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Highlighting type A RRs as potential regulators of the dkHK1 multi-step phosphorelay pathway in *Populus*

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ABSTRACT

In previous studies, we highlighted a multistep phosphorelay (MSP) system in poplars composed of two hybrid-type Histidine aspartate Kinases, dkHK1a and dkHK1b, which interact with three Histidine Phosphotransfer proteins, dkHPt2, 7, and 9, which in turn interact with six type B Response Regulators. These interactions correspond to the dkHK1a-b/dkHPts/dkRRBs MSP. This MSP is putatively involved in an osmosensing pathway, as dkHK1a-b are orthologous to the Arabidopsis osmosensor AHK1, and able to complement a mutant yeast deleted for its osmosensors. Since type A RRs have been characterized as negative regulators in cytokinin MSP signaling due to their interaction with HPt proteins, we decided in this study to characterize poplar type A RRs and their implication in the MSP. For a global view of this MSP, we isolated 10 poplar type A RR cDNAs, and determined their subcellular localization to check the *in silico* prediction experimentally. For most of them, the *in planta* subcellular localization was as predicted, except for three RRAs, for which this experimental approach gave a more precise localization. Interaction studies using yeast two-hybrid and *in planta* BiFC assays, together with transcript expression analysis in poplar organs led to eight dkRRAs being singled out as partners which could interfere the dkHK1a-b/dkHPts/dkRRBs MSP identified in previous studies. Consequently, the results obtained in this study now provide an exhaustive view of dkHK1a-b partners belonging to a poplar MSP.

1. Introduction

Living organisms are exposed to fluctuating environmental conditions, and in the context of global climatic change, those with efficient perception mechanisms will be able to adapt more efficiently. To sense and respond to environmental stimuli, prokaryotic organisms, such as bacteria, employ a well characterized signaling pathway, the canonical two component system (TCS). This system is specific to the stimuli and is comprised of a sensor protein corresponding to a Histidine Kinase and a Response Regulator (RR) [1]. A more elaborate system is also found in prokaryotic and eukaryotic organisms, which involves a His-Asp-His-Asp phosphorelay called a multistep phosphorelay (MSP) system. The system is comprised of sensors, RRs, and a histidine phosphotransfer protein (HPt), which shuttles between them [2]. In plants, the MSP comprises a receptor, a hybrid-type histidine aspartate kinase (HK), HPt proteins, and four types of RR: type A, type B, type C, and pseudo RRs. These signaling pathways are known to mediate plant responses to light, abiotic stresses, and phytohormones such as cytokinin (CK) and ethylene, which regulate growth and plant development [3–7].

The CK pathway is the most studied and characterized MSP in plants. In Arabidopsis, CK receptors AHK2, AHK3 and AHK4 activated by CK binding can autophosphorylate and trigger a phosphorelay involving five HPt proteins (AHP1-5), and 11 type B RRs (AtRRB1-2, AtRRB10-14, AtRRB18-21), which once activated by phosphorylation can activate the transcription of target genes such as type A RR genes [8–12]. These type A RR genes are primary response genes, and once activated by phosphate transfer via AHK and AHP proteins, act as negative regulators of CK signaling [9,10,13–15]. Type A RRs are consequently in competition with type B RRs for the phosphate transfer by AHP proteins in CK signaling and could also function via phosphorylation-dependent protein-protein interactions with type B RRs, or as yet non-identified regulators [14].

Type A RRs are also regulated by abiotic stresses such as drought, salinity, dehydration and cold [16–18]. For example, the expression of

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the *OsRRA1-2-5-6-7* and *9* from *Oryza sativa* are induced by salt, dehydration and low temperature treatments [16,17]. In *Arabidopsis thaliana*, *AtRRA7* is cold inducible [18], and also acts as a transcriptional repressor for a variety of early CK-regulated genes such as genes encoding transcription factors, or signal transmitters [19]. Other results suggest that AtRRA3-4-5 and 6 may function as positive regulators whereas AtRRA8 and 9 function as negative regulators under osmotic stress [20]. In *Glycine max*, there are six dehydration-repressed type A RR genes (*GmRRA07-08-09* and *GmRRA11-12-13*) which encode AtRRA8 and AtRRA9 homologues respectively, providing evidence that these GmRRAs function in stress response and may act as negative regulators in a similar fashion to their orthologues AtRRA8 and AtRRA9 [21]. Similarly, *GmRRA01* and *GmRRA02* genes, *AtRRA4* and *AtRRA6* homologues respectively, are up-regulated in response to drought, suggesting that they may function as positive regulators in this stress response [21]. This regulation by abiotic stresses has led to the proposition that type A RRs may also form a complex network that is predominantly responsible for integration, fine-tuning and cross-talk of many plant signaling pathways [22]. To date, target genes of activated type A RRs which negatively regulate the CK pathway remain to be identified.

Less data is available for tree plant models. In *Malus domestica*, 19 type A RRs were identified by *in silico* analysis and a number of them are up-regulated by CK treatments [23]. In *Pinus pinea*, a type A RR gene *PipiRRA1* was cloned and shown to be up-regulated in cotyledons after CK exposure, suggesting that it could play a crucial role in adventitious meristem formation [24]. Moreover, the *PipiRRA1* homologous gene *PipsRRA1*, cloned in *Pinus pinaster*, also seems to be involved in meristem formation and may play a role in adventitious shoot meristem formation and somatic embryo development [25]. In *Populus trichocarpa*, 11 type A RRs, PtRRA1 to PtRRA11, were identified *in silico,* whereas only four were detected in *Prunus persica* [26,27]. Some of them are up-regulated in detached mature leaves after 1 h of CK treatment in *Populus tremula* x *Populus alba* [26]. Regarding type A RRs in tree models, some studies have been conducted, although none of them investigate the role of these proteins in MSP regulation.

In previous works, we identified poplar HK, dkHK1a and dkHK1b [28,29]. Both dkHK1s can functionally complement a *Saccharomyces cerevisiae* deletion mutant for its two osmosensors Sln1 and Sho1, which demonstrates their kinase and osmosensor functions in yeast. Protein interaction studies alongside transcript co-expression analysis *in planta* have been carried out to determine dkHK1 partners among the 10 poplar HPt proteins identified. Hence, three HPt partners have been retained [29,30]. A similar study led to the identification of six type B RRs, which could participate in a poplar MSP [31–33]. Taken together, these studies highlight a network for dkHK1a-b/dkHPt2-7-9/dkRRB12-13, 16, 18–19 MSP, which could potentially be involved in drought stress response in poplars. As type A RRs are negative regulators of CK MSP and participate in the regulation of CK signaling, the role of these proteins in other MSP pathways remains to be elucidated.

To complete the dkHK1 MSP network previously identified, we decided to identify type A RRs in poplars and to study their putative implication in this signaling pathway, as potential regulators of this MSP. We managed to isolate 10 cDNAs encoding type A RRs in the poplar 'Dorskamp'. Then we identified the subcellular localization, and studied the interactions with the three dkHPts, the preferential interacting partners for dkHK1, by performing two-hybrid assays in yeast. Some interactions were validated by BiFC assay in plant cells, and the relevance of these interactions has been strengthened by co-expression analysis of transcripts of all the studied proteins in poplar organs (roots, stems, petioles and leaf blades). Taken together, these results define a protein network linked to dkHK1 in poplar and highlight that at least eight dkRRAs may participate in the MSP dkHK1a-b/dkHPts/dkRRBs as type B RR competitors through their interactions with HPt proteins.

2. Materials and methods

2.1. Isolation of type A RR CDSs and phylogeny analysis

We used the references of genes from *Populus trichocarpa* type A RRs (PtRRA1 to PtRRA11) [26] to search for their nucleotidic sequences in JGI *Populus trichocarpa* (v1.1). We also designed a specific primer pair corresponding to each RR, in order to isolate their coding sequence (CDS) from the poplar clone 'Dorskamp' (*Populus deltoides* (Bartr.) Marsh x *P. nigra* L.). Apart from for *dkRRA11*, all PCRs were performed using a root cDNA library constructed using the Marathon cDNA Amplification Kit (Clontech) and Taq Advantage polymerase (Clontech), with primers at a final concentration of 0.2 µM. PCR products were cloned into pGEM-T easy vector (Promega), and sequenced and compared with *P. trichocarpa* type A RR sequences using ClustalW [34]. PCR was performed under the same conditions for *dkRRA11*, but using cDNA libraries from stressed leaves [31], and a nested PCR (Results Section 4).

Deduced amino acid sequences of dkRRAs were aligned with those from *Arabidopsis thaliana* using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The alignment was represented with a phylogram constructed using the neighbour-joining method in the phylogenetic software MEGA (v 6.06) (Pennsylvania State University, State College, PA, USA).

2.2. Yeast two-hybrid assays

The yeast two-hybrid assays were performed using a LexA DNA-binding domain encoding bait vector (pBTM116 referred to as pLex), and a Gal4 activation domain encoding prey vector (pGADT7, Clontech). RRA CDSs were cloned into the pGAD vector as *Eco*RI-*Xho*I fragments for dkRRA1 to dkRRA10, and *Xma*I-*Xho*I for dkRRA11; dkHPt CDSs were cloned into the pLex vector as previously described [29]. The yeast strain L40Δ (MATa ade2-101 his3-200 leu2-3,112 trp1-901 ura3-52 LYS2::(lexA op)x4-HIS3 URA3:: (lexA op)x8-lacZ gal4Δ) was used for co-transformations according to the lithium acetate method from [35]. Co-transformed yeasts were selected onto leucine-trytophan lacking medium (-LW) for 4 days at 30 °C. For each interaction, overnight cell cultures with an Optical Density at 600 nm (OD_{600}) of 0.5 for three dilutions (1:10, 1:100 and 1:1000) were prepared. Five microliters of each cell suspension were dropped onto control medium -LW (-Leu, -Trp) and interaction selective medium -LWH (-Leu, -Trp, -His). Due to autoactivation of dkHPts, 3-amino-1, 2, 4-triazole (3 A T) was supplemented to -LWH medium at either 20 (dkHPt7 and 9) or 60 mM (dkHPt2) according to [31]. Yeast cells grew for two or four days at 30 °C for all interactions which were tested using two different reporter genes, HIS3 and LacZ (data not shown). All interactions were tested at least twice with 8 positive yeast clones.

2.3. BiFC assays

BiFC assays were conducted using the pSPYCE(MR) [36] and pSPYNE173 plasmids [37], which allow the expression of a protein fused to the C- or N-terminal of the split-yellow fluorescent protein (YFP) fragments, respectively. The CDSs of dkRRA8 and dkRRA10 were cloned into the *Spe*I site in frame with the C-terminal fragment of YFP. DkHPt2, dkHPt7 and dkHPt9 CDSs were cloned into the *Spe*I site in frame with the N-terminal fragment of YFP [30]. Transient transformation of *Catharanthus roseus* cells by particle bombardment and YFP imaging were performed according to [38] with adaptation for BiFC assays [37] and negative control used is described in [39].

2.4. DkRRA transcript detection by RT-PCR

This study was performed using the poplar clone 'Dorskamp'. Roots, stems, petioles and leaf blades of one month-old hydroponically grown

rooted cuttings [40] were harvested and frozen. RNA extractions were carried out using the NucleoSpin RNA Plant mini kit (Macherey-Nagel). One µg of total RNA was reverse transcribed using M-MuLV Reverse Transcriptase RNase H- (Finnzyme), according to the manufacturer's procedure, and used as a template for PCR amplifications. Thirty or forty PCR cycles were performed to detect *dkRRA* transcripts, and *clathrin* was used as an expression control gene. The amplified fragments were separated by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and analyzed under UV light. All PCRs were performed in triplicate at least, and three independent biological replicates were performed.

2.5. DkRRA localization by GFP-fused protein expression

To express RRA-GFP fusion proteins, the CDSs of RRAs were amplified by PCR using specific primers extended by *Spe*I restriction sites at both extremities. The amplified CDSs were subsequently cloned using the pGEM-T Easy Vector (Promega) and checked by sequencing. After *Spe*I digestion, CDSs were cloned into the *Spe*I restriction site of pSCA-cassette GFP [38], upstream to and in frame with the CDS of GFP. Transient transformation of *C. roseus* cells by particle bombardment and GFP imaging were performed using the nucleocytoplasmic CFP (pSCA-cassette CFP-GUS) and nuclear mcherry (pSCA-cassette mcherry-GUS-NLS) markers [37]. These two plasmids have been created by substituting the YFP coding sequence of the pSCA-cassette YFP-GUS plasmid (described in [38]) by the CFP or mcherry coding sequence followed by the addition of a bipartite nuclear localization sequence (NLS) through primers annealing at the 5′ end of the mcherry GUS coding sequence [37].

3. Results

3.1. Isolation of 10 type A RRs in the poplar clone 'Dorskamp'

Using sequence information from [26] and the JGI *Populus trichocarpa* (v1.1) database, we isolated 10 CDSs which encode type A RRs in the poplar clone 'Dorskamp': *dkRRA1* to *dkRRA11*. In our plant materials (root or leaves) and PCR conditions we were unable to isolate cDNA encoding for *dkRRA9*. Among the 10 CDSs, identities ranged from 51 to 89%. All the deduced amino acid sequences of isolated *dkRRA* CDSs share the DDK conserved residues characteristic of the receiver domain (RD) of RRs (type A and type B), belonging to the MSP. As already observed by [26], some *dkRRA* genes are duplicated and constitute sister pairs (Fig. 1). The C-terminal end of the dkRRA1/2 pair is characterized by a serine and proline rich zone (Fig. 2) as described for the Arabidopsis homologues, AtRRA3 and AtRRA4 [13]. DkRRA10, encoded by an unduplicated gene, is characterized by a charged serine and a proline rich zone, resembling AtRRA7 and AtRRA15, as its charged C-terminal end is also enriched by serine residues, although with threonine instead of proline residues. As observed in the unrooted tree (Fig. 1), five dkRRAs, dkRRA3-7, group together but present different characteristics. DkRRA3-5 are characterized by a charged C-terminal end as observed for AtRRA8 and 9, whereas dkRRA6 and 7 are characterized by a charged proline and glutamine rich C-terminal end without corresponding type A RRs in Arabidopsis (Fig. 2). A last group is composed of dkRRA8 and dkRRA11 which are not characterized by a C-terminal end like for the pairs AtRRA5/6 and AtRRA16/17.

3.2. DkRRA localization

DkRRA subcellular localizations were determined by transient expression of GFP-fused dkRRAs in *C. roseus* cells. GFP and a nuclear mCherry marker were used as nucleocytoplasmic and nuclear markers respectively (Fig. 3A1, A2). All dkRRA proteins fused with GFP at their C-terminal ends displayed a strict nuclear localization (Fig. 3B1, D1-H1, J1, K1) that was confirmed by the co-localization of the GFP fluorescent signal with the signal of the nuclear mCherry marker (Fig. 3B3, D3-H3, J3, K3), except dkRRA2 and dkRRA8. Indeed, both dkRRA2- and dkRRA8-GFP fusion proteins showed an additional diffuse pattern of fluorescence, characteristic of a cytosolic localization (Fig. 3C1, I1). Using the nuclear mCherry marker (Fig. 3C2, I2), a nucleocytoplasmic localization was observed for both proteins, as they perfectly merged in the nucleus (Fig. 3C3, I3). Therefore, many of the dkRRA proteins showed a strict subcellular localization in the nucleus, except for dkRRA2 and dkRRA8 which exhibited an additional cytoplasmic localization.

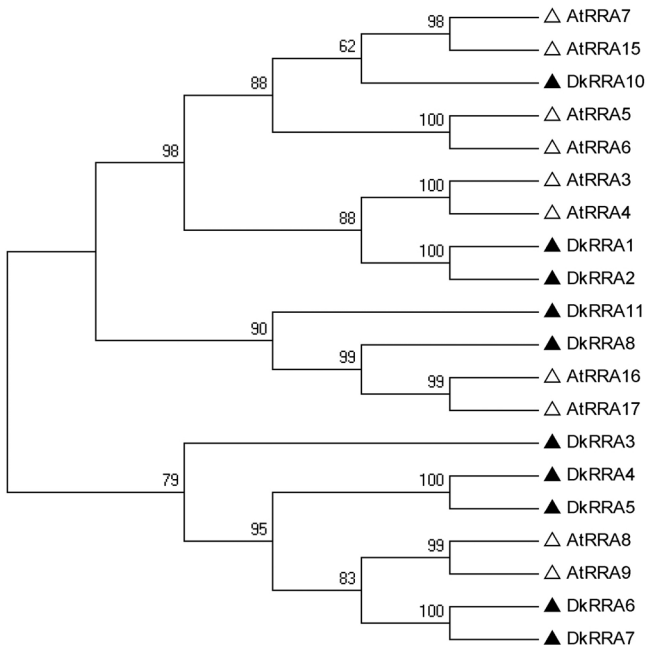

Fig. 1. Unrooted relationship tree of RRAs from *Arabidopsis thaliana* and *Populus*.
The full-length protein sequences of poplar type A RRs deduced from CDS sequences were aligned with those from Arabidopsis type A RRs using Clustal Omega, and the alignment was represented by a phylogram constructed with the neighbour-joining method in the phylogenetic software MEGA (v 6.06). Numbers indicate bootstrap support (1000 replicates). Δ: AtRRAs, ▲: dkRRAs.

3.3. DkRRAs interact with dkHPt2, dkHPt7 and dkHPt9

To determine whether dkRRAs could compete the interaction between dkHPt2-7-9 and dkRRB12-13, 16, 18–19, we performed two-hybrid assays in yeast with all isolated type A RRs and the three dkHPt partners of dkHK1a-b, dkHPt2, 7 and 9. This study revealed reporter gene activation for all interactions tested except for dkRRA8, which only interacts with dkHPt7 (Fig. 4). Yeast expressing both dkRRA8 and dkHPt2 or 9 showed a similar growth pattern with the negative control (Fig. 4A). For the dkRRA1/dkRRA2 pair, a different behavior was observed, as yeast expressing dkRRA2 needed two extra days before cell growth was observed (Fig. 4B). This delay probably reflects a weaker interaction between dkRRA2 and the three dkHPts, compared to dkRRA1. In the same way, a differential behavior was observed for dkRRA4/dkRRA5 and dkRRA6/dkRRA7 pairs with weaker growth observed for yeast expressing dkRRA5, compared to dkRRA4 and dkRRA6 to dkRRA7. The lack of dkRRA9 prevented a study into dkRR9/dkRR11 pair interactions. These two-hybrid assays showed that dkRRAs can be classed into four different categories according to reporter gene activation. The first group comprised dkRRA2, which presented weak interaction with the three dkHPt proteins. A second group included dkRRA1, 3, 4 and 7, and presented more pronounced interactions. An intermediate group was composed of dkRRA5, 6, 10 and 11, and the

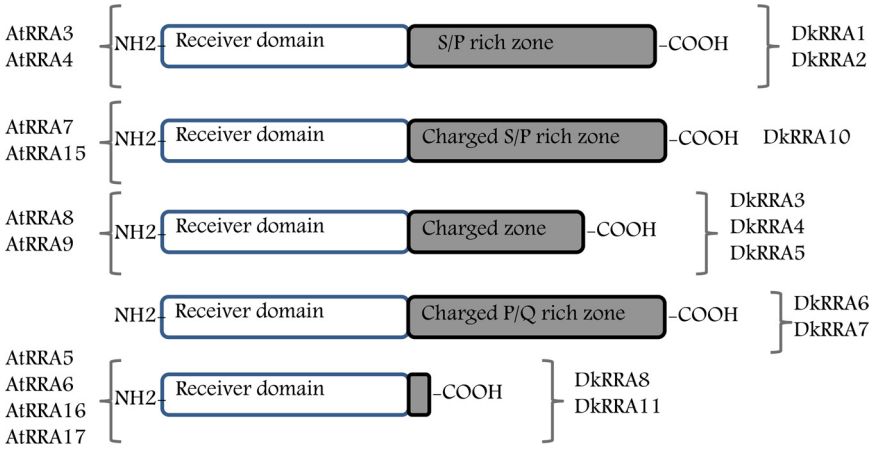

Fig. 2. Proteic characteristics of poplar type A RRs and correspondences with type A RRs from *Arabidopsis thaliana*.

last group was comprised of dkRRA8, which only interacted with dkHPt7.

3.4. Validation of dkRRA8/dkHPt interaction by a BiFC approach

To validate the lack of interaction observed in yeast two-hybrid assays between dkRRA8 and dkHPt2 and 9, BiFC assays were conducted between dkRRA8 and the three dkHPt proteins *in planta*. DkHPt2, 7 and 9, dkRRA8, and dkRR10 CDSs were fused either to the N-terminal (YFP^N) or C-terminal (YFP^C) fragments of yellow fluorescent protein (YFP) at their C-terminal end, to produce YFP^C-dkHPt2/7/9 and dkRRA8/10-YFP^N. As observed in yeast two-hybrid tests, the BiFC approach substantiates the interaction between dkRRA8 and dkHPt7 by observation of the YFP complex reconstitution (Fig. 5B1). Interactions between dkRRA8 and dkHPt9 and dkHPt2 were observed using this second approach when co-expressing dkRRA8-YFP^N, and YFP^C-dkHPt2 (Fig. 5A1), and dkRRA8-YFP^N and YFP^C -dkHPt9 (Fig. 5C1). As a positive control, we tested dkRRA10-YFP^N with YFP^C-dkHPt2/7/9. As expected, the YFP complex reconstitution was observed for all interactions tested (Fig. 5D1, E1, F1). This signal using dkRRA10 merged perfectly with the CFP nuclear marker (Fig. 5D2, E2, F2) leading to the observation of a nuclear localization of the interaction due to the nuclear localization of dkRRA10 (Fig. 5D3, E3, F3). In contrast, BiFC complex reconstitution for dkRRA8-YFP^N and YFP^C-dkHPt2/7/9 was observed both in nuclear and cytosolic compartments (Fig. 5A1, B1, C1). The localization in the nucleus was confirmed by the CFP nuclear marker (Fig. 5A2, B2, C2) and the merge observed (Fig. 5A3, B3, C3). This experiment led to the validation of the nuclear and cytoplasmic localization for the interactions of both the partners respectively. To confirm our results, negative controls were realized using CrTHAS1, a nuclear protein (Fig. 6A1, A2, A3). No interaction was observed either with YFP^C-dkHPt2/7/9 or with dkRRA8/10-YFP^N (Fig. 6B1, C1, D1, E1, F1) respectively.

3.5. DkRRA transcript expression analysis by RT-PCR

To be physiologically pertinent in plants, all interactions observed using yeast two-hybrid or BiFC assays need to be validated by the observation of the concomitant co-expression of both partners in the same organs. To validate the relevance of observed interactions, the *dkRRA* transcript expression pattern was studied by RT-PCR analysis with *Clathrin* as the reference gene (Fig. 7C). A constitutive expression was observed for six *dkRRA* transcripts (*dkRRA2*, *3*, *5*, *6*, *7* and *10*) in all studied organs (Fig. 7A) in 30 PCR cycles. To refine these results, we performed 10 more cycles and were able to detect two other RRs, *dkRRA1* and *dkRRA4* (Fig. 7B). These two RRs are less abundant than the others, as they were detected after 40 PCR cycles, and *dkRRA1* was not detected in leaf blades (Fig. 7B). Furthermore, a differential gene expression can be observed for both gene pairs, *dkRRA1/dkRRA2* and *dkRRA4/dkRRA5*. In contrast, the gene pair *dkRRA6/dkRRA7* shows a similar expression pattern. Among the most expressed *dkRRA*s (*2*, *3*, *5*, *6*, *7*, and *10* Fig. 7A) *dkRRA10* shows the weakest expression in roots, with expression being slightly stronger in leaf blades (Fig. 7B).

4. Discussion

In this study, we successfully isolated 10 cDNA encoding type A RRs from the poplar clone 'Dorskamp'. These RRAs correspond to *dkRRA1* to *dkRRA11* (apart from *dkRRA9*), among the 11 genes identified in JGI *Populus trichocarpa* (v1.1).

In Arabidopsis, all type A RR genes are duplicated [41,27], whereas in poplar three genes (*dkRRA3*, *dkRRA8* and *dkRRA10*) don't seem to be duplicated [26]. However, as observed in Arabidopsis, genes homologous to these three genes are duplicated in another tree species: *Malus domestica* [23]. Gene duplication is now well known to contribute to the evolution of novel functions. In plants, about 64.5% of genes are paralogs, ranging from 45.5% in *Physcomitrella patens* to 84.4% in *M. domestica,* and the longevity of duplicated genes may be influenced by various factors [42]. For example, duplicate loss could be observed for weakly expressed genes with uncomplex promoters [43]. A similar lack of pairwise genes is observed for rice RRAs, probably due to frequent gene loss events [44]. Thus, the hypothesis that these three genes in poplar have never been duplicated or undergone a duplicate loss during poplar evolution could be considered.

The dkRRA1/dkRRA2 pair, homologous to the AtRRA3/AtRRA4 pair, shares the same characteristics, *i.e.* a RD followed by a serine and proline rich domain in the C-terminal end. In Arabidopsis, AtRRA4, was shown to interact with phytochrome B (PHY-B) and found to be involved in a phase delay of the circadian rhythm [45,46]. Moreover, AtRRA4 plays a central role in the interaction between cytokinin signaling and light signal transduction. Phosphorylation of the conserved aspartate residue in the RD is important for AtRRA4 activity during photomorphogenesis [47], but this activity is also controlled by its protease-mediated degradation [48]. This degradation is mediated by DEG9 interaction with AtRRA4's C-terminal end, leading to AtRRA4 specific degradation, which is not observed for AtRRA3 due to differences in the C-terminal end [48]. A similar C-terminal extension, characterized by a serine/proline rich domain, was found in dkRRA1/dkRRA2, which suggests a possible involvement in circadian rhythm regulation in poplar for these two RRAs.

In the phylogenetic tree, dkRRA3, the dkRRA4/dkRRA5 and dkRRA6/dkRRA7 pairs group together with AtRRA8/AtRRA9. However, they do not share similar characteristics in their C-terminal ends. DkRRA3 and dkRRA4/dkRRA5 have charged C-terminal ends

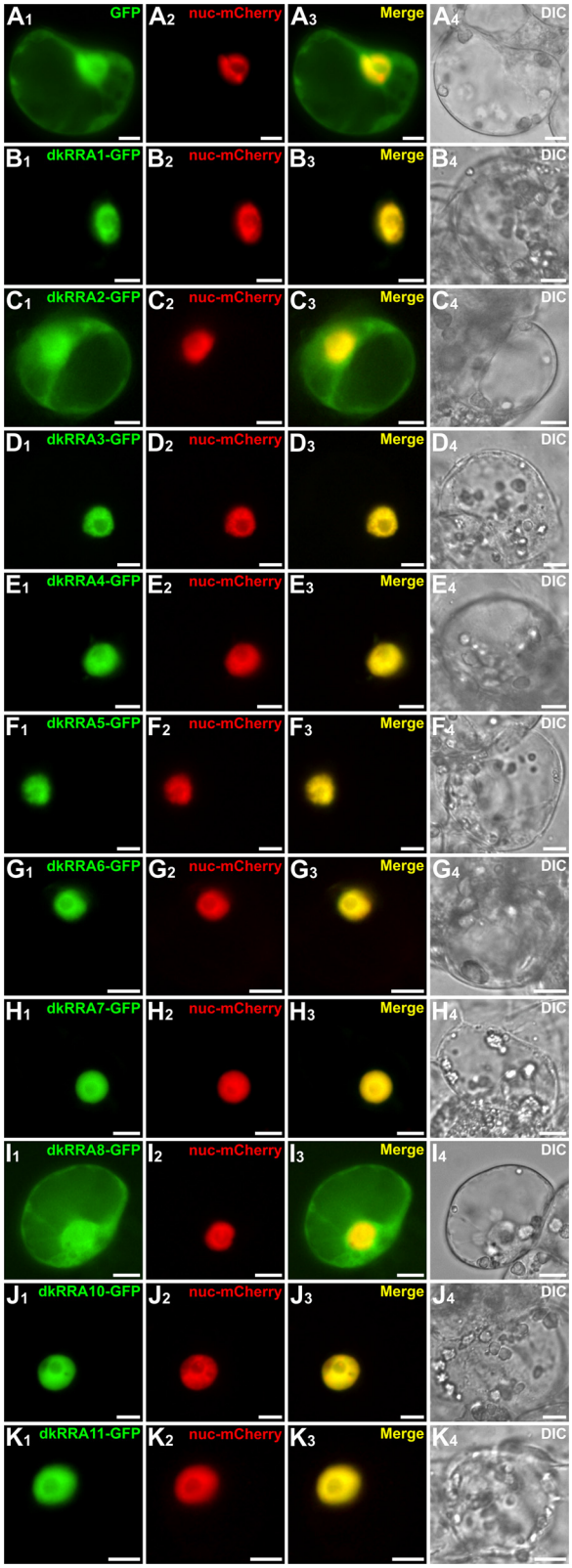

Fig. 3. Subcellular localization of poplar type A RRs.
C. roseus cells were transiently transformed with GFP (A1) and dkRRAs-GFP (B1-K1) expressing vectors in combination with a nuclear-mcherry marker (A2-K2). Co-localization of the two fluorescence signals is shown in the merged image (A3-K3). The morphology was observed by differential interference contrast (DIC) microscopy (A4-K4). Scale bar = 10 µm.

similar to AtRRA8 and AtRRA9, whereas the dkRRA6/dkRRA7 pair contains a short, charged proline/glutamine rich domain. No type A RRs from Arabidopsis have these domains. While type B RRs are characterized by a C-terminal end enriched in proline/glutamine residues involved in transactivation function, such domains are lacking in type A RRs [49,3], and this domain in dkRRA6 and dkRRA7 is not long enough to function as a transactivation domain. However, proline and glutamine residues are often associated with protein interactions [50], raising the question as to whether this domain could be involved in protein-protein interactions leading to specific function for these type A RRs as observed for AtRRA4's C-terminal end.

In the same way, dkRRA8 and dkRRA11 group with the AtRRA16/AtRRA17 pair in the phylogenetic tree, as expected due to their common architecture, *i.e.* a short C-terminal end (< 30 amino acids). However, the AtRRA5/AtRRA6 pair does not group with these dkRRAs even though they share the same architecture. Moreover, dkRRA10, which is homologous to AtRRA5, shares common architecture, a serine and proline/threonine rich domain, with the AtRRA7/AtRRA15 pair. It should be mentioned that RR phylogenetic trees are commonly constructed using only the RD, and do not include the C-terminal end [41]. However, the C-terminal end of these proteins is clearly important for the protein function or regulation, as shown for AtRRA4 [48]. Thus, even though these proteins are phylogenetically distant, they could share common functional mechanisms thanks to their common architecture.

In our study, we failed to isolate one CDS, *dkRRA9,* but we did succeed in isolating *dkRRA11* using a nested PCR on cDNAs from poplar drought stressed leaves. *PtRRA9*, homologous to *dkRRA9*, was also undetectable in roots, young and mature leaves, nodes and internodes, phloem and xylem of two other poplar genotypes, *Populus balsamifera* ssp. *trichocarpa* genotype Nisqually 1 and *Populus tremula* × *Populus alba* INRA-clone 717-1-B4 [26]. In these two genotypes, *PtRRA3*, *PtRRA9* and *PtRRA11* were undetectable in these organs, although clearly expressed in catkins, from which only *PtRRA9* was strictly specific. The *PtRRA9* tissue specific expression in catkins linked to its involvement in sex determination could explain our unsuccessful attempt to isolate this *RRA* from our poplar material (roots, stems, petioles and leaf blades). It was shown that the sex-linked specific region in *Populus trichocarpa* contains 13 genes, with at least two candidate genes involved in sex determination: a methyltransferase, *PtMET1* and a type A RR, *PtRRA9*, homologous to *AtRRA17* [51]. In *P. balsamifera*, authors showed that *PbRRA9* was more heavily methylated in males than in females and consequently probably less expressed in males, leading to the hypothesis that the *PbRRA9* gene could be involved in poplar sex determination [52]. It was also demonstrated that male poplars adapt more efficiently during drought stress, as water deficiency inhibits growth, photosynthesis and ROS protection more strongly in females than in males [53]. Indeed, this difference was previously observed between two *Populus x euramericana* clones, the male genotype 'Dorskamp' and the female genotype 'Luisa Avanzo' [54]. Growth and photosynthesis in female poplars were affected via a clear leaf area decrease during drought [55]. Furthermore, in Arabidopsis, leaf differentiation and consequently leaf area is controlled through *AtRRA16* activation by the complex of the chromatin remodeler BRM and TCP4 CIN-TCP leading to CK decrease [56,57]. It seems that type A RRs could be involved in sex-specific drought response (AtRRA17) or leaf development and leaf area control (AtRRA16). In the poplar clone 'Dorskamp', dkRRA9 could be also involved in sex determination, and therefore indirectly in drought response. The fact that it was impossible to isolate this gene in vegetative tissue could argue in favor of this hypothesis. Moreover, the increase of *dkRRA11* transcript levels in drought stressed leaves compared to the control in nested RT-PCR (data not shown, condition to isolate the corresponding CDS) raised the question about this RRA's involvement in leaf area control during drought. As neofunctionalization after whole genome duplication has been proposed for *PtRRA9/PtRRA11* [51,52],

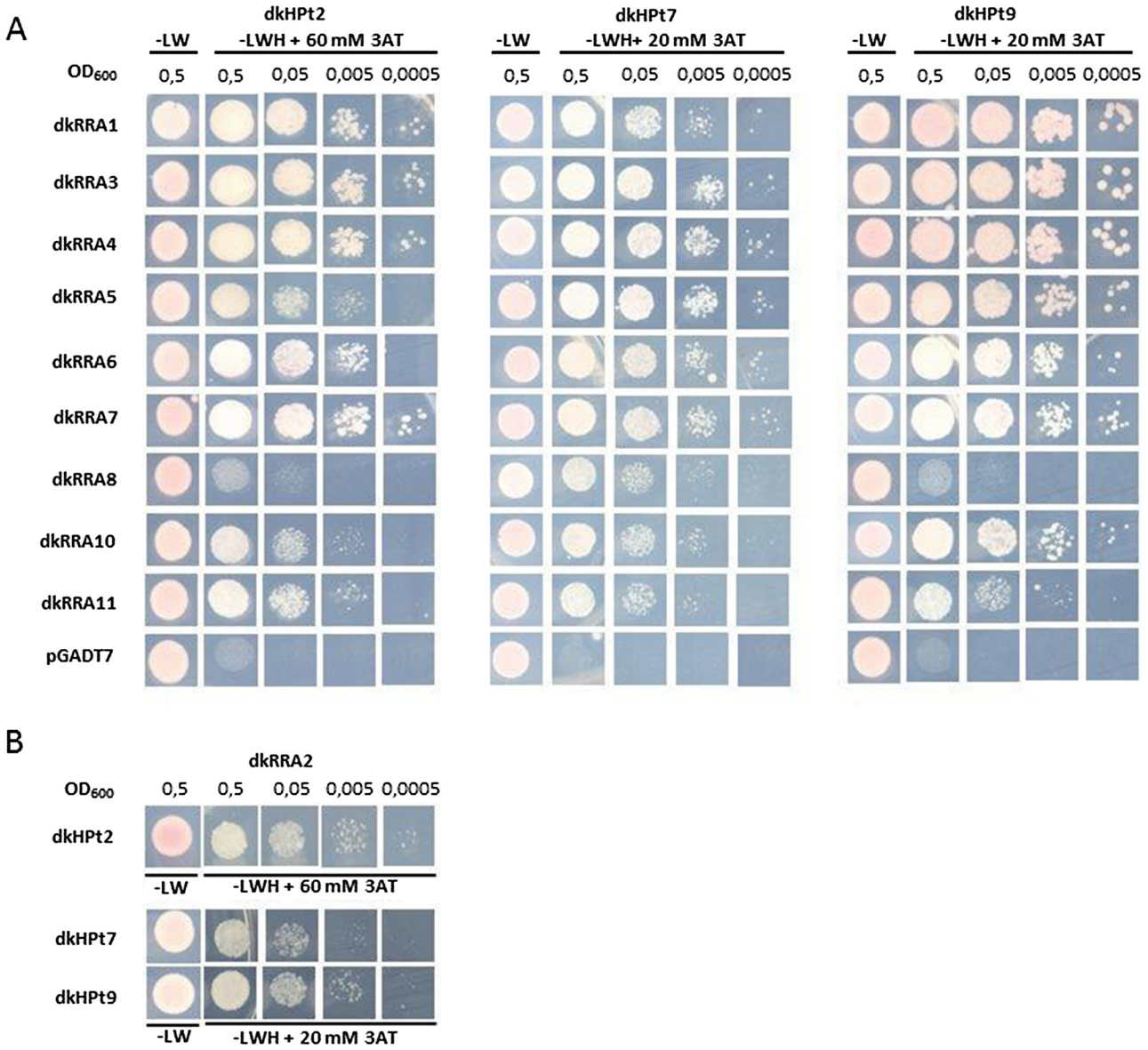

Fig. 4. Interaction between dkRRAs and dkHPt2-7-9 in the yeast two-hybrid system. Overnight cultures of yeast co-transformed with pLex-dkHPts and pGAD-dkRRAs were adjusted to an Optical Density at 600 nm (OD600) of 0.5. This culture and three dilutions (OD_{600} 0,05 to 0,0005) were spotted onto -LWH medium supplemented with 3 A T as indicated and grown for two (A) or four (B) days.

more studies are needed to determine the involvement of this RRA in poplar drought response.

Since only *in silico* prediction methods have been used to analyze poplar type A RR subcellular localization until now, we have done an exhaustive study to confirm or reject these predictions *in planta*. These assays showed a refined localization for dkRRA2, 4 and 5 compared to *in silico* results obtained by Ramirez-Carvajal et al. [26], hence pointing out the necessity to perform experimental validation. DkRRA1 is a nuclear protein, whereas AtRRA4, dkRRA2 and AtRRA3 are all nucleocytoplasmic proteins [58]. Because nuclear AtRRA4 is involved in mediating cross-talk between light and CK signaling through modulation of PHY B activity, and is involved in the circadian period, dkRRA1 could be involved in a similar process [59,47]. This observation, as well as common structural characteristics, could assume a similar cellular function of poplar proteins. DkRRA4, 5, 6 and 7 are nuclear proteins as is observed for their Arabidopsis homologues, AtRRA8 and AtRRA9 [58]. DkRRA8 is localized in the nucleus and cytoplasm, as is observed for AtRRA16, probably due to their same short C-terminal ends [60].

In the past, type A RRs were identified as primary response genes, and are known to be negative regulators in CK pathways by applying a negative feedback control in CK sensibility. This feedback control is probably due to their activation by phosphorylation, leading to a reduced degradation and an increased stability [10,14,15,61]. This activation is due to direct interaction with HPt proteins in the nucleus [15,62,63].

In previous work, we identified a specific network of interactions composed of dkHK1a-b/dkHPt2-7-9/dkRRB12-13, 16, 18–19 [29–31,33]. To determine if type A RRs could interfere with this network, we decided to study potential interactions between all isolated type A RRs, and the three dkHPt protein partners of dkHK1. All type A dkRR proteins were able to interact with these dkHPt proteins. However, a surprising behavior was observed for type A RR pairs. Indeed, all pairs are composed of a strongly and a weakly interacting RRA. In Arabidopsis, AtRRA3/AtRRA4 and AtRRA8/AtRRA9 pairs showed similar interaction patterns for AHP5, and some differences were detected for AtRRA5/AtRRA6 and AtRRA7/AtRRA15 pairs [64]. These different binding properties of proteins making up each pair could reflect the redundancy already observed in other plant models. A different

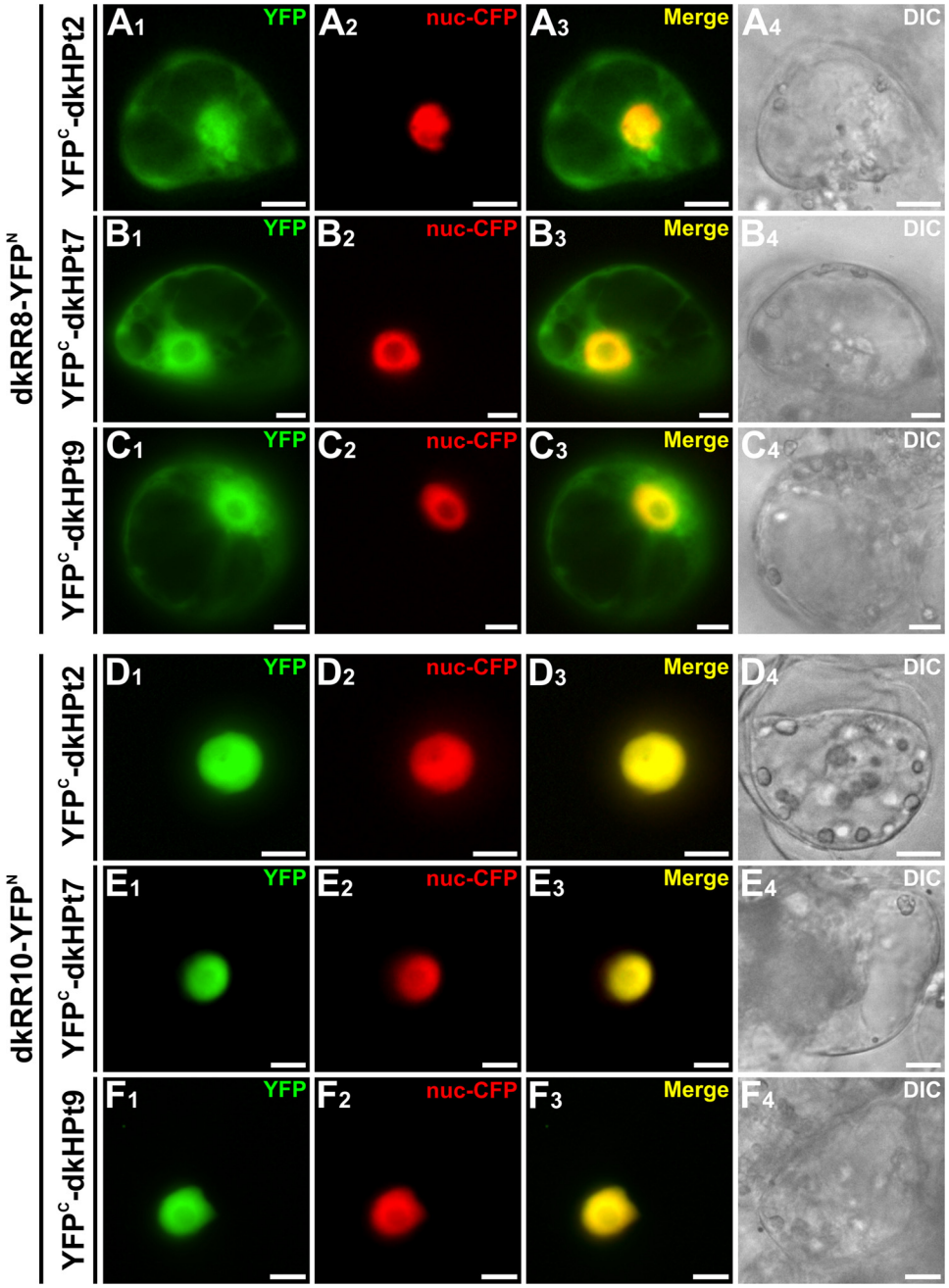

Fig. 5. Analysis of dkRRA8/dkHPt2-7-9 interactions in *C. roseus* cells using BiFC assays.
C. roseus cells were co-transformed with plasmids expressing dkRRA8-YFP^N and YFP^C-dkHPt2, -dkHPt7 and -dkHPt9 (A1-C1). An additional co-transformation with the CFP nuclear marker (A2-C2) confirms the co-localization of the two fluorescence signals (A3-C3). *C. roseus* cells were also co-transformed with plasmids expressing dkRR10-YFP^N and YFP^C-dkHPt2 (D1-D3), -dkHPt7 (E1-E3) and -dkHPt9 (F1-F3) as positive interaction controls. The morphology was observed by differential interference contrast (DIC) microscopy (A4-F4). Scale bar = 10 µm.

experimental approach is often necessary to confirm two-hybrid results [65,31]. The lack of interactions observed between dkRRA8 and dkHPt2/dkHPt9 was checked by BiFC and revealed interactions between dkRRA8 and these HPts. The strong sequence similitude between the dkHPt7 and dkHPt9 (96.7%) pair was in favor of such results with dkHPt9. Moreover, the BiFC approach showed that the interactions of dkRRA8/dkHPts and dkRRA10/dkHPts exhibited a nucleocytoplasmic and a nuclear localization respectively.

The interactions observed in yeast two-hybrid or *in planta* BiFC assays are only biologically relevant if tested proteins are spatio-temporally co-expressed in the plant. As the expression of all proteins of the dkHK1a-b/dkHPt2-7-9/dkRRB12-13, 16, 18–19 network was studied in roots, stems, petioles and leaf blades [31,29], we decided to study the type A RR expression from the exact same biological material.

As previously discussed, the *dkRRA9* CDS was not isolated and consequently undetectable in our experiment. Surprisingly, constitutive expression for *PtRRA9* transcripts was detected in leaves by RT-PCR in the genotype *P. tremula x P. alba*, although three aberrant transcripts shorter than the expected one were found [26]. Consequently, in these poplar genotypes, *Populus RRA9* is probably not expressed in leaves. *DkRRA8* and *dkRRA11* transcripts were also undetectable in our experiment, even though we managed to isolate them from the cDNA library due to its enrichment in mRNA. In *P. trichocarpa* and *P. tremula x P. alba*, *PtRRA11* transcripts were also undetectable in all vegetative

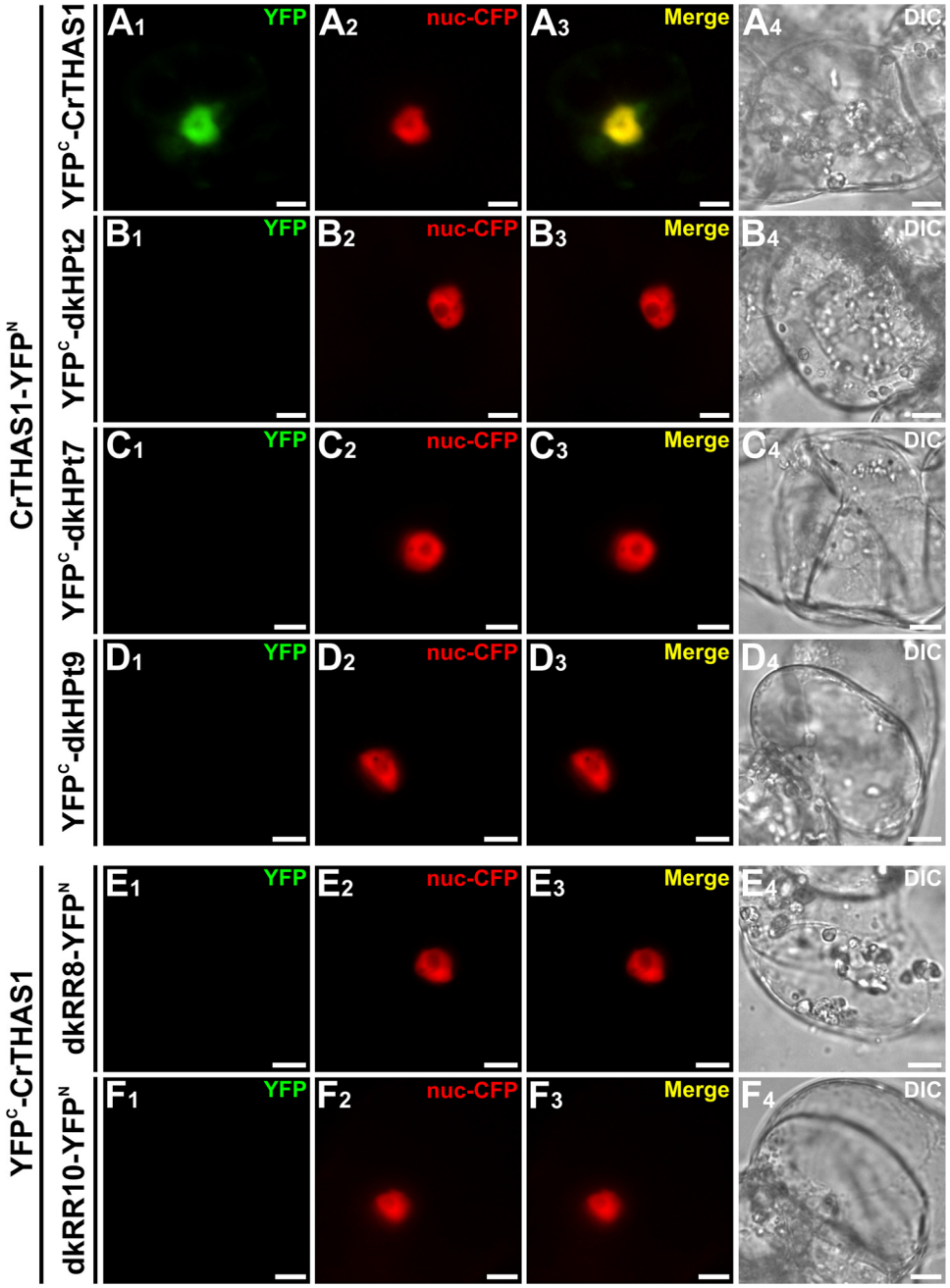

Fig. 6. Control of dkRRA8-10/dkHPt2-7-9 interaction specificity in *C. roseus* cells using BiFC assays. *C. roseus* cells were co-transformed with plasmids expressing YFP^C-CrTHAS1 with dkRRA8 (E1) and dkRRA10YFP^N (F1), and CrTHAS-YFP^N with YFP^C-dkHPt2 (B1), -dkHPt7 (C1) and -dkHPt9 (D1). An additional co-transformation with the CFP nuclear marker (A2-F2) specifies the nuclear localization. A positive control is observed by transformation with YFP^C-CrTHAS1 with CrTHAS-YFP^N (A1, B1, C1). The morphology was observed by differential interference contrast (DIC) microscopy (A4-F4). Scale bar = 10 µm.

organs studied (leaves and detached leaves, nodes, internodes and roots) but were slightly expressed in phloem, xylem and in catkins [26]. The same pattern of expression could most probably be expected in the 'Dorskamp' clone for *dkRRA11*. However, the isolation of *dkRRA11* from drought stressed leaves after a nested PCR supports the hypothesis that *dkRRA11* is slightly expressed in leaves and may be regulated by drought (data not shown). On the other hand, *dkRRA8* transcripts were detected in all vegetative tissues and precatkins in *P. trichocarpa,* but not in *P. tremula x P. alba* leaves [26]. Another difference observed was that *dkRRA3* was expressed in the 'Dorskamp' clone, but not in *P. trichocarpa* or *P. tremula x P. alba* [26]. Consequently, the lack of *dkRRA8*, *9* and *11* gene expression in vegetative tissues studied led us to suppose that these three type A RRs could not interfere in the dkHPt2-7-9/dkRRB12-13, 16, 18–19 network during the early drought response, since their mRNAs are not detected in control conditions. Surprisingly, *dkRRA4-5-7* and *10* are similarly expressed in control leaves in these three genotypes (*RRA9* and *11* are not detected and present a common response in these genotypes), whereas *dkRRA1-2-3-6*, and *8* present various expression patterns, creating the hypothesis that there are genotype specific responses to stress (Table S1). Could these differences, observed between genotypes for the expression level of type A RRs under control conditions, explain the different adaptability of the genotypes to environmental constraints? A comparative study of genotype response during environmental constraints could help answer this question. In *Populus x canescens* stems (corresponding to *P. tremula x P. alba*) *PtaRRA3* has been shown to be down regulated by drought [66].

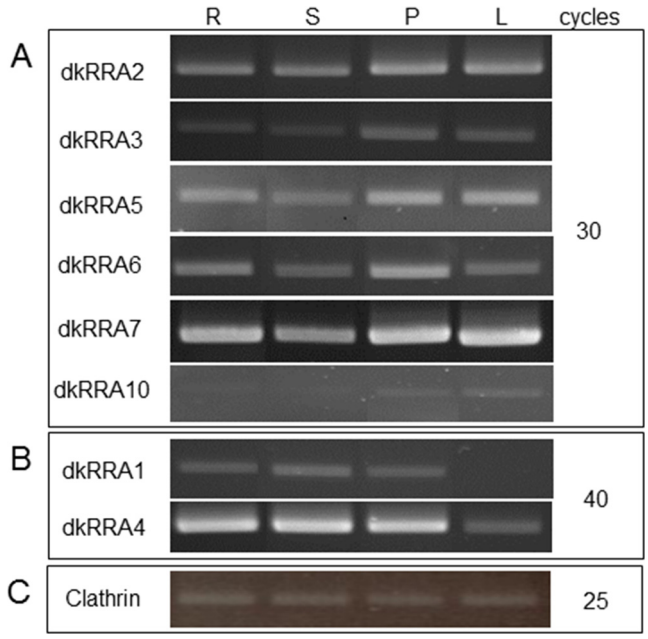

Fig. 7. Expression analysis of poplar type A RRs by RT-PCR.
RNAs isolated from roots (R), stems (S), petioles (P) and leaf blades (L) were reverse transcribed and used as templates for PCR amplification. PCR reactions were performed using *dkRRA* specific primers under optimal conditions for each primer set (A: 30 cycles, B: 40 cycles). Expression profile of *Clathrin*, used as a housekeeping gene, was realized with 25 cycles of PCR amplification (C).

Due to this *RRA3* regulation and the interaction between dkRRA3 and dkHPt2, 7 and 9, further studies should be conducted to investigate a possible involvement of this RRA as a regulatory protein during water stress. As shown in Arabidopsis seedlings, a slight increase in *AtRRA5*, *7* and *15* expression was observed during dehydration stress [67,68]. Since *AtRRA5* is the homologue of *PtRRA10*, it could be interesting to investigate type A RR transcript regulation during drought in all organs by a semi-quantitative PCR approach during a more complete time-course experiment.

In the present work, the aim of our analysis was to check if type A RRs could interfere with the dkHK1a-b/dkHPt2-7-9/dkRRB12-13, 16, 18–19 pathway previously characterized in poplar [28–33]. We successfully isolated 10 type A RRs, and defined their subcellular localizations experimentally, which weren't exactly as predicted by *in silico* tools. Moreover, we determined that since they were co-expressed with dkHPt proteins, eight of them could interfere in the MSP dkHK1a-b/dkHPt2-7-9/dkRRB12-13, 16, 18–19 (Fig. 8). Amongst these eight interacting proteins, dkRRA1 and dkRRA10 could not interfere with MSP partners in leaves and roots respectively as they were either poorly expressed, or not expressed at all in these organs under our experimental conditions. Our results, together with literature data, show a variability of type A RR gene expression for three *Populus* genotypes under control conditions, leading to the possible conclusion that there is a genotypic variability for these proteins belonging to MSP. Consequently, variable poplar responses to unfavorable environmental conditions could be explained, at least in part, by this MSP genotypic variability which could lead to a better tolerance to stress. This hypothesis emphasizes the importance of a study into the genotypic variability of poplar MSP partners in this context of global climate change.

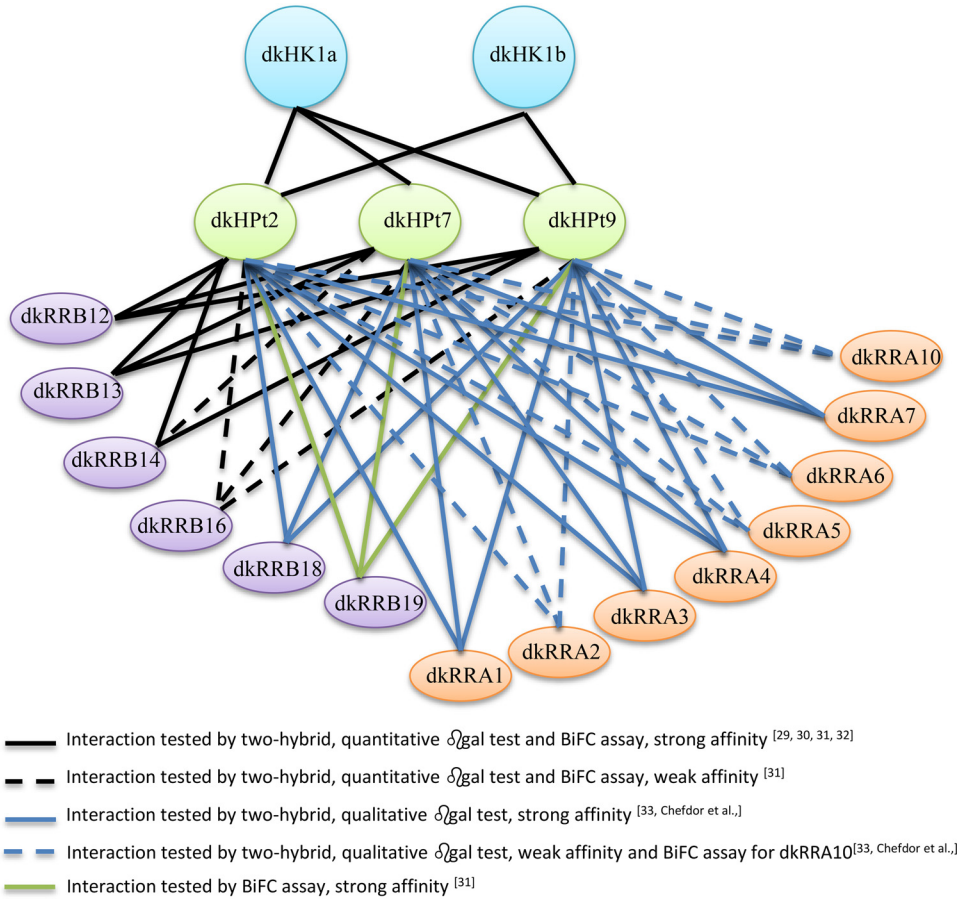

Fig. 8. Interaction network in dkHK1 multistep phosphorelay pathway.

Conflicts of interest

The authors declare no conflict of interest.

The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.plantsci.2018.09.010.

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