



**HAL**  
open science

## Evaluation of type-B RR dimerization in poplar: A mechanism to preserve signaling specificity?

I. Djeghdir, F. Chefdor, L. Bertheau, K. Koudounas, I. Carqueijeiro, P. Lemos Cruz, V. Courdavault, C. Depierreux, M. Larcher, F. Lamblin, et al.

### ► To cite this version:

I. Djeghdir, F. Chefdor, L. Bertheau, K. Koudounas, I. Carqueijeiro, et al.. Evaluation of type-B RR dimerization in poplar: A mechanism to preserve signaling specificity?. *Plant Science*, 2021, 313, pp.111068. 10.1016/j.plantsci.2021.111068 . hal-03536824

**HAL Id: hal-03536824**

<https://univ-tours.hal.science/hal-03536824>

Submitted on 16 Oct 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Evaluation of type-B RR dimerization in poplar: a mechanism to preserve signaling**  
2 **specificity?**

3  
4 I. Djeghdir<sup>1</sup>, F. Chedfor<sup>1</sup>, L. Bertheau<sup>1</sup>, K. Koudounas<sup>2</sup>, I. Carqueijeiro<sup>2</sup>, P. Lemos Cruz<sup>2</sup>, V.  
5 Courdavault<sup>2</sup>, C. Depierreux<sup>1</sup>, M. Larcher<sup>1</sup>, F. Lamblin<sup>1</sup>, F. Héricourt<sup>1</sup>, G. Glévarec<sup>2</sup>, A.  
6 Oudin<sup>2</sup>, S. Carpin<sup>1\*</sup>

7  
8 <sup>1</sup> LBLGC, Université d'Orléans, INRAE, USC1328, 45067 Orléans Cedex 2, France

9 <sup>2</sup> Biomolécules et Biotechnologies Végétales (BBV), EA 2106, Université de Tours, 31  
10 avenue Monge, 37200 Tours, France

11  
12 \*Corresponding author: Email: [sabine.carpin@univ-orleans.fr](mailto:sabine.carpin@univ-orleans.fr)

13 Tel: 00 +33 2 38 49 48 04 / Fax: 00 +33 2 38 49 40 89

14  
15 **Abstract**

16 Plants possess specific signaling pathways, such as the MultiStep Phosphorelay  
17 (MSP), which is involved in cytokinin and ethylene sensing, and light, drought or osmotic  
18 stress sensing. These MSP comprise histidine-aspartate kinases (HKs) as receptors, histidine  
19 phosphotransfer (HPTs) proteins acting as phosphorelay proteins, and response regulators  
20 (RRs), some of which act as transcription factors (type-B RRs). In previous studies, we  
21 identified partners of the poplar osmosensing signaling pathway, composed of two HKs, three  
22 main HPTs, and six type-B RRs. To date, it is unresolved as to how cytokinin or osmotic stress  
23 signal specificity is achieved in the MSP in order to generate specific responses. Here, we  
24 present a large-scale interaction study of poplar type-B RR dimerization. Using the two-  
25 hybrid assay, we were able to show the homodimerization of type-B RRs, the  
26 heterodimerization of duplicated type-B RRs, and surprisingly, a lack of interaction between  
27 some type-B RRs belonging to different duplicates. The lack of interaction of the duplicates  
28 RR12-14 and RR18-19, which are involved in the osmosensing pathway has been confirmed  
29 by BiFC experiments. This study reveals, for the first time, an overview of type-B RR  
30 dimerization in poplar and makes way for the hypothesis that signal specificity for cytokinin  
31 or osmotic stress could be in part due to the fact that it is impossible for specific type-B RRs  
32 to heterodimerize.

33  
34 **Keywords** : MultiStep Phosphorelay, type-B Response Regulators, *Populus*, Dimerization

35

## 36 **1 Introduction\***

37

38 All organisms interact with their environment, and any modification to this  
39 environment must be perceived in order to allow them to adapt to changes, and therefore  
40 ultimately for them to survive. Bacteria, archaea, fungi, and plants share a common group of  
41 signaling proteins. These proteins are involved in a wide range of fundamental developmental  
42 processes and are able to sense environmental changes, thereby inducing gene regulations  
43 leading to adaptive response. The prototypical bacterial signaling pathway, or the Two-  
44 Component System (TCS) comprises a Histidine Kinase (HK) sensor protein characterized by  
45 a Transmitter Domain (TD), and its cognate response regulator (RR) which contains a  
46 Receiver Domain (RD) [2]. A variant of this TCS exists in bacteria and is called a MultiStep  
47 Phosphorelay (MSP). This pathway is initiated by a hybrid type HK with an RD fused to its  
48 C-terminus end. These proteins act as homodimers and autophosphorylate by inter- or intra-  
49 molecular reactions, and the phosphate residue is transferred by the TD to the RD. The  
50 phosphoryl group is then shuttled to a Histidine Phosphotransfer (Hpt) protein, and from there  
51 to a terminal RR [10,2]. In plants, the MSP signaling pathway is involved in phytohormone  
52 signal transduction, such as cytokinin and ethylene signaling [34,47], red light responses *via*  
53 phytochrome receptors and in osmosensing *via* osmosensors [2,58,59]. There are four types of  
54 RRs in plants: type-A, type-B, type-C and pseudo RRs. Type-C RRs present a similar  
55 structure to the type-A RRs, with a receiver domain, but their gene expression is not induced  
56 by cytokinins, unlike type-A RRs. Type-A and -B RRs are both involved in the cytokinin  
57 signaling pathway. The last group is composed of pseudo-RRs which lack the canonical  
58 histidine phosphorylation site in their RD and are involved in circadian rhythm [40,24].

59 Out of all these RRs, type-B RRs are clearly characterized as transcription factors due to the  
60 presence of a DNA-binding domain and a transactivation sequence in the carboxy-terminal  
61 end. They are widely involved in the transcriptional regulation of cytokinin-dependent genes,  
62 and control intrinsic developmental plant programs, or responses to abiotic stresses, and biotic  
63 interactions involving cytokinins [31,32,62]. For instance, in *Arabidopsis*, ARR2 is known to  
64 be involved in shoot development [11], and ARR1 and ARR12 in cell division and  
65 differentiation of the root meristem [12,43]. However, their possible involvement in  
66 osmosensing and the transcriptional control of osmotic-responsive genes have not yet been  
67 addressed.

68           These transcription factors are found in all organisms, from unicellular green algae  
69 such as *Ostreococcus tauri* and *Chlamydomonas reinhardtii*, to higher plants [48]. They are  
70 composed of an RD characterized by the conserved D-D-K motif and a large C-terminal  
71 extension including a Myb-like DNA-binding domain, called the GARP domain or B motif,  
72 followed by a glutamine-rich transactivation domain [51,52,28].

73           In the plant cytokinin signaling pathway, these transcription factors regulate the  
74 phytohormone signal through the RD, since this domain is thought to inhibit the DNA-  
75 binding activity of the GARP domain in the absence of the phosphorylated Aspartate residue  
76 associated with cytokinin signaling [52,53,34,56,20,50,36]. HPt proteins phosphorylate an  
77 Aspartate conserved residue in their RD of the transcription factor, which leads to its  
78 activation and transactivation capacity.

79           In bacteria, almost all RRs are able to homodimerize through the RD (RR<sub>RD</sub>), and are  
80 characterized by a structure composed of five  $\alpha$  helix and five  $\beta$  sheets [57,17,39,10,5,64]. In  
81 these prokaryotic organisms, RR dimerization is known to promote DNA binding and  
82 transcription activation. Only homo-dimerization has been selected during evolution, as no  
83 evidence of physiologically relevant heterodimerization was observed [10]. Furthermore, RR  
84 homo-dimerization is typically accompanied by the RRs having an increased affinity for  
85 specific DNA-binding motifs and then typically required for output generation [64].

86           In plants, the crystal structure of three HK RDs (HK<sub>RDs</sub>), namely AHK5<sub>RD</sub>, ETR1<sub>RD</sub>  
87 and CKII<sub>RD</sub>, have been determined [4,44,45]. These data revealed a high level of structural  
88 conservation between plant HK<sub>RDs</sub> and bacterial RR<sub>RDs</sub>, with dimerization interfaces  
89 characterized by the  $\alpha 4$ - $\beta 5$ - $\alpha 5$  region [18,46]. Other studies reported HK and HPt  
90 dimerization in *Arabidopsis* and in rice [13,14,21,49,63,54]. In poplar the two HK1  
91 osmosensors (HK1a and HK1b) linked to drought perception were shown to be able to  
92 homodimerize [26,7,27]. However, only a few studies concerning RR dimerization have been  
93 conducted, due to the strong auto-transactivation capacity of the type-B RRs in the yeast two-  
94 hybrid system (except for the homo-dimerization of ARR14, ARR18 and heterodimerization  
95 between ARR14 and ARR2 [13,30]). While RR homo-dimerization process is well  
96 established in bacteria, similar data in plants are missing.

97  
98           Among the 11 type-B RRs of *Populus trichocarpa* genome, we previously isolated  
99 nine RRs (RR12-RR16, RR18-19, RR21-22) from *Populus deltoides* (Bartr.) Marsh x *P.*  
100 *nigra* L. Dorskamp genotype and determined that RR12-14, RR16, and RR18-19 could be  
101 involved in the osmosensing signaling pathway controlled by HK1 osmosensors [6,7].

102 In the present work, our aim was to provide an overview of homo- and  
103 heterodimerization of these type-B RRs in poplar. We studied protein-protein interactions for  
104 all type-B RRs isolated from this plant model by performing yeast two-hybrid (Y2H) assays.  
105 In the case of a lack of interactions being observed with this technique, we studied these  
106 potential interactions using Bimolecular Fluorescence Complementation (BiFC) assay in plant  
107 cells. When combined, our results showed homo-dimerization for all type-B RR proteins. As  
108 type-B RRs are duplicated genes, the heterodimerization within each corresponding protein  
109 pair (intra-pairs) was also observed. On the other hand, heterodimerization of RRs belonging  
110 to different pairs (inter-pairs) was not observed systematically, revealing that some duplicated  
111 gene products were unable to interact. This study is the first to give a full overview of plant  
112 type-B RR dimerization and leads us to hypothesize that heterodimerization, at least in plants,  
113 could be a way of modulating the signal specificity induced by the HK sensor.

114

## 115 **2 Material and Methods**

116

### 117 **2.1 Yeast two-hybrid (Y2H) assays**

118

119 The Y2H assays were performed using a LexA DNA-binding domain encoding bait  
120 vector (pBTM116 referred as pLex) and a Gal4 activation domain encoding prey vector  
121 (pGADT7, Clontech). DDK domains from each B-type RR were cloned into the pLex and  
122 pGAD vectors as *XmaI-SalI* and *XmaI-XhoI* fragments respectively. The yeast strain L40Δ  
123 (MATa *ade2-101 his3-200 leu2-3,112 trp1-901 ura3-52 LYS2::(lexA op)x4-HIS3 URA3::*  
124 *(lexA op)x8-lacZ gal4Δ*) and Y187 (MATα *gal4Δ gal80Δ ade2-101 his3-200 leu2-3,-112 trp1-901*  
125 *ura3-52 URA3::GAL1UAS-lacZ met-*) was used for transformations with pLex and pGAD  
126 constructions respectively, according to the lithium acetate method [19]. Transformed yeasts  
127 were selected onto a tryptophan (-W) or leucine (-L) lacking medium for 3 to 4 days at 30°C.  
128 MATa and MATα yeasts were then mated and resulting diploids were selected onto -LW  
129 medium. For each diploid strain, an overnight cell culture with Optical Density wavelength of  
130 600 nm (OD<sub>600</sub>) of 0.2 and three dilutions (1:10, 1:100 and 1:1000) were prepared. Five  
131 microliters of each cell suspension were dropped onto -LW control medium and -LW -His (-  
132 LWH) interaction selective medium. Yeast cells grew for three days at 30°C for the growth  
133 test and were then tested for LacZ activity. X-Gal tests were performed according to the  
134 overlay method derived from Fromont-Racine [16] by applying 10 mL of X-Gal mixture  
135 (0.5% agar, 0.25 M phosphate buffer, 0.1% SDS, 0.04% X-Gal) onto -LWH plates. The blue

136 color was estimated after 5 hours of incubation at 30°C. Thus, all interactions were tested  
137 using two reporter genes (*HIS3* and *LacZ*) and at least twice with 8 positive yeast clones.  
138 Negative controls were performed using mating between each MATa (pLexDDK) and MATα  
139 (pGADØ) yeast.

140

141

## 142 2.2 Bimolecular Fluorescence Complementation (BiFC) assays

143

144 BiFC assays were conducted using the pSPYCE(MR) [61] and pSPYNE(R)173  
145 plasmids (23) which allow the expression of a protein fused to the C- or N-terminal of the  
146 split-yellow fluorescent protein (YFP) fragments, respectively. The full length coding  
147 sequences from RR-12, -14, -18 and -21 were cloned as *SalI-KpnI* fragments and those from  
148 RR-13, -19 as *SpeI-SpeI* fragments. Each RR was cloned in frame with the N-terminal  
149 fragment (pSPYNE(R)173) and the C-terminal fragment (pSPYCE(MR)) of YFP. Transient  
150 transformation of *Catharanthus roseus* cells through particle bombardment and YFP imaging  
151 were performed according to Guirimand [22] with adaptation for BiFC assays [23].

152

## 153 3 Results

154

### 155 3.1 Dimerization screen of type-B RRs from Dorskamp poplar genotype

156

157 A Y2H system was used to study interactions between the nine isolated type-B RRs  
158 from the Dorskamp poplar genotype (Figure 1). Yeast growth observed on the non-selective  
159 medium (-LW) indicates the success of the cell co-transformation in all tested conditions. For  
160 all pLex-DDK-RRs tested with pGAD empty vector as a negative control, no auto-activation  
161 was observed. On interactive-selective medium (-LWH), the analysis showed homo-  
162 dimerization of the nine tested type-B RRs, revealed by the activation of at least one reporter  
163 gene. Except for yeast expressing RR15, yeast growth on -LWH medium revealed strong  
164 interactions for all homo-dimerizations.

165 With the exception of RR22, RR genes are duplicated in the poplar genome, thus  
166 constituting pairs of RRs (Fig S1). The Y2H study revealed interactions between all type-B  
167 RRs from intra-pair genes studied (RR12/RR13, RR14/RR21, RR15/RR16, RR18/RR19).  
168 Interactions were also detected between RRs from inter-pair genes, although each RR within a  
169 pair did not show the same interaction pattern. For example, within the RR12/RR13 pair,

170 RR12 interacted strongly with RR15 and RR16, whereas RR13 only interacted strongly with  
171 RR16. In the same way, within the RR14/RR21 pair, only RR14 was able to interact with  
172 RR15 and RR16. More interestingly, a complete lack of interaction was observed between  
173 some inter-pair RRs such as between RR12/RR13 and RR14/RR21, and also between  
174 RR18/RR19 and RR14/RR21, whatever the configuration tested (prey or bait configuration  
175 for all RRs tested). Moreover, among the six type-B RRs identified as partners in the poplar  
176 osmosensing pathway (RR12, RR13, RR14, RR16, RR18 and RR19), only RR16 was able to  
177 interact with all five of the other RRs since a clear lack of interaction was observed in some  
178 cases. All data are summarized in table S1.

179

### 180 **3.2 Validation of the interaction screen of osmosensing type-B RRs by a BiFC analysis**

181

182 Due to a lack of interaction between some RRs belonging to different pairs involved in  
183 the poplar osmosensing pathway (RR12/RR13:RR14 and RR18/RR19:RR14) being observed  
184 in the Y2H analysis, a BiFC analysis was conducted in order to validate this specific RR's  
185 behavior. Firstly, as previously observed by the Y2H assay, the homo-dimerization of RR12,  
186 13, 18 and 19 was validated using the BiFC approach (Figure 2) but not for RR14 (Fig S2).  
187 Similarly, the heterodimerization for the intra-pairs, RR12-RR13 and RR18-RR19 was  
188 confirmed (Figure 3). Interestingly, using this BiFC approach, we detected an interaction  
189 between RR13/RR18 and RR14/RR12 which was not previously observed in the Y2H assay  
190 (Figure 4, Figure 5A). Finally, the complete lack of interaction shown by the Y2H assay was  
191 confirmed using the BiFC approach between RR14 and the RR18-RR19 pair (Figure 5I and  
192 5M), as well as between RR14 and RR13 (Figure 5E). All these data allowed us to draw up a  
193 dimerization map of poplar type-B RRs (Figure 6).

194

## 195 **4 Discussion**

196

197 In Arabidopsis, the dimerization of HKs has been reported for cytokinin receptors  
198 (AHK2, 3, 4 and 5), CKI1, the ethylene receptor (ETR1), and for AHK1 [13,25,9,44,45,4].  
199 Expressed in *Nicotiana benthamiana*, AHP1 to 3 are also able to homodimerize [37]. With the  
200 exception of the homo-dimerization of ARR14, ARR18 and the heterodimerization between  
201 ARR14 and ARR2, only scarce data are available concerning RR dimerization in plants  
202 [13,42,30]. Recently, in bacteria, it has been shown that heterodimerization could be used to  
203 integrate signals from two distinct developmental pathways [1]. Such a system using RR

204 homo- or hetero-dimerization could also be used in plants as part of the response specificity to  
205 abiotic stresses.

206 In plants, HKs are signal specific receptors, with AHK2 to 5 involved in cytokinin  
207 signaling, ETR1 in ethylene signaling and AHK1 in putative osmosensing in dehydration  
208 avoidance and low water-potential responses [58]. However, using the same HPT and RR  
209 proteins as partners, the question of signal specificity conservation is raised. In poplar, the  
210 lack of interactions between osmosensors HK1, and cytokinin receptors HK2 to 4, suggests  
211 that interconnection between the two signaling pathways may not be mediated by direct HK  
212 hetero-dimerization [27]. Moreover, some HPT proteins have been shown to be specific to  
213 cytokinin signaling or common to both osmotic stress and cytokinin signaling [27]. These  
214 results suggest that some HPT proteins could participate in the preservation of the signal  
215 specificity. In this case, type-B RRs must also participate in this preservation *via* homo-  
216 and/or hetero-dimerization. It seems that a deeper understanding on how MSP elements  
217 oligomerize would allow us to determine the functional importance of homo- and hetero-  
218 dimerization in plant MSP signaling regulation.

219 In the Dorskamp poplar genotype, we previously isolated nine type-B RRs, RR12-16,  
220 18-19, 21 and 22. Based on interaction studies between these RRs, and HPT protein partners of  
221 the HK1 receptor, and the co-expression analysis of transcripts of these potential partners in  
222 poplar organs, only six of them (RR12-14, 16, 18-19) have been retained as potentially  
223 involved in drought response [7,8]. In this work, we showed the homo-dimerization for all  
224 type-B RRs and the heterodimerization in each RR pair. However, surprisingly, among the six  
225 type-B RRs belonging to the HK1 pathway, some RRs are unable to interact with each other,  
226 such as RR18-19 with RR14. As reported for interaction study between R and GL3 ACT  
227 domains by Y2H assay, it is difficult to assess interactions with *Kd* values above 400 nM [35].  
228 In order to assess the robustness of the Y2H results for RR14, we used a second *in vivo*  
229 method, the BiFC assay, to study protein-protein interactions (PPIs). Then we showed a weak  
230 interaction between RR12 and RR14 but observed once again a lack of interaction between  
231 RR13, RR18 and RR19 with RR14. The use of this second method combined with that of the  
232 Y2H makes it possible to reinforce the idea of an absence of interaction between RR14 and  
233 the three other RR13, 18 and 19. Consequently, RR14 is the least interactive RR from this  
234 group of six RRs, as it only interacts with RR12 and RR16. In Arabidopsis, ARR11,  
235 homologous to the poplar RR14, is a positive transcriptional regulator of the cytokinin  
236 signaling pathway [53,34,56] and is involved in biological processes controlled by cytokinins,  
237 such as root stem cell maintenance [38].

238           Recently, it has been shown that ARR11 was able to interact with the Sucrose  
239 nonfermenting1-related kinase 2.6 (SnRK2.6), a key kinase of the ABA signaling pathway  
240 which is also involved in stomata closure and regulation of ABA-responsive genes leading to  
241 drought tolerance. It has been shown that this interaction repressed the kinase activity of  
242 SnRK2.6. Finally, ARR11 is a negative regulator of drought stress tolerance as it inhibits  
243 SnRK2.6. However, it is a positive regulator of plant growth development induced by  
244 cytokinins in normal conditions [29]. At the same time, it has been shown that ARR18,  
245 homologous to the poplar RR18, is able to interact with the transcription factor bZIP63, which  
246 is a negative regulator of seed germination upon osmotic stress. This interaction interfered  
247 negatively with the transcriptional activity of bZIP63 on the PDH1 promoter. This study has  
248 led to the conclusion that ARR18 is a positive osmotic stress RR in Arabidopsis seeds [60].  
249 Interestingly, in Arabidopsis, ARR11 and ARR18 are negative and positive regulators of the  
250 drought stress response, respectively. In poplar, RR14 and RR18-19 (homologous to ARR11  
251 and ARR18 respectively) are unable to interact with each other. The biological function of  
252 these poplar RRs has not yet been determined. However, RR18 could only be isolated in  
253 drought stressed leaves and is up-regulated by osmotic stress [8].

254           Altogether, the specificity in transcription factor dimerization could be an efficient  
255 mechanism for avoiding unwanted crosstalk between pathways. Indeed, transcription factors  
256 of signaling pathways involved in antagonistic developmental processes, such as growth  
257 maintenance versus drought tolerance could have the same spatio-temporal expression  
258 pattern, and in this case the dimer specificity could avoid unwanted signaling crosstalk and  
259 maintain specific responses [3, 55]. The lack of interaction observed in our study between  
260 RR14 and RR18-19 could participate in the maintenance of this specificity. Indeed, poplar  
261 RR18 and probably also RR19 could be involved in drought tolerance in the same way as  
262 their Arabidopsis counterpart ARR18, and poplar RR14 could be involved in the cytokinin  
263 signaling pathway and plant growth development in the same way as its Arabidopsis  
264 counterpart ARR11. In this case, the lack of interaction between them could be particularly  
265 important in order to participate specifically in the trade-off between plant growth and stress  
266 tolerance. Neofunctionalization, and the forming of heterodimers may be considered as an  
267 example of subfunctionalization [63].

268  
269 Drought stress is one of the environmental stresses which has the most impact on plants. To  
270 cope with water-deficit conditions during the current global warming, plants will have to  
271 adapt to increasing drought stress events by regulating a variety of physiological processes. In

272 this context, the efficiency of the signaling pathway involved in the trade-off between plant  
273 growth and stress adaptation will play a crucial role. The maintenance of specificity in  
274 signaling pathways (including the cytokinin pathway) is a key point in this balance. In our  
275 study, although the homo- and heterodimerization of the paralogs of type-B RRs were  
276 expected, the lack of interaction between some specific type-B RRs inter-pairs was not. It is  
277 now well known that gene duplication and divergence is a major mechanism of the  
278 evolutionary process by which new proteins and functions emerge in biology, and provide  
279 cells with diverse functions. However, regulatory complexity and additional specificity can  
280 arise through other mechanisms, such as through homodimeric or heterodimeric interactions  
281 [41]. To establish new and insulated pathways, substitutions that eliminate the  
282 heterodimerization of the diverging paralogous proteins, while maintaining homodimerization  
283 are needed [10]. Our study suggests that the specificity conservation could be due, in part, to  
284 the fact that it is impossible for specific type-B RRs to heterodimerize. However, this  
285 hypothesis needs to be further validated. As has been done for bacteria, a global study of  
286 conserved residues at the dimerization interface characterized by the  $\alpha 4$ – $\beta 5$ – $\alpha 5$  region would  
287 help us to better understand how plants could maintain a specific response to cytokinin or  
288 drought stress using such a signaling pathway.

289

290 **Funding:** This research was funded by the Conseil Régional du Centre-Val de Loire  
291 (SiSCyLi project, 2013-00085478).

292

293 **Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in  
294 the design of the study; in the collection, analyses, or interpretation of data; in the writing of  
295 the manuscript, or in the decision to publish the results.

296

297 **Acknowledgement:** We thank Ms Deki Fourcin from “Infinity English” for manuscript  
298 reviewing.

299

## 300 **References**

301

302 [1] M.M. Al-Bassam, M.J. Bibb, M.J. Bush, G. Chandra, M.J. Buttner, Response Regulator  
303 Heterodimer Formation Controls a Key Stage in Streptomyces Development, PLoS Genet.  
304 10(8), (2014), e1004554.

305

- 306 [2] A.F. Alvarez, C. Barba-Ostria, H. Silva-Jimenez, and D. Georgellis, Organization and  
307 mode of action of two component system signaling circuits from the various kingdoms of life,  
308 Environ. Microbiol. 18, (2016), 3210-3226.  
309
- 310 [3] G.D. Amoutzias, D.L. Robertson, Y. Van de Peer and S. G. Oliver, Choose your partners:  
311 dimerization in eukaryotic transcription factors, Trends Biochem. Sci. 33, (5), (2008), 220-  
312 229.  
313
- 314 [4] J. Bauer, K. Reiss, M. Veerabagu, M. Heunemann, K. Harter, T. Stehle, Structure-  
315 function analysis of *Arabidopsis thaliana* Histidine Kinase AHK5 bound to its cognate  
316 phosphotransfer protein AHP1, Mol. Plant 6, (2013), 959-970.  
317
- 318 [5] C.M. Barbieri, T. Wu, A.M. Stock, Comprehensive analysis of OmpR phosphorylation,  
319 dimerization, and DNA binding supports a canonical model for activation, J. Mol. Biol.  
320 27;425 (10), (2013), 1612-1626.  
321
- 322 [6] L. Bertheau, F. Cheddor, G. Guirimand, V. Courdavault, C. Depierreux, D. Morabito, F.  
323 Brignolas, F. Héricourt, S. Carpin, Identification of five B-type response regulators as  
324 members of a multistep phosphorelay system interacting with histidine-containing  
325 phosphotransfer partners of *Populus* osmosensor, BMC Plant Biol. 12, (2012), 241.  
326
- 327 [7] L. Bertheau, M. Miranda, E. Foureau, L.F. Rojas Hoyos, F. Cheddor, F. Héricourt, C.  
328 Depierreux, D. Morabito, N. Papon, M. Clastre, G.S. Scippa, F. Brignolas, V. Courdavault, S.  
329 Carpin, *In planta* validation of HK1 homodimerization and recruitment of preferential HPt  
330 downstream partners involved in poplar multistep phosphorelay systems, Plant Biosyst. 147,  
331 (2013), 991-995.  
332
- 333 [8] L. Bertheau, I. Djeghdir, E. Foureau, F. Cheddor, G. Glevarec, A. Oudin, C. Depierreux,  
334 D. Morabito, F. Brignolas, V. Courdavault, F. Héricourt, D. Auguin, S. Carpin, Insights into  
335 B-type RR members as signaling partners acting downstream of HPt partners of HK1 in the  
336 osmotic stress response in *Populus*, Plant Physiol. Bioch. 94, (2015), 244-252.  
337

- 338 [9] K. Caesar, A.M.K. Thamm, J. Witthöft, K. Elgass, P. Huppenberger, C. Grefen, J. Horak,  
339 K. Harter, Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and  
340 AHK4 in the endoplasmic reticulum, *J. Exp. Bot.*, 62, (2011), 5571–5580.
- 341
- 342 [10] E.J. Capra, M.T. Laub, Evolution of two-component signal transduction systems *Annu*  
343 *Rev. Microbiol.*, 66, (2012), 325-47.
- 344
- 345 [11] P. Che, S. Lall, S.H. Howell, Acquiring competence for shoot development in  
346 Arabidopsis: ARR2 directly targets A-type ARR genes that are differentially activated by  
347 CIM preincubation, *Plant Signal. Behav.*, (2), (2008), 99-101.
- 348
- 349 [12] R. Dello Ioio, K. Nakamura, L. Moubayidin, S. Perilli, M. Taniguchi, M.T. Morita, T.  
350 Aoyama, P. Costantino, S. Sabatini, A genetic framework for the control of cell division and  
351 differentiation in the root meristem, *Science* 28, 322, (5906), (2008), 1380-4.
- 352
- 353 [13] H. Dortay, N. Mehnert, L. Bürkle, T. Schmülling, A. Heyl, Analysis of protein  
354 interactions within the cytokinin-signaling pathway of *Arabidopsis thaliana*, *FEBS J.* 273,  
355 (2006), 4631-4644.
- 356
- 357 [14] H. Dortay, N. Gruhn, A. Pfeifer, M. Schwerdtner, T. Schmülling, A. Heyl, Toward an  
358 interaction map of the two-component signaling pathway of *Arabidopsis thaliana*, *J.*  
359 *Proteome Res.* 7, (2008), 3649-3660.
- 360
- 361 [15] R.C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high  
362 throughput, *Nuc. Acids Res.* 32, (2004), 1792e1797.
- 363
- 364 [16] M. Fromont-Racine, J-C. Rain, P. Legrain, Toward a functional analysis of the yeast  
365 genome through exhaustive two-hybrid screens, *Nat. Genet.*, 16, (1997), 277-282.
- 366
- 367 [17] R. Gao and A.M. Stock, Biological insights from structures of two-component proteins,  
368 *Annu. Rev. Microbiol.*, 63, (2009), 133-54.
- 369
- 370 [18] R. Gao and A.M. Stock, Molecular strategies for phosphorylation-mediated regulation of  
371 response regulator activity, *Curr. Opin. Microbiol.* 13(2), (2010), 160-167.

372

373 [19] R.D. Gietz, R.H. Schiestl, A.R. Willems, R.A. Woods, Studies on the transformation of  
374 intact yeast cells by the LiAc/SS-DNA/PEG procedure, *Yeast* 11, (1995) 355-360.

375

376 [20] O. Ginis, A. Oudin, G. Guirimand, M. Chebbi, V. Courdavault, G. Glévarec, N. Papon, J.  
377 Crèche, M. Courtois, A type-B response regulator drives the expression of the  
378 hydroxymethylbutenyl diphosphate synthase gene in periwinkle, *J. Plant Physiol.* 169,  
379 (2012),1571-1574.

380

381 [21] C. Grefen, K. Städele, K. Ruzicka, P. Obrdlik, K. Harter, J. Horak, Subcellular  
382 localization and *in vivo* interactions of the *Arabidopsis thaliana* ethylene receptor family  
383 members, *Mol. Plant* 1, (2008), 308-320.

384

385 [22] G. Guirimand, V. Burlat, A. Oudin, A. Lanoue, B. St-Pierre, V. Courdavault,  
386 Optimization of the transient transformation of *Catharanthus roseus* cells by particle  
387 bombardment and its application to the subcellular localization of hydroxymethylbutenyl 4-  
388 diphosphate synthase and geraniol 10-hydroxylase, *Plant Cell. Rep.* 28, (2009), 1215-1234.

389

390 [23] G. Guirimand, V. Courdavault, A. Lanoue, S. Mahroug, A. Guihur, N. Blanc, ,  
391 Strictosidine activation in Apocynaceae: towards a “nuclear time bomb”?, *BMC Plant Biol.*  
392 10, (2010), 182.

393

394 [24] S.L. Harmer, The circadian system in higher plant, *Annu. Rev. Plant Biol.* 60, (2009),  
395 357-377

396

397 [25] J. Hejátko, H. Ryu, G.-T. Kim, R. Dobesová, S. Choi, S.M. Choi, P. Soucek, J. Horák, B.  
398 Pekárová, K. Palme, B. Brzobohaty, I. Hwang, The histidine kinases CYTOKININ-  
399 INDEPENDENT1 and ARABIDOPSIS HISTIDINE KINASE2 and 3 regulate vascular tissue  
400 development in *Arabidopsis* shoots, *Plant Cell* 21, (2009), 2008-2021.

401

402 [26] F. Héricourt, F. Chefdor, L. Bertheau, M. Tanigawa, T. Maeda, G. Guirimand, V.  
403 Courdavault, M. Larcher, C. Depierreux, H. Bénédicti, D. Morabito, F. Brignolas, S. Carpin,  
404 Characterization of histidine-aspartate kinase HK1 and identification of histidine

405 phosphotransfer proteins as potential partners in a *Populus* multistep phosphorelay, *Physiol.*  
406 *Plant.* 149, (2013),188-199.

407

408 [27] F. Héricourt, M. Larcher, F. Cheddor, K. Koudounas, I. Carqueijeiro, P. Lemos Cruz, V.  
409 Courdavault, M. Tanigawa, T. Maeda, C. Depierreux, F. Lamblin, G. Glévarec, S. Carpin,  
410 New Insight into HPTs as Hubs in Poplar Cytokinin and Osmosensing Multistep  
411 Phosphorelays: Cytokinin Pathway Uses Specific HPTs, *Plants* 11;8(12), (2019), 591.

412

413 [28] K. Hosoda, A. Imamura, E. Katoh, T. Hatta, M. Tachiki, H. Yamada, Molecular structure  
414 of the GARP family of plant Myb-Related DNA binding motifs of the Arabidopsis response  
415 regulators, *Plant Cell.* 14, (2002), 2015-2029.

416

417 [29] X. Huang, L. Hou, J. Meng, H. You, Z. Li, Z. Gong, S. Yang, Y. Shi, The Antagonistic  
418 Action of Abscisic Acid and Cytokinin Signaling Mediates Drought Stress Response in  
419 Arabidopsis, *Mol. Plant.* 11, (2018), 970-982.

420

421 [30] R. Huo, Z. Liu, X. Yu, Z. Li, The Interaction Network and Signaling Specificity of Two-  
422 Component System in Arabidopsis, *Int. J. Mol. Sci.* 21, (2020), 4898.

423

424 [31] I. Hwang, H.C. Chen, J. Sheen, Two-component signal transduction pathways in  
425 Arabidopsis, *Plant Physiol.* 129, (2002), 500-515.

426

427 [32] I. Hwang, J. Sheen, B. Müller, Cytokinin Signaling Networks, *Annu. Rev. Plant Biol.* 63,  
428 (2012), 353-380.

429

430 [33] A. Imamura, T. Kiba, Y. Tajima, T. Yamashino, Mizuno, *In vivo* and *in vitro*  
431 characterization of the ARR11 response regulator implicated in the His-to-Asp phosphorelay  
432 signal transduction in *Arabidopsis thaliana*, *Plant Cell Physiol.* 44, (2003), 122-131.

433

434 [34] J.J. Kieber and G.E. Schaller, Cytokinin signaling in plant development, *Development*  
435 27, 145 (4), (2018), dev149344.

436

437 [35] Y. S. Lee, A. Herrera-Tequia, J. Silwal, J. H. Geiger, E. Grotewold, A hydrophobic  
438 residue stabilizes dimers of regulatory ACT-like domains in plant basic helix–loop–helix  
439 transcription factors, *J. Biol. Chem.* 296, (2021), 100708.

440

441 [36] Y. Liang, X. Wang, S. Hong, Y. Li, J. Zuo, Deletion of the initial 45 residues of ARR18  
442 induces cytokinin response in *Arabidopsis*, *J. Genet. Genom.* 39, (2012), 37-46.

443

444 [37] S.N. Lomin, Y.A. Myakushina, D.V. Arkhipov, O.G. Leonova, V.I. Popenko, T.  
445 Schmülling, G.A. Romanov, Studies of cytokinin receptor-phosphotransmitter interaction  
446 provide evidences for the initiation of cytokinin signalling in the endoplasmic reticulum,  
447 *Func. Plant Biol.* 45, (2017), 192-202.

448

449 [38] B. Lv, K. Hu, T. Tian, K. Wei, F. Zhang, Y. Jia, H. Tian, Z. Ding, The pre-mRNA  
450 splicing factor RDM16 regulates root stem cell maintenance in *Arabidopsis*, *J. Integr. Plant*  
451 *Biol.* 63, (2021), 662- 675.

452

453 [39] T.R. Mack, R. Gao, A.M. Stock, Probing the roles of the two different dimers mediated  
454 by the receiver domain of the response regulator PhoB, *J. Mol. Biol.* 389, (2009) 349-364.

455

456 [40] S. Makino, T. Kiba, A. Imamura, N. Hanaki, A. Nakamura, T. Suzuki, M. Taniguchi, C.  
457 Ueguchi, T. Sugiyama, T. Mizuno, Genes Encoding Pseudo-Response Regulators: Insight  
458 into His-to-Asp Phosphorelay and Circadian Rhythm in *Arabidopsis thaliana*, *Plant Cell*  
459 *Physiol.* 41, (6), (2000), 791-803

460

461 [41] C.J. McClune, M.T. Laub, Constraints on the expansion of paralogous protein families,  
462 *Curr. Biol.*, 30, (2020), 460-464.

463

464 [42] V. Mira-Rodado, New Insights into Multistep-Phosphorelay (MSP)/Two-Component  
465 System (TCS) Regulation: Are Plants and Bacteria That Different?, *Plants* 8, 12, (2019), 590.  
466

467 [43] L. Moubayidin, S. Perilli, R. Dello Ioio, R. Di Mambro, P. Costantino, S. Sabatini, The  
468 rate of cell differentiation controls the Arabidopsis root meristem growth phase. *Curr. Biol.*  
469 20, (2010), 1138-1143.  
470

471 [44] H.-J. Müller-Dieckmann, A.A. Grantz, S.-H. Kim, The structure of the signal receiver  
472 domain of the *Arabidopsis thaliana* ethylene receptor ETR1, *Structure* 7, (1999), 1547-1556.  
473

474 [45] B. Pekárová, T. Klumpler, O. Třísková, J. Horák, S. Jansen, R. Dopitová, P. Borkovcová,  
475 V. Papoušková, E. Nejedlá, V. Sklenář, J. Marek, L. Žídek, J. Hejátko, L. Janda, Structure and  
476 binding specificity of the receiver domain of sensor histidine kinase CKII from *Arabidopsis*  
477 *thaliana*, *Plant J.* 67, (2011), 827-839.  
478

479 [46] B. Pekarova, A. Szmitkowska, R. Dopitova', O. Degtjarik, L. Zidek, J. Hejatko,  
480 Structural Aspects of Multistep Phosphorelay-Mediated Signaling in Plants, *Mol. Plant.* 9,  
481 (2016), 71-85.  
482

483 [47] B. Pekarova, A. Szmitkowska, J. Houser, M. Wimmerova, J. Hejátko, Cytokinin and  
484 Ethylene Signaling. In: Hejátko J., Hakoshima T. (eds) *Plant Structural Biology: Hormonal*  
485 *Regulations*. Springer, Cham. (2018), 165-200.  
486

487 [48] B. Pils, A. Heyl, Unraveling the evolution of cytokinin signaling, *Plant Physiol.* 151,  
488 (2009), 782-791.  
489

490 [49] J.A. Punwani, J.J. Kieber, Localization of the Arabidopsis histidine phosphotransfer  
491 proteins is independent of cytokinin, *Plant Signal. Behav.* 5, (2010) 896-898.  
492

493 [50] G.A. Ramírez-Carvajal, A.M. Morse, C. Dervinis, J.M. Davis, The Cytokinin Type-B  
494 Response Regulator PtRR13 Is a Negative Regulator of Adventitious Root Development in  
495 *Populus*, *Plant Physiol.* 150, (2), (2009), 759-771  
496

497 [51] J.L. Riechmann, J. Heard, G. Martin, L. Reuber, C.Z. Jiang, J. Keddie, L. Adam, O.  
498 Pineda, O.J. Ratcliffe, R.R. Samaha, R. Creelman, M. Pilgrim, P. Broun, J.Z. Zhang, D.  
499 Ghandehari, B.K. Sherman, G-L. Yu, Arabidopsis transcription factors: Genome-wide  
500 comparative analysis among eukaryotes, Science 290, (2000), 2105-2110.  
501

502 [52] H.Sakai, T. Aoyama, A. Oka, Arabidopsis ARR1 and ARR2 response regulators operate  
503 as transcriptional activators, Plant J. 24, (2000), 703-711.  
504

505 [53] H. Sakai, T. Honma, T. Aoyama, S. Sato, T. Kato, S. Tabata, A. Oka, ARR1, a  
506 transcription factor for genes immediately responsive to cytokinins, Science 294, (2001),  
507 1519-1521.  
508

509 [54] A. Sharan, P. Soni, R.C. Nongpiur, S. L. Singla-Pareek, A. Pareek, Mapping the ‘Two-component  
510 system’ network in rice, Sci. Rep. 7, (2017) 9287.  
511

512 [55] J. Skalak, K.L. Nicolas, R. Vankova, J. Hejatko, Signal Integration in Plant Abiotic  
513 Stress Responses via Multistep Phosphorelay Signaling, Front. Plant Sci. 12, (2021), 644823.  
514

515 [56] Y. Tajima, A. Imamura, T. Kiba, Y. Amano, T. Yamashino, T. Mizuno, Comparative  
516 Studies on the Type-B Response Regulators Revealing their Distinctive Properties in the His-  
517 to-Asp Phosphorelay Signal Transduction of *Arabidopsis thaliana*, Plant Cell Physiol. 45, (1),  
518 (2004), 28-39.  
519

520 [57] A. Toro-Roman, T.R. Mack, A.M. Stock, Structural Analysis and Solution Studies of  
521 the Activated Regulatory Domain of the Response Regulator ArcA: A Symmetric Dimer  
522 Mediated by the  $\alpha 4$ - $\beta 5$ - $\alpha 5$  Face, J. Mol. Biol. (2005), 349, 11-26.  
523

524 [58] L-S.P. Tran, T. Urao, F. Qin, K. Maruyama, T. Kakimoto, K. Shinozaki, K. Yamaguchi-  
525 Shinozaki, Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in  
526 response to abscisic acid, drought, and salt stress in Arabidopsis, Proc. Natl. Acad. Sci. 104,  
527 (2007), 20623-20628.  
528

529 [59] T. Urao, B. Yakubov, R. Satoh, K. Yamaguchi-Shinozaki, M. Seki, T. Hirayama, A  
530 transmembrane hybrid-type Histidine Kinase in Arabidopsis functions as an osmosensor,  
531 Plant Cell 11, (1999), 1743-1754.  
532

533 [60] M. Veerabagu, T. Kirchler, K. Elgass, B. Stadelhofer, M. Stahl, K. Harter, V. Mira-  
534 Rodado, C. Chaban, The interaction of the Arabidopsis response regulator ARR18 with  
535 bZIP63 mediates the regulation of PROLINE DEHYDROGENASE expression, Mol. Plant 7,  
536 (2014), 1560-1577.  
537

538 [61] R. Waadt, L.K. Schmidt, M Lohse, K. Hashimoto, R. Bock, J. Kudla, Multicolor  
539 bimolecular fluorescence complementation reveals simultaneous formation of alternative  
540 CBL/CIPK complexes *in planta*, Plant J. 56, (2008), 505-516.  
541

542 [62] T. Werner, T. Schmülling, Cytokinin action in plant development, Curr. Opin. Plant Biol.  
543 12, (2009), 527-538.  
544

545 [63] K. Wulfetange, S.N. Lomin, G.A. Romanov, A. Stolz, A. Heyl, and T. Schmülling, The  
546 cytokinin receptors of Arabidopsis are located mainly to the endoplasmic reticulum, Plant  
547 Physiol. 156, (2011), 1808-1818.  
548

549 [64] C.P. Zschiedrich, V. Keidel, H. Szurmant, Molecular Mechanisms of Two-Component  
550 Signal Transduction, J. Mol. Biol. 428, (19), (2016), 3752-3775.  
551

552 **Figure legends:**

553

554 **Figure 1:** Dimerization study of type-B RRs in a yeast two-hybrid system. Overnight cultures  
555 of yeast co-transformed with pLex-DDK and pGAD-DDK for each RRs were adjusted to an  
556 Optical Density at wavelength of 600 nm (OD<sub>600</sub>) of 0.2 and three dilutions (1:10, 1:100 and  
557 1:1000) were spotted onto -LWH medium and grown during three days at 30°C for growth  
558 test and were then tested for LacZ activity (X-Gal test).

559

560 **Figure 2 :** Analysis of homo-dimerization of RR12, 13, 18 and 19 in *C. roseus* cells using  
561 BiFC assays. *C. roseus* cells were co-transformed with plasmids expressing YFP<sup>N</sup>-RRs and  
562 YFP<sup>C</sup>-RRs (A, E, I, M). An additional co-transformation with the CFP nuclear marker (B, F,  
563 J, N) confirms the co-localization of the two fluorescence signals (C, G, K, O). The  
564 morphology was observed by differential interference contrast (DIC) microscopy (D, H, L, P).  
565 Scale bar = 10 μm.

566

567 **Figure 3:** Hetero-dimerization analysis for the intra-pairs RR12-RR13 and RR18-RR19 in *C.*  
568 *roseus* cells using BiFC assays. *C. roseus* cells were co-transformed with plasmids expressing  
569 YFP<sup>N</sup>-RRs and YFP<sup>C</sup>-RRs (A, E). An additional co-transformation with the CFP nuclear  
570 marker (B, F) confirms the co-localization of the two fluorescence signals (C, G). The  
571 morphology was observed by differential interference contrast (DIC) microscopy (D, H).  
572 Scale bar = 10 μm.

573

574 **Figure 4:** Hetero-dimerization analysis for the intra-pairs RR12-RR13 and RR18-RR19 in *C.*  
575 *roseus* cells using BiFC assays. *C. roseus* cells were co-transformed with plasmids expressing  
576 YFP<sup>N</sup>-RRs and YFP<sup>C</sup>-RRs (A, E, I, M). An additional co-transformation with the CFP nuclear  
577 marker (B, F, J, N) confirms the co-localization of the two fluorescence signals (C, G, K, O).  
578 The morphology was observed by differential interference contrast (DIC) microscopy (D, H,  
579 L, P). Scale bar = 10 μm.

580

581 **Figure 5:** Hetero-dimerization analysis for RR14 and the two intra-pairs RR12-RR13 and  
582 RR18-RR19 in *C. roseus* cells using BiFC assays. *C. roseus* cells were co-transformed with  
583 plasmids expressing YFP<sup>N</sup>-RR12,-RR13, -RR18, -RR19 and YFP<sup>C</sup>-RR14 (A, E, I, M). An  
584 additional co-transformation with the CFP nuclear marker (B, F, J, N) confirms the co-

585 localization of the two fluorescence signals (C, G, K, O). The morphology was observed by  
586 differential interference contrast (DIC) microscopy (D, H, L, P). Scale bar = 10  $\mu$ m.

587

588 **Figure 6:** Dimerization map of Dorskamp poplar genotype type-B RRs. Blue boxes gather  
589 duplicated RR genes. Homo-dimerization is represented by red arrows, hetero-dimerization  
590 intra-pairs are represented by solid red lines. Continuous black lines represent strong hetero-  
591 dimerization inter-pairs and dashed lines represent weak interactions observed by Y2H. The  
592 green dashed line represents a weak interaction observed only by BiFC assay.

593











