1 **Functional analysis of the chromosomal passenger complex in** 2 **Arabidopsis**

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4 Shinichiro Komaki^{1,2}, Hidenori Takeuchi^{3,4}, Yuki Hamamura², Maren Heese², Takashi
5 Hashimoto¹, and Arp Schnittger^{2,*}

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9 ¹Nara Institute of Science and Technology, Graduate School of Biological Sciences,
10 8916-5 Takayama, Ikoma, Nara 630-0192, Japan

11 ²University of Hamburg, Institute for Plant Sciences and Microbiology, Department
12 of Developmental Biology, Ohnhorststr. 18, D-22609 Hamburg, Germany

13 ³Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Furo-
14 cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan.

15 ⁴ Institute for Advanced Research, Nagoya University, Furo-cho, Chikusa-ku,
16 Nagoya, Aichi 464-8602, Japan

17 *For correspondence arp.schnittger@uni-hamburg.de, Phone: +49 40 428 16 502,

18 Fax: +49 40 428 16 503

19 **SUMMARY**

20 A key regulator of cell division in all eukaryotes is the kinase Aurora B, which is
 21 encoded by the Aurora 3 (AUR3) gene in Arabidopsis. Aurora B has at least two
 22 central functions during cell division. On the one hand, it is essential for the
 23 correct, i.e. balanced, segregation of chromosomes in mitosis and meiosis by
 24 controlling kinetochore function. On the other hand, Aurora B acts at the division
 25 plane, where it is necessary to complete cytokinesis. To accomplish these two
 26 spatially distinct functions, Aurora B in animals is guided to its sites of action by
 27 Borealin, INCENP, and Survivin that build together with Aurora B the chromosome
 28 passenger complex (CPC). However, besides Aurora homologs, only a candidate
 29 gene with restricted homology to INCENP has so far been described in Arabidopsis
 30 raising the question whether there exists a full complement of the CPC in plants
 31 and how Aurora homologs are targeted subcellularly. Here, we have identified and
 32 functionally characterized a Borealin homolog, BOREALIN RELATED (BORR), in
 33 Arabidopsis. This, together with detailed localization studies including the putative
 34 Arabidopsis INCENP homolog, supports the existence of a CPC in plants.

35 INTRODUCTION

36 Equal chromosome segregation during cell division is crucial for survival, growth,
37 and reproduction of every organism. Chromosome segregation is assured by the M-
38 phase checkpoint, which involves two regulatory units, the spindle assembly
39 checkpoint (SAC) and the chromosomal passenger complex (CPC) (Carmena et al.,
40 2012).

41 Chromatids are held together by Cohesin, a proteinaceous ring-like
42 structure, which needs to be cleaved by Separase to allow their separation in
43 anaphase. The SAC inhibits the Anaphase Promoting Complex/Cyclosome (APC/C),
44 an E3 ubiquitin ligase, which mediates the degradation of Securin, an inhibitor of
45 Separase, until all kinetochores are properly attached to microtubules of opposing
46 poles (London and Biggins, 2014; Komaki and Schnittger, 2016).

47 The CPC has been well characterized in yeast and mammals, where it has
48 been found to consist of four proteins: Aurora kinase B (Aurora B), Borealin, Inner
49 centromere protein (INCENP), and Survivin. The CPC fulfills several functions
50 during mitosis. In particular, it is involved in ensuring that all kinetochores are
51 attached to microtubules emanating from opposing poles (Kitagawa and Lee,
52 2015). The CPC localizes to inner centromere where it activates Aurora B in

53 response to low inter-kinetochore tension. Active Aurora B phosphorylates
54 kinetochore proteins leading to the destabilization of erroneous microtubule
55 attachments. Once proper kinetochore-microtubule attachments are established
56 from opposing poles, which gives high inter-kinetochore tension, Aurora B is
57 spatially separated from kinetochores resulting in proper bistable spindle
58 formation.

59 Aurora B belongs to the Aurora kinase family of serine/threonine kinases that
60 are highly conserved in the eukaryotic kingdom (van der Waal et al., 2012; Weimer
61 et al., 2016). While yeast possesses a single Aurora homolog, mammals have three
62 Aurora kinases, Aurora A, Aurora B, and Aurora C, among which only Aurora C acts
63 in meiosis (Goldenson and Crispino, 2015). Since these three kinases share a
64 common consensus phosphorylation motif, it is thought that interacting proteins
65 are important for their localization and substrate specificity.

66 Aurora A interacts with the spindle assembly factor TPX2, and localizes to
67 spindle microtubules to regulate spindle assembly (Gruss and Vernos, 2004). In
68 contrast, Aurora B and Aurora C, the catalytic subunit of the CPC, is involved in the
69 correction of erroneous kinetochore-microtubule attachments, activation of the
70 SAC, and cytokinesis in mitosis and meiosis. These diverse functions are based on

71 its dynamic localization pattern controlled by the three non-catalytic subunits of
72 the CPC (van der Horst and Lens, 2014).

73 INCENP is the largest non-catalytic subunit of the CPC, which in animals and
74 yeast directly binds to all other components of the complex. Borealin and Survivin
75 interact with the conserved N-terminal region of INCENP while Aurora B binds to
76 the C-terminal domain of INCENP, called IN-box (Carmena et al., 2012). The IN-box
77 is required for interaction with and activation of Aurora B (Honda et al., 2003).

78 While the N-terminus of Borealin acts as the INCENP-binding region its C-
79 terminus contains a homo-dimerization domain that is involved in a stable CPC
80 localization at centromeres (Bekier et al., 2015). In addition, the phosphorylation
81 status of the central part affects the centromere localization and steady-state level
82 of Borealin itself (Kaur et al., 2010; Date et al., 2012).

83 Aurora kinases in plants are categorized into two groups, alpha-Aurora
84 and beta-Aurora, based on the localization pattern and sequences (Weimer et al.,
85 2016). Interestingly, these two groups have mixed features of the animal Aurora A
86 and Aurora B/C groups. For instance, AUR1 and AUR2 in Arabidopsis, both
87 belonging to the alpha class, localize to spindle microtubules, which is reminiscent
88 of Aurora A, but they also localize to the central region of phragmoplasts

89 paralleling Aurora B/C at the cell cleavage site. AUR3, a member of the beta-Aurora
90 group, localizes to kinetochores, similar to Aurora B (Komaki and Schnittger, 2017),
91 but in contrary to Aurora B, it does not accumulate at the division plane (Demidov
92 et al., 2005).

93 While *aur1* and *aur2* single mutants do not show any obvious growth
94 alterations, the *aur1 aur2* double null mutant is gametophytic lethal (Van Damme
95 et al., 2011). A weak loss-of-function *aur1 aur2* double mutant exhibits altered
96 division plane orientation, reduced pollen viability, and enhanced vascular cell
97 differentiation. These defects can be rescued by expressing either AUR1 or AUR2
98 but not AUR3, indicating that alpha-Auroras and beta-Auroras have distinct
99 functions (Van Damme et al., 2011; Demidov et al., 2014; Lee et al., 2019).

100 Given the sparse information about the non-catalytic subunits of the plant
101 CPC, it is not even clear whether there is a conserved CPC function in plants. The
102 putative homolog of INCENP in Arabidopsis has a long N-terminal region of
103 unknown function, which is conserved only in plants. Although Arabidopsis *incenp*
104 mutants, also known as *wyrd* (*wyr*), show defects in gametophytic cell division
105 (Kirioukhova et al., 2011), it is still unclear whether Arabidopsis INCENP acts as
106 part of a putative plant CPC because of missing information about its localization

107 and binding partners.

108 Here, we present the identification and functional characterization of an
 109 Arabidopsis Borealin homolog, which colocalizes with the INCENP homolog to the
 110 inner centromere and the central domain of the phragmoplast. We also observed
 111 that only AUR3 acts as the catalytic subunit of the plant CPC. These data
 112 underscore the mixed features of plant Aurora kinases.

113

114

115 **RESULTS**

116 **Identification of a putative Borealin homolog in plants**

117 As a first step to determine whether plants have a functional CPC, we searched for
 118 Borealin and Survivin homologs in the Arabidopsis genome. Given that the putative
 119 INCENP homolog of Arabidopsis, WYR, shares only very weak similarities with its
 120 animal counterpart (Kirioukhova et al., 2011), we expected the same to be the case
 121 for Borealin and Survivin. Indeed, standard BLAST searches did not result in the
 122 identification of likely candidates. Therefore, we made use of the fact that Borealin
 123 is transcriptionally controlled by the tumor suppressor protein Retinoblastoma in
 124 animals (Cam et al., 2004; Date et al., 2007). Mining a dataset of the genome-wide
 125 binding sites of the Arabidopsis Retinoblastoma homolog RETINOBLASTOMA-
 126 RELATED (RBR1), revealed several unknown genes likely involved in cell division
 127 control (Bouyer et al., 2018) (Sup Fig. 1). One of these genes, *AT4G39630*, showed a
 128 weak similarity to Borealin and will be referred to as *BOREALIN-RELATED (BORR)*
 129 (Supplemental Fig. S1A).

130 The C-terminal region of the corresponding protein shows 37% similarity
 131 with the central region of human Borealin, which includes highly conserved
 132 consensus sites for CDK phosphorylation (Fig. 1A). A second stretch of homology

133 can be found in the N-terminus, which is predicted to adopt an alpha-helical
134 structure. In animals and yeast this N-terminal alpha-helix forms a three-helical
135 bundle with INCENP and Survivin (Fig. 1A) (Jeyaprakash et al., 2007). Notably, the
136 Arabidopsis BORR is considerably shorter than the human homolog (233 aa versus
137 280 aa), partly as the result of a truncated C-terminal domain. Using *BORR* as a
138 template, we found putative Borealin genes in all branches of the plant kingdom,
139 including angiosperms, gymnosperms, pteridophytes, bryophytes, and algae (Fig.
140 1B - phylogenetic tree).

141

142 **Phenotypic analysis of *borr* mutants**

143 Since no mutants for *BORR* were available in the public mutant collections of
144 Arabidopsis, we generated a mutant by CRISPR/Cas9. The resulting *borr-1* allele
145 has a T insertion in the 2nd exon, i.e. between nucleotides 280 and 281 downstream
146 of the start codon leading to a stop codon and a predicted truncated protein of 67
147 amino acids (Supplemental Fig. S1A). While the heterozygous *borr-1* plants grow as
148 the wildtype, we could never obtain homozygous mutant plants. Consistently, we
149 observed aborted seeds and undeveloped ovules in siliques of *borr-1 +/-* (Fig.
150 2A,B).

151 To assess whether this lethal phenotype was indeed due to the frameshift
152 mutation in the *BORR* gene, we constructed reporter lines in which the genomic
153 region of *BORR* was fused to an ORF encoding for GFP. Expression of either an N-
154 terminal (GFP:BORR) or C-terminal (BORR:GFP) GFP fusion to BORR
155 complemented the lethal phenotype of *borr-1* mutant plants corroborating that
156 loss of BORR affects plant reproduction.

157 To address the nature of the lack of homozygous *borr-1* mutants, we
158 conducted reciprocal crosses of heterozygous mutants with the wildtype. When we
159 used *borr-1* +/- as male parent, the transmission rate was determined to be 44%.
160 Consistently, 96% of *borr1*+/- pollen resembled wild-type pollen and based on
161 DAPI-staining, contained two sperm cells.

162 In contrast, when we used *borr-1* +/- as female parent, the transmission
163 rate was reduced to 29%, indicating that *BORR* is especially needed for the
164 development and/or function of the female gametophyte. However, when analyzing
165 the mature female gametophyte no obvious developmental defects were observed
166 suggesting that the reduced transmission of *borr-1* through the female
167 gametophyte originated shortly before or after/during fertilization. In accordance
168 with this, we found 12% unfertilized ovules/early arrested seeds and 19% late

169 aborted seeds when we used *borr-1 +/-* as the female (n=250) in contrast to 3%
 170 unfertilized ovules/early arrested seeds and 0.3% late aborted seeds in control
 171 crosses when we used the wildtype as the female and male parent (n=299)(Fig.
 172 2A,B).

173 In addition, we observed abnormal embryo development, including
 174 delayed and distorted embryos or embryo-like structures in approximately 10% of
 175 all ovules/seeds analyzed when *borr-1 +/-* was used as the female parent in crosses
 176 with the wildtype as a male versus 4% in the control crosses supporting a female
 177 gametophytic effect of *borr* (n=313 and n=408 for the control cross) (Fig. 2C,D).
 178 One likely explanation for this is that the divisions leading to the development of
 179 the embryo sac cause aneuploidy in *borr* that preclude and/or severely interfere
 180 with embryo development.

181 In addition, *BORR* is needed during embryo development since the number
 182 of seeds with embryonic defects almost doubled in self-fertilized *borr-1* mutants
 183 (Fig. 2D). Thus, *BORR* is an essential gene needed for cell proliferation and
 184 development during early stages of the plant life cycle.

185 To address the *BORR* function after embryo development, we generated
 186 *BORR* knockdown plants by expressing two artificial microRNAs (amiRNA)

187 targeted against the 2nd (*amiBORR#1*) and 3rd exon (*amiBORR#2*) of *BORR*,
 188 respectively (Fig. 3A). Most transgenic plants expressing *amiBORR#1* (19 out of 25)
 189 and *amiBORR#2* (15 out of 20) showed a dwarf phenotype. For the following
 190 analyses, we selected two transgenic plants for each construct (*amiBORR#1-1*,
 191 *amiBORR#1-2*, *amiBORR#2-1* and *amiBORR#2-2*). All four knockdown plants had a
 192 similar level of *BORR* transcript reduction (Fig. 3B) and had a dwarf phenotype
 193 with curled leaves during the vegetative stage (Supplemental Fig. S2A). At the
 194 flowering stage, all *BORR* knockdown plants were bushy and exhibited a typical
 195 *bonsai* phenotype (sometimes also called *broom stick* phenotype), which is
 196 commonly observed in mutants with low APC/C activity and is characterized by
 197 short inflorescences, which are often curled at the very end with only a few
 198 developing siliques (Supplemental Fig. S2B and C) (Saze and Kakutani, 2007;
 199 Zheng et al., 2011).

200 Primary root growth was also compromised in all *BORR* knockdown plants
 201 (Fig. 3C and D). Microscopic analyses of *amiBORR#1-1* revealed that the root
 202 meristem size was reduced (Fig. 3E-G). Moreover, an aberrant pattern of cell
 203 divisions was found in the columella of all roots analyzed (25 out of 25). In 40% of
 204 all seedlings (10 out of 25), altered division patterns were also present in the

205 epidermal, cortex and endodermal layers underlining that *BORR* is required for
206 proper cell division (Fig. 3E).

207 To assess whether Arabidopsis *BORR* has a function in chromosome
208 segregation as well, *amiBORR#1-1* was introgressed into a transgenic line
209 expressing both a microtubule (RFP:TUA5) and a centromere (GFP:CENH3) marker
210 (Komaki and Schnittger, 2017). Indeed, we could frequently observe lagging
211 chromosomes in *amiBORR#1-1* plant cells (9 out of 50), a phenotype that hardly
212 occurred in the wildtype control plant cells (1 out of 50) (Fig. 3H and I). To
213 examine whether the lagging chromosomes are related to a compromised AUR3
214 localization, we introduced a previously generated *AUR3:GFP* reporter into
215 *amiBORR#1-1* plants (Komaki and Schnittger, 2017). While some AUR3:GFP signal
216 could be still detected at the kinetochores in *amiBORR#1-1* plants, the signal
217 intensity was much weaker than in wild-type plants, suggesting that Arabidopsis
218 BORR ensures chromosome segregation through AUR3 localization (Fig. 3J and K).
219 Taken together, corresponding to Borealin function in animals, *BORR* is required for
220 proper chromosome segregation and cell division in Arabidopsis.

221

222 **Interaction scheme of CPC components in Arabidopsis**

223 To reveal the molecular network of the Arabidopsis CPC, we investigated the
 224 interaction of BORR with INCENP and the three Aurora kinases of Arabidopsis.
 225 Although Borealin has a conserved coiled-coil domain, which is known as an
 226 INCENP binding site in other organisms (Jeyaprakash et al., 2007), an interaction
 227 between BORR and INCENP was not detected by a yeast two-hybrid interaction
 228 assay (Y2H assay) (Fig. 4A). We next performed an *in vivo* co-immunoprecipitation
 229 (IP) assay. To this end, anti-GFP immuno-precipitates from total protein extracts of
 230 plants expressing both *BORR:RFP* and *GFP:INCENP* or from plants expressing
 231 *BORR:RFP* and free *GFP* as a negative control were probed with an anti-RFP
 232 antibody. BORR:RFP was only detected in the extract of plants co-expressing GFP-
 233 INCENP, indicating that Arabidopsis BORR could be part of a CPC *in vivo* (Fig. 4C).

234 Consistent with the topology of the CPC in animal and yeast, we further
 235 found that INCENP interacts specifically with AUR3 through its C-terminal region
 236 while no interaction was detected with AUR1 or AUR2 (Fig. 4A, B). The C-terminal
 237 region of INCENP contains an Aurora B binding domain, called IN-box, which is
 238 conserved from yeast to mammals (Adams et al., 2000). Since Arabidopsis INCENP
 239 also possesses a putative IN-box in its C-terminus, we exchanged Trp1723 to Gly
 240 and Phe1745 to Ala. These residues are conserved in yeast and animals, and the

241 Trp1723 mutation leads to loss of interaction with Aurora B in *in vitro* assays
 242 (Sessa et al., 2005), as well as in chicken culture cells (Xu et al., 2009). Consistent
 243 with a key role of the putative IN-box, Arabidopsis INCENP^{F1745A} partially and
 244 INCENP^{W1723G} completely failed to interact with AUR3 (Fig. 4B).

245

246 **Expression pattern and subcellular localization of the CPC components in** 247 **mitosis**

248 To investigate the spatial and temporal expression pattern of *BORR*, we generated
 249 transgenic plants harboring a genomic fragment of the *BORR* gene fused to a beta-
 250 glucuronidase (GUS) gene before the STOP codon. As expected, strong GUS activity
 251 was observed in both the shoot and root meristems of seedlings, indicating that
 252 *BORR* is expressed in proliferating cells (Fig. 5A-C). In addition, *BORR* was also
 253 found to be strongly expressed in flowering tissues including both the male and
 254 female reproductive organs (Fig. 5D-F).

255 To reveal the subcellular localization of the CPC components in plants, we
 256 made use of the functional *BORR* reporter line used for the complementation
 257 studies above. In addition, we constructed reporter lines in which the genomic
 258 region of *INCENP* was fused to an ORF encoding for GFP. In contrast to *BORR*, only

259 the N-terminal (GFP:INCENP) GFP fusion of INCENP could complement the lethal
260 phenotype of *incenp* homozygous mutants (Supplemental Fig. S1D and E). For
261 AUR3, we used a previously published reporter line (*AUR3:GFP*) in the wildtype
262 background (Komaki and Schnittger, 2017).

263 First, we crossed the three CPC reporter lines with *RFP:TUA5* expressing
264 plants to check the localization of CPC components during mitosis. All three CPC
265 components showed the same localization pattern (Fig. 5G; Supplemental Movies
266 S1-3). In interphase, they localized to the nucleus. Before nuclear envelope
267 breakdown (NEB), the CPC components strongly accumulated at the kinetochores
268 until anaphase onset. Once chromosomes moved toward the spindle poles, they
269 localized to the middle part of the phragmoplast. Interestingly, in early telophase,
270 the CPC components moved back to the nucleus even though there still was an
271 expanding phragmoplast.

272 To corroborate that all three CPC components co-localize, we created
273 transgenic plants which expressed BORR:RFP and GFP:INCENP, BORR:RFP
274 together with AUR3:GFP and RFP:INCENP along with AUR3:GFP (Table 1).
275 Microscopical analyses revealed a tight co-localization pattern through the cell
276 cycle in all three lines, suggesting that BORR, INCENP and AUR3 work together as a

277 plant CPC (Fig. 5H; Supplemental Fig. S3 and 4; Supplemental Movies S4-6).

278 In other organisms, it has been reported that the CPC localizes to the inner
279 centromere region to monitor kinetochore-microtubule attachments (Hindriksen
280 et al., 2017). To reveal the localization of the CPC precisely, we crossed the
281 *BORR:GFP* line with the inner kinetochore marker *RFP:CENH3*. Just after NEB, these
282 two fluorescent reporters showed a high level of overlapping signal. After
283 prometaphase, BORR:GFP localized closer to the inner region of the kinetochore
284 than RFP:CENH3. Yet, both proteins still overlapped in their localization pattern.
285 Shortly before anaphase onset, RFP:CENH3 formed two lines along the metaphase
286 plate, and BORR:GFP localized between these two lines with no overlap (Fig. 6;
287 Supplemental Movie S7). This result demonstrates that the CPC localizes to the
288 inner centromere region in plants.

289 In humans, Borealin function is regulated by phosphorylation. Especially the
290 phosphorylation of S219, a putative Cdk1 target residue, in the central region of the
291 protein affects its stability and centromere localization (Kaur et al., 2010; Date et
292 al., 2012). Since this Cdk1 phosphorylation site resides in the most conserved
293 region between human and plant Borealin is conserved in BORR (Figure 1B), we
294 generated a phospho-mimic (*BORR^{S214D}:GFP*) and a dephospho variant

295 (BORR^{S214A}:GFP) (Dissmeyer and Schnittger, 2011), and transformed them into the
296 heterozygous *borr-1* mutants to evaluate their functionality. Notably, both
297 constructs could complement the lethal phenotype of *borr-1* mutant plants, and
298 showed a normal BORR localization pattern (Supplemental Fig. S1A-C). Thus, the
299 physiological importance of this conserved CDK target motif has yet to be resolved
300 in plants.

301

302 **Expression pattern and subcellular localization of the CPC components in** 303 **meiosis**

304 Since the plant meiotic spindle checkpoint seems to be less stringent (Wijnker and
305 Schnittger, 2013; Komaki and Schnittger, 2016), we asked whether BORR and
306 INCENP are present in meiosis. Analyzing male meiosis, we found that both
307 proteins localized to kinetochores until the onset of anaphase I (Fig. 7A and B;
308 Supplemental Movies S8 and S9). Interestingly, although no cytokinesis occurs
309 after meiosis I in Arabidopsis meiocytes, both proteins localized to the division
310 plane shortly after anaphase I either representing a late anaphase midzone or a
311 phragmoplast midzone-like structure (Fig. 7A and B; Supplemental Movies S8 and
312 S9). The transition between both phases is currently only poorly defined but the

313 localization of BORR and INCENP could possibly contribute to the understanding of
314 the composition and dynamics of these structures.

315 The localization of BORR and INCENP in the second meiotic division
316 resembled the localization of both proteins in mitosis, i.e. at the kinetochores and
317 subsequently at the phragmoplast (Fig. 7A and B; Supplemental Movies S8 and S9).

318

319

320 **Discussion**

321 The CPC is essential for proper cell division in animals and yeast. Whether CPC
322 activity exists in plants and how the potential complex is composed was up to the
323 presented work not clear. Here, we have identified and characterized *BORR*, a
324 Borealin homolog in Arabidopsis. While the presence of a functional homolog of
325 *Survivin* in plants is currently still unclear, the existence of *BORR* together with
326 localization data for AUR3 and an INCENP homolog demonstrates that a CPC is
327 present in plants and of equal importance as in animals.

328 BORR and INCENP localize to kinetochores in mitosis and meiosis. Both
329 proteins could also be co-precipitated from seedlings indicating that BORR and
330 INCENP indeed work in one complex. Consistent with previous reports, we
331 observed that AUR3 accumulates at mitotic kinetochores (Fig. 5G; Supplemental
332 Movie S3) (Demidov et al., 2005), while AUR1 and AUR2 localize to the mitotic
333 spindle (Van Damme et al., 2011). In addition, out of the three AUR proteins in
334 Arabidopsis, only AUR3 interacted with INCENP in our Y2H assays (Fig. 4). These
335 data suggest that only AUR3 acts as the catalytic subunit of the CPC at the inner
336 centromere in plants.

337 In animals, the CPC localizes to inner centromere to monitor inter-

338 kinetochore tension. Since proper kinetochore-microtubule attachments are not
339 established during prophase, the distance between inner centromere and
340 kinetochores is very small allowing the CPC-dependent centromere-localized
341 Aurora B activity to act on kinetochores and by to destabilize erroneous
342 attachments of microtubules.

343 Once proper attachments are formed and inter-kinetochore tension is built
344 up, Aurora B localized to inner centromere is spatially separated from kinetochores
345 leading to the formation of a stable bipolar spindle. Since we found that the
346 kinetochore signals moved away from BORR signals during cell-cycle progression,
347 we propose that the plant CPC also acts as a tension sensor.

348 At anaphase onset, the animal CPC translocates from kinetochores to the
349 cell division plane. Although plant cell division is strikingly different (Müller and
350 Jürgens, 2016), we could show that Arabidopsis BORR, INCENP and AUR3 also
351 accumulate at the division plane at the beginning of cell division (Fig. 5G;
352 Supplemental Movie S1-3). Interestingly, they accumulate the reforming nuclei at
353 telophase before the expanding phragmoplast is completely disassembled, i.e.
354 cytokinesis is finished, while in animals the CPC stays at the cleavage site until the
355 two daughter cells are formed (Carmena et al., 2012).

356 Remarkably, previous studies revealed that the two alpha-Aurora members,
357 AUR1 and AUR2, localize to the division plane until the end of cytokinesis
358 (Demidov et al., 2005; Kawabe et al., 2005). Therefore, the plant CPC might be
359 needed for the initiation but may not be necessary for later steps of cell division. In
360 animals, TPX2 recruits Aurora A to the division plane (Kufer et al., 2002). However,
361 TPX2 does not localize to the division plane in plants although it and its homologs
362 also interact with alpha-Aurora members in Arabidopsis (Petrovská et al., 2012;
363 Boruc et al., 2019). Thus, it is still not clear how alpha-Aurora localization to the
364 division plane is controlled.

365 It seems likely that there is a yet unidentified interaction partner of the CPC
366 in plants that has affinity to the plus-end of microtubules and causes the
367 localization of the CPC, i.e. AUR3 to kinetochores. After separation of the
368 chromosomes in anaphase, this or a different factor then promotes the
369 accumulation in the spindle midzone/early phragmoplast.

370 Since each component of the CPC is needed for its activity, loss of any CPC
371 component leads to the same mutant phenotypes as seen in Aurora B mutants
372 (Honda et al., 2003; Vader et al., 2006). CPC mutants typically exhibit cell division
373 defects and lagging chromosomes resulting from incorrect microtubule-

374 kinetochore attachments. These phenotypes are frequently coupled with ploidy
 375 changes causing cancer in mammals (Tang et al., 2017). Notably, a complete loss-
 376 of-function of any of the CPC components leads to lethality (Cutts et al., 1999; Uren
 377 et al., 2000; Lu et al., 2008; Yamanaka et al., 2008). The sporophyte of *borr-1*
 378 heterozygous knockout plants grew like the wildtype. However, no homozygous
 379 *borr* mutants could be recovered and the heterozygous mutants harbor
 380 undeveloped ovules, aborted seeds, and embryonic defects in the forming siliques.
 381 Similar effects were observed in the *INCENP* mutant *wyr* (Kirioukhova et al., 2011).
 382 However, in contrast to *borr*, the loss of *INCENP* causes an arrest in development of
 383 both the female and the male gametophytes. We can currently not rule out whether
 384 *INCENP* has a specific and *BORR*-independent function during the gametophyte life
 385 phase. It also possible that *INCENP* has a shorter half-life than *BORR*, given that it is
 386 a large protein, and that hence the gametophytes run out of sporophytically
 387 inherited protein levels much earlier than it is the case for the much smaller *BORR*
 388 protein.

389 Using knock-down plants, we could further reveal that reduction of *BORR*
 390 results in mitotic defects including lagging chromosomes and abnormal cell
 391 division which appear to be likely caused by a compromised localization of AUR3. A

392 previous study reported that hesperadin treatment, which inhibits the AUR3
393 kinase activity *in vitro*, induces lagging chromosomes in tobacco BY-2 cells
394 (Kurihara et al., 2006), suggesting that the AUR3 function in chromosome
395 segregation is conserved in the plant lineage.

396 Interestingly, the *BORR* knockdown plants showed a typical *bonsai*
397 phenotype, which is characterized by the inhibition of internode elongation and
398 the premature termination of the shoot apical meristem. Although the molecular
399 mechanism is still not known, the *bonsai* phenotype is associated with a reduction
400 of APC/C activity (Saze and Kakutani, 2007; Zheng et al., 2011).

401 The SAC is another M-phase checkpoint, which works together with the CPC
402 to ensure faithful chromosome segregation. The primary role of the SAC is delaying
403 APC/C activity until all kinetochores are properly attached to the spindle
404 microtubules. Therefore, one possible explanation of the *bonsai* phenotype is that
405 the SAC is over-activated in *BORR* knockdown plants. Indeed, these two M-phase
406 checkpoints are directly or indirectly connected in other organisms (Trivedi and
407 Stukenberg, 2016). Further studies are needed to understand the relationship
408 between SAC and CPC in plants.

409 The final non-catalytic CPC subunit, Survivin, remains elusive in plants.

410 Survivin localizes to the inner centromere upon phosphorylation of histone H3 at
411 Thr3, which in animals is catalyzed by the Haspin kinase (Kelly et al., 2010).
412 Survivin localization is required for recruiting the entire CPC to the inner
413 centromere. Therefore, inhibition of Haspin kinase activity leads to the dissociation
414 of the CPC from inner centromere in mammals. Recently, it was shown that
415 inhibition of Haspin kinase activity with 5-iodotubercidin induces the disruption of
416 AUR3 localization at the inner centromere in BY-2 tobacco culture cells as well
417 (Kozgunova et al., 2016). Although this result indicates that Haspin kinase activity
418 is important for proper AUR3 localization at the inner centromere, no Survivin
419 homolog could be identified in plants on a sequence level. However, a functional
420 homolog might still exist. Alternatively, plants might employ a different mechanism
421 for CPC localization.

422 The phosphorylation status of its different components is of key importance
423 for the regulation of the CPC in animals and yeast. For instance, yeast INCENP is
424 cooperatively phosphorylated by Cdk1 and Aurora, which prevents the CPC binding
425 to the spindle before anaphase (Goto et al., 2006; Nakajima et al., 2011). In
426 addition, Casein kinase 2 phosphorylates the human Survivin, which leads to its
427 exclusion from the nucleus in interphase (Barrett et al., 2011). Borealin is also

428 phosphorylated by many kinases, including Cdk1 that is required for the targeting
429 of the CPC to kinetochores (Kaur et al., 2010; Date et al., 2012). However, the
430 mutation of a conserved CDK phosphorylation site neither obviously altered BORR
431 localization nor reduced the activity of the protein in a way that it would result in a
432 mutant phenotype. Hence, further work is needed to shed light on the regulation of
433 the plant CPC with the present study opening the door for an in-depth analysis of
434 the CPC function in plants with focus on kingdom-specific aspects of its regulation
435 and activity.

436

437 **MATERIALS AND METHODS**

438 **Plant Materials and Growth Conditions**

439 The *Arabidopsis thaliana* accession Columbia (Col-0) was used as the wildtype in
440 this study. All mutants are in the Col-0 background. Plants were grown on a solid
441 medium containing half-strength Arabidopsis nutrient solution (Haughn and
442 Somerville, 1986), 1% (w/v) sucrose and 1.5% (w/v) agar in a growth chamber
443 (16h of light; 21°C/8h of dark; 18°C). The T-DNA insertion line GABI_65B09 (*wyr-*
444 2) was obtained from the Nottingham Arabidopsis Stock Center. The *borr-1* line
445 was generated by CRISPR/CAS9 (Fauser et al., 2014). Primer pairs for genotyping

446 are described in Supplemental Table S1 and Supplemental Fig. S1.

447

448 **Plasmid Construction and Transgenic Plants**

449 The plasmid construction for the CRISPR/CAS9 system was performed as
 450 described in Fauser et al. (2014). To obtain the *borr* knockout plants, 20-bp gene-
 451 specific spacer sequences of *BORR* gene (Supplemental Table S1) were cloned into
 452 the pEn-Chimera, followed by LR recombination reactions with the destination
 453 vector pDe-CAS9. The plasmid construction for the artificial microRNA system was
 454 performed as described in Carbonell et al. (Carbonell et al., 2014). To obtain the
 455 *BORR* knockdown plants, 75-bp gene-specific sequences of *BORR* gene with
 456 *AtMIR390a* backbone (Carbonell et al., 2014) (Supplemental Table S2) were
 457 synthesized and cloned into *pDONR221*, followed by LR recombination reactions
 458 with the destination vector *pGWB602*. To create the *PRO_{BORR}:BORR:GUS* construct, 2
 459 kb upstream of the start codon and 1 kb downstream of the stop codon of the *BORR*
 460 gene were amplified by PCR and cloned into *pENTR2B* by SLiCE. The *SmaI* site was
 461 inserted in front of the stop codon of the *BORR* construct. The resulting construct
 462 was linearized by *SmaI* digestion and was ligated to the *GUS* gene, followed by LR
 463 recombination reactions with the destination vector *pGWB501* (Nakagawa et al.,

2007). To create *PRO_{BORR}:BORR:FPs* constructs, the *GUS* gene in *PRO_{BORR}:BORR:GUS* construct was replaced by the ORF for monomeric *GFP* (*mGFP*) or *TagRFP-T*. To create the *PRO_{BORR}:GFP:BORR* construct, the *SmaI* site was inserted in front of the start codon of *BORR* construct. To create *PRO_{INCENP}:FPs:INCENP*, the genomic fragment of *INCENP* gene was amplified by PCR and cloned into *pENTR2B* by SLiCE method. The *SmaI* site was inserted in front of the start codon of *INCENP*. The resulting construct was linearized by *SmaI* digestion and was ligated to the *mGFP* or *mRUBY3* gene, followed by LR recombination reactions with the destination vector *pGWB501*. Primer pairs for plasmid construction are described in Supplemental Table S1. Transgenic plants were generated by the floral dip method. The *Agrobacterium tumefaciens* strain *GV3101* (*pMP90*) harboring the gene of interest on a binary plasmid was grown in 3 ml of LB media at 28°C. Agrobacteria were resuspended in 3 ml of transformation buffer containing 5% sucrose and 0.05% silwet L-77, and used for plant transformation.

478

479 **Expression Analysis by qRT-PCR**

480 Total RNA was isolated from 7 day-old seedlings with the RNeasy Plant Mini Kit
481 (Qiagen). 300 mg of total RNA were reverse transcribed with ReverTra Ace qPCR

RT Master Mix with gDNA Remover (TOYOBO) according to the manufacturer's instructions. Real-time PCR was performed using the Roche LightCycler 480 and the TB Green Premix Ex Taq (TaKaRa). PP2AA3 (*AT1G13320*) was used as the reference gene (Czechowski et al., 2005). Primer pairs for *BORR* and *PP2AA3* are described in Supplemental Table S1. All experiments were performed in three biological replicates.

Confocal Microscopy and Image Analysis

Root tips of 5 day-old seedlings were used for live cell imaging. Samples were put on glass bottom dishes and covered with a solid medium containing half-strength Arabidopsis nutrient solution, 1% sucrose and 1.5% agar. Confocal images of mitotic cells were acquired by an inverted Nikon ECLIPSE Ti-U microscope equipped with a YOKOGAWA CSU-X spinning disc detector unit connected to an EM-CCD camera (iXon3 DU897; Andor) and a laser combiner system (500 series; Andor), using a Plan Apo 60x/1.20 water immersion objective. GFP was excited at 488 nm with a 520/35 emission filter and TagRFP-T and mRUBY3 at 561 nm with a 617/73 emission filter. Images were obtained at 20 second intervals and corrected for sample drift using the StackReg plugin (ImageJ version 1.49). To obtain line

500 profile data, images were analyzed by the RGB Profiler plugin (ImageJ version
501 1.49).

502 For meiotic live cell imaging, flower buds from 1 month-old plants were
503 used. Sample preparation was performed as described in Prusicki et al., (2019)
504 (Prusicki et al., 2018).

505 Images were obtained every 1 min for BORR:GFP and every 3 min for GFP:INCENP.
506 Images were corrected for the sample drift by the StackReg plugin (ImageJ version
507 1.49).

508

509 **GUS Histochemical Analysis**

510 Samples were fixed in 90% acetone for 15 min and were washed in 50 mM sodium
511 phosphate buffer. The fixed samples were incubated in GUS solution (50 mM
512 sodium phosphate buffer, pH 7.0, 0.5% (v/v) Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5
513 mM $K_4Fe(CN)_6$ and 0.5 mg ml⁻¹ X-gluc) for 1 h at 37°C. After staining, the samples
514 were cleared in chloral hydrate solution (8g chloral hydrate, 1 mL 100% glycerol, 2
515 mL distilled water).

516

517 **Yeast Two-Hybrid Assay (Y2H)**

518 Y2H assays were performed as described in Komaki and Schnittger (2017). All of
519 the cDNAs tested were amplified by PCR using gene specific primers from cDNA
520 made from total RNA of wildtype Arabidopsis, followed by PCR with universal *attB*
521 primers, and cloned into *pDONR221*. The subcloned *BORR* cDNA was recombined
522 into *pGBT9-C* (DNA-BD), which *GAL4-BD* is fused with C-terminus of *BORR* by LR
523 recombination reactions. The other subcloned cDNAs were recombined into the
524 conventional vector *pGBT9* (DNA-BD) or *pGAD424* (AD). Primer pairs for plasmid
525 construction are described in Supplemental Table S1.

526

527 **Protein Extraction and Co-Immunoprecipitation Assay**

528 1 g of 7 day-old seedlings expressing *BORR:RFP* with *GFP:INCENP* or *GFP* was
529 ground to a fine powder in liquid nitrogen with mortar. Total protein was extracted
530 in 2 mL of extraction buffer containing 50 mM Tris-HCl, pH8.0, 150 mM NaCl, 1%
531 IGEPAL CA-630, and a EDTA-Free Protease Inhibitor Cocktail (Roche) for 30 min on
532 ice, and centrifuged for 10 min at 20,000 g at 4°C. The supernatant was incubated
533 for 1 h on ice with anti-GFP magnetic beads (Miltenyi Biotec), and beads were
534 washed 4 times with extraction buffer on the magnetic field. Then, the beads were
535 boiled in SDS sample buffer to release the proteins. Protein samples were detected

536 with 1:2,000 diluted anti-GFP (A6455; Thermo Scientific) and 1:1,000 diluted anti-
537 RFP (AB233; evrogen) as primary antibodies and subsequently with 1:10,000
538 diluted anti-rabbit IgG, HRP-linked antibody (NA934; GE Healthcare) as secondary
539 antibody.

540

541 **Accession Numbers**

542 Sequence data from this article can be found in Arabidopsis Information Resource
543 (TAIR) – database under the following accession numbers: *AUR1* (*AT4G32830*),
544 *AUR2* (*AT2G25880*), *AUR3* (*AT2G45490*), *BORR* (*AT4G39630*), *INCENP/WYRD*
545 (*AT5G55820*). Borealin protein sequence data of other organisms from this article
546 can be found in the NCBI data libraries under the following accession numbers:
547 *Brachypodium distachyon* (XP_003570304.1), *Citrus clementina* (XP_006428073.1),
548 *Drosophila melanogaster* (NP_609279.1), *Homo sapiens* (NP_001243804.1),
549 *Marchantia polymorpha* (PTQ34788.1), *Micromonas commoda* (XP_002503544.1),
550 *Mus musculus* (NP_080836.3), *Oryza sativa* (XP_015650359.1), *Physcomitrella*
551 *patens* (XP_024357151.1), *Populus trichocarpa* (XP_024458072.1), *Saccharomyces*
552 *cerevisiae* (AJV31725.1), *Schizosaccharomyces pombe* (CAA22184.2), *Selaginella*
553 *moellendorffii* (XP_024537708.1), *Vitis vinifera* (XP_002280684.1), *Xenopus*

554 *tropicalis* (NP_001002902.1), *Zea mays* (NP_001142076.1).

555

556 **SUPPLEMENTAL DATA**

557 The following supplemental materials are available.

558 Supplemental Figure S1. Complementation test of BORR and INCENP mutants.

559 Supplemental Figure S2. Both BORR and AUR3 localize to inner centromeres.

560 Supplemental Table S1. Primers used in this study.

561 Supplemental Table S2. Synthesized oligonucleotides used in this study.

562 Supplemental Movie S1. Subcellular localization of GFP:INCENP during mitosis.

563 Supplemental Movie S2. Subcellular localization of BORR:GFP during mitosis.

564 Supplemental Movie S3. Subcellular localization of AUR3:GFP during mitosis.

565 Supplemental Movie S4. Co-localization of AUR3:GFP and BORR:RFP.

566 Supplemental Movie S5. Co-localization of AUR3:GFP and RFP:INCENP.

567 Supplemental Movie S6. Co-localization of GFP:INCENP and BORR:RFP.

568 Supplemental Movie S7. Comparison of subcellular localization between BORR:GFP

569 and RFP:CENH3.

570 Supplemental Movie S8. Subcellular localization of BORR:GFP during meiosis.

571 Supplemental Movie S9. Subcellular localization of GFP:INCENP during meiosis.

572

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576

577 **Footnotes**

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582

583

Functional analysis of the chromosomal passenger complex in Arabidopsis

Shinichiro Komaki^{1,2}, Hidenori Takeuchi^{3,4}, Yuki Hamamura², Maren Heese², Takashi Hashimoto¹, and Arp Schnittger^{2,*}

Key words: Cell division, spindle assembly checkpoint, phragmoplast, Aurora B

¹ Nara Institute of Science and Technology, Graduate School of Biological Sciences, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan

² University of Hamburg, Institute for Plant Sciences and Microbiology, Department of Developmental Biology, Ohnhorststr. 18, D-22609 Hamburg, Germany

³ Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan.

⁴ Institute for Advanced Research, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8602, Japan

* For correspondence arp.schnittger@uni-hamburg.de, Phone: +49 40 428 16 502, Fax: +49 40 428 16 503

19 SUMMARY

20 A key regulator of cell division in all eukaryotes is the kinase Aurora B, which is
 21 encoded by the Aurora 3 (AUR3) gene in Arabidopsis. Aurora B has at least two
 22 central functions during cell division. On the one hand, it is essential for the
 23 correct, i.e. balanced, segregation of chromosomes in mitosis and meiosis by
 24 controlling kinetochore function. On the other hand, Aurora B acts at the division
 25 plane, where it is necessary to complete cytokinesis. To accomplish these two
 26 spatially distinct functions, Aurora B in animals is guided to its sites of action by
 27 Borealin, INCENP, and Survivin that build together with Aurora B the chromosome
 28 passenger complex (CPC). However, besides Aurora homologs, only a candidate
 29 gene with restricted homology to INCENP has so far been described in Arabidopsis
 30 raising the question whether there exists a full complement of the CPC in plants
 31 and how Aurora homologs are targeted subcellularly. Here, we have identified and
 32 functionally characterized a Borealin homolog, BOREALIN RELATED (BORR), in
 33 Arabidopsis. This, together with detailed localization studies including the putative
 34 Arabidopsis INCENP homolog, supports the existence of a CPC in plants.

INTRODUCTION

Equal chromosome segregation during cell division is crucial for survival, growth, and reproduction of every organism. Chromosome segregation is assured by the M-phase checkpoint, which involves two regulatory units, the spindle assembly checkpoint (SAC) and the chromosomal passenger complex (CPC) (Carmena et al., 2012).

Chromatids are held together by Cohesin, a proteinaceous ring-like structure, which needs to be cleaved by Separase to allow their separation in anaphase. The SAC inhibits the Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase, which mediates the degradation of Securin, an inhibitor of Separase, until all kinetochores are properly attached to microtubules of opposing poles (London and Biggins, 2014; Komaki and Schnittger, 2016).

The CPC has been well characterized in yeast and mammals, where it has been found to consist of four proteins: Aurora kinase B (Aurora B), Borealin, Inner centromere protein (INCENP), and Survivin. The CPC fulfills several functions during mitosis. In particular, it is involved in ensuring that all kinetochores are attached to microtubules emanating from opposing poles (Kitagawa and Lee, 2015). The CPC localizes to inner centromere where it activates Aurora B in

response to low inter-kinetochore tension. Active Aurora B phosphorylates kinetochore proteins leading to the destabilization of erroneous microtubule attachments. Once proper kinetochore-microtubule attachments are established from opposing poles, which gives high inter-kinetochore tension, Aurora B is spatially separated from kinetochores resulting in proper bistable spindle formation.

Aurora B belongs to the Aurora kinase family of serine/threonine kinases that are highly conserved in the eukaryotic kingdom (van der Waal et al., 2012; Weimer et al., 2016). While yeast possesses a single Aurora homolog, mammals have three Aurora kinases, Aurora A, Aurora B, and Aurora C, among which only Aurora C acts in meiosis (Goldenson and Crispino, 2015). Since these three kinases share a common consensus phosphorylation motif, it is thought that interacting proteins are important for their localization and substrate specificity.

Aurora A interacts with the spindle assembly factor TPX2, and localizes to spindle microtubules to regulate spindle assembly (Gruss and Vernos, 2004). In contrast, Aurora B and Aurora C, the catalytic subunit of the CPC, is involved in the correction of erroneous kinetochore-microtubule attachments, activation of the SAC, and cytokinesis in mitosis and meiosis. These diverse functions are based on

its dynamic localization pattern controlled by the three non-catalytic subunits of the CPC (van der Horst and Lens, 2014).

INCENP is the largest non-catalytic subunit of the CPC, which in animals and yeast directly binds to all other components of the complex. Borealin and Survivin interact with the conserved N-terminal region of INCENP while Aurora B binds to the C-terminal domain of INCENP, called IN-box (Carmena et al., 2012). The IN-box is required for interaction with and activation of Aurora B (Honda et al., 2003).

While the N-terminus of Borealin acts as the INCENP-binding region its C-terminus contains a homo-dimerization domain that is involved in a stable CPC localization at centromeres (Bekier et al., 2015). In addition, the phosphorylation status of the central part affects the centromere localization and steady-state level of Borealin itself (Kaur et al., 2010; Date et al., 2012).

Aurora kinases in plants are categorized into two groups, alpha-Aurora and beta-Aurora, based on the localization pattern and sequences (Weimer et al., 2016). Interestingly, these two groups have mixed features of the animal Aurora A and Aurora B/C groups. For instance, AUR1 and AUR2 in Arabidopsis, both belonging to the alpha class, localize to spindle microtubules, which is reminiscent of Aurora A, but they also localize to the central region of phragmoplasts

paralleling Aurora B/C at the cell cleavage site. AUR3, a member of the beta-Aurora group, localizes to kinetochores, similar to Aurora B (Komaki and Schnittger, 2017), but in contrary to Aurora B, it does not accumulate at the division plane (Demidov et al., 2005).

While *aur1* and *aur2* single mutants do not show any obvious growth alterations, the *aur1 aur2* double null mutant is gametophytic lethal (Van Damme et al., 2011). A weak loss-of-function *aur1 aur2* double mutant exhibits altered division plane orientation, reduced pollen viability, and enhanced vascular cell differentiation. These defects can be rescued by expressing either AUR1 or AUR2 but not AUR3, indicating that alpha-Auroras and beta-Auroras have distinct functions (Van Damme et al., 2011; Demidov et al., 2014; Lee et al., 2019).

Given the sparse information about the non-catalytic subunits of the plant CPC, it is not even clear whether there is a conserved CPC function in plants. The putative homolog of INCENP in Arabidopsis has a long N-terminal region of unknown function, which is conserved only in plants. Although Arabidopsis *incenp* mutants, also known as *wyrd* (*wyr*), show defects in gametophytic cell division (Kirioukhova et al., 2011), it is still unclear whether Arabidopsis INCENP acts as part of a putative plant CPC because of missing information about its localization

and binding partners.

Here, we present the identification and functional characterization of an Arabidopsis Borealin homolog, which colocalizes with the INCENP homolog to the inner centromere and the central domain of the phragmoplast. We also observed that only AUR3 acts as the catalytic subunit of the plant CPC. These data underscore the mixed features of plant Aurora kinases.

RESULTS

Identification of a putative Borealin homolog in plants

As a first step to determine whether plants have a functional CPC, we searched for Borealin and Survivin homologs in the Arabidopsis genome. Given that the putative INCENP homolog of Arabidopsis, WYR, shares only very weak similarities with its animal counterpart (Kirioukhova et al., 2011), we expected the same to be the case for Borealin and Survivin. Indeed, standard BLAST searches did not result in the identification of likely candidates. Therefore, we made use of the fact that Borealin is transcriptionally controlled by the tumor suppressor protein Retinoblastoma in animals (Cam et al., 2004; Date et al., 2007). Mining a dataset of the genome-wide binding sites of the Arabidopsis Retinoblastoma homolog RETINOBLASTOMA-

RELATED (RBR1), revealed several unknown genes likely involved in cell division control (Bouyer et al., 2018) (Sup Fig. 1). One of these genes, *AT4G39630*, showed a weak similarity to Borealin and will be referred to as *BOREALIN-RELATED (BORR)* (Supplemental Fig. S1A).

The C-terminal region of the corresponding protein shows 37% similarity with the central region of human Borealin, which includes highly conserved consensus sites for CDK phosphorylation (Fig. 1A). A second stretch of homology can be found in the N-terminus, which is predicted to adopt an alpha-helical structure. In animals and yeast this N-terminal alpha-helix forms a three-helical bundle with INCENP and Survivin (Fig. 1A) (Jeyaprakash et al., 2007). Notably, the Arabidopsis BORR is considerably shorter than the human homolog (233 aa versus 280 aa), partly as the result of a truncated C-terminal domain. Using *BORR* as a template, we found putative Borealin genes in all branches of the plant kingdom, including angiosperms, gymnosperms, pteridophytes, bryophytes, and algae (Fig. 1B - phylogenetic tree).

Phenotypic analysis of *borr* mutants

Since no mutants for *BORR* were available in the public mutant collections of

Arabidopsis, we generated a mutant by CRISPR/Cas9. The resulting *borr-1* allele has a T insertion in the 2nd exon, i.e. between nucleotides 280 and 281 downstream of the start codon leading to a stop codon and a predicted truncated protein of 67 amino acids (Supplemental Fig. S1A). While the heterozygous *borr-1* plants grow as the wildtype, we could never obtain homozygous mutant plants. Consistently, we observed aborted seeds and undeveloped ovules in siliques of *borr-1 +/-* (Fig. 2A,B).

To assess whether this lethal phenotype was indeed due to the frameshift mutation in the *BORR* gene, we constructed reporter lines in which the genomic region of *BORR* was fused to an ORF encoding for GFP. Expression of either an N-terminal (GFP:BORR) or C-terminal (BORR:GFP) GFP fusion to BORR complemented the lethal phenotype of *borr-1* mutant plants corroborating that loss of BORR affects plant reproduction.

To address the nature of the lack of homozygous *borr-1* mutants, we conducted reciprocal crosses of heterozygous mutants with the wildtype. When we used *borr-1 +/-* as male parent, the transmission rate was determined to be 44%. Consistently, 96% of *borr1+/-* pollen resembled wild-type pollen and based on DAPI-staining, contained two sperm cells.

In contrast, when we used *borr-1 +/-* as female parent, the transmission rate was reduced to 29%, indicating that *BORR* is especially needed for the development and/or function of the female gametophyte. However, when analyzing the mature female gametophyte no obvious developmental defects were observed suggesting that the reduced transmission of *borr-1* through the female gametophyte originated shortly before or after/during fertilization. In accordance with this, we found 12% unfertilized ovules/early arrested seeds and 19% late aborted seeds when we used *borr-1 +/-* as the female (n=250) in contrast to 3% unfertilized ovules/early arrested seeds and 0.3% late aborted seeds in control crosses when we used the wildtype as the female and male parent (n=299)(Fig. 2A,B).

In addition, we observed abnormal embryo development, including delayed and distorted embryos or embryo-like structures in approximately 10% of all ovules/seeds analyzed when *borr-1 +/-* was used as the female parent in crosses with the wildtype as a male versus 4% in the control crosses supporting a female gametophytic effect of *borr* (n=313 and n=408 for the control cross) (Fig. 2C,D). One likely explanation for this is that the divisions leading to the development of the embryo sac cause aneuploidy in *borr* that preclude and/or severely interfere

with embryo development.

In addition, *BORR* is needed during embryo development since the number of seeds with embryonic defects almost doubled in self-fertilized *borr-1* mutants (Fig. 2D). Thus, *BORR* is an essential gene needed for cell proliferation and development during early stages of the plant life cycle.

To address the *BORR* function after embryo development, we generated *BORR* knockdown plants by expressing two artificial microRNAs (*amiRNA*) targeted against the 2nd (*amiBORR#1*) and 3rd exon (*amiBORR#2*) of *BORR*, respectively (Fig. 3A). Most transgenic plants expressing *amiBORR#1* (19 out of 25) and *amiBORR#2* (15 out of 20) showed a dwarf phenotype. For the following analyses, we selected two transgenic plants for each construct (*amiBORR#1-1*, *amiBORR#1-2*, *amiBORR#2-1* and *amiBORR#2-2*). All four knockdown plants had a similar level of *BORR* transcript reduction (Fig. 3B) and had a dwarf phenotype with curled leaves during the vegetative stage (Supplemental Fig. S2A). At the flowering stage, all *BORR* knockdown plants were bushy and exhibited a typical *bonsai* phenotype (sometimes also called *broom stick* phenotype), which is commonly observed in mutants with low APC/C activity and is characterized by short inflorescences, which are often curled at the very end with only a few

developing siliques (Supplemental Fig. S2B and C) (Saze and Kakutani, 2007; Zheng et al., 2011).

Primary root growth was also compromised in all *BORR* knockdown plants (Fig. 3C and D). Microscopic analyses of *amiBORR#1-1* revealed that the root meristem size was reduced (Fig. 3E-G). Moreover, an aberrant pattern of cell divisions was found in the columella of all roots analyzed (25 out of 25). In 40% of all seedlings (10 out of 25), altered division patterns were also present in the epidermal, cortex and endodermal layers underlining that *BORR* is required for proper cell division (Fig. 3E).

To assess whether Arabidopsis *BORR* has a function in chromosome segregation as well, *amiBORR#1-1* was introgressed into a transgenic line expressing both a microtubule (RFP:TUA5) and a centromere (GFP:CENH3) marker (Komaki and Schnittger, 2017). Indeed, we could frequently observe lagging chromosomes in *amiBORR#1-1* plant cells (9 out of 50), a phenotype that hardly occurred in the wildtype control plant cells (1 out of 50) (Fig. 3H and I). To examine whether the lagging chromosomes are related to a compromised AUR3 localization, we introduced a previously generated *AUR3:GFP* reporter into *amiBORR#1-1* plants (Komaki and Schnittger, 2017). While some AUR3:GFP signal

could be still detected at the kinetochores in *amiBORR#1-1* plants, the signal intensity was much weaker than in wild-type plants, suggesting that Arabidopsis BORR ensures chromosome segregation through AUR3 localization (Fig. 3J and K). Taken together, corresponding to Borealin function in animals, *BORR* is required for proper chromosome segregation and cell division in Arabidopsis.

Interaction scheme of CPC components in Arabidopsis

To reveal the molecular network of the Arabidopsis CPC, we investigated the interaction of BORR with INCENP and the three Aurora kinases of Arabidopsis. Although Borealin has a conserved coiled-coil domain, which is known as an INCENP binding site in other organisms (Jeyaparakash et al., 2007), an interaction between BORR and INCENP was not detected by a yeast two-hybrid interaction assay (Y2H assay) (Fig. 4A). We next performed an *in vivo* co-immunoprecipitation (IP) assay. To this end, anti-GFP immuno-precipitates from total protein extracts of plants expressing both *BORR:RFP* and *GFP:INCENP* or from plants expressing *BORR:RFP* and free *GFP* as a negative control were probed with an anti-RFP antibody. BORR:RFP was only detected in the extract of plants co-expressing GFP-INCENP, indicating that Arabidopsis BORR could be part of a CPC *in vivo* (Fig. 4C).

Consistent with the topology of the CPC in animal and yeast, we further found that INCENP interacts specifically with AUR3 through its C-terminal region while no interaction was detected with AUR1 or AUR2 (Fig. 4A, B). The C-terminal region of INCENP contains an Aurora B binding domain, called IN-box, which is conserved from yeast to mammals (Adams et al., 2000). Since Arabidopsis INCENP also possesses a putative IN-box in its C-terminus, we exchanged Trp1723 to Gly and Phe1745 to Ala. These residues are conserved in yeast and animals, and the Trp1723 mutation leads to loss of interaction with Aurora B in *in vitro* assays (Sessa et al., 2005), as well as in chicken culture cells (Xu et al., 2009). Consistent with a key role of the putative IN-box, Arabidopsis INCENP^{F1745A} partially and INCENP^{W1723G} completely failed to interact with AUR3 (Fig. 4B).

Expression pattern and subcellular localization of the CPC components in mitosis

To investigate the spatial and temporal expression pattern of *BORR*, we generated transgenic plants harboring a genomic fragment of the *BORR* gene fused to a beta-glucuronidase (GUS) gene before the STOP codon. As expected, strong GUS activity was observed in both the shoot and root meristems of seedlings, indicating that

BORR is expressed in proliferating cells (Fig. 5A-C). In addition, *BORR* was also found to be strongly expressed in flowering tissues including both the male and female reproductive organs (Fig. 5D-F).

To reveal the subcellular localization of the CPC components in plants, we made use of the functional *BORR* reporter line used for the complementation studies above. In addition, we constructed reporter lines in which the genomic region of *INCENP* was fused to an ORF encoding for GFP. In contrast to *BORR*, only the N-terminal (GFP:INCENP) GFP fusion of *INCENP* could complement the lethal phenotype of *incenp* homozygous mutants (Supplemental Fig. S1D and E). For *AUR3*, we used a previously published reporter line (*AUR3:GFP*) in the wildtype background (Komaki and Schnittger, 2017).

First, we crossed the three CPC reporter lines with *RFP:TUA5* expressing plants to check the localization of CPC components during mitosis. All three CPC components showed the same localization pattern (Fig. 5G; Supplemental Movies S1-3). In interphase, they localized to the nucleus. Before nuclear envelope breakdown (NEB), the CPC components strongly accumulated at the kinetochores until anaphase onset. Once chromosomes moved toward the spindle poles, they localized to the middle part of the phragmoplast. Interestingly, in early telophase,

the CPC components moved back to the nucleus even though there still was an expanding phragmoplast.

To corroborate that all three CPC components co-localize, we created transgenic plants which expressed BORR:RFP and GFP:INCENP, BORR:RFP together with AUR3:GFP and RFP:INCENP along with AUR3:GFP (Table 1). Microscopical analyses revealed a tight co-localization pattern through the cell cycle in all three lines, suggesting that BORR, INCENP and AUR3 work together as a plant CPC (Fig. 5H; Supplemental Fig. S3 and 4; Supplemental Movies S4-6).

In other organisms, it has been reported that the CPC localizes to the inner centromere region to monitor kinetochore-microtubule attachments (Hindriksen et al., 2017). To reveal the localization of the CPC precisely, we crossed the *BORR:GFP* line with the inner kinetochore marker *RFP:CENH3*. Just after NEB, these two fluorescent reporters showed a high level of overlapping signal. After prometaphase, BORR:GFP localized closer to the inner region of the kinetochore than RFP:CENH3. Yet, both proteins still overlapped in their localization pattern. Shortly before anaphase onset, RFP:CENH3 formed two lines along the metaphase plate, and BORR:GFP localized between these two lines with no overlap (Fig. 6; Supplemental Movie S7). This result demonstrates that the CPC localizes to the

inner centromere region in plants.

In humans, Borealin function is regulated by phosphorylation. Especially the phosphorylation of S219, a putative Cdk1 target residue, in the central region of the protein affects its stability and centromere localization (Kaur et al., 2010; Date et al., 2012). Since this Cdk1 phosphorylation site resides in the most conserved region between human and plant Borealin is conserved in BORR (Figure 1B), we generated a phospho-mimic (BORR^{S214D}:GFP) and a dephospho variant (BORR^{S214A}:GFP) (Dissmeyer and Schnittger, 2011), and transformed them into the heterozygous *borr-1* mutants to evaluate their functionality. Notably, both constructs could complement the lethal phenotype of *borr-1* mutant plants, and showed a normal BORR localization pattern (Supplemental Fig. S1A-C). Thus, the physiological importance of this conserved CDK target motif has yet to be resolved in plants.

Expression pattern and subcellular localization of the CPC components in meiosis

Since the plant meiotic spindle checkpoint seems to be less stringent (Wijnker and Schnittger, 2013; Komaki and Schnittger, 2016), we asked whether BORR and

INCENP are present in meiosis. Analyzing male meiosis, we found that both proteins localized to kinetochores until the onset of anaphase I (Fig. 7A and B; Supplemental Movies S8 and S9). Interestingly, although no cytokinesis occurs after meiosis I in Arabidopsis meiocytes, both proteins localized to the division plane shortly after anaphase I either representing a late anaphase midzone or a phragmoplast midzone-like structure (Fig. 7A and B; Supplemental Movies S8 and S9). The transition between both phases is currently only poorly defined but the localization of BORR and INCENP could possibly contribute to the understanding of the composition and dynamics of these structures.

The localization of BORR and INCENP in the second meiotic division resembled the localization of both proteins in mitosis, i.e. at the kinetochores and subsequently at the phragmoplast (Fig. 7A and B; Supplemental Movies S8 and S9).

Discussion

The CPC is essential for proper cell division in animals and yeast. Whether CPC activity exists in plants and how the potential complex is composed was up to the presented work not clear. Here, we have identified and characterized *BORR*, a Borealin homolog in Arabidopsis. While the presence of a functional homolog of

Survivin in plants is currently still unclear, the existence of *BORR* together with localization data for AUR3 and an INCENP homolog demonstrates that a CPC is present in plants and of equal importance as in animals.

BORR and INCENP localize to kinetochores in mitosis and meiosis. Both proteins could also be co-precipitated from seedlings indicating that *BORR* and INCENP indeed work in one complex. Consistent with previous reports, we observed that AUR3 accumulates at mitotic kinetochores (Fig. 5G; Supplemental Movie S3) (Demidov et al., 2005), while AUR1 and AUR2 localize to the mitotic spindle (Van Damme et al., 2011). In addition, out of the three AUR proteins in Arabidopsis, only AUR3 interacted with INCENP in our Y2H assays (Fig. 4). These data suggest that only AUR3 acts as the catalytic subunit of the CPC at the inner centromere in plants.

In animals, the CPC localizes to inner centromere to monitor inter-kinetochore tension. Since proper kinetochore-microtubule attachments are not established during prophase, the distance between inner centromere and kinetochores is very small allowing the CPC-dependent centromere-localized Aurora B activity to act on kinetochores and by to destabilize erroneous attachments of microtubules.

Once proper attachments are formed and inter-kinetochore tension is built up, Aurora B localized to inner centromere is spatially separated from kinetochores leading to the formation of a stable bipolar spindle. Since we found that the kinetochore signals moved away from BORR signals during cell-cycle progression, we propose that the plant CPC also acts as a tension sensor.

At anaphase onset, the animal CPC translocates from kinetochores to the cell division plane. Although plant cell division is strikingly different (Müller and Jürgens, 2016), we could show that Arabidopsis BORR, INCENP and AUR3 also accumulate at the division plane at the beginning of cell division (Fig. 5G; Supplemental Movie S1-3). Interestingly, they accumulate the reforming nuclei at telophase before the expanding phragmoplast is completely disassembled, i.e. cytokinesis is finished, while in animals the CPC stays at the cleavage site until the two daughter cells are formed (Carmena et al., 2012).

Remarkably, previous studies revealed that the two alpha-Aurora members, AUR1 and AUR2, localize to the division plane until the end of cytokinesis (Demidov et al., 2005; Kawabe et al., 2005). Therefore, the plant CPC might be needed for the initiation but may not be necessary for later steps of cell division. In animals, TPX2 recruits Aurora A to the division plane (Kufer et al., 2002). However,

TPX2 does not localize to the division plane in plants although it and its homologs also interact with alpha-Aurora members in Arabidopsis (Petrovská et al., 2012; Boruc et al., 2019). Thus, it is still not clear how alpha-Aurora localization to the division plane is controlled.

It seems likely that there is a yet unidentified interaction partner of the CPC in plants that has affinity to the plus-end of microtubules and causes the localization of the CPC, i.e. AUR3 to kinetochores. After separation of the chromosomes in anaphase, this or a different factor then promotes the accumulation in the spindle midzone/early phragmoplast.

Since each component of the CPC is needed for its activity, loss of any CPC component leads to the same mutant phenotypes as seen in Aurora B mutants (Honda et al., 2003; Vader et al., 2006). CPC mutants typically exhibit cell division defects and lagging chromosomes resulting from incorrect microtubule-kinetochore attachments. These phenotypes are frequently coupled with ploidy changes causing cancer in mammals (Tang et al., 2017). Notably, a complete loss-of-function of any of the CPC components leads to lethality (Cutts et al., 1999; Uren et al., 2000; Lu et al., 2008; Yamanaka et al., 2008). The sporophyte of *borr-1* heterozygous knockout plants grew like the wildtype. However, no homozygous

borr mutants could be recovered and the heterozygous mutants harbor undeveloped ovules, aborted seeds, and embryonic defects in the forming siliques. Similar effects were observed in the *INCENP* mutant *wyr* (Kirioukhova et al., 2011). However, in contrast to *borr*, the loss of *INCENP* causes an arrest in development of both the female and the male gametophytes. We can currently not rule out whether *INCENP* has a specific and *BORR*-independent function during the gametophyte life phase. It also possible that *INCENP* has a shorter half-life than *BORR*, given that it is a large protein, and that hence the gametophytes run out of sporophytically inherited protein levels much earlier than it is the case for the much smaller *BORR* protein.

Using knock-down plants, we could further reveal that reduction of *BORR* results in mitotic defects including lagging chromosomes and abnormal cell division which appear to be likely caused by a compromised localization of AUR3. A previous study reported that hesperadin treatment, which inhibits the AUR3 kinase activity *in vitro*, induces lagging chromosomes in tobacco BY-2 cells (Kurihara et al., 2006), suggesting that the AUR3 function in chromosome segregation is conserved in the plant lineage.

Interestingly, the *BORR* knockdown plants showed a typical *bonsai*

phenotype, which is characterized by the inhibition of internode elongation and the premature termination of the shoot apical meristem. Although the molecular mechanism is still not known, the *bonsai* phenotype is associated with a reduction of APC/C activity (Saze and Kakutani, 2007; Zheng et al., 2011).

The SAC is another M-phase checkpoint, which works together with the CPC to ensure faithful chromosome segregation. The primary role of the SAC is delaying APC/C activity until all kinetochores are properly attached to the spindle microtubules. Therefore, one possible explanation of the *bonsai* phenotype is that the SAC is over-activated in *BORR* knockdown plants. Indeed, these two M-phase checkpoints are directly or indirectly connected in other organisms (Trivedi and Stukenberg, 2016). Further studies are needed to understand the relationship between SAC and CPC in plants.

The final non-catalytic CPC subunit, Survivin, remains elusive in plants. Survivin localizes to the inner centromere upon phosphorylation of histone H3 at Thr3, which in animals is catalyzed by the Haspin kinase (Kelly et al., 2010). Survivin localization is required for recruiting the entire CPC to the inner centromere. Therefore, inhibition of Haspin kinase activity leads to the dissociation of the CPC from inner centromere in mammals. Recently, it was shown that

inhibition of Haspin kinase activity with 5-iodotubercidin induces the disruption of AUR3 localization at the inner centromere in BY-2 tobacco culture cells as well (Kozgunova et al., 2016). Although this result indicates that Haspin kinase activity is important for proper AUR3 localization at the inner centromere, no Survivin homolog could be identified in plants on a sequence level. However, a functional homolog might still exist. Alternatively, plants might employ a different mechanism for CPC localization.

The phosphorylation status of its different components is of key importance for the regulation of the CPC in animals and yeast. For instance, yeast INCENP is cooperatively phosphorylated by Cdk1 and Aurora, which prevents the CPC binding to the spindle before anaphase (Goto et al., 2006; Nakajima et al., 2011). In addition, Casein kinase 2 phosphorylates the human Survivin, which leads to its exclusion from the nucleus in interphase (Barrett et al., 2011). Borealin is also phosphorylated by many kinases, including Cdk1 that is required for the targeting of the CPC to kinetochores (Kaur et al., 2010; Date et al., 2012). However, the mutation of a conserved CDK phosphorylation site neither obviously altered BORR localization nor reduced the activity of the protein in a way that it would result in a mutant phenotype. Hence, further work is needed to shed light on the regulation of

the plant CPC with the present study opening the door for an in-depth analysis of the CPC function in plants with focus on kingdom-specific aspects of its regulation and activity.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* accession Columbia (Col-0) was used as the wildtype in this study. All mutants are in the Col-0 background. Plants were grown on a solid medium containing half-strength Arabidopsis nutrient solution (Haughn and Somerville, 1986), 1% (w/v) sucrose and 1.5% (w/v) agar in a growth chamber (16h of light; 21°C/8h of dark; 18°C). The T-DNA insertion line GABI_65B09 (*wyr-2*) was obtained from the Nottingham Arabidopsis Stock Center. The *borr-1* line was generated by CRISPR/CAS9 (Fauser et al., 2014). Primer pairs for genotyping are described in Supplemental Table S1 and Supplemental Fig. S1.

Plasmid Construction and Transgenic Plants

The plasmid construction for the CRISPR/CAS9 system was performed as described in Fauser et al. (2014). To obtain the *borr* knockout plants, 20-bp gene-

specific spacer sequences of *BORR* gene (Supplemental Table S1) were cloned into the pEn-Chimera, followed by LR recombination reactions with the destination vector pDe-CAS9. The plasmid construction for the artificial microRNA system was performed as described in Carbonell et al. (Carbonell et al., 2014). To obtain the *BORR* knockdown plants, 75-bp gene-specific sequences of *BORR* gene with *AtMIR390a* backbone (Carbonell et al., 2014) (Supplemental Table S2) were synthesized and cloned into *pDONR221*, followed by LR recombination reactions with the destination vector *pGWB602*. To create the *PRO_{BORR}:BORR:GUS* construct, 2 kb upstream of the start codon and 1 kb downstream of the stop codon of the *BORR* gene were amplified by PCR and cloned into *pENTR2B* by SLiCE. The *SmaI* site was inserted in front of the stop codon of the *BORR* construct. The resulting construct was linearized by *SmaI* digestion and was ligated to the *GUS* gene, followed by LR recombination reactions with the destination vector *pGWB501* (Nakagawa et al., 2007). To create *PRO_{BORR}:BORR:FPs* constructs, the *GUS* gene in *PRO_{BORR}:BORR:GUS* construct was replaced by the ORF for monomeric *GFP* (*mGFP*) or *TagRFP-T*. To create the *PRO_{BORR}:GFP:BORR* construct, the *SmaI* site was inserted in front of the start codon of *BORR* construct. To create *PRO_{INCENP}:FPs:INCENP*, the genomic fragment of *INCENP* gene was amplified by PCR and cloned into *pENTR2B* by SLiCE

method. The *SmaI* site was inserted in front of the start codon of INCENP. The resulting construct was linearized by *SmaI* digestion and was ligated to the *mGFP* or *mRUBY3* gene, followed by LR recombination reactions with the destination vector *pGWB501*. Primer pairs for plasmid construction are described in Supplemental Table S1. Transgenic plants were generated by the floral dip method. The *Agrobacterium tumefaciens* strain *GV3101* (*pMP90*) harboring the gene of interest on a binary plasmid was grown in 3 ml of LB media at 28°C. Agrobacteria were resuspended in 3 ml of transformation buffer containing 5% sucrose and 0.05% silwet L-77, and used for plant transformation.

Expression Analysis by qRT-PCR

Total RNA was isolated from 7 day-old seedlings with the RNeasy Plant Mini Kit (Qiagen). 300 mg of total RNA were reverse transcribed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) according to the manufacturer's instructions. Real-time PCR was performed using the Roche LightCycler 480 and the TB Green Premix Ex Taq (TaKaRa). *PP2AA3* (*AT1G13320*) was used as the reference gene (Czechowski et al., 2005). Primer pairs for *BORR* and *PP2AA3* are described in Supplemental Table S1. All experiments were performed in three

biological replicates.

Confocal Microscopy and Image Analysis

Root tips of 5 day-old seedlings were used for live cell imaging. Samples were put on glass bottom dishes and covered with a solid medium containing half-strength Arabidopsis nutrient solution, 1% sucrose and 1.5% agar. Confocal images of mitotic cells were acquired by an inverted Nikon ECLIPSE Ti-U microscope equipped with a YOKOGAWA CSU-X spinning disc detector unit connected to an EM-CCD camera (iXon3 DU897; Andor) and a laser combiner system (500 series; Andor), using a Plan Apo 60x/1.20 water immersion objective. GFP was excited at 488 nm with a 520/35 emission filter and TagRFP-T and mRUBY3 at 561 nm with a 617/73 emission filter. Images were obtained at 20 second intervals and corrected for sample drift using the StackReg plugin (ImageJ version 1.49). To obtain line profile data, images were analyzed by the RGB Profiler plugin (ImageJ version 1.49).

For meiotic live cell imaging, flower buds from 1 month-old plants were used. Sample preparation was performed as described in Prusicki et al., (2019) (Prusicki et al., 2018).

Images were obtained every 1 min for BORR:GFP and every 3 min for GFP:INCENP.

Images were corrected for the sample drift by the StackReg plugin (ImageJ version 1.49).

GUS Histochemical Analysis

Samples were fixed in 90% acetone for 15 min and were washed in 50 mM sodium phosphate buffer. The fixed samples were incubated in GUS solution (50 mM sodium phosphate buffer, pH 7.0, 0.5% (v/v) Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 0.5 mg ml⁻¹ X-gluc) for 1 h at 37°C. After staining, the samples were cleared in chloral hydrate solution (8g chloral hydrate, 1 mL 100% glycerol, 2 mL distilled water).

Yeast Two-Hybrid Assay (Y2H)

Y2H assays were performed as described in Komaki and Schnittger (2017). All of the cDNAs tested were amplified by PCR using gene specific primers from cDNA made from total RNA of wildtype Arabidopsis, followed by PCR with universal *attB* primers, and cloned into *pDONR221*. The subcloned *BORR* cDNA was recombined into *pGBT9-C* (DNA-BD), which *GAL4-BD* is fused with C-terminus of *BORR* by LR

recombination reactions. The other subcloned cDNAs were recombined into the conventional vector *pGBT9* (DNA-BD) or *pGAD424* (AD). Primer pairs for plasmid construction are described in Supplemental Table S1.

Protein Extraction and Co-Immunoprecipitation Assay

1 g of 7 day-old seedlings expressing *BORR:RFP* with *GFP:INCENP* or *GFP* was ground to a fine powder in liquid nitrogen with mortar. Total protein was extracted in 2 mL of extraction buffer containing 50 mM Tris-HCl, pH8.0, 150 mM NaCl, 1% IGEPAL CA-630, and a EDTA-Free Protease Inhibitor Cocktail (Roche) for 30 min on ice, and centrifuged for 10 min at 20,000 g at 4°C. The supernatant was incubated for 1 h on ice with anti-GFP magnetic beads (Miltenyi Biotec), and beads were washed 4 times with extraction buffer on the magnetic field. Then, the beads were boiled in SDS sample buffer to release the proteins. Protein samples were detected with 1:2,000 diluted anti-GFP (A6455; Thermo Scientific) and 1:1,000 diluted anti-RFP (AB233; evrogen) as primary antibodies and subsequently with 1:10,000 diluted anti-rabbit IgG, HRP-linked antibody (NA934; GE Healthcare) as secondary antibody.

Accession Numbers

Sequence data from this article can be found in Arabidopsis Information Resource (TAIR) – database under the following accession numbers: *AUR1* (AT4G32830), *AUR2* (AT2G25880), *AUR3* (AT2G45490), *BORR* (AT4G39630), *INCENP/WYRD* (AT5G55820). Borealin protein sequence data of other organisms from this article can be found in the NCBI data libraries under the following accession numbers: *Brachypodium distachyon* (XP_003570304.1), *Citrus clementina* (XP_006428073.1), *Drosophila melanogaster* (NP_609279.1), *Homo sapiens* (NP_001243804.1), *Marchantia polymorpha* (PTQ34788.1), *Micromonas commoda* (XP_002503544.1), *Mus musculus* (NP_080836.3), *Oryza sativa* (XP_015650359.1), *Physcomitrella patens* (XP_024357151.1), *Populus trichocarpa* (XP_024458072.1), *Saccharomyces cerevisiae* (AJV31725.1), *Schizosaccharomyces pombe* (CAA22184.2), *Selaginella moellendorffii* (XP_024537708.1), *Vitis vinifera* (XP_002280684.1), *Xenopus tropicalis* (NP_001002902.1), *Zea mays* (NP_001142076.1).

SUPPLEMENTAL DATA

The following supplemental materials are available.

Supplemental Figure S1. Complementation test of BORR and INCENP mutants.

Supplemental Figure S2. Both BORR and AUR3 localize to inner centromeres.

Supplemental Table S1. Primers used in this study.

Supplemental Table S2. Synthesized oligonucleotides used in this study.

Supplemental Movie S1. Subcellular localization of GFP:INCENP during mitosis.

Supplemental Movie S2. Subcellular localization of BORR:GFP during mitosis.

Supplemental Movie S3. Subcellular localization of AUR3:GFP during mitosis.

Supplemental Movie S4. Co-localization of AUR3:GFP and BORR:RFP.

Supplemental Movie S5. Co-localization of AUR3:GFP and RFP:INCENP.

Supplemental Movie S6. Co-localization of GFP:INCENP and BORR:RFP.

Supplemental Movie S7. Comparison of subcellular localization between BORR:GFP and RFP:CENH3.

Supplemental Movie S8. Subcellular localization of BORR:GFP during meiosis.

Supplemental Movie S9. Subcellular localization of GFP:INCENP during meiosis.

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Footnotes

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Table 1. The genetic material generated in this study.

Construct	Genetic background
amiRNAi lines	
PRO _{35S} :amiBORR#1	Wild type
PRO _{35S} :amiBORR#2	Wild type
FP expression lines	
PRO _{BORR} :mGFP:BORR	<i>borr-1</i>
	PRO _{RPS5A} :TagRFP:TUA5
PRO _{BORR} :BORR:mGFP	<i>borr-1</i>
	PRO _{RPS5A} :TagRFP:TUA5
	PRO _{CENH3} :TagRFP:CENH3
PRO _{BORR} :BORR ^{S214A} :mGFP	<i>borr-1</i>
	PRO _{RPS5A} :TagRFP:TUA5
PRO _{BORR} :BORR ^{S214D} :mGFP	<i>borr-1</i>
	PRO _{RPS5A} :TagRFP:TUA5
PRO _{INCENP} :mGFP:INCENP	<i>wyr-2</i>
	PRO _{RPS5A} :TagRFP:TUA5
	PRO _{BORR} :BORR:TagRFP
PRO _{INCENP} :INCENP:mGFP	<i>wyr-2</i>
PRO _{AUR3} :AUR3:mGFP	PRO _{RPS5A} :TagRFP:TUA5
	PRO _{RPS5A} :TagRFP:TUA5 (<i>amiBORR#1-1</i>)
	PRO _{BORR} :BORR:TagRFP
	PRO _{INCENP} :mRUBY3:INCENP
PRO _{CENH3} :TagRFP:CENH3 PRO _{RPS5A} :TagRFP:TUA5	<i>amiBORR#1-1</i>
GUS expression line	
PRO _{BORR} :BORR:GUS	Wild type

Figure 1

BOREALIN-RELATED (BORR) gene structure in plants. A, Protein sequence of BORR in Arabidopsis. Predictions of the N-terminal helix in BORR are shown in (a) and alignment of the most sequence conserved region of the protein in (b). Arrow heads in (b) indicate the conserved CDK consensus sites. B, Phylogenetic analysis of the Borealin family in yeast, animals and plants. The tree was constructed using MEGA X by the neighbor-joining method.

Figure 2

***BORR* is required for seed development.** A, Wildtype or heterozygous *borr* mutant plants pollinated with pollen from wildtype or heterozygous *borr* mutants. Black and gray arrowheads indicate tiny white ovules (unfertilized ovules/early arrested seeds) and collapsed brown seeds or seeds without green embryo (late aborted seeds), respectively. B, Proportion of the seed phenotypes shown in (A). Total number (*n*) of seeds and ovules from five hand-pollinated siliques are shown in each cross. C, Seeds and ovules from the heterozygous *borr* siliques cleared in chloral hydrate solution. At the globular embryo stage, developing seeds with abnormal embryos, developing seeds with no embryo, and unfertilized ovules were observed. D, Classification of the embryo phenotype for each cross. Total number (*n*) of developing seeds and unfertilized ovules from 6-8 hand-pollinated pistils are shown.

Figure 3

Phenotypal analysis of artificial miRNA-mediated BORR knockdown plants. A, Sequence alignments of artificial miRNAs and their target sites on BORR mRNA. B, Relative expression level of BORR in the amiBORR plants was confirmed by qRT-PCR analysis with three biological replicates. C, 9-day-old wild-type and amiBORR seedlings. D, Root growth measurements of the wildtype and amiBORR plants. 4-day-old seedlings were transferred to a MS plate and root lengths were measured for 5 days. ** $P < 0.01$, Student's t -test. Error bars indicate SD ($n = 10$). E, Confocal images of 7-day-old wild-type and amiBORR#1-1 roots stained with propidium iodide. amiBORR#1-1 roots were categorized into 'Mild' and 'Severe' groups based on the phenotype. Arrows indicate the boundary between the dividing region and the elongation region of the root. The Panels Columella region and Dividing region show close-up views of the regions marked by white and yellow boxes, respectively. F and G, Root meristem size (F) and number of meristematic cortex cells (G). ** $P < 0.01$, Student's t -test. Error bars indicate SD ($n = 15$). H, Representative images of normally distributed and lagging chromosomes in 7-day-old WT and amiBORR#1-1 root cells. Microtubules and centromeres are visualized by TagRFP:TUA5 and GFP:CENH3, respectively. The arrow indicates a lagging chromosome. I, Frequency of lagging chromosomes in anaphase in (H). Error bars indicate SD ($n = 50$). J, Representative images of AUR3 accumulation levels at kinetochores in 7-day-old wild-type and amiBORR#1-1 root cells. Microtubules and AUR3 are visualized by TagRFP:TUA5 and AUR3:GFP, respectively. K, AUR3 signal intensity at kinetochores in (J). 40 AUR3-GFP signals at kinetochores from 10 cells were measured. Median (center line), IQR (box), 1.5 x IQR (error bars), and outliers (circles) are shown. ** $P < 0.01$, Student's t -test. Error bars indicate SD.

Figure 4

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Interaction among the CPC components. A, Interaction among the CPC components were revealed by yeast two-hybrid assays. B, Interaction between AUR3 and various regions of INCENP or IN-box-mutated INCENP. Monomeric GFP (mGFP) was used as a negative control. Each strain was spotted on SD plates without tryptophan and leucine (-TL; control media) or without tryptophan, leucine, and histidine (-TLH; selection media) and photographed after incubation at 30°C for 2 days. C, Co-immunoprecipitation of INCENP with BORR. 7-day-old Arabidopsis seedlings expressing BORR:RFP and mGFP:INCENP or BORR:RFP and mGFP were used for IP with an anti-GFP antibody. Both input and IP fraction were subjected to immunoblotting with an anti-RFP antibody. The asterisk on the input panel indicates a nonspecific band.

Figure 5

Expression and subcellular localization of CPC components. A to F, Expression pattern of BORR:GUS in the shoot (A), root (B), lateral roots (C), inflorescence (D), ovules (E), and young flower buds (F). 10-day-old seedlings were used for (A) to (C), and 4-week old plants were used for (D) to (F). Three independent transgenic lines were analyzed and representative images are shown. G, Subcellular localization of GFP:INCENP, BORR:GFP, and AUR3:GFP during the cell cycle. Each reporter line was crossed with TagRFP:TUA5-expressing plants to visualize microtubule structures. H, Co-localization of the CPC components during the cell cycle. For live imaging of (G) and (H), root tips of 5-day-old seedlings were used.

Figure 6

CPC localizes to inner centromeres. Localization of the CPC from prophase to immediately before anaphase onset. Inner kinetochores and CPC complexes are visualized by TagRFP:CENH3 and BORR:GFP, respectively. For live cell imaging, root tips of 5-day-old seedlings were used. Blue bar indicates the position where the line profiles were obtained.

Figure 7

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Subcellular localization of CPC components in meiosis. A and B, Subcellular localization of BORR:GFP (A) and GFP:INCENP (B) during meiosis. Each reporter line was crossed with TagRFP:TUA5-expressing plants to visualize the microtubule structures. For live imaging of (A) and (B), flower buds of 1-month-old plants were used.

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