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

3

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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_{RD}), and are
80 characterized by a structure composed of five α helix and five β 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_{RDs}), namely AHK5_{RD}, ETR1_{RD}
87 and CKII_{RD}, have been determined [4,44,45]. These data revealed a high level of structural
88 conservation between plant HK_{RDs} and bacterial RR_{RDs}, 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 (OD₆₀₀) 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 MAT α (pLexDDK) and MAT α
139 (pGAD \emptyset) 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

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292

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299

300 **References**

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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 (OD₆₀₀) 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^N-RRs and
562 YFP^C-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^N-RRs and YFP^C-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^N-RRs and YFP^C-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^N-RR12,-RR13, -RR18, -RR19 and YFP^C-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 μ 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











