, which is a component of the abscisic acid (ABA) receptor (Miyazono et al., 2009) or the nodulin MtN13 (Ruszkowski et al., 2013), which takes part in cytokinin signaling in nodulating legumes. Other examples include proteins such as the birch pollen allergen Bet v 1 (Kofler et al., 2012), peanut panallergen Ara h 8 (Hurlburt et al., 2013), or yellow lupine isoform LlPR-10.2B (Fernandes et al., 2008, 2009), with likely storage or transport functions, as they are capable of accommodating numerous copies of chemically diversified ligand molecules in their huge internal cavity. In an attempt to correlate the PR-10 fold with function, Sliwiak et al. (2013) proposed a taxonomy of PR-10 proteins according to their cavities. In this classification, PR-10 members that bind ligands in a highly specific manner and are often involved in signaling, have cavity of type 1, which is small and shallow, has only one entrance (E1) and usually accommodates only one ligand molecule. On the other hand, PR-10 proteins that bind chemically different ligands nonspecifically and thus tend to have storage function, possess a huge cavity of type 2, which spans the entire body of the protein interior from E1 to E2, and is capable of accommodating two or more ligand molecules. Proteins from the latter group bind their ligands in an unusual manner, not only because of the variable number of the cargo molecules, but also because the number of strong, specific and anchoring interactions between the ligand and protein residues is very limited. However, despite this lack of specific interactions, atomic-resolution crystal structures have revealed in a number of cases a perfect order of the different ligand molecules, most often plant hormones.

Notably, plant hormones from the cytokinin group have been reported to be bound by numerous PR-10 proteins. In plant physiology, cytokinins stimulate cell division and additionally control the symbiotic root nodulation in legumes (Hwang et al., 2012). The most common among these adenine N6-derivatives is *trans*-zeatin (Fig. 2). Interestingly, synthetic urea derivatives, e.g. diphenylurea (DPU) or *N*-(3-chloropyridyl)-*N*′-phenylurea (CPPU), show very strong physiological activity almost identical to that of natural cytokinins (Ricci and Bertoletti, 2009) despite no chemical similarity.

PR-10 proteins were first linked to cytokinin binding by Fujimoto et al. (1998), who isolated a cytosolic fraction from *Vigna radiata* with high cytokinin affinity. The protein responsible for this interaction, named Cytokinin Specific Binding Protein (CSBP), was later shown to bind cytokinins with lower affinity (Pasternak et al., 2006) than originally reported and the binding mode (within a type 1 cavity) turned out to have a puzzling diversity. Moreover, a recent study revealed that the CSBP proteins bind gibberellin, which is an entirely different phytohormone, with higher affinity and specificity (Ruszkowski et al., 2014). Accordingly, the term Phytohormone Binding Protein (PhBP) has been proposed to replace CSBP as more appropriate. Nevertheless, another PR-10 protein with type 1 cavity, *Medicago truncatula* nodulation protein MtN13, involved in cytokinin signaling (Ruszkowski et al., 2013), was shown to bind cytokinins in a highly specific and reproducible manner.

The PR-10 members that have a large type 2 cavity and are promiscuous in ligand selection are also capable of cytokinin