Inositol Polyphosphate Binding Specificity of the Jasmonate Receptor Complex

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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; Gillaspy, 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 with inositol hexakisphosphate (InsP$_6$) (Tan et al., 2007). TIR1 mutants defective in InsP$_6$ 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 InsP$_6$ binding might be important for auxin receptor function. Interestingly, the ASK1-COI1 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 $\alpha$- and/or $\gamma$-myo-inositol-1,2,4,5,6-pentakisphosphate (Sheard et al., 2010), also referred to as Ins$_{(2,4,5)}$P$_6$ or short Ins$_{P_6}$ [3-OH] and Ins$_{(2,3,4,5,6)}$P$_5$ or short Ins$_{P_5}$ [1-OH], respectively. Unfortunately, NMR cannot discriminate between enantiomers; therefore, the structure of the insect-purified Ins$_{P_6}$ isomer remains unresolved. Dia-lyzed ASK1-COI1 protein depleted of inositol polyphosphate failed to reconstitute the jasmonate receptor complex in vitro, while addition of Ins$_{P_6}$ [3-OH] robustly stimulated complex formation (Sheard et al., 2010). Interestingly, Ins$_{(1,4,5,6)}$P$_4$ and Ins$_{P_5}$ also stimulated complex formation, although Ins$_{P_6}$ stimulated with lower efficiency (Sheard et al., 2010). Other Ins$_{P_5}$ isomers (including the possible alternative Ins$_{P_5}$ [1-OH] enantiomer) were not tested in this study.

In plants, three Ins$_{P_5}$ 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 Ins$_{P_5}$ [2-OH] was determined, while the identity of the other two Ins$_{P_5}$ 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 Ins$_{P_7}$ and Ins$_{P_8}$ 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 COI1, the F-box component of an SCF ubiquitin E3 ligase complex. COI1 recruits Jasmonate ZIM-domain (JAZ) transcriptional
receptors 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 InsP5-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 InsP5 by the ASK1-COII-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 InsP5 [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 INSP5 ISOMERS TO THE JASMONATE RECEPTOR COMPLEX

To investigate inositol polyphosphate binding specificity, we performed in vitro reconstitution experiments with insect cell-purified ASK1-COII, recombinant JAZ proteins, the JA-Ile mimic coronatine, and [3H]InsP5 to determine IC50 values (50% inhibition of radioligand binding) for different InsP5 isomers. This approach was chosen because radiolabeled InsP5 isomers are not commercially available. A similar strategy was recently employed to investigate relative binding affinities of mammalian casein kinase-2 to InsP6,5 and a nonhydrolyzable InsP7 derivative (Rao et al., 2014). We used His8-tagged recombinant JAZ protein to pull down ASK1-COII in the presence of coronatine via Ni-NTA affinity chromatography and then determined [3H]InsP5-derived activity (see “Supplemental Data”). For JAZ1, the following relative order of effectiveness of InsP5 and the various InsP5 isomers in competing with [3H-InsP5] binding was observed (Fig. 1, A and B): InsP5 [3-OH] (IC50: 56 nm) ≈ InsP5 [5-OH] (IC50: 58 nm) > InsP5 [4-OH] (IC50: 66 nm) > InsP5 [2-OH] (IC50: 146 nm) > InsP5 [5-OH] (IC50: 205 nm) > InsP5 [6-OH] (IC50: 363 nm) > InsP5 [1-OH] (IC50: 902 nm). The data suggest strong differences in the relative binding affinity of different InsP5 isomers (including enantiomers) to the jasmonate receptor complex. For instance, the IC50 value of InsP5 [1-OH] is 16-fold higher than that of InsP5 [3-OH], suggesting a much higher affinity of the jasmonate receptor to InsP5 [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 IC50 value for InsP5 [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 InsP5 [3-OH] and InsP6, 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 InsP6 and selected InsP5 isomers at a fixed concentration of “cold” inositol polyphosphate. The effectiveness of InsP5 isomers to compete with [3H]InsP6 binding largely recapitulated the observations from the experiment using the JAZ1 protein, showing binding affinities in the following order: InsP5 [3-OH] ≥ InsP5 > InsP5 [2-OH]. Binding experiments with JAZ4 further indicate that, as in the case of JAZ1, InsP5 [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 InsP5 [2-OH] has a weaker relative affinity than InsP5 was surprising, as an increase of InsP5 [2-OH] at the cost of InsP5 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 INSP5 [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 InsP5 [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 InsP5 (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 ± SEM. 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 ± SEM. 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 ± SEM. F, Compromised defenses of vih2-4, ipk1-1, and coi1-t against a necrotrophic fungus corroborates a role of higher inositol polyphosphates (≥InsP5) 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.
ipkl-1 line (Supplemental Fig. S1B) in complete agreement with Murphy et al. (2008). Collectively, these data question the idea that InsP₅ [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 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-InsP₈ to be a better ligand of the jasmonate receptor complex as compared with InsP₅ [3-OH], supporting a physiological role of InsP₈ jasmonate perception in agreement with defective defense gene expression and defective defense against insect herbivores and necrotrophic fungi in plants compromised in InsP₇ synthesis (Laha et al., 2015). Unfortunately, the isomeric identity of plant InsP₈ remains unknown. While our previous work indicates that VIH proteins convert 5-InsP₂ to 1,5-InsP₈ in yeast and thus have the ability to catalyze 1-PP bond formation (Laha et al., 2015), the isomeric identity of VIH-dependent InsP₈ in plants remains elusive. This is mainly because the isomeric nature of plant InsP₈ is unknown. Since plant genomes do not encode Kcs1/IP₆K enzymes (which are responsible for 5-InsP₂ production in nonplant eukaryotes) and since plant InsP₂ synthetases have not yet been identified, the structure identification of plant InsP₂ and InsP₈ 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 InsP₇ substrate, we also performed in silico docking experiments with the remaining 1,X-InsP₈ isomers. All 1,X-InsP₈ 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-InsP₈ and InsP₅ [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-InsP₈ 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-InsP₈: Lys-79, Lys-81, His-118, Arg-120, and Arg-121. Additional residues at the distal face are Arg-85 (for 1,3-InsP₇; 1,4-InsP₇; 1,5-InsP₇), Lys-144 (for 1,3-InsP₇; 1,4-InsP₇; 1,6-InsP₇), and Lys-147 (for 1,2-InsP₇; 1,3-InsP₇; 1,4-InsP₇; 1,6-InsP₇). 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-InsP₇; 1,3-InsP₇; 1,5-InsP₇; 1,2-InsP₇) and Arg-492 (for 1,3-InsP₇; 1,4-InsP₇; 1,5-InsP₇; 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 carboxyl 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 carboxyl of Leu-201 (JAZ1), a hydrogen bond donated by COI1 residue Arg-351 to the JAZ1 backbone carboxyl 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 carboxyl 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 carboxyl of Met-203 and the side chain of JAZ residue Arg-211, as well as hydrogen bonds between the backbone carboxyl 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 at the site of infection; Class III, spreading necrosis; Class IV, leaf maceration; Class V, sporulation. The distribution of data were analyzed with a χ² test (no. of leaves, n ≥ 29 classes contained at least 2.5% of total scorings per genotype), * P < 0.05. The experiments were repeated independently with similar results.
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 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
To distinguish InsP$_5^+$ and InsP$_5^−$-dependent interaction of COI1-JAZ in the yeast system (in which, based on the catalytic activities of Kcs1/IP6K and Vip1/PPPIP5K enzymes, the identity of InsP$_5^+$ is likely to represent 1,5-InsP$_5^+$; 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 selected since all three are predicted to interact with 1,5-InsP$_5^+$ 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 antagonists 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$_5^-$ isomers and, more importantly, because the isomer identity of plant InsP$_5^-$ 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: V122 (At3g01310), JAZ1 (At1g19130), JAZ2 (At1g74950), JAZ4 (At1g48500), JAZ9 (At1g70700), JAZ12 (At5g20900), ASK1 (At1g78950), COI1 (At2g39940), and IPK1 (At5g42810). Accession numbers for T-DNA insertion lines are as follows: vih2-4 (GK-080A07), ipk1-1 (SALK_065337C), and coi-1 (SALK_035548).

**Supplemental Data**

The following supplemental materials are available.

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

**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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**LITERATURE CITED**


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