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Heterologous expression of a lycophyte protein enhances angiosperm seedling vigor

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Running title: *BRX variants boost growth vigor*

22 **Abstract**

23 Seedling vigor is a key agronomic trait that determines juvenile plant performance. Angiosperm seeds
24 develop inside fruits and are connected to the mother plant through vascular tissues. Their formation
25 requires plant-specific genes, such as *BREVIS RADIX (BRX)* in *Arabidopsis thaliana* roots. BRX family proteins
26 are found throughout the euphyllophytes but also occur in non-vascular bryophytes and non-seed
27 lycophytes. They consist of four conserved domains, including the tandem “BRX-domains”. We found that
28 bryophyte or lycophyte BRX homologs can only partially substitute for Arabidopsis BRX (AtBRX) because
29 they miss key features in the linker between the BRX-domains. Intriguingly however, expression of a BRX
30 homolog from the lycophyte *Selaginella moellendorffii* (SmBRX) in *A. thaliana* wildtype background confers
31 robustly enhanced root growth vigor that persists throughout the life cycle. This effect can be traced to a
32 substantial increase in seed and embryo size, is associated with enhanced vascular tissue proliferation, and
33 can be reproduced with a modified, “SmBRX-like” variant of AtBRX. Our results thus suggest that BRX
34 variants can boost seedling vigor and shed light on the activity of ancient, non-angiosperm BRX family
35 proteins.

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37

38 **Keywords:** *Arabidopsis*, *Selaginella*, *Marchantia*, *Physcomitrium*, vigor, seed, embryo, root, phloem, BRX;

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41 Introduction

42 Plant evolution is marked by major transitions that have led to the angiosperms, the flowering seed plants
43 that dominate the extant terrestrial biosphere (Amborella Genome, 2013; Rensing, 2020; Spencer et al.,
44 2021). Key inventions include the evolution of vascular tissues, which separates lycophytes from the
45 bryophytes; enclosure of the embryo in a seed, which separates spermatophytes from ferns; and protection
46 of the seeds inside fruits, which separates angiosperms from gymnosperms. The development of such
47 evolutionary novelties often entails plant-specific gene families (Armisen et al., 2008; Guo, 2013; Jiao et al.,
48 2020; Pfannebecker et al., 2017; Rensing, 2020). Among them, the *BREVIS RADIX (BRX)* gene family
49 comprises five members in the angiosperm model plant *Arabidopsis thaliana*, *AtBRX* and its homologs
50 *AtBRX-LIKE (AtBRXL) 1-4* (Beuchat et al., 2010a; Briggs et al., 2006). The encoded BRX family proteins consist
51 of four distinct, highly conserved domains that are not found outside the green lineage (Briggs et al., 2006;
52 Koh et al., 2021). They include the signature tandem “BRX-domains”, which are connected by a linker of
53 variable sequence and size (Koh et al., 2021). Originally, *AtBRX* had been identified based on a rare natural
54 loss-of-function variant that might confer an adaptative advantage in certain conditions (Gujas et al., 2012;
55 Mouchel et al., 2004; Shindo et al., 2008). Moreover, a rare *AtBRX* gain-of-function allele that carries a
56 deletion in the linker between the BRX-domains is associated with slightly increased root growth vigor
57 (Beuchat et al., 2010a). *BRX* family gene variants have also been implicated in *Brassica* domestication
58 (Zhang et al., 2021). These independent evidences suggest that allelic variations in *BRX* family genes are
59 relevant for the evolution of trait diversity.

60 All BRX family proteins monitored to date are primarily plasma-membrane-associated and display
61 polar cellular localization (Bringmann and Bergmann, 2017; Koh et al., 2021; Marhava et al., 2020; Marhava
62 et al., 2018; Rowe et al., 2019; Scacchi et al., 2009). The biological functions of *BRX* family genes are diverse
63 and point to sub-functionalization of individual family members (Koh et al., 2021; Li et al., 2019; Marhava
64 et al., 2020; Rowe et al., 2019; Zhang et al., 2021). For instance, the best-characterized members, *AtBRX*
65 and *AtBRXL2*, are interchangeable in stomata development (Rowe et al., 2019) but not in root protophloem
66 development (Koh et al., 2021; Marhava et al., 2020), and this unequal redundancy has recently been
67 associated with differences in protein behavior (Koh et al., 2021; Marhava et al., 2020). In the root, *AtBRX*
68 guides the progression of protophloem sieve element differentiation by modulating the local trans-cellular
69 flux of the phytohormone, auxin (Marhava et al., 2018; Moret et al., 2020). In *brx* loss-of-function mutants,
70 protophloem differentiation is thus impaired and consequently, root growth vigor is strongly reduced (Anne
71 and Hardtke, 2017; Moret et al., 2020; Mouchel et al., 2004; Rodrigues et al., 2009). *AtBRX* protein is polarly
72 localized at the rootward end of developing protophloem sieve elements, where it interacts with an AGC-

73 type kinase regulator of the auxin transport machinery in an intricate, feedback-regulated “molecular
74 rheostat” (Aliaga Fandino and Hardtke, 2022; Bassukas et al., 2021; Marhava et al., 2018). A key feature of
75 this molecular rheostat is the auxin-responsive plasma-membrane-dissociation of AtBRX (Marhava et al.,
76 2018; Scacchi et al., 2009), which is a quantitative determinant of BRX family protein activity in the
77 developing protophloem context (Koh et al., 2021; Marhava et al., 2020). It has been mapped to AGC kinase
78 target phosphosites, including a key site in the linker sequence, which are present in AtBRX but absent in
79 AtBRXL2 (Koh et al., 2021). Engineering these sites into AtBRXL2 renders the modified protein auxin-
80 responsive and augments its biological activity in the protophloem (Koh et al., 2021). Conversely, in a BRX
81 family protein from the lycophyte *Selaginella moellendorffii* (SmBRX), the phosphosites are missing and the
82 linker is much shorter than in *A. thaliana* BRX family proteins (Koh et al., 2021). Thus, SmBRX plasma-
83 membrane-association is not auxin-responsive and can only partially rescue the protophloem
84 differentiation defects of *brx* loss-of-function mutants (Koh et al., 2021). In summary, the available data
85 suggest that sub-functionalization of BRX family proteins is at least in part determined by the sequence of
86 the linker between the tandem BRX-domains.

87 Despite their incapacity to fully complement the *brx* mutant, both AtBRXL2 and SmBRX can confer
88 significant rescue of average root growth vigor when expressed under control of the *AtBRX* promoter
89 (Beuchat et al., 2010a; Briggs et al., 2006; Koh et al., 2021). This might reflect the significant yet partial
90 rescue of protophloem defects, which manifests in a strongly reduced proportion of seedlings that display
91 visibly impaired differentiation in both sieve element strands and suggests that at least one strand is often
92 functional (Breda et al., 2017; Koh et al., 2021). Nevertheless, compared to other BRX family proteins that
93 lack the key AGC kinase target phosphosite in the linker and were monitored previously (Beuchat et al.,
94 2010a; Briggs et al., 2006; Marhava et al., 2020), the rescue of *brx* root growth vigor obtained with SmBRX
95 was remarkable and statistically indistinguishable from the Columbia-0 (Col-0) wildtype control (Koh et al.,
96 2021). Here, we explored this phenomenon in detail and found that SmBRX expression in *A. thaliana*
97 wildtype substantially enhances seedling vigor.

98

99 **Results**

100 *The linker between the BRX-domains determines BRX protein family sub-functionalization*

101 Alignment of 300 full length *bona fide* BRX family proteins retrieved from across the green lineage (One
102 Thousand Plant Transcriptomes, 2019) shows that the key AGC target phosphosite (corresponding to S228
103 in AtBRX) is embedded in a 20 amino acid motif within the linker (SAXXSPVTPPLXKERLPRNF) that is
104 conserved in the vast majority of BRX family proteins (Dataset S1 and Figure S1). Although the phosphosite
105 serine shows the highest level of conservation (89%) within this motif, the AGC kinase consensus (R[D/E]S)
106 is only present in a subset of ca. 10% of BRX family proteins that are all exclusively from angiosperms.
107 Moreover, in AtBRX and its interchangeable homolog AtBRXL1 (Briggs et al., 2006; Koh et al., 2021), the
108 R[D/E]S site is present but the motif is only partly conserved. Finally, the motif is notably absent from all
109 lycophyte and bryophyte BRX family proteins examined.

110 To further determine the functional relevance of this region, we chose to investigate a few
111 representative BRX family proteins from different phylogenetic branches that display a combination of
112 linker features (Figure 1A and Figure S1). First, we identified two *BRX* family genes in the early diverging
113 angiosperm lineage, *Amborella trichopoda* (*AmbBRXL1* and *AmbBRXL2*). Both encoded proteins have linkers
114 of a size comparable to *A. thaliana* BRX family proteins (125 and 120 amino acids, respectively), and in both
115 the 20 amino acid motif is conserved. However, only *AmbBRXL2* carries the R[D/E]S consensus phosphosite
116 (Figure S1). When expressed under control of the *AtBRX* promoter, an *AmbBRXL1*-CITRINE fusion protein
117 codon-optimized for *A. thaliana* at best partially complemented the root growth (Figure S2A) or
118 protophloem defects (Figure S2B) of *A. thaliana brx* mutants. By contrast, a codon-optimized *AmbBRXL2*-
119 CITRINE fusion protein fully complemented all *brx* mutant defects (Figure S2C and D), and consistently,
120 *AmbBRXL2*, but not *AmbBRXL1*, displayed auxin-induced plasma-membrane-dissociation (Figure S2K-M).
121 These functional assays reiterate the importance of the S228 phosphosite for AtBRX-like activity.

122 Next, using the same complementation approach, we monitored three BRX family proteins
123 identified in bryophytes, the two proteins identified in the *Physcomitrium patens* genome (*PpBRXL1* and
124 *PpBRXL2*), and the single protein found in the *Marchantia polymorpha* genome (*MpBRXL1*). All three
125 proteins lack the R[D/E]S phosphosite as well as the 20 amino acid motif, but whereas *PpBRXL1* and
126 *PpBRXL2* linker sizes are comparable to *A. thaliana* BRX family proteins (127 and 122 amino acids,
127 respectively), the *MpBRXL1* linker is considerably shorter (61 amino acids) (Figure S1). As expected, all three
128 proteins only partially rescued root growth vigor or protophloem defects of *brx* mutants (Figure S2E-J), and
129 none of them was auxin-responsive (Figure S2N-P). Since all five proteins assayed displayed protophloem-
130 specific expression and polar localization similar to AtBRX (Figure S2Q-U), we conclude that the linker

131 sequence between the BRX-domains is a major determinant of specific activity, consistent with previous
132 findings (Beuchat et al., 2010a; Koh et al., 2021); and that the entire functional spectrum represented by
133 AtBRX is only contained in a subgroup of angiosperm BRX family proteins.

134 *Seedling growth vigor is enhanced by heterologous SmBRX expression*

135 Our assays of bryophyte BRX family proteins reiterate that the linker region contains the functional features
136 that make AtBRX unique and are required for proper protophloem sieve element differentiation (Koh et al.,
137 2021). Notably however, neither of the three bryophyte proteins consistently conferred the essentially
138 wildtype root growth vigor observed previously in complementation experiments with SmBRX (Koh et al.,
139 2021), which has the shortest (37 amino acids) linker identified so far and lacks any of the conserved linker
140 sequences recognizable in most other BRX family proteins (Figure 1A and Dataset S1). To further explore
141 this phenomenon, we expressed SmBRX-CITRINE fusion protein under control of the *AtBRX* promoter in
142 wildtype background. Corroborating its growth-promoting effect, these transgenic lines displayed
143 significantly increased growth vigor that manifested in ca. 25% longer roots in 7-day-old seedlings (Figure
144 1B). Such root growth promotion was neither observed in similar experiments with AmbBRXL1, MpBRXL1
145 and AtBRXL1 (Figure 1C-E), nor upon copy number increase of AtBRXL1 or AtBRXL2 (Figure 1F and G), nor
146 upon ectopic overexpression of AtBRX or AtBRXL2 under control of the ubiquitous *35S* promoter (Figure
147 1H and I). To exclude that the growth-promoting property of SmBRX was due to its codon-optimization for
148 *A. thaliana*, we introduced a similar construct expressing a non-codon-optimized version (SmBRX^{noP}) into
149 *brx* mutants and Col-0 wildtype. Similar to codon-optimized SmBRX, this protein displayed sieve element-
150 specific expression and polar localization (Figure S2V). Again, we observed partial complementation of
151 protophloem defects and wildtype-level root growth vigor in *brx* (Figure S3A and B), and larger-than-
152 wildtype root growth vigor in Col-0 (Figure S3C). Conversely, a codon-optimized version of AtBRX (AtBRX^{opt})
153 essentially behaved like the wildtype protein (Figure S3D-F). In summary, these experiments suggest that
154 structural features of the SmBRX protein are responsible for its growth-promoting property.

155 Next, we tested whether SmBRX mediates enhanced root growth across a range of conditions. We
156 found that both wildtype and SmBRX transgenics responded to variations in sucrose concentration in the
157 media, but in all conditions, the SmBRX transgenics displayed longer roots than wildtype (Figure 2A-C). In
158 tendency, the difference in growth vigor was even amplified in the absence of sucrose (Figure 2A). Likewise,
159 SmBRX transgenics maintained their growth advantage in various adverse conditions, such as absence of
160 any nutrients (Figure 2D), sub-optimal pH (Figure 2E), or challenge by peptides that suppress protophloem
161 formation (Depuydt et al., 2013) (Figure 2F). Finally, although Col-0 seeds are essentially non-dormant, we
162 stimulated germination by application of gibberellic acid to exclude that the root growth differences could

163 result from a premature germination of SmBRX transgenics (Figure 2G). Finally, SmBRX transgenics also kept
164 their advantage when treated with the gibberellic acid antagonist, abscisic acid (Topham et al., 2017)
165 (Figure S4A). In summary, our experiments suggest that heterologous expression of SmBRX in *A. thaliana*
166 results in a robust increase in seedling growth vigor.

167 *SmBRX confers increased seed and embryo size*

168 To determine whether the growth advantage of SmBRX transgenics in tissue culture translates into the soil
169 environment, we first tested the capacity of their roots to vertically penetrate a ca. 2 cm thick barrier of
170 densely packed pebbles embedded in media. In this assay, SmBRX transgenics performed as well as
171 wildtype (Figure 2H), suggesting that they maintained their capacity to navigate a complex environment.
172 We then monitored adult root system growth in a greenhouse setting. In replicate experiments with a setup
173 of 96 tubes, SmBRX transgenics maintained their growth advantage until at least 31 days after germination.
174 The mean difference to wildtype remained relatively constant in comparisons between 21-day-old (Figure
175 2I) and 31-day-old (Figure 2J) plants however (between 3-4 cm, with average wildtype length ca. 28 and 37
176 cm, respectively), suggesting that SmBRX transgenics do not display a permanently higher growth rate but
177 might carry over an early advantage. Since we had excluded a causative role of premature or accelerated
178 germination (Figure 2G), we closely inspected the seeds. Indeed, seeds from SmBRX transgenics were
179 visibly bigger than wildtype seeds (Figure 2K). Size approximation through image analysis of flatbed scans
180 of dried seeds confirmed this notion and its statistical significance for a sample of independent transgenic
181 lines and wildtype seed batches harvested at different times (Figure 2L and M). By contrast, seed batches
182 of *brx* mutants were similar in size to wildtype (Figure 2L and M). Moreover, a high resolution image analysis
183 (Figure S4B and C) of thousands of individual seeds confirmed these results in a comparison of wildtype
184 with independent transgenic lines that either expressed an AtBRX-CITRINE fusion protein or an SmBRX-
185 CITRINE fusion protein under control of the *AtBRX* promoter: wildtype and AtBRX transgenics were largely
186 similar in the distributions of surface projection (Figure 3A), maximum seed length (Figure 3B) and
187 maximum seed width (Figure 3C), whereas the distribution of SmBRX transgenics was skewed to higher
188 values for all parameters tested (Figure 3A-C). Based on the average length and width, we estimated that
189 the derived idealized ellipsoid seed volume of SmBRX transgenics is increased by about 75% compared to
190 wildtype or AtBRX transgenics (ca. 0.190 vs ca. 0.107 cubic micrometers). In summary, our data suggest
191 that the seeds of SmBRX transgenics are substantially bigger than in wildtype. Dried *A. thaliana* seeds
192 largely represent the mature embryo, because the endosperm is essentially consumed as embryogenesis
193 progresses (Doll and Ingram, 2022; Lafon-Placette and Kohler, 2014). This suggested that mature SmBRX
194 transgenic embryos are bigger than wildtype embryos, which turned out to be the case (Figure 4A and B).

195 Finally, the observation matches the reported activity of the *AtBRX* promoter during embryogenesis (Bauby
196 et al., 2007; Scacchi et al., 2009). Investigation of SmBRX transgenics by confocal microscopy imaging
197 matched these earlier findings and revealed expression of the SmBRX-CITRINE fusion protein in the shoot
198 apical meristem of early and late heart stage embryos (Figure 4C and D) that ceased in torpedo stage (Figure
199 4E), whereas expression in the developing (phloem) vasculature of the hypocotyl-root axis and the
200 cotyledons was observed from the bent cotyledon stage onwards (Figure 4F) and persisted in maturing
201 embryos (Figure 4G and H). Moreover, stele width, measured at the hypocotyl-radicle junction, was
202 significantly wider in SmBRX transgenics than in wildtype (Figure 4I). Consistently, cell counts across tissue
203 layers showed an increase specifically in vascular cell files in SmBRX transgenics (Figure 4J), which might
204 thus drive the size increase.

205 Next we asked whether the increased seed size in SmBRX transgenics is associated with tradeoffs
206 in seed or biomass productivity? In greenhouse-grown plants, we did not observe any difference in fruit
207 size (silique length, Figure 2N), however we observed a statistically significant, ca. 10% reduction in seed
208 number (Figure 2O). Nevertheless, total dry seed weight per plant was similar if not in tendency higher in
209 SmBRX transgenics (Figure 2P). Collectively, our data therefore suggest that the *SmBRX* transgene triggers
210 a substantial increase in seed size, accompanied by a small decrease in seed number, but does not adversely
211 affect overall plant productivity.

212 *An AtBRX in-frame deletion variant recapitulates SmBRX gain-of-function effects*

213 Because SmBRX does not only diverge from AtBRX in the linker between the BRX-domains, but also in the
214 non-conserved sequences that connect the conserved domains in the N-terminal regions, we sought to
215 determine whether the reduced linker size of 37 amino acids was causative for the SmBRX-mediated
216 growth promotion. To this end we engineered an AtBRX variant in which residues 219 to 266 were deleted,
217 thereby removing the 20 amino acid motif including the RES phosphosite (Figure S1) and reducing the linker
218 size from 93 to 45 amino acids. This “SmBRX-like” AtBRX (*AtBRX^{Sml}*) was then introduced into Col-0 wildtype
219 plants and *brx* mutants as a CITRINE fusion protein, expressed under control of the *AtBRX* promoter.
220 Intriguingly, *AtBRX^{Sml}* transgenics displayed essentially similar features as SmBRX transgenics: in *brx*
221 background, protophloem defects were only partially rescued (Figure 5A) although root growth vigor was
222 similar to wildtype (Figure 5B); in wildtype background, *AtBRX^{Sml}* promoted root elongation as much as
223 SmBRX (Figure 5C) and again this could be traced back to a bigger seed size (Figure 5D). In summary, these
224 data suggest that removal of the regulatory phosphosite in conjunction with a size reduction in the linker
225 between the BRX-domains confers a dominant, growth-promoting effect on AtBRX.

226

227 Discussion

228 Plants represent a variation of eukaryotic multicellularity that is distinct from animals in its unique
229 structural, physiological and cellular characteristics. They evolved independently and it is therefore not
230 surprising that their genomes encode proteins that are specific to the green lineage (Armisen et al., 2008;
231 Guo, 2013; Jiao et al., 2020; One Thousand Plant Transcriptomes, 2019; Rensing, 2020). The BRX family
232 proteins are a prime example in this context, since their highly conserved domains are not found outside
233 plants, not even in some rudimentary form. Besides the two conserved domains in the N-terminal half of
234 BRX proteins, the tandem BRX-domains in the C-terminal half are most prominent and can also be found in
235 single copy in another plant-specific group of proteins that typically also contain, among others, a lipid-
236 binding domain (Briggs et al., 2006; Furutani et al., 2020; van Leeuwen et al., 2004). The exact molecular
237 function of the BRX-domains remains somewhat obscure, but evidence from various independent contexts
238 suggests that they primarily mediate both homologous and heterologous protein-protein interactions and
239 membrane attachment (Briggs et al., 2006; Furutani et al., 2020; Li et al., 2019; Marhava et al., 2020; Rowe
240 et al., 2019; Wang et al., 2022). BRX-domains might therefore primarily represent versatile scaffolds to
241 recruit other proteins to the plasma membrane and/or regulate their trafficking, which could explain their
242 involvement in various processes, such as subcellular polarity establishment and maintenance or
243 modulation of phytohormone transport (Furutani et al., 2020; Li et al., 2019; Marhava et al., 2020; Marhava
244 et al., 2018; Moret et al., 2020; Muroyama et al., 2020; Rowe et al., 2019).

245 The founding member of the BRX protein family in *A. thaliana* has been identified via a loss-of-
246 function allele, through a natural variation approach (Mouchel et al., 2004). Thus, despite the high
247 conservation of AtBRX and its essential role in root protophloem development (Aliaga Fandino and Hardtke,
248 2022; Rodriguez-Villalon et al., 2014), the *AtBRX* gene is apparently dispensable in particular circumstances.
249 Indeed, additional, extant loss-of-function alleles isolated from natural settings suggest that in acidic soils,
250 where root growth is generally inhibited, such alleles might even confer a competitive advantage (Gujas et
251 al., 2012). Conversely, *AtBRX* was also identified as a quantitative trait locus that conferred slightly yet
252 significantly increased root growth vigor (Beuchat et al., 2010a). The underlying, causative natural *AtBRX*
253 allele carries a small, seven amino acid in-frame deletion in the linker between the BRX-domains (Beuchat
254 et al., 2010a). This deletion does not affect the phosphosite that is necessary for comprehensive AtBRX
255 function in the protophloem (Koh et al., 2021), but supports the conclusion from our current study that the
256 linker has a pivotal influence on the activity of BRX family proteins.

257 Among the BRX family proteins that we identified, SmBRX has the shortest linker sequence and
258 lacks the critical phosphosite required to fully replace AtBRX in root protophloem development (Koh et al.,

259 2021). However, additional, heterologous SmBRX expression in *A. thaliana* wildtype confers increased seed
260 and embryo size, and subsequently enhanced seedling vigor. The engineered AtBRX^{sml} variant, which we
261 synthesized using AtBRX as the backbone, has the same effects in both Col-0 wildtype and *brx* mutant
262 backgrounds. This supports the idea that the unique amino acid sequence of SmBRX is not responsible for
263 the observed gain-of-function effects, but rather structural features that depend on the distance between
264 the two BRX-domains. Notably, this effect was observed upon expression under control of the relatively
265 weak *AtBRX* promoter (Bauby et al., 2007; Beuchat et al., 2010b; Scacchi et al., 2009). Whether the
266 observed gain-of-function effects are directly related to the expression in the protophloem remains unclear
267 however, because the *AtBRX* promoter confers initially wider expression during embryogenesis (including
268 the apical shoot meristem and the root stem cell niche) (Bauby et al., 2007; Scacchi et al., 2009), and
269 because importantly, the gene body also shapes the *AtBRX* expression pattern (Koh et al., 2021).
270 Nevertheless, the observed SmBRX protein expression pattern in embryogenesis is consistent with the
271 increased vascular proliferation, and also with the observation that *AtBRX* loss-of-function mutants display
272 smaller cotyledons (Beuchat et al., 2010b).

273 Interestingly, the knock-out allele of *brx* in Col-0 background used in this study was originally
274 identified because of its hypersensitive response to the phytohormone abscisic acid (Rodrigues et al.,
275 2009). One of the various biological functions of abscisic acid is the promotion of seed dormancy, however
276 our experiments do not support premature or accelerated germination as causative for the enhanced
277 seedling root growth upon SmBRX or AtBRX^{sml} expression. Nevertheless, SmBRX/AtBRX^{sml}-conferred gain-
278 of-function might reflect reduced abscisic acid sensitivity, because loss-of-function mutants in the
279 downstream abscisic acid effector ABSCISIC ACID-INSENSITIVE 5 form bigger seeds (Cheng et al., 2014; Li
280 and Li, 2016), and because the abscisic acid antagonist, gibberellic acid, promotes post-germination root
281 meristem growth (Achard et al., 2009; Moubayidin et al., 2010). However, our observation that SmBRX
282 transgenics respond normally to abscisic acid does not support this scenario. Nevertheless, these leads
283 could be investigated in follow up experiments, which might also clarify why exactly SmBRX/AtBRX^{sml} seeds
284 are bigger, since multiple interactions between seed coat, endosperm and embryo contribute to the final
285 outcome of seed development (Doll and Ingram, 2022; Lafon-Placette and Kohler, 2014; Li and Li, 2016).
286 Given the high conservation of BRX family proteins in both non-vascular and non-seed plants, such analyses
287 might also aid in the characterization of their ancestral function. Phylogenetic analyses of linker sequence
288 evolution could yield additional insight and clarify whether there is an evolutionary trend in linker length
289 or sequence. Protein homology searches suggest that within the euphyllophytes, BRX family proteins are
290 prevalent in angiosperms and might be less common in gymnosperms or ferns. This could, however, also

291 reflect sampling bias (One Thousand Plant Transcriptomes, 2019). Combined with experimental
292 verification, phylogenetic analyses could also clarify whether there is a linker threshold length or absent
293 sequence feature associated with the gain-of-function effect, which could for example explain why it was
294 not observed with the relatively short linker of MpBRXL1. Finally, irrespective of the mechanism through
295 which SmBRX/AtBRX^{smi} gain-of-function operates, and which we did not identify here, our data suggest that
296 BRX family protein variants might be applied as tools to robustly modify seedling vigor without yield penalty.
297

298 **Materials and Methods**

299 *Sequences*

300 The protein sequences analyzed in this paper are provided in the supplemental information, Dataset S1.
301 The open reading frame coding sequences used for the creation of transgenes are provided in the
302 supplemental information, Dataset S2.

303 *Plant material and growth conditions*

304 The *A. thaliana* Columbia-0 (Col-0) accession was the wildtype background for all lines produced in this
305 study. Transgenes were assayed in Col-0 or the *brx-2* mutant allele (Rodrigues et al., 2009) background. For
306 tissue culture phenotyping assays, seeds were surface sterilized and then stratified for 2 d in the dark at ca.
307 4°C. Seeds were then germinated and grown in continuous white light of ca. 120 μ E intensity at ca. 22°C,
308 on vertically placed Petri dishes that contained 0.5 X Murashige and Skoog (MS) media supplemented with
309 1% agar and 0.3% sucrose, or variations as indicated in figure panel labels. Adult plants were either
310 monitored under controlled conditions in a walk-in chamber (16h light – 8h dark cycle with ca. 130 μ E light
311 intensity, ca. 22°C, ca. 60% humidity) or a greenhouse (ca. 16h light – 8 h dark cycle with variable light
312 intensity between ca. 120-360 μ E, ca. 24°C, ca. 50% humidity).

313 *Generation of transgenic lines*

314 Transgenic constructs for plant transformation were created in the pH7m34GW binary vector using the
315 GatewayTM cloning technology as previously described for *AtBRX* and *SmBRX* (Koh et al., 2021). Coding
316 sequences for the BRX family proteins and variants assayed in this study are provided in the supplemental
317 information, Dataset S2. DNA fragments for transgene construction were either obtained by gene synthesis
318 (*AtBRX^{opt}*, *AtBRX^{SmI}*, *AmbBRXL1*, *AmbBRXL2*, *MpBRXL1*) (*GeneArtTM*, using the codon optimization tool where
319 pertinent), or by RT-PCR amplification from mRNA isolated from *P. patens* or *S. moellendorffii* plants using
320 standard procedures (*SmBRX^{nop}*, *PpBRXL1*, *PpBRXL2*). All BRX family protein variants tested were expressed
321 as C-terminal CITRINE fusion proteins to permit verification of transgene expression. All binary constructs
322 were verified by Sanger sequencing and introduced into *Agrobacterium tumefaciens* strain GV3101pMP90
323 for plant transformation using the floral dip method.

324 *Phenotyping*

325 For root length or seed size measurements, plates or seeds were imaged using a high resolution (1'200 dpi)
326 flatbed scanner. Seedling root length or seed area was determined with *Fiji* image analysis software (version
327 2.0.1/1.53i), using suitable plug-ins. For quantification of gap cells in protophloem sieve element cell files

328 or visualization of fluorescent protein localization, roots were imaged by confocal microscopy as described
329 (Koh et al., 2021). High throughput and high resolution seed size measurements were performed on the
330 *Boxeed* platform run by *Labdeers Ltd*. For trait quantification in adult *A. thaliana*, plants were grown in soil
331 individually, in 50 cm long open-ended polyvinyl chloride tubes of 3.5cm diameter that were arranged in a
332 96 (8 x 12) set up. The tubes were lined with thin plastic sheets to facilitate removal of the root system and
333 subsequent careful soil washout. Root systems were then imaged together with a ruler under a fixed
334 distance camera set up and measured. Shoot productivity (seed number, silique length, seed weight) was
335 monitored at end-of-life. All phenotyping assays were performed using homozygous T3 or T4 transgenic
336 lines with verified transgene expression.

337 *Embryo microscopy and cellular analysis*

338 To visualize the SmBRX-Citrine fusion protein during embryogenesis, embryos at different developmental
339 stages were excised, fixed, cleared, and stained as described (Imoto et al., 2021). For cell counts and other
340 quantitative measures, mature embryos were dissected, fixed, stained, and imaged as previously described
341 (Bassel et al., 2014; Truernit et al., 2008). Images were acquired with a *Leica Stellaris 5* confocal microscope.

342 *Sequence analyses*

343 BRX family protein sequences were retrieved from the “One KP” transcriptome dataset (One Thousand
344 Plant Transcriptomes, 2019) and aligned and analyzed using *SnapGene* software (version 6.0.2).

345 *Quantification and statistical analysis*

346 Analyses to determine statistical significance were performed in *Graphpad Prism* software, version 9.3.1.
347 Specific statistical tests used are indicated in the figure legends and were always two-tailed.

348

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352 Methodology, S.W.H.K., H.N.D.A. and C.S.B.; Investigation, S.W.H.K., H.N.D.A. and C.S.B.; Validation,
353 S.W.H.K., H.N.D.A., C.S.B. and E.B.; Visualization, S.W.H.K., H.N.D.A. and C.S.H.; Writing – Original Draft,
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356 **Competing interests**

357 The authors declare no competing interests.

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360

361 **Figure legends**

362 **Figure 1. Heterologous expression of the *Selaginella moellendorffii* BRX family protein enhances root**
363 **growth vigor.** (A) Alignment of the linker in BRX family proteins (flanked by ten amino acids of each flanking
364 BRX-domain in bold) from *Arabidopsis thaliana* (AtBRX), *Amborella trichopoda* (AmbBRXL1 and AmbBRXL2),
365 *Selaginella moellendorffii* (SmBRX), *Physcomitrium patens* (PpBRXL1 and PpBRXL2), and *Marchantia*
366 *polymorpha* (MpBRXL1). The AGC kinase target phosphosite of AtBRX and AmbBRXL2 is highlighted in red.
367 (B) Average root length of 7-day-old seedlings from Col-0 wildtype, *brx* mutant, and transgenic lines
368 expressing the *S. moellendorffii* BRX homolog under control of the *A. thaliana* BRX promoter (*BRX::SmBRX*).
369 n=5-10 independent (transgenic) lines and experiments. (C-E) Root length of 7-day-old seedlings from Col-
370 0, *brx*, and two representative independent transgenic lines each, expressing the indicated BRX family
371 proteins under control of the *A. thaliana* BRX promoter in Col-0. (C) n=24-39 roots; (D) n=17-33 roots; (E)
372 n=17-40 roots. (F-G) Root length of 7-day-old seedlings from Col-0, *brx*, and two representative
373 independent transgenic lines with a dosage increase in the indicated *A. thaliana* BRX family gene in Col-0.
374 (F) n=15-29 roots; (G) n=23-30 roots. (H-I) Root length of 7-day-old seedlings from Col-0, *brx*, and two
375 representative independent transgenic lines each, ectopically over-expressing the indicated *A. thaliana* BRX
376 family protein under control of the constitutive 35S promoter in Col-0. (H) n=20-38 roots; (I) n=17-44 roots.
377 Box plots display 2nd and 3rd quartiles and the median, bars indicate maximum and minimum. All BRX
378 family proteins were expressed as C-terminal CITRINE fusions. Statistically significant different samples
379 (lower case letters) were determined by ordinary one-way ANOVA.

380 **Figure 2. Enhanced seedling vigor in lines expressing SmBRX is robust.** (A-C) Root growth progression in Col-
381 0 wildtype and transgenic lines expressing the *S. moellendorffii* BRX homolog under control of the *A.*
382 *thaliana* BRX promoter in Col-0 (*BRX::SmBRX*), on media with different sucrose supplements. n=30-51 roots
383 per time point. Error bars indicate standard deviation. (D) Root length of 7-day-old seedlings from Col-0
384 and *BRX::SmBRX* plants grown on plain agar media, three independent seed batches each. n=19-32 roots.
385 (E-G) Root length of 7- or 9-day-old seedlings from Col-0 and *BRX::SmBRX* plants, grown on media adjusted
386 for different pH (E) or supplemented with CLE45 peptide (F) or gibberellic acid (G). (E) n=51-74 roots; (F)
387 n=127-159 roots; (G) n=35-50 roots. (H) Number of roots that successfully penetrated a dense ca. 2 cm
388 layer of pebbles embedded in agar media at 10 days after germination. n=6 replicates, n=20 plants per
389 replicate. (I-J) Root length of 21- or 31-day-old Col-0 and *BRX::SmBRX* plants, grown in soil in individual
390 rhizotrons. n=36-42 roots. Note that rhizotron length was 50 cm and *BRX::SmBRX* plants in (J) had
391 frequently reached the bottom of the set up box. (K) Flatbed scanner images of seeds obtained from

392 greenhouse-grown Col-0 and *BRX::SmBRX* plants, equal scale. (L) Projected area of Col-0, *brx*, and
393 *BRX::SmBRX* seeds in flatbed scanner images, for seed batches harvested from independent lines at
394 different times. n=357-628 seeds. (M) Projected area of Col-0, *brx*, and *BRX::SmBRX* seeds in flatbed
395 scanner images, averages per genotype for the data shown in (L). n=3-9. (N-P) Silique length (N), seed
396 number per silique (O), and total dry seed weight per plant (P) for greenhouse-grown Col-0 and *BRX::SmBRX*
397 plants. (N) n=45 siliques each; (O) n=41-44 siliques; (P) n=15 plants each. Box plots display 2nd and 3rd
398 quartiles and the median, bars indicate maximum and minimum. Statistically significant different samples
399 (lower case letters) were determined by ordinary one-way ANOVA.

400 **Figure 3. Heterologous SmBRX confers increased seed size.** (A-C) High throughput, high resolution seed size
401 parameter measurements obtained on a *Boxeed* platform for Col-0 wildtype and three independent
402 transgenic lines each expressing either AtBRX or SmBRX under control of the *A. thaliana* BRX promoter in
403 Col-0 background. Dry seeds were harvested from mother plants grown in parallel.

404 **Figure 4. SmBRX expression in embryogenesis.** (A-B) Confocal microscopy images of calcofluor white-
405 stained mature embryos, dissected from a Col-0 (A) or SmBRX transgenic seed (B). (C-H) Confocal
406 microscopy images of SmBRX-CITRINE fusion protein (green fluorescence) expression (orange arrows) in
407 calcofluor white-stained SmBRX transgenic embryos, in early (C) or late (D) heart stage, torpedo stage (E),
408 bent cotyledon stage (F) or maturing embryos (G-H). (G) and (H) show different confocal planes of the same
409 embryo. (I-J) Stele width (I) and cell file counts (J) at the hypocotyl-radicle junction of mature embryos.
410 n=17 (I) or 10 (J) mature embryos. Box plots display 2nd and 3rd quartiles and the median, bars indicate
411 maximum and minimum. Statistically significant different samples (lower case letters) were determined by
412 ordinary one-way ANOVA.

413 **Figure 5. An AtBRX variant recapitulates SmBRX gain-of-function effects.** (A) Quantification of protophloem
414 sieve element differentiation defects (“gap cells”) in 7-day-old roots from Col-0 wildtype, *brx* mutant, and
415 three independent transgenic lines expressing an AtBRX variant with an in-frame deletion of amino acids
416 219-266 (AtBRX^{smi}) under control of the *A. thaliana* BRX promoter in the *brx* background. n=24-36 roots.
417 (B) Root length of 7-day-old seedlings corresponding to the genotypes assayed in (A). n=15-35 roots. (C)
418 Root length of 7-day-old Col-0 seedlings and transgenic seedlings expressing either SmBRX or AtBRX^{smi}
419 under control of the *A. thaliana* BRX promoter in Col-0. n=20-40 roots. (D) Projected area of seeds from
420 Col-0 and five independent transgenic AtBRX^{smi} in flatbed scanner images, for seed batches harvested from
421 mother plants grown in parallel. n=147-216 seeds. Box plots display 2nd and 3rd quartiles and the median,

422 bars indicate maximum and minimum. Statistically significant different samples (lower case letters) were
423 determined by Fisher's exact test (A) or ordinary one-way ANOVA (B-D).

424

425 **Supplemental items**

426 **Supplemental Dataset 1** Protein sequence alignment of 302 BRX family proteins retrieved from a cross the
427 green lineage with scaffold IDs (One Thousand Plant Transcriptomes, 2019) and species names, html file.

428 **Supplemental Dataset 2** Open reading frame coding sequences of BRX family genes and variants used for
429 the creation of transgenes, pdf file.

430

431 **References**

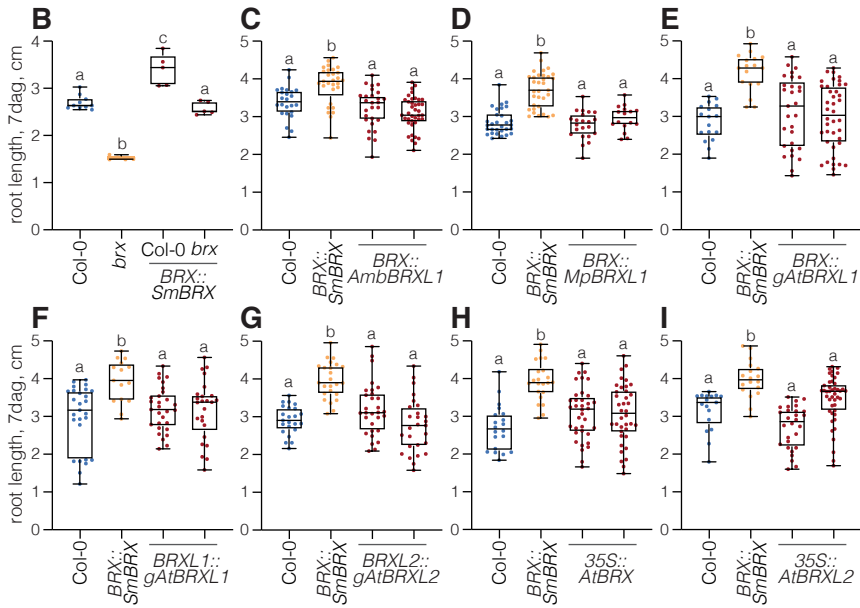
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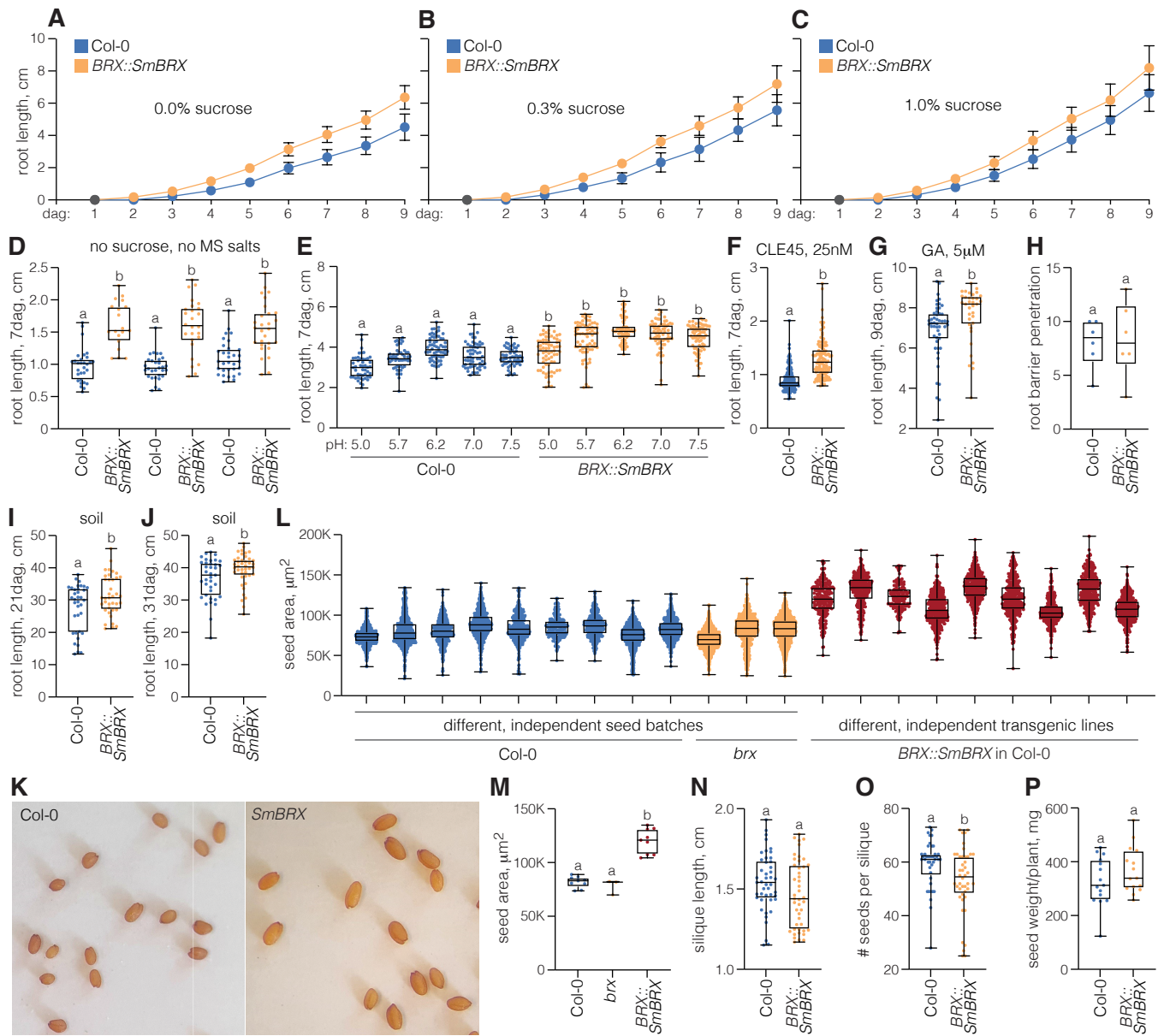
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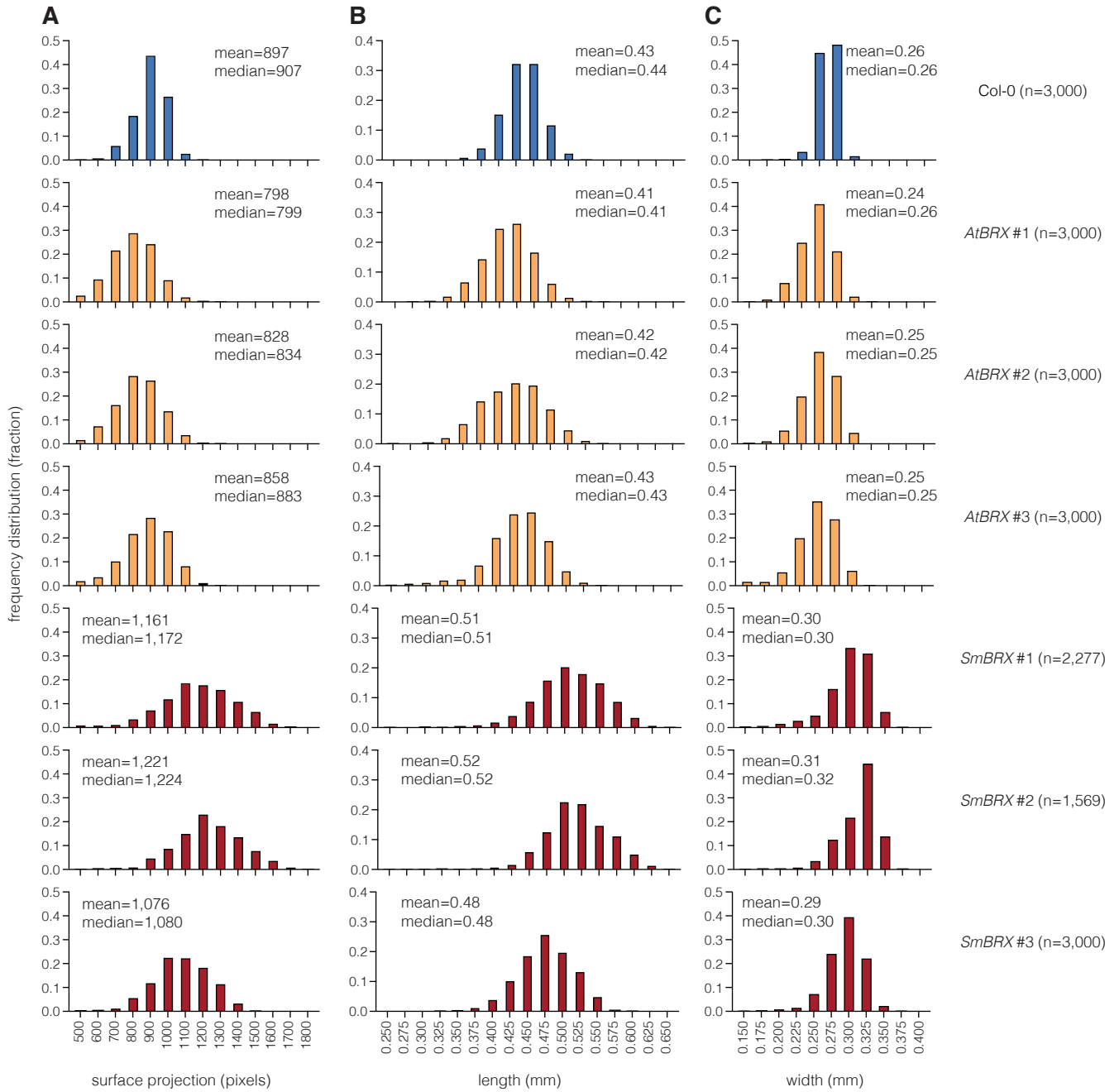
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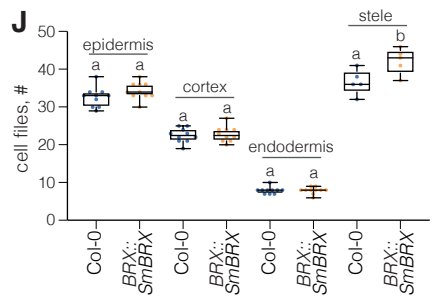
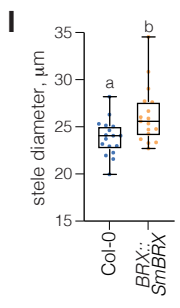
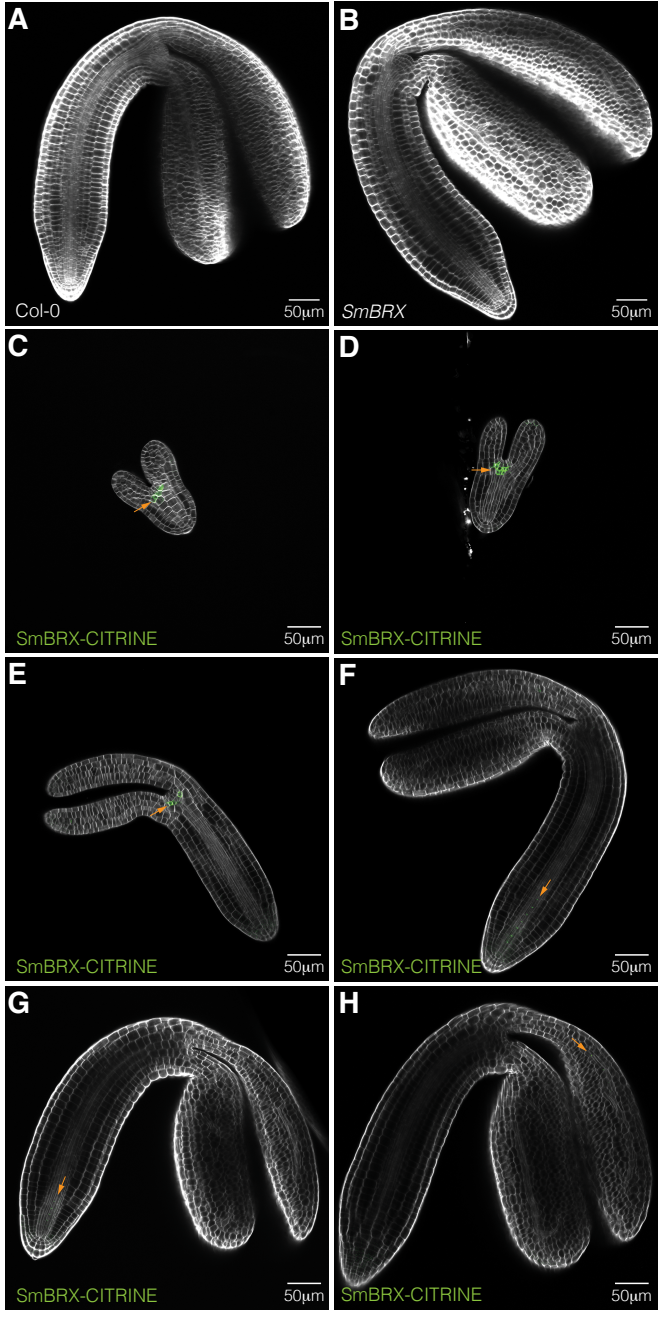
A AtBRX **DKIVELYNVQR**FNRRQALQT- PARSDDQSQRDSTYSKMDS**ARES**-----KDWTPRHNFRFP-----
 AmbBRXL1 **DKVMELYNVHK**FNRRQTVPL-PTPPRSEDES---SKMESAEDSPVTPPLNGERLPRNFQRPGGGLMSYSSSDSME
 AmbBRXL2 **DRIMELYNVVER**FRTRQAVNL-PTPPRSEDERDAS-SRVNSA**RES**PVTPPLAKERTHRNFYRPT---GKGYFSDSAE
 SmBRX **ERVHELINVS**G-----
 PpBRXL1 **NRVREATYNVPA**FERTTTANVHQVTSNSEEEQVSGVSGYATPAYSPQGSRGASMWNSPAGYGSG--ISRGASLRDTS
 PpBRXL2 **NRVREYVNVPA**FERTTTNGHQATSSSEEEQVSGVSGYATPSYSPQGSRGASTRDSFAGYSSG-----ISRGAS
 MpBRXL1 **AMVRELYNVRP**-----PSSSTAVDDDRFS--SGYATPD-----

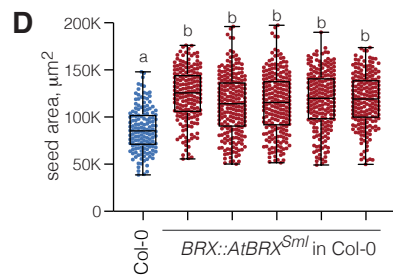
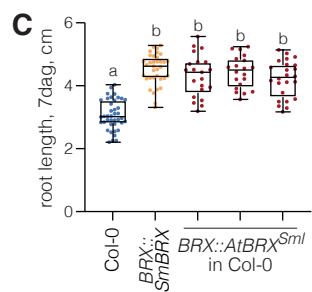
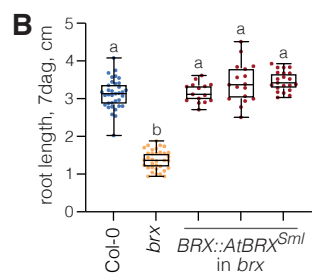
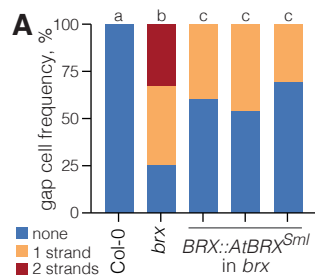
AtBRX -----GSPVPHHFYGGSSNYGPGSYHG-GPPMDAARTTTS---SRDDPP-SMSNASEMQ**AEWIEEDEPGV**
 AmbBRXL1 HHHHASCARQYDCGLASTPNLSGISAKTERSSSMDASRTSSSRDADRSDEVSVGASNVSDQ**MEWVEQDEPGV**
 AmbBRXL2 KWASAGSSYGGYNSHIFNSNSNINNNRCE-TSSIDAGRRTSS---SRDEASVSYNSASEA**ETWVEQDEPGV**
 SmBRX -----NDHSFPNSQKQVQEAQTSQQNHAEVTKSNTT-----S**DELTEWVEEDEPGV**
 PpBRXL1 SREASIREAAMREASIREAMKESMRDAVSEHSESVTCTERATETDTVAGSEAG-SDRTFDGE**ETWVEEDVPGV**
 PpBRXL2 LRDTSSREASMREPSIRESIRQSMRDAVSEHSEATCTERETETDTVAGSVAG-SDRTYDGE**ESTWVEEDVPGV**
 MpBRXL1 ----AYSNSHKSPCHSYNGDYQLTKRKVATDARSITSEKSETG-----**NEWVEQDEPGV**











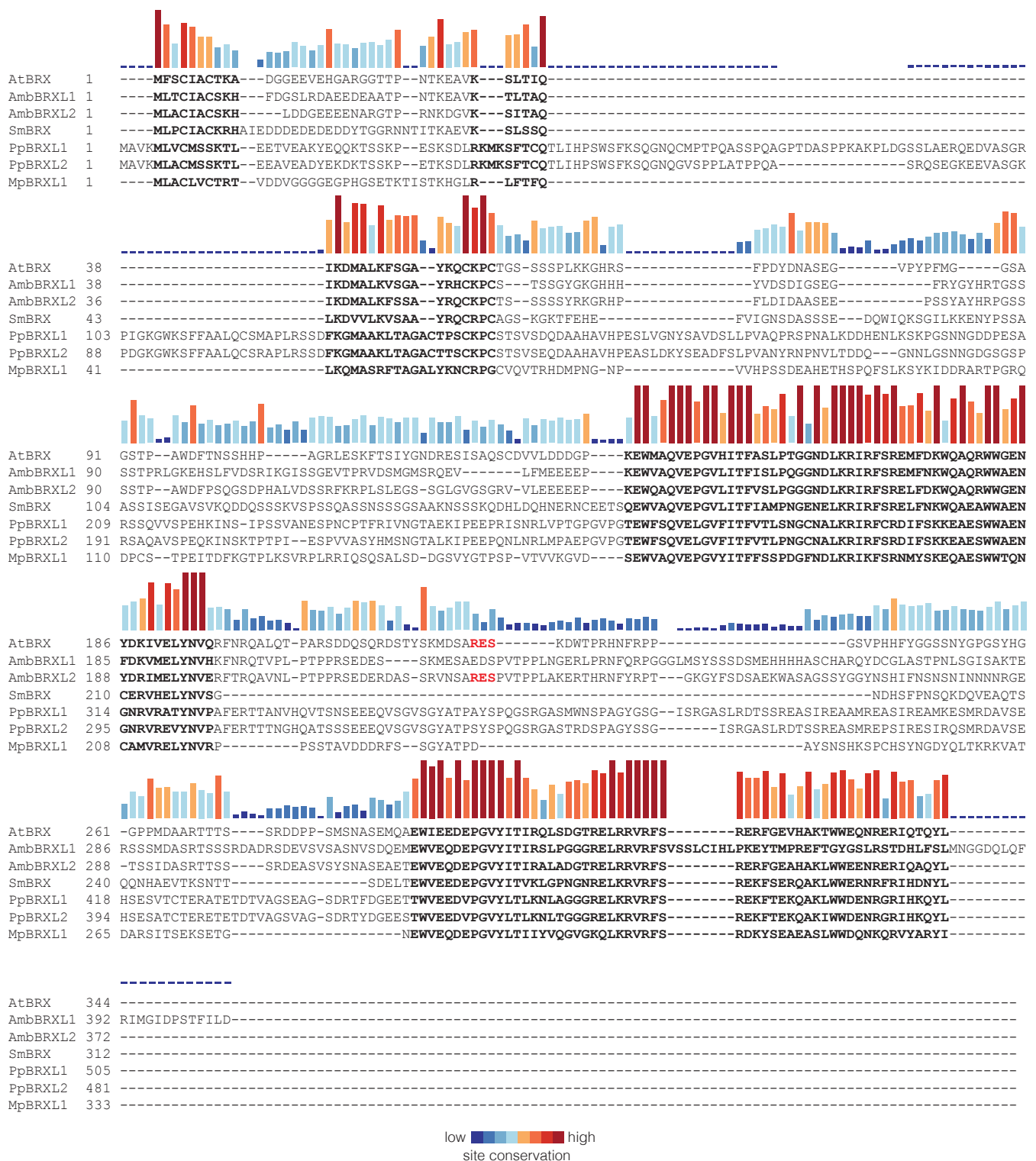


Figure S1. Sequence alignment. Alignment of BRX family proteins from *Arabidopsis thaliana* (AtBRX), *Amborella trichopoda* (AmbBRXL1 and AmbBRXL2), *Selaginella moellendorffii* (SmBRX), *Physcomitrium patens* (PpBRXL1 and PpBRXL2), and *Marchantia polymorpha* (MpBRXL1). The AGC kinase target phosphosite of AtBRX and AmbBRXL2 in the linker between the BRX-domains is highlighted in red.

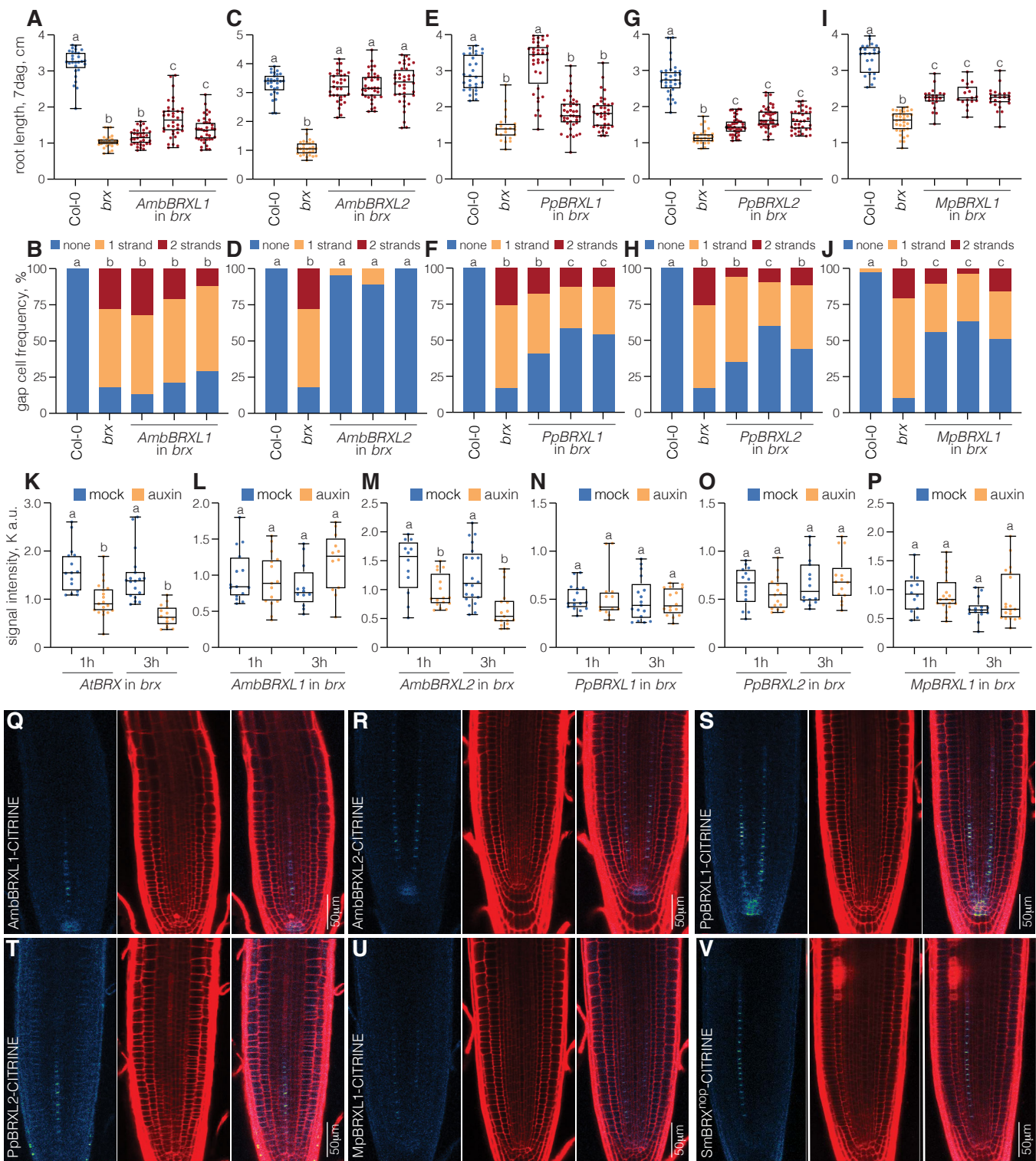


Figure S2. Functional assays of BRX family proteins across the green lineage. (A-J) Root length of 7-day-old seedlings and corresponding quantification of protophloem sieve element differentiation defects ("gap cells") in 7-day-old roots from Col-0 wildtype, *brx* mutant, and three representative independent transgenic lines each expressing the indicated BRX family proteins under control of the *A. thaliana* *BRX* promoter in *brx* background. (A) n=26-38 roots; (B) n=17-31 roots; (C) n=28-38 roots; (D) n=26-41 roots; (E) n=21-40 roots; (F) n=22-31 roots; (G) n=25-40 roots; (H) n=17-25 roots; (I) n=17-33 roots; (J) n=29-45 roots. (K-P) Signal intensity quantification of indicated CITRINE fusion proteins (expressed under control of the *A. thaliana* *BRX* promoter) at the rootward plasma membrane of developing protophloem sieve elements, 1h or 3h after mock (DMSO) or auxin (10 μ M 1-naphthalene-acetic acid) treatment. (Q-V) Confocal microscopy images of indicated CITRINE fusion proteins (expressed under control of the *A. thaliana* *BRX* promoter), showing their protophloem-specific expression and polar localization (green fluorescence, left panels). Central panels show propidium iodide-stained cell wall outline (red fluorescence), right panels show the overlay. Box plots display 2nd and 3rd quartiles and the median, bars indicate maximum and minimum. Statistically significant different samples (lower case letters) were determined by Fisher's exact test (B, D, F, H, J) or ordinary one-way ANOVA (A, C, E, G, I, K-P).

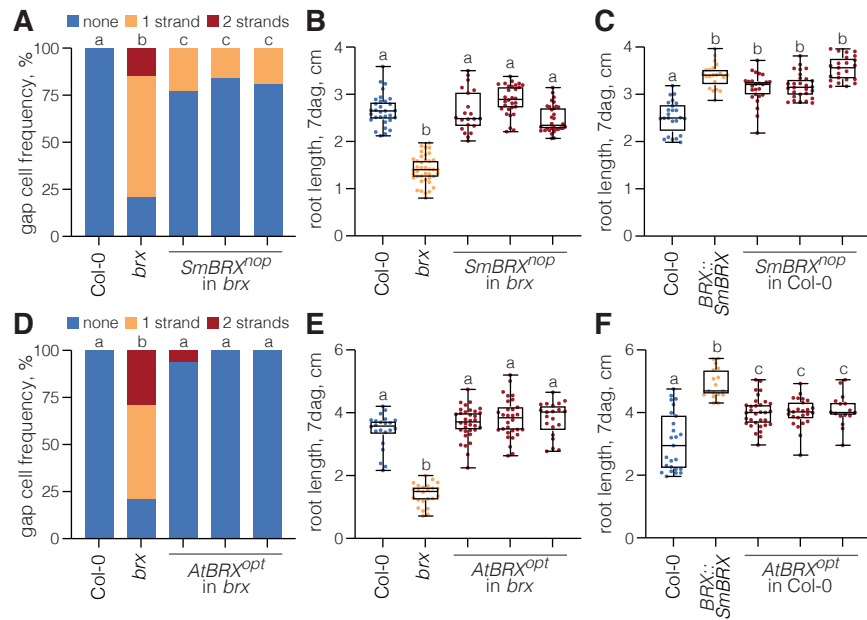


Figure S3. Functional assays of codon-(non)-optimized SmBRX and AtBRX variants. (A) Quantification of protofloem sieve element differentiation defects ("gap cells") in 5-day-old roots from Col-0 wildtype, *brx* mutant, and three independent transgenic lines expressing a non-codon-optimized SmBRX variant (*SmBRX^{nop}*) under control of the *A. thaliana BRX* promoter in the *brx* background. n=27-48 roots. (B) Root length of 7-day-old seedlings corresponding to the genotypes assayed in (A). n=21-40 roots. (C) Root length of 7-day-old seedlings from Col-0, *brx*, and three independent transgenic lines expressing *SmBRX^{nop}* under control of the *A. thaliana BRX* promoter in Col-0 background. n=21-28 roots. (D-F) Similar to (A-C), for an *A. thaliana* codon-optimized AtBRX variant (*AtBRX^{opt}*). (D) n=14-27 roots; (E) n=21-34 roots; (F) n=16-32 roots. Box plots display 2nd and 3rd quartiles and the median, bars indicate maximum and minimum. Statistically significant different samples (lower case letters) were determined by Fisher's exact test (A, D) or ordinary one-way ANOVA (B, C, E, F).

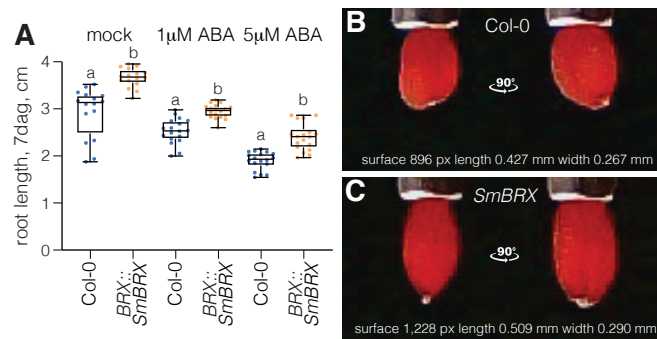


Figure S4. Illustration of increased seed and embryo size upon heterologous *SmBRX* expression. (A) Root length of 7-day-old seedlings from Col-0 and *BRX::SmBRX* plants, grown on media supplemented with increasing amounts of abscisic acid. n=16-18 roots; (B-C) Illustration of high resolution seed imaging with the *Boxeed* platform, for a Col-0 wildtype (B) and an *SmBRX* transgenic seed (C). Measured parameter values for the seeds shown are indicated.