Molecular modelling and BiFC studies of protein-protein interactions in cytokinin signaling

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Cytokinins are phytohormones that regulate various physiological processes in higher plants. Three main proteins are involved in the cytokinin signaling. First of them is transmembrane cytokinin receptor – hybrid sensor histidine kinase (AHK in Arabidopsis). Upon cytokinin binding, AHK in dimeric form autophosphorylates itself and transmits the acquired phosphate as a signal to the second protein – histidine-containing phosphotransfer protein (in Arabidopsis AHP, or phosphotransmitter). AHP in turn transfers the phosphate to the third type of protein – response regulator (ARR in Arabidopsis). B-type ARR interacts with target genes culminating the cytokinin signaling pathway. This pathway uses the histidine-aspartate phosphorelay coupled with multiple protein-protein interactions (AHK dimerization, AHK-AHP interaction, AHP dimerization and AHP-ARR interaction). Our research was aimed to investigate the molecular mechanism of some of these interactions.

Previously, using the bimolecular fluorescence complementation (BiFC), we experimentally showed that all of Arabidopsis cytokinin receptors (AHK2-4) can interact with three phosphotransmitters (AHP1-3) without prominent preference. Also we demonstrated that AHPs can form homo- and heterodimers, mainly in the nucleus of the plant cell. To gain insight into promiscuity of receptor-phosphotransmitter interaction at the atomic level, we performed molecular modeling of this complex. Using the Modeller software, homology models for all combinations of complexes of Arabidopsis phosphotransmitters AHP1-3 bound to receiver domains of Arabidopsis cytokinin receptors AHK2-4 were built. The crystal structures of CKI2-AHP1 complex (PDB ID: 4EUK) and CKI1 monomer (PDB ID: 3MMN) were used as templates. All modelled complexes were energy minimized in AMBER ff14SB force field and validated by ProCheck software. The obtained models had acceptable stereochemical quality.

Distribution of hydrophobic and hydrophilic regions on the interfaces of all investigated AHP-AHK complexess is very similar. The promiscuity of receptor-phosphotransmitter interaction might be explained, at least partly, by this similarity. According to QtPISA assessment, total binding energy of the interfaces varies in different complexes from -9.1 to -14.2 kcal/mol. Interface area of different complexes ranges between 777.5 and 873.4 $Å^2$. Number of hydrogen bonds within the interaction interfaces of modelled complexes varies from 5 to 13, and number of salt bridges varies from 1 to 9. To reveal the key amino acids and their interactions in the interfaces that could be critical for the cytokinin receptor–phosphotransfer binding, a virtual alanine scanning was performed using Rosetta implemented in Robetta with default settings. As a result, three main “hot spot” residues (producing the largest change of ΔGf when mutated to alanine) were identified on the receiver domain side of the interface: K1013 (AHK3rd-AHP2 numbering is used) that does not form any intermolecular hydrogen bonds or salt bridges, N898 that interacts with Q83 and S87 phosphotransmitter’s residues, and N901 forming hydrogen bond with S90 of AHP. S87 was independently highlighted by the alanine scanning.

The role of the predicted key interactions in the receptor-phosphotransmitter interface was studied experimentally. The putative interacting residues of AHK3 and AHP2 were consecutively replaced by Ala: N898A and N901A in AHK3rd, and Q83A, S87A and S90A in AHP2, resulting in a removal of three predicted hydrogen bonds. Three-stages PCR was employed for site-directed mutagenesis. According to

our BiFC data, mutations in the phosphotransmitter counterpart decreased the fluorescence (i.e. interaction). Triple mutation (Q83A-S87A-S90A) reduced it almost to the level of control untransformed leaf, regardless of the presence or absence of mutations in the partner protein AHK3, while the expression level of both partners, controlled by Western-blot, stayed quite stable. These data confirmed our prediction about the key role of these hydrogen bonds in the receptor-phosphotransmitter interaction. But in cases when AHK mutants was co-expressed with wild type AHP, only N898A mutation in AHK3 was effective to reduce the fluorescence.

The next purpose of our investigation was AHP-AHP interface and its comparison to AHP-AHKrd interface. OsHP1 (PDB ID's: 1YVI, 2Q4F) – is the only structure in PDB of a homodimer form of histidine-containing phosphotransfer protein. To use the OsHP1 crystal structure as a template for modelling AHP-AHP complexes, we checked if this crystal structure has a biological relevance. Blind docking of AHP2 homology model monomers was performed using ClusPro and PatchDock servers. Both ClusPro and PatchDock best structures have very similar conformation to OsHP1 crystal structure and homology model of AHP2 dimer. According to accepted conformation of AHP2-homodimer, the AHP-AHP interface is located on the same side of AHP surface as AHP-AHKrd interface. Alanine scanning for AHP2-AHP2 complex shows that main "hot spots" for it are Q83, S87 and D54, located in the center of interaction interface. So at least one of "hot spot" amino acids is the same as in the AHKrd-AHP interaction. According to PISA interface summary, 34 residues of AHP2 are involved in the interaction with AHK3 receiver domain, and 25 in case of homodimerization; 18 of them are identical in both cases. Validation of the complexes with PISA and PRODIGY services showed that AHP2 binds to AHK3rd with lower affinity than to another AHP2. To investigate the conservation of AHKrd and AHP surface residues, multiple alignment was performed in ClustalX. Orthologs and paralogs for AHP and AHK proteins were found using OMA browser. Only phosphotransmitters that have a conserved histidine able to phosphorylation, and histidine kinases that have a conserved aspartate in the receiver domain, were included in alignment. Totally, 103 sequences were used for multiple alignment of phosphotransmitters and 94 for receiver domains. Multiple alignments were implemented in Chimera session and surfaces of the AHP2 and AHK3rd was rendered by conservation percent to show that interaction interfaces are the most conserved regions of both proteins. This is an additional explanation for the interaction promiscuity between various HKs and HPs. Also it can support correctness of AHP-AHP interface choice.