

Inositol Polyphosphate Binding Specificity of the Jasmonate Receptor Complex^{1[OPEN]}

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Recent findings that receptor complexes for auxin and jasmonate bind inositol polyphosphates stimulated the idea that plant hormone perception is regulated by inositol-derived molecules (Tan et al., 2007; Sheard et al., 2010). Inositol polyphosphates regulate critical cellular functions in eukaryotic cells (Munnik and Nielsen, 2011; Munnik and Vermeer, 2010; Gillaspay, 2013; Tsui and York, 2010; Kuo et al., 2014; Lee et al., 2015), and the discovery that these molecules bind to plant hormone receptors provides an interesting case model to study plant hormone perception. For instance, the ASK1-TIR1 component of the auxin receptor complex was copurified and cocrystallized with insect cell-derived inositol hexakisphosphate (InsP₆; Tan et al., 2007). TIR1 mutants defective in InsP₆ binding failed to interact with the IAA7 transcriptional repressor in the presence of auxin in yeast two-hybrid assays and in pull-down experiments using tagged-recombinant Aux/IAA protein (Calderón Villalobos et al., 2012), suggesting that InsP₆ binding might be important for auxin receptor function. Interestingly, the ASK1-COII component of the jasmonate receptor complex also copurified with inositol polyphosphate (Sheard et al., 2010). Here, NMR analyses revealed that insect cell-

purified, nondialyzed protein contained either D- and/or L-myo-inositol-1,2,4,5,6-pentakisphosphate (Sheard et al., 2010), also referred to as Ins(1,2,4,5,6)P₅ or short InsP₅ [3-OH] and Ins(2,3,4,5,6)P₅ or short InsP₅ [1-OH], respectively. Unfortunately, NMR cannot discriminate between enantiomers; therefore, the structure of the insect-purified InsP₅ isomer remains unresolved. Dialyzed ASK1-COII protein depleted of inositol polyphosphate failed to reconstitute the jasmonate receptor complex *in vitro*, while addition of InsP₅ [3-OH] robustly stimulated complex formation (Sheard et al., 2010). Interestingly, Ins(1,4,5,6)P₄ and InsP₆ also stimulated complex formation, although InsP₆ stimulated with lower efficiency (Sheard et al., 2010). Other InsP₅ isomers (including the possible alternative InsP₅ [1-OH] enantiomer) were not tested in this study.

In plants, three InsP₅ species with distinct chromatographic mobilities have been identified (Stevenson-Paulik et al., 2005; Hanke et al., 2012; Laha et al., 2015; Brearley and Hanke, 1996). Among them, only the isomeric nature of the symmetrical molecule InsP₅ [2-OH] was determined, while the identity of the other two InsP₅ isomers remains unknown (Stevenson-Paulik et al., 2005; Brearley and Hanke, 1996). Independent work in amoeba and in a pancreatoma cell line showed that inositol polyphosphates can be further phosphorylated at an existing phosphate position to give rise to inositol pyrophosphates, molecules such as InsP₇ and InsP₈ that contain energy-rich diphosphate bonds and have important cellular functions in amoeba, animal, and yeast cells (Menniti et al., 1993; Stephens et al., 1993; Shears et al., 2012; Mulugu et al., 2007; Wilson et al., 2013; Thota and Bhandari, 2015). Inositol pyrophosphates have also been detected in different plant species (Desai et al., 2014; Lemtiri-Chlieh et al., 2000; Brearley and Hanke, 1996; Laha et al., 2015), and recent work suggests an important function of these molecules in regulating jasmonate-dependent responses (Laha et al., 2015).

Jasmonate perception is regulated by COII, the F-box component of an SCF ubiquitin E3 ligase complex. COII recruits Jasmonate ZIM-domain (JAZ) transcriptional

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repressors upon binding to the bioactive jasmonic acid (JA) conjugate JA-Ile, resulting in polyubiquitylation and proteasomal degradation of the JAZ repressors and subsequent activation of jasmonate-dependent gene expression (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008; Pauwels and Goossens, 2011). A combinatorial approach analyzing InsP_8 -deficient *vih2* mutant plants and using in vitro reconstitution and in silico molecular docking experiments suggested that coincidence detection (i.e. simultaneous detection) of active jasmonate and the inositol pyrophosphate InsP_8 by the ASK1-COI1-JAZ receptor complex is critical for the activation of defense gene expression and for defenses against insect herbivores and necrotrophic fungi (Laha et al., 2015). Another study proposed InsP_5 [2-OH] to be involved in jasmonate perception (Mosblech et al., 2011). Collectively, these reports raise the question whether the jasmonate receptor shows selectivity for distinct inositol polyphosphates.

COMPETITIVE BINDING ASSAYS REVEAL LARGE DIFFERENCES IN RELATIVE BINDING AFFINITIES OF DISTINCT INSP_5 ISOMERS TO THE JASMONATE RECEPTOR COMPLEX

To investigate inositol polyphosphate binding specificity, we performed in vitro reconstitution experiments with insect cell-purified ASK1-COI1, recombinant JAZ proteins, the JA-Ile mimic coronatine, and [^3H] InsP_6 to determine IC_{50} values (50% inhibition of radioligand binding) for different InsP_5 isomers. This approach was chosen because radiolabeled InsP_5 isomers are not commercially available. A similar strategy was recently employed to investigate relative binding affinities of mammalian casein kinase-2 to InsP_6 , 5- InsP_7 , and a nonhydrolyzable InsP_7 derivative (Rao et al., 2014). We used His₈-tagged recombinant JAZ protein to pull down ASK1-COI1 in the presence of coronatine via Ni-NTA affinity chromatography and then determined [^3H] InsP_6 -derived activity (see "Supplemental Data"). For JAZ1, the following relative order of effectiveness of InsP_6 and the various InsP_5 isomers in competing with [^3H]- InsP_6 binding was observed (Fig. 1, A and B): InsP_5 [3-OH] (IC_{50} : 56 nM) \geq InsP_6 (IC_{50} : 58 nM) > InsP_5 [4-OH] (IC_{50} : 66 nM) > InsP_5 [2-OH] (IC_{50} : 146 nM) > InsP_5 [5-OH] (IC_{50} : 205 nM) > InsP_5 [6-OH] (IC_{50} : 363 nM) > InsP_5 [1-OH] (IC_{50} : 902 nM). The data suggest strong differences in the relative binding affinity of different InsP_5 isomers (including enantiomers) to the jasmonate receptor complex. For instance, the IC_{50} value of InsP_5 [1-OH] is 16-fold higher than that of InsP_5 [3-OH], suggesting a much higher affinity of the jasmonate receptor to InsP_5 [3-OH]. This is remarkable as both isomers are enantiomers that are chemically indistinguishable and for which a method to determine enantiomer identity has not yet been developed. Furthermore, the IC_{50} value for InsP_5 [2-OH], an isomer previously suggested to play a role in the activation of the jasmonate receptor (Mosblech et al., 2011), is

2.5-fold higher than that of InsP_5 [3-OH] and InsP_6 , suggesting it is less effective in potentiating jasmonate receptor assembly (Fig. 1B).

COI1 LARGELY DETERMINES THE INOSITOL POLYPHOSPHATE BINDING SPECIFICITY

To investigate the contribution of the JAZ protein to the inositol polyphosphate binding specificity, we performed similar experiments as described above with JAZ2, JAZ4, and JAZ9 (Fig. 1, C–E) with InsP_6 and selected InsP_5 isomers at a fixed concentration of "cold" inositol polyphosphate. The effectiveness of InsP_5 isomers to compete with [^3H] InsP_6 binding largely recapitulated the observations from the experiment using the JAZ1 protein, showing binding affinities in the following order: InsP_5 [3-OH] \geq InsP_6 > InsP_5 [2-OH]. Binding experiments with JAZ4 further indicate that, as in the case of JAZ1, InsP_5 [1-OH] has the weakest affinity, suggesting that the jasmonate receptor complex retains its ability to discriminate between the two enantiomers (1/3-OH) when using another JAZ protein. Altogether these data corroborate the idea that COI1, not the JAZ protein, determines inositol polyphosphate binding specificity.

The observation that InsP_5 [2-OH] has a weaker relative affinity than InsP_6 was surprising, as an increase of InsP_5 [2-OH] at the cost of InsP_6 in the Arabidopsis (*Arabidopsis thaliana*) *ipk1-1* mutant was previously proposed to activate COI1 function and to cause increased resistance to *Plutella xylostella* caterpillars (Mosblech et al., 2011).

INCREASE OF INSP_5 [2-OH] BY DEACTIVATION OF THE INOSITOL 1,3,4,5,6-PENTAKISPHOSPHATE 2-KINASE (IPK1) DOES NOT GLOBALLY ACTIVATE COI1 FUNCTIONS IN ARABIDOPSIS

To investigate whether findings by Mosblech et al. (2011) reflect a global role of InsP_5 [2-OH] in increasing COI1 functions, we analyzed the resistance of *ipk1-1* against *Alternaria brassicicola*, a fungal necrotroph that plants contain by COI1-dependent defenses (Leon-Reyes et al., 2010). In agreement with previous observations suggesting that InsP_8 (which is strongly reduced in *ipk1-1*) is critical for COI1 activation (Laha et al., 2015), the *ipk1-1* line showed a severe increase in susceptibility in this assay, similar to *coi1* mutant plants (Fig. 1F; Supplemental Fig. S1A). This is also in agreement with a previous report showing increased susceptibility of the *ipk1-1* line to *Botrytis cinerea*, another fungal necrotroph, in an assay where whole plants were sprayed with fungal spores and analyzed for plant survival (Murphy et al., 2008). We have repeated this assay with a complementary approach in which we spotted fungal spores onto the leaf surface and subsequently classified disease symptoms 72 h postinoculation. We again found increased susceptibility of the

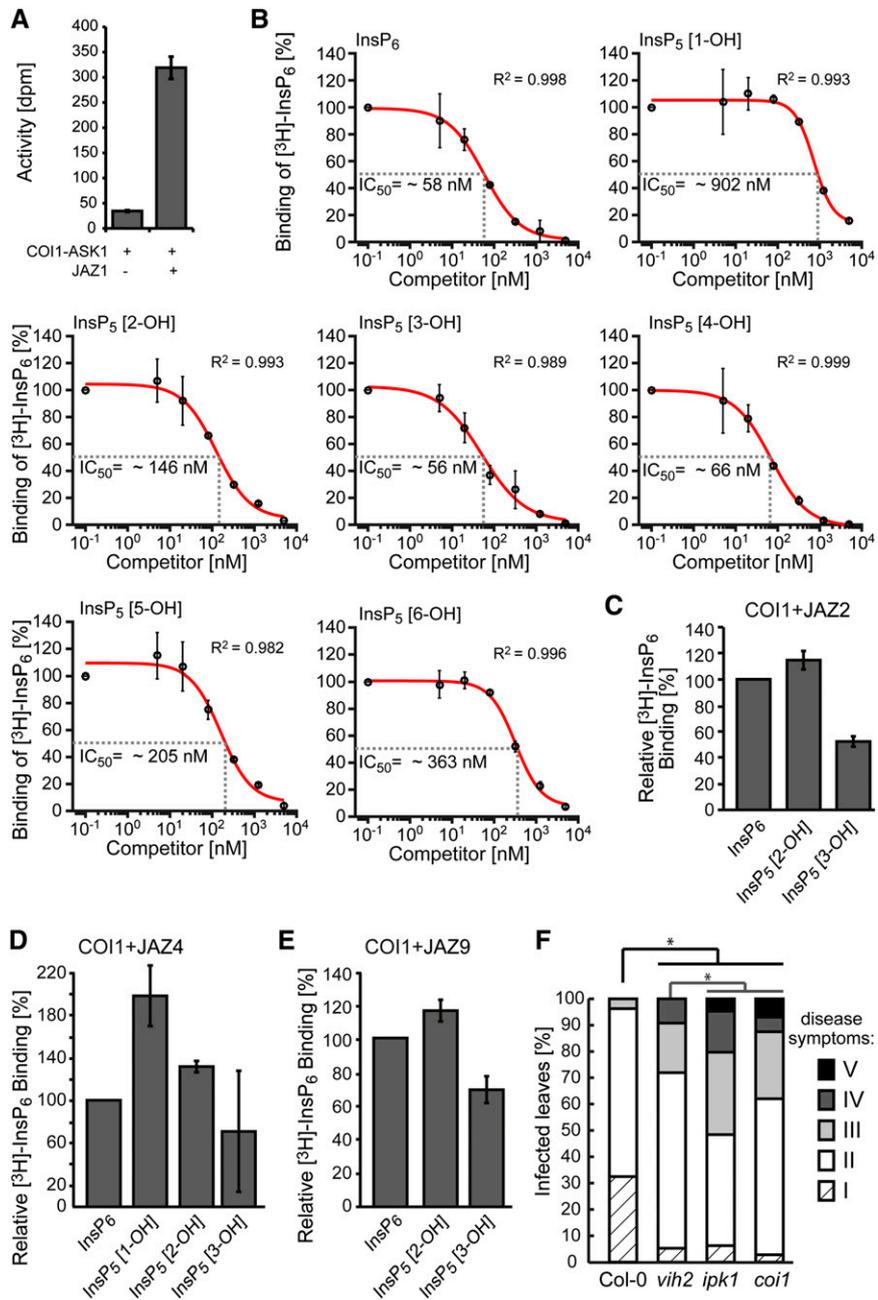


Figure 1. COI1 determines the inositol polyphosphate binding specificity of the jasmonate receptor complex. A, JAZ-dependent binding of [³H]InsP₆ to ASK1-COI. Insect cell-purified ASK1-COI1 was incubated with recombinant His₈-MBP-JAZ1 and [³H]InsP₆ in the presence of 1 μM coronatine. The complex was then purified by immobilized Ni²⁺ affinity chromatography (taking advantage of JAZ1's N-terminal His₈ tag), and the immobilized activity was determined by scintillation counting. A reaction in the absence of JAZ protein served as a negative control. Values show background-subtracted means ± SE. B, Competitive binding assays with [³H]InsP₆ and unlabeled inositol polyphosphates as indicated. Results are presented as percentage of total binding. Nonlinear regression analysis was employed to fit data to a sigmoidal model, which allowed the determination of IC₅₀ values. R² values given in the plots provide estimations for goodness of fit. Error bars represent ± SE. C to E, Relative [³H]InsP₆ binding to the ASK1-COI1 complex in the presence of 1 μM coronatine and different InsP₅ isomers and JAZ proteins as depicted. For the JAZ2 experiment, all competing InsP species were at 150 nM; for JAZ4, we used 80 nM of all InsP species; and for JAZ9, all competing InsP were at 50 nM. The average of [³H]InsP₆ binding to the jasmonate receptor complex in the absence of unlabeled inositol polyphosphate was set to 100%. The experiment was repeated with similar results. Error bars denote ± SE. F, Compromised defenses of *vih2-4*, *ipk1-1*, and *coi1-t* against a necrotrophic fungus corroborates a role of higher inositol polyphosphates (≥InsP₆) in COI1-dependent responses. All genotypes were treated with 5 μL of an *A. brassicicola* spore suspension (1 × 10⁶ spores/mL). Disease symptoms were scored in a double-blinded manner after 10 d of spore inoculation and categorized as different classes.

ipk1-1 line (Supplemental Fig. S1B) in complete agreement with Murphy et al. (2008). Collectively, these data question the idea that InsP_5 [2-OH] globally activates COI1 functions in vivo.

ANISOTROPIC COORDINATION OF THE COI1 SOLENOID BY INOSITOL POLYPHOSPHATE SUGGESTS ACTIVATION OF THE JASMONATE RECEPTOR BY AN ALLOSTERIC SWITCH

Our findings that the JAZ component of the jasmonate receptor has little, if any, effect on the relative inositol polyphosphate binding affinities (Fig. 1, B–E) suggest that COI1 largely determines the inositol polyphosphate binding specificity of the jasmonate receptor. This may be explained by *in silico* molecular docking experiments, which predicted the solenoid-fold of the F-box protein COI1 to provide an intricate network of electrostatic interactions engaging in inositol polyphosphate coordination (Laha et al., 2015). These docking experiments also predicted 1,5- InsP_8 to be a better ligand of the jasmonate receptor complex as compared with InsP_5 [3-OH], supporting a physiological role of InsP_8 jasmonate perception in agreement with defective defense gene expression and defective defenses against insect herbivores and necrotrophic fungi in plants compromised in InsP_8 synthesis (Laha et al., 2015). Unfortunately, the isomer identity of plant InsP_8 remains unknown. While our previous work indicates that VIH proteins convert 5- InsP_7 to 1,5- InsP_8 in yeast and thus have the ability to catalyze 1-PP bond formation (Laha et al., 2015), the isomer identity of VIH-dependent InsP_8 in plants remains elusive. This is mainly because the isomeric nature of plant InsP_7 is unknown. Since plant genomes do not encode Kcs1/IP6K enzymes (which are responsible for 5- InsP_7 production in nonplant eukaryotes) and since plant InsP_7 synthetases have not yet been identified, the structure identification of plant InsP_7 and InsP_8 remains a challenging task for future research. In addition, low amounts of these molecules in plant extracts complicate a thorough analysis. Assuming that VIH proteins retain their 1-PP synthetase activity independent of the InsP_7 substrate, we also performed *in silico* docking experiments with the remaining 1,X- InsP_8 isomers. All 1,X- InsP_8 isomers occupy largely overlapping sites of the presumptive inositol polyphosphate binding pocket (Fig. 2A; Supplemental Table S1). As we have previously seen for 1,5- InsP_8 and InsP_5 [3-OH], all inositol polyphosphates are coordinated by a single electrostatic interaction with the JAZ1 degron residue Arg-206 (Fig. 2, A and B; Supplemental Table S1). All 1,X- InsP_8 isomers

are furthermore predicted to form extensive interactions with the highly basic concave surface of the COI1 solenoid. Interestingly, these interactions stabilize and hold together the two faces of the inner wall of the Leu-rich repeat (LRR) solenoid that are distal and proximal to the hormone binding pocket (Fig. 2, A and B). At the distal face, the following COI1 residues are predicted to coordinate 1,X InsP_8 : Lys-79, Lys-81, His-118, Arg-120, and Arg-121. Additional residues at the distal face are Arg-85 (for 1,3- InsP_8 ; 1,4- InsP_8 ; 1,5- InsP_8), Lys-144 (for 1,3- InsP_8 ; 1,4- InsP_8 ; 1,6- InsP_8), and Lys-147 (for 1,2- InsP_8 ; 1,3- InsP_8 ; 1,4- InsP_8 ; 1,6- InsP_8). COI1 residues at the proximal face near the hormone binding site that are predicted to coordinate inositol polyphosphate are Arg-409, Arg-440, and additionally Arg-346 (for 1,2- InsP_8 ; 1,3- InsP_8 ; 1,5- InsP_8 ; 1,2- InsP_8) and Arg-492 (for 1,3- InsP_8 ; 1,4- InsP_8 ; 1,5- InsP_8 ; Fig. 2, A and B; Supplemental Table S1). The anisotropic nature of these interactions (which are partially compensated for by four single phosphate ions in the inositol polyphosphate-free crystal structure; PDB ID: 3OGM) is likely to have a strong effect on the elliptical shape of the LRR solenoid. Coronatine forms a salt bridge and hydrogen bond network with COI1 residues Arg-85, Arg-348, Arg-409, Tyr-444 and Arg-496. Two of these residues coordinate all (Arg-409) or most (Arg-85) inositol polyphosphate isomers and further stabilize the shape of the solenoid (Fig. 2, A and B). The elliptical shape in turn is likely critical for efficient recruitment of the JAZ1 degron to the top surface of the carboxy-terminal LRR domain: besides hydrophobic packing, a number of polar interactions stabilize the COI1-JAZ1 interface. For instance, strong interactions are mediated by a hydrogen bond formed between the backbone carbonyl of Ala-207 in JAZ1 and the backbone amide of COI1 residue Met-88, by the hydrogen bond interaction of Tyr-472 (COI1) with the backbone carbonyl of Leu-201 (JAZ1), a hydrogen bond donated by COI1 residue Arg-351 to the JAZ1 backbone carbonyl of Ile-203, a salt bridge formed between the side chain of JAZ1 residue Arg-205 and the carboxyl group of Glu-355 (COI1), a hydrogen bond donated by the same JAZ1 residue to the backbone carbonyl of Gly-352, a salt bridge formed between side chains of COI1 residue Glu-359 and Arg-216 in JAZ1, salt bridges formed between COI1 residues Glu-203/Asp-229 and JAZ1 residue Lys-215, hydrogen bonds between the COI1 backbone carbonyl of Met-203 and the side chain of JAZ1 residue Arg-211, as well as hydrogen bonds between the backbone carbonyl of Pro-202 in JAZ1 and the coronatine-interacting COI1 residue Arg-496 (Fig. 2C; Supplemental Fig. S2). The interaction is further stabilized by a hydrogen bond between the backbone

Figure 1. (Continued.)

Classes are defined as follows: Class I, light brown spots at the site of infection; Class II, dark brown spots on the site of infection; Class III, spreading necrosis; Class IV, leaf maceration; Class V, sporulation. The distribution of data were analyzed with a χ^2 test (no. of leaves, $n \geq 29$ classes contained at least 2.5% of total scorings per genotype), * $P < 0.05$. The experiments were repeated independently with similar results.

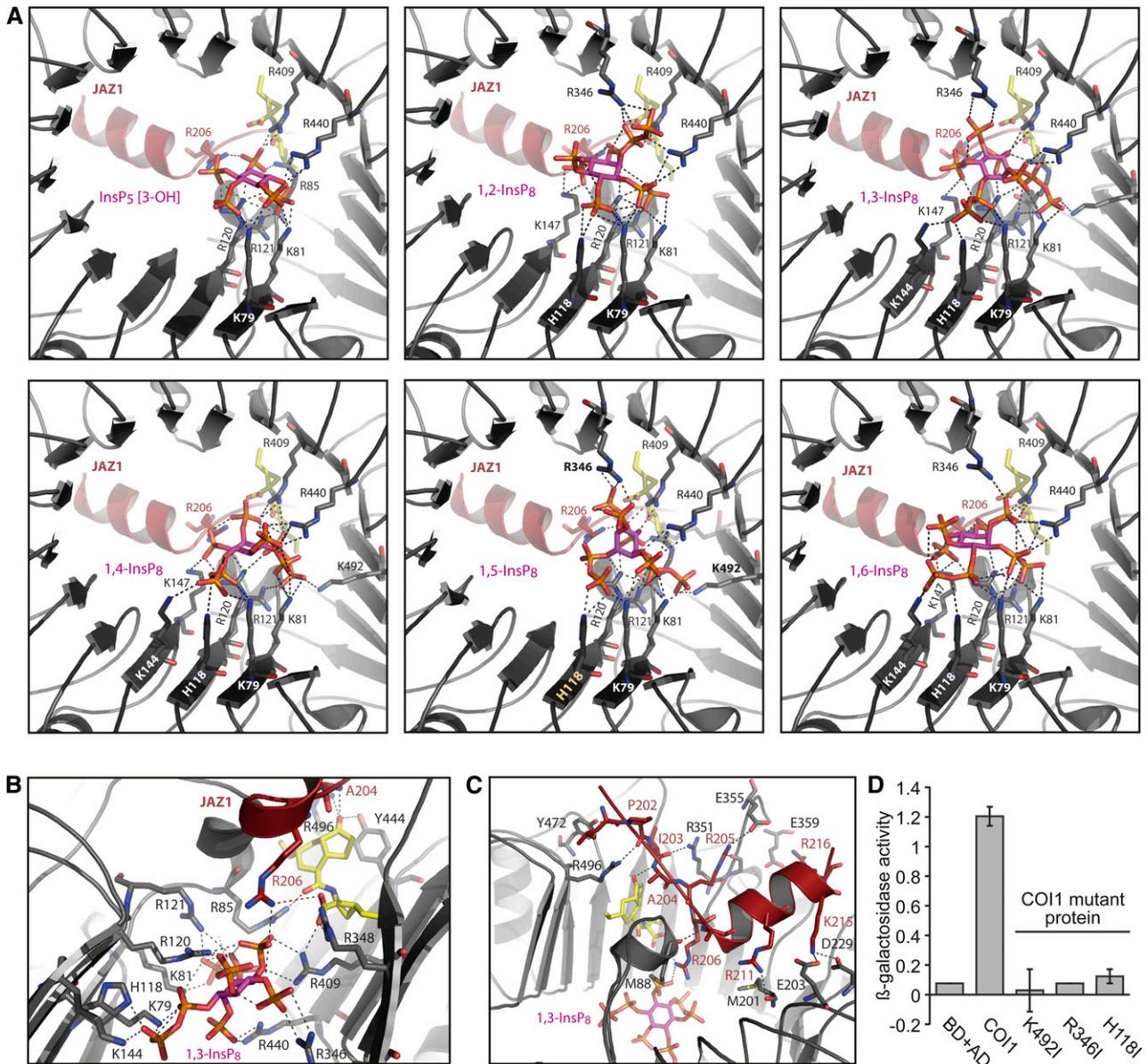


Figure 2. Anisotropic coordination of the COI1 solenoid by inositol polyphosphate suggests activation of the jasmonate receptor by an allosteric switch. A to C, Structural snapshots of the COI1-ASK1-JAZ1 degnon complex bound to coronatine and different inositol polyphosphates (as indicated) generated from in silico docking experiments. COI1 (gray), JAZ1 degnon (dark red), inositol polyphosphates (magenta stick), and coronatine (yellow stick) are presented. Residues employed for mutagenesis and yeast two-hybrid assays are depicted in bold (Arg-346, Lys-492) or orange (His-118) in the 1,5-InsP₈ structure. Bottom views (A) of InsP₅ [3-OH] and 1,X InsP₈ structures, as well as side view (B) and top view (C) of the presumed 1,3-InsP₈ and coronatine binding pockets, are shown. Dashed gray lines represent strong polar contacts; dashed firebrick lines mark a weak polar contact between the side chain of JAZ1 residue Arg-206 and the carboxy group of coronatine. D, JAZ12 interaction with wild-type or mutant COI1 in yeast was evaluated in the presence of 50 μ M coronatine by coexpression of pGBKT7-COI1 (and mutated versions as indicated) with pGADT7-JAZ12 in yeast strain Y187 (Clontech) and subsequent quantification of β -galactosidase-mediated hydrolysis of *ortho*-nitrophenyl- β -D-galactopyranoside. Values represent means of two independent biological replicas \pm SE. BD+AD, yeast strain harboring the empty vector controls pGBKT7 (expressing the Gal4 DNA-binding domain) and pGADT7 (expressing the Gal4 activation domain).

amide group of JAZ1 residue Ala-204 and the keto moiety of the hormone mimic coronatine, as well as by the interaction between JAZ1 residue Arg-206 and a phosphate group of the inositol polyphosphate ligand as mentioned above (Fig. 2C; Supplemental

Fig. S2). Because of the involvement of several strong polar backbone interactions (eight in total), it seems likely that small changes in the elliptical shape of the COI1 solenoid will have a strong effect on JAZ recruitment because backbone interactions cannot adjust

easily compared with interactions mediated by amino acid side chains. We therefore propose that inositol polyphosphate might induce a conformational change or allosteric switch of the COI1 carboxy-terminal LRR solenoid that, with the help of coronatine/JA-Ile, allows docking of the JAZ degenon.

To distinguish InsP_5 - and InsP_8 -dependent interaction of COI1-JAZ in the yeast system (in which, based on the catalytic activities of Kcs1/IP6K and Vip1/PPIP5K enzymes, the identity of InsP_8 is likely to represent 1,5- InsP_8 ; Wang et al., 2011; Draskovic et al., 2008), we have previously engineered single COI1 mutant proteins affected in residues His-118, Lys-492, and Arg-346. These residues were chosen since all three are predicted to interact with 1,5- InsP_8 , but for geometrical reasons, not all three residues can interact simultaneously with an InsP_5 molecule (irrespective of InsP_5 isomer identity). The observation that all single mutant COI1 proteins failed to interact with JAZ1 in a yeast two-hybrid assay suggested that, at least in yeast, InsP_5 isomers are not critically involved in COI1-JAZ1 interaction (Laha et al., 2015). We have now extended these analyses to the interaction between COI1 and JAZ12. We have chosen JAZ12 because it is, together with JAZ11, most distantly related to JAZ1 (Cuéllar Pérez et al., 2014). While wild-type COI1 interacted robustly with JAZ12, all three single Ile substitutions of COI1 residues His-118, Lys-492, and Arg-346 strongly compromised COI1-JAZ interaction despite stable protein being made in all cases (Fig. 2D; Supplemental Fig. S3). These results suggest that for the interaction of COI1 with JAZ12, like JAZ1, in yeast, InsP_5 isomers are unlikely to play a major role, providing further evidence that COI1, not the JAZ partner, determines inositol polyphosphate binding specificity.

FUTURE TASKS

We envisage that the ability of the jasmonate receptor to discriminate between inositol polyphosphate enantiomers might be employed as a tool to reveal isomer identity of these molecules in biological samples, independent of their precise role in activating jasmonate perception. To address more directly the role of inositol pyrophosphates in triggering an allosteric switch of the COI1 carboxy-terminal LRR solenoid, we propose molecular dynamics simulations and/or crystallization of various ASK1-COI1 complexes in the presence and absence of ligands, as well as traditional biochemical measurements of affinity. The latter two approaches are currently complicated by the lack of commercially available InsP_8 isomers and, more importantly, because the isomer identity of plant InsP_8 remains unknown. It will be a major task for future research to develop technologies to determine the structure of inositol polyphosphates when present in only small amounts in biological extracts. Additionally, it will be important to identify the proteins that generate InsP_7 in plants so as

to allow in vitro reactions to produce sufficient amounts of InsP_7 and InsP_8 for proper structure determination.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *VIH2* (At3g01310), *JAZ1* (At1g19180), *JAZ2* (At1g74950), *JAZ4* (At1g48500), *JAZ9* (At1g70700), *JAZ12* (At5g20900), *ASK1* (At1g75950), *COI1* (At2g39940), and *IPK1* (At5g42810). Accession numbers for T-DNA insertion lines are as follows: *vih2-4* (GK-080A07), *ipk1-1* (SALK_065337C), and *coi1-t* (SALK_035548).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Increased susceptibility of the Arabidopsis *ipk1-1* mutant to fungal necrotrophs.

Supplemental Figure S2. Polar backbone interactions between JAZ1 and COI1 suggest strong influence of COI1 carboxy-terminal LRR solenoid shape on JAZ recruitment.

Supplemental Figure S3. Immunoblots of soluble lysates prepared from yeast transformants.

Supplemental Table S1. List of presumptive electrostatic interactions between inositol polyphosphate and the jasmonate receptor complex; script for plotting sigmoidal curves.

Supplemental Methods.

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