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Abstract

In poplar, we highlighted a multistep phosphorelay (MSP) system composed by two hybrid-type Histidine aspartate Kinases, HK1a and HK1b, interacting with three Histidine Phosphotransfer proteins, HPt2, 7 and 9, interacting themselves with six type-B Response Regulators (RR), corresponding to the HK1a-b/HPts/RRs-B MSP. This MSP is putatively involved in an osmosensing pathway since HK1a-b are homologous 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 signaling MSP through their interaction with HPt proteins, we decided to characterize poplar type-A RRs and their implication in the MSP studied to determine which one could regulate the HK1a-b/HPts/RRs-B MSP. To have a global view of this MSP, we isolated 10 poplar type-A RR cDNAs and a subcellular localization study was conducted to check experimentally the in silico prediction. For most of them, the in planta subcellular localization was as predicted except for three RRs for which this experimental approach gave a more precise localization. Interaction studies by yeast two-hybrid and BiFC assays together with transcripts expression analysis in poplar organs led to point out eight type-A RRs as potentially involved in disruption of the HK1a-b/HPts/RRs-B MSP identified in previous studies. Consequently, the results obtained in this study now provide an exhaustive view of HK1a-b partners belonging to a poplar MSP.

Keywords	multistep phosphorelay, type-A Response Regulators, Populus

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09 April 2018

Cover letter

Dear Editor

You will find enclosed our manuscript entitled "Highlighting type-A RRs as potential regulators of HK1 multi-step phosphorelay pathway in Populus" for publication in Plant Sciences as original research paper. This work concerns the identification of type-A response regulators (type-A RRs) belonging to MSP in *Populus* to determine what type-A RRs could interfere the HK1a-b/HPts/RRs-B MSP previously identified in poplar as potential competitors to type-B RRs.

Sincerely yours

Sabine Carpin

Highlights

- Isolation of ten type-A RRs from poplar clone “Dorskamp”
- Nucleocytoplasmic and nuclear localization for eight and two of them respectively
- Eight type-A RRs could interfere the HK1a-b/HPts/RRs-B MSP

Highlighting type-A RRs as potential regulators of HK1 multi-step phosphorelay pathway in *Populus*

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Abstract

In poplar, we highlighted a multistep phosphorelay (MSP) system composed by two hybrid-type Histidine aspartate Kinases, HK1a and HK1b, interacting with three Histidine Phosphotransfer proteins, HPt2, 7 and 9, interacting themselves with six type-B Response Regulators (RR), corresponding to the HK1a-b/HPts/RRs-B MSP. This MSP is putatively involved in an osmosensing pathway since HK1a-b are homologous 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 signaling MSP through their interaction with HPt proteins, we decided to characterize poplar type-A RRs and their implication in the MSP studied to determine which one could regulate the HK1a-b/HPts/RRs-B MSP. To have a global view of this MSP, we isolated 10 poplar type-A RR cDNAs and a subcellular localization study was conducted to check experimentally the *in silico* prediction. For most of them, the *in planta* subcellular localization was as predicted except for three RRs for which this experimental approach gave a more precise localization. Interaction studies by yeast two-hybrid and BiFC assays together with transcripts expression analysis in poplar organs led to point out eight type-A RRs as potentially involved in disruption of the HK1a-b/HPts/RRs-B MSP identified in previous studies. Consequently, the results obtained in this study now provide an exhaustive view of HK1a-b partners belonging to a poplar MSP.

Keywords: multistep phosphorelay, type-A Response Regulators, *Populus*

1 Introduction

Living organisms have to face fluctuant environmental conditions and in the context of global climatic change, organisms endowed with efficient perception mechanisms will be able to adapt themselves more efficiently. To sense and response to environmental stimuli, prokaryotic organisms, such as bacteria, employ a well characterized signaling pathway, the

canonical two component system (TCS) comprising a sensor protein corresponding to a Histidine Kinase, and a response regulator (RR), specific to the stimuli [1]. A more elaborate system is also described in prokaryotic and eukaryotic organisms, involving a His-Asp-His-Asp phosphorelay called multistep phosphorelay (MSP) system, comprising sensors and RRs but completed by a third partner shuttling between them, called histidine phosphotransfer protein (HPt) [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, phytohormones such as cytokinin (CK) and ethylene which regulate growth and plant development [3,4,5,6,7]

The CK pathway is the most studied and characterized MSP in plants. In Arabidopsis, CK receptors AHK2, AHK3 and AHK4 activated by CK fixation can autophosphorylate and trigger a phosphorelay involving five HPt proteins (AHP1-5) and 11 type-B RRs (ARR12-22), which once activated by phosphorylation can activate transcription of target genes such type-*A RR* genes [8,9,10,11,12] These latter genes are primary response genes and once activated by phosphate transfer *via* AHK and AHP proteins, act as negative regulators of CK signaling [13,9,10,14,15]. Type-A RRs are consequently in competition with type-B RRs for the phosphate transfer by AHP proteins in CK signaling.

Type-A RRs are also regulated by abiotic stresses such as drought, salinity, dehydration and cold [16,17,18]. For example, the expression of the type-A *OsRR1-2-5-6-7* and *9* from *Oryza sativa* are induced by salt, dehydration and low temperature treatments [16,17]. In *Arabidopsis thaliana*, *ARR7* is cold inducible [18] and acts also 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 ARR3-4-5 and 6 may function as positive regulators whereas ARR8 and 9 function as negative regulators in osmotic stress [20]. In *Glycine max*, there are six dehydration-repressed type-A RR genes (*GmRR07-08-09* and *GmRR11-12-13*) which encode *ARR8*- and *ARR9-like* RR respectively, providing evidence that these GmRRs function in stress response and may act as negative regulators in a similar fashion as their ARR8 and ARR9 orthologues [21]. Similarly, *GmRR01* and *GmRR02* genes, *ARR4*- and *ARR6-like* 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 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 in tree plant models. In *Malus domestica*, 19 type-A RRs were identified by *in silico* analysis and some of them are up-regulated by CK treatments [23]. In *Pinus pinea*, a type-A RR gene *PipiRR1* 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 *PipiRR1* homologous gene *PipsRR1*, cloned in *Pinus pinaster*, seems also 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, PtRR1 to PtRR11, 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 1h of CK treatment in *Populus tremula* x *Populus alba* [26]. Regarding type-A RRs in tree models, few studies have been conducted but none of them investigate the role of these proteins in MSP regulation.

In previous works, we first identified poplar hybrid-type histidine aspartate kinases, HK1a and HK1b [28,29]. Both HK1 succeed to functionally complement a *Saccharomyces cerevisiae* deletion mutant for its two osmosensors sln1 and sho1, demonstrating to have kinase and osmosensor functions in yeast. Protein interaction studies jointly to transcripts co-expression analysis *in planta* have been carried out to determine HK1 partners among the 10 poplar HPt proteins identified. Hence, three HPt partners have been retained [29,30]. In a same way, a similar study led to the identification of six type-B RRs, which could participate in a poplar MSP [31,32,33]. Taken together, these studies highlight a MSP partnership, HK1a-b/HPt2-7-9/RR-B12-13, 16, 18-19, potentially involved in drought stress response in poplar. As type-A RRs are negative regulators of CK MSP and participate to the regulation of CK signal, the role of these proteins in other MSP pathways remains to be elucidated.

In order to complete the MSP network identified in partnership with HK1, we decided to identify type-A RRs in poplar and to study their putative implication in this signaling pathway, as potential regulators of this MSP. We succeeded in isolating 10 cDNAs encoding type-A RRs in the poplar clone "Dorskamp". Then we identified the subcellular localization, and studied the interactions with the three HPt, preferential interacting partners of HK1, by performing two-hybrid assays in yeast. Some interactions were validated by BiFC assay in *Catharanthus roseus* cells and the relevance of these interactions has been strengthened by co-expression analysis of transcripts of all the studied proteins in poplar organs, under control and osmotic stress conditions. Taken together, these results define an interactome linked to HK1 in poplar and highlights that at least eight type-A RRs can participate to the MSP HK1a-b/HPts/RRs-B as type-B RRs competitors *via* 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 (PtRR1 to PtRR11) [26] to search their nucleotidic sequences in JGI *Populus trichocarpa* (v1.1) and designed a specific primers pair corresponding to each RR (Additional file 1), in order to isolate their coding sequence (CDS) from the poplar clone "Dorskamp" (*Populus deltoides* (Bartr.) Marsh x *P. nigra* L.). Except for *dkRR11*, PCRs were performed using a root cDNAs library constructed with 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 vector (Promega), sequenced and compared with *P. trichocarpa* type-A RRs sequences using ClustalW [34]. For *dkRR11*, PCR was performed in the same conditions, but using stressed leaves cDNAs library and a nested PCR (Results section 4).
Amino acid sequences of type-A dkRRs were aligned with those from *Arabidopsis thaliana* using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the alignment was represented by a phylogram constructed with 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 as pLex) and a Gal4 activation domain encoding prey vector (pGADT7, Clontech). RR CDSs were cloned into the pGAD vector as *Eco*RI-*Xho*I fragments

for dkRR1 to dkRR10, and *Xma*I-*Xho*I for dkRR11; HPt CDSs were cloned into 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, an overnight cell culture with an Optical Density at wavelength of 600 nm (OD_{600}) of 0.5 and 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 HPts, 3-amino-1, 2, 4-triazole (3AT) was supplemented to -LWH medium at 20 (HPt7 and 9) or 60mM (HPt2) according to [31]. Yeast cells grew during 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 coding sequences of RR8 and RR10 were cloned into the *Spe*I site in frame with the C-terminal fragment of YFP. HPt2, HPt7 and HPt9 were cloned into the SpeI 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].

2.4 Type-A *dkRR* transcripts detection by RT-PCR

This study was performed using the poplar clone “Dorskamp”. An osmotic stress was applied to one month-old hydroponically grown rooted cuttings by supplementing the growth medium with PEG 6000 at 50 g/L [39]. Roots, stems, petioles and leaf blades were harvested and frozen after 0 and 10 minutes of stress. RNA extractions were carried out with 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 template for PCR amplifications. Thirty or fourty PCR cycles were performed in order to detect type-A *dkRR* 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 Type-A dkRR localization by GFP-fused protein expression

To express RR-GFP fusion proteins, the coding sequences of RRs were amplified by PCR using specific primers extended by *Spe*I restriction sites at both extremities. The amplified CDS was subsequently cloned into pGEM-T vector (Promega) and checked by sequencing. After *Spe*I digestion, CDSs were cloned into *Spe*I restriction site of pSCA-cassette GFP [38] upstream of and in frame with the coding sequence of GFP. Transient transformation of *C. roseus* cells by particle bombardment and GFP imaging were performed using the nuclear mcherry and nucleocytoplasmic CFP markers according to [38].

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) data base, we isolated 10 CDSs encoding type-A RRs in the poplar clone "Dorskamp", *dkRR1* to *dkRR11*, with the exception of *dkRR9* CDS. Among the 10 CDSs, identities ranged from 51 to 89 %. All the deduced amino acid sequences of isolated type-A *dkRR* CDSs share the DDK conserved residues characteristic of the receiver domain (RD) of RR belonging to MSP. As already observed by [26], some type-A *dkRR* genes are duplicated and constitute sister pairs (Fig. 1). The C-terminal end of dkRR1/2 pair is characterized by a serine and proline rich zone (Fig. 2) as described for the *Arabidopsis* homologues, ARR3 and ARR4 [13]. DkRR10, encoded by an unduplicated gene, is characterized by a charged serine and proline rich zone, similar to ARR 7 and ARR15 charged C-terminal end which is also enriched by serine residues, but with threonine instead of proline residues. As observed in the unrooted tree (Fig 1), five type-A *dkRRs*, dkRR3-7, group together but present different characteristics. DkRR3-5 are characterized by a charged C-terminal end as observed for ARR8 and 9 whereas dkRR6 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 by dkRR8 and dkRR11 which are not characterized by a C-terminal end as for both pairs ARR5/6 and ARR16/17.

3.2 Type-A dkRRs localization

Type-A dkRRs subcellular localization were determined by transient expression of GFP-fused RRs in *C. roseus* cells. GFP and a nuclear mCherry marker were used as nucleocytoplasmic and nuclear marker respectively (Fig. 3A1, A2). All type-A dkRR 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 dkRR2 and dkRR8. Indeed, both dkRR2- and dkRR8-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 since they perfectly merged in the nucleus (Fig. 3C3, I3). Therefore, many of the type-A dkRR proteins showed a strict subcellular localization in the nucleus, except for dkRR2 and dkRR8 which exhibited an additional cytoplasmic localization.

3.3 Type-A dkRRs interact with HPt2, HPt7 and HPt9

To determine if type-A dkRRs could compete for the partnership between HPt2-7-9 and RR-B12-13, 16, 18-19, we performed two-hybrid assays in yeast with all isolated type-A RRs and the three HPt partners of HK1a/b, HPt2, 7 and 9. This study revealed reporter genes activation for all interactions tested except for dkRR8 which interacts with HPt7 only (Fig. 4). Yeast expressing both dkRR8 and HPt2 or 9 showed a similar growth pattern with the negative control (Fig 4A). For dkRR1/dkRR2 pair, a different behavior was observed since yeast expressing dkRR2 needed two extra days before cell growth was observed (Fig. 4B). This delay reflects probably a weaker interaction between dkRR2 and the three HPts compared to

dkRR1. In the same way, a differential behavior was also observed for dkRR4/dkRR5 and dkRR6/dkRR7 pairs with weaker growth observed for yeast expressing dkRR5 compared to dkRR4 and dkRR6 to dkRR7. The lack of dkRR9 prevented studying dkRR9/dkRR11 pair interactions. These two-hybrid assays showed that type-A dkRRs could be separated in four categories according to reporter genes activation. The first group only included dkRR2 which presented weak interaction with the three HPt proteins; a second group with dkRR1, 3, 4 and 7, presenting more pronounced interaction; and an intermediate group composed of dkRR5, 6, 10 and 11. The last group is comprised of dkRR8 interacting only with HPt7.

3.4 Validation of dkRR8/HPts interaction by a BiFC approach

To validate the lack of interaction observed in yeast two-hybrid assay between dkRR8 and HPt2 and 9, BiFC assays were conducted between dkRR8 and the three HPt proteins *in planta*. HPt2, 7, and 9, dkRR8, and 10 coding sequences 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-HPt2/7/9 and dkRR8/10-YFP^N. As observed in yeast two-hybrid tests, the BiFC approach substantiates the interaction between dkRR8 and HPt7 by observation of the YFP complex reconstitution (Fig. 5B1). Interactions between dkRR8 and HPt9 and HPt2 were observed using this second approach when co-expressing dkRR8-YFP^N and YFP^C-HPt2 (Fig. 5A1) and dkRR8-YFP^N and YFP^C -HPt9 (Fig. 5C1). As positive control of interaction, we tested dkRR10-YFP^N with YFP^C-HPt2/7/9. As expected, the YFP complex reconstitution was observed for all interactions tested (Fig. 5D1, El, Fl). This signal using dkRR10 perfectly merged 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 dkRR10 (Fig. 5D3, E3, F3). By contrast, BiFC complex reconstitution for dkRR8-YFP^N and YFP^C-HPt2/7/9 was observed both in nuclear and cytosolic compartment (Fig. 5A1, B1, C1) and the localization in the nucleus was confirmed by the merge observed with the CFP-nuclear marker (Fig. 5A3, B3, C3). This experiment led to the conclusion of a nuclear and cytoplasmic localization of the interaction of both partners.

3.5 Type-A *DkRR* genes expression analysis by RT-PCR

To be physiologically pertinent in plants, all partnerships observed using yeast two-hybrid or BiFC assays need to be validated by the observation of the concomitantly co-expression of both partners in same organs. In order to validate the relevancy of observed interactions, type-A *dkRR* transcripts expression pattern was studied by RT-PCR analysis with *Clathrin* as reference gene (Fig. 6C). A constitutive expression was observed for six *dkRR* transcripts (*dkRR2*, *3*, *5*, *6*, *7* and *10*) in all studied organs (Fig 6A) at 30 PCR cycles. To refine these results, we performed 10 more cycles and were able to detect two other RRs, *dkRR1* and *dkRR4* (Fig. 6B). These two latter are less abundant than the others since they were detected after 40 PCR cycles and *dkRR1* was not detected in leaf blades (Fig. 6B). Furthermore, a differential gene expression can be observed for both pairs of gene, *dkRR1/dkRR2* and *dkRR4/dkRR5*. By contrast, the gene pair *dkRR6*/*dkRR7* shows a similar expression pattern. Among the most expressed type-A *dkRR*s (*2*, *3*, *5*, *6*, *7*, and *10* Fig. 6A) *dkRR10* shows the weakest expression in roots and this expression is slightly stronger in leaf blades (Fig. 6B). In our experimental conditions (PEG 50 g/L during 10 minutes), two type-A *dkRR* transcripts, *dkRR8* and *dkRR11,* were not detected (data not shown) and osmotic stress applied did not impact transcription levels of studied genes.

4 Discussion

In this study, we successfully isolated 10 cDNAs encoding type-A RRs from the poplar clone "Dorskamp". These RRs correspond to *dkRR1* to *dkRR11*, except *dkRR9* among the 11 genes identified in JGI *Populus trichocarpa* (v1.1).

In Arabidopsis, type-A RR genes are all duplicated [40, 27], while in poplar three genes (*dkRR3*, *dKRR8* and *dKRR10*) seem not to be duplicated [26]. However, as observed in Arabidopsis, genes homologous of these three genes are duplicated in *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 [41]. For example, duplicate loss could be observed for weakly expressed genes with uncomplex promoter [42]. Such a lack of pairwise genes is observed for rice RRs and is probably due to frequent gene loss events [43]. Thus, hypothesis that these three genes in poplar have never been duplicated or have undergone a duplicate loss during poplar evolution could be considered.

The dkRR1/dkRR2 pair, homologous to the ARR3/ARR4 pair, shares the same characteristics, *i.e.* a RD followed by a serine and proline rich domain in the C-terminal end. In Arabidopsis, ARR4, was shown to interact with PHY-B and found to be involved in a phase delay of the circadian rhythm [44,45]. Moreover, ARR4 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 ARR4 activity during photomorphogenesis [46], but this activity is also controlled by its protease-mediated degradation [47]. This degradation is mediated by DEG9 interaction with ARR4 C-terminal end leading to ARR4 specific degradation which is not observed for ARR3 due to differences in the C-terminal end [47]. Since a similar C-terminal extension, characterized by a serine/proline rich domain, was found in dkRR1/dkRR2, a possible involvement in circadian rhythm regulation in poplar could be envisaged for these two RRs.

In the phylogenetic tree, dkRR3, the dkRR4/DkRR5 and dkRR6/dkRR7 pairs, group together with ARR8/ARR9. However, they do not share similar characteristics in their C-terminal ends. DkRR3 and dkRR4/dkRR5 have a charged C-terminal end as ARR8 and ARR9 whereas dkRR6/dkRR7 pair contains a short charged proline/glutamine rich domain. No type-A RRs from Arabidopsis have such domains. While type-B RRs are characterized by a C-terminal end enriched in proline/glutamine residues involved in transactivation function, such domain is lacking in type-A RRs [48,3], and this domain in dkRR6 and dkRR7 is not long enough to function as a transactivation domain. However, proline and glutamine residues are often associated with protein interactions [49] raising the question whether this domain could be involved in protein-protein interactions leading to specific function for these type-A RRs as observed for ARR4 C-terminal end.

In the same way, dkRR8 and dkRR11 group with the ARR16/ARR17 pair in the phylogenetic tree as expected since they share a common architecture, *i.e*. a short C-terminal end (< 30 amino acids). However, the ARR5/ARR6 pair does not gather with these RRs although they share the same architecture. Moreover, dkRR10, homologous to ARR5, shares common architecture, a serine and proline/threonine rich domain, with the ARR7/ARR15 pair. It is noteworthy that phylogenetic trees of RRs are commonly built with the RD only and does not include the C-terminal end [40]. However, the C-terminal end of these proteins seems clearly important for the protein function or regulation as shown for ARR4 [47]. Thus, even though these proteins are phylogenetically distant, they could share common functional mechanism according to their common architecture.

In our study, we failed to isolate one gene, *dkRR9* cDNA but we did succeed to isolate *dkRR11* thanks to the use of a nested PCR on cDNAs from poplar drought stressed leaves. *PtRR9*, homologous to *dkRR9*, 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 no. 717-1-B4 [26]. In these two genotypes, *PtRR3*, *PtRR9* and *PtRR11* were undetectable in these organs but clearly expressed in catkins, from which only *PtRR9* was strictly specific. The *PtRR9* tissue specific expression in catkins linked to its involvement in sex determination could explain our unsuccessful attempt to isolate this RR 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, *PtRR9*, homologous to *ARR17* [50]. In *P. balsamifera*, authors showed that *PbRR9* was more heavily methylated in males than in females and consequently probably less expressed in males leading to the hypothesis that *PbRR9* gene could be involved in poplar sex determination [51]. Yet, it was demonstrated that male poplars adapt more efficiently during drought stress since water deficiency inhibits growth, photosynthesis and ROS protection more strongly in females than in males [52]. Indeed, this difference was previously observed between two *Populus x euramericana* clones, the male genotype Dorskamp and the female genotype Luisa Avanzo [53]. Growth and photosynthesis were affected *via* a clear leaf area decrease during drought in female poplars [54]. Furthermore, in Arabidopsis, leaf differentiation and consequently leaf area is controlled through *ARR16* activation by the complex of the chromatin remodeler BRM and TCP4 CIN-TCP leading to CK decrease [55,56]. It seems that type-A RRs could be involved in drought response *via* sex determination (ARR17) or leaf development and leaf area control (ARR16). In the poplar clone "Dorskamp", dkRR9 could be also involved in sex determination and indirectly in drought, by controlling sex determination. The impossibility to isolate this gene in vegetative tissue argues in favor of this hypothesis. Moreover, the increase of *dkRR11* transcript levels in drought stressed leaves compared to control in nested RT-PCR (data not shown) raised the question about an involvement of this RR in leaf area control during drought. As neofunctionalization after whole genome duplication is proposed for *PtRR9*/*PtRR11* [50,51], more studies are needed to determine the involvement of this RR in poplar drought response.

Since only *in silico* prediction methods have been used until now to analyze poplar type-A RRs subcellular localization, we have done an exhaustive study to confirm or not these predictions *in planta*. These assays showed a different localization for dkRR2, 4 and 5 compared to *in silico* results obtained by Ramirez-Carvajal *et al*. [26] hence pointing out the necessity to perform experimental validation. DkRR1 is a nuclear protein as is ARR4 and dkRR2 is a nucleocytoplasmic protein as is ARR3 [57]. Considering the fact that nuclear ARR4 is involved in mediating cross-talk between light and CK signaling through modulation of phytochrome B activity and involved in circadian period, dkRR1 could be involved in a similar process [58,46]. This observation as well as common structural characteristics could assume a similar cellular function of poplar proteins. DkRR4, 5, 6 and 7 are nuclear proteins as is observed for their Arabidopsis homologs, ARR8 and ARR9 [57]. DkRR8 localizes in the nucleus and cytoplasm as is observed for ARR16, probably due to their same short C-terminal ends [59].

In the past, type-A RRs were identified as primary response genes and are known to be negative regulators in CK pathway, 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 [10,14,15,60]. This activation is due to direct interaction with HPt proteins in the nucleus [61,15,62].

In previous work, we identified a specific partnership composed by HK1a-b/HPt2-7-9/RR-B12-13, 16, 18-19 [29,30,31,33]. To determine if type-A RRs could interfere with this partnership, we decided to study potential interactions between all isolated type-A RRs and the three HPt proteins partners of HK1. All type-A RR proteins were able to interact with these HPt proteins. However, a surprising behavior was observed for type-A RR pairs. Indeed, all pairs are composed of a strongly and a weakly interacting RR. In Arabidopsis, ARR3/ARR4 and ARR8/ARR9 pairs showed similar interaction patterns for AHP5 and some differences were detected for ARR5/ARR6 and ARR7/ARR15 pairs [63]. These different binding properties of proteins making up each pair could reflect the redundancy already observed in other plant models. A different experimental approach is often necessary to confirm two-hybrid results [64,31]. The lack of interactions observed between dkRR8 and HPt2/HPt9 was checked by BiFC and revealed interactions between dkRR8 and these HPts. The strong sequence similitude between HPt7 and HPt9 (96.7%), which constitute a pair, was in favor of such results with HPt9. Moreover, BiFC approach showed that the interactions of dkRR8/HPts and dkRR10/HPts exhibited a nucleocytoplasmic and a nuclear localization respectively.

The interactions observed in yeast two-hybrid or BiFC assays are biologically relevant only if tested proteins are spatio-temporally co-expressed in plant. As the expression of all proteins of the HK1/HPt2-7-9/RR-B12-13, 16, 18-19 partnership was studied in roots, stems, petioles and leaf blades [31,29], we decided to study the type-A RRs expression from exactly the same biological material.

As previously discussed, *dkRR9* cDNA was not isolated and consequently undetectable in our experiment. Surprisingly, constitutive expression for *PtRR9* transcripts was detected in leaves by RT-PCR, in the genotype *P. tremula x P. alba*, however three aberrant transcripts shorter than the expected one were found [26]. Consequently, in these poplar genotypes, *Populus RR9* is probably not expressed in leaves. *DkRR8* and *dkRR11* transcripts were also undetectable in our experiment even if we succeed to isolate them from cDNA library due to its enrichment in mRNA. In *P. trichocarpa* and *P. tremula x P. alba*, *PtRR11* transcripts were also undetectable in all vegetative organs studied, leaves and detached leaves, nodes, internodes and roots but were slightly expressed in phloem, xylem and in catkins [26]. Probably the same pattern of expression could be expected in "Dorskamp" clone for *dkRR11*. However, the isolation of *dkRR11* from drought stressed leaves after a nested PCR supports the hypothesis that *dkRR11* is slightly expressed in leaves and may be regulated by drought (data not shown). On the other hand, *dkRR8* transcripts were detected in all vegetative tissues and precatkins of *P. trichocarpa* but not in *P. tremula x P. alba* leaves [26]. Another difference is also observed since *dkRR3* was expressed in "Dorskamp" clone but not in *P. trichocarpa* or *P. tremula x P. alba* [26]. Consequently, the lack of *dkRR8*, *9* and *11* genes expression in vegetative tissues studied led us to suppose that these three type-A RRs could not interfere in the HPt2-7-9/RR-B12-13, 16, 18-19 partnership during the first ten minutes of drought signal transduction. Surprisingly, type-A *RR4-5-7* and *10* are similarly expressed in control leaves in these three genotypes (type-A *RR9* and *11* are not detected and also present a common response in these genotypes) whereas type-A *RR1-2-3-6*, and *8* present various expression patterns, suggesting the hypothesis of genotype specific responses to stress (Table S1). Could these differences, observed between genotypes for type-A RRs expression level under control conditions, explain the different adaptability of the genotypes to

environmental constraints? A comparative study of genotypes response during environmental constraints could help to answer to this question. In our experimental conditions, drought stress did not induce detectable transcript regulation. Nonetheless, in *Populus x canescens* stems (corresponding to *P. tremula x P. alba*) *PtaRR3* has been shown to be down regulated by drought [65]. Due to this *RR3* regulation and the interaction between dkRR3 and HPt2, 7 and 9, further studies should be conducted about a possible involvement of this RR as a regulatory protein during water stress. As shown in Arabidopsis seedlings, a slight increase in *ARR5*, *7* and *15* expression was observed during dehydration stress [66]. Since *ARR5* is the homolog of *PtRR10*, it could be interesting to investigate type-A RR transcripts 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 compete in the HK1/HPt2-7-9/RR-B12-13, 16, 18-19 pathway previously characterized in poplar [28, 29, 30, 31, 32, 33]. We successfully isolated ten type-A RRs, experimentally defining their subcellular localizations which was not exactly as predicted previously. Moreover, we determined that since they were co-expressed with HPt proteins, eight of them could interfere in the MSP HK1a-b/HPt2-7-9/RR-B12-13, 16, 18-19 (Fig. 7). Among these eight interacting proteins, dkRR1 and dkRR10 could not interfere in leaves and roots respectively since they are not or poorly expressed in these organs in our experimental conditions. Besides, the variability of type-A RR gene expression observed for the three *Populus* genotypes under control conditions led 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 on the genotypic variability of poplar MSP partners in this context of global climate change.

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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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Figure Legends

Figure 1: Unrooted relationship tree of RRAs from *Arabidopsis thaliana* and *Populus*.
The full-length protein sequences of poplar type-A RRs deduced from cDNA sequences were aligned 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: ARRs, ▲: dkRRs.

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

Figure 3: Subcellular localization of poplar type-A RRs
C. roseus cells were transiently transformed with GFP (A1) and type-A dkRRs-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.

Figure 4: Interaction between type-A dkRRs and HPt2-7-9 in the yeast two-hybrid system.
Overnight cultures of yeast co-transformed with pLex-HPts and pGAD-type-A RRs were adjusted to an Optical Density at wavelength of 600 nm (OD600) of 0.5. This culture and three dilutions (OD600 0,05 to 0,0005) were spotted onto -LWH medium supplemented with 3AT as indicated and grown two (A) or four (B) days.

Figure 5: Analysis of dkRR8/HPt2-7-9 interactions in *C. roseus* cells using BiFC assays.
C. roseus cells were co-transformed with plasmids expressing dkRR8-YFP^N and YFP^C-HPt2, -HPt7 and -HPt9 (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-HPt2 (D1-D3), -HPt7 (E1-E3) and -HPt9 (F1-F3) as positive control of interaction. The morphology was observed by differential interference contrast (DIC) microscopy (A4-F4). Scale bar = 10 µm.

Figure 6: expression analysis of poplar type-A RRs by RT-PCR.
RNAs isolated from roots (R), stems (S), petioles (P) and leaf blades (L) under control (c) or stressed (s) conditions, were reverse transcribed and used as template for PCR amplification. PCR reactions were performed using type-A *dkRRs* specific primers under optimal conditions for each primer set (A : 30 cycles, B: 40 cycles). Expression profile of *Clathrin* (*Clath*), used as housekeeping gene, was realized with 25 cycles of PCR amplification (C).

Figure 7: Interaction network in HK1 multi-step phosporelay pathway.

Table S1: Summary table of type-A *RRs* expression in leaves from three different poplar genotypes by RT-PCR. D : Detected, ND : Not Detected. a : Ramírez-Carvajal *et al*., 2008. b : This study (Chefdor *et al*.,). ☑ : Common response.

Figure 1

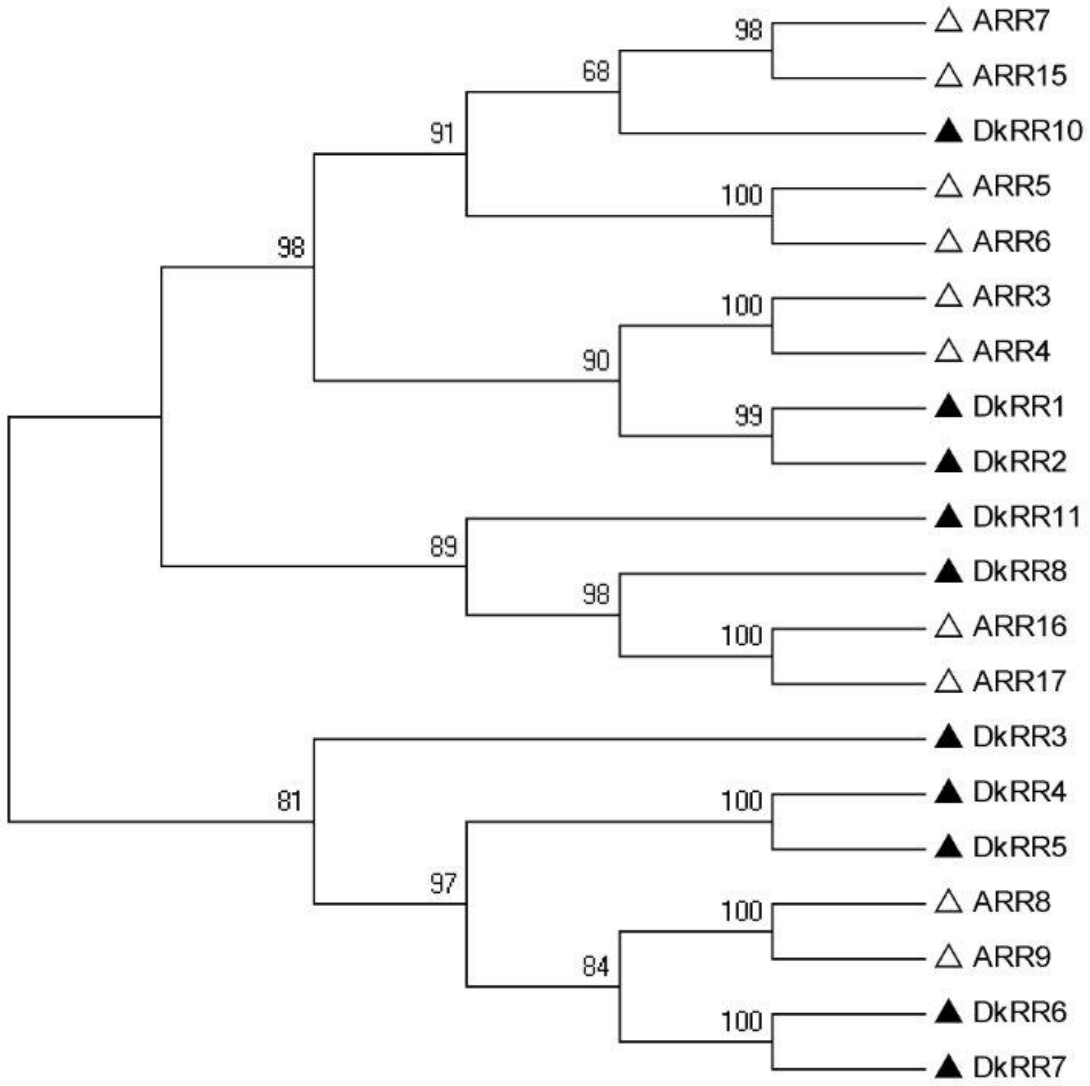
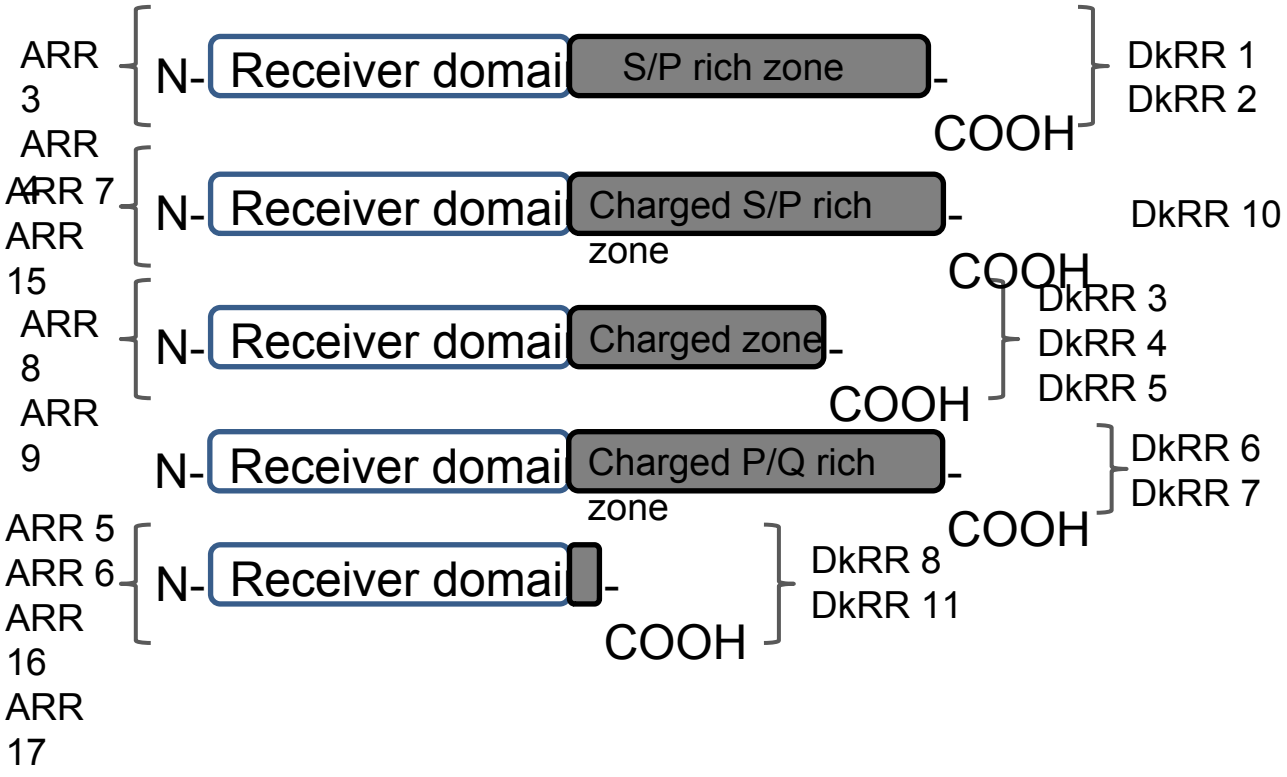
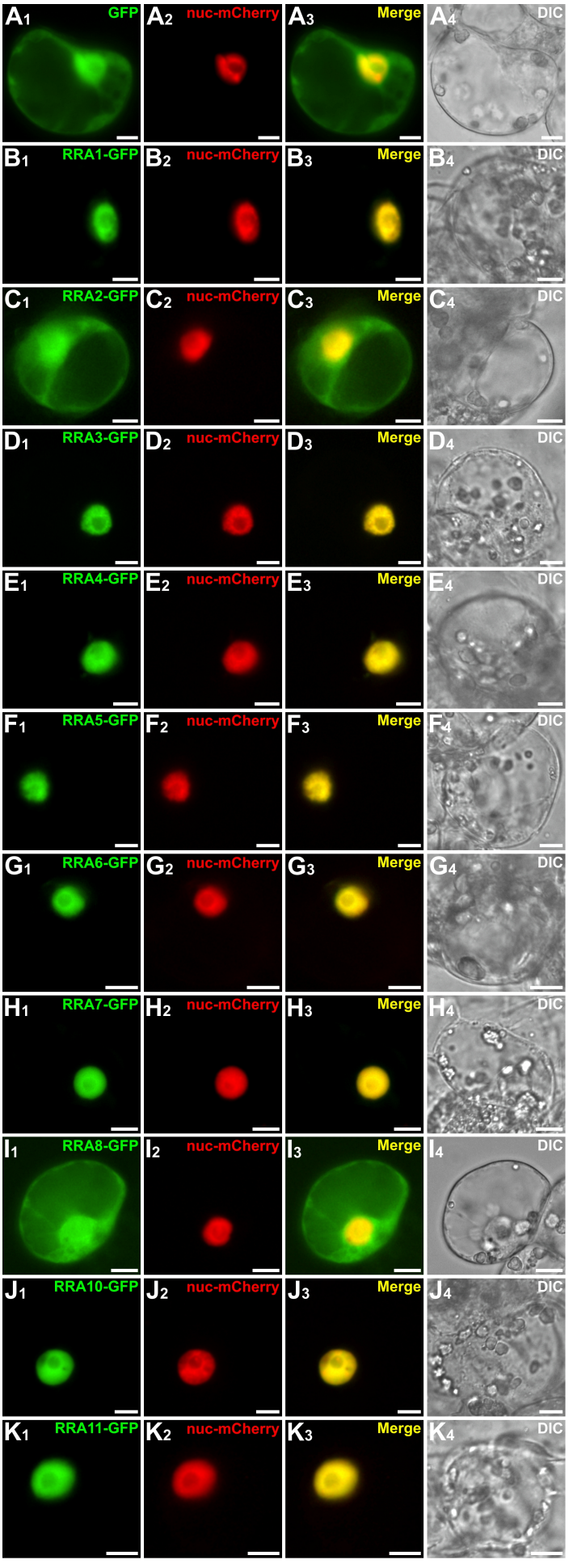
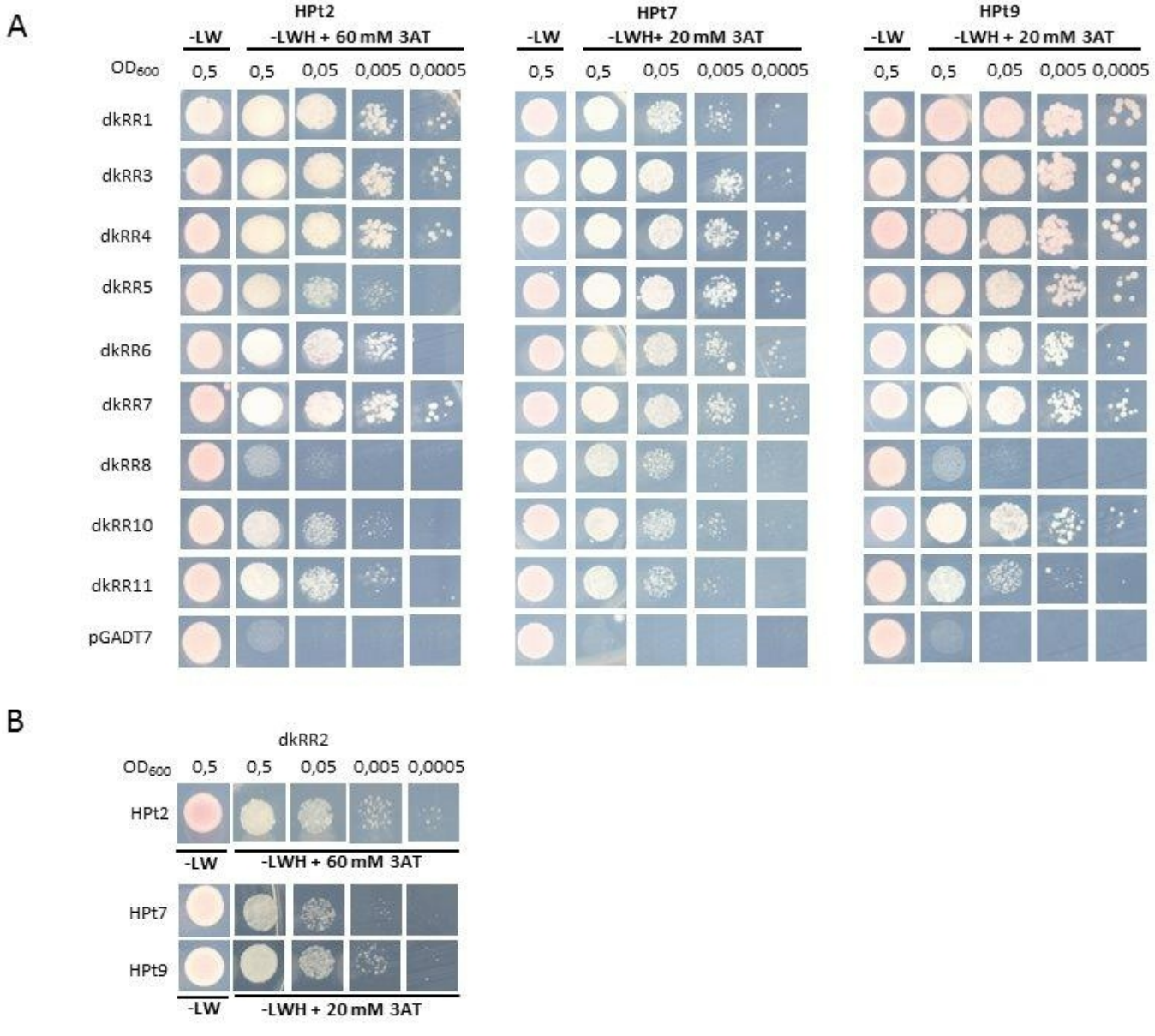
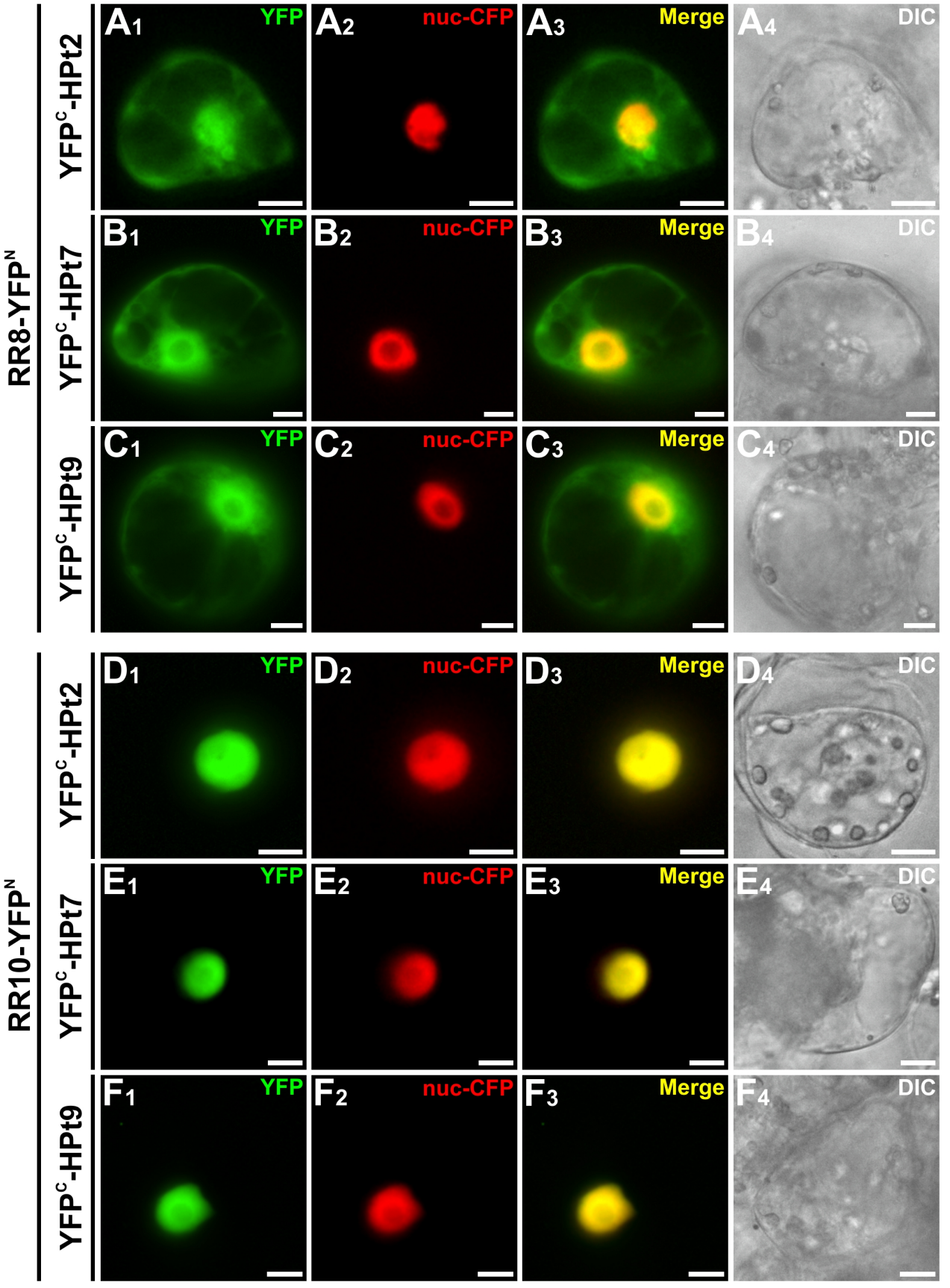

98
68
91
100
98
100
90
99
89
98
100
81
100
97
100
84
100
△ ARR7
△ ARR15
▲ DkRR10
△ ARR5
△ ARR6
△ ARR3
△ ARR4
▲ DkRR1
▲ DkRR2
▲ DkRR11
▲ DkRR8
△ ARR16
△ ARR17
▲ DkRR3
▲ DkRR4
▲ DkRR5
△ ARR8
△ ARR9
▲ DkRR6
▲ DkRR7

Figure 2


ARR 3
N-
Receiver domai
S/P rich zone
-COOH
DkRR 1
DkRR 2
ARR 7
ARR 15
N-
Receiver domai
Charged S/P rich zone
-COOH
DkRR 10
ARR 8
N-
Receiver domai
Charged zone
-COOH
DkRR 3
DkRR 4
DkRR 5
ARR 9
N-
Receiver domai
Charged P/Q rich zone
-COOH
DkRR 6
DkRR 7
ARR 5
ARR 6
ARR 16
ARR 17
N-
Receiver domai
-COOH
DkRR 8
DkRR 11


A1 GFP
A2 nuc-mCherry
A3 Merge
A4 DIC
B1 RRA1-GFP
B2 nuc-mCherry
B3 Merge
B4 DIC
C1 RRA2-GFP
C2 nuc-mCherry
C3 Merge
C4 DIC
D1 RRA3-GFP
D2 nuc-mCherry
D3 Merge
D4 DIC
E1 RRA4-GFP
E2 nuc-mCherry
E3 Merge
E4 DIC
F1 RRA5-GFP
F2 nuc-mCherry
F3 Merge
F4 DIC
G1 RRA6-GFP
G2 nuc-mCherry
G3 Merge
G4 DIC
H1 RRA7-GFP
H2 nuc-mCherry
H3 Merge
H4 DIC
I1 RRA8-GFP
I2 nuc-mCherry
I3 Merge
I4 DIC
J1 RRA10-GFP
J2 nuc-mCherry
J3 Merge
J4 DIC
K1 RRA11-GFP
K2 nuc-mCherry
K3 Merge
K4 DIC


A
HPt2
-LW
-LWH + 60 mM 3AT
HPt7
-LW
-LWH+ 20 mM 3AT
HPt9
-LW
-LWH + 20 mM 3AT
OD600
0,5
0,5
0,05
0,005
0,0005
dkRR1
dkRR3
dkRR4
dkRR5
dkRR6
dkRR7
dkRR8
dkRR10
dkRR11
pGADT7
B
dkRR2
OD600
0,5
0,5
0,05
0,005
0,0005
HPt2
-LW
-LWH + 60 mM 3AT
HPt7
HPt9
-LW
-LWH + 20 mM 3AT


RR8-YFP^N
RR10-YFP^N
YFP^C-HPt2
YFP^C-HPt7
YFP^C-HPt9
A1 YFP
A2 nuc-CFP
A3 Merge
A4 DIC
B1 YFP
B2 nuc-CFP
B3 Merge
B4 DIC
C1 YFP
C2 nuc-CFP
C3 Merge
C4 DIC
D1 YFP
D2 nuc-CFP
D3 Merge
D4 DIC
E1 YFP
E2 nuc-CFP
E3 Merge
E4 DIC
F1 YFP
F2 nuc-CFP
F3 Merge
F4 DIC

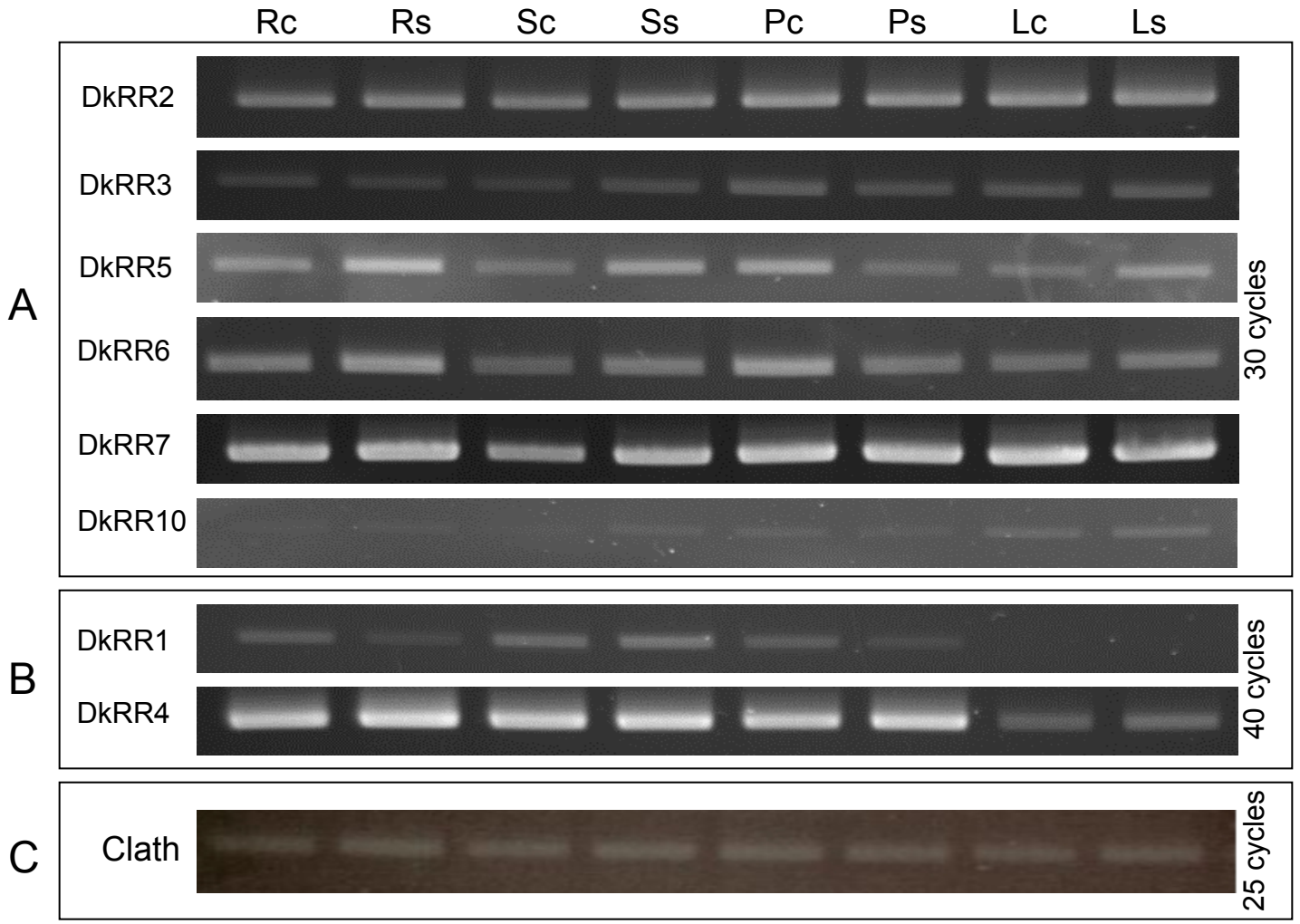

Rc
Rs
Sc
Ss
Pc
Ps
Lc
Ls
A
DkRR2
DkRR3
DkRR5
DkRR6
DkRR7
DkRR10
30 cycles
B
DkRR1
DkRR4
40 cycles
C
Clath
25 cycles

Figure 7

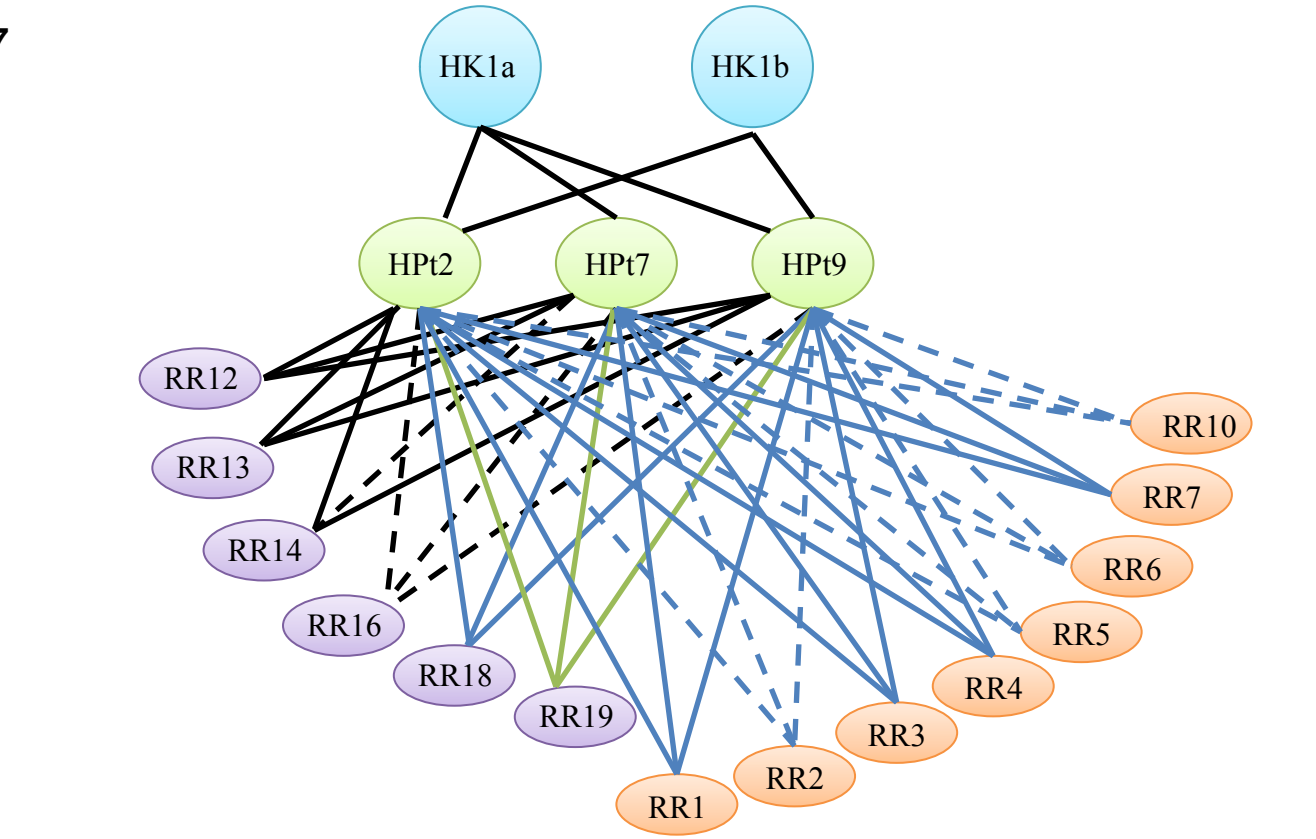

HK1a
HK1b
HPt2
HPt7
HPt9
RR12
RR13
RR14
RR16
RR18
RR19
RR1
RR2
RR3
RR4
RR5
RR6
RR7
RR10
Interaction tested by two-hybrid growth, β galactosidase activity and BiFC assay, strong affinity
Interaction tested by two-hybrid growth, β galactosidase activity and BiFC assay, weak affinity
Interaction tested by two-hybrid growth, and BiFC assay, strong affinity
Interaction tested by two-hybrid growth, and BiFC assay, weak affinity
Interaction tested by BiFC assay, strong affinity

Table S1

Type-A RRs	*P. trichocarpa*[a]	*P. tremula* x *P. alba*[a]	*P. deltoides* x *P. nigra*[b]	Common responses detected by RT-PCR in leaves
RR1	D	ND	ND	
RR2	D	ND	D	
RR3	ND	ND	D	
RR4	D	D	D	☑
RR5	D	D	D	☑
RR6	D	ND	D	
RR7	D	D	D	☑
RR8	D	ND	ND	
RR9	ND	ND	ND	☑
RR10	D	D	D	☑
RR11	ND	ND	ND	☑