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1THE CELL SURFACE IS THE PLACE TO BE FOR BRASSINOSTEROID2PERCEPTION AND RESPONSES

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14 Abstract

Adjusting the micro-environment around the cell surface is critical to respond to external cues 15 or endogenous signals and to maintain cell activities. In plant cells, the plasma membrane is 16 covered by the cell wall and scaffolded with cytoskeletal networks, which altogether compose 17 the cell surface. It has long been known that these structures mutually interact, but the 18 mechanisms that integrate the whole system are still obscure. Here, we spotlight the 19 20 brassinosteroid (BR) plant hormone receptor BRI1 since it represents an outstanding model for understanding cell surface signaling and regulation. We summarize how BRI1 activity and 21 22 dynamics are controlled by plasma membrane components and their associated factors to finetune signaling. The downstream signals, in turn, manipulate cell surface structures by 23 transcriptional and post-translational mechanisms. Moreover, the changes in these 24 architectures impact BR signaling, resulting in a feedback loop formation. This review will 25 discuss how BRI1 and BR signaling function as central hubs to integrate cell surface regulation. 26 27 Keywords 28

29 Brassinosteroid receptor (BRI1) / Brassinosteroid signaling / Cell surface regulation / Post-

- 30 translational modification / Cytoskeletons / Cell wall / Feedback loop / Cell growth
- 31

32 Introduction

The plasma membrane (PM) provides a unique barrier and interface for exchanging chemicals 33 or information between extra- and intra-cellular environments to maintain cellular homeostasis. 34 These contradictory functions of the PM are supported by a water-impermeable lipid bilayer 35 and numerous membrane-integral or -surface proteins that facilitate the exchange of molecules 36 or signals. Cell surface receptors, such as G-protein coupled receptors (GPCRs), receptor 37 tyrosine kinases (RTKs), or receptor-like kinases (RLKs), are representative classes of 38 transmembrane proteins that play a crucial role in signal transduction at the cell surface. 39 Animals expanded GPCR and RTK families (over 800 GPCRs¹ and 58 RTKs² in humans), 40 while plants developed a wide variety of RLKs (over 600 RLK-family genes in Arabidopsis³ 41 and over 1100 RLK-family genes in rice⁴). Among them, the brassinosteroid (BR) hormone 42 receptor BRASSINOSTEROID INSENSITIVE1 (BRI1)⁵ has been widely used as a model 43 receptor to better understand the molecular mechanisms of signal transduction in plants and its 44 45 interplay with the cell surface.

46

BRs are a group of polyhydroxylated sterols that show phytohormonal activities in many 47 aspects of the plant life cycle. For instance, Arabidopsis bril or other BR-defective mutants 48 show extreme dwarfism^{5,6}, cell proliferation defects⁷, abnormal vascular tissue differentiation⁸, 49 or impaired male fertility^{9,10}. Not limited to developmental or reproductive defects, BR-related 50 mutants also display impaired resistance to biotic and abiotic stresses^{11,12}. Similar to what is 51 observed in dicots, BRs also play significant roles in monocots. Several rice BR biosynthetic 52 mutants indeed show dwarf and fertility-related phenotypes^{13,14}. Null mutation of the rice 53 ortholog OsBRI1 leads to a severe defect in organ growth in shoots but a mild effect in root 54 growth, most likely due to the strong expression of its homologs¹⁵. A genetic screen in wheat 55 also identified a high-yield semi-dwarf strain, which is impaired in BR signaling, corroborating 56 the high importance of BR signaling in agriculture¹⁶. 57

58

59 BRI1 belongs to the LRR-RLK (leucine-rich repeat receptor kinase) subfamily X and plays a 60 central role in triggering BR signaling upon BR perception. BRI1 has both Ser/Thr and Tyr 61 kinase activities, and both enzymatic activities are critical for its functions^{17,18}. As already 62 reviewed in many articles^{19–21}, the BR signaling pathway is one of the best-characterized 63 pathways in plant receptor signaling, particularly in the control of genomic responses. In the 64 absence of BRs, BIN2 (BRASSINOSTEROID INSENSITIVE 2) kinase²² suppresses BR-

triggered transcriptional responses by phosphorylating the transcription factors BES1 and 65 BZR1 (BRASSINOSTEROID-INSENSITIVE EMS SUPPRESSOR1 and BRASSINAZOLE 66 RESISTANT 1)^{23,24}. This phosphorylation has been shown to control BES1 and BZR1 at 67 different levels, all preventing downstream genomic responses. These include the inability to 68 bind target promoters²⁵, nuclear export/cytoplasmic retention^{26–29}, or proteasome-dependent 69 degradation³⁰. The presence of BRs induces the formation of the BRI1-BAK1 (BRI1 70 ASSOCIATED KINASE, from LRR-RLK subfamily II) receptor complex^{31–33}. Full activation 71 of the BRI1-BAK1 complex allows signal transduction to BSKs (BR-SIGNALING KINASEs) 72 and CDGs (CONSTITUTIVE DIFFERENTIAL GROWTH proteins) RLCKs (receptor-like 73 cytoplasmic kinases)^{34–36}. These RLCKs, in turn, phosphorylate and activate the BSU1 (BRI1 74 SUPPRESSOR 1) phosphatase that dephosphorylates BIN2, leading to BIN2 deactivation and 75 destabilization ^{37,38}. Consequently, BES1 and BZR1 transcription factors are dephosphorylated 76 by PP2A phosphatases³⁹, resulting in their accumulation in the nucleus where they control the 77 transcription of thousands of BR-responsive genes^{25,27,28,38,40-42}. The intricate mechanism of 78 BR signal transduction from the cell surface to the nucleus is very well understood, but how 79 BR signaling regulates cell surface components or structures remains quite elusive compared 80 to other RLK signaling⁴³⁻⁴⁷. Recent advances shed light on how BR signaling impacts the 81 membrane surface environment and how cell surface cues regulate BRI1 and BR signaling. 82 This review will focus on the dynamics of BRI1 at the PM and the mutual relationships between 83 84 BR signaling and cell surface structures, such as cortical microtubules (MTs) and the cell wall. 85

86 BRI1 regulation at the cell surface

87 *Quantitative control of BRI1 at the PM*

BRI1 spans over diverse subcellular compartments, including PM, early endosomes (EE), 88 multivesicular bodies (MVB), and the vacuolar lumen⁴⁸. It has already been shown in animals 89 that the spatial distribution of membrane-bound receptors within cells is a critical determinant 90 of signaling activity⁴⁹. Several studies demonstrated that stabilization of BRI1 at the cell 91 surface by genetic perturbation yields hypersensitivity to BRs both in terms of physiological 92 signatures and BES1 phosphorylation status^{50–53}, supporting the idea that BRI1 initiates 93 signaling from the PM. Only one pharmacological approach where BRI1 is trapped into 94 endosome-containing cellular structures suggested that BRI1 could fire from endosomes, but 95 this hypothesis lacks physiological evidence⁵⁴. 96

97 Intracellular trafficking appears to be one of the major ways to adjust the PM pool of BRI1. BRI1 is believed to be constitutively internalized from the PM to EE in a ligand-independent 98 manner together with BAK1 since a BRI1 variant unable to bind BRs still shows comparable 99 internalization to the wild-type form⁵⁴⁻⁵⁶ (Fig. 1a), while some other receptors, such as FLS2 100 (FLAGELLIN INSENSITIVE 2) and SGN3 (SCHENGEN 3, also called GSO1 (GASSHO1)), 101 show ligand-induced endocytosis^{57,58}. This concept, however, remains obscure for BRI1 102 considering that several mechanisms driving BRI1 endocytosis, such as ubiquitination or the 103 recruitment of the E3 ligases PUB12/13 (Plant U-box type E3 ligase 12 and 13), appear to be 104 themselves BR-regulated^{51,59}. 105

Recent studies highlighted the numerous components involved in BRI1 endocytic machinery. 106 A pair of SNARE (Soluble N-ethylmaleimide sensitive factor attachment protein receptors) 107 family proteins, SYP22 (Q-SNARE) and VAMP727 (R-SNARE), regulates the PM pool of 108 BRI1⁶⁰. BRI1 endocytosis is executed by the AP-2- or T-PLATE-dependent clathrin-mediated 109 endocytosis (CME)^{50,61}, and the clathrin-independent pathway through FLOT1 (flotillin1)⁶² 110 (Fig. 1a). A recent report showed that a Tyr motif (YXX Φ , where Y stands for Tyr, X is any 111 amino acid, and Φ is a bulky hydrophobic residue) of BRI1 was critical for the CME-dependent 112 internalization through the interaction with an adaptor AP-2 subunit AP2M, similar to what has 113 also been characterized for plant nutrient transporters^{52,63}. 114

One important mechanism leading to BRI1 receptor internalization is polyubiquitination (Fig. 115 1b). More precisely, endocytosis of BRI1 largely relies on Lys63 (K63) polyubiquitination⁵¹, 116 as seen for other PM proteins⁶⁴⁻⁶⁶. The PUB12/13 E3 ubiquitin ligases and UBC35/36 117 (UBIQUITIN CONJUGATING ENZYMES 35 and 36) E2s have been demonstrated as main 118 actors of BRI1 Lys63 polyubiquitination in vivo and in vitro 53,59,65. In vitro experiments 119 showed that PUB13 phosphorylation by BRI1 is necessary for activating its ubiquitin ligase 120 activity⁵⁹. This result is consistent with the fact that the BRI1 kinase activity and its co-receptor 121 BAK1 are required for BRI1 constitutive ubiquitination and that BR depletion decreases BRI1 122 ubiquitination^{51,59}. So far, the precise ubiquitination sites on BRI1 are yet to be identified, but 123 introducing 25 Lys mutations in a BRI1 cytosolic domain severely abolished its internalization 124 and degradation, and yielded hyper-stimulated BR signaling⁵¹. Furthermore, BRI1 Lys63 125 polyubiquitination is reversibly removed by deubiquitination enzymes UBP12/13 (Ubiquitin 126 specific protease 12 and 13), enabling fine-tuning the degree of receptor polyubiquitination and 127 endocytosis⁶⁷ (Fig. 1b). These factors coordinately dictate the levels of active BRI1 at the cell 128 surface. Interestingly, PUB12/13 components also significantly impact the polyubiquitination 129

of the immune signaling FLS2 receptor⁶⁸, but the precise mechanisms determining the level of
ubiquitination for distinct receptors through PUB12/13 remain to be clarified.

A remarkable example of quantitative control of BRI1 at the PM takes place under higher 132 ambient growth temperatures. Elevated ambient temperature downregulates BR signal by 133 ubiquitin-mediated selective BRI1 degradation, which tunes root cell elongation under high 134 temperature condition^{69,70}. Recent work, however, revealed the interplay between 135 ubiquitination and another post-translational modification targeting BRI1, SUMOylation 136 (SMALL UBIQUITIN MODIFIER), in the control of BRI1 at warmer temperatures⁷⁰ (Fig. 1b). 137 Interestingly, SUMO was also reported in the case of FLS2⁷¹. *In vitro* experiments showed that 138 the SCE1 E2 SUMO conjugation enzyme SUMOylated FLS2, and the DESUMOYLATING 139 ISOPEPTIDASE 3a (Desi3a) SUMO protease removes SUMO from FLS2. In planta, the 140 SUMOvlation level of FLS2 is increased in an flg22-dependent manner by degrading Desi3a, 141 which unleashes BIK1 (BOTRYTIS-INDUCED KINASE1) from the FLS2-BAK1 complex 142 and activates downstream pathways⁷¹. BRI1 is also SUMOylated *in planta*, but the identity of 143 the SUMO E2 or E3 catalyzing the SUMO conjugation to BRI1 is still uncharacterized. 144 Elevated temperature induces Desi3a protein accumulation, recruitment to BRI1, and BRI1 145 deSUMOvlation⁷⁰. DeSUMOvlated BRI1 shows higher affinity to BIK1, a negative regulator 146 of BR signaling⁷², and promotes BRI1 internalization. Both mechanisms limit the activation of 147 BRI1 upon elevated temperature and prevent an over-elongation of hypocotyls under such 148 conditions. An attractive possibility is that SUMOylation counteracts ubiquitination, as 149 SUMOylation sites on BRI1 likely overlap with ubiquitination targets, allowing plants to 150 modulate BR sensitivity in response to warmth. This regulatory mechanism represents an 151 excellent model for understanding how multi-posttranslational modifications compete, 152 converge, and determine BRI1 abundance at the PM. 153

154

155 Cell surface nanodomains and BR signaling

Receptor-like kinases are embedded in the PM where uncountable components meet⁷³. Recent advances in microscopic techniques unveiled that specific proteins or lipids form nanodomains at the PM within a sub-micrometer order. A prominent example is ROP6 nanodomain formation upon auxin or hyperosmotic stress stimulation, leading to downstream activation^{74,75}. BRI1 also forms nanodomains at the PM, serving as hot spots for flotillin-dependent endocytosis⁶². Pharmacological disruption of sterol or sphingolipid distribution attenuates BRI1 endocytosis and BES1 dephosphorylation, implying these membrane components are crucial for BRI1 function by stabilizing nanodomain formation⁶². The BRI1 co-receptor, BAK1, also forms nanodomains at the PM, but its relevance for BR signaling remains to be characterized⁷⁶.

Some components in the BR pathways, like BIK1, are indeed shared by immune signaling 166 receptors, such as FLS2 or EFR^{72,77–79}, enabling plants to cross-regulate each pathway and to 167 provide trade-off mechanisms between defense and growth^{72,80,81}. A major question arises in 168 such a context: how do plants disentangle the different signaling pathways by sharing the same 169 170 components? Part of the answer relies on nanodomains that spatially separate signaling complexes to avoid unfavorable signal interference (Fig. 1c). Single particle analysis by 171 variable angle epifluorescence microscopy (VAEM) revealed that BRI1 and FLS2 reside, in 172 fact, in distinct nanoclusters within the PM⁸². These particles show different dynamics at the 173 PM, and of note, the BRI1-BIK1 complex in nanodomains tends to localize along cortical MTs, 174 while the FLS2-BIK1 complex appears to be in distinct nanodomains with no correlation with 175 MTs (Fig. 1c). This observation might give us a clue to grasp how plants regulate distinct 176 signaling pathways using the same components. Similarly, BSK1 forms nanodomains with 177 BRI1 or FLS2, and the complexes behave differently after stimulation by corresponding 178 ligands⁸³. Overall, nanodomain formation now emerges as a critical step to emit different 179 outputs while sharing the same signaling components. 180

181

182 Scaffolding function in BR signaling

Quantitative proteomics estimated that 25.8 billion protein molecules are found in one plant 183 mesophyll cell, and 3.2 billion protein molecules exist in its cytosol⁷³. Under such crowded 184 conditions, gathering signaling components by scaffolding proteins or in nanodomains allows 185 them to initiate quick, efficient, and precise signal transduction from the PM to the nucleus, as 186 seen in yeast or humans⁸⁴. In plants, only a few examples are found for signal scaffolding 187 proteins, but the TTL (TETRATRICOPEPTIDE THIOREDOXIN-LIKE) family proteins⁸⁵ 188 and BSK3 (BR-SIGNALLING KINASE 3)⁸⁶ serve such a function in BR signaling. *ttl3* and 189 bsk3 show hyposensitivity to BRs, supporting the positive roles of TTLs and BSK3 in BR 190 signaling^{85,86}. Mechanistically, both interact with central molecules in BR signaling, such as 191 BRI1, BSK1, BSU1, BIN2, and BZR1 for TTL3^{85,86}, enabling smooth signal relay. TTL 192 proteins interact with these components through TPR (TETRATRICOPEPTIDE REPEAT) 193 domains⁸⁵, while TPR domains in BSK3 are not required for its scaffolding activity, except 194

- hetero/homo-oligomerization with BSK1/3⁸⁶. This is consistent with the fact that TPRs are not
 absolutely required for its functions since a TRP-deleted BSK3 construct could complement
- 197 $bsk3^{86}$. BR signaling enhances this scaffolding mechanism by upregulating *TTL* transcription
- and promoting relocation from the cytosol to the PM^{85} .
- TTL1, 3, and 4 proteins were also recently reported as CESA1 (Cellulose synthase A1) interactors and confer abiotic stress tolerance to plants by maintaining CESAs at the PM during stress^{87,88}. Therefore, TTL family scaffolding proteins also offer a chance for a deeper understanding of a crossing point of stress response and BR signaling.
- 203

204 Activity regulation by homo and heterodimerization of BRI1 and interaction with other

205 *regulators*

Besides the quantitative regulation of BRI1 at the PM, BRI1 is also tightly controlled at the 206 activity level. BRI1 was early demonstrated to undergo BR-dependent homodimerization using 207 in planta biochemical approaches⁸⁹. BRI1 homodimerization is further supported by in vitro 208 data where purified BRI1 cytoplasmic kinase domain can homodimerize¹⁸, but not the 209 extracellular domain in vitro, regardless of the presence of ligands⁹⁰. Additionally, a BR-210 independent homodimerization of BRI1 was brought to light using imaging analysis^{62,91}. BRI1 211 also possesses an autoinhibitory domain in its C-terminal domain. This autoinhibitory lock is 212 released by BR-induced inter-molecular phosphorylation of the BRI1 C-ter domain, suggesting 213 homodimerization might be important for this regulation^{89,92}. BRI1 Another 214 autophosphorylation site, Ser891, in the ATP-binding domain strongly suppresses the auto-215 phosphorylation on Tyr residues, resulting in BR signal inhibition⁹³. 216

The BRI1-BAK1 complex formation represents another important regulatory mechanism. BAK1 is sequestered away from BRI1 by BIR3 (BAK1-INTERACTING RECEPTOR-LIKE KINASE 3) in the absence of ligand, as also observed for other BAK1 partners^{94–96} (**Fig. 1a**). Ligand binding to BRI1 shifts the affinity of BAK1 toward BRI1 and allows BAK1 to form the BR receptor complex with BRI1⁹⁷. Interestingly, BIR3 also binds to BRI1, arguing for the existence of additional BIR3-dependent regulatory mechanisms targeting BRI1⁹⁸.

BRI1 interactors such as BKI1 (BRI1 KINASE INHIBITOR 1), a MEMBRANE-ASSOCIATED KINASE REGULATOR (MAKR) family protein, also participate in the confinement of BRI1 activity⁹⁹ (**Fig. 1a**). BKI1 prevents the complex formation of BRI1 and BAK1, and its inhibitory activity largely depends on its association with PM¹⁰⁰. Four doublets of KR (Lys-Arg) motifs of BKI1 ensure electrostatic interaction with specific lipids of the PM in a "membrane hook" domain^{100,101}. Upon BR perception by BRI1, BKI1 is phosphorylated

by BRI1 on a tyrosine next to one KR motif and quickly disassociates from the PM, unlocking 229 the BR signaling pathway activation^{99,100}. Furthermore, a recent report demonstrated that an 230 evolutionary-conserved RING-type E3 Ub ligase in plants, namely ZnF, induces degradation 231 of the wheat TaBKI1, resulting in activating the downstream pathway¹⁶. Other RLK proteins 232 are known to be regulated either positively or negatively by MAKR family proteins^{102,103}, 233 including the BKI1-interacting partner ER (ERECTA). BKI1 inhibits ER kinase activity and 234 disassociates from ER upon BR signaling activation, but not after EPF1/2 (EPIDERMAL 235 PATTERNING FACTOR 1/2) perception by ER. This mechanism adds one more complexity 236 to plant architecture development¹⁰⁴. 237

After the activation of the BR pathway, a PP2A phosphatase complex attenuates BR signaling 238 by dephosphorylating BRI1, as attested by PP2A mutant and overexpression phenotypes^{105,106}. 239 Of note, PP2A localization is regulated by reversible methylation on an A subunit of PP2A 240 through SBI1 (SUPPRESSOR OF BRI1) leucine carboxylmethyltransferase activity. Subunits 241 A, such as RCN1 (ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID1), are generally 242 distributed in the cytosol, but methylation on RCN1 leads to PP2A complex formation to 243 membrane fractions at PM and/or endosomes, where BRI1 resides¹⁰⁵. BR signaling also 244 transcriptionally upregulates SBI1 expression, which in turn dampens signaling¹⁰⁵. SBI1 and 245 PP2A are also required for BRI1 degradation¹⁰⁵, implying that dephosphorylation of BRI1 is 246 critical for diverting BRI1 to MVBs or lytic vacuolar compartments for degradation, although 247 248 SBI1 and PP2A might lead to BRI1 degradation by other mechanisms. These multi-lock systems at distinct layers tightly regulate BRI1 activity or complex formation to suppress the 249 250 basal or excess BRI1 activity.

The activities of BR signaling components are not solely under the control of BR perception. 251 252 For example, other phytohormones crosstalk with BR signaling factors through posttranslational mechanisms. Salicylic acid, one of the stress-induced phytohormones, promotes 253 the detachment of BSKs from the PM by upregulating de-S-acylation enzymes, resulting in BR 254 pathway downregulation¹⁰⁷. The core regulators of abscisic acid (ABA) signaling ABI1 and 2 255 (ABA INSENSITIVE 1 and 2) phosphatases dephosphorylate BIN2, and BIN2 phosphorylates 256 SnRK2s (SNF1-Related Kinase 2)¹⁰⁸, thereby oscillating both signaling mechanisms. Further 257 study in such growth/stress response signaling cross points is critical to understand plant 258 resilience under complex environments. 259

260

261 Interplay between BR signaling and cytoskeletal networks

Two interplaying cytoskeletal networks in the cytosol and close contact with the PM, MTs and the actin microfilaments (MF), participate in essential cell activities such as cell elongation, cell division, and vesicle/organelle trafficking¹⁰⁹.

The cytoskeleton is a highly dynamic biopolymer, and its organization is tightly controlled by 265 external and internal cues^{110–113}. This plastic nature of the cytoskeleton provides the essential 266 basis for morphogenesis, directional growth, and stress adaptation, as seen in stomatal closure 267 under high salinity stress^{114,115}, for example. Phytohormones, including BRs, are representative 268 endogenous regulators of the cytoskeleton¹¹⁶. BRs modulate the organization and dynamics of 269 MFs in response to gravity in Arabidopsis¹¹⁷, and other plants such as Zea mays and Medicago 270 *truncatula*¹¹⁸. However, the detailed molecular mechanisms underlying such regulation are still 271 obscure¹¹⁹. Here, we highlight recent advances in how BRs impact cytoskeletal network 272 regulation, particularly focusing on MTs. 273

274

275 Transcriptional regulation

Transcriptome analyses have already connected BRs and MTs/MFs, since the expression of 276 some tubulin (TUB1 and TUB8) and actin (ACT11) genes are upregulated in the presence of 277 BRs¹⁹. A couple of studies have also highlighted the BR-induced transcriptional upregulation 278 of MAPs (Microtubule-Associated Proteins) that impacts MT dynamics and organ 279 developmental processes. The BZR1 transcription factor protein directly binds to promoter 280 regions of MDP40 (MICROTUBULE DESTABILIZING PROTEIN40), KATANIN1 (KT1, 281 encoding p60 subunit), and KATANIN80(1/2/3/4) (KT80s) and upregulates their transcription. 282 MDP40 promotes hypocotyl elongation, and KATANINs (KTN) support stamen filament cell 283 elongation for efficient fertilization through BR-triggered MT reorganization^{120,121}. 284

Not limited to uni-directional transcriptional regulation, BR signaling and MTs form a 285 feedback loop by controlling CLASP (CLIP170-associated protein) transcription (Fig. 2d). 286 CLASP encodes a MAP acting in transfacial MT bundle formation, particularly important to 287 maintain proper cell population in root division zone¹²². Ruan et al. demonstrated that the 288 transcription of CLASP was negatively regulated by BRs, which prolongs the transition from 289 the division zone to the elongation zone in the root meristems through the reorganization of 290 MTs. Conversely, CLASP modulates BR signaling through the delivery of BRI1 to the PM by 291 bridging BRI1-containing SORTING NEXIN1 (SNX1)-labeled endosomes and MTs¹²²⁻¹²⁵. 292 Thus, CLASP plays a pivotal role in the negative feedback loop in BR sensing by adjusting the 293 levels of BRI1 (Fig. 2d, Fig. 3a). Further analyses using super-resolution microscopy will 294

reveal how transfacial MTs, where CLASP preferentially accumulates, contribute to BRI1secretion and distribution on the PM.

297

298 Non-transcriptional regulation

Several studies have shown that BRs also control MT organization through post-translational modifications. Such effects prominently appear in lobe-indent formation during leaf pavement cell development. Genetic or pharmacological perturbation of BR biosynthesis/signaling^{126,127} leads to MT disorganization and altered jigsaw puzzle shapes of pavement cells¹²⁸. However, the detailed molecular mechanisms underlying the impact of BRs on MT regulation are still missing.

305 Two recent complementary papers have nicely connected BR signaling to cortical MTs via regulating ROP (Rho of plant) small GTPase signaling during pavement cell formation (Fig. 306 $(2a)^{129,130}$. ROPs, which regulate pavement cell shapes, are small GTPases acting as molecular 307 switches from active to inactive states depending on GTP or GDP binding, respectively¹³¹. The 308 ROP activation cycle is carried out by GEFs (Guanine nucleotide Exchange Factors), and the 309 inactivation of ROPs is finely tuned by GAPs (GTPase-Activating Proteins) and GDIs (GDP 310 Dissociation Inhibitors). BRs were shown to impact MT organization through BIN2-mediated 311 phosphorylation of PHGAP1 and 2 (Pleckstrin Homology GAP1 and 2), whose double mutant 312 demonstrates vague lobe-indent patterns in pavement cells^{129,130}. PHGAPs target ROP2, which 313 prevents MT alignment in indent areas by inhibiting RIC1 (ROP Interactive CRIB motif-314 containing protein1)¹³². PHGAPs are preferentially accumulated at the anticlinal face of the 315 pavement cell indent regions in MT- and BIN2-dependent manners^{129,130}. In the absence of 316 BRs, BIN2 activates and stabilizes PHGAPs, leading to ROP2 suppression, allowing MTs to 317 form well-ordered networks in indent regions through the ROP6-RIC1-KTN module¹³³. On the 318 other hand, when the BR signal is on, BIN2 is inactivated and degraded, allowing PHGAPs to 319 stay dormant¹²⁹. Then, active ROP2 prevents MT bundle formation by inhibiting ROP6, 320 leading to lobe formation. Thus, BRs modulate pavement cell lobe-indent formation through 321 322 MT regulation. This model also implies that local BR signal modulation occurs since BIN2 stays active only on the indent sides, possibly through feedback signal from ROP-related 323 factors, bundled MTs, or tensile stress to the BR pathways (Fig. 2a, Fig. 3b). 324

Previous reports also show that BIN2 phosphorylates both α and β-tubulins *in vitro*¹²⁸, and some cytoskeletal proteins, such as TUA6, TUB4, and ACT2, showed mobility shifts on 2D electrophoresis gels after BR treatments, implying that BRs induce some modifications on these proteins that are yet to be characterized¹³⁴. Furthermore, a recent proximal labeling screen identified potential substrates of BIN2¹³⁵. The candidate list includes various cytoskeletonassociated proteins and PM proteins, suggesting the BR pathway impacts cell surface regulation in several ways.

332

333 Interplay between BR signaling and cell wall homeostasis

334

Cell growth is an intricate process that requires precise regulation from both the inner- and 335 extracellular sides. As mentioned, BR signaling leads to complex cell shape formation in the 336 leaf epidermis by spatially controlling tip growth through cortical MT network regulation. BRs 337 also dictate cell growth by global transcriptional changes in cell wall biogenesis and 338 modification enzymes. For instance, several cell wall-remodeling genes, such as CESA6 339 A6), XTH9, 18, 33 340 (CELLULOSE **SYNTHASE** (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASEs), EXPA8 (EXPANSINA8), and PME2 PME3 341 (PECTIN METHYLESTERASEs), are direct targets of BES1/BZR1^{41,42}. In addition to the 342 transcriptional down-regulation of CESA1⁴², BIN2 also phosphorylates CESA1 and negatively 343 regulates its activity¹³⁶. BR treatment also suppresses gravitropism response in etiolated 344 hypocotyls by reorientating cellulose microfibrils and changes in cell wall components¹³⁷. 345 346 Besides, the BR pathway plays multiple roles in determining the onset timing of secondary cell wall deposition^{138–140}. In this part, we will focus on how BRI1 engages the modulation and 347 sensing of the extracellular environment. 348

349 Negative feedback loop between plasmodesmata aperture and BR signaling/biosynthesis

Changes in cell wall composition determine the activity of cell surface structures such as 350 351 plasmodesmata (PD). PDs function as narrow channels for micro- and macro-molecules, such as nutrients, signaling molecules, bacterial effectors, or viruses, between the cytoplasm of 352 neighboring cells. Their permeability responds to internal and external stimuli and is 353 predominantly regulated by callose deposition on the PD necks.^{141,142}. BR signaling 354 manipulates the PD permeability by inducing callose accumulation, thereby restricting the 355 diffusion of molecules through PD¹⁴³. BR-induced callose deposition appears to be mediated 356 by a transcriptional pathway, still, this does not exclude a possibility that the BRI1 complex 357 directly regulates callose synthase activity or other non-transcriptional mechanisms at the 358 PM¹⁴³. 359

Of note, PD permeability impacts BR biosynthesis¹⁴³. The metabolic pathway of BRs from 360 campesterol to brassinolide, the most bioactive BR, is well-documented, and a reductase and 361 several cytochrome P450 family mono-oxygenases are assigned to each step¹⁴⁴. Each enzyme 362 is expressed in different tissue layers around root meristem, despite some overlaps, indicating 363 that BR precursors should relocate across the layers to complete the full biosynthesis 364 pathwav¹⁴⁵. Furthermore, full complementation of the cpd BR biosynthetic mutant by 365 epidermis-specific expression of *CPD* also supports such relocation idea⁸. This model is further 366 reinforced by elegant experiments combining a dominant callose synthase transgenic line that 367 over-accumulates callose and a fluorescently tagged BR precursor to directly visualize the 368 precursor movements¹⁴³. Together with the negative transcriptional feedback of biosynthesis 369 genes¹⁴⁴, callose deposition at plasmodesmata regulates BR production, constituting another 370 feedback in BR signaling regulation via the relocation of BRs and their precursors between 371 cells. 372

373

374 *Control of cell wall extensibility by acidification*

Acidifying apoplastic spaces is a critical step in expanding plant cells by increasing the 375 extensibility of the cell walls. Some cell wall loosening enzymes, such as expansins, possess 376 higher activity under acidic conditions¹⁴⁶. Auxins are well-known inducers of apoplast 377 acidification acting through the ABP1 (AUXIN BINDING PROTEIN1)-TMKs 378 (TRANSMEMBRANE KINASEs)-H⁺ATPase module. TMKs interact with the AHA1/2 H⁺-379 ATPase and phosphorylate their penultimate residues (Thr948/947, respectively)^{46,47,147}, 380 (PROTEIN PHOSPHATASE2C-D) deactivates whereas PP2C-D AHA1/2 381 by dephosphorylation¹⁴⁸. 382

Similar to auxin, BR treatments trigger apoplastic acidification by inducing phosphorylation of 383 the AHA1/2 H⁺-ATPases^{149–151}. Mechanistically, two non-mutually exclusive models are 384 proposed to explain the proton ATPase activation (Fig. 2b)¹⁴⁹⁻¹⁵¹. In the first model, the 385 activation of H⁺-ATPases relies on the canonical BR pathway through transcriptional 386 upregulation of PP2C inhibitors, SAUR9/19 (SMALL AUXIN UP RNA 9/19)¹⁵⁰. In the second 387 model, BRI1 directly interacts with AHA1/2, and SAUR15 enhances the complex formation 388 to activate the proton ATPases by enhancing BRI1 activity¹⁵¹. Genetic evidence supports both 389 scenarios since SAUR15 overexpression increases hypersensitivity to BRs by enhancing 390 391 extracellular acidification through upregulating H⁺-ATPase activity, and a saur15 mutant shows reduced sensitivity to BRs in root length¹⁵¹. Similarly, *SAUR19* overexpression leads to
extracellular acidification¹⁴⁸. It is still unclear how much both pathways contribute to apoplast
acidification and whether they crosstalk to coordinate proper cell elongation by adjusting cell
wall extensibility.

A modeling approach predicted that cation channels were involved in this pathway to 396 compensate for positive charges when protons are transported toward extracellular spaces. 397 CNGC10 (CYCLIC NUCLEOTIDE GATED CHANNEL 10) was proposed as a candidate 398 since cngc10 mutants show defects in apoplastic acidification upon BR treatment. CNGC10 399 forms a complex with BRI1-BAK1, suggesting direct regulation by the receptor complex¹⁵². 400 Recent reports described that the altered pH changed receptor-peptide ligand binding¹⁵³ or 401 ligand activities¹⁵⁴. Remains to be determined whether the acidification matters to the BRI1-402 BR binding capacity or BRI1-BR-BAK1 complex formation, and these points might be 403 essential to understand the homeostasis of apoplastic spaces. 404

405 Sensing cell wall integrity

Plant cell walls consist of carbohydrates, proteins, and other secondary metabolites. Cellulose, 406 cross-bridged by hemicellulose, provides tensile strength, while pectin confers elasticity to cell 407 walls. Transcriptional regulations and enzymatic activity changes allow plants to dynamically 408 remodel plant cell walls depending on developmental cues or stress responses, but how plant 409 cells monitor the cell wall status is still obscure. Yeast, which is also encapsulated in a cell 410 wall, has cell wall sensors, Wsc1-3, Mid1, and Mtl1 that monitor its mechanical properties and 411 trigger transcriptional changes to adapt to acute osmotic changes or heat shock^{155,156}. Plants do 412 not have obvious homologs of these molecules, but solid evidence indicates that plants do have 413 cell wall surveillance systems^{157,158}. Recent studies indeed revealed that different classes of 414 proteins, such as mechano-sensitive Ca²⁺ channels¹⁵⁹, cell wall proteins¹⁶⁰, receptor-like 415 proteins¹⁶¹, GPI-anchored proteins¹⁶², or receptor-like kinases¹⁶³⁻¹⁶⁶, function in cell wall 416 sensing. Mutations in these proteins often lead to abnormal cell shapes, defects in cell growth, 417 or pollen tube rupture¹⁵⁸. 418

An unexpected role of BRI1 in cell wall sensing emerged from a forward genetic approach (Fig. 2c). Recent reports demonstrated that a BRI1-mediated cell wall surveillance system monitors the homeostasis of pectin methyl esterification, affecting the biophysical properties of pectins. Demethylesterified pectin binds to Ca^{2+} through its free carboxy groups, promoting bridge formation between pectin chains. Of interest, super-resolution microscopic observation

clearly highlighted the biased distribution of methyl- and demethyl-esterified pectins¹⁶⁷. PMEs 424 (pectin methylesterases) and PMEIs (PME inhibitors) are critical proteins that determine the 425 pectin methyl esterification ratio. Disruption of the pectin methylesterification balance by 426 overexpression of *PMEI5* leads to wavy root growth¹⁶⁸. Wolf *et al.* used this wavy growth as a 427 phenotypic proxy to the methylesterification status of the cell walls and conducted a forward 428 suppressor screen of the PMEIox phenotype¹⁶⁸. One of the suppressor mutants, called 429 comfortable numb 1 (cnu1), has a mutation in the kinase domain of BRI1. Other bri1 mutant 430 alleles, or the det2 BR biosynthesis mutant, also suppress the PMEIox phenotype^{168,169}, 431 suggesting the phenotype of PMEI5 overexpression is a result of the enhanced BR signaling 432 pathway¹⁶⁸. 433

Another component in this pathway was identified as CNU2, encoding RLP44 (Receptor Like 434 Protein 44), in the same suppressor screen. RLP44 stabilizes a ternary complex with BRI1-435 BAK1 and enhances BR transcriptional responses as an auxiliary component of the pathway¹⁶¹ 436 (Fig. 2c). RLP44 is phosphorylated in BR/cell wall homeostasis-dependent manners, which is 437 required for its functionality in cell wall maintenance^{161,170}. Since phosphorylated RLP44 tends 438 to stay at the PM, while a non-phosphorylated form mainly resides in endosomes, phospho-439 status of RLP44 may affect BR signal initiation in this context¹⁷¹. In parallel, RLP44 also 440 strengthens the PSKR (phytosulfokine receptor)-BAK1 complex formation, which mediates 441 procambium/xylem patterning together with BRI1¹⁷². This fact raises the question of how 442 RLP44 balances BRI1 and PSKR activities. Indeed, plants harboring the bril^{cnu4} mutation 443 display abnormal xylem pattern formation due to the imbalance of competition between 444 BRI1^{cnu4}, which has tighter interaction with RLP44, and PSKR¹⁶⁹. Distinct from BR signaling, 445 the phospho-status of RLP44 does not affect PSK signaling, suggesting RLP44 446 phosphorylation status may not explain the competition¹⁷⁰. Moreover, a recent study also 447 suggested that BRI1 plays a role in the xylem patterning process partially independent of BRs⁵⁵. 448 Further study may reveal the nature of microenvironmental cues or components that govern 449 the competition over RLP44 during xylem patterning. 450

A recent report provides mechanistic insights into how BRI1 functions in cell-surface
continuum signaling with another RLK in rice. OsWAK11 (WALL ASSOCIATED KINASE
11) was identified as a negative growth regulator and as an interactor of OsBRI1. OsWAK11
monitors pectin methylation status by preferentially binding to methylesterified pectins.
Demethylesterified pectin suppresses BR signaling through BRI1 phosphorylation by
OsWAK11. On the other hand, the methylesterified rich situation leads to OsWAK11 reduction,

allowing BRI1 to initiate signals¹⁷³. OsWAK11 phosphorylates a monocot-specific motif in 457 OsBRI1, suggesting this pectin sensing module is unique to monocots. How the pectin status 458 enhances BR signaling is still an open question in Arabidopsis, but the recent identification of 459 RFO1 (RESISTANCE TO FUSARIUM OXYSPORUM1), a WAK-like receptor-like kinase, 460 may fill the gap (Fig. 2c). rfo1 mutant suppresses the PMEIox phenotype like BR-defective 461 mutants by modulating the transcription of BR-related genes. In addition, RFO1 directly binds 462 to demethylesterified pectins, supporting the idea that RFO1 may connect pectins to BR-463 signaling¹⁷⁴. Further studies on direct interaction between RFO1 and BRI1-BAK1-RLP44, and 464 divergent mechanisms in cell wall sensing among plant species are awaited. 465

466

467 **Coordination of organ growth**

468 Coordinated cell growth in each tissue is one of the critical processes for multicellular 469 organisms to determine organ shape, to keep their integrity, and to achieve biased organ growth. 470 For example, the root endodermis-specific expression of a dominant negative form of DELLA 471 protein, a gibberellin and other growth signal integrating factor, severely attenuates whole root 472 growth¹⁷⁵. On the other hand, ERECTA-EPFL4/6 (EPIDERMAL PATTERNING FACTOR-473 LIKE 4/6) receptor-peptide pairs determine inflorescence stem height by modulating cortex 474 cell proliferation and growth¹⁷⁶.

475 Several lines of evidence prove that BR signaling coordinates organ growth by adjusting physical constraint forces. For example, the proper balance of responses to BRs in root hair 476 477 and non-hair cells is crucial since an imbalanced BR-induced cell growth triggers ethylene responses and accumulation of crystalline cellulose¹⁷⁷, resulting in inadequate whole root 478 growth. Quantitative 3D cell growth analysis also revealed that cells in each tissue layer display 479 distinct responses to BRs, which are essential for proper radial root meristem growth. 480 Additionally, BR treatment decreases the stele area in WT roots, while BR signaling 481 perturbation increases the stele size¹⁷⁸. Of note, epidermal specific BRI1 expression in *bri1* 482 mutant rescues the root growth and limits the stele area⁸. These observations suggest that the 483 epidermal layer provides BR-dependent mechanical constraint. A recent study uncovered that 484 BRs are also involved in organ integrity through coordinated tissue growth in the carnivorous 485 aquatic plant Utricularia gibba (commonly called floating bladderwort) and terrestrial plant 486 shoots¹⁷⁹. In this study, a genetic screen identified dwarf and extra-dwarf mutants, both 487 presenting short internodes, small leaves, small traps, and distorted vascular tissue. 488

Computational modeling predicted that the wavy vascular tissue phenotype in the dwarf mutant 489 resulted from the physical constraint of retarded epidermal layer growth. Consistent with the 490 dwarf phenotypes, the responsible gene was a cytochrome P450 90B1 enzyme, a homolog of 491 DWF4 (DWARF4) in Arabidopsis^{179–181}. In line with this observation, treatment with a BR 492 biosynthesis inhibitor recapitulates *dwarf* mutant phenotypes of *U. gibba*, while BR treatment 493 rescued the mutant phenotypes. Furthermore, a mutation in Arabidopsis DWF4 enhances the 494 495 crack phenotype in *qual-2* (*quasimodo 1-2*) in hypocotyl epidermal cells, suggesting BRs reduce epidermal constraint¹⁷⁹. Modeling analysis revealed that cell wall extensibility through 496 BR-induced apoplast acidification could be one of the determinants of such coordination, but 497 this does not exclude the possibility that other BR-related mechanisms also drive the process. 498 Decoding BR response under tension in each tissue may support the unwiring of these 499 interactions. Thus, BRs impact not only the adjustment of the microenvironment around the 500 cell surface but also regulate the whole apoplast continuum to coordinate organ growth. 501

502

503 Conclusion and perspectives

Since the identification of BRs as "growth-promoting chemicals" from *B.napa*¹⁸², forward 504 genetic approaches in Arabidopsis or rice, biochemical characterization, and many other 505 physiological analyses unraveled the critical roles of BRs in plant life cycles and the detailed 506 underlying molecular mechanisms²¹. Besides these developmental and physiological aspects, 507 BRI1 serves as a model of choice to many cell biologists because of its unique dynamic 508 regulation through post-translational modifications, particularly in the vicinity of the cell 509 surface (Fig. 1). Together with the dynamic quantitative regulation of BRI1, a multi-layered 510 511 interaction network fine tunes the activity of BRI1 or BRI1-BAK1 complex formation at the PM (Fig. 1), oscillating the level of BR signaling to regulate cell surface structures through 512 513 transcriptional and non-transcriptional mechanisms (Fig. 2).

514 Many questions are yet to be answered, from the factors binding to and decoding the post-515 translational modification codes of BRI1 controlling its activity and dynamics, to the 516 mechanisms driving the diffusion, nanodomain formation/maintenance, or intracellular sorting 517 of BRI1. Recent technical advances in high- and super-resolution microscopy uncovered 518 nanodomain formation of BRI1 and will be instrumental in understanding better this new facet 519 of BRI1 biology.

Signals from BRI1 are emitted from nanodomains, but how these signals can modulate the 520 spatially restricted cell wall status changes is still obscure. For example, methyl- and demethyl-521 esterified pectins distribute ununiformly, suggesting pectin modification status sensing is 522 locally activated. As mentioned above, transcriptional feedback is thought to maintain cell wall 523 status, but it is still unclear how transcriptional feedback can achieve local cell wall 524 modifications. Combining recent advanced methods, such as proximal labeling-based spatial 525 proteomes, super-resolution microscopy techniques, and all conventional approaches will help 526 us approach the molecular machinery for local cell wall modification with unprecedented 527 528 resolutions. Thus, BRI1 and its signaling are still inspiring us to discover novel concepts in cell 529 biology.

530

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539

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543 **Declaration of interests**

544 No interests are declared.

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- 546
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Figure 1 Schematic representation of BRI1 levels and activity controls at the plasma membrane

- **a.** BRI1 activity is negatively regulated by numerous protein partners such as BSKs, BKI1, and BIK1, all interacting with BRI1 at the plasma membrane (PM) when the BR pathway is off. BAK1 is also sequestered away from BRI1 by BIR3. BRs induce the formation of the BRI1-BAK1 complex and initiate downstream signaling, resulting in the release of inhibitors. The BRI1 pool at the PM is adjusted by the balance of exocytosis, endocytosis and degradation. Two endocytotic pathways, the clathrin-mediated and -independent endocytosis, remove BRI1 from the PM. Endocytosed BRI1 reaches the trans-Golgi network/early endosomes (TGN/EE) and is transported to the lytic vacuole for degradation.
- b. Post-translational modifications on lysine residues (K) of BRI1 cytoplasmic domain are signals regulating its dynamics. The UBC35/36 (E2) and PUB12/13 (E3) machinery leads to Lys63 (K63) polyubiquitination of BRI1, which is enhanced by BRs, driving BRI1 endocytosis. The UBP12/13 deubiquitinating enzymes stabilize BRI1 at the PM by removing ubiquitin moieties from BRI1. SUMOylation also stabilizes BRI1 at the PM, and deSUMOylation by DeSi3a enhances BRI1 endocytosis. Since both PTMs target the same residues, competition between PTMs likely happens to fine tune BRI1 cell surface levels.
- c. BRI1 and FLS2 form distinct nanodomains within the PM, acting as platforms to amplify signal and drive responses towards development or defense. BIK1 has different impacts on the two pathways. BRI1-BIK1 interaction colocalizes with CMTs and negatively affects the BR pathway. After sensing BRs, the BRI1-BAK1 complex and BIK1 trans-phosphorylate each other, leading to the release of the BIK1 in the cytosol. Consequently, the negative regulator BIN2 is degraded by the proteasome, allowing BES1/BZR1 transcription factors to initiate transcriptional changes. Conversely, the BIK1 phosphorylation induced by FLS2 activates the MAPK cascade and defense responses.

Figure 2 Schematic representation of cell surface regulation by the BR pathway in *Arabidopsis thaliana* seedling development

- **a.** BRs modulate the formation of lobes and indentations in the leave pavement cells. In indent areas, the BR pathway is locally off, allowing BIN2 to be active and to phosphorylate PHGAPs, inhibitors of ROP2, specifically localized at the anticlinal face of indent regions in a microtubuleand BIN2-dependent manner. ROP6 activation acts on the CMTs through its effector RIC1/KTN, making the convex form. BIN2 also phosphorylates tubulins *in vitro*, but its effects are still unknown. In the lobe regions, BIN2 is inhibited by the canonical BR pathway, leading to the accumulation of an active form of ROP2, which acts on the actin cytoskeleton through RIC4 and promotes expansion. Reciprocal inhibitions between ROP2 and ROP6 also maintain spatially restricted ROP activation.
- **b.** BR-induced apoplastic acidification through AHA1 promotes cell wall swelling in hypocotyl cells. In the absence of BRs, PP2C inhibits AHA1, leading to less acidic pH conditions. When BR signaling turns on, *SAUR9/19*, targets of BES1/BZR1, are transcriptionally upregulated and inhibit PP2C, supporting apoplastic acidification. Furthermore, BRI1 forms a complex with SAUR15 and AHA1, increasing AHA1 phosphorylation and enhancing proton ATPase activity, resulting in altered CW enzyme activities and membrane polarization.
- **c.** Cell wall integrity sensing in root epidermal cells partially depends on BRI1. PMEs and their inhibitor PMEIs control the degree of homogalacturonan methylation. PME inhibition results in a higher ratio of methylated pectin. The altered pectin methylation ratio activates the RLP44-BAK1-BRI1 complex, probably through RFO1. *PMEs* are transcriptionally upregulated by BES1/BZR1 directly, balancing the ratio between the two forms of homogalacturonan.
- **d.** BRI1 and CLASP form a negative feedback loop. When the BR pathway is off, CLASP allows the transport of BRI1 to the PM via the SNX1 retromer that depends on the MT network. In the presence of BRs, *CLASP* transcription is directly downregulated by BES1/BZR1, leading to the

reorientation of the MTs and less BRI1 transport to the PM. This feedback loop finetunes the division zone size in the root tips.

Figure 3 BR controls the feedback loop coupled by several mechanisms

- **a.** BRI1 and CLASP constitute a negative feedback loop by a transcription-vesicle transport coupled mechanism. Please also refer to Fig. 2d for the detailed mechanisms.
- **b.** BIN2 kinase enhances the ROP-MT feedback loop through PHGAP focalization at indent sites and activation (left). In the lobe areas, active ROP2 promotes actin cytoskeleton network formation (right). Active ROP2 and ROP6 are highlighted by color. Please also refer to Fig. 2a for the detailed mechanisms.



Delesalle et al. Figure 1





Delesalle et al. Figure 3