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Crystal structure of a PR-10 nodulin in complex with *trans*-zeatin

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Abstract

Nodules are unique root organs of legume plants. Nodulation (nodule formation) is an extraordinary symbiotic process which eventually allows a plant to assimilate atmospheric nitrogen. The nitrogen-fixing bacteria that colonize the nodules convert molecular nitrogen N_2 into compounds suitable for plant metabolism. A class of plant hormones called cytokinins are known to affect the nodulation process. A small protein, Nodulin 13 from *Medicago truncatula* (MtN13), is expressed in the outer cortex of the nodule at early stages of nodulation. MtN13 belongs to class 10 of plant pathogenesis-related proteins (PR-10). PR-10 members have been shown to bind cytokinins. This paper focuses on the involvement of cytokinins in nodulation and presents a brief overview of the crystal structure of MtN13 in complex with *trans*-zeatin, a naturally occurring cytokinin.

Introduction

Plants, like all living organisms, require nitrogen to synthesize vital compounds, such as amino acids, nucleotides, and many others. Plants are unable to assimilate nitrogen from the atmosphere, but a family of legumes (*Fabaceae*) has developed an intriguing solution to this shortcoming. They encapsulate nitrogen-fixing bacteria in special root organs called nodules. This symbiotic interaction, called nodulation, is specific for both species, the plant host and the bacteria, meaning that the partners have to precisely recognize each other. The nitrogen-fixing bacteria belong to the taxon *Rhizobia*. The bacteria assimilate atmospheric, molecular nitrogen, convert it into inorganic or organic form, and supply the plant host with either ammonia or glutamine. Thanks to this symbiotic association, legumes do not require either any or only a very small amount of nitrogen fertilization. Historically, this made legumes perfect intercrops, and so important to humans. Bean, pea, soybean, peanut and lentil are just a few examples of legumes, whose importance and influence on our life on this planet cannot be overestimated.

Cytokinins (CKs) are plant hormones (phytohormones) that promote cell division (cytokinesis) and differentiation in various developmental processes. These processes include, for instance, apical dominance, axillary bud growth, leaf senescence and nodulation (Hwang et al., 2012). Their impact on nodulation is described in a separate section. From the chemical point of view, naturally occurring CKs are N6-substituted adenine derivatives (Fig. 1). There are also synthetic compounds, eg. phenylurea derivatives, that have the same properties *in planta*. The CK signal transduction pathway is a phosphorylation cascade (Hwang and Sheen, 2001; Kakimoto, 1996). It is composed of three consecutive steps. Signal transduction is triggered upon binding of the hormone to a plasma-membrane-anchored receptor kinase. The kinase phosphorylates a histidine-containing phosphotransfer protein (HPt). The latter migrates to the nucleus and activates a response regulator (RR). Until recently, it was thought that cytokinin perception is only extracellular and that the cytokinin-binding moiety was extracellular. Recent detection of cytokinin receptors in endoplasmic reticulum (ER) shed new light on cytokinin signaling (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011). However, despite the relatively thorough functional characterization of the CK transduction pathway, it is still not clear how exactly the transport of CKs occurs in the cytoplasm and whether there are any factors interfering with this process.

Proteins involved in nodulation are commonly termed nodulins. Gamas et al. (1998) reported that Nodulin

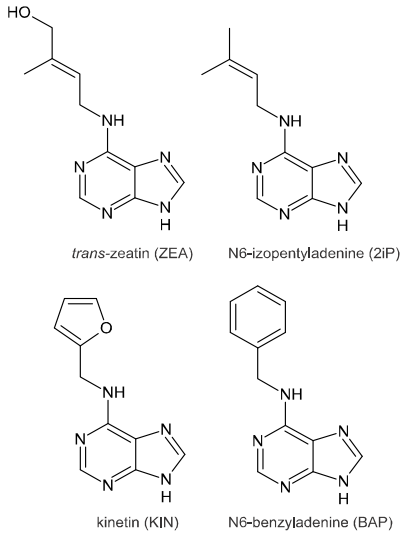

Fig. 1. Structures of four naturally occurring cytokinins

13 from *Medicago truncatula* (MtN13) is expressed only at early stages of nodulation. MtN13 belongs to class 10 of plant pathogenesis-related proteins (PR-10). Some proteins from this class are known to bind cytokinins (Fernandes et al., 2009; Fernandes et al., 2008; Pasternak et al., 2006). Two observations, namely the expression pattern of the MtN13 protein and the ability of some PR-10 proteins to bind CKs, suggest that MtN13 may bind these hormones. In this paper, a preliminary view is presented of the crystal structure of MtN13 in complex with *trans*-zeatin.

The role of cytokinin in the molecular mechanisms of nodulation

The pathway of nodulation events is Nod Factor (NF) dependent. NF is a variously modified lipooligosaccharide with a chitin skeleton, secreted by *Rhizobia* into the soil in the vicinity of a legume root. NF is perceived in the root epidermis by the Nod factor perception/Lys-M kinase receptor (NFP/LYK). Calcium signaling that involves Ca^{2+} and calmodulin dependent kinase (CCaMK) is then triggered. At this stage, the pathway diverges into two independent branches, both leading to an increased rate of cell division in the nodule primordium. The first pathway activates Nod factor signaling pathways 1 and 2 (NSP1/2), the ethylene response transcription factor (ERN) and the nodule inception transcription factor (NIN). The second pathway involves increased production of a cytokinin that activates the cytokinin receptor and starts the phosphorylation relay, known as the regular response to these hormones (Hwang and Sheen, 2001; Kakimoto, 1996). NSP2 is present in both the NF and the cytokinin dependent pathways.

There have been several studies which show that cytokinins are involved in nodulation. Studies on gain-of-function mutants of the CCaMK (Gleason et al., 2006; Tirichine et al., 2006) and the cytokinin receptor histidine kinase 1 from *L. japonicus* (LHK1) (Murray et al., 2007; Tirichine et al., 2007) have demonstrated that the pathways involving either the CCaMK or LHK1 are not only necessary but also sufficient for nodule organogenesis. Nodule formation in these cases does not involve actual infection by *Rhizobia* and such barren nodules obviously lack their function of nitrogen fixation. Moreover, the gain-of-function mutations of these genes have an additive effect and a double mutant generates more nodules than a single mutant. Also, other studies indicate that cytokinins are the key signaling molecules in the nodulation process. For example, non-symbiotic bacteria that carried a CK biosynthesis gene, coding isopentyl transferase (IPT), were able to rescue the morphogenesis of nodules (Cooper and Long, 1994). On the other hand, overexpression of genes responsible for cytokinin catabolism led to decreased organogenesis of nodules in *Lotus japonicus* (Lohar et al., 2004). Experiments with the cytokinin receptor (CRE1) from *M. truncatula* revealed that, when CRE1 is suppressed, the formation of nodules is defective (Gonzalez-Rizzo et al., 2006). The latter result is most relevant in our context, as it was obtained using CRE1 from the same organism as our MtN13 protein.

Structures of proteins involved in nodulation

In the Protein Data Bank (PDB) (Berman et al., 2000) there are only a few structures of proteins in-volved in nodulation. All of them, apart from MtN13, are from nitrogen-fixing bacteria, and not from the plant host. None of them show either structural or functional similarity to the MtN13 protein. Fowler et al. (2000), using nuclear magnetic resonance spectroscopy (NMR), reported the solution structure of a very small NodF protein from *Rhizobium leguminosarum*. Unfortunately, that report is not focused on the NodF protein but on methodology. Detailed structural information is available for only two proteins from NF biosynthesis. The first,

Table 1. Crystal structures of PR-10 proteins in complexes with cytokinins*

Protein name	Source organism	Cytokinin	Resolution [Å]	PDB code; Reference
LlPR-10.2B	*Lupinus luteus*	*trans*-zeatin	1.35	2qim; (Fernandes et al., 2008)
LlPR-10.2B	*Lupinus luteus*	diphenylurea (DPU)	1.95	3e85; (Fernandes et al., 2009)
Bet v 1a	*Betula pendula*	kinetin	1.40	4a85; (Kofler et al., 2012)
CSBP	*Vigna radiata*	*trans*-zeatin	1.20	2flh; (Pasternak et al., 2006)

* In the cases of Bet v 1a and CSBP only a single representative structure is listed. Simultaneous complexes with a cytokinin and non-cytokinin ligand are not included.

NodZ α-1,6-fucosyltransferase, catalyzes the transfer of fucose from GDP-fucose to the oligosaccharide core during NF biosynthesis. The crystal structures of the NodZ protein from *Bradyrhizobium* sp. (Brzezinski et al., 2007) and from its complexes with guanosine diphosphate, GDP, and GDP-fucose (Brzezinski et al., 2012) were determined. They revealed the residues responsible for binding the substrate and product molecules. The second protein, NodS, is an S-adenosyl-L-methionine (SAM)-dependent N-methyltransferase. Two crystal structures of the NodS protein from *Bradyrhizobium* sp. were reported: the unliganded form and its complex with S-adenosyl-L-homocysteine (SAH) (Cakici et al., 2010). With these crystal structures, two steps of NF biosynthesis are now clearer. The entire NF biosynthetic pathway is, however, far from being understood. A huge amount of work is still needed to fully elucidate the structural basis of this process. Many of the enzymes involved in NF biosynthesis are membrane proteins, and this makes them even more difficult to study.

Structural features of the MtN13 protein and its complex with *trans*-zeatin

The PR-10 group, of which MtN13 is a member, is a collection of diverse proteins with a generally poorly understood function but with a clear common fold (Fernandes et al., 2013). PR-10 group members are small (up to 19 kDa), slightly acidic proteins, found in the cytosol of various plants. Even though some of them share less than 20% amino acid identity, their overall fold is still preserved and consists of a seven-stranded antiparallel β-sheet and three α-helices. A hydrophobic cavity, recognized as a ligand-binding site, is formed between the β-sheet and the longest C-terminal α-helix. The structural diversity of the cavity between PR-10 proteins is expected to be a crucial determinant of protein-ligand interactions.

PR-10 proteins are key elements of plant defense systems. They have been studied for more than two decades because some of them are potent allergens. Despite many attempts, no exact, reliable and common function has been proposed for PR-10 proteins. One agreed conclusion is that PR-10 proteins have evolved to bind versatile small molecules that are partly hydrophobic, but each case has to be investigated individually.

In general, PR-10 proteins exist in solution as monomers. Even though some exceptions have been reported (Ma et al., 2006; Scholl et al., 2005), they have not been examined from the structural point of view. We have examined the oligomeric state of the MtN13 protein in solution using two methods. Firstly, dynamic light scattering (DLS) was used, which indicated the dimeric form of this protein (data not shown). This result has been confirmed to a very high degree of confidence using small angle X-ray scattering (SAXS) (data not shown). The dimerization of the MtN13 protein is an important issue to remember in the context of its cytokinin binding (*vide infra*).

There are several crystal PR-10 protein structures in complexes with cytokinins; most of them reported from our laboratory (Table 1). One should note the high resolution of all these structures. The existing complex structures show, however, an incoherent picture with no common pattern of cytokinin binding by PR-10 proteins.

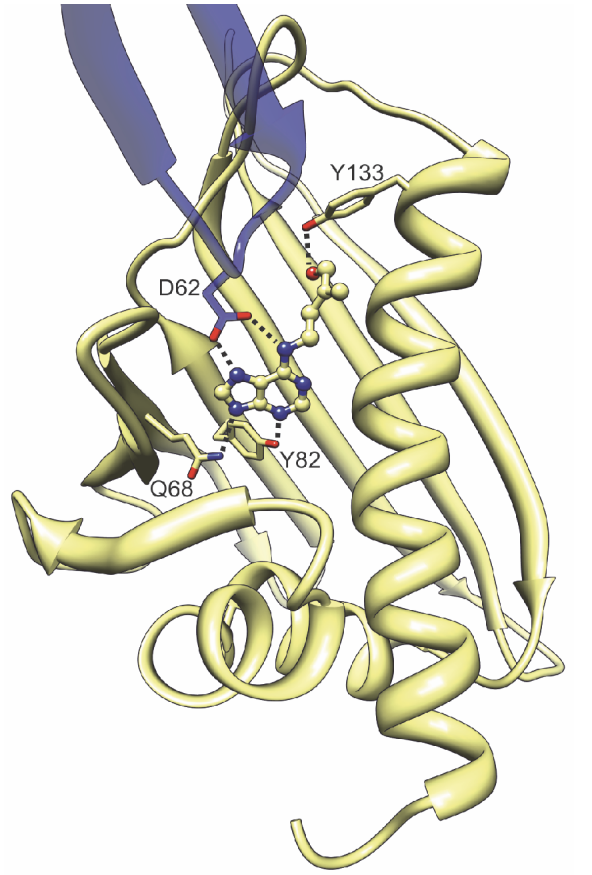

Fig. 2. The overall fold of MtN13 with *trans*-zeatin (in ball-and-stick representation) bound in the protein cavity. The loop from a second MtN13 molecule that also participates in the creation of the binding site is shown in semitransparent blue color. Hydrogen bonds are marked by dotted lines

In this context, it is very important to note that the *trans*-zeatin binding pattern of MtN13 suggests a strong and specific interaction (Fig. 2) and the *trans*-zeatin molecule is precisely defined in electron density maps. There is no other PR-10 protein, including the Cytokinin-Specific Binding Protein CSBP, that has more hydrogen-bond interactions with a cytokinin molecule than MtN13. In MtN13 there are five hydrogen bonds with a single *trans*-zeatin molecule. Three of these bonds are formed by the residues Gln68, Tyr82 and Tyr133 in the lumen of the cavity of the protein molecule where the cytokinin ligand is bound. In addition, Asp62 from the second subunit of the MtN13 dimer creates a fork with the two hydrogen bonds with the N6-N7 synthon of the cytokinin skeleton. The loops carrying Asp62 are mutually exchanged by the protein subunits, pushing the hormone molecules towards the core of the complementary protein molecule. The biological assembly is, therefore, formed by two protein molecules and two cytokinin ligands and the complex stoichiometry is best described as 2:2.

Conclusions and future perspectives

This paper has presented the initial findings of our preliminary studies on the structural features of legume plant nodulin MtN13 in complex with the phytohormone *trans*-zeatin. In a crystalline complex, the protein forms a dimer with a protein:ligand stoichiometry of 2:2. A binding site is created within the protein cavity with the participation of a structural element from the second subunit. Further crystallographic work is in progress to investigate if this hormone binding mode is reproduced by other cytokinins and other similar proteins from the PR-10 class. Along with these crystallographic studies, we are trying to determine the binding properties of MtN13 in solution. Comparison of the results obtained for different cytokinins will be of significant interest.

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