1 **Microtubule-associated ROP interactors both delimit and transduce ROP signaling and**

2 **regulate microtubule dynamics**

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4 **Gil Feiguelman[1], Xiankui Cui[2], Hasana Sternberg[1], Ying Fu[2,3], and Shaul Yalovsky[1*]**

5 **[1]School of Plant Sciences and Food Security, Tel Aviv University, Israel, [2]State Key**

6 **Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China**

7 **Agricultural University, China, [3]Joint Laboratory for International Cooperation in**

8 **Crop Molecular Breeding, Ministry of Education, China**

9 *Corresponding author: Shaul Yalovsky, School of Plant Sciences and Food Security, Tel

10 Aviv University, Tel Aviv 6997801, Israel; Email: shauly@tauex.tau.ac.il

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13 **Abstract**

14 Evidence suggests that ICR proteins function as adaptors that mediate ROP signaling. Here,

15 we studied the functions of ICR2 and its homologs ICR5 and ICR3. We showed that ICR2 is

16 a microtubule-associated protein that regulates microtubule dynamics. ICR2 can retrieve

17 activated ROPs from the plasma membrane, and it is recruited to a subset of ROP domains.

18 Secondary cell wall pits in the metaxylem of *icr2* and *icr5 Arabidopsis* single mutants and

19 *icr2*/*icr5* double and *icr2*/*icr5*/*icr3* triple mutants were denser and larger than those in wild-

20 type Col-0 seedlings, implicating these three ICRs in restriction of ROP function. The *icr2*

21 but not the *icr5* mutants developed split root hairs further implicating ICR2 in restriction of

22 ROP signaling. Taken together, our results show that ICR2, and likely also ICR5 and ICR3,

23 have multiple functions as ROP effectors and as regulators of microtubule dynamics.

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Introduction

27 ROP (Rho Of Plants) are the plant-specific subfamily of RHO superfamily of small G
28 proteins. ROPs function as plasma membrane-anchored molecular switches that cycle
29 between active, GTP-bound and inactive, GDP-bound states (Feiguelman et al., 2018). ROP
30 activity is spatiotemporally controlled by regulatory proteins in plasma membrane-associated
31 microdomains that contain active ROP. Guanine nucleotide Exchange Factors (GEFs) are
32 known to positively regulate the activation of ROPs by facilitating nucleotide exchange
33 (Basu et al., 2008; Berken et al., 2005; Thomas and Berken, 2010). GTPase Activating
34 Proteins (GAPs) down-regulate ROP activity, and ROPs are recycled by GDP Dissociation
35 Inhibitors (GDIs) (Boulter and Garcia-Mata, 2010; DerMardirossian and Bokoch, 2005;
36 Schaefer et al., 2011; Wu et al., 2000). In the active, GTP-bound state, ROPs interact with
37 target effector proteins to perform their biological functions (Dvorsky and Ahmadian, 2004;
38 Feiguelman et al., 2018). ROPs regulate a variety of cellular processes such as the
39 organization and dynamics of the actin and microtubule cytoskeleton, endocytosis and
40 exocytosis, and activation of NADPH oxidase and intracellular kinase cascades. ROP
41 functions impact cell growth and shape, cytokinesis, subcellular protein localization, and
42 responses to pathogens and abiotic stresses (Bloch and Yalovsky, 2013; Feiguelman et al.,
43 2018; Kawano et al., 2014; Nagawa et al., 2010; Oda and Fukuda, 2014; Rivero et al., 2017;
44 Yalovsky et al., 2008; Yang, 2008).

45 We have previously identified a family of ROP effectors that we designated "Interactors of
46 Constitutively active ROP" (ICRs) (Lavy et al., 2007). The ICRs are coiled-coil domain-
47 containing proteins that do not contain additional known structural or catalytic domains
48 (Lavy et al., 2007). The ICRs do contain two conserved sequence motifs, an N-terminal
49 QEEL and a C-terminal QWRKAA (Lavy et al., 2007). The ICRs are subdivided into two
50 clades, which differ in molecular mass. In *Arabidopsis*, ICR1 (At1g17140, 38 kDa) and ICR4
51 (At1g78430, 36 kDA) represent the lower molecular weight clade, whereas ICR2
52 (At2g37080, 65 kDa), ICR3 (At5g60210, 63 kDa), and ICR5 (At3g53350, 45 kDa) represent
53 the higher molecular weight clade.

54 Studies on ICR1 have shown that it is a microtubule-associated protein (MAP) that integrates
55 auxin and Ca^{2+} signaling. It functions as a ROP-associated scaffold that interacts with
56 specific group of proteins (Hazak et al., 2010; Hazak et al., 2019; Hazak et al., 2014; Lavy et

57 al., 2007). ICR1 is recruited to the plasma membrane by ROPs and subsequently recruits the

58 EF-hand calcium binding protein Calcium-dependent Modulator of ICR1 (CMI1) to cortical

59 microtubules. This effect on CMI1 subcellular distribution influences its function (Hazak et

60 al., 2019; Lavy et al., 2007).

61 Studies on ICR5 (also known as ROP Interacting Partner 3 (RIP3) and Microtubule Depletion

62 Domain 1 (MIDD1)) revealed that it is a MAP that interacts with the microtubule-

63 destabilizing kinesin, Kinesin13A (Mucha et al., 2010). Functional analysis of

64 dedifferentiating tracheary elements showed that ICR5 regulates secondary cell wall

65 deposition in differentiating metaxylem cells through an association with depolymerizing

66 cortical microtubules in future secondary cell wall pits (Oda and Fukuda, 2012; Oda and

67 Fukuda, 2013; Oda et al., 2010). It was proposed that ICR5 is recruited to plasma membrane

68 domains by ROP11, where it promotes local microtubule breakdown, which in turn results in

69 the formation of cell wall pits (Oda and Fukuda, 2012; Oda and Fukuda, 2013). A recent

70 study showed that that ICR2 and ICR5 interact with the AGC1.5 protein kinase, which in turn

71 phosphorylates ROPGEF4 and ROPGEF10 to promote root hair growth (Li et al., 2020).

72 Plant microtubules function dynamically to regulate cellular functions related to cell division,

73 cell growth, cell shape formation, pathogen invasion, and abiotic stresses. Microtubule

74 dynamics, including extension, shrinkage, catastrophe, and rescue, have been studied in plant

75 cells (Ehrhardt and Shaw, 2006; Elliott and Shaw, 2018; Shaw et al., 2003). Although plant

76 cells have unique plant microtubule structures and organization processes, microtubule

77 dynamics are conserved in eukaryotes (Hamada, 2014a). Microtubules organize in several

78 typical structures in the course of the cell cycle. During interphase, microtubules form

79 cortical arrays beneath the plasma membrane. In contrast to animal and yeast cells, in plant

80 cells, interphase microtubules organize without an organizing center and their plus and minus

81 ends are distributed throughout the cell cortex (Ehrhardt, 2008; Ehrhardt and Shaw, 2006;

82 Elliott and Shaw, 2018; Wasteneys and Ambrose, 2009; Yagi et al., 2018; Yi and Goshima,

83 2018).

84 The dynamic nature of the cortical microtubules and their ability to respond to diverse stimuli

85 is governed by MAPs that regulate their nucleation, stability, crosslinking, severing,

86 membrane interaction, and orientation (Hamada, 2014b). For example, movement of

87 cellulose synthases (CesAs) in the membrane is driven by the synthesis of cellulose chains

88 and overlays with cortical microtubules (Paredez et al., 2006). Cortical microtubules have

89   been suggested to affect CesAs localization in the plasma membrane and to regulate their

90   movement (Gutierrez et al., 2009). Several MAPs are known to mediate CesA and

91   microtubule colocalization (Bringmann et al., 2012; Endler et al., 2015; Gu et al., 2010;

92   Kesten et al., 2019; Lei et al., 2013; Li et al., 2012).

93   Though ICR proteins are MAPs (Hazak et al., 2019; Mucha et al., 2010; Oda and Fukuda,

94   2012; Oda and Fukuda, 2013; Oda et al., 2010) their effect on microtubule organization and

95   how they affect ROP signaling are not well understood. In this work, we characterized the

96   functions of ICR2 and ICR5 and analyzed the *icr2/icr5* double and *icr2/icr5/icr3* triple

97   mutant phenotypes. Our results indicate that ICR2 function is associated with restriction of

98   ROP signaling domains and that the function ICR5 in differentiating metaxylem cell differs

99   from that previously proposed.

Results
ICR2 expression pattern

102   To analyze the expression pattern of ICR2 protein, the genomic sequence of *ICR2*, including

103   2225 bp of upstream promoter sequence, was fused to the sequence encoding the β-

104   glucuronidase (GUS) reporter (*pICR2::ICR2$_{genomic}$-GUS*). At 7 days after germination (DAG)

105   ICR2 expression was observed near the root tip, specifically in the cell division zone, and in

106   lateral root initials, lateral roots, vascular tissues, and root hairs (Figure 1A-D). In the

107   hypocotyl and the cotyledons, ICR2-GUS expression was strong in vascular tissues, leaf

108   primordia, and stomata linage cells (Figure 1E-F). Interestingly, although detected, the

109   expression was lower in mature guard cells than in stomata linage cells (Figure 1G and H). In

110   reproductive organs, ICR2-GUS was observed in developing floral tissue, the vasculature of

111   pedicels and receptacles, sepals, the stamen filament, ovary and ovules, and developing seeds

112   and siliques (Figure 1I-L).

113   In agreement with the ICR2-GUS reporter data, gene expression data from the *Arabidopsis*

114   eFP Browser (https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al., 2007)) indicates

115   that *ICR2* expression is higher in the shoot apex and in seeds than other tissues and higher

116   during flower development than other stages (Figure 1-Supplement 1). A co-expression

117   analysis using GENEVESTIGATOR (www.genevestigator.com/gv/ (Hruz et al., 2008))

118   indicated that the expression of *ICR2* is highly correlated with various MAPs and actin-

119   associated proteins (Table S1). The co-expression data suggested that *ICR2* is involved in, or

120   at the very least, up-regulated, during cell division, cytokinesis by cell plate formation, cell

121 proliferation, regulation of the cell cycle, and cytoskeletal organization. Although many of

122 the co-expressed genes are uncharacterized, the strong correlations of *ICR2* levels with levels

123 of *ICR3* and *ICR4* suggest that they either function together or that there is some functional

124 redundancy among these ICR family members. Interestingly, *MAP65-2*, which is the mRNA

125 with expression most highly correlated with *ICR2* levels, is a coiled coil-containing

126 microtubule-stabilizing protein involved in microtubule bundling in both interphase and

127 cytokinetic microtubule arrays (Guo et al., 2009; Lucas et al., 2011; Lucas and Shaw, 2012).

128 The GUS reporter expression data are in line with the transcriptomic data and indicate that

129 *ICR2* is highly expressed in meristem and dividing cells, developing stomata, flower organs,

130 ovules, and seeds. The relatively high expression detected in vascular tissues and root hairs

131 suggests that ICR2 may function in these tissues and cells.

Generation of single and double mutants *of ICR2* and *ICR5* and the *ICR2*, *ICR5*, and *ICR3* triple mutant

134 In order to characterize the function of ICR2, mutants were either obtained or generated. The

135 *icr2-1* (*GK567F02*), *icr2-2* (*GK281B01*), and *icr2-3* (*GK159B08*) *T-DNA* mutants, which are

136 part of the GABI KAT seed stock (Kleinboelting et al., 2012), were obtained from the

137 European *Arabidopsis* Stock Centre (NASC) and are in the Columbia-0 (Col-0) background

138 (Figure 2-Supplement 1). Further, multiplex genome editing by means of CRISPR/Cas9

139 (Bortesi and Fischer, 2015) was carried out in order to generate multiple mutant alleles in

140 *ICR2*, *ICR3*, and *ICR5*. We identified two independent *icr5* single-mutant alleles, two

141 independent *icr2/icr5* double-mutant alleles, and a single *icr2/icr3/ic*r5 triple mutant (Figure

142 2-Supplement 2). The CRISPR/Cas9-mediated genome editing generated InDels that resulted

143 in mutant genes that encoded truncated proteins from which most residues were missing and

144 were therefore very likely inactive. Hence all mutants were considered nulls.

ICR2, ICR5, and ICR3 negatively regulate metaxylem pit formation

146 Previous work indicated that ICR5 is required for the formation of secondary cell wall pits in

147 the metaxylem (MX) (Oda and Fukuda, 2012; Oda et al., 2010), but the phenotype of an *icr5*

148 mutant has not been previously described. The creation of *icr2* and *icr5* single and double

149 mutant plants, as well as the *icr2/icr3/icr5* triple mutant, enabled analysis of the functions of

150 these ICRs in MX pit formation (Figure 2).

151 Surprisingly, contrary to previous predictions regarding the function of ICR5, the analysis of

152 *icr2* and *icr5* single mutants revealed that they have significantly larger and denser pits than

153   Col-0 plants. Hence pit formation was enhanced rather than suppressed in the *icr5*-null

154   background. The size and the density of the pits in *icr2/icr5* and *icr2/icr3/icr5* double and

155   triple mutants are increased compared to *icr2* and *icr5* single mutants, indicating that these

156   three ICRs have redundant functions in the regulation of pit formation. Importantly, pit size

157   and density were partially complemented in double transgenic plants expressing a genomic

158   clone of ICR2 fused to three repeats of the YFP variant YPet, under regulation of the *ICR2*

159   promoter, and ICR2 fused to the microtubule marker RFP-MBD (*icr2-1 X UBQ10::RFP-*

160   *MBD X ICR2-3xYPet* and *icr2-2 X UBQ10::RFP-MBD X ICR2-3xYPet*).

161   **ICR5 but not ICR2 regulates protoxylem secondary cell wall deposition**

162   To analyze whether ICR2, ICR3 and ICR5 have additional roles during vascular

163   differentiation, specifically in secondary cell wall deposition, the density of developing

164   protoxylem (PX) lignin coils was measured by imaging lignin auto-fluorescence (Figure 3A-

165   J). The PX lignin coils in *icr2* mutants were similar to those of Col-0, whereas the *icr5* single

166   mutants as well as the *icr2/icr5* and *icr2/icr3/icr5* mutants had denser lignin deposition than

167   Col-0 (Figure 3K). This finding implicated ICR5 in regulation of secondary cell wall

168   deposition in PX. As there was no additive phenotype in double and triple mutants, we reason

169   that ICR2 and ICR3 do not function in the PX.

170   *icr2* **mutants exhibit a deformed, branched root hair phenotype**

171   ICR2 expression was detected in root hair (Figure 1D). Because ROP signaling plays central

172   role in root hair tip growth (Bloch et al., 2005; Bloch et al., 2011; Carol et al., 2005; Chai et

173   al., 2016; Denninger et al., 2019; Duan et al., 2010; Jones et al., 2002; Kang et al., 2017;

174   Molendijk et al., 2001; Nakamura et al., 2018; Wan et al., 2017), we asked whether single,

175   double, and triple *icr* mutants develop abnormal root hairs. All the plants with *ICR2* mutant

176   alleles exhibited deformed, branched root hair phenotypes (Figure 4A-K). In contrast, *icr5*

177   root hairs were normal, and the double and triple mutants showed no additive effects (Figure

178   4L). Further, there was partial complementation of the split root hair phenotype in *icr2-1* and

179   *icr2-2* by ICR2-YPet (Figure 4L). These data indicate that the function of ICRs in root hair

180   growth regulation is not redundant. To observe whether mutations in genes encoding ICRs

181   have any other effect on root hair development, we measured the distance of the first root hair

182   bulge from the root tip (Figure 4-Supplement 1A), the root hair length (Figure 4-Supplement

183   1B) and root hair density (Figure 4-Supplement 1C). None of these characteristics differed in

184   the *icr* mutants compared to Col-0 plants. This finding suggested that ICR2 is involved

185   specifically in polarity maintenance of growing root hairs but not in root hair initiation. This

186   is the first evidence that a ROP interactor affects root hair polarity.

187

188   **Interaction of ICR2 with microtubules and ROPs *in vivo***

189   Similar to secondary cell wall pits in MX, the split root hair phenotype has been associated

190   with perturbation in microtubules and was described for several MAP mutants (Kang et al.,

191   2017; Sakai et al., 2008; Whittington et al., 2001; Yang et al., 2007; Zhang et al., 2015). We

192   discovered ICR2 in a yeast two-hybrid screen with constitutively active ROP10 (rop10^{CA}) as

193   bait (Lavy et al., 2007). Transient expression in *Nicotiana benthamiana* leaf epidermis cells

194   showed that, when individually expressed, ICR2 localizes along cortical microtubules (Figure

195   5A, Figure 5-Supplement 1), whereas ROP11 localizes to the plasma membrane (Figure 5B)

196   as has previously been demonstrated (Bloch et al., 2005; Lavy et al., 2002; Lavy and

197   Yalovsky, 2006). The localization of ICR2 on microtubules was verified by treatment with

198   the microtubule inhibitor oryzalin, which resulted in the disappearance of ICR2-labeled

199   microtubules and a shift of ICR2 to the cytoplasm (Figure 5-Supplement 1B). Localization of

200   ICR2 to microtubules was further verified by co-expression with the microtubule marker

201   RFP-MBD with and without oryzalin treatment (Figure 5-Supplement 1F-H).

202   The interaction of ICR2 with ROPs was examined using bimolecular fluorescence

203   complementation (BiFC). ICR2 fused at its carboxy terminus to the C-terminal half of YFP

204   (ICR2-YC) was transiently expressed in *N. benthamiana* leaf epidermis cells along with

205   various ROPs fused at their N-termini to the N-terminal half of YFP (YN-ROPs). ICR2

206   interacted with constitutively active versions of the type-I ROP6 or type-II ROP9, ROP10, or

207   ROP11 or wild-type ROP2, ROP4, or ROP6 (type-I ROPs) or wild-type ROP9, ROP10, or

208   ROP11 (type-II ROPs). The complexes localized along cortical microtubules (Figure 5C-L).

209   Furthermore, in yeast two-hybrid assays, ICR2 interacted with both type-I ROP2, ROP4, and

210   ROP6 and with type-II ROP9, ROP10, and ROP11 (Figure 5M). Taken together, these results

211   suggest that ICR2 is a microtubule-associated ROP interactor that may recruit ROPs to

212   microtubules from the plasma membrane.

213   To further explore the interaction between different ROPs and ICR2, GFP-tagged ROP2,

214   ROP4, ROP6, ROP9, ROP10, and ROP11 were co-expressed in *N. benthamiana* leaf

215   epidermis with the catalytic Plant-specific ROP Nucleotide Exchanger (PRONE) domain of

216   ROPGEF3 (GEF3p), GAP1, and mCherry-tagged ICR2 (ICR2mCh). No changes in the

217   organization of ICR2mCh-labeled microtubules were found around ROP2 domains (Figure

218   6A), and faint ICR2mCh labeling was detected around the ROP4 domains (Figure 6B).

219   However, significant clustering of ICR2mCh-tagged microtubules was found around domains

220   containing ROP6, ROP9, and ROP10 (Figure 6C-E) and weaker but clearly visible

221   microtubule reorganization was found around ROP11 domains (Figure 6F). These results

222   suggest that ROPs may differ in their abilities to recruit ICR2-associated microtubules to

223   specific domains in the plasma membrane. We hypothesize that ICR2 has a dual role: On one

224   hand, it restricts ROP activity to domains in the plasma membrane, and on the other hand, it

225   functions as a scaffold for ROP interactions with microtubules and possibly with other

226   proteins.

ICR1 and ICR2 bind microtubules *in vitro*

Both ICR1 and ICR2 localize to microtubules *in vivo* (Hazak et al., 2019; Mucha et al., 2010; Oda and Fukuda, 2012; Oda and Fukuda, 2013; Oda et al., 2010), but it is possible that their localization could have resulted from interaction with a third component rather than direct interaction with microtubules. To examine whether ICR1 and ICR2 are indeed MAPs, we tested their interactions with microtubules *in vitro* using three independent assays.

Escherichia coli-expressed, affinity-purified ICR1-His$_6$ and ICR2-His$_6$ at concentrations ranging between 1 to 10 μM were incubated with preformed taxol-stabilized microtubules. The protein mixtures were precipitated by centrifugation at 100,000 x *g*, and the precipitated proteins were separated by SDS-PAGE and visualized by Coomassie blue staining (Figure 7A, 7-Supplement 1A). The levels of precipitated ICR1-His$_6$ and ICR2-His$_6$ were quantified by densitometry of the relevant bands (Figure 7B, 7-Supplement 1B). MAP65, a known microtubule-interacting protein, was used a positive control. The binding of recombinant ICRs to microtubules was saturated at stoichiometries of 0.4 mol ICR1-His$_6$ per mol of tubulin and 0.85 mol ICR2-His$_6$ per mol of tubulin. This is in agreement with our findings that both ICR1 and ICR2 interact directly with microtubules and that the binding of ICR2 with microtubules is stronger than that of ICR1.

Second, *in vitro* immuno-fluorescence assays were used to examine whether ICR1 and ICR2 colocalize with polymerized microtubules. To visualize microtubules, rhodamine-labeled tubulin was mixed with non-labeled tubulin and polymerized into microtubules in the presence of ICR1-His$_6$ or ICR2-His$_6$. Visualized by *in vitro* immuno-localization established that ICR1 and ICR2 are MAPs (Figure 7C-E, 7-Supplement 2A-C). Incubation with denatured ICR1-His$_6$/ICR2-His$_6$ were used as negative controls (Figure 7F-H, 7-Supplement 2D-F). ICR1 was distributed in individual punctae, whereas ICR2 was more evenly distributed along microtubule filaments suggesting stronger binding, in line with results of co-precipitation assays.

Third, we carried out an *in vitro* microtubule bundling assay. To this end, rhodamine-labeled tubulin was polymerized into microtubules in the presence of increasing concentrations of ICR1 or ICR2. With ICR1, microtubule bundling was detected only at the high concentration of 5 μM, whereas ICR2 caused bundling even at 0.1 μM (Figure 7I-K, 7-Supplement 3). Taken together, the bundling assays further confirmed that that ICR1 and ICR2 are MAPs and that binding of ICR1 to microtubules is weaker than ICR2 binding.

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ICR2 co-localizes with microtubules in all stages of the cell cycle

To characterize the subcellular localization of ICR2, we generated a marker composed of the genomic sequence of *ICR2* (including introns) with its promoter fused with the sequence for 3xYPet. To reduce potential steric hindrance a 33 amino acid linker was placed between ICR2 and the 3xYPet tag. To avoid potential mis-localization due to overexpression, the *pICR2::ICR2$_{genomic}$:3xYpet* construct was transformed into two *icr2 T-DNA* insertion mutants, *icr2-1* and *icr2-2*, that also express the microtubule marker RFP-MBD (*icr2-1 X UBQ10::RFP-MBD* and *icr2-2 X UBQ10::RFP-MBD*). Importantly, the *pICR2::ICR2$_{genomic}$:3xYPet* fusion complemented the *icr2-1* and *icr2-2* pit formation and root hairs phenotypes, confirming its functionality (Figure 2-4). In the lateral root cap, root hairs, and in root epidermis cells, the ICR2:3xYPet fusion protein was observed on cortical microtubules (Figure 8A-I). The localization of ICR2 on microtubules was confirmed by colocalization with the microtubule marker RFP-MBD (Figure 8C, F, I, and J). The localization of ICR2 on microtubules in root hairs suggested that the split root hair phenotype of the *icr2* mutants is associated with ICR2 function on microtubules. Given that ICR2 can retrieve ROP2 from the plasma membrane to microtubules (Figure 5G) and given the similarities between the phenotypes of the *icr2* null and ROP2 gain-of-function mutants (Jones et al., 2002; Kang et al., 2017), ICR2 may restrict ROP2 signaling by recruiting it to microtubules.

In dividing cells, ICR2 was colocalized with microtubules during mitosis (Figure 9A). ICR2-3xYPet colocalized with RFP-MBD in all mitotic stages including the preprophase band, the spindle during metaphase and anaphase, and the expanding phragmoplast microtubules in telophase (Figure 9B). Interestingly, the localization of ICR2 on microtubules during cell division coincided with the co-expression data, which showed high correlations with cell division cytoskeleton genes (Table S1).

The pit phenotype of *icr2* mutants prompted us to examine ICR2 localization in vascular tissues. Unfortunately, with the experimental setup available to us for imaging of microtubules in the vasculature and the low expression levels of ICR2 made direct imaging impossible. To overcome this difficulty, we used bikinin-induced xylem/phloem dedifferentiation of leaf/cotyledon mesophyll cells (Kondo et al., 2016). The dedifferentiation

291   of the mesophyll cells to cells harboring secondary cell wall occurred within 4 days and was

292   visible using lignin auto fluorescence (Figure 10A-D). ICR2 expression was detected in

293   differentiating xylem cells during the beginning of secondary cell wall deposition in cells that

294   were still had chloroplasts (Figure 10A-D). ICR2-3xYPet fluorescence was not detected in

295   fully differentiated cells (Figure 10A-D). Hence, ICR2 was expressed during relatively short

296   time window at the onset of dedifferentiation indicating that its likely has specific function

297   during xylem differentiation. The short time window in which ICR2 was expressed may also

298   explain why it was difficult to detect it in root vascular tissues.

299   In the dedifferentiating xylem cells, ICR2-3xYPet colocalized with lignified secondary cell

300   wall (Figure 10E), whereas no fluorescence was detected in the pitted areas. Because the

301   ICR2-3xYPet fluorescence was detected only in few cells and was absent in most of the cells

302   that had secondary cell walls, it could not be a results of fluorescence channel spillover.

303   Furthermore, the images were generated using spectral separation to ensure the presence of

304   the YPet fluorescence. The strict colocalization of ICR2 with the secondary cell walls

305   strongly suggests that it was localized along cortical microtubules. The pit phenotype of the

306   *icr2* mutant implicates ICR2 in restriction of pit formation and regulation of pit size. In the

307   developing MX, ICR2 may function by recruiting ROPs from the plasma membrane to

308   microtubules.

309   **icr2 mutants display altered microtubule organization and dynamics**

310   The localization of ICR2 to microtubules at all stages of the cell cycle suggested that it may

311   affect the organization and dynamics of microtubules. To test this, *icr2-1* and *icr2-2* plants

312   were crossed with *UBQ10::RFP-MBD*, and analysis of microtubule dynamics was carried out

313   on non-segregating double homozygous plants using high-frequency time-lapse imaging and

314   tracking of individual microtubule filaments. The tracking data (Figure 11A) was used to

315   create kymographs (Figure 11B), which were then used to calculate microtubule growth and

316   shrinkage rates, the time spent in each condition, transition times, and pauses in

317   growth/shrinkage. In root epidermal cells as well as root hairs, microtubule growth rates were

318   significantly slower in the *icr2* mutants than Col-0 plants ($p \leq 0.001$) (Figure 11C). In contrast,

319   shrinkage rates were lower only in the epidermis (Figure 11C). Additionally, time spent at

320   pause was higher in mutant root epidermal cells than Col-0 plants (Figure 11-Supplement 1),

321   and the transitions between filament growth, shrinkage, and pause occurred at higher

322   frequency in the *icr2* mutants than in Col-0 plants (Figure 11-Supplement 2).

323   Taken together the analysis of microtubule dynamics indicated that ICR2 regulates

324   microtubule stability differently in different cell types. This cell-specific regulation of

325   microtubule dynamics by ICR2 is consistent with the developmental phenotypes in root hair

326   growth in *icr2* mutants but not the *icr5* single mutant (Figure 4). The secondary cell wall

327   deposition phenotype of *icr5* but not *icr2* in PX (Figure 3) further supports cell- or tissue-

328   specific function of ICR2. The MX pit patterning phenotype (Figure 2) suggests that ICR5,

329   like ICR2, functions in a cell-specific manner.

330

Discussion

The functions of ICR2, ICR5, and ICR3

ICR proteins have been suggested to function as adaptors that mediate the interaction of ROPs with distinct target proteins (Lavy et al., 2007; Li et al., 2020; Mucha et al., 2010; Oda and Fukuda, 2012). The phenotypic analysis of the mutants presented in this work indicate that the function of the ICRs is more complex than previously thought. The mutant phenotypes indicate that at least ICR2, ICR3, and ICR5 also function as regulators that restrict ROP signaling, likely by recruiting ROPs from the plasma membrane to microtubules. Furthermore, the phenotypic analysis showed that the function of ICR2 and ICR5 is partially cell specific and that at least ICR2 may have ROP-independent functions in the regulation of microtubule dynamics. The data also suggest that ICR2 functions as effector for some ROPs. By recreating the naturally occurring active ROP domains in the plasma membrane, we were able to visualize how a subset of plasma-membrane anchored, active ROPs recruit the microtubule-associated ICR2 to these domains, thus relaying spatiotemporally regulated signal transduction into the cell.

ICR2 is a MAP

The *in vitro* assays confirmed the ICR2 is a MAP. Our *in vitro* system also enabled us to compare the microtubule binding strength of ICR1 and ICR2. The co-sedimentation with microtubules revealed that ICR2 has a higher affinity for microtubules than ICR1. Whereas the binding of recombinant ICR2-His$_6$ to microtubules was not saturated until 0.85 mol per mol of tubulin, saturation of ICR1-His$_6$ was reached at only 0.35 mol per mol of tubulin. Similarly, the bundling assays showed that ICR1 caused microtubule bundling at 5 µM, whereas ICR2 induced microtubule bundling at the much lower concentration of 0.1 µM. The tighter binding of ICR2 to microtubules was also detected in the *in vitro* colocalization assays, which revealed an even distribution of ICR2 along microtubules at 0.5 µM, whereas ICR1 was detected as discrete punctae at 1 µM. It will be interesting to examine whether and how the differential binding of ICR1 and ICR2 to microtubules affects their functions as MAPs and as ROP signaling effectors.

The involvement of ICR2, ICR5, and ICR3 in secondary cell wall patterning

ROP11 was previously implicated in regulation of secondary cell wall pits. A model involving ICR5 in the process was suggested previously (Oda and Fukuda, 2012; Oda et al., 2010). In this model, locally activated ROP11 recruits ICR5, leading to depolymerization of

363 cortical microtubules in the future pit regions. Here we found that *icr2* and *icr5* single
364 mutants have larger, denser pits compared to *Col-0*, whereas the *icr2/icr5* double and the
365 *icr2/icr3/icr5* triple mutants have even larger pits and higher pit densities than the single
366 mutants. These data indicate that ICR2, ICR3, and ICR5 have redundant functions in the
367 regulation of pit formation in the MX. Additionally, expression of *ICR2-3xYPet* restored pit
368 density in *icr2-1* and *icr2-2* backgrounds further confirming the function of ICR2 in
369 regulation of pit formation. In dedifferentiating vascular cells ICR2 colocalized with
370 secondary cell wall, indicating that its function is associated with the microtubules and
371 secondary cell wall deposition rather than microtubule destabilization.

372 Furthermore, the microtubule dynamics analysis showed decreased microtubule growth rates
373 in the *icr2* mutant background. If the primary function of ICR5 is induction of local
374 microtubule destabilization through the recruitment of Kinesin13A, we would have expected
375 that the size and number of pits would be reduced and not increased as seen in the *icr5*
376 mutants. Thus, our data indicate that the functions of ICR5 as well as ICR2 and ICR3 are
377 more complex than previously proposed. Possibly, ICR2, ICR3, and ICR5 restrict ROP
378 domain activity by moving ROPs from the plasma membrane to microtubules. The higher
379 density of secondary cell wall coils in the PX of *icr5* is in line with a role of ICR5 in
380 destabilization of microtubules. Based on a combination of experimental work and computer
381 simulation, Schneider et al. recently proposed that microtubule destabilization takes place
382 during secondary cell wall formation in PX (Schneider et al., 2020). It is possible that ICR5
383 functions during this microtubule destabilization. It could be that the VND6-induced
384 dedifferentiating cell culture system that has been used in previous studies (Oda and Fukuda,
385 2012) does not fully recapitulate the differentiating MX in the root and that these cells harbor
386 characteristics of PX, which would have made it difficult to identify cell-specific functions of
387 ICR5.

The function of ICR2 in root hair growth

389 The split root hair phenotype of *icr2* mutant is not associated with changes in root hair
390 density or position in the trichoblasts. This indicates that ICR2 function is required for
391 maintenance of polar root hair elongation. The localization of ICR2 on microtubules in
392 growing root hairs and the altered microtubule dynamics of *icr2* mutants (i.e., slower
393 microtubule growth rate and increased rate of transitions between filament extension pause
394 and shrinkage) indicate that ICR2 is necessary for stability of microtubules in root hairs. A
395 split root hair phenotype has been associated with perturbation of microtubules and was

396   described for several MAP mutants (Kang et al., 2017; Sakai et al., 2008; Whittington et al.,
397   2001; Yang et al., 2007; Zhang et al., 2015). In root hairs, ICR2 on was found to localize on
398   microtubules along the shank and not in active ROP domains at the root hair tip characterized
399   previously (Jones et al., 2002).

400   Furthermore, although ICR2 was not recruited to active ROP2 domains, ROP2 gain-of-
401   function mutations lead to formation of split root hairs (Jones et al., 2002; Kang et al., 2017),
402   similar to the *icr2* mutant phenotype. Hence, ICR2 may function by recruiting active ROP2
403   from the plasma membrane to microtubules (Jones et al., 2002). Additionally, ROP10 was
404   recently shown to regulate secondary cell wall formation in the shank leading to root hair
405   shank hardening (Hirano et al., 2018). As we showed that ICR2 is recruited to active ROP10
406   domains, it is possible that ICR2 functions as a ROP10 effector in root hair. A recent study
407   implicated ICR2 in recruitment of AGC1.5 where it phosphorylates ROPGEF4 and
408   ROPGEF10 to promote root hair growth (Li et al., 2020). However, the split root hair
409   phenotype of the *icr2* mutants, the localization of ICR2 on microtubules in root hairs, and the
410   inability of active ROP2 domains to recruit ICR2 are not compatible with the proposed
411   function of ICR2 in the activation of ROP2 function via AGC1.5 and ROPGEF4/10.
412   Importantly, the distribution of ICR2 reported by Li *et al.* (Li et al., 2020) was determined by
413   analysis of ICR2 with GFP fused to the N-terminal end; this fusion likely disrupted the
414   interaction with microtubules, which takes place via the N-terminal end of ICR2. Hence,
415   although the interaction of ICR2 with AGC1.5 is intriguing, its functional role will require
416   additional investigation.

417   The analysis presented here together with previous works suggest that the ICR family
418   proteins have multiple unique roles as ROP effectors, ROP regulators, and as MAPs that
419   regulate microtubule dynamics. ICR2 and ICR5 may regulate microtubule destabilization
420   through their interactions with proteins such as Kinesin13A. On the other hand, the analysis
421   of microtubule dynamics and pit formation indicate that ICR2, and likely ICR3 and ICR5,
422   stabilize microtubules and restrict ROP-mediated signaling by moving ROPs from the plasma
423   membrane to microtubules. The tissue- and cell-specific functions of ICR2 and ICR5 may
424   reflect interactions with different proteins in different cells. In addition, that only a subset of
425   ROPs recruit microtubule-associated ICR2 to ROP domains may also affect the signaling
426   specificity. Previously, we showed that ICR1 and ICR2 do not interact with the same proteins
427   (Lavy et al., 2007), supporting the hypothesis that they function as differential adaptors of
428   ROP signaling. The differences in the binding affinity of ICR1 and ICR2 to microtubules

429   indicate that, in addition to interactions with different proteins, the two ICRs affect

430   microtubules differently and may have different distributions in the cell. Although ICR2

431   localized to microtubules in all stages of the cell cycle, we did not detect any cell division

432   abnormalities in the *icr2* single mutants or in the *icr2*/*icr5* double or *icr2*/*icr5*/*icr3* triple

433   mutants. The function of ICR2 during cell division may be redundant with proteins outside

434   the ICR family or may be required under specific conditions; this will be the focus of future

435   studies.

436

Materials and methods

Molecular procedures

Plasmid DNA purification. Plasmid purification was carried out with a DNA-spin™ Plasmid DNA Purification Kit (iNtRON biotechnology) according to the manufacturer's protocol.

Polymerase chain reaction (PCR). PCR was used for gene detection and cloning. For general uses such as colony screening, Taq DNA polymerase (Fermentas) was used. To eliminate error, for cloning purposes, PCR reactions were carried with the proof-reading Phusion DNA polymerase (NEB). Reaction conditions were according to the enzyme manufacturer's instructions with annealing temperatures chosen based on primers.

DNA fragment extraction from agarose gel. DNA extraction from agarose gels was done using the Gel extraction kit Qiaex II (Qiagen).

Cloning for co-expression, yeast two-hybrid, and BiFC assays. For imaging, *ROP6*, *ROP9*, *ROP10*, *ROP11* were subcloned downstream of *GFP* into *pGFP-MRC*. The resulting cassettes, containing a cauliflower mosaic virus (CaMV) *35S* promoter, the gene of interest, and a *NOS* transcriptional terminator, were subcloned into *pCAMBIA 2300* expression vector. Sequences encoding GFP-ROP2, GFP-ROP4, His-GAP1, His-GEF3$_{PRONE}$, and ICR2-mCherry were cloned into expression vectors *pB7m34GW* or *pK7m34GW* by the Three-Way Gateway standard protocol (Invitrogen). The expression cassette included the *35S* promoter, the tag, the gene, and the NOS terminator. To obtain constitutively active ROP mutants, respective genes were mutated by site-directed mutagenesis. ROP6 and ROP10 were mutated to Q67L, ROP9 and ROP11 were mutated to G15V. For yeast two-hybrid analysis, ROP2, ROP4, ROP6, ROP9, ROP10, and ROP11 coding sequences were subcloned into *pGBT9.BS* and the ICR2 coding sequence was cloned into *pGAD GH*. For BiFC assays, YN-ROP2, YN-ROP4, YN-ROP6, YN-ROP9, YN-ROP10, YN-ROP11, ICR2-YC sequences were subcloned into pB7m34GW by the Three-Way Gateway standard protocol (Invitrogen). The expression cassette included the CaMV *35S* promoter, tag, gene of interest, and *NOS* terminator.

Creation of *pB7-pICR2::ICR2*. Intermediate vectors were created using Gateway BP Clonase. A 2493-bp fragment harboring the entire genomic sequence of *ICR2* (AT2G37080) from the ATG initiation codon through the stop codon was amplified from genomic DNA and subcloned into pDONR221. The promoter sequence of *ICR2* (2225 bp upstream of the

468   *ICR2* initiation codon) was likewise amplified and subcloned into *pDONR-P4R1*. The *NOS*

469   terminator was subcloned into *pDONR-P2R3*. All three intermediate vectors were further

470   cloned into *pB7m34GW* destination vector using the Gateway LR Clonase II Plus Enzyme

471   Mix for MultiSite LR recombination reaction.

472   **Creation of *pK7-pICR2::ICR2-3xYPet* (EYFP variant)**. Intermediate vectors were created

473   using Gateway BP Clonase. A 2490-bp fragment harboring the entire *ICR2* (AT2G37080)

474   genomic sequence from the ATG initiation codon but without the final stop codon was

475   amplified from genomic DNA and subcloned into *pDONR221*. The promoter sequence of

476   *ICR2* (2225 bp upstream of the *ICR2* initiation codon) was likewise amplified and subcloned

477   into *pDONR-P4R*. 3xYPet-3xHA (Marquès-Bueno et al., 2016) was received from NASC

478   (NASC code N2106295). This vector contains a 33-amino acid linker

479   (DPAFLYKVARLEEFGTPGSKSISLDPLPAAAAA) between ICR2 and the three repeats of

480   the fluorescent protein YPet to reduce potential steric hindrance. All three intermediate

481   vectors were further cloned into *pK7m34GW* using the Gateway LR Clonase II Plus Enzyme

482   Mix for MultiSite LR recombination reaction.

483   **Creation of ICR1-His$_6$ and ICR2-His$_6$**. *pET28b-ICR1-His$_6$* was created by amplifying a

484   1050-bp fragment of the coding sequence of *ICR1* (AT1G17140) without a terminating stop

485   codon from cDNA with flanking Nco1 and Not1 sites, and was subcloned into pJET1.2

486   (Thermo Scientific). The plasmid was than digested with Nco1 and Not1, and the *ICR1*

487   fragment was subcloned into *pET28b*. *pET28b-ICR2-His$_6$* was created by amplifying a 1749-

488   bp fragment of the coding sequence of *ICR2* without the stop codon and subcloning into

489   *pJET1.2*. It was than integrated into *pET28b* using primers containing overlapping sequence

490   to *pET28b* and amplifying the entire vector by Transfer PCR (T-PCR) (Erijman et al., 2014).

491   **Multiplex genome editing design and constructs**. The polycistronic tRNA-gRNA system

492   (PTG) was used to generate multiple sgRNAs with different target sequences by flanking the

493   sgRNAs with a tRNA precursor sequence as previously described (Xie et al., 2015). A pJET-

494   gRNA-tRNA plasmid, which contains a gRNA-tRNA-fused fragment, was used as a template

495   to synthesize the PTG construct. The gRNA scaffold fragment was amplified by PCR using a

496   pair of specific primers (Bsa-gRNA-F and gRNA-R), whereas the tRNA$_{Gly}$ fragment was

497   amplified as an overlapping fragment of the primers g-tRNA-F and tRNA-R. Then these two

498   fragments were fused as a gRNA-tRNA by overlapping extension PCR using primers Bsa-

499   gRNA-F and tRNA-R. The overlapping PCR product was separated and purified from an

500 agarose gel, and then inserted into *pJET1.2* (Thermo Scientific) to generate the template

501 plasmid. The specific spacer sequences targeting *ICR2*, *ICR3*, and *ICR5* were selected using

502 the CRISPR-PLANT database (www.genome.arizona.edu/crispr/). The PTG clones were

503 created using Golden Gate (GG) for the assembly of DNA fragments. In order to ligate

504 multiple DNA fragments in a desired order, GG assembly requires distinct 4-bp overhangs to

505 ligate two DNA fragments after digestion with BsaI. The gRNA spacers are the only unique

506 sequences in the PTG and were used for this purpose. Each part was amplified with spacer-

507 specific primers containing the BsaI adaptor, except two terminal parts using gRNA spacer

508 primer and terminal specific primers containing BbsI site. These PCR fragments were ligated

509 together using GG assembly to produce the PTG with complete gRNA spacers targeting

510 *ICR2*, *ICR3*, and *ICR5*. The assembled product was amplified with short terminus specific

511 primers containing the BbsI adaptor. Next, using a second GG assembly step, the PTG

512 fragment was inserted into the BbsI digested *pEntr_L1L2_AtU6gRNA*. The PTG cassette was

513 than inserted into *pMR294_pKGCAS9PLUS-1* by Gateway LR Clonase (Thermo Fisher

514 Scientific). The *pEntr_L1L2_AtU6gRNA* and *pMR294_pKGCAS9PLUS-1* vectors were gifts

515 from Professor Gitta Coaker, University of California, Davis.

516 In all cloning, PCR-generated fragments were sequenced to verify that no PCR-generated

517 errors were introduced. In the cases of gene fusions, following cloning, the borders between

518 fragments were sequenced to verify that fragments were in frame. All primers and plasmids

519 used and generated in this work are listed in Tables S4-S6.

520 **Sequencing.** DNA sequencing was performed at the Tel Aviv University DNA sequencing

521 facility and was carried using the BigDye Terminator Cycle Sequencing Kit (Applied

522 Biosystems).

523 **Plant genomic DNA isolation**

524 Typically, 100 mg of liquid N_2 batch-frozen leaf tissue were ground with a mortar and pestle,

525 and genomic DNA was isolated using the GenElute Plant Gemomic DNA Kit (Sigma)

526 according to the manufacturer's protocol.

527 **Total RNA isolation from plants**

528 *Arabidopsis* seedlings were batch frozen using liquid N_2, and tissue was ground with a mortar

529 and pestle. Total RNA was isolated from the ground material using the RNeasy SV total

530 RNA isolation kit (QIAGEN) according to the manufacturer's instructions.

RT-PCR (cDNA synthesis)

cDNA synthesis for standard RT-PCR experiments was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems): 1 µg RNA dissolved in 10 µl H_2O was added to 10 µl of the 2X Reverse Transcription Master Mix, containing 2 µl 10X RT buffer, 0.8 µl 25X dNTPs mix, 2 µl 10X RT Random Primers, 1 µl MultiScribe™ Reverse Transcriptase, 1 µl RNase Inhibitor, and 3.2 µl Nuclease-free H_2O. The reaction was performed in a thermal cycler for 10 min at 25 °C, then 120 min at 37 °C, and 5 min at 85 °C (for inactivation).

Sequence analysis

Sequence analysis was carried out using the SnapGene® (GSL Biotech; available at snapgene.com) sequence analysis software package. The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) was used to search the DNA and protein database for similarity. Multiple sequence analysis was done using JALVIEW (Waterhouse et al., 2009) with the Clustal (Thompson et al., 1994) algorithm.

Bacterial strains and growth conditions

E. coli DH5α(F')-*F'* was used for heat shock transformation and molecular cloning. *Agrobacterium tumefaciens* strain *GV3101/pMP90* was used for transient and stable expression of recombinant genes in *N. benthamiana* and *Arabidopsis* as previously described (Lavy et al., 2007). Growth media for bacteria was prepared as previously described (Ausubel et al., 1995). For solid media, 1.5% w/v of agar was added to the medium. *E. coli* cells were selected on 100 µg/mL ampicillin or 50 µg/mL kanamycin. *Agrobacterium tumefaciens GV3101/pMP90* was selected on 100 µg/mL gentamycin and 50 µg/mL spectinomycin.

Yeast two-hybrid assays

Saccharomyces cerevisiae strain *PJ69-4a* was used as host. Plasmids for expression of ROPS (*pGBT ROPs*) were co-transformed with *pGAD-ICR2* into yeast cells via a standard lithium acetate transformation protocol. Four decimal dilutions of colonies expressing both plasmids were grown on a medium lacking leucine (L), tryptophan (T), and histidine (H) supplemented with 1 mM 3-amino-1,2,4-triazole (3AT) for interaction detection or on -LT for growth monitoring. The plates were incubated at 28 °C.

Plant materials and transformation

Plant materials. *Arabidopsis Col-0* ecotype was used as wild type in all experiments and was used for all transformation for generation of transgenic plants. *N. benthamiana* was used for transient expression in leaf epidermal cells. The *icr2-1(GK567F02), icr2-2(GK281B01),* and *icr2-3(GK159B08)* T-DNA mutants were obtained from NASC and are in the *Col-0* background. In generating the CRISPR/Cas9-mediated genome edited mutants, transgenic plants were created by expression of appropriate gRNAs in a single transcriptional unit, spaced by tRNAs under the control of the *AtU6* promoter as described (Xie et al., 2015). The Cas9 in this system was expressed under the control of the *GEX1* egg-specific promoter, and therefore the genomic editing events identified were heritable and not somatic, thus improving the screening process. For analysis, the T-DNA containing the *pGEX1::Cas9-AtU6::tRNA-gRNA* expression cassette was crossed out from all mutants. Seeds for *UBN::RFP-MBD* were a gift from Dr. Sabine Müller, University of Tübingen and were previously described (Lipka et al., 2014). Plants used and generated in this work are listed in Table S7.

Plant growth conditions. Seeds of wild-type *Col-0*, transgenic, and mutant *Arabidopsis* plants were sown on the soil (Weizmann Institute mix, Pele Shaham, Ashkelon) and moved to stratification at 4 °C for 48 h in the dark in order to increase the uniformity of germination. The seeds were then moved to a growth chamber under long-day conditions (16 h light/8 h dark, light intensity 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) at ~22 °C. *N. benthamiana* plants were grown in 10-cm pots. Seeds were sown on a mixture of 70% soil with vermiculite (Avi Saddeh mix, Pecka Hipper Gan). Plants were grown in an environmental growth chamber under conditions of long days (16 h light/8 h dark-light intensity 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) at ~25 °C. For growth of *Arabidopsis* on plates, plates contained 0.5X Murashige Skoog (MS) medium (Duchefa) titrated to pH 5.7 with MES and KOH and 0.8% plant agar (Duchefa). In some cases, the medium was prepared with 1% sucrose. The seeds were then moved to a growth chamber and placed vertically in most cases, or horizontally for germination assays to grow under long-day conditions (16 h light/8 h dark-light intensity 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) at ~22 °C. Prior to the transfer to the growth chamber, the sown seeds were stratified at 4 °C for 48 h in darkness. In both cases, seeds were surface sterilized by evaporation of HCl (6 mL) in sodium hypochlorite (100 mL) in a closed container for 1 h.

Stable transformation in *Arabidopsis*. Transformation was performed by the floral dip method (Clough and Bent, 1998).

594 **Transient expression in *N. benthamiana*.** Transient expression was carried out as
595 previously described (Lavy et al., 2007).

Vascular Cell Induction Culture System Using Arabidopsis Leaves (VISUAL)
597 Vascular cell dedifferentiation was carried out with the VISUAL system as previously
598 described (Kondo et al., 2016) with the following modifications. In brief, 10-15 seedlings
599 were grown in 10 ml liquid growth media and (2.2 g/L MS Basal Medium, 10 g/L sucrose
600 and 0.5 g L^{-1} MES adjusted to pH to 5.7 using KOH) in 6-wells-plates at 25 ºC under a long-
601 day regime (16 h light, 8 h dark) for 6 days until true leaves appeared. The central part of the
602 hypocotyl was cut, and the roots were removed. For VISUAL induction, the aerial parts of
603 the seedlings were incubated in induction medium (2.2 g/L MS Basal Medium containing 50
604 g/L D(+)-glucose adjusted to pH 5.7 with KOH) supplemented with 1.2 mg/L 2,4D, 0.25
605 mg/L kinetin and 10 μM bikinin in 12-well-plates for 4 additional days. Differentiating cells
606 were imaged for lignin autofluorescence by excitation at 405 nm. Emission was detected with
607 a spectral detector set between 410 nm and 524 nm. For simultaneous detection of lignin
608 autofluorescence, chloroplasts, and ICR2-3xYPet, lambda mode was used with excitation at
609 514 nm. Emission was detected with a spectral detector set between 520 nm and 690 nm. Z-
610 stacks were taken, and maximum intensity images were created.

Analysis of microtubule dynamics
612 Microtubule dynamics was analyzed by high frequency time-lapse imaging of seedlings of
613 *RFP-MBD*, *icr2-1xRFP-MBD*, and *icr2-2xRFP-MBD* genotypes at 8 DAG. Seedlings were
614 grown on CellView[TM] cell culture dishes, 35/10 mm, glass bottom (Grainer 627860) at a 45º
615 angle, so that roots grew along the glass bottom between the growth medium and the glass.
616 Imaging of root hairs and adjacent root epidermis cells was done at 2-s intervals, 60 frames
617 total, using LSM 780-NLO confocal laser scanning microscope (Zeiss) in fast scanning mode
618 with a 63X water immersion objective, and were visualized by excitation with an argon laser
619 at 561 nm and spectral GaAsP detector set between 570 nm and 695 nm. Quantification of
620 microtubule dynamics was done by tracking of individual microtubule filaments. The
621 tracking data were used to create kymographs, which were then used to calculate microtubule
622 growth and shrinkage rates, the time spent at each condition, as well as the transitions
623 between them and pauses in growth/shrinkage. This analysis of imaging data was performed
624 using the KymoToolBox ImageJ plugin (Zala et al., 2013). Typically, 5-10 microtubule
625 filaments were analyzed per cell and five cells, each from a different plant, were analyzed for

626 each genotype and cell type. Overall, the number microtubule filaments analyzed was 77-
627 113.

Secondary cell wall pits area and pit density per area

629 Analysis of secondary cell wall of the MX pits was carried out on seedling roots at 8 DAG.
630 Roots were imaged using differential interference contrast (DIC) light microscopy after
631 clearing with chloral hydrate:lactic acid (2:1) for 1-3 days. To quantify the area of secondary
632 cell wall pits, pits were manually selected in DIC images and analyzed using ImageJ. Pit
633 density was calculated as the number of secondary cell wall pits divided by the area of MX
634 vessel cells and expressed as the number of pits per 1000 μm^2. Two or three cells were
635 imaged for each plant, and four or five plants were analyzed for each genotype.

Protoxylem lignification

637 Roots at 7 DAG were imaged for lignin autofluorescence by excitation at 405 nm. Emission
638 was detected with a spectral detector set between 410 nm and 524 nm. Z-stacks were taken of
639 6-10 focal planes, and maximum intensity images were created. Analysis was carried out in
640 the maturation zone of the root on maturing PX cells, which at this region have a well-
641 defined spiral pattern. No MX differentiation was detected. Mean distance between lignified
642 spirals was measured using the semi-automated Cell-o-Tape macro for ImageJ (Fiji). Five
643 roots were analyzed for each genotype, and in each plant two PX cells were imaged and
644 quantified.

Analysis of root hairs deformation

646 Root hairs were observed in seedlings grown on 0.5X MS agar medium at 8 DAG. Ten
647 seedlings for each genotype were compared, and the percent of normal and deformed root
648 hairs was scored.

Root hair measurements

650 Root hairs in seedlings at 7 DAG were imaged and measured as previously described
651 (Denninger et al., 2019). The first visible swelling of the cell outline was defined as first
652 bulge, and distance to root tip was measured. Root hair density was analyzed in the next
653 2 mm. Root hair length was measured in a region 3-6 mm away from the root tip.

Light and confocal laser scanning microscopy

655 Brightfield and Nomarsky DIC imaging was performed with an Axioplan-2 Imaging
656 microscope (Zeiss) equipped with an Axio-Cam and a cooled charge-coupled device camera

657   using either 10X, 20X dry, or 63X water immersion objectives with numerical aperture

658   values of 0.5, 0.9, and 1.2, respectively. Laser scanning confocal microscopy and associated

659   brightfield and DIC imaging was performed using LSM 780-NLO confocal laser scanning

660   microscope (Zeiss) with 10X and 20X air objectives and 40X and 63X water immersion

661   objectives with numerical apertures of 0.3, 0.8, 1.2, and 1.15. Fluorescein was visualized by

662   excitation with an argon laser at 488 nm; emission was detected between 493 and 556 nm.

663   Rhodamine was visualized by excitation with an argon laser at 561 nm; emission was

664   detected between 566 and 685 nm. 3xYPet was visualized by excitation with an argon laser

665   set at 514 nm; emission was detected 526 and 570 nm.

Image analysis

667   Image analyses were performed with ZEN 2012 Digital Imaging, Photoshop CS5.1 (Adobe

668   Systems) and ImageJ (FIJI).

Quantifications and statistical analyses

670   Stacked charts and box plots were prepared using JMP (SAS) or Office Excel 2016

671   (Microsoft). Statistically significant differences were determined using the Student's t-test or

672   one-way ANOVA and Tukey-HSD post-hoc analysis, as noted in the figure legends and

673   Tables S2 and S3.

Expression of ICR1-His$_6$ and ICR2-His$_6$ in *E. coli*

675   ICR1-His$_6$ and ICR2-His$_6$ were transformed into the BL21 (Rosetta) *E.coli* strain. Cells were

676   grown at 37 °C to an OD$_{600}$ of 0.5-0.7 and then induced with 1 mM isopropyl-beta-D-

677   thiogalactopyranoside overnight at 16 °C. Immediately after induction cells were harvested

678   by centrifugation at 5000 X g for 15 min at 4 °C, and stored at -80 °C until further use.

Purification of ICR1-His$_6$ and ICR2-His$_6$

680   Protein purification was carried out with the AKTA-prime protein purification system (GE

681   Healthcare). First, cells were homogenized by sonication using VCX500 ultrasonic processor

682   (Sonics & Materials, Inc.) in washing buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM

683   imidazol, and 5% glycerol, pH 8.0) containing 1 mM DTT. ICR1-His$_6$ and ICR2-His$_6$

684   recombinant proteins were purified over a His-TRAP FF column (GE Healthcare) with 1-mL

685   bed volume. The column was washed with 30 mL of washing buffer. The proteins were

686   released with Imidazole Elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole

687   and 5% glycerol, pH 8.0). The proteins were concentrated with Amicon Ultra-15 filters

688   (Millipore) with molecular weight cutoffs of 30 kDa and 50 kDa for ICR1-His$_6$ and ICR2-

689   His$_6$, respectively, at 4,000 g and 4 °C to a final volume of approximately 500 µl. The

690   concentrated protein samples were filtrated through Millex 0.22-µm syringe filters

691   (Millipore) and loaded onto a Superdex 200 HR 10/30 gel-filtration column (GE Healthcare)

692   and eluted with a gel filtration column buffer (50 mM NaH$_2$PO$_4$, pH 7.0). To concentrate the

693   protein, an Amicon Ultra-15 Centrifugal Filter Device was used, protein was centrifuged, and

694   the buffer was exchanged to PEM buffer (0.1 M PIPES, 1 mM EGTA, and 1 mM MgCl$_2$, pH

695   6.9). Purified proteins were again concentrated using the Amicon Ultra-15, divided into

696   aliquots, batch frozen in liquid nitrogen, and kept at −80 °C until further use. Protein

697   concentrations were determined using the BCA Protein Assay kit (Pierce) according to the

698   manufacturer's protocol.

Microtubule co-sedimentation assay

700   Porcine brain tubulin was purified as described (Castoldi and Popov, 2003). For the co-

701   sedimentation assay, 0, 1, 2, 4, 6, 8, and 10 µM purified ICR1-His$_6$ or ICR2-His$_6$ were added

702   to taxol-stabilized microtubules (5 µM tubulin) in PEMT (100 mM PIPES, 1 mM EGTA, 1

703   mM MgCl$_2$, 1 mM GTP, and 20 µM taxol, pH 6.9). The samples were centrifuged at 100,000

704   g at 25 °C for 15 min. Pellets and supernatants were analyzed by 10% SDS-PAGE and

705   visualized by staining the gels with Coomassie Brilliant Blue R 250. His-NtMAP65-1c and

706   BSA were used as positive and negative controls, respectively.

Microtubule immunofluorescence co-localization and *in vitro* bundling assays

708   Rhodamine-labeled tubulin was prepared as previously described (Hyman, 1991). For the co-

709   localization assay, taxol-stabilized microtubules composed of tubulin mixed with rhodamine-

710   labeled tubulin (molar ratio, 1:4) in PEMT were incubated with 3 µM ICR1-His$_6$ or 0.5 µM

711   ICR2-His$_6$ for 15 min at 37 °C and then crosslinked with 20 mM 1-ethyl-3-(3-

712   dimethylaminopropyl) carbodiimide (Pierce Biotechnology) for 5 min at 37 °C. The mixture

713   was then centrifuged at 12,000 g for 5 min, and the pellet was resuspended in PEM buffer

714   preheated to 37 °C. ICR1-His$_6$ and ICR2-His$_6$ were stained with an anti-His antibody

715   (1:5,000) and secondary antibody conjugated with fluorescein (Sigma; F0257; 1:5,000). The

716   solution was then centrifuged at 12,000 g for 5 min, and the pellet was resuspended with

717   PEM buffer preheated to 37 °C. An aliquot of 1 µl was put on a poly-L-lysine coated glass

718   slide (Sigma, P0425) and observed by confocal microscopy. For the *in vitro* bundling assay,

719   the same taxol-stabilized rhodamine-labeled microtubules were incubated with 0.1, 0.5, 1,

720   and 5 µM ICR1-His$_6$ or 0.1, 0.5, 1, and 2 µM ICR2-His$_6$ for 30 min at 37 °C and then treated

721   with 0.005% glutaraldehyde. A 1-µl aliquot of each sample was put on a poly-L-lysine coated

722   glass slide (Sigma, P0425) and observed by confocal microscopy.

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730   **References**

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952   **Figures and legends**

953   **Figure 1: The expression pattern of ICR2.** Expression of ICR2 was analyzed in

954   *pICR2::ICR2-GUS* plants. Expression was detected in **(A)** the root tip, **(B)** lateral root

955   initials, **(C)** developing lateral root, **(D)** stele and root hairs of root differentiation zone, **(E)**

956   vascular tissues and developing stomata in cotyledons, **(F)** vasculature tissues and developing

957   leaves hypocotyl (ICR2 is indicated by arrowheads), **(G and H)** meristemoids and

958   developing guard cells (ICR2 is indicated by arrowheads), **(I)** vasculature in pedicels and

959   receptacles, **(J)** vascular tissues in in sepals, **(K)** the stamen filaments, and **(L)** ovules,

960   developing seeds, and siliques. Scale bars correspond to 50 µm for panels A-F and 20 µm for

961   panels G-H. See also Figure 1-Supplement 1.

962   **Figure 2: Metaxylem secondary cell wall pit size and density is regulated by ICRs. (A-**

963   **K)** DIC images of seedlings at 8 DAG, taken 3 or 4 cells shootward from the initiation of

964   metaxylem differentiation. Scale bars, 10 µm. **(L)** Examples of pit area (yellow) and density

965   (number of pits divided by area of red polygon) measurements. Scale bar, 10 µm. **(M)** Mean

966   pit area (μm^2). n>100 pits. **(N)** Pit density (number of pits per 1000 μm^2). n>10 cells.

967   Statistical analyses by one-way ANOVA. For panel M: $F_{(10, 1400)}=49.4836$, $p<0.0001$ and

968   for panel N: $F_{(10, 122)}=17.0351$, $p<0.0001$. Means with different letters are significantly

969   different (Tukey's HSD, $p<0.05$). The boxes are the interquartile ranges, the whiskers

970   represent the 1st and 4th quartiles, and the lines are the averages. See source data-Figure 2M,

971   Figure 2N. See also Figure 2-Supplement 1, Figure 2-Supplement 2.

972   **Figure 3: Protoxylem secondary cell wall coil density is regulated by ICR5. (A-I)** Lignin

973   auto-fluorescence in PX cells, imaged 3 or 4 cells shootward for initiation of differentiation.

974   Scale bars, 20 µm. **J)** An exemplary image showing how the distance between the lignin coils

975   was measured along white line. Images are Z-stacks of 8-11 confocal planes, 20x lens. **K)**

976   Mean distance in µm between the lignin coils. Statistical analysis by one-way ANOVA: $F_{(8,}$

977    $_{86)}=34.4972$, $p<0.0001$.   Means with different letters are significantly different (Tukey's

978   HSD, $p<0.05$). The boxes are the interquartile ranges, the whiskers represent the 1st and 4th

979   quartiles, and the lines are the averages. n=10 for each line. See source data-Figure 3K.

980   **Figure 4: *icr2* mutants develop deformed split root hairs. (A-K)** Representative images

981   for each genotype. Arrowheads highlight split root hairs. Scale bars, 50 µm. **(L)** Percentage

982   of abnormal root hairs in seedlings at 8 DAG. n=5 seedlings for each line. See source data-

983   Figure 4L. See also Figure 4-Supplement 1.

Figure 5: ICR2 is a microtubule-associated protein that interacts with ROP GTPases.
(A) Image of *N. benthamiana* leaf epidermis that transiently express ICR2-3xYPet. Scale bar, 10 μm. **(B)** Image of *N. benthamiana* leaf epidermis that transiently express GFP-ROP11. Scale bar, 10 μm. **(C-L)** BiFC images of *N. benthamiana* leaf epidermis that transiently express ICR2-YC and C) YN-rop6^{CA}, **D)** YN-rop9^{CA}, **E)** YN-rop10^{CA}, **F)** YN-rop11^{CA}, **G)** YN-ROP2, **H)** YN-ROP4, **I)** YN-ROP6, **J)** YN-ROP9, **K)** YN-ROP10, and **L)** YN-ROP11. Scale bars for panels C-F, 10 μm and for panels G-L, 20 μm. YFP signal for panels G-I was separated by linear unmixing. Images for panels A, C-F are Z-projections of multiple confocal sections. **(M)** Yeast two-hybrid assays of ICR2 with ROP2, ROP4, ROP6, ROP9, ROP10, and ROP11. -LT, Leu-, Trp-deficient medium; -LTH, Leu-, Trp-, His-deficient medium; 3AT – 3-amino-1,2,4-triazole. Numbers above the panels denote dilution series. See also Figure 5-Supplement 1.

Figure 6: Microtubule-associated ICR2 is recruited to GEF3$_{PRONE}$-GAP1-ROP domains.
Images of *N. benthamiana* leaf epidermis that transiently express GFP-tagged ROPs (green) **(A)** ROP2, **(B)** ROP4, **(C)** ROP6, **(D)** ROP9, **(E)** ROP10, and **(F)** ROP11 with ICR2-mCherry (red), untagged GAP1, and the GEF3 PRONE domain (GEF3p). Scale bars, 10 μm in all panels.

Figure 7: ICR2 interacts with microtubules *in vitro*. (A) Coomassie brilliant blue stained SDS-PAGE of recombinant ICR2-His$_6$ co-sedimented with taxol-stabilized microtubules pre-polymerized from 5 μM tubulin. His-AtMAP65-1 was used as a positive control and GFP-His as a negative control. **(B)** Quantification of the ICR2-His$_6$ band in panel A. The plot averages three replicates. **(C-H)** Immunofluorescence images of rhodamine-labeled tubulin (red) mixed with non-labeled tubulin and polymerized into microtubules in the presence of fluorescein-labeled ICR2-His$_6$ (green). Arrowhead in panel D indicates ICR2 on a microtubule. Denatured ICR2-His was used as control. Scale bars, 10 μm. **(I-K)** Images of rhodamine-labeled tubulin bundling in the presence of 0, 0.1, or 2 μM ICR2. See source data-Figure 7B. See also Figure 7-Supplement 1, Figure 7-Supplement 2, Figure 7-Supplement 3.

Figure 8: ICR2-3xYPet colocalizes with microtubules. (A-C) Images of *icr2-2* roots that express ICR2-3xYPet (green) and RFP-MBD (magenta). ICR2 was detected during interphase at the root tip, in the lateral root cap, and in dividing cells in the root cortex, as indicated by arrowheads. Scale bars, 10 μm. **(D-F)** Images of root hair shank in *icr2-2* plants that express ICR2-3xYPet (green) and RFP-MBD (magenta). Scale bars, 10 μm. **(G-I)**

1016 Images of differentiation/elongation zone epidermis in *icr2-2* plants that express ICR2-
1017 3xYPet (green) and RFP-MBD (magenta). Scale bars, 10 μm. **(J)** Fluorescence intensity
1018 profile of MRF-MBD and ICR2-3xYPet signals along the white lines in panels G-I. See
1019 source data-Figure 8J.

1020 **Figure 9: ICR2-3xYPet co-localizes with microtubules during all stages of cell division.**
1021)**A)** Image of lateral root cap of *icr2-2* plant expressing ICR2-3xYPet (green) and RFP-MBD
1022 (magenta). The expanding phragmoplast is indicated by arrowheads. Scale bars, 5 μm. **(B)**
1023 Tracking of a single cell undergoing cell division and cytokinesis in *icr2-2* plant expressing
1024 ICR2-3xYPet (green) and RFP-MBD (magenta). Colocalization is observed in all mitotic
1025 stages: preprophase band in prophase, spindle during metaphase and anaphase, and
1026 phragmoplast in telophase. Scale bars, 5 μm.

1027 **Figure 10: ICR2 is detected in differentiating xylem cells but not differentiated cells. (A-**
1028 **D)** Images of secondary cell walls of cotyledon mesophyll cells undergoing dedifferentiation
1029 to tracheary elements: **A)** lignin autofluorescence (cyan), **B)** ICR2-3xYPet (yellow), **C)**
1030 chlorophyll (red), and **D)** overlay. Newly differentiating cells are indicated by arrows and
1031 fully differentiated cells by asterisks). Scale bars, 10 μm. **(E)** Fluorescence intensity profile of
1032 ICR2-3xYPet (yellow) lignin autofluorescence (cyan) along the line in panels A and B. See
1033 source data-Figure 10E.

1034 **Figure 11: ICR2 affects microtubule dynamics in the root epidermis and root hairs**. **(A)**
1035 Representative time-lapse imaging of RFP-MBD-labeled microtubules. Filament extension,
1036 pause, and shrinkage are labeled with green, blue, and red dots, respectively. Scale bars, 5
1037 μm. **B)** Kymographs tracking RFP-MBD-labeled microtubule tips in *Col-0, icr2-1, icr2-2*
1038 seedlings. **(C)** Quantification of microtubule extension and shrinkage rates. Means with
1039 different letters are significantly different (Tukey's HSD, $p<0.05$). One-way ANOVA results
1040 are in Table S3. The boxes are the interquartile ranges, the whiskers represent the 1st and 4th
1041 quartiles, and the lines are the averages. n>77 filaments per genotype. See source data-Figure
1042 11. See also Figure 11-Supplement 1, Figure 11-Supplement-2.

1043 **Supplement figures**
1044 **Figure 1-Supplement 1: *ICR2* expression pattern and during development.**
1045 Transcriptomics data of *ICR2* expression levels. Figure adapted from the *Arabidopsis* eFP
1046 Browser (https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Figure 2-Supplement 1: Map of the *ICR2* locus showing three *icr2* T-DNA insertion mutant alleles. (A) *icr2-1* (*GK567F02*) T-DNA insertion is in the third exon, 297 bp before the stop codon; *icr2-2* (*GK281B01*) insertion is in the first exon, 19 bp after the initiation codon; *icr2-3* (*GK159B08*) insertion is in the third exon, 472 bp after the start of the exon. **(B)** *icr2* mutant plants have no *ICR2* mRNA transcript (1,750 bp). *Ubiquitin 5* (UBQ) was detected as a control (650 bp).

Figure 2-Supplement 2: The CRISPR/Cas9 generated mutations in *ICR2*, *ICR3,* and *ICR5*. (A) Positions of the gRNA target sequence for each gene. **(B)** Sequences of InDels in the mutant alleles aligned with the WT *Col-0* allele. Inserted bases are marked in red. Dashed lines indicate deletions. **C)** Predicted amino acid sequences of the mutants. Asterisk indicates stop codon.

Figure 4-Supplement 1: Root hair initiation sites, density, and length in WT and single, double, and triple mutants. Quantification of **(A)** normalized distance of first bulge from root tip (n≥16), **(B)** length of root hairs (n≥142), and **(C)** density of root hairs (n≥16). No significant differences were identified between the lines using ANOVA. The boxes are the interquartile ranges, the whiskers represent the 1st and 4th quartiles, and the lines are the averages. See source data-Figure 4-Supplement 1 A,B,C.

Figure 5-Supplement 1: ICR2 localization with a microtubule marker is disrupted by oryzalin. (A) Image of *N. benthamiana* leaf epidermis that transiently expresses ICR2-3xYPet. **(B)** Image of *N. benthamiana* leaf epidermis that transiently expresses ICR2-3xYPet after oryzalin treatment, which disrupts microtubules. **(C-E)** Image of *N. benthamiana* leaf epidermis that transiently expresses ICR2-3xYPet and RFP-MBD. **(F-H)** Image of *N. benthamiana* leaf epidermis that transiently expresses ICR2-3xYPet and RFP-MBD after oryzalin treatment. Scale bars are 10 µm.

Figure 7-Supplement 1: ICR1 interacts with microtubules *in vitro*. (A) Coomassie brilliant blue stained SDS-PAGE of recombinant ICR1-His$_6$ co-sedimented with taxol-stabilized microtubules pre-polymerized from 5 µM tubulin. His-AtMAP65-1 was used as a positive control and GFP-His as a negative control. **(B)** Quantification of the ICR1-His$_6$ band. The plot averages three replicates. See source data-Figure 7-Supplement 1 B.

Figure 7-Supplement 2: ICR1 co-localizes with microtubules *in vitro*. Immunofluorescence images showing the specific binding of **(A-C)** recombinant fluorescein-

1078   labeled ICR1-His$_6$ (green) along rhodamine-labeled microtubules (red). Inset in panel B is an

1079   enlargement of the region highlighted by arrowheads in B and C. (**D-F**) Imaging of denatured

1080   ICR1-His used as control. Scale bars, 10 μm in all panels.

1081   **Figure 7-Supplement 3: ICR1 and ICR2 induce microtubule bundling.** Rhodamine-

1082   labeled microtubules (red) formed bundles when incubated with a range of concentrations of

1083   recombinant ICR1-His$_6$ and ICR2-His$_6$. The panels displaying mock, 0.1 μM ICR2, and 2

1084   μM ICR2 samples are duplicates of Figure 7 panels I, J, and K, respectively, and are

1085   presented here for the sake of clarity.

1086   **Figure 11-Supplement 1. ICR2 affects pause time of microtubules between growth and**

1087   **shrinkage.** Fraction of the time microtubule filaments were extending (blue), pausing (green)

1088   or shrinking (red). Means with different letters are significantly different (Tukey's HSD,

1089 $p < 0.05$). One-way ANOVA results are in Table S3. The boxes are the interquartile ranges,

1090   the whiskers represent the 1st and 4th quartiles, and the lines are the averages. $n \geq 77$ for each

1091   genotype. See source data-Figure 11-Supplement 1.

1092   **Figure 11-Supplement-2: The effects of ICR2 on microtubule dynamics as detected by**

1093   **frequency of transitions.** Number of transitions per second for each genotype at each cell

1094   type. In, shrinkage; Out, extension, Pause, pause. Means with different letters are

1095   significantly different (Tukey's HSD, $p < 0.05$). One-way ANOVA values are in Table S3.

1096   The boxes are the interquartile ranges, the whiskers represent the 1st and 4th quartiles, and the

1097   lines are the averages. $n \geq 77$ for each genotype. See source data-Figure 11-Supplement 2.

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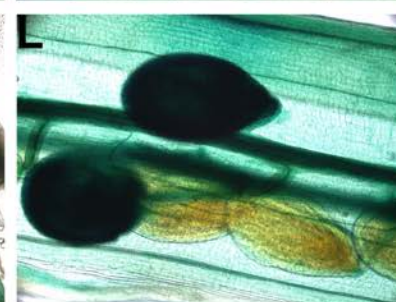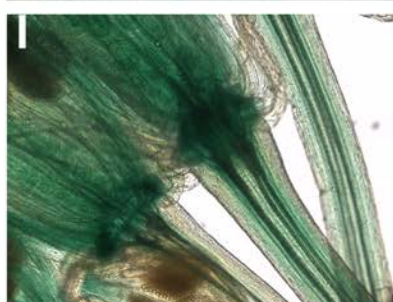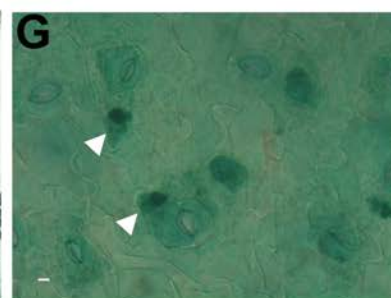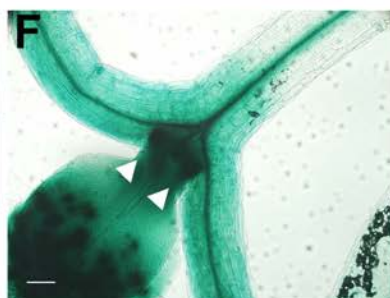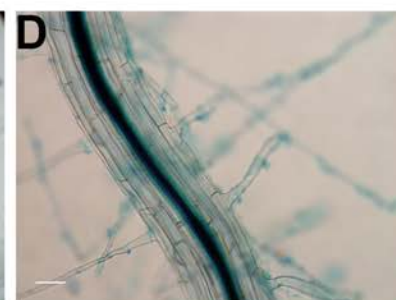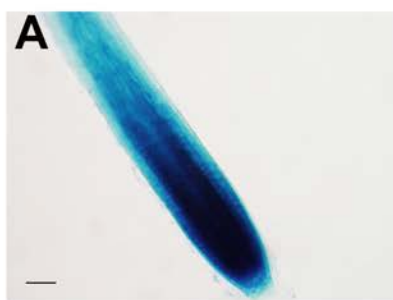
GCOS expression signal (TGT=100, Bkg=20)

Tissue/Stage	
Dry seed	
Imbibed seed, 24 h	
1st Node	
Flower Stage 12, Stamens	
Cauline Leaf	
Cotyledon	
Root	
Entire Rosette After Transition to Flowering	
Flower Stage 9	
Flower Stage 10/11	
Flower Stage 12	
Flower Stage 15	
Flower Stage 12, Carpels	
Flower Stage 12, Petals	
Flower Stage 12, Sepals	
Flower Stage 15, Carpels	
Flower Stage 15, Petals	
Flower Stage 15, Sepals	
Flower Stage 15, Stamen	
Flowers Stage 15, Pedicels	
Leaf 1 + 2	
Leaf 7, Petiole	
Leaf 7, Distal Half	
Leaf 7, Proximal Half	
Hypocotyl	
Root	
Rosette Leaf 2	
Rosette Leaf 4	
Rosette Leaf 6	
Rosette Leaf 8	
Rosette Leaf 10	
Rosette Leaf 12	
Senescing Leaf	
Shoot Apex, Inflorescence	
Shoot Apex, Transition	
Shoot Apex, Vegetative	
Stem, 2nd Internode	
Mature Pollen	
Seeds Stage 3 w/ Siliques	
Seeds Stage 4 w/ Siliques	
Seeds Stage 5 w/ Siliques	
Seeds Stage 6 w/o Siliques	
Seeds Stage 7 w/o Siliques	
Seeds Stage 8 w/o Siliques	
Seeds Stage 9 w/o Siliques	
Seeds Stage 10 w/o Siliques	
Vegetative Rosette	

A ICR2 genomic locus

Exon 1 Exon 2 Exon 3

icr2-2 icr2-3 icr2-1

B UBQ ICR2

WT 1 2 3 WT 1 2 3

1750bp

650bp

A — Col-0
B — icr2-1
C — icr2-2
D — icr2-3
E — icr5-1
F — icr5-2
G — icr2-4/icr5-1
H — icr2-5/icr5-3
I — icr2-5/icr3-1/icr5-3
J

K

Mean distance (microns)

A   A   A   A   B   B   B   B   B

Col-0   icr2-1   icr2-2   icr2-3   icr5-1   icr5-2   icr2-4/icr5-1   icr2-5/icr5-3   icr2-5/icr3-1/icr5-3

A Col-0 B icr2-1 C icr2-2 D icr2-3

E icr2-1xICR2-Ypet F icr2-2xICR2-Ypet G icr5-1 H icr5-2

I icr2-4/icr5-1 J icr2-5/icr5-3 K icr2-5/icr3-1/icr5-3

L

A ICR2-3xYpet

B GFP-ROP11

C ICR2-YC + YN-rop6CA

D ICR2-YC + YN-rop9CA

E ICR2-YC + YN-rop10CA

F ICR2-YC + YN-rop11CA

G ICR2-YC + YN-ROP2

H ICR2-YC + YN-ROP4

I ICR2-YC + YN-ROP6

J ICR2-YC + YN-ROP9

K ICR2-YC + YN-ROP10

L ICR2-YC + YN-ROP11

M

ICR2

-LT
1 1:10 1:10² 1:10³

-LTH + 1 mM 3AT
1 1:10 1:10² 1:10³

ROP2
ROP4
ROP6
ROP9
ROP10
ROP11

Empty vector

A   ICR2-3xYpet

B   ICR2-3xYpet

C   ICR2-3xYpet

D   RFP-MBD

E   O\L

F   ICR2-3xYpet

G   RFP-MBD

H   O\L

A GAP1 GEF3p — GFPROP2 / ICR2mCh / O/L

B GAP1 GEF3p — GFPROP4 / ICR2mCh / O/L

C GAP1 GEF3p — GFPROP6 / ICR2mCh / O/L

D GAP1 GEF3p — GFPROP9 / ICR2mCh / O/L

E GAP1 GEF3p — GFPROP10 / ICR2mCh / O/L

F GAP1 GEF3p — GFPROP11 / ICR2mCh / O/L

A

+ICR2

| | 0 | 1 | 2 | 4 | 6 | 8 | 10 | 10 | +MAP65 10 | +GFP-His 10 | (µM) |
Tubulin (5 µM): + + + + + + + − + +

ICR2-His
Tubulin

B

ICR2-His bound mol/mol tubulin bound vs ICR2-His (µM)

C Rh-tubulin
D ICR2-His 0.5µM
E O\L

F Rh-tubulin
G ICR2-His 0.5µM denatured
H O\L

I Mock
J ICR2-His 0.1µM
K ICR2-His 2µM

A

+ICR1

+MAP65 +GFP-His

	0	1	2	4	6	8	10	10	10	10	(μM)

Tubulin (5 μM)   + + + + + + + − + +

Tubulin
ICR1-His

B

ICR1-His

A — Rh-tubulin
B — ICR1-His 1µM
C — O\L
D — Rh-tubulin
E — ICR1-His 1µM denatured
F — O\L

ICR1-His	ICR2-His
mock	mock
0.1µM	0.1µM
0.5µM	0.5µM
1µM	1µM
5µM	2µM

A RFP-MBD   B ICR2-3xYPet   C Overlay

D RFP-MBD   E ICR2-3xYPet   F Overlay

G RFP-MBD   H ICR2-3xYPet   I Overlay

J

— ICR2-Ypet   — RFP-MBD

A

B

	Prophase	Metaphase	Anaphase	Telophase

A — Lignin

B — ICR2-Ypet
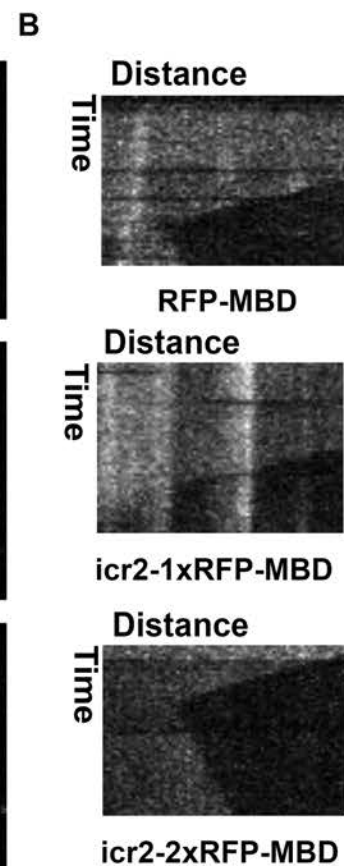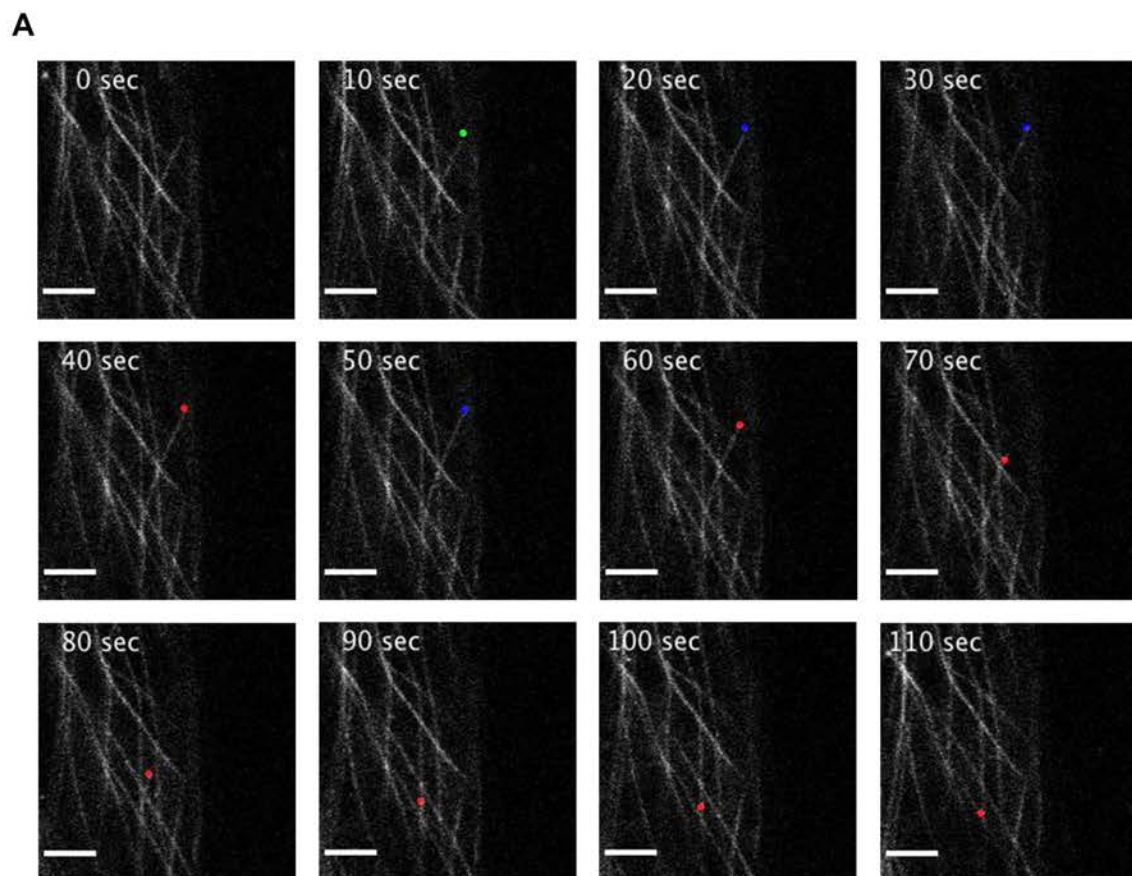
C — Chlorophyl

D — O/L

E

ICR2-YFP — Lignin

A

B

RFP-MBD

icr2-1xRFP-MBD

icr2-2xRFP-MBD

C

Transitions per second