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ORIGINAL ARTICLE

In planta validation of HK1 homodimerization and recruitment of preferential HPt downstream partners involved in poplar multistep phosphorelay systems

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Abstract
Multistep phosphorelays involve a phosphate transfer from sensor histidine-aspartate kinases (HKs) to response regulators (RRs), via histidine-containing phosphotransfer proteins (HPts). In *Arabidopsis*, some AHK receptors are organized as homodimers and able to interact with HPts (AHPs). However, there are no data available concerning the dimerization of the *Arabidopsis* osmosensor AHK1. Although only AHP2 is able to interact with AHK1 in yeast, validation of this interaction remains to be clarified in planta. The ability of poplar HK1 osmosensor, homologous to AHK1, to homodimerize and interact with three HPts (HPt2, 7 and 9) as preferential partners has been previously shown by yeast two-hybrid assay. However, protein interaction studies need to use complementary approaches to avoid interaction artifacts. Here, we confirmed *in planta* homodimerization of the cytoplasmic part of HK1 (HK1-CP) and the functional relevance of HK1-CP/HPt interactions by bimolecular fluorescence complementation assays. This work led us to validate these partnerships and to propose them as probably involved in osmosensing pathway in *Populus*.

Keywords: *Histidine-aspartate kinase, histidine-containing phosphotransfer protein, osmosensing pathway,* Populus*, protein interactions*

Introduction

Adverse environmental conditions, such as mechanical stress, high or low temperatures, heavy metals or drought, affect plant functions and growth (Scippa et al. 2008; Durand et al. 2011a, 2011b, 2012; Pal'ove-Balang & Mistrík 2011; Rahman et al. 2012; Trupiano et al. 2012a, 2012b). Plant response and adaptation to water stress are dependent on its perception by the multistep phosphorelay system that enables to perceive also plant hormone such as cytokinins. In *Arabidopsis thaliana*, this system is composed of histidine-aspartate kinases (AHKs), histidine-containing phosphotransfer proteins (AHPs) and response regulators (ARRs) (Hwang et al. 2002). In this model plant, five ethylene receptors (Grefen & Harter 2004); three cytokinin receptors AHK2, AHK3 and AHK4/CRE1 (Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006); one osmosensor AHK1 (Urao et al. 1999; Tran et al. 2007) and two cytokinin-independent AHKs CKI1 (Pischke et al. 2002; Hejátko et al. 2003, 2009; Deng et al. 2010) and AHK5/CKI2 (Iwama et al. 2007; Desikan et al. 2008) have been identified. Several AHKs are able to dimerize as described for AHK2, 3 and 4 (Dortay et al. 2006; Caesar et al. 2011;

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Wulfetange et al. 2011) and CKI1 (Hejátko et al. 2009). The downstream members of this pathway are represented by 5 authentic AHPs and 11 B-type RRs, proteins of ARR family including also the A-type, C-type and pseudo-RRs (Pils & Heyl 2009). Binding of cytokinin to AHKs sensory CHASE domain initiates a phosphate transfer from these receptors to shuttle AHPs, and finally to B-type ARRs, transcription factors constituting the final output of cytokinin pathway. In addition to cytokinin signalling, multistep phosphorelays are also involved in other signalling pathways as osmosensing. AHK1 acts as an osmosensor in both yeast (Urao et al. 1999) and plant (Tran et al. 2007). This receptor seems to be involved in the control of the stomatal density to avoid dehydration during drought (Kumar et al. 2013) and also plays a role in the regulation of desiccation processes during seed formation (Wohlbach et al. 2008). Among the five authentic AHPs, only AHP2 has been shown to interact with AHK1 in yeast two-hybrid (Y2H) system (Urao et al. 2000). Although interaction studies between some HKs (CKI1 and AHK5) and HPts have been shown *in planta* (Pekárová et al. 2011; Mira-Rodado et al. 2012), no information is available concerning the AHK1/AHP interactions *in planta*.

In *Populus*, some multistep phosphorelay members have been identified such as HK1, 10 HPts and 8 B-type RRs. HK1 is able to function as osmosensor in yeast. The homodimerization of the cytoplasmic part of HK1 (HK1-CP) has been also demonstrated and three HPts (HPt2, 7 and 9) were identified as downstream partners of HK1 by only Y2H interaction studies (Chefdor et al. 2006; Héricourt et al. 2013). Moreover, we showed the functional relevance of interactions between these three HPts and B-type RRs by both Y2H and biomolecular fluorescence complementation (BiFC) analyses (Bertheau et al. 2012). A recent study in *Arabidopsis* showed that some interactions between AHK5 and AHPs established in yeast were not confirmed *in planta* constituting a yeast artifact (Mira-Rodado et al. 2012). Here, we confirmed, in plant cells, the interactions observed in yeast, highlighting their pertinence in a possible multistep phosphorelay in *Populus*.

Materials and methods

Y2H assays

Y2H assays were performed according to Héricourt et al. (2013). A cell suspension with an Optical Density at wavelength of 600 nm (OD_{600}) of 0.2 was prepared for each type of interaction. Ten microliters of each cell suspension were dropped on control medium -LW (-Leu, -Trp) and interaction selective medium -LWH (-Leu, -Trp, -His) supplemented with 5 mM of 3AT (3-amino-1,2,4-triazole). In addition, two dilutions (1:10 and 1:100) of each cell suspension (OD_{600} of 0.2) were prepared and dropped on -LWH medium. Yeast cells grew during four days at 30°C.

Plasmids construction

BiFC assays were conducted using the pSPYNE(R) 173 and pSPYCE(MR) plasmids (Waadt et al. 2008), and pSPYNE173 and SPYCE(M) plasmids (Guirimand et al. 2010) which allowed the expression of a protein fused to the C- or N-terminal of the split-yellow fluorescent protein (YFP) fragments, respectively. The cDNA of HK1-CP was cloned via *Spe*I in frame with the N- or C-terminal fragments of YFP, generating four distinct fusion proteins. The coding sequences of HPt2, 7 and 9 were cloned via *Spe*I in frame with the C-terminal fragment of YFP, generating one fusion protein by using pSPYCE(MR) plasmid only.

Bimolecular fluorescent complementation

Transient transformation of *Catharanthus roseus* cells by particle bombardment and YFP imaging were performed according to Guirimand et al. (2009) with adaptation for BiFC assays (Guirimand et al. 2010).

Results

Validation of HK1-CP dimerization in planta

To confirm whether HK1 is also able to homodimerize *in planta*, we conducted a BiFC analysis of this interaction. As shown in Figure 1(A), HK1-CP is able to self-interact in yeast. In *C. roseus* cells, co-transformations of plasmids expressing the split-YFP fragments fused to the N-terminal extremity of HK1-CP (e.g. YFP^N-HK1-CP/YFP^C-HK1-CP) allowed the formation of a BiFC complex visualized as a fluorescent signal within the cytosol of cells (Figure 1(D),(E)). On the other hand, no fluorescence was detected when the split-YFP fragments were fused to the C-terminal of HK1-CP (Figure 1(B),(C)) or in opposite orientations (Figure 1(F),(G)). No YFP reconstitution could be visualized when co-expressing the fusion protein YFP^N-HK1-CP with YFP^C, thereby validating the specificity of this interaction (Figure 1(H),(I)). These results show that HK1-CP is able to homodimerize *in planta*.

Validation of partnerships between HK1 and HPt2, 7 and 9 in planta

Héricourt et al. (2013) realized an exhaustive interaction study in yeast between HK1-CP and HPts highlighting three of them as preferential

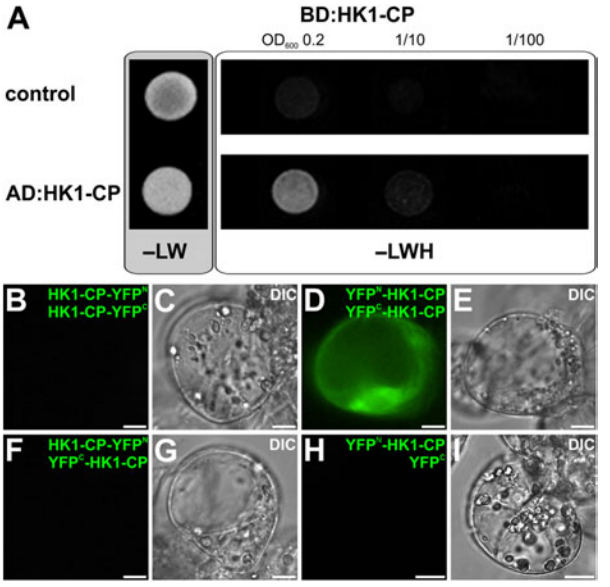

Figure 1. HK1-CP forms homodimer in Y2H and BiFC assays. (A) Y2H analysis with yeast cells co-expressing HK1-CP fused to the LexA binding domain (BD: HK1-CP) and fused to the GAL4 activation domain (AD: HK1-CP). Yeast cells were dropped on -LW or -LWH (5 mM of 3AT) media. Two dilutions of each culture were dropped on -LWH (5 mM of 3AT) medium. (B) Cells of *C. roseus* were co-transformed using the indicated YFP-N and YFP-C fusion proteins. Panels B, D, F and H show the fluorescence signal, and C, E, G and I the morphology by differential interference contrast (DIC) microscopy. Scale bar: 10 μm.

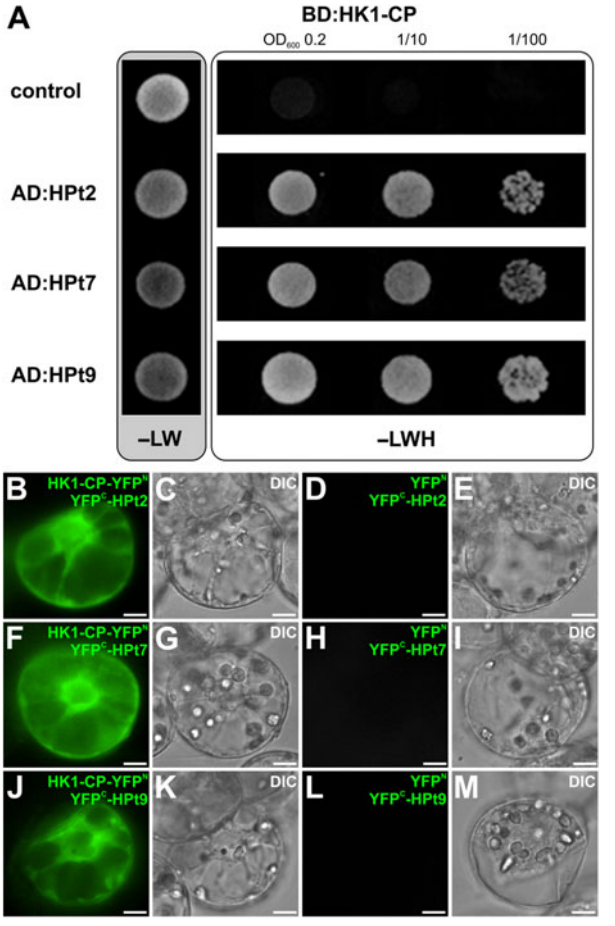

Figure 2. HK1-CP interacts with HPt2, 7 and 9 in Y2H and BiFC assays. (A) Y2H analysis with yeast cells co-expressing HK1-CP fused to the LexA binding domain (BD: HK1-CP) and HPt2, 7 or 9 fused to the GAL4 activation domain (AD: HPts). Yeast cells were dropped on -LW or -LWH (5 mM of 3AT) media. Two dilutions of each culture were dropped on -LWH (5 mM of 3AT) medium. (B) Cells of *C. roseus* were co-transformed using the indicated YFP-N and YFP-C fusion proteins. Panels B, D, F, H, J and L show the fluorescence signal, and C, E, G, I, K and M the morphology by DIC microscopy. Scale bar: 10 μm.

partners: HPt2, 7 and 9. We decided to confirm in plant cells the pertinence of these interactions by BiFC assays. As shown in Figure 2(A), HK1-CP interacted with HPt2, 7 and 9 in yeast as expected (Héricourt et al. 2013). When HK1-CP protein was co-expressed with HPts, a BiFC signal was observed in the cytosol for HPt2 (Figure 2(B),(C)), HPt7 (Figure 2(F),(G)) and HPt9 (Figure 2(J),(K)), indicating an interaction between these different proteins in plant cells. Regarding these interactions, the fusion protein orientations were also essential because no fluorescence was detected when we used YFP-HK1-CP orientation (data not shown). The specificity of HK1-CP/HPt interactions was validated by the absence of signal when the split N-terminal of YFP alone was co-expressed with HPts (YFP^C-HPts) (Figure 2(D),(E),(H),(I),(L),(M)). These results show that HK1-CP is able to interact with these three HPt proteins *in planta*.

Discussion

In the present study, we confirmed *in planta* HK1 homodimerization and HK1 interactions with its preferential downstream HPt proteins.

The capacity of HK1-CP to self-interact observed via BiFC assays corroborates the results obtained by the heterologous yeast system (Héricourt et al. 2013), showing that HK1-CP is organized as homodimer. It was previously shown that only the transmitter domain (TD) of HK1 was necessary for this interaction (Héricourt et al. 2013) due to the presence of a coiled-coil motif upstream of the TD, in agreement with our observations since a YFP reconstitution is only visualized when the split-YFP is fused to the N-terminal extremity of HK1-CP (i.e. upstream of the TD). Such homodimerization seems to be a common feature of AHK receptors, such as ethylene receptors (Gao et al. 2008; Grefen et al. 2008). The cytokinin receptors AHK2, AHK3, AHK4 and CKI1 are also reported to form dimers (Dortay et al. 2006; Hejátko et al. 2009; Caesar et al. 2011; Wulfetange et al. 2011). However, in *Arabidopsis* and other plants, no interaction study

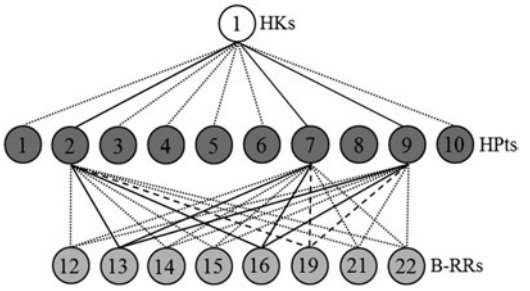

Figure 3. Protein–protein interactions model of HK1 pathway in *Populus*. The gene number designation of different family protein is noted in a circle. The verified interactions by both Y2H and BiFC are indicated by a line. Dashed lines correspond to the interactions observed only by Y2H system (.....) or by BiFC (--). The white circle indicates a self-interaction protein.

about the capacity of an osmosensor to form dimers was conducted *in planta*. Thus, up to now, these data on poplar HK1 constitute the first evidence of an osmosensor homodimerization *in planta*.

Furthermore, in this study we showed that HK1, a poplar osmosensor, is also able to interact with HPt2, 7 and 9 *in planta*. Only the configuration of the split-YFP fused to the C-terminal of HK1-CP, downstream the receiver domain (RD) which is involved in interaction interface of HK1 with HPt proteins, allows a complete reconstitution of YFP at the time of interaction with HPts. These observations corroborate results obtained in *Arabidopsis* by Pekárová et al. (2011) showing that CKI1-RD was necessary and sufficient to determine the interaction specificity with AHPs.

Several recent interaction studies using a combination of Y2H and BiFC approaches emerged. Some studies were able to substantiate protein–protein interaction *in planta* despite the lack of detected interaction in yeast (Cutcliffe et al. 2011; Bertheau et al. 2012). Pekárová et al. (2011) confirmed their interaction results by showing similar interactions between CKI1-RD and AHP2, 3, 5 in yeast and *in planta*. On the other hand, Mira-Rodado et al. (2012) showed that some interactions between AHK5 and AHPs detected in yeast did not have functional relevance in *Arabidopsis*. Thus, the confirmation of protein–protein interactions using different techniques appears essential. Until now, in *Arabidopsis*, only Y2H assays indicated that AHP2 seems to be a potential partner for AHK1 (Urao et al. 2000), but without interaction study *in planta* to corroborate this result.

Thus, the present work confirms and validates HPt2, 7 and 9 as potential downstream partners of HK1 in a multistep phosphorelay pathway in poplar. Figure 3 summarizes all protein interactions among the poplar HK1 signalling pathway between HK1/HPts (Chefdor et al. 2006; Héricourt et al. 2013) and HPts/B-RRs (Bertheau et al. 2012) observed by Y2H and/or BiFC.

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