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Ubiquitin-mediated endocytosis of the plant steroid hormone receptor BRI1 and regulation by elevated ambient temperature

Sara Martins

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THESE DE DOCTORAT
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ÉCOLE DOCTORALE N° 567
Sciences du Végétal : du Gène à l'Ecosystème

Spécialité de doctorat : Biologie

Par

Sara Martins

Ubiquitin-mediated endocytosis of the plant steroid hormone receptor BRI1 and
regulation by elevated ambient temperature

Thèse présentée et soutenue à Gif-sur-Yvette, le 31 octobre 2016 :

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Acknowledgments

I used to joke and say that if things come easy to us, we'll probably not value it as we should. I think I can joke about it, but I also believe it is quite often truth! ☺

My PhD had its ups and downs as many others. There were some particular hard time I've been through, but with some perseverance and the help of my family and friends, those closer and far, I've overcome them. I would like to start to thank them all for the support and positive words and advices they shared with me. I apologize in advance those I might not mention.

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Abbreviations

(Includes the abbreviations organized by alphabetic order)

aa: amino acid

ABC: ATP-binding cassette

AFCS: Alexa Fluor 647–castasterone

ALIX: Apoptosis-Linked Gene-2 Interacting Protein

AMSH: Associated Molecule with the SH3 domain of STAM

AP-2: Adaptor Protein 2

BAK1: BRI1-Associated Receptor Kinase 1

BAS1: phyBactivation-tagged Suppressor1

BES1: *bri1*-EMS-Supressor 1

BFA: Brefeldin A

bHLH: basic Helix Loop Helix

BIM1: BES1-Interacting Myc-like 1

BIN2: Brassinosteroid Insensitive 2

BKI1: BRI1 Kinase Inhibitor 1

BL: Brassinolide

BNST: *Brassica napus* sulfotransferase

BOR1: Boron Transporter 1

BPCS: biotin-tagged photoaffinity castasterone

BR: Brassinosteroids

BRI1: Brassinosteroid Insensitive 1

BRL: BRI1-Like

BRRE: BR responsive elements

BRZ: Brassinazole

BSK1: BR-Signaling Kinase 1

BSU1: BRI1-Supressor 1

BZR1: Brassinazole-Resistant 1

CDG1: Constitutive Differential Growth 1

ChIP: Chromatin-Immunoprecipitation
CHMP1: Charged Multivesicular Body Protein/Chromatin Modifying Protein 1
CHX: Cyclohexamide
CLC: Clathrin Light Chain
CME: Clathrin-Mediated Endocytosis
CIE: Clathrin-Independent Endocytosis
ConA: Concanamycin A
CPD: Constitutive Photomorphogenesis and Dwarfism
DET2: De-etiolated 2
DRP: Dynamin-Related Protein
DUB: Deubiquitinating Enzymes
DWF4: Dwarf 4
EE: Early Endosomes
EGFR: Epidermal Growth Factor Receptor
EGL3: Enhancer of Glabra 3
EMS: Ethyl-MethaSulfonate
ESCRT: Endosomal Sorting Complex Required for Transport
FLS2: Flagellin Sensing 2
FREE1: FYVE domain protein required for endosomal sorting 1
FRET-FLIM: Förster Resonance Energy Transfer – Fluorescence Lifetime Imaging Microscopy
GFP: Green Fluorescent Protein
GSK3: Glycogen Synthase Kinase 3
HSP90: Heat Shock Protein90
IRAK: Interleukin 1-Receptor Associated Kinase
IRT1: Iron Transporter 1
LE: Late Endosome
LC-MS/MS: Liquid-Chromatography tandem Mass Spectrometry
LRR-RLK: Leucine-Rich Repeat Receptor-Like Kinase
MudPIT: Multidimensional Protein Identification Technology
MVB: Multivesicular Bodies
OPS: Octopus

PI4P: PtdIns(4)P
PIF: Phytochrome-Interacting Factors
PIP: Plasma membrane Intrinsic Protein
PM: Plasma Membrane
PP2A: Protein Phosphatase 2A
QC: Quiescent Center
RLCK: Receptor-Like Cytoplasmic Kinases
SERK: Somatic Embryogenesis Receptor-like Kinase
SNX1: Sorting Nexin 1
TAA1: Tryptophan Aminotransferase of Arabidopsis 1
TAK1: Transforming growth factor- β Activating Kinase 1
TGF β R: Transforming Growth Factor- β Receptor
TGN/EE: *trans*-Golgi Network/Early Endosomes
TIR1/AFB: Transport Inhibitor Response1/Auxin Response F-Box
TIRF: Total Internal Reflection Fluorescence Microscopy
TML: TPLATE-Muniscin-Like
TPC: TPLATE complex
TTG1: Transparent Testa Glabra 1
TyrA23: Tyrphostin A23
Ub: Ubiquitin
YUC8: YUCCA8

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Résumé

Les brassinostéroïdes (BR) sont des hormones stéroïdes végétales qui jouent un rôle dans la croissance et le développement des plantes. Ils sont perçus à la surface cellulaire par le récepteur kinase BRI1 (BR insensitive 1) situé au niveau de la membrane plasmique. La voie de signalisation des BRs implique la cis et trans-phosphorylation de BRI1 et de son corécepteur BAK1 (BRI1 Associated Receptor Kinase 1) et aboutit à la déphosphorylation des facteurs de transcription BZR1 (Brassinazole Resistant 1) et BES1 (bri1-EMS-Supressor 1) et l'activation des réponses génomiques aux BRs. Les mécanismes permettant la dé-activation du complexe de perception des BRs sont encore peu connus mais peuvent impliquer la déphosphorylation de BRI1, la fixation de régulateur négatif comme BKI1, et l'endocytose de BRI1. Mon travail de doctorat a consisté à étudier les mécanismes d'endocytoses et de dégradation de BRI1 et leurs régulations par les conditions environnementales.

L'ubiquitination est une modification post-traductionnelle impliquant l'attachement d'un polypeptide d'ubiquitine (Ub) sur une ou plusieurs lysines (K) d'une protéine cible. L'ubiquitine elle-même peut être sujette à l'ubiquitination, créant ainsi des chaînes de poly-ubiquitine (polyUb) qui peuvent adopter des topologies différentes selon le résidu K de l'ubiquitine impliqué dans la formation de la chaîne. Parmi ces chaînes de polyUb, la chaîne impliquant la lysine-63 (K63) est connue pour être impliquée dans la dégradation des protéines par endocytose chez les levures et les mammifères. Néanmoins, peu de choses sont connues sur l'endocytose dépendante de l'ubiquitine chez les plantes. Durant la première partie de ma thèse, j'ai démontré que BRI1 est modifié *in vivo* par des chaînes de polyUb K63 et j'ai pu identifier des sites putatifs d'ubiquitination dans BRI1. En utilisant une forme artificiellement ubiquitinée de BRI1 ainsi qu'une forme non ubiquitinable de BRI1, j'ai montré que l'ubiquitination joue un rôle sur

l'internalisation de BRI1 à la surface cellulaire et est essentielle pour son adressage à la vacuole. Par ailleurs, j'ai établi que la dynamique de la protéine BRI1 médiée par son ubiquitination joue un rôle important dans le contrôle des réponses des BR.

La deuxième partie de ma thèse m'a permis de découvrir une connexion entre l'endocytose dépendante de l'ubiquitine de BRI1 et la réponse des plantes à une élévation de température. J'ai notamment montré que l'accumulation de la protéine BRI1 est réduite dans les racines lorsque les plantes sont cultivées à une température plus élevée (i.e. 26°C). Cependant, la forme non ubiquitinable de BRI1 ne répond pas à cette élévation de la température suggérant une implication de l'endocytose dépendant de l'ubiquitine dans de telles conditions. Cette déstabilisation de BRI1 observée à température plus élevée se traduit au niveau moléculaire par une inhibition de la voie de signalisation des BRs et une hypersensibilité à des traitements BRs exogènes. Les plantes répondent à une montée subite de la température par une augmentation de la longueur de l'hypocotyle, des pétioles et de la racine principale ; processus largement sous le contrôle de l'auxine. J'ai accumulé au cours de ma thèse des évidences génétiques et génomiques montrant que la déstabilisation du BRI1 et l'inhibition de la signalisation des BR à plus hautes températures contrôle l'élongation des racines indépendamment de l'auxine. En conclusion, les résultats obtenus indiquent que BRI1 intègre les informations de température et de signalisation des BRs pour ajuster la croissance des racines lors de variations des conditions environnementales.

Chapter 1:

Introduction

Chapter 1: Introduction

Similar to animals, plants need hormones to complete their life cycle. Regardless the nature of the hormone or the organism considered, studying a hormone requires a better understanding of its metabolism (biosynthesis, inactivation, and degradation), its transport, its perception and signaling, and its physiological and molecular responses. Among all plant hormones, the plant steroid hormones called brassinosteroids (BR) are one of the best studied hormones. Much information about the mechanisms of perception and signaling of BRs, BR responses and BR roles during plant growth and development have been gained over the past two decades (Vert et al., 2005; Wang et al., 2012). The main goal of my thesis was to study the downregulation mechanisms of the BR receptor BRI1 (Brassinosteroid insensitive 1) activity in the cell, and its importance for root responses to environmental signals in the model plant *Arabidopsis thaliana*. I will therefore focus my introduction on the role of BRs in plants, the BR signaling pathway and the mechanisms of plasma membrane proteins degradation.

1.1. Brassinosteroids : from physiology to signaling

1.1.1. Identification and characterization of brassinosteroids

BRs were originally isolated from the bee-collected pollen of *Brassica napus* plants, for their ability to promote growth. Among BRs, the most active form is brassinolide (BL). The chemical structure of BL closely resembles those of steroid hormones found in the animal kingdom, such as the sex hormone testosterone from humans or insect ecdysone (Bishop and Koncz, 2002). BL is however more hydroxylated than its animal counterparts (Figure 1), and this likely explains the radical difference in steroid signaling mechanisms between plants and animals that I will develop later.

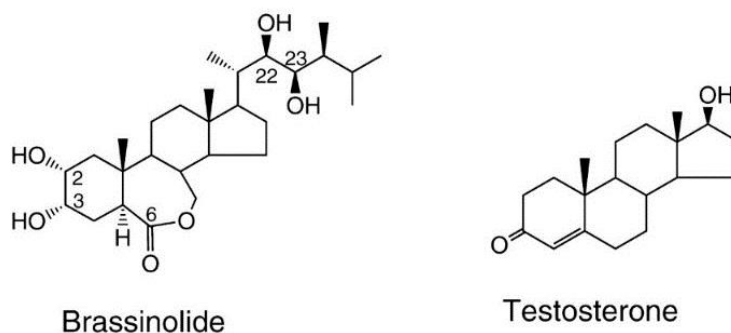


Figure 1. Brassinolide and testosterone chemical structures (adapted from (Bishop and Koncz, 2002)).

Exogenous application of BL, and BRs in general, promotes elongation of light-grown tissues. Whether this represented the physiological role of endogenous BRs was only elucidated when BR-defective mutants, impaired in BR biosynthesis or signaling, were identified. Several studies showed that BR-defective mutants have pleiotropic effects on plant growth and development. Plants with genomes containing mutations altering BR biosynthesis or signaling are dwarf, present dark green round and epinastic leaves, have short petioles, reduced male fertility, and don't properly respond to light fluctuations when grown in the light (Figure 2a) (Li and Chory, 1997; Thummel and Chory, 2002). This clearly highlights the importance of BRs for proper growth and a wide variety of developmental processes. When grown in the dark, BR mutants mimic light-grown plants with a short and thick hypocotyl, open expanded cotyledons and constitutive expression of light-regulated genes (Thummel and Chory, 2002). On the other hand, BR mutants showing constitutive BR responses, or plant treated with of high concentrations of BL are known to show light green expanded and narrow leaves, long bending petioles in light conditions (Figure 2b). The BR-mediated growth promotion of hypocotyls clearly argue for a major role of BRs in cell elongation (Szekeres et al., 1996), since hypocotyls only grow after germination by cell expansion. In the dark, BR responses are close to saturation, explaining that constitutive mutants or plants challenged with very low levels of BRs usually show hypocotyls close to wild-type, while the use of

higher BR concentrations yields shorter hypocotyls. Therefore, one can assume that BR amount in plants is tightly controlled for a proper development (Mussig et al., 2003).

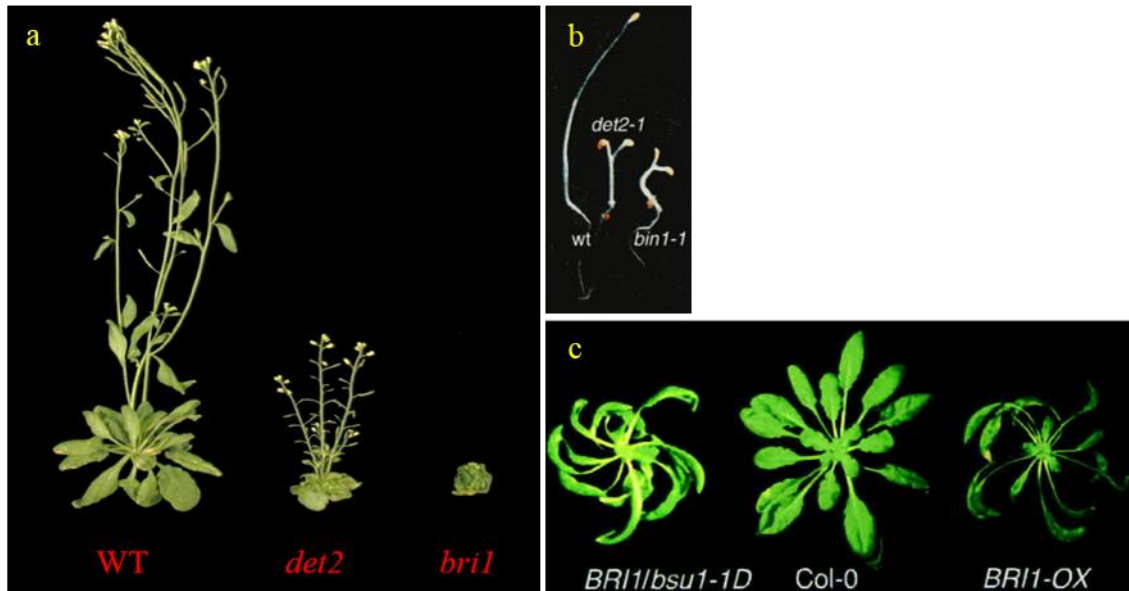


Figure 2. Phenotypes of BR-related mutants impaired in BR biosynthesis (*det2*), perception (*bri1* – first identified as *bin1*), or showing hypersensitivity to BRs (*BRI1/bsu1-1D*, *BRI1-OX*) BR mutant phenotypes (adapted from (Mora-Garcia et al., 2004) and (Li and Chory, 1997)). (a) and (b) Dark-grown phenotypes. (c) Light-grown phenotypes.

1.1.2. Identification of the main steps involved in brassinosteroid biosynthesis

The biosynthetic pathway of BRs is extremely complex and contains hundreds of intermediates (Figure 3).

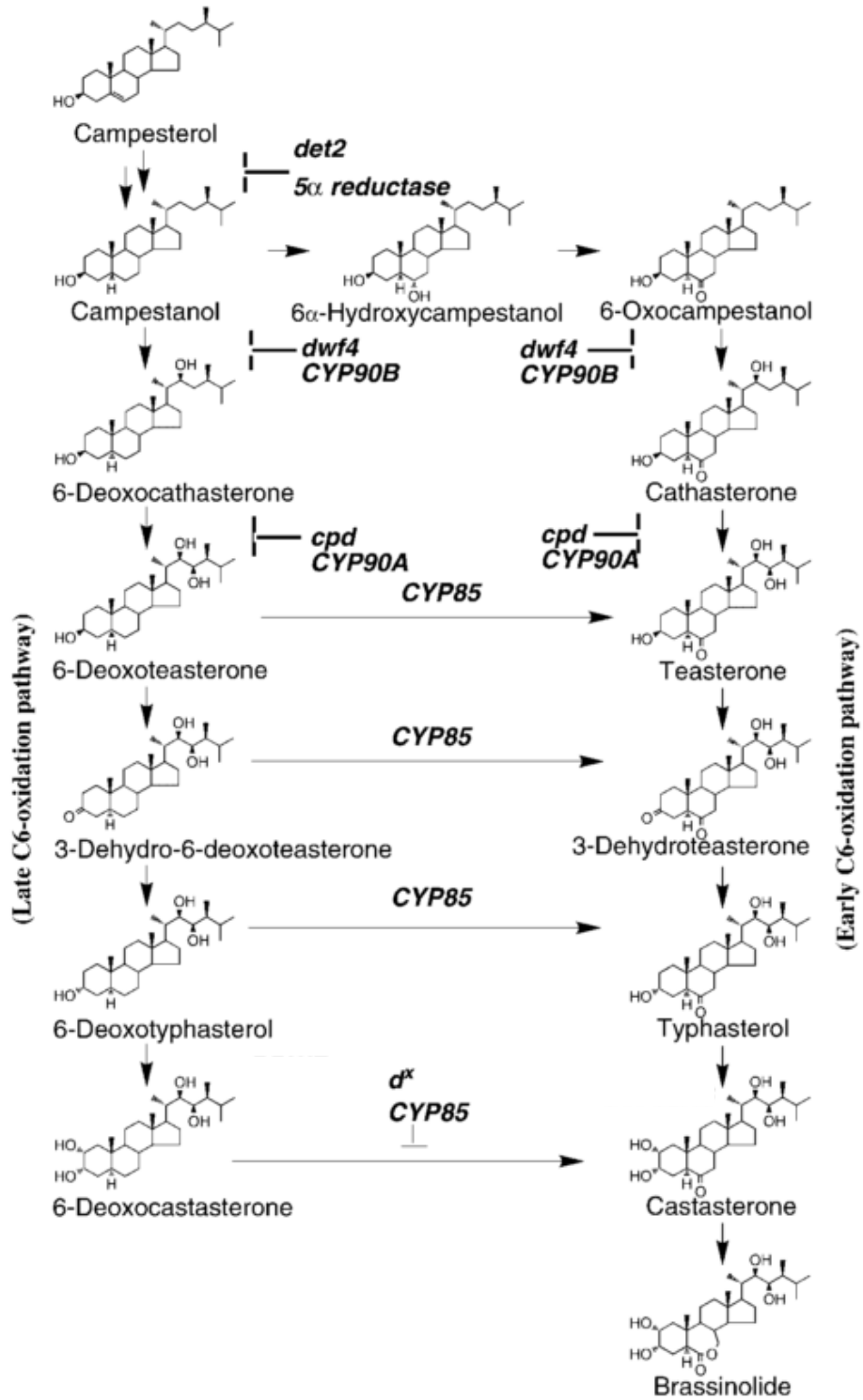


Figure 3. A simplified representation of the BR biosynthesis pathway (adapted from (Bishop and Koncz, 2002)).

One of the first BR biosynthetic gene identified was *DET2* (De-etiolated 2). *DET2* was cloned by forward genetic screening of mutants that show characteristics of light-grown plants even when grown in the dark, a phenotype called de-etiolated (Li et al., 1996). As previously mentioned, *det2* mutants are severely dwarf when grown in the light (Figure 2). The *DET2* gene presents high similarity to a steroid 5 α -reductase enzyme from mammals. Exogenous application of BL on *det2* restores a wild-type phenotype in both light and dark conditions, indicating the *det2* is impaired in BR biosynthesis (Li et al., 1996). Using radioactively labelled precursors of campestanol, *det2* mutants were shown to be impaired in the conversion of campesterol into campestanol (Fujioka et al., 1997). The *DWF4* (Dwarf 4) and *CPD* (Constitutive Photomorphogenesis and Dwarfism) genes encode cytochromes P450s and were also shown to act in the BR biosynthetic pathway (Szekeres et al., 1996; Choe et al., 1998). *CPD* was cloned by reverse genetic analyses of a T-DNA tagged mutant collection for mutants defective in the elongation of hypocotyls. Plants mutated in this gene present short hypocotyls in the dark and are dwarf in the light, presenting similar phenotypes to the ones described before for *det2* mutants (Figure 2). The hypocotyl elongation defect of *cpd* could not be rescued by cathasterone, an intermediate in the BR biosynthetic pathway. However, C23-hydroxylated derivatives of cathasterone, such as teasterone or catasterone, could fully rescue *cpd* mutants proving its role in BR biosynthesis (Szekeres et al., 1996). These results also pointed to the importance of BRs in hypocotyl cell elongation (Szekeres et al., 1996), as previously mentioned. *DWF4* was also identified by reverse genetic analyses of T-DNA lines showing a dwarf phenotype similar to *det2* (Choe et al., 1998). *DWF4* was cloned and shown to encode for a cytochrome P450. When *dwf4* mutants were treated with various BR intermediates, the 22 α -hydroxylated BRs were the only intermediates able to rescue its dwarf phenotype (Choe et al., 1998). Later, using a similar approach and measuring BR levels in plants, *DWF4* was shown to be responsible for the 22 α -hydroxylation of campestanol (Choe et

al., 1998). Plants overexpressing *DWF4* present bigger plants in dark and light, longer hypocotyls, prolonged flowering, and increased seed yield and higher levels of BR (Choe et al., 2001).

The DWF4 enzyme is the target of the BR biosynthesis inhibitor. Recombinant DWF4 protein expressed in *E. coli* was indeed shown to be directly bound by BRZ *in vitro* (Asami et al., 2001). Arabidopsis plants treated with BRZ are dwarf, consistent with lower levels of BRs, and show increased levels of feedback-regulated biosynthetic genes like *CPD* (Asami et al., 2001), consistent with a downregulation of BR signaling upon BRZ treatment. For the above mentioned reasons, BRZ turned out to be instrumental to characterize further BR responses by forward genetic and genomic approaches (Wang et al., 2002).

1.1.3. Transport and inactivation of brassinosteroids

The above mentioned genetic studies were crucial to characterize the BR biosynthetic pathway and to shed light on the roles of BR in plant growth and development. However much was still left to know about the mechanisms controlling the levels of active BRs in the plant, and notably on the transport and the inactivation of BRs.

Although the biosynthetic pathway is well described, it is still largely unknown where BRs are produced in the plant, and where are the corresponding enzymes localized in the cell. As I will discuss later in my introduction, the perception of BRs occurs at the cell surface (Irani et al., 2012), raising the question of how the BR molecules reach the receptors. It is likely that BR enzymes are associated with the secretory pathway, allowing BRs to be secreted out of the cell in the apoplastic compartment. Alternatively, BRs may be actively transported out of the cell and the ABC (ATP-binding cassette) class of transporters are likely candidates for this job. ABC transporters recently appeared in plants as major transporters of several plant hormones (Ruzicka et al., 2010; Kubes et al., 2012). More importantly, in mammals the ABC

transporters have also been shown to be able to transport steroids (Cho et al., 2014).

Overall, this largely contrasts the situation in mammals where the highly hydrophobic steroids can diffuse through the membrane (Mangelsdorf et al., 1995) and are perceived inside the cell. The higher degree of hydroxylation of plant steroids may explain this radical difference in BR perception mechanisms.

To test the long distance transport of BRs, simple grafting experiments were performed in tomato (Montoya et al., 2005) and pea (Symons and Reid, 2004). Both experiments failed to demonstrate the long distance transport of BR, indicating that BR action occurs at the site of synthesis.

Different promoters were used to express the *CPD* and *BRI1* in the biosynthetic mutant *cpd* and BR responsive mutant *bri1* respectively, in order to examine the role in shoot growth (Savaldi-Goldstein et al., 2007). Only when using the epidermal specific promoter ML1, plants expressing CPD or BRI1 could recover the dwarf phenotype, indicating a possible transport from the epidermis to the inner layers (Savaldi-Goldstein et al., 2007). These findings support the idea that indeed BRs are capable to be transported. It is possible though, that the transport of BR is restricted to a short distance. Moreover, when BAS1 (an enzyme capable of inactivation of BR, described in the following paragraph) is expressed in epidermis, the shoot growth is restricted (Savaldi-Goldstein et al., 2007).

The free concentration of active BRs is also determined by the ability of plant cells to inactivate BRs. The best characterized way to regulate active BR levels is by the inactivation of the steroids through BAS1-mediated hydroxylation. *bas1-D* (phyBactivation-tagged suppressor1-dominant) was identified by activation tagging looking for suppressors of the missense mutation *phyB-4*. Plants with shorter hypocotyl than *phyB-4* were selected and

analyzed. Activation tagging screens involve generating random T-DNA insertions using *Agrobacterium*-mediated transformation of a transgene that contains strong transcriptional enhancers capable of increasing the expression of nearby genes (Weigel et al., 2000). Feeding experiments using *basI-D* revealed that this mutant has no detectable free BL. However, transgenic lines with reduced expression of *basI* show hypocotyls with enhanced responses to BL and reduced responses to light (Neff et al., 1999).

Another way to inactivate the BR signaling pathway is by *O*-sulfonation of the brassinosteroids (Rouleau et al., 1999). Isolation of a sulfotransferase of *Brassica napus* (BNST) and recombinant expression in *E. coli* is capable of catalyzing sulfonation of BR and of mammalian estrogenic steroids (Rouleau et al., 1999). The sulfonation of 24-Epibrassinolide by the BNST3 enzyme abolishes its biological activity (Rouleau et al., 1999).

1.1.4. Brassinosteroid perception at the plasma membrane

To better identify components of the BR signaling pathway, two different screens were performed. The first screen searched for mutants resistant to the root elongation inhibition mediated by exogenous application of BL and identified the *briI* mutant (Clouse et al., 1996). The second screen allowed the identification of dwarf mutants with phenotypic resemblance to *det2* and *cpd* (Li and Chory, 1997). Isolated mutants were further screened for their inability to respond to exogenously applied BL, to eliminate biosynthetic mutants. The *bin1* mutant (for BR insensitive 1) was the first reported mutant isolated from this screen (Li and Chory, 1997). Map-based cloning *bin1* allowed the cloning of the *BIN1/BR11* gene subsequently named *BR11*. *briI* mutants show characteristics of a BR-insensitive mutant i.e. dwarfism, dark green leaves with reduced apical dominance and male fertility, and delay of leaf senescence in light (Li and Chory, 1997). In the dark, *briI* mutants present short and thick hypocotyls and open, expanded cotyledons and accumulated anthocyanins (Li and

Chory, 1997).

BRI1 encodes for a Leucine-Rich Repeat (LRR) receptor-like kinase (RLK). *BRI1* is composed of an extracellular domain that contains 25 LRRs with an island domain of 70 amino acids (aa) between the 21st and 22nd LRRs (Li and Chory, 1997). The identification of *BRI1* as a LRR-RLK protein strongly suggested that *BRI1* was acting as a receptor for BRs. Several lines of evidence confirmed this. First, *BRI1* protein was localized to the plasma membrane, consistent with extracellular perception of a signal. Second, the extracellular domain of *BRI1*, including the 70-aa island domain, was fused to the kinase domain of the rice LRR-RLK disease resistance *XA21*, and the activity of the chimeric kinase was tested upon application of BL (He et al., 2000). BL application promoted plant defense responses in rice cells, revealing the importance of the extracellular domain for the perception of BRs, and suggesting a general signaling mechanism for the LRR receptor kinases in plants (He et al., 2000). The ability of *BRI1* to directly perceive BRs was further established by Wang and colleagues (Wang et al., 2001). They used plants overexpressing *BRI1*-GFP to test the specificity of BL binding. Overexpression of *BRI1* promoted cell elongation and increased the number of radiolabeled BL binding sites in membrane fractions. Finally, binding assays of active brassinosteroids to *BRI1*, using a biotin-tagged photoaffinity castasterone (BPCS) precursor of BL and recombinant fragments of the extracellular domain of *BRI1* produced in *E. coli*, demonstrated the capacity of the island domain together with LRR21 and LRR21 alone to strongly and directly binding BR ligands (Kinoshita et al., 2005). Recent crystallographic studies of the extracellular domain of *BRI1* purified from insect cells confirmed the direct binding and identified the residues involved in BR binding (Hothorn et al., 2011; She et al., 2011).

The intracellular domain of *BRI1* shows serine/threonine (S/T) kinase activity *in vitro* (Wang et al., 2005). More recent work showed that *BRI1* also displays tyrosine kinase activity (Oh et

al., 2009). The tyrosines located on the cytoplasmic domain of BRI1, Tyr-831 and Tyr-956, were shown to be important for the autophosphorylation of BRI1 *in vitro* and *in vivo*. Tyr-831 is not essential for kinase activity, but it was shown to be important for BR signaling (Oh et al., 2009). A schematic representation of BRI1 is presented in Figure 4.

Consistent with its role as receptor, *BRI1* overexpression under the strong 35S promoter led to phenotypes similar with the overexpression of the BR biosynthetic enzyme DWF4, and increased BR-binding affinity in BR-binding assays (Wang et al., 2001)

Based on sequence similarity and complementation studies of *bri1* mutants, three other LRR-RLKs, BRL1, BRL2 and BRL3 were also identified as BRI1 homologs in the Arabidopsis genome. Both BRL1 and BRL3 are also capable of BR perception at the PM and rescue the dwarf phenotype of *bri1*, while the BRL2 protein that missed the island domain failed to do so (Cano-Delgado et al., 2004).

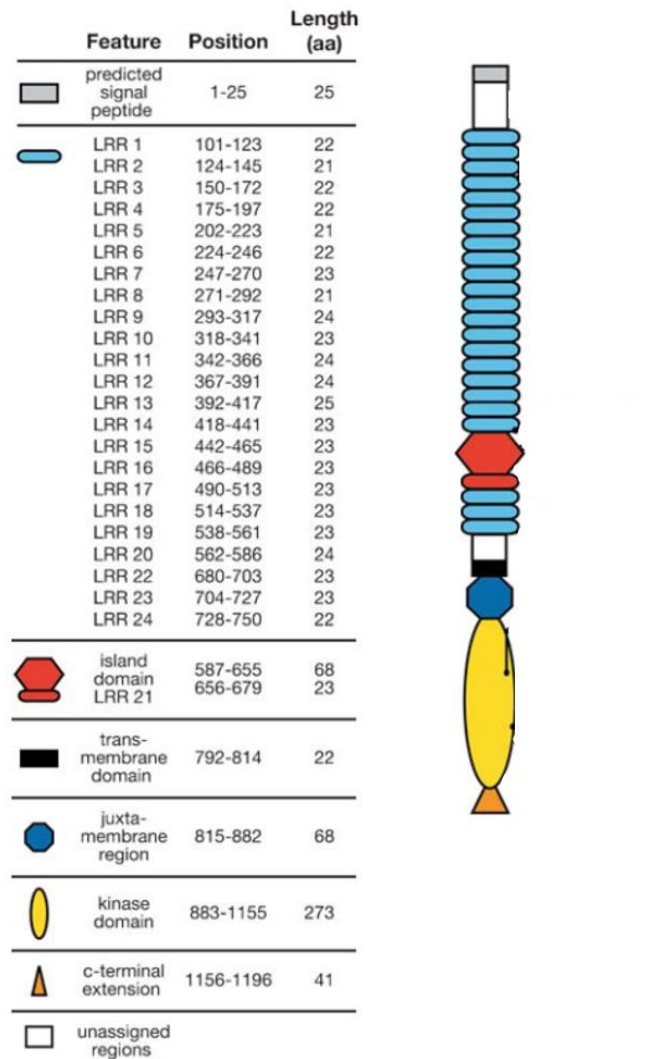


Figure 4. Schematic domain architecture of the receptor kinase BRI1 (adapted from (Vert et al., 2005))

The perception of BRs and the initiation of the BR signaling cascade from the PM is not only dependent of the perception of BRs by BRI1, but also of the interaction of BRI1 with other LRR-RLKs from the SERK (Somatic Embryogenic Receptor-like Kinase) family (Gou et al., 2012). Among these SERK proteins, BAK1 (*bri1*-associated receptor kinase 1; also known as SERK3) was studied in more details. BAK1 was identified by activation tagging looking for suppressors of a weak *bri1* allele (Li et al., 2002). The *bak1-1D* allele has elongated organs while a null allele of *BAK1* carrying a T-DNA in the *BAK1* gene shows a semi-dwarf phenotype reminiscent of weak *bri1* alleles (Li et al., 2002). BAK1 on its own do not

participate to BR binding (Cano-Delgado et al., 2004), but recent crystallographic data on the BR receptor complex composed of BRI1 and SERK1 indicates that both participate in BR binding (Santiago et al., 2013). BAK1 also came out of a yeast two hybrid screen using the BRI1 kinase domain as bait (Nam and Li, 2002). Similar to BRI1, BAK1 displays S/T kinase activity and is localized to the PM (Li et al., 2002). BRI1 and BAK1 are able to auto- and transphosphorylate each other (Li et al., 2002), consistent with their ability to interact in yeast (Russinova et al., 2004). The extracellular domains of BRI1 and BAK1 were also shown to interact (Jaillais et al., 2011; Santiago et al., 2013).

Despite the large amount of evidence suggesting a role of BAK1 and SERKs in BR signaling, the ultimate proof came from the severe de-etiolated *det2*-like phenotype and complete BR-insensitivity of a *bak1/serk1/4* triple mutant (Gou et al., 2012). However, in addition to their crucial role in BR receptor complex activation and initiation of BR signaling, BAK1 and related SERK members have been co-opted to serve as co-receptor of a large number of LRR-RLK and therefore are involved in a wide-variety of signaling pathways and responses such as plant defense, light and reproduction (Geldner and Robatzek, 2008; Clouse, 2011) .

Considering the multiple role of BAK1 and SERKs as co-receptors, it was thought that ligand binding to the corresponding receptors would induce conformational changes necessary to activate the receptor complex. In the case of BRI1, BR binding would trigger a change in BRI1 conformation promoting BRI1 and BAK1 interaction and transphosphorylation (Wang et al., 2008; Jaillais et al., 2011). Crystallographic studies helped highlight the mechanisms of interaction of this hetero-oligomeric complex. The extracellular domain of BRI1 presents an helical solenoid structure with an island of 70 aa in between the 21st and 22nd LRR repeat that create a non-polar surface upon BR binding, creating a docking for the interaction with the co-receptor required for further activation of the signaling pathway (Hothorn et al., 2011; She

et al., 2011). Besides BAK1, within the SERK family, SERK1 is also known to interact with BRI1 in its ectodomain and to form heterodimers (Santiago et al., 2013). The fact that the extracellular and intracellular domains of BRI1 were crystalized separately prevents from drawing final conclusions on the possible conformational changes associated with BR binding to the extracellular domain (Hothorn et al., 2011; She et al., 2011).

Altogether, BRs bind to the BR receptor complex composed of BRI1 and SERKs. Whether BRI1 only exists as a monomer or dimer and whether the BRI1-BAK1 heterodimerization is strictly dependent on ligand binding is still debated. BRI1 has been reported to be able to interact with itself (Friedrichsen et al., 2000). In addition, a small portion of preformed heterodimers BRI1/BAK1 at the PM were observed by FRET-FLIM experiments in the absence of endogenous BRs (Bucherl et al., 2013). BRI1 and BAK1 may interact in the absence of ligand, and BL may stabilize their interaction.

1.1.5. Downstream events in BR signaling

The BR signaling pathway is initiated by the perception of BRs by the extracellular domain of BRI1 and its co-receptors at the cell surface that triggers a series of auto and trans-phosphorylation reactions of the BR receptor complex and activation of further downstream partners. An overview of the signaling pathway cascade can be observed Figure 5.

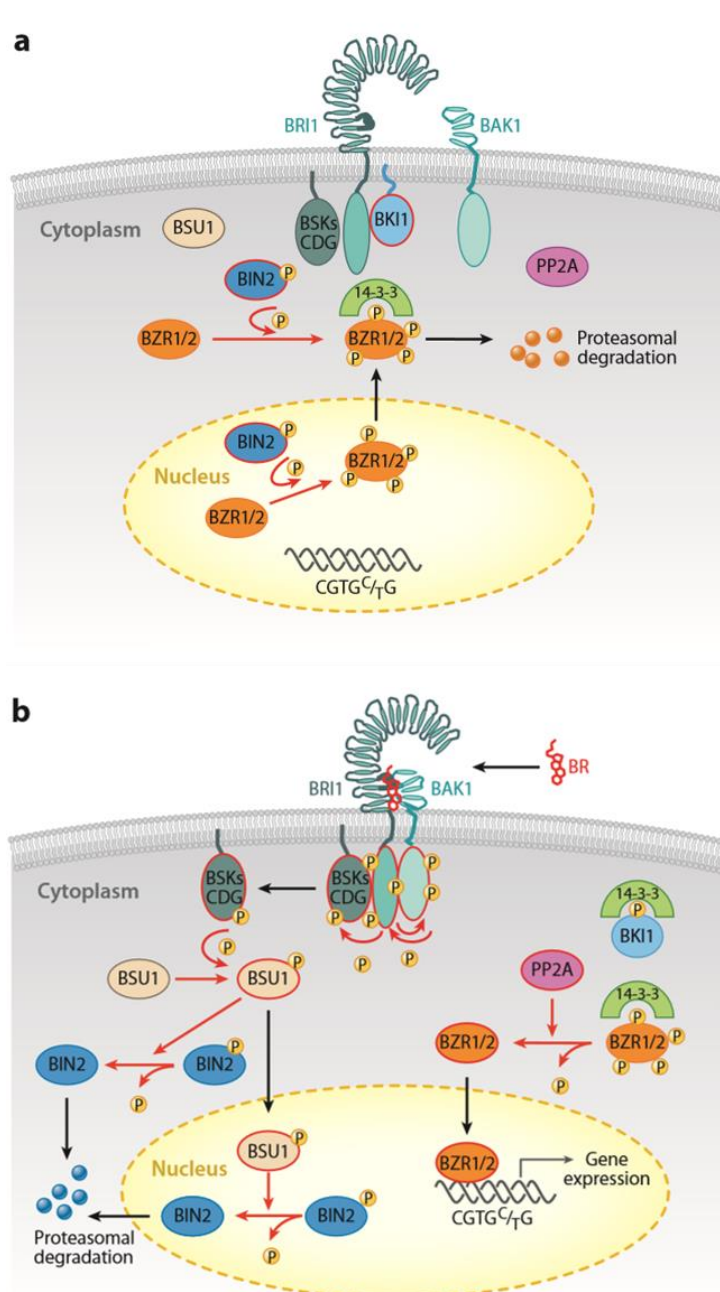


Figure 5. Two-state model of the BR signaling pathway (Adapted from (Wang et al., 2012)).
(a) In the absence of the ligand, BRI1 is associated with BKI1 and BSKs/CDG. The BIN2 kinase is activated and phosphorylates de transcription factors BZR1 and BES1 (BZR2). Phosphorylated BZR1 and BES1 lose their DNA binding activity and follow proteasomal degradation. **(b)** In the presence of the ligand, sequential phosphorylation events promote BRI1 association with its co-receptor BAK1 and dissociation of BIN2. Phosphorylation of BSKs/CGD by the complex BRI1/BAK1, promote the activation of BSU1 phosphatase. Which in turn dephosphorylates and inactivate BIN2. Dephosphorylation of BZR1/BES1 by PP2A allows the activation of these transcription.

BKI1 (BRI1 Kinase Inhibitor1) is a protein localized at the PM that works as a negative regulator of the BR signaling pathway (Jaillais et al., 2011). A recent study demonstrated that the recruitment of BKI1 to the PM by its membrane hook results from electrostatic interactions between positively charged residue (lysines and arginines) with PI4P (PtdIns(4)P, an anionic phospholipid), showing this way for the first time the importance of phosphoinositides in BR signaling (Simon et al., 2016). BR binding to BRI1 promotes the release of BKI1 from PM by phosphorylation of a conserved tyrosine on its membrane hook, thereby releasing the inhibitory effect of BKI1 on BRI1 (Jaillais et al., 2011). The dissociation of BRI1 and BKI1 is important to allow the interaction of BRI1 to BAK1, full activation of the BR receptor complex and activation of the BR signaling cascade (Belkhadir and Jaillais, 2015). Once fully activated, the BR receptor complex will phosphorylate BSKs (BR-signaling kinases) and of the CDG1 (Constitutive Differential Growth 1), belonging to two subfamily of receptor-like cytoplasmic kinases (RLCK) (Tang et al., 2008)(Kim et al., 2011). The phosphorylation of CDG1 in three different Serines (Ser) residues activates this kinase that will in turn phosphorylate BSU1 (BRI1-Supressor 1) (Kim et al., 2011). And the phosphorylation of the BSK1 Ser230 by BRI1 promotes the interaction with BSU1 and its activation (Kim et al., 2009). BSU1 is a protein phosphatase 1-like protein showing Ser/Thr phosphatase activity that was identified as a dominant suppressor of *bri1* (Mora-Garcia et al., 2004). BSU1 in turns dephosphorylates the downstream negative regulators BIN2 and homologs from the GSK3 (Glycogen Synthase Kinase 3). *BIN2* was isolated as a gain-of-function mutant (*bin2-1*) in the same genetic screen that identified *bri1* (Li et al., 2001). The *BIN2* gene was cloned by map-based cloning and shown to encode a GSK3-like kinase that function as a negative regulator of the BR signaling (Li and Nam, 2002). BIN2 has several homologs that also negatively regulate the BR signaling pathway (Li et al., 2001; Vert and Chory, 2006; De Rybel et al., 2009). In the absence of ligand, BIN2 undergoes

autophosphorylation on residue tyrosine 200, sustaining its negative regulator role by phosphorylation and inhibition of two related downstream transcription factors BZR1 (Brassinazole Resistant 1) and BES1 (*bri1*-EMS-Suppressor 1, also known as BZR2) (Wang et al., 2002; Yin et al., 2002). When phosphorylated BES1 and BZR1 remain inactive and are targeted to degradation by the 26S-proteasome (He et al., 2002). BIN2 binding of the 14-3-3 proteins that are known to be required for a more efficient deactivation of BZR1 (Gampala et al., 2007). Phosphorylation of BZR1 and BES1 impairs the migration of these transcription factors from the cytoplasm to the nucleus (Gampala et al., 2007; Ryu et al., 2007) The accumulation of the dephosphorylated BES1 and BZR1 in the nucleus promotes the DNA-binding of this protein to the to their target genes(Yin et al., 2002; Vert and Chory, 2006). Upon ligand binding, BSU1 dephosphorylates BIN2 and homologs (BR-Insensitive 2), leading to their inactivation (Kim et al., 2011). The sole inactivation of BIN2 and homologs by dephosphorylation is likely sufficient to release the downstream pathway from their inhibitory effect, but another regulatory mechanism was also proposed for BIN2. Dephosphorylated BIN2 has indeed been shown to be targeted to degradation in the 26S-proteasome (Peng et al., 2008). Inactivated GSK3s allowed dephosphorylation of BES1 and BZR1 by the phosphatase PP2A (Tang et al., 2011). A recent study showed that the protein phosphatase 2A B' (PP2A B') regulatory subunits also bind and dephosphorylate BRI1 (Wang et al., 2016). The authors showed that depending on the PP2A B' subunit nuclear or cytoplasmic localization, this phosphatase can act in the nucleus to dephosphorylate BZR1, activating the BR signaling or in the cytoplasm to dephosphorylate BRI1 contributing for the upstream inactivation of the BR signaling (Wang et al., 2016). BZR1 has also been reported to target at least 5 biosynthetic BR genes repressing them, and also BRI1, contributing for the negative feedback regulation of BR signaling (Sun et al., 2010). BR-related genes are involved in several cellular, developmental and regulatory processes that are involved in cell

elongation and differentiation (Sun et al., 2010). Microarray works reported crosstalk between BR signaling and other regulatory pathways such as auxin at a nuclear level (Goda et al., 2004; Nemhauser et al., 2004).

Despite of BES1 and BZR1 similarities and homology, these basic helix loop helix-type (bHLH) transcription factors bind to different elements in the promoters of BR-regulated genes. While BZR1 binds the BRRE (BR responsive elements) (CGTG(T/C)G) sequences, BES1 interacts with BIM1 (BES1-interacting Myc-like 1), another bHLH (Yin et al., 2005) in order to bind more efficiently the E-boxes (CACGTG and CACTTG) motifs and the BRRE (BR responsive elements) (Yu et al., 2011). Although BZR1 and BES1 were originally considered as transcriptional repressors and activators, respectively, more recent work showed that these transcription factors act as both activators and repressors, respectively (Sun et al., 2010). Chromatin immunoprecipitation (ChIP) analysis pointed to the preferential recruitment of BRRE to mediate BR-repression of gene expression, whereas the E-boxes are more enriched in BR-induced genes. Both BZR1 and BES1 interact with other transcription factors, pointing to the role of BRs in various cell processes and highlighting the connections with other signaling pathways (Sun et al., 2010; Yu et al., 2011). Accordingly, BZR1 signaling was shown to interact with transcription factors belonging to other signaling pathways, such as gibberellins (GA) and light (Jaillais and Vert, 2012; Fridman and Savaldi-Goldstein, 2013). BZR1 and PIF4 (phytohormone interacting factor 4) interact through their DNA-binding domains and N-terminal domain of PIF4 (Oh et al., 2012). Both transcription factors have been to synergistically regulate a plethora of genes, including the bHLH factors belonging to the PRE family that is required to promote cell elongation. Genetics studies using a quadruple mutant (*pifq*) lacking PIFs (PIF1, PIF3, PIF4 and PIF5) and *bzr1-ID* suggest that PIFs are required to promote hypocotyl elongation in the dark (Oh et al., 2012). PIF4 was previously

shown to be required for temperature response in hypocotyls (Koini et al., 2009). In the same way, the *pifq;bzr1-ID* mutant is resistant to the response to high temperature, showing the interdependence of BZR1 and PIF4 to promote hypocotyl elongation (Oh et al., 2012). Recently, it was demonstrated that DELLAs negatively regulate the abundance of four PIF proteins through the ubiquitin–proteasome system (Li et al., 2016). However, only PIF3 downregulation by DELLA had an effect in hypocotyl elongation (Li et al., 2016). The DELLA family are transcriptional regulators that are known to inhibit the GA signaling pathway in *Arabidopsis*. BZR1 was also shown to interact with RGA (Repressor of ga1-3), a member of DELLA family (Li et al., 2012). GA-induced degradation of DELLAs enhances BR-regulated cell elongation through activation of BES1 and BZR1. Which makes sense since ectopic expression of DELLAs destabilize and inactivate BZR1 (Li et al., 2012). On the other hand, active BZR1 and BES1 attenuate the transcriptional activity of DELLAs, promoting GA-regulated cell elongation (Li et al., 2012). Therefore, BR and GA signaling crosstalk occurs at the level of their transcriptional factors.

1.1.6. Emerging roles of brassinosteroids in roots

BRs are involved in many aspects of plant development, as described previously in this chapter by the dwarfism described in the biosynthetic and signaling mutants (Szekeres et al., 1996; Li and Chory, 1997). BRs can also affect other aspects of the plant development such as male fertility, shoot branching, stomata development, etc (Clouse et al., 1996; Gudesblat et al., 2012; Kim et al., 2012; Wang et al., 2013).

The roles of BRs in controlling root growth and development however emerged only recently. Roots present different types of cells and are divided in different zones along the longitudinal axis, namely the division zone, the elongation zone, and the differentiation zone. A representation of the *Arabidopsis* root can be observed in Figure 6.

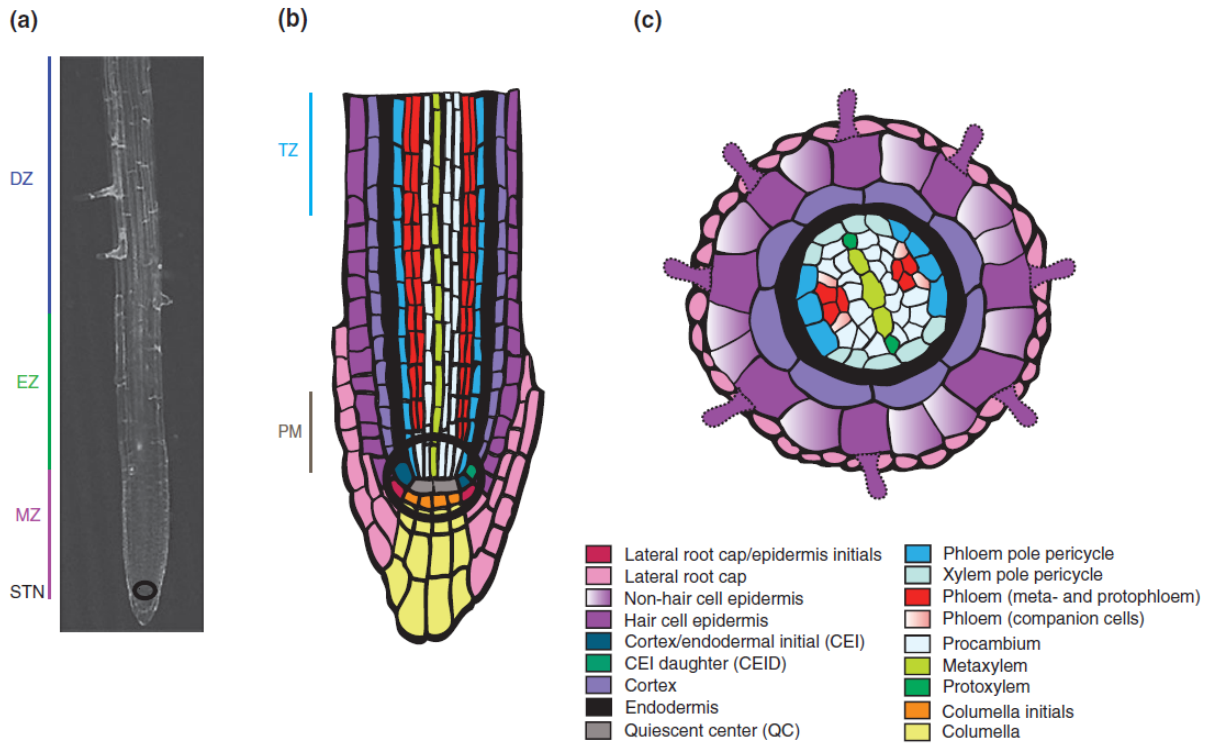


Figure 6. Cell types and developmental zones of a primary root (Adapted from (Cederholm et al., 2012)). (a) Image of 5-day-old root showing developmental zones in the root. Stem cell niche (STN) can be observed inside the black oval outlines, meristematic zone (MZ), elongation zone (EZ), and differentiation zone (DZ). (b) Schematic of a longitudinal section through the root. (c) Schematic of a transverse section of the root showing symmetry about the radial axis for the outer cell layers. The distinct cell types are demarcated in colors.

BRs impact root growth by modulating not only the elongation of differentiated cells but also the meristem size (Gonzalez-Garcia et al., 2011; Hacham et al., 2011). Hacham and colleagues (2011) suggest that BR control of meristem size depends on the gene expression that is specific of the cell-type. They conclude that BRI1 activity induces signals that communicate with the inner cells to promote cell expansion and cell proliferation, this signal is independent of BES1 and BZR1 that might act locally (Hacham et al., 2011). Accordingly, studies using different BR mutants shown that an adequate level of BL and BR signaling is necessary for the control of normal root growth. BRs promote indeed elongation of differentiated cells and reduce the number of meristematic cells and meristem size. At the

same time the absence of BR mutants also present a shorter meristem size. However, BL seems to promote cell division in the QC (quiescent center) and differentiation of distal columella stem cells (Gonzalez-Garcia et al., 2011). Another study suggests that the BR transcription factors BES1 and BZR1 repress the MYB transcription factor BRAVO that is required for repression of the division of the QC (Hacham et al., 2011). Analysis of BZR1 direct targets showed that BZR1 inactivate genes in the QC while activates genes in the elongation zone. Using a transcriptomic and genetic approaches, the same authors conclude that auxin and BR act antagonistically in a gradient manner to control root growth and cell elongation, BR gradient increases from root tip to elongation zone and auxin high levels at the meristematic might repress BZR1 transcription factor (Chaiwanon and Wang, 2015). Translatome-based work of Vragovic and colleagues with *bri1* mutants expressing BRI1 at the epidermis and where the BR genomic responses were analyzed in wild type showing that BR indeed has a tissue-dependent action and that meristematic zones have an extended gradient of auxin. They also showed that BR-induced genes in epidermis are enriched in auxin-related genes and that auxins modulate BR-response in this tissue (Vragovic et al., 2015).

BR were shown to be involved in vascular differentiation in shoots. The BRI1-like BRL1 and BRL3 were described to be expressed in vascular tissues in shoots, the *bri1brl1brl3* triple mutants exhibit disturbed phloem:xylem differentiation rates (Cano-Delgado et al., 2004). The PM protein Octopus (OPS) involved in the specification and differentiation of phloem is expressed in provascular tissue, and it was shown to bind BIN2 in roots and to sequester it at the PM activating this way the BR signaling. Mutants defective in *ops* present discontinuous phloem. This phenotype can be rescued by the dominant positive mutants of *bes1-D* and *bzr1-D* confirming a role of BR in phloem differentiation (Anne et al., 2015).

The patterning into trichoblast (root hair cells) and atrichoblast (non-root hair cells) in the root

epidermis are determined by the transcriptional complex MYB-bHLH-WD40. BIN2 is capable of interaction, phosphorylation and inactivation of the bHLH transcription factors EGL3 (Enhancer of Glabra 3) and TTG1 (Transparent Testa Glabra 1 – a WD40 repeat protein), both expressed in trichoblast. These transcription factors negatively regulate the trichoblast formation. It is postulated that BIN2 inactivation of EGL3 inhibits the nuclear accumulation of this transcription factor and facilitates its cell-to-cell movement from the trichoblast to atrichoblast (Cheng et al., 2014). The activity of BIN2 is modulated by the amount BR perceived at the PM. More BR leads to active BR signaling and inactive BIN2. Therefore, BR signaling and inactive BIN2 promotes atrichoblast while active BIN2 allows the formation of trichoblast, which means that BR signaling is important for root epidermal cell fate (Cheng et al., 2014).

A lot of recent works provided new information about the BR signaling, BRI1 trafficking, and BR responses came from studies in roots (Reviewed in Jaillais and Vert, 2016). This is especially true for cell biology analyses of the BR receptor complex since root tip cells offer very nice imaging properties including a limited vacuole that allows imaging of endocytic compartments, and the availability of a large collection of intracellular markers allowing a precise characterization of the distribution of proteins in root tip cells (Geldner et al., 2009).

1.2. BRI1: a model for studying the dynamics and degradation of plasma membrane proteins in plants

1.2.1. Endocytic trafficking and mechanisms in plants

Endocytosis can be defined as a process that consists in the uptake of different proteins or material from the plasma membrane by invagination of the PM and internalization into

endocytic compartments (Valencia et al., 2016). As such, endocytosis is involved in a wide array of biological functions including nutrition, turnover of plasma membrane proteins, downregulation of receptors, etc (Bache et al., 2004; Barberon et al., 2011; Luschnig and Vert, 2014). Internalized vesicles fused to cytoplasmic compartments, known as early endosomes (EE) have been shown overlap with the trans-Golgi Network in plants (TGN), and hereafter called TGN/EE (Dettmer et al. 2006). The TGN/EE then matures into the late endosomes (LE) and multivesicular bodies (MVB) and fuse with the lytic vacuole, that in plants is the equivalent to the lysosome in mammals (Reyes et al., 2011). Endocytic cargo internalized from the PM and received by the EE/TGN can also recycle back to the PM (Geldner, 2004).

The use of the amphiphilic styryl dye FM4-64 helped the visualization of endocytic compartments such as EE by microscopy (Bolte et al., 2004) and it was used thereafter to help on the identification of several proteins that are involved in the endocytosis and endosomal trafficking (Dettmer et al., 2006). A general overview of the endosomal trafficking in plants is illustrated in Figure 7.

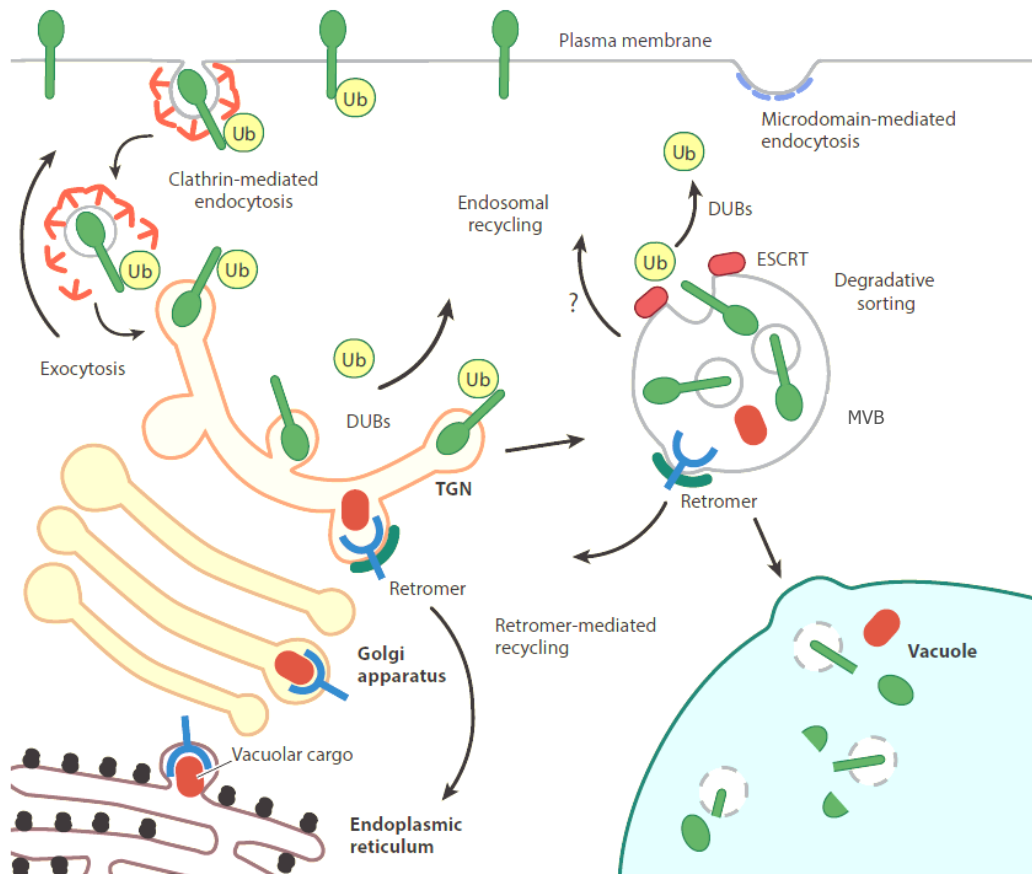


Figure 7. Overview of the endosomal trafficking in plants (Adapted from (Valencia et al., 2016)). ER, endoplasmic reticulum; TGN, trans-Golgi network/early; MVB, multivesicular bodies; Ub, Ubiquitin; DUBs, Deubiquitinating enzymes; ESCRT, Endosomal Sorting Complex Required for Transport. Black arrows represent trafficking pathways.

Studies using drugs such as Brefeldin A (BFA) and concanamycin A (Conc A – a V-ATPase inhibitor that blocks the trafficking to the vacuole) showed a localization of the subunit VHA-a1 at the EE and the TGN level, suggesting a convergence of the early endocytic and secretory pathways (Dettmer et al., 2006). Other proteins such as the Rab GTPases are also involved in the regulation and identity of the different endosomal compartments. The Rab GTPases RabA2/A3 are markers of the EE/TGN (Chow et al., 2008), whereas the Rab GTPases ARA6 (RabF1) and ARA7 (RabF2b) are good markers of the LE/MVB (Haas et al., 2007). Proteins that are addressed to vacuolar degradation usually are target to the vacuole

from the LE/MVB through the complex ESCRT (endosomal sorting complexes required for transport) (Spitzer et al., 2006; Spitzer et al., 2009).

The endosomal trafficking is not linear and besides the recycling from EE/TGN to the PM, it was also identified another recycling pathway that is believed to be involved in the recycling from the LE/MVB to the EE/TGN, the retromer complex (Valencia et al., 2016). The marker ARF-GEF GNOM has been shown to play a role on the recycling of PIN1 efflux-carrier of auxin from the EE to the PM (Geldner et al., 2003). The Sorting Nexin 1 (SNX1) and the VPS (vacuolar protein sorting) 29 belonging to the retromer complex were also shown to be important to the cycling of PIN1, but at the level of the LE/MVB (Jaillais et al., 2006; Jaillais and Gaude, 2007).

The mechanisms for internalization of plasma membrane proteins can be divided in clathrin-mediated (CME) and clathrin-independent endocytosis (CIE) (Doherty and McMahon, 2009). The CME has been pointed as the predominant pathway for internalization of numerous PM proteins in plants (Dhonukshe et al., 2007). Overexpression of the C-terminal part of clathrin heavy chain (Hub1) impairs the clathrin cage formation and leads to a strong dominant-negative effect on clathrin-mediated processes (DN-Hub1) and cause defects in CME in protoplasts (Dhonukshe et al., 2007). Co-expression of DN-Hub1 and the PM proteins PIN1 and PIN2 associated with a photoconvertible fluorescent protein EosFP in protoplasts confirm the importance of CME on the internalization of these two auxin efflux carriers (Dhonukshe et al., 2007).

Although the CME is best studied, there are still a lot of gaps in the understanding of this mode of endocytosis in plants. Essentially there are some differences between plants and animals in CME. For instance, in plants, there is a lack of some subunits of the AP-2 complex that exist in animals (Valencia et al., 2016). Plants mutated in the AP2 σ subunit

present several stages of plant growth and development defects. The cotyledons of the *ap2* σ mutants exhibited defective organogenesis associated with an altered vascular pattern. *ap2* σ mutants grown in soil have dwarf phenotypes, with reduced leaf size and stem height and shorter siliques than wild-type plants (Fan et al., 2013). Another essential difference of CME in plants is the presence of the ancestral adaptor complex TPLATE in plants. A T-DNA insertion on *TPLATE* causes male sterility by interfering with pollen development or germination (Dettmer et al. 2006). The TPLATE complex (TPC) consists of eight core subunits that are required for the CME initiation preceding the recruitment of the AP-2 complex, the clathrin and the dynamin-related proteins (DRP) (Gadeyne et al., 2014). Similar to *tplate* mutants, the *tpc* mutants also present pollen development defect and male sterility (Gadeyne et al., 2014). Both proteins as expected are localized at the PM (Dettmer et al. 2006; Gadeyne et al., 2014). Besides these differences on the CME machinery, plants present orthologs for the most ESCRT complex proteins, except for the ESCRT-0 subunit (Valencia et al., 2016). The existence of CIE in plants only emerged recently, but its functional importance compared to CME remains obscure. CIE has also been shown to play a role in the internalization of PM proteins (Valencia et al., 2016). The aquaporin PIP2;1 (Plasma membrane intrinsic protein) undergoes both CME and CIE. Using variable angle epifluorescence microscopy it was shown that PIP2;1 co-localize both with CLC (clathrin light chain) and Flot1 (a flotillin protein involved in PM microdomain formation) (Li et al., 2011).

The recognition and selection of the cargo can be grouped by conformational motifs, linear amino acid motifs and posttranslational modifications, such as ubiquitination and phosphorylation (Kelly and Owen, 2011). The existence of one type of sorting doesn't exclude the possibility of another recognition in the same protein. Moreover, the sorting can

happen in different compartments along the endocytic pathway (Traub and Bonifacino, 2013). In animals AP-2 is known to recognize two internalization sorting linear motifs in cargo proteins: the di-Leucine based motif [DE]xxxL[LI] and the Tyrosine (Tyr)-based motif Yxx ϕ (where ϕ is a bulky hydrophobic amino acid) (Ohno et al., 1995; Kelly and Owen, 2011). The role of AP2 in plant endocytosis was assessed either by mutating AP2 subunits, or by interfering genetically or pharmacologically with AP2-mediated endocytosis of cargo proteins. Biochemical and confocal works revealed the role of the AP2 complex subunit σ in endocytosis and polar localization of PIN2 and consequently in plant development (Fan et al., 2013). Upon high supply of Boron (B) the Boron Transporter 1 (BOR1) is internalized and degraded by the vacuole (Takano et al., 2005). The internalization of BOR1 seems to depend on its Tyr-based motifs (Y373QLL, Y398DNM, and Y405HHM) (Takano et al., 2010). BOR1 is a PM transporter that presents three tyrosine motifs and a polar localization. When these BOR1 tyrosine motifs are mutated to alanine, BOR1 is no longer polar and cannot be degraded by the vacuole (Takano et al., 2005). The use of BFA shows that BOR1(Y373A/Y398A/Y405A)-GFP still accumulates in the BFA bodies. Nevertheless, the authors point for the role of other endocytosis mechanisms in BOR1 internalization (Takano et al., 2005). Other examples using pharmacological approaches and notably Tyrphostin A23, pointed to the importance of AP2 in the internalization of several cargo proteins (Barberon et al., 2011; Irani et al., 2012). However, Tyrphostin A23 was recently reported to be a protonophore and conclusions drawn from its use must therefore be used with caution (Dejonghe et al., 2016).

1.2.2. BRI1 as a model for endocytosis and receptor signaling in plants

The localization of BRI1 and its dynamics within the endocytic pathway has long been studied and served as a model in the study of receptor dynamics in plants. BRI1 serves as a

classic receptor kinase in plants and can be considered as the best model for its different advantages such as: i) it's a transmembrane protein that can be easily tagged on its C-terminal without interfering with its function, ii) the ligand is well known, iii) detailed activation mechanisms, iv) existence of several loss- and gain-of-function mutants, v) profound knowledge of BR signaling mechanisms and downstream factors that can serve as readout of BR signaling such as the dephosphorylation of BES1 or the hypocotyl elongation.

BRI1 has been originally found at the cell surface, consistent with its role in the perception of BRs (Friedrichsen et al., 2000). However, the transgenic line used strongly overexpressed BRI1, and the functionality of the corresponding BRI1-GFP fusion protein was not demonstrated. More recent work provided a better picture on the localization of BRI1, using a transgenic line expressing a functional BRI1-GFP fusion protein at the level of the endogenous BRI1 protein (Geldner, 2007). Using this refined tool, BRI1-GFP was shown to localize to the plasma membrane as well as in the TGN/EE. Further studies by immunogold analyses revealed the presence of BRI1 in late endosomal compartments (Viotti et al., 2010). The localization and *in vivo* interaction of the BR receptor complex was tested very early (Ruscinova et al., 2004). In cowpea protoplasts, BRI1 indeed appears to be internalized with its co-receptor BAK1 to the TGN/EE (Ruscinova et al., 2004). Considering that the interaction between BRI1 and BAK1 is considered to be ligand-dependent, this strongly argued for the ligand-dependent endocytosis of BRI1 in response to BR binding. This concept was challenged by the fact that neither the localization, nor the half-life of BRI1-GFP expressed at endogenous levels were affected by BRs (Geldner et al., 2007). However, a recent study using the variable angle - total internal reflection fluorescence microscopy (VA-TIRF) that allows to follow PM proteins events such as endocytosis, showed that exogenous applications of BL promote BRI1 endocytosis (Wang et al., 2015). Endocytosed BRI1 can

recycle back to the PM or be target for degradation in the vacuole (Geldner et al., 2007).

To get a hint on about the turnover of BRI1 protein Geldner and colleagues treated Arabidopsis plants expressing BRI1-GFP with BRZ and BL. This treatments didn't show any difference on the localization of BRI1, therefore the plants were treated with BFA (Geldner et al., 2007). BFA is a fungal toxin that inhibits the recycling pathway to the PM at the level of the TGN (Nebenfuhr et al., 2002) creating the BFA compartments/bodies (Satiat-Jeunemaitre et al., 1996). The plants treated with BFA showed an increased BR signaling, measured by the dephosphorylation of BES1, showing that the endocytosed and trapped BRI1 on the BFA bodies can still signal. When the BR signaling is activated BRI1 dissociates from BKI1, and BKI1 is released from the PM to the cytosol (Jaillais et al., 2011). The plants treated with BFA, did not show BKI1 on the BFA bodies (Geldner et al., 2007), suggesting BKI1 is not integral membrane protein. In order to understand better where the BR signal is initiated, a study using a fluorescent BR analog, Alexa Fluor 647–castasterone (AFCS) that helped visualize the endocytic pathway of the receptor BRI1 and the ligand BR. It was demonstrated that BR signaling is mainly initiated at the PM and not in endosomes (Irani et al., 2012). Accordingly, another study using confocal microscopy showed that addition of BL increased the basal co-localization of BRI1 and its co-receptor BAK1 (Bucherl et al., 2013). And using FRET-FLIM these authors showed that BRI1 and BAK1 interact at endosomal level, including in BFA bodies. It is hypothesized that these complexes are originated at the PM (Bucherl et al., 2013). In conclusion, works using microscopy confirm that after perception of its ligand, BRI1 is internalized to the EE/TGN (Ruscinova et al., 2004; Irani et al., 2012; Bucherl et al., 2013), and is targeted to degradation in the vacuole (Geldner et al., 2007) though the LE/MVB (Viotti et al., 2010), contributing for the downregulation of BR signaling.

As referred before, the BR hormone receptor, BRI1 is internalized and target to the vacuole. Recently, genetic works with the help of confocal microscopy showed co-localization of BRI1 with a CLC in roots and AP-2. Interaction of BRI1 and AP-2 was shown by co-immunoprecipitation and plants defective in AP2A subunit of the AP-2 complex exhibit decreased endosomal pool in epidermal cells. This work was the first strong evidence of the involvement of CME in BRI1 internalization (Di Rubbo et al., 2013). More recently the TPLATE-Muniscin-Like (TML) protein, member of the TPLATE complex (TPC) was also shown to be important for BRI1 internalization. Downregulation of TML impacts negatively in the internalization of BRI1 and reduced accumulation of AP2A at the PM (Gadeyne et al., 2014). Finally, BRI1 was also co-localized with the Flot1 flotillin protein involved in PM microdomain formation, suggesting that BRI1 may also be internalized via CIE mechanisms. Consistently, plants defective in CIE seem to affect BRI1 endocytosis and lead to an increase of BRI at the PM (Wang et al., 2015). To test the effect of BR in CME and CIE, Wang and colleagues treated plants expressing either BRI1-GFP/CLC-mCherry or BRI1-GFP/AtFlot1-mCherry and checked the proximity of BRI1 and the markers for CME and CIE in the presence of BR. When BL is added, there's an increase of proximity between BRI1 and AtFlot1. Therefore, authors suggested that exogenous application of BL triggers CIE of BRI1. BRI1 may be internalized via alternative endocytic pathways, depending on BR level (Wang et al., 2015).

Overall, the study of both CME and CIE mechanisms of BRI1 endocytosis still needs to be expanded to understand their relative contribution. Different cell types or concentrations of ligand may indeed drive BRI1 internalization through different pathway.

1.2.3. The role of ubiquitination in endocytosis

Another important mechanism of endocytosis and degradation of proteins is ubiquitination. In

yeast and mammals ubiquitination has been shown to be important for internalization and/or degradation of several plasma membrane proteins (Lauwers et al., 2010).

Ubiquitination is a posttranslational modification that consists on the conjugation of the small protein ubiquitin (Ub) to the target proteins and that is involved in several cellular physiologic processes (Clague et al., 2015). Ubiquitination is a cascade process that involves 3 different enzymes: the Ub-activating enzyme, E1; the Ub-conjugating enzyme, E2; and the Ub-ligase enzyme, also known as E3-ligase that allows the recognition of the target protein to be ubiquitinated. Together, these enzymes are responsible for the catalysis of the covalent conjugation of the Ub to a target lysine(s) (K) of the specific protein. As the Ub also contains lysine residues in its primary sequence, it can also be a target for further ubiquitination forming this way a polyubiquitin (polyUb) chains through one of seven lysine residues from ubiquitin (K6, K11, K27, K29, K33, K48 and K63) (Adhikari and Chen, 2009). Therefore, ubiquitination can occur in different forms, such as mono or polyUb. Each type of ubiquitination might lead a different role of ubiquitination in the cell.

Ubiquitination in yeast and mammals have been associated to endocytosis and trafficking to the vacuole/lysosomes. An original work in yeast has shown for the first time that Ub is necessary for ligand-induced endocytosis of the receptor Ste2p (Hicke and Riezman, 1996). Mono-ubiquitination has been postulated to be enough to trigger internalization and sorting in MVB of the Phm5 protein in yeast (Reggiori and Pelham, 2001). However, posterior works in yeast, have established the K63-polyUb chains as responsible for the proper sorting at the MVB of different PM proteins (Erpapazoglou et al., 2008; Lauwers et al., 2009). Similarly, in mammals ubiquitination of RTKs (receptor tyrosine kinases) like the EGFR (Epidermal growth factor receptor) have been first linked to internalization (Haglund et al., 2003). Later, it was demonstrated that EGFR not only carries mono-Ub but also K63-polyUb chains (Huang et al., 2006) and that ubiquitination was not essential for internalization of EGFR

(Huang et al., 2007). The K63-polyubiquitination but not multi-monoubiquitination, was shown to be required for lysosomal degradation of EGFR (Huang et al., 2013).

Ubiquitination has also been shown to be an important for endocytosis and targeting to degradation by the vacuole of several transporters in plants (Barberon et al., 2011; Kasai et al., 2011; Leitner et al., 2012). Mono-ubiquitination has been reported to be sufficient for internalization of the iron transporter IRT1 and to its target to the vacuole. Mutation of two lysines in the intracellular loop of this protein abolish IRT1 ubiquitination and impair its internalization from the PM (Barberon et al., 2011). The boron transporter (BOR1), on the other hand is known to be internalized via CME, where the tyrosine motifs play an essential role (Takano et al., 2010). However, the trafficking to the lytic vacuole is dependent of ubiquitination. A mutation in lysine 590 (Lys590) of BOR1 did not affect internalization of this mutated form, but prevented the sorting and degradation by the vacuole (Kasai et al., 2011). Work with the auxin carrier PIN2 showed that this protein carries of K63-polychains and that this type of ubiquitination is important for the targeting to the vacuole (Leitner et al., 2012).

In addition, several E3 ubiquitin ligases have been shown to interact with cell surface proteins, including receptors (Mbengue et al., 2010; Lu et al., 2011), suggesting a role for ubiquitin-mediated endocytosis for these proteins. This will however will have to be demonstrated experimentally since ubiquitination can mediate a wide variety of biological functions. To date, the degradation of the plant flagellin receptor FLS2 by ubiquitination has been shown to involve K48-linked polyubiquitin chains, pointing to a role of proteasome (Lu et al., 2011). However, considering the extensive literature arguing for endocytosis of FLS2 (Robatzek et al., 2006; Spallek et al., 2013), it is likely that FLS2 undergoes ubiquitin-mediated endocytosis.

The ESCRT complex has an important role on the degradation of ubiquitinated proteins by the vacuole/lysosome since it is responsible for the recognition of ubiquitinated proteins in the lysosomes and trafficking to the vacuole/lysosomes (Raiborg and Stenmark, 2009; Shields and Piper, 2011; Gao et al., 2014). Although the plant ESCRT complex shows different subunit composition compared the yeast and mammals, several evidence point to their crucial role in sorting of ubiquitinated cargo proteins. The auxin carriers AUX1, PIN1 and PIN2 have been shown to be cargo of the ESCRT complex (Spitzer et al., 2009). A recent study showed that subunits of the ESCRT-I regulate the internalization of FLS2 into the lumen of the MVB (Spallek et al., 2013). Recently, a new ESCRT component incorporated into the ESCRT-I complex was identified: FREE1 (FYVE domain protein required for endosomal sorting 1). FREE1 is essential for plant growth and endosomal vesiculation and trafficking (Gao et al., 2014). The domain FYVE has also been demonstrated to be important for polar localization of the iron transporter IRT1 (Barberon et al., 2014). This is consistent with the fact that ubiquitin has mostly been involved in the intracellular sorting of endocytosed proteins to the vacuole. Whether ubiquitin can mediate the early internalization steps during endocytosis is still unclear and is reminiscent of the contradictory results accumulated in the mammalian literature for the ubiquitin-mediated endocytosis of EGFR.

As mentioned above ubiquitination was thought to play a role on internalization of EGFR (Haglund et al., 2003) and later on the sorting to the lysosome only (Huang et al., 2006; Huang et al., 2007). However, several evidences for multiple mechanisms including ubiquitination promoting internalization and degradation by the lysosome have been unraveled (Haglund and Dikic, 2012). Works using EGFR with 15 lysines mutated to arginines (15KR) or 21KR shown that the Lys residues in the kinase domain of EGFR promote interaction of the receptor with the clathrin adaptor protein (AP)-2 complex, interaction with the adaptor protein GRB2 and acetylation of C-terminal Lys residues (Goh et

al., 2010). Moreover, it is thought that the ligand might interfere with the ubiquitination status and role of EGFR (Sigismund et al., 2005). Low concentrations of EGF promote CME of EGFR. But, at high concentrations of EGF, EGFR seems to follow CIE via ubiquitination (Sigismund et al., 2005). It is thought that EGFR endocytosed via CME is recycled back to the PM, whereas at high concentration of EGF, EGFR is targeted to degradation by the vacuole (Sigismund et al., 2008).

Context

When I arrived in France for my PhD at the end of the year of 2012, the lab was just getting established in at the Institut des Sciences du Végétal (ISV) in Gif-sur-Yvette.

The Cell Signaling and Ubiquitin Team is focused on the roles of a subtype of poly-ubiquitin chains involving the lysine residue K63 of ubiquitin. This post-translational modification has been implicated in proteasome-independent functions in yeast and mammals, but is overall very poorly characterized. One of the major goals from the lab is to study of the role of K63 polyubiquitination in the endocytosis of plasma membrane proteins.

The study of ubiquitination in plants is relatively recent, except for K48 polyubiquitination which drives proteasome-mediated degradation (Pickart and Fushman, 2004). Much is still to discover about K63 polyubiquitination and its role in plants. In particular, work in yeast and mammals established a role of K63 polyubiquitin chains in endocytosis, although its precise action is still debated as previously discussed. Work from the lab demonstrated that ubiquitination triggers the endocytosis of plasma membrane proteins in plants as well. This was achieved using the IRT1 root iron transporter as model (Barberon et al., 2011). This findings were further confirmed by two other studies using the BOR1 boron transporter and the PIN2 auxin efflux carrier (Kasai et al., 2011; Leitner et al., 2012). So far, only PM transporters have been shown to undergo endocytosis and vacuolar degradation through ubiquitination.

To broaden the study of ubiquitin-mediated endocytosis to other types of PM proteins, and to evaluate the interplay between endocytosis and signaling, the lab established the BRI1 as another model PM protein. As mentioned during my introduction, BRI1 is the BR receptor localized at the PM, which the mechanisms of ligand perception, activation, and endocytic traffic has been well characterized (Vert et al., 2005; Wang et al., 2012; Jaillais and Vert,

2016). Furthermore, the BR signaling pathway has been extensively studied, offering a unique opportunity to understand, if any the connections between BR perception by BRI1, deactivation of BRI1 and signaling.

The general goal of my PhD was to study the ubiquitination of BRI1, evaluate its functional importance for BRI1 dynamics and more generally for BR signaling.

Prior my arrival to the team, preliminary results had shown that BRI1 is indeed ubiquitinated *in vivo*. This was first assessed by immunoprecipitation of the tagged version of BR1 with posterior probing of the blot with a specific antibodies of the different types of ubiquitin. I had the chance to describe the technique that will be published in *Methods in Molecular Biology* (MiMB, *in press*, Annex 1 of this thesis). To evaluate the importance of BRI1 ubiquitination, the precise sites of linkage were searched by mass-spectrometry. A single lysine was identified, but the sole mutation on this lysine showed little or no decrease of the BRI1 ubiquitination profile, indicating that other lysines were likely ubiquitinated. I therefore undertook several complementary approaches during my PhD to tackle the role of BRI1 ubiquitination, including a gain-of-function approach using a translational BRI1-Ub fusion and a loss-of-function strategy where all possible ubiquitination sites are knocked out. I notably had the opportunity to use biochemical and imaging techniques to study the ubiquitination and cellular localization of the different plant material available or generated. For the first time in the team, I used the TIRF imaging technique in order to study the role of ubiquitination in the internalization of BRI1 from the PM. This study resulted in a paper that composes the Chapter 2 of this thesis manuscript.

While investigating the role of ubiquitination in BRI1 endocytosis, trafficking to the vacuole and impact on BR signaling, we had the chance to uncover a role of temperature in BRI1

degradation. I then focused the second part of my thesis on study the impact of elevated ambient temperature in BR-dependent root responses. This was a great opportunity for me to develop my critical judgment and to study something new, broadening my field of expertise. Until recently, not much was known about the impact of mild increase temperature in hormone signaling, especially in roots. The main reports about impact of temperature in plant development come from studies done in hypocotyl and include mainly PIFs and auxin-mediated responses (Franklin et al., 2011; Sun et al., 2012). Therefore, I used different kind of techniques to sort out the role of BRs and in particular BRI1 and BRI1 ubiquitination in root development at elevated environmental temperature. This work also allowed me to be more involved in the preparation of the manuscript of a paper that was also submitted to Nature communications and composes now the chapter 3 of my thesis manuscript.

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Chapter 2:

Internalization and vacuolar targeting of the brassinosteroid hormone receptor BRI1 are regulated by ubiquitination

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Chapter 2

Internalization and vacuolar targeting of the brassinosteroid hormone receptor BRI1 are regulated by ubiquitination

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Abstract

Brassinosteroids (BRs) are plant steroid hormones that control many aspects of plant growth and development. BRs are perceived at the cell-surface by the plasma membrane-localized receptor kinase BRI1. Here we show that BRI1 is post-translationally modified by K63 polyubiquitin chains *in vivo*. Using both artificial ubiquitination of BRI1 and generation of an ubiquitination-defective BRI1 mutant form, we demonstrate that ubiquitination promotes BRI1 internalization from the cell surface and is essential for its recognition at the *trans*-Golgi Network/Early Endosomes (TGN/EE) for vacuolar targeting. Finally, we demonstrate that the control of BRI1 protein dynamics by ubiquitination is an important control mechanism for BR responses in plants. Altogether, our results identify ubiquitination and K63-linked polyubiquitin chain formation as a dual targeting signal for BRI1 internalization and sorting along the endocytic pathway, and highlight its role in hormonally controlled plant development.

Introduction

Brassinosteroids (BRs) are polyhydroxylated steroid hormones that regulate plant growth and development (Vert et al., 2005). Studies of mutants with defects in BR biosynthesis or signaling demonstrated that BRs play essential roles in nearly all phases of plant development, as these mutants show multiple developmental defects, such as reduced seed germination, extreme dwarfism, photomorphogenesis in the dark, altered distribution of stomata, delayed flowering and male sterility (Vert et al., 2005; Fridman and Savaldi-Goldstein, 2013). BRs are perceived at the cell surface by the membrane-bound receptor complex composed of the BRASSINOSTEROID INSENSITIVE1 (BRI1) receptor kinase and BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) (Hothorn et al., 2011; She et al., 2011; Santiago et al., 2013). Ligand-dependent auto- and trans-phosphorylation of BRI1 and BAK1 participate in receptor complex activation and modulate a cellular cascade of kinases and phosphatases (Kim et al., 2009; Tang et al., 2011), which ultimately culminates in the dephosphorylation and activation of two transcription factors, BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS SUPPRESSOR1 (BES1) (He et al., 2005; Yin et al., 2005). BES1 and BZR1 bind to target promoters in the presence of different interacting partners to regulate BR genomic responses (Vert and Chory, 2006; Sun et al., 2010; Yu et al., 2011).

Adjustments in subcellular distribution of cell surface receptors are critical to modulate their signaling activity. Over the past decade, BRI1 has served as model for a better understanding of the interplay between receptor trafficking, signal transduction and deactivation. BRI1 constitutively cycles between the plasma membrane and the *trans*-Golgi network/early endosome (TGN/EE), and it is targeted to the vacuole for degradation *via* the late endosomes/multivesicular bodies (MVB), independently of its ligand (Geldner et al., 2007;

Jaillais et al., 2008). Internalization of BRI1 involves clathrin-mediated endocytosis, the adaptor complex AP-2 and the ARF-GEFs GNOM and GNL1 (Irani et al., 2012; Di Rubbo et al., 2013). The drug-mediated trapping of BRI1 in endosomes was shown to enhance BR responses, leading to a model whereby BRI1 signals preferentially from endosomal compartments (Geldner et al., 2007). However, blocking BRI1 internalization by genetic or pharmacological interferences with AP2 was shown to activate BR signaling, indicating that BRI1 signals from the plasma membrane (Irani et al., 2012; Di Rubbo et al., 2013).

The post-translational modification of proteins by ubiquitination is well-known in yeast and mammals to target proteins to proteasome-mediated degradation, and this involves K48-linked polyubiquitin chains (Deshaies and Joazeiro, 2009). Ubiquitination also serves numerous proteasome-independent roles, including the endocytosis of membrane proteins (MacGurn et al., 2012). Notably, K63-linked polyubiquitin chains are essential for endosomal sorting and lysosome/vacuolar delivery of cargos *via* recognition by the ESCRT complex (Raiborg and Stenmark, 2009). Ubiquitination also controls early steps of plasma membrane protein internalization. In yeast, Ub-mediated internalization is the major mechanism allowing retrieval of plasma membrane proteins (Lauwers et al., 2010), while several other redundant Ub-independent pathways exist in mammals (Goh et al., 2010). In plants, Ub-mediated endocytosis emerged only very recently with the study of ion and hormone transporters (Barberon et al., 2011; Kasai et al., 2011; Leitner et al., 2012; Lin et al., 2013; Shin et al., 2013). To date, the role of ubiquitination in the dynamics of plant transporters appears restricted to their targeting to the vacuole (Kasai et al., 2011; Leitner et al., 2012). Whether Ub-mediated endocytosis has also been co-opted for driving the trafficking of other types of plant plasma membrane proteins, such as receptors, and for triggering early steps of internalization from the cell-surface is unclear. Although several plant E3 ubiquitin ligases have been shown to interact with receptors (Gu et al., 1998; Kim et al., 2003; Wang et al.,

2006; Samuel et al., 2008), their direct role in receptor ubiquitination has not been shown. Only the ubiquitination of the cell-surface flagellin receptor FLS2 by the PUB12 and PUB13 E3 ligases has been experimentally demonstrated, but its link with FLS2 endocytosis is still undocumented (Lu et al., 2011).

Here we demonstrate that BRI1 is post-translationally modified by K63 polyubiquitin chains *in vivo*. Artificial ubiquitination of BRI1 negatively regulates its activity by enhanced vacuolar targeting from TGN/EE. Mass spectrometry analyses identify residue K866 of BRI1 as a target of ubiquitination *in vivo*. Loss of BRI1 ubiquitination at residue K866 is associated with subtle BR hypersensitivity, confirming the negative role of BRI1 ubiquitination on BR signaling, and pointing to the existence of other ubiquitination sites in BRI1. Model-based prediction of cell surface-exposed lysine residues in BRI1 allowed the generation of a functional but ubiquitination-defective BRI1. Global loss of BRI1 ubiquitination is associated with major defects in endosomal sorting and vacuolar delivery. In addition, using Total Internal Reflection Fluorescence Microscopy (TIRF), we also shed light on the role of BRI1 ubiquitination in the BRI1 internalization from the cell-surface. Altogether, our results identify K63-linked polyubiquitin chain formation as a dual targeting signal for BRI1 internalization and sorting along the endocytic pathway, and uncover its role in hormonally controlled plant development.

Results

BRI1 receptor is ubiquitinated *in vivo*

Earlier work demonstrated that BRI1 undergoes endocytosis and trafficking to the vacuole, independently of its activation state (Geldner et al., 2007). However, little is known about the mechanisms driving BRI1 protein dynamics in the cell. Ubiquitination of cell-surface receptors in yeast and mammals has been shown to control internalization and/or

vacuolar/lysosomal targeting (MacGurn et al., 2012). To investigate BRI1 ubiquitination and its possible role in BR signaling, we used previously characterized transgenic lines expressing the functional BRI1-mCitrine fusion protein in complemented *bri1* null mutants (Jaillais et al., 2011), or in its isogenic wild-type background (Belkhadir et al., 2012), under the control of *BRI1* promoter to reach endogenous expression level (Supplementary Fig. 1a). We first used anti-GFP antibodies to immunoprecipitate BRI1 from BRI1-mCitrine-expressing plants or wild-type plants as control. Immunoprecipitates from BRI1-mCitrine-expressing plants were enriched in BRI1-mCitrine, as attested by the strong signal observed at the expected size of BRI1-mCitrine fusion protein (~170 kDa; Fig. 1, top left panel). To evaluate if a fraction of BRI1 is post-translationally modified by ubiquitination, immunoprecipitates were probed with the general P4D1 anti-Ubiquitin antibodies that recognizes monoubiquitin and several forms of polyubiquitin chains. A high molecular weight smear, typical of ubiquitinated proteins, was specifically observed from ~170kDa and higher in immunoprecipitates from BRI1-mCitrine plants (Fig.1, top right panel). A comparable high molecular weight smear was observed when using the widely-used Apu3 K63 polyubiquitin chain-specific antibody (Newton et al., 2008), indicating that BRI1 is decorated by K63 polyubiquitin chains *in vivo* (Fig. 1, bottom left panel). In contrast, BRI1 immunoprecipitates showed no signal when probed with a K48 polyubiquitin chain-specific Apu2 antibody (Newton et al., 2008), highlighting the fact that BRI1 does not carry K48-linked polyubiquitin chains (Fig. 1, bottom right panel).

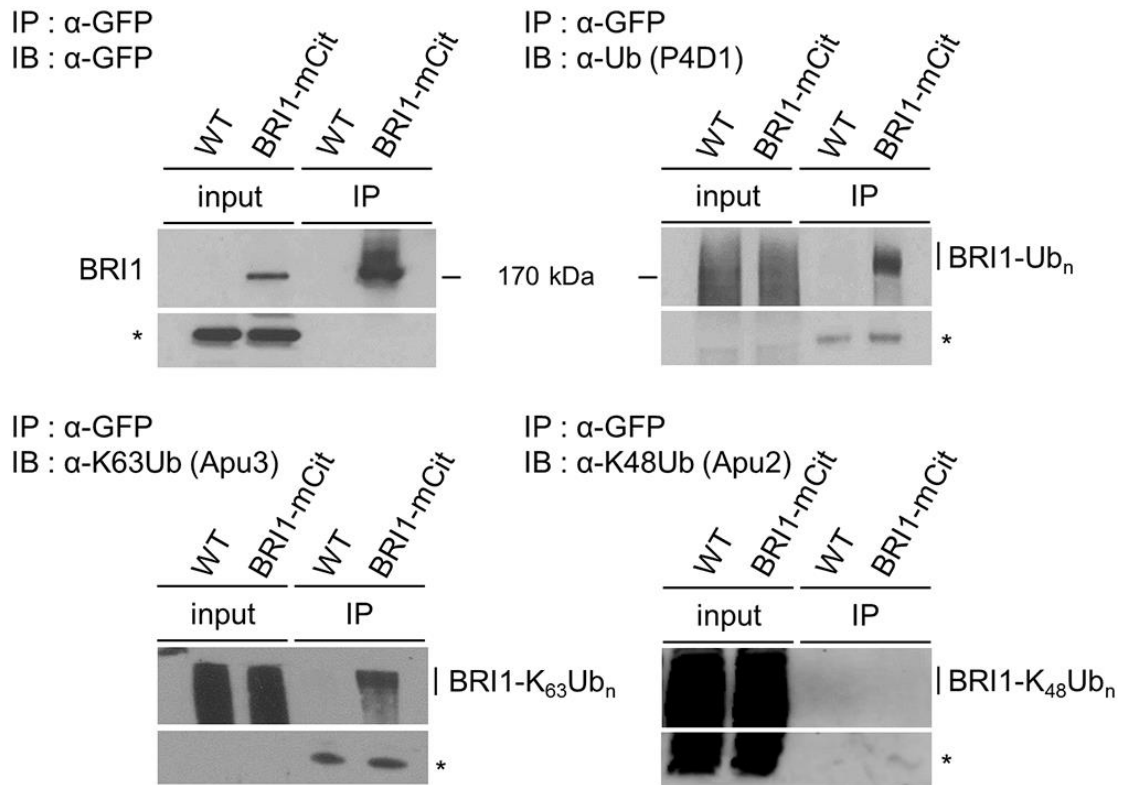


Figure 1. BRI1 carries K63 polyubiquitin chains *in vivo*. *In vivo* ubiquitination analyses of BRI1. Immunoprecipitation was performed using anti-GFP antibodies on solubilized protein extracts from wild-type and mono-insertional homozygous BRI1-mCitrine plants and subjected to immunoblotting with anti-GFP (top left), anti-Ub P4D1 (top right), anti-K63 polyUb Apu3 (bottom left) and anti-K48 polyUb Apu2 antibodies (bottom right). IB, immunoblotting; IP, immunoprecipitation. The asterisk indicates non-specific signals used as loading control.

We next addressed whether ubiquitination of BRI1 was regulated by steroid hormone perception. Challenging BRI1-mCitrine-expressing plants for 1 hour with 1 μ M brassinolide (BL), the most active brassinosteroid form, had no detectable effect on the ubiquitination profile of BRI1-mCitrine (Fig. 2a; Supplementary Fig. 1b). This concentration and duration of treatment is routinely used to monitor BR responses, such as the dephosphorylation of BES1 and BZR1 transcription factors (He et al., 2002; Yin et al., 2002; Mora-Garcia et al., 2004; Vert and Chory, 2006), suggesting that BRI1 ubiquitination does not correlate with ligand binding. It is possible however that in our growth conditions such young growing BRI1-mCitrine plants have close to maximal levels of activated receptors, thus preventing the detection of ligand-dependent changes in BRI1 ubiquitination. Two lines of evidence argue against this hypothesis. First, BRI1-mCitrine plants are fully responsive to exogenously applied BL, as observed by the marked change in BES1 phosphorylation status (Supplementary Fig. 1c), indicating that BR responses are not saturated. Second, growing plants on the BR biosynthetic inhibitor brassinazole (BRZ) to deplete the endogenous pool of BRs prior to a 1 hour-BL treatment had no effect on BRI1 ubiquitination (Supplementary Fig. 1d, 1e). Interestingly, a 15-minute-BL-treatment revealed a modest increase in BRI1 ubiquitination (Supplementary Fig. 1f, 1g). Such a short BL treatment was sufficient to trigger total dephosphorylation of BES1 (Supplementary Fig. 1h), highlighting the fact that BRI1 kinase is activated within minutes after ligand binding. Altogether, these observations indicate that BRI1 ubiquitination appears to be largely independent of ligand-binding with its regulation by BRs restricted to early BR receptor complex activation.

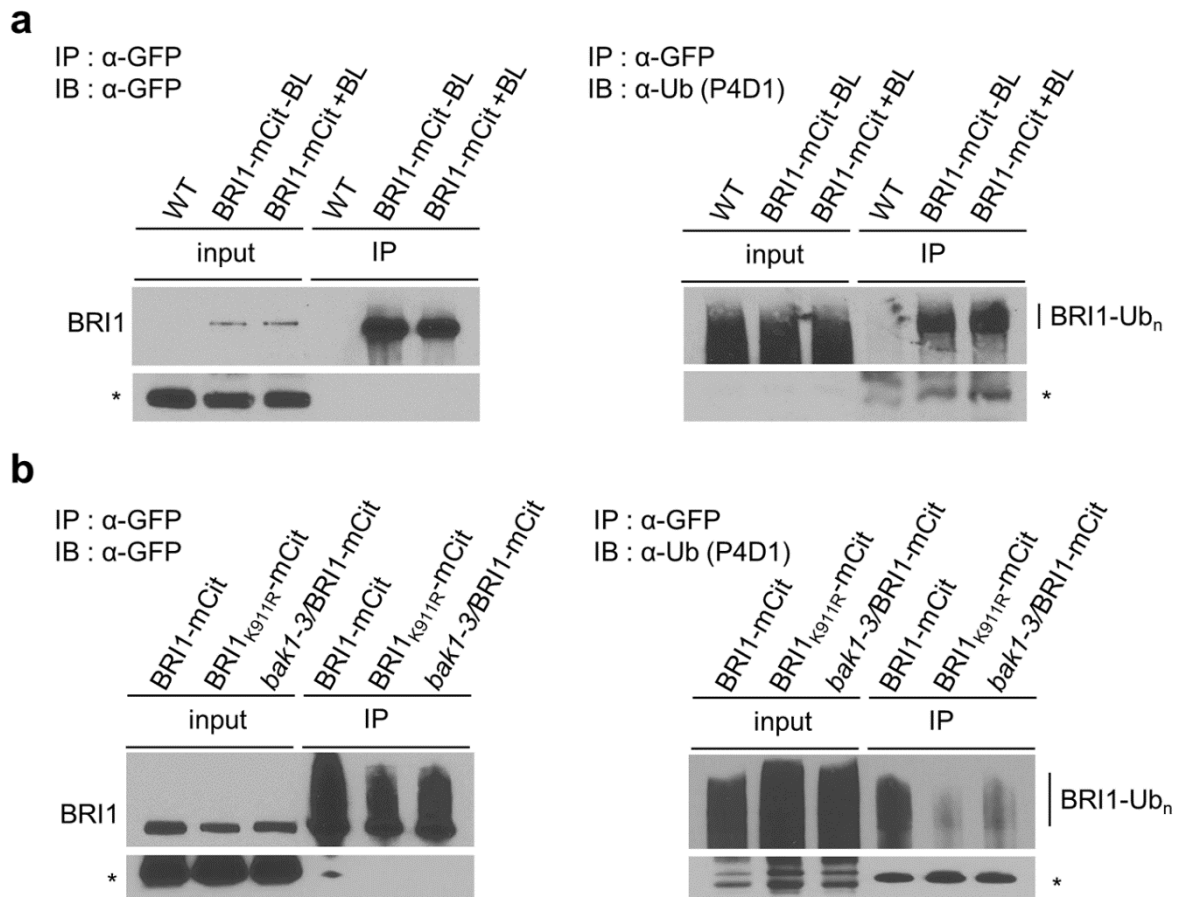


Figure 2. BRI1 is ubiquitinated largely independently of ligand binding. (a) Ligand-dependency of BRI1 ubiquitination. Ubiquitination assays were performed on wild-type and BRI1-mCitrine plants treated with mock (-BL) or 1 μ M BL (+BL) for 1 hour. The asterisk indicates non-specific signals used as loading control. (b) BRI1 ubiquitination in mutants affected in receptor complex activation. Ubiquitination assays were performed on mono-insertional homozygous BRI1-mCitrine, kinase-dead BRI1_{K911R}-mCitrine, and *bak1-3*/BRI1-mCitrine lines. The asterisk indicates non-specific signals used as loading control.

Mechanism of BRI1 ubiquitination

We next investigated the molecular mechanisms driving BRI1 ubiquitination. In particular, we focused on the possible role played by receptor activation, although BRI1 ubiquitination appears to be mostly independent of ligand binding. Lysine residue K911 of BRI1 is an invariant residue in subdomain II of kinases and is critical for BRI1 kinase activity and BR signaling. Consequently, *bri1* mutant expressing the kinase-dead K911R mutation are not complemented and show extreme dwarfism (Wang et al., 2005). Transgenic plants expressing the non-functional BRI1_{K911R} mutant form under the control of *BRI1* promoter were assayed for BRI1 ubiquitination. Care was taken to normalize the immunoprecipitation carried out from BRI1-mCitrine- and BRI1_{K911R}-mCitrine-expressing plants to compensate for difference in transgene expression level. In contrast to what is observed for the wild-type form of BRI1, BRI1_{K911R}-expressing plants showed reduced ubiquitination (Fig. 2b, Supplementary Fig. 1i). This suggests that BRI1 kinase activity is required for BRI1 ubiquitination. The possibility that residue K911 itself is a major ubiquitination site in BRI1 protein is very unlikely since it is not surface-exposed (Supplementary Fig. 5a). Mutation in K911 of BRI1 was shown to drastically affect BRI1-BAK1 receptor complex formation (Wang et al., 2008). We therefore addressed whether the co-receptor BAK1 was also required for BRI1 ubiquitination. To this purpose, we expressed BRI1-mCitrine in a *bak1* null mutant background. *bak1-3*/BRI1-mCitrine showed a reduction in BRI1 ubiquitination compared to BRI1-mCitrine plants, although not statistically significant (Fig. 2b, Supplementary Fig. 1i). The defect in BRI1 ubiquitination observed in *bak1-3* is less severe than what is observed for kinase-dead BRI1, likely due to genetic redundancy with other BAK1 homologs (Gou et al., 2012). The defect of BRI1 ubiquitination observed in genetic backgrounds impaired in receptor complex activation is however not explained by a different subcellular distribution of BRI1 in the cell. BRI1 is indeed found both at the plasma membrane and in endosomal compartments in BRI1-

mCitrine and *bak1-3/BRI1*-mCitrine, as well as in *BRI1_{K911R}*-mCitrine plants although the expression level of kinase-dead *BRI1* is very low (Supplementary Fig. 1j). Altogether, these results indicate that although *BRI1* ubiquitination is largely independent of steroid binding, it requires *BRI1* kinase activity and to a lesser extent its co-receptor *BAK1*, two hallmarks of ligand perception by *BRI1* (Vert et al., 2005).

***BRI1* artificial ubiquitination triggers vacuolar targeting**

Extensive work in yeast and mammals demonstrated the importance of mono- and K63 polyubiquitination for plasma membrane protein internalization, and sorting in MVB (Mukhopadhyay and Riezman, 2007; Lauwers et al., 2010). However, K63 polyubiquitination has also been involved in the regulation of kinases. *TAK1* (transforming growth factor- β activating kinase 1) mediates NF- κ B activation in response to the activation of TGF- β receptor. Upon stimulation with TGF- β , *TAK1* undergoes TRAF6-dependent K63-linked ubiquitination on residue K34. Modification of *TAK1* by ubiquitination is critical for *TAK1* autophosphorylation and subsequent activation (Sorrentino et al., 2008). To get a first glimpse into the biological role of *BRI1* post-translational modification by K63 polyubiquitination, we took a gain-of-function approach where *BRI1*-mCitrine was translationally fused to a single Ub moiety. Although *BRI1* is artificially linked to a single Ub, seven lysine residues are found in Ub including lysine K63 that may engage in K63 polyUb chain formation. We used the complementation of the dwarfism of the *bri1* null T-DNA knock-out mutant (Jaillais et al., 2011), as readout for *BRI1* activity (Supplementary Fig. 2a). As controls, we also generated *bri1/BRI1*-mCitrine, as well as *bri1/BRI1*-mCitrine-Ub_{I44A} carrying a point mutation in a hydrophobic patch necessary for recognition by ubiquitin-binding domain proteins (Hicke et al., 2005). Care was taken at that stage to use transgenic lines expressing similar amounts of RNA for the respective transgenes (Supplementary Fig. 2b). Both

bri1/BRI1-mCitrine and *bri1*/BRI1-mCitrine-Ub_{I44A}, expressing a non-functional ubiquitin, fully complemented the severe dwarfism of *bri1* null mutant (Fig. 3a).

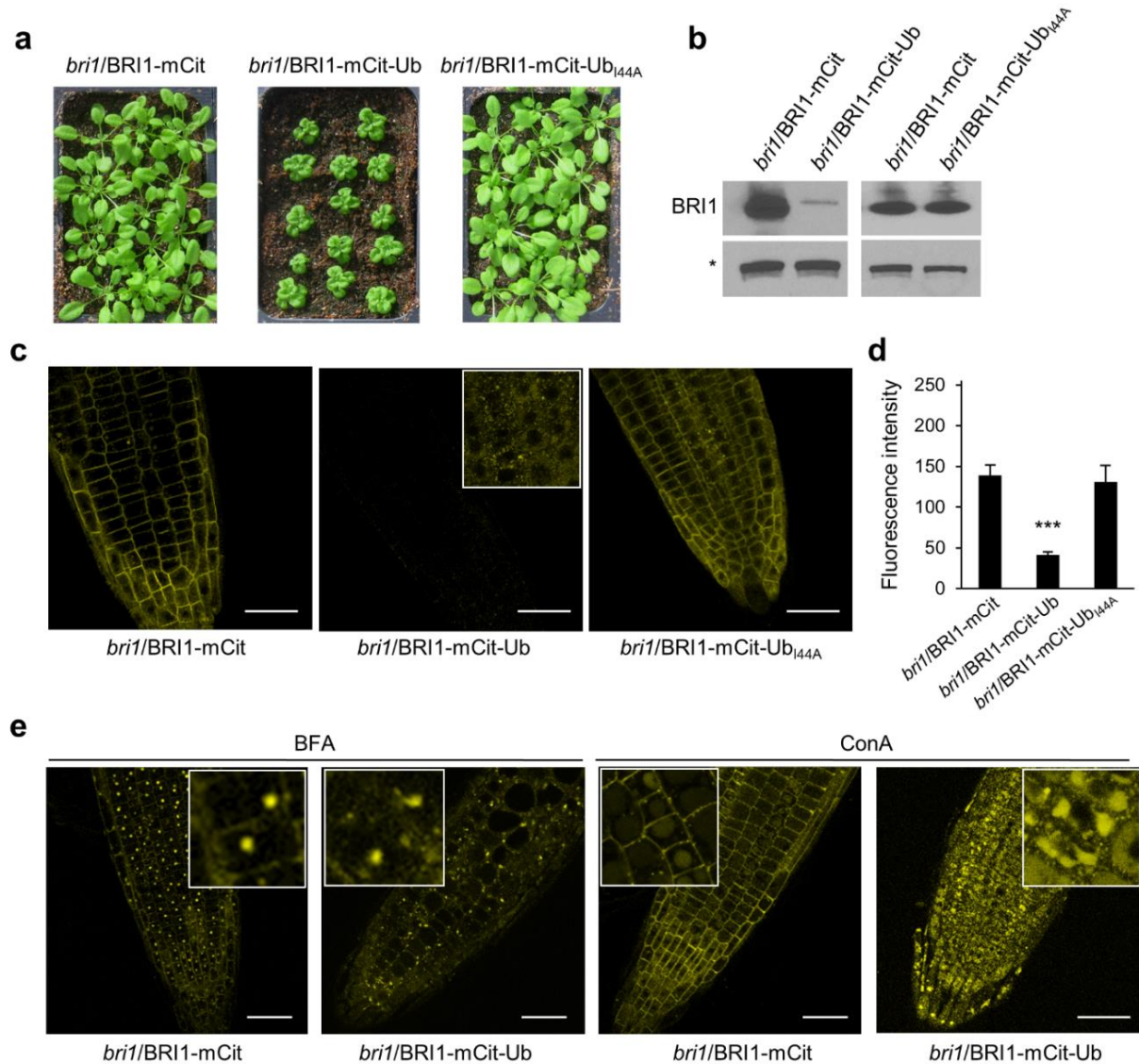


Figure 3. Artificial ubiquitination of BRI1 triggers vacuolar targeting from TGN/EE. (a) Phenotypic analysis of transgenic plants expressing BRI1-mCitrine, BRI1-mCitrine-Ub and BRI1-mCitrine-Ub_{I44A} in the *bri1* null background. All transgenic lines are mono-insertional and homozygous for the transgene. A representative transgenic line is shown for each genotype (b) Western blot analyses monitoring BRI1 protein accumulation in *bri1*/BRI1-mCitrine, *bri1*/BRI1-mCitrine-Ub and *bri1*/BRI1-mCitrine-Ub_{I44A} plants using anti-GFP antibodies. The asterisk indicates non-specific signals used as loading control. (c) Confocal microscopy analyses of *bri1*/BRI1-mCitrine, *bri1*/BRI1-mCitrine-Ub and *bri1*/BRI1-

mCitrine-Ub_{I44A} roots. Similar confocal detection settings were used to compare the different lines. Inset, higher laser power and gain to visualize BRI1-mCitrine-Ub. Scale bars = 50µm. (d) Quantification of the total fluorescence intensity of *bri1*/BRI1-mCitrine, *bri1*/BRI1-mCitrine-Ub and *bri1*/BRI1-mCitrine-Ub_{I44A} roots. Experiments were done in triplicates. Error bars represent standard deviation (n=15). The asterisks indicate a statistically significant difference between *bri1*/BRI1-mCit-Ub and the *bri1*/BRI1-mCit control by Kruskal-Wallis one-way analysis of variance and Dunnett multiple testing (P < 0.0001). (e) Drug sensitivity of *bri1*/BRI1-mCitrine and *bri1*/BRI1-mCitrine-Ub plants. Plants were exposed to BFA (50µM) and ConA (2µM) for 1 hour. Similar confocal detection settings were used to compare the different lines. Inset, higher magnification to visualize the accumulation of BRI1-mCitrine and BRI1-mCitrine-Ub fluorescent proteins in BFA bodies and vacuoles upon BFA and ConA treatment, respectively. Scale bars = 50µm.

In contrast, BRI1-mCitrine-Ub did not complement *bri1* phenotype, indicating that artificial ubiquitination of BRI1 down-regulates its activity. Artificial ubiquitination of BRI1-mCitrine protein led to a strong decrease in total BRI1 protein levels compared to wild-type BRI1-mCitrine and BRI1-mCitrine-Ub_{I44A} (Fig. 3b, Supplementary Fig.2c). Confocal microscopy observations, using similar detection settings to compare the fluorescence levels of the three transgenic lines and to quantify total fluorescence intensity, clearly indicate that BRI1-mCitrine-Ub fails to accumulate in the cell (Fig. 3c, 3d). Higher laser intensity and gain however allowed detection of close to background levels of BRI1-mCitrine-Ub as punctate pattern, with little or no accumulation at the plasma membrane (Fig. 3c, inset). These observations point out that ubiquitination of BRI1 is sufficient to trigger its degradation.

To investigate deeper the mechanisms driving the loss of BRI1-mCitrine-Ub protein, we took advantage of drugs interfering with vesicular trafficking. The fungal toxin Brefeldin A (BFA) is a widely used inhibitor of endosomal trafficking, creating large aggregates of *trans*-Golgi network/early endosomal compartments in the Arabidopsis root. Although BRI1-mCitrine-Ub failed to accumulate under normal conditions, BRI1-mCitrine-Ub levels rapidly built up in BFA bodies after BFA treatment to reach the levels observed for BRI1-mCitrine (Fig. 3e, Supplementary Fig. 3a). This suggests that BRI1-mCitrine-Ub is translated, exits the endoplasmic reticulum and passes through the TGN/EE on its way to degradation. We next assessed the influence of the vacuolar ATPase inhibitor Concanamycin A (ConA), which prevents the degradation of proteins targeted to the vacuole (Takano et al., 2005). ConA treatment led to the dramatic accumulation of BRI1-mCitrine-Ub in the vacuole (Fig. 3e, Supplementary Fig. 3b), compared to BRI1-mCitrine, thus revealing enhanced vacuolar delivery triggered by ubiquitin. This indicates that artificial ubiquitination of BRI1-mCitrine is sufficient for vacuolar targeting, consistent with what has been recently observed for other plant cargos (Herberth et al., 2012; Leitner et al., 2012; Scheuring et al., 2012). We next

investigated whether BRI1-mCitrine-Ub was trafficking *via* the plasma membrane on its way to the vacuole by inhibiting clathrin-mediated endocytosis, the major endocytic road in plants (Dhonukshe et al., 2007). Treatment with the inhibitor of clathrin-mediated endocytosis TyrA23 was ineffective at stabilizing BRI1-mCitrine-Ub at the cell surface, but rather led to a cytosolic-like pattern of fluorescence (Supplementary Fig. 3c). In contrast, TyrA23 prevented the formation of BFA bodies in BRI1-mCitrine plants (Supplementary Fig. 3d), attesting that TyrA23 treatment was active. Taken together, these results indicate that artificial ubiquitination is recognized before reaching the plasma membrane, likely at the TGN/EE, and serves as a targeting signal for degradation in the vacuole.

To discriminate further the form of Ub associated with vacuolar targeting, we generated *bri1*/BRI1-mCitrine-Ub_{7KR} plants where all seven lysines from Ub were mutated to arginines to prevent K63 polyubiquitin chain formation. Confocal microscopy observations of *bri1*/BRI1-mCitrine and *bri1*/BRI1-mCitrine-Ub_{7KR} using the same detection settings showed that artificial monoubiquitination of BRI1 also impairs BRI1 protein accumulation (Supplementary Fig. 3e, 3f). Monoubiquitination of BRI1 therefore appears sufficient to trigger its vacuolar delivery, similarly to what has been reported for the plant ATPase PMA or the yeast polyphosphate endophosphatase Phm5 (Reggiori and Pelham, 2001; Herberth et al., 2012).

BRI1 is ubiquitinated at residue K866 *in vivo*

To identify ubiquitination sites in BRI1, we performed immunoprecipitation of BRI1 in the presence of deubiquitinase inhibitors followed by mass spectrometry analyses. Immunopurified BRI1-mCitrine was digested by trypsin and subjected to liquid-chromatography tandem mass spectrometry analyses (LC-MS/MS). Multidimensional Protein Identification Technology (MudPIT) analyses on BRI1-mCitrine immunoprecipitates detected

9 BRI1 peptides, including the EALSINLAAFEK^{GG}PLR peptide modified by the tryptic ubiquitin remnant G-G isopeptide on the lysine residue K866. This residue lies on the cytosolic face of BRI1 protein, in the juxtamembrane domain (Fig. 4a). To examine the biological role of K866 ubiquitination, we generated transgenic plants expressing BRI1 where residue K866 is substituted by the positively charged but non-ubiquitinatable R residue (BRI1_{K866R}), under the control of its own promoter in *bri1* background. *bri1*/BRI1_{K866R}-mCitrine transgenic plants expressing similar BRI1-mCitrine levels than *bri1*/BRI1-mCitrine showed no macroscopic phenotypes at the adult stage (Fig. 4b). To better characterize BRI1_{K866R}-expressing plants, we monitored two well-established readouts of BR signaling such as the phosphorylation state of BES1 and hypocotyl length. Compared to plants expressing wild-type BRI1, BRI1_{K866R}-expressing plants reproducibly showed more dephosphorylated BES1 (Fig. 4c; Supplementary Fig. 4a) and longer hypocotyls when grown under both normal conditions and BRZ (Fig. 4d). These observations suggest that expression of BRI1_{K866R} leads to a mild activation of BR signaling. No striking difference on the localization and the levels of BRI1_{K866R}-mCitrine protein could be observed (Fig. 4e). Altogether, ubiquitination of residue K866 appears to play a negative role on BRI1 activity. The fact that *bri1*/BRI1_{K866R}-mCitrine transgenic plants only show a slight activation of BR signaling compared to what has been reported for BRI1 overexpressing plants (Belkadir et al., 2012), and still harbors massive ubiquitination (Supplementary Fig. 4b, 4c) suggests that many more ubiquitination sites exist in BRI1.

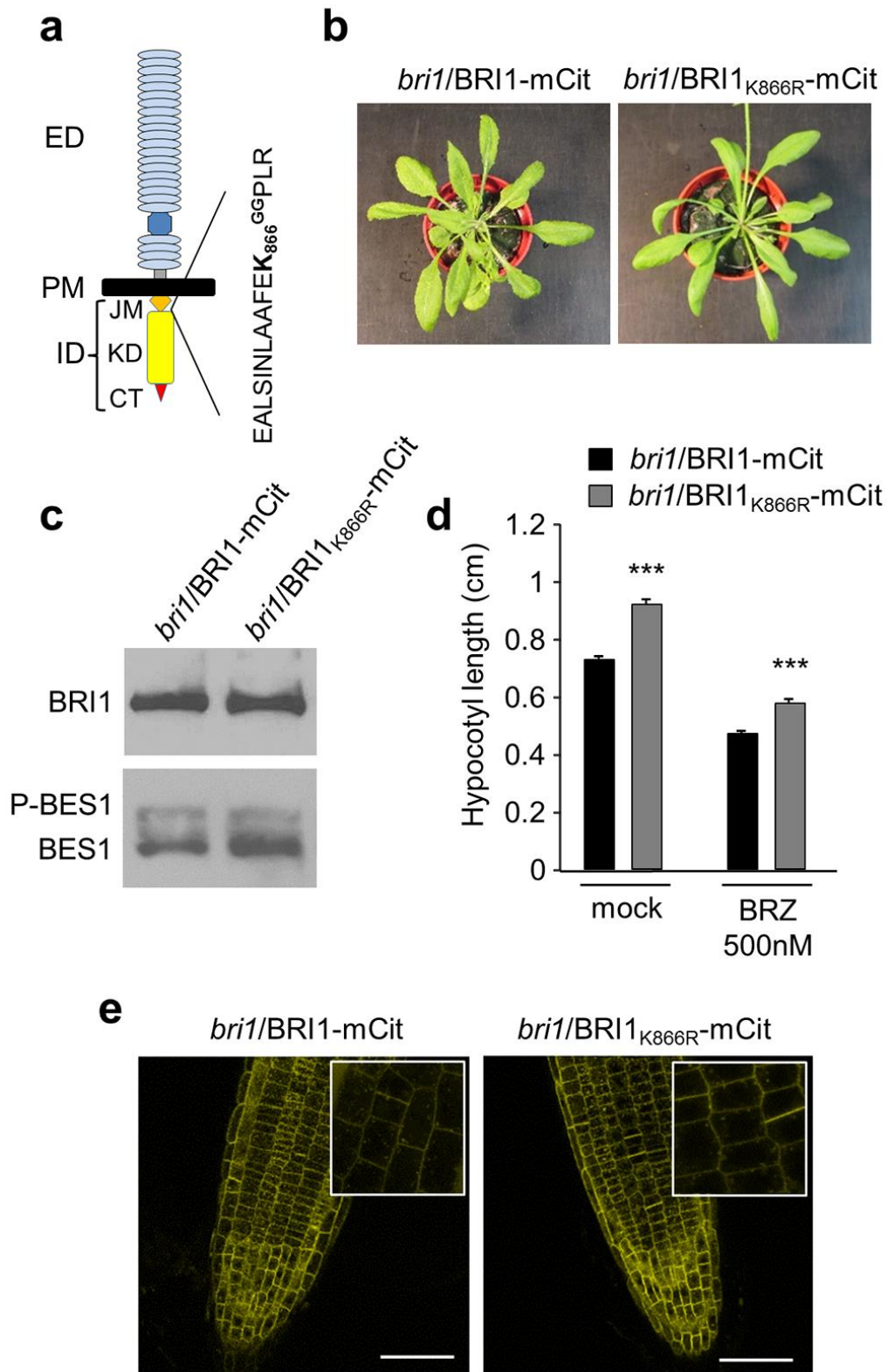


Figure 4. Ubiquitination of residue K866 negatively regulates BRI1. (a) Identification of *in vivo* ubiquitination sites in BRI1. ED, extracellular domain; PM, plasma membrane, ID, intracellular domain; JM, juxtamembrane domain; KD, kinase domain; CT, C-terminal domain. The ubiquitinated peptide carrying the GG di-glycine signature on K866 is shown.

(b) Phenotypic analysis of 4-week-old mono-insertional homozygous *bri1/BRI1*-mCitrine and *bri1/BRI1_{K866R}*-mCitrine plants. A representative transgenic line is shown for both genotypes **(c)** Western blot analyses on *bri1/BRI1*-mCitrine and *bri1/BRI1_{K866R}*-mCitrine plants. Protein levels were detected with anti-GFP and anti-BES1 antibodies, respectively. **(d)** Average hypocotyl lengths of 3-day-old etiolated *bri1/BRI1*-mCitrine and *bri1/BRI1_{K866R}*-mCitrine seedlings. Experiments were done in triplicates. Error bars indicate standard deviation (n=15). The asterisks indicate a statistically significant difference between *bri1/BRI1*-mCit and the *bri1/BRI1_{K866R}*-mCit control (Mann-Whitney, $P < 0.0001$). **(e)** Confocal microscopy analyses of *bri1/BRI1*-mCitrine and *bri1/BRI1_{K866R}*-mCitrine roots. Similar confocal detection settings were used to compare the two representative transgenic lines. Inset, higher magnification. Scale bars = 50 μ m.

Loss of BRI1 ubiquitination impacts endocytosis and sorting

The intracellular domain of BRI1 contains 29 lysine residues. Sequence alignment of BRI1 with i) the BRI1-like homologs from Arabidopsis, ii) BRI1 from other plant species, iii) Arabidopsis receptor kinases belonging to different subfamilies such as FLS2 and CLV1 and iv) human IRAK4 indicate that four lysines from BRI1 kinase are highly conserved in all kinases analyzed. These include the ATP-binding pocket-located K899, K911 and K912, and residue K1011 in the catalytic loop, that are mostly buried in the recently resolved BRI1 kinase structure (Bojar et al., 2014) (Supplementary Fig. 5a), and are likely critical for kinase activity since highly conserved. To pinpoint the role of BRI1 ubiquitination, we sought to generate an ubiquitination-defective BRI1 that retains its kinase activity. We therefore mutated 25 lysines to arginines in BRI1 intracellular domain, and kept only the four strictly conserved lysine residues K899, K911, K912 and K1011. Expression of the resulting BRI1_{25KR} mutants under the control of *BRI1* promoter complemented the dwarf phenotype of *bri1* null mutant (Fig. 5a). This result indicates that BRI1_{25KR}-mCitrine was still functional and that none of these 25 lysines are required for receptor activation. BRI1_{25KR}-mCitrine-expressing plants were severely compromised for BRI1 ubiquitination compared to BRI1-mCitrine (Supplementary Fig. 5b, 5c). Interestingly, BRI1_{25KR}-expressing plants showed long bending petioles and narrow leaf blades (Fig. 5a, 5b; Supplementary Fig. 5d), reminiscent of what is observed in BRI1-overexpressors (Belkhadir et al., 2012). This correlates with increased accumulation of dephosphorylated BES1 which positively regulates BR signaling (Vert et al., 2005) (Fig. 5c, 5d). The BR hypersensitivity phenotype of BRI1_{25KR} is observed for plants that express less BRI1 proteins than *bri1*/BRI1-mCitrine plants (Fig. 5c, 5d), highlighting further the negative role played by ubiquitination on BRI1 activity.

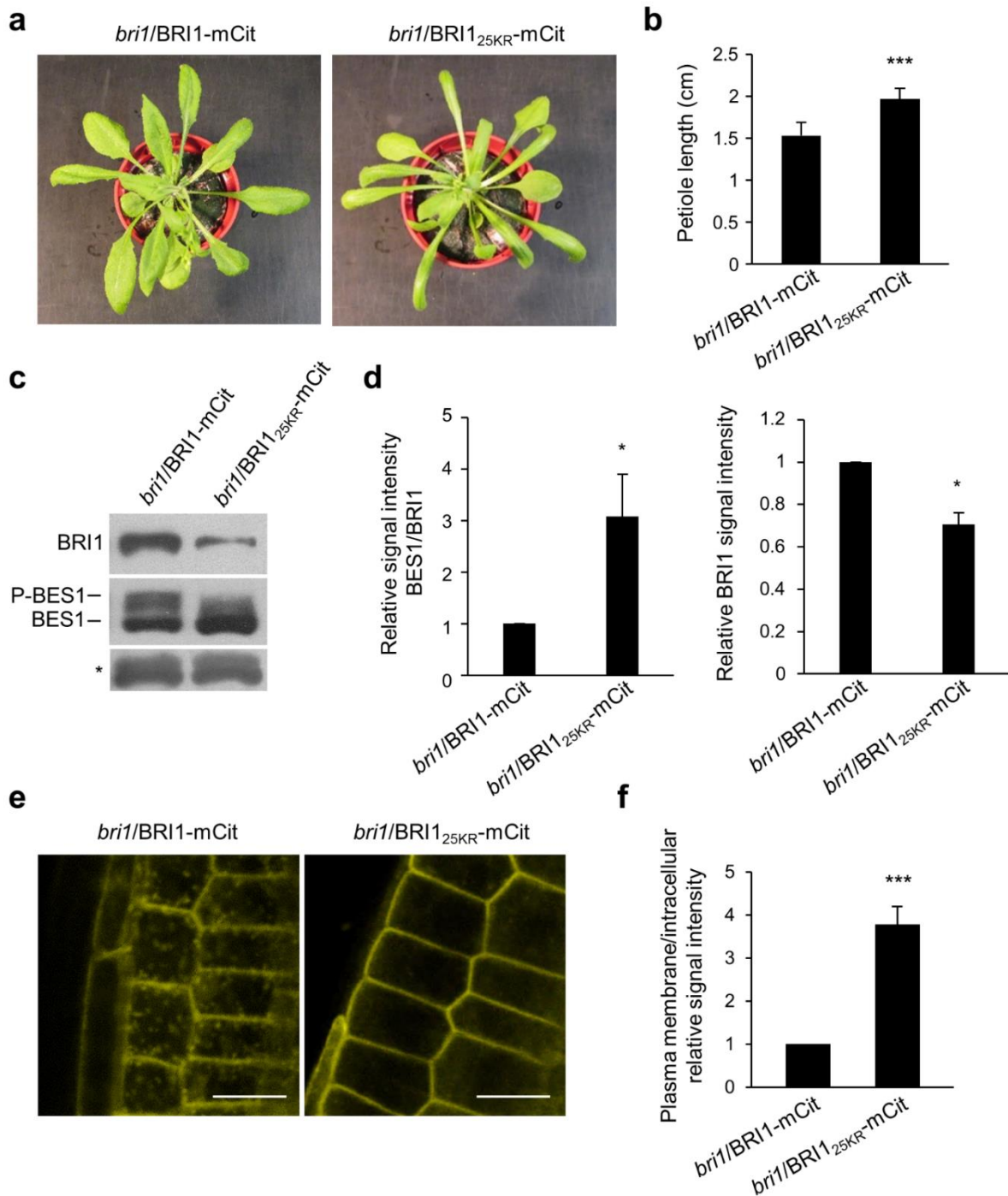


Figure 5. BRI1 ubiquitination controls brassinosteroid signaling and BRI1 localization.

(a) Phenotypic analysis of 4-week-old *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* mono-insertional homozygous plants. A representative transgenic line is shown for both genotypes (b) Average petiole lengths of the fourth true leaf from 4-week-old *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* plants. Experiments were done in triplicates. Error bars represent standard deviation (n=20). The asterisk indicates a statistically significant difference between *bri1/BRI1-mCit* and the *bri1/BRI1_{25KR}-mCit* control (Mann-Whitney, P < 0.0001). (c) Western blot analyses on *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* plants using

anti-GFP and anti-BES1 antibodies. The asterisk indicates non-specific signals used as loading control. **(d)** Quantification of dephosphorylated BES1 (BES1, left) and BRI1 (right) protein levels in *bri/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit*. Experiments were done in triplicates. Error bars represent standard deviation (n=3). The asterisk indicates a statistically significant difference in phosphorylated BES1 and BRI1 levels between *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* (Mann-Whitney, $P < 0.05$). **(e)** Confocal microscopy analyses of *bri1/BRI1-mCitrine* and *bri1/BRI1_{25KR}-mCitrine* roots. Scale bars = 7 μ m. **(f)** Quantification of the ratio between plasma membrane and intracellular fluorescence signal intensities of *BRI1-mCitrine* and *BRI1_{25KR}-mCitrine*. Experiments were done in triplicates. Error bars represent standard deviation (n=15). The asterisk indicates a statistically significant difference between *bri1/BRI1-mCit* and the *bri1/BRI1_{25KR}-mCit* (Mann-Whitney, $P < 0.0005$).

BRI1 protein is found under normal conditions at the plasma membrane, but also in endosomal compartments (TGN/EE and MVB) (Fig. 5e) (Geldner et al., 2007; Jaillais et al., 2008). In contrast, BRI1_{25KR}-mCitrine was found mostly at the cell-surface, with little intracellular compartments observed (Fig. 5e, 5f). To unravel the mechanism leading the different intracellular distribution of BRI1_{25KR}-mCitrine, we compared the endocytic trafficking of BRI1-mCitrine and BRI1_{25KR}-mCitrine. BFA inhibits the trafficking of membrane proteins from TGN/EE to MVB and recycling to the plasma membrane, but allows endocytosis. Both BRI1-mCitrine and BRI1_{25KR}-mCitrine accumulated in BFA compartments in absence of *de novo* protein synthesis (Fig. 6a, 6b), although the size of BFA bodies appeared smaller for BRI1_{25KR}-mCitrine (Fig. 6c). This suggests that BRI1_{25KR}-mCitrine undergoes internalization from the plasma membrane, but presumably to a lesser extent than its wild-type counterpart so that less BRI1 proteins is trapped in BFA bodies. To obtain more resolution and quantitative data on the dynamics of BRI1 and BRI1_{25KR} at the cell surface, we implemented Total Internal Reflection Fluorescence microscopy (TIRF). TIRF generates high contrast images and allows studying the dynamic behavior of proteins at the plasma membrane. We were unable to perform reproducible TIRF imaging at the root tip likely due to the ovoid shape of the root tip and the presence of root hairs in root samples preventing good contacting with the coverslip, a prerequisite for TIRF imaging. Hypocotyls from etiolated seedlings have flat cells suited with TIRF imaging and their elongation strongly depends on BR perception by BRI1 (Vert et al., 2005). BRI1-mCitrine localized to the plasma membrane in etiolated hypocotyl cells, but the TGN/EE pool of BRI1 was difficult to observe due to the large central vacuole (Supplementary Fig. 6a). BRI1 trafficking was found to be sensitive to BFA in etiolated hypocotyl cells, as highlighted by the intracellular aggregation of BRI1-mCitrine upon BFA treatment. Finally, artificially ubiquitinated BRI1 failed to accumulate in hypocotyl cells. Altogether, this indicates that BRI1 trafficking in etiolated

hypocotyl cells mirrors what has been described in roots. BRI1-mCitrine-expressing plants were therefore imaged by TIRF microscopy over time and showed small diffraction-limited fluorescent spots (Supplementary Movies 1, 2). These spots were immobile on the (x,y) axis but dynamically appeared and disappeared from the focal plane, thus representing fusion and fission events at the cell surface. A similar pattern was observed for BRI1_{25KR}-mCitrine, although the density of spots at the cell surface was higher (Supplementary Fig. 6b). To grasp the role of ubiquitination in BRI1 internalization from the plasma membrane, we further analyzed the dynamic behavior of wild-type BRI1 and BRI1_{25KR} by kymograph analysis. Kymographs monitor on a single image the intensity at a given (x,y) location over time, and allow determination of surface time residence for wild-type BRI1 and BRI1_{25KR} by measuring track length (Fig. 6d). The time of residency of BRI1-mCitrine at the cell surface was widely distributed (Fig. 6e), but over 60% of the spots resided less than 10s at the plasma membrane. The distribution observed for BRI1_{25KR} was markedly different, with most spots persisting longer at the plasma membrane. The surface residence time median values for BRI1 and BRI1_{25KR} were 7.2s and 28.6s, respectively (Supplementary Fig. 6c). These observations clearly argue for a direct role of BRI1 ubiquitination in internalization from the plasma membrane.

We next monitored the role of ubiquitination in BRI1 vacuolar targeting. BRI1-mCitrine and BRI1_{25KR}-mCitrine were transferred to dark conditions, which impair vacuolar lytic activity and allow visualization of the pH-resistant mCitrine fluorescent fusion proteins targeted to the vacuole (Tamura et al., 2003), and monitored by confocal microscopy. Consistently, BRI1-mCitrine showed vacuolar accumulation after 2 hours of darkness (Fig. 6f, 6g). However, BRI1_{25KR}-mCitrine exhibited no vacuolar targeting within the same time frame. Consistently, the ubiquitination-defective BRI1_{25KR} is more stable than wild-type BRI1 (Supplementary Fig. 6d, 6e). The inability of BRI1_{25KR} to reach the vacuole combined to the increased cell surface

accumulation suggest that BRI1_{25KR} undergoes enhanced recycling to the cell surface. Endocytosed BRI1 protein was previously shown to recycle to the cell surface by BFA washout experiments (Wang et al., 2013), in which the recovery of BRI1 at the plasma membrane is monitored after BFA removal in the absence of protein synthesis. We confirmed that BRI1 both recycles at the plasma membrane and is also targeted to the vacuole after BFA washout, and observed that BRI1_{25KR} recycles more to the cell surface (Supplementary Fig. 6f, 6g). Altogether, our results unravel the dual role of BRI1 ubiquitination in BRI1 receptor internalization from the plasma membrane and also in its endosomal sorting and vacuolar targeting.

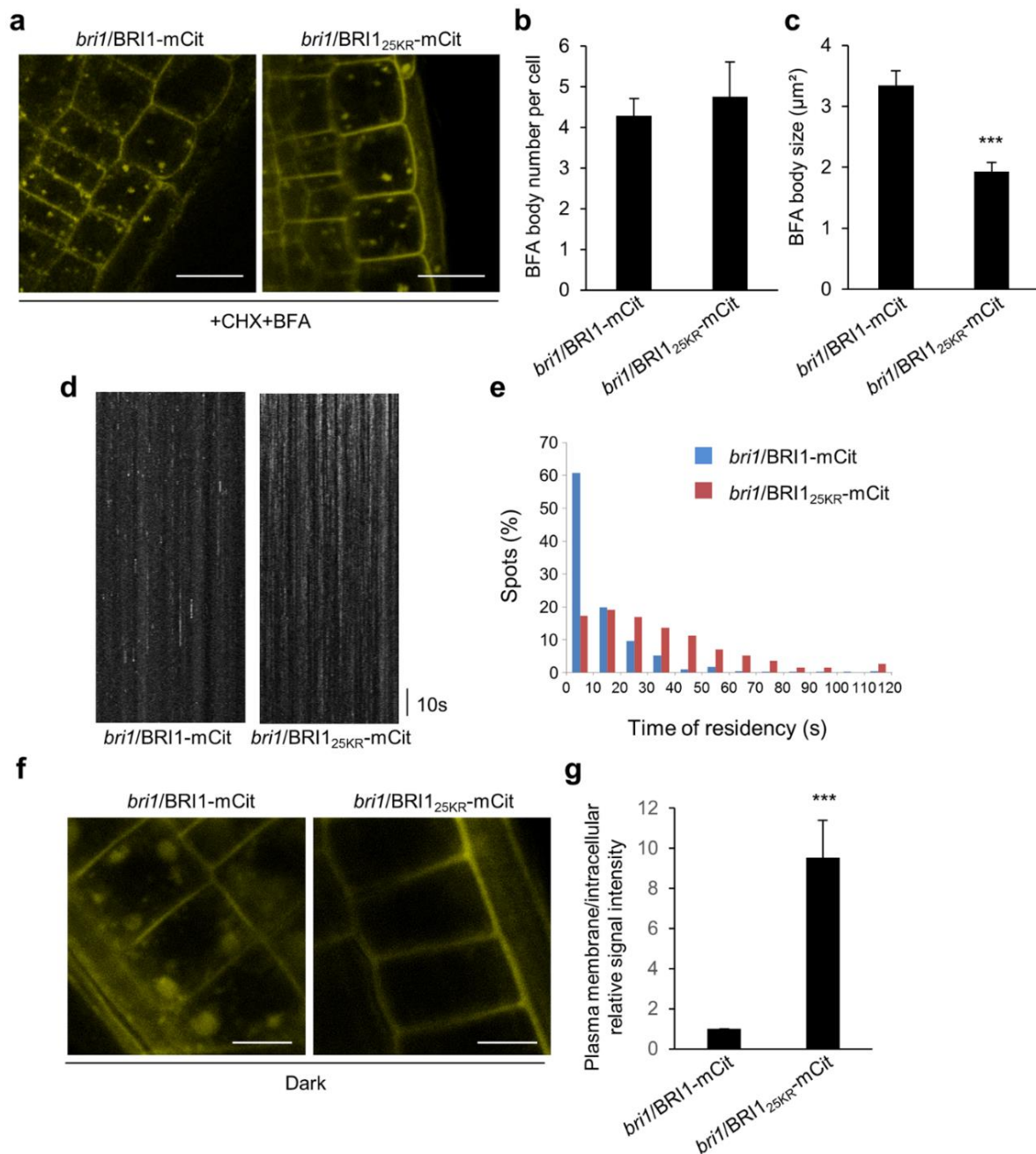


Figure 6. Loss of BRI1 ubiquitination impairs its internalization and vacuolar targeting.

(a) Sensitivity of *bri1/BRI1-mCitrine* and *bri1/BRI1_{25KR}-mCitrine* plants to BFA. Plants were pretreated with 100 μM CHX for 1 hour and exposed to 100 μM CHX and 50 μM BFA for 1 hour. Scale bars = 7 μm . (b) Quantification of BFA body number in *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* roots. Experiments were done in triplicates. Error bars represent standard deviation (n=15). No statistical difference if observed between *bri1/BRI1-mCit* and the *bri1/BRI1_{25KR}-mCit* (Mann-Whitney, $P > 0.05$). (c) Quantification of BFA body size in *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* roots. Experiments were done in triplicates. Error bars represent standard deviation (n=15). The asterisk indicates a statistically significant

difference between the two genotypes (Mann-Whitney, $P < 0.0001$). **(d)** Kymograph analysis from TIRF movies of *bri1*/BRI1-mCitrine and *bri1*/BRI1_{25KR}-mCitrine. The time scale is shown. **(e)** Time of residency at the plasma membrane of *bri1*/BRI1-mCitrine and *bri1*/BRI1_{25KR}-mCitrine. The distribution was obtained from kymograph-based track length analyses (n=350). **(f)** Sensitivity of BRI1-mCitrine and BRI1_{25KR}-mCitrine to dark growth conditions. Light-grown seedlings were kept in the dark for 2 hours before confocal imaging. Scale bars = 20 μ m. **(g)** Quantification of the ratio between relative plasma membrane and intracellular fluorescence signal intensities of BRI1-mCitrine and BRI1_{25KR}-mCitrine. Experiments were done in triplicates. Error bars represent standard deviation (n=15). The asterisk indicates a statistically significant difference between BRI1-mCit and the BRI1_{25KR}-mCit (Mann-Whitney, $P < 0.0001$).

Discussion

The BR receptor BRI1 is the best-studied plant receptor and serves as the archetypical receptor to study the activation/deactivation mechanisms involved in receptor-mediated signaling. Previous studies have shed light on the ligand-dependent oligomerization and phosphorylation of the BRI1/BAK1 receptor complex and have identified components of the BR signaling pathway controlling BR genomic responses (Zhu et al., 2013). However, not much is known about the mechanisms driving the trafficking of BRI1 and its turnover in relationship with signaling. In this report, we show that BRI1 dynamics in the cell is controlled by ubiquitination and unravel its role for BR-mediated plant growth.

Ubiquitination of proteins has been associated with many different cellular outputs in yeast and mammals, depending on the type of ubiquitination (Mukhopadhyay and Riezman, 2007). In the case of membrane proteins, ubiquitination triggers endocytosis and vacuolar/lysosomal degradation (MacGurn et al., 2012). In plants, only a handful of integral membrane proteins have been shown to undergo Ub-mediated endocytosis. These examples are so far exclusively restricted to multispan transporters involved in iron, boron or phosphate nutrition or in auxin transport (Barberon et al., 2011; Kasai et al., 2011; Leitner et al., 2012; Lin et al., 2013). Both monoubiquitination and K63 polyubiquitination has been implicated in these examples, consistent with their described roles in yeast and mammals. Ubiquitination of plant receptors is still poorly documented, although several E3-Ub ligases interacting with receptors have been identified (Gu et al., 1998; Kim et al., 2003; Wang et al., 2006; Samuel et al., 2008). Only the Flagellin receptor FLS2 involved in plant immune responses have been shown to be ubiquitinated (Lu et al., 2011). However, FLS2 carries K48 polyubiquitin chains and has been shown to be degraded by the proteasome.

In this study, we first demonstrated using a gain-of-function approach that the artificial ubiquitination of BRI1 is sufficient for destabilizing the corresponding protein. Artificial

monoubiquitination of BRI1 appeared sufficient to prevent accumulation of BRI1. Destabilization of BRI1-mCitrine-Ub protein involves trafficking through the TGN/EE and degradation in the vacuole, based on the strong accumulation of BRI1-mCitrine-Ub following treatments with BFA and ConA. Inhibition of clathrin-mediated endocytosis, the major BRI1 endocytic route (Irani et al., 2012; Di Rubbo et al., 2013), with TyrA23 failed to stabilize BRI1-mCitrine-Ub at the cell-surface, suggesting that the artificially ubiquitinated BRI1 variant never reaches the plasma membrane. Formally, artificially ubiquitinated BRI1 could be rapidly endocytosed from the plasma membrane using a clathrin-independent TyrA23-insensitive pathway. However, we favor the hypothesis that BRI1-mCitrine-Ub is recognized at the TGN/EE and diverted away from the secretory pathway before it reaches the cell surface, being directly routed to the vacuole for degradation. In yeast and mammals, GGA proteins at the TGN work to divert ubiquitinated cargos from the secretory pathway towards endosomes, preventing them from reaching the cell surface (Scott et al., 2004). Late endocytic sorting of ubiquitinated cargos in intraluminal vesicles of MVB and vacuolar/lysosomal targeting require the late endosome-located ESCRT complex (MacGurn et al., 2012). In plants, the TOL GGA-related proteins and several subunits of the ESCRT complex have been recently detected in TGN/EE (Scheuring et al., 2011; Korbei et al., 2013), indicating that the sorting of artificially ubiquitinated BRI1 indeed occurs at the TGN/EE.

We demonstrated that BRI1 is K63 polyubiquitinated *in vivo*, and does not carry K48 polyubiquitin chains. The combination of ubiquitination site identification by mass spectrometry and model-based prediction allowed us to generate a functional ubiquitination-defective BRI1_{25KR} mutant form. In contrast to wild-type BRI1 that shows significant endosomal localization (Geldner et al., 2007), BRI1_{25KR} was mostly found at the cell surface. To decipher whether the increased plasma membrane localization resulted from internalization defects and/or intracellular sorting defects, we implemented TIRF microscopy

to specifically monitor BRI1 dynamics at the cell surface. Such analysis first highlighted the highly dynamic behavior of wild-type BRI1, with extremely rapid fusion and fission events within the seconds range. Interestingly, BRI1_{25KR} shows much slower fusion/fission events at the plasma membrane, within the tens of second range, indicating that ubiquitination of BRI1 triggers its internalization from the cell surface. Detailed analysis of BRI1_{25KR} trafficking also highlighted the crucial role of ubiquitination in BRI1 intracellular sorting and vacuolar targeting. Overall, ubiquitination of BRI1 therefore appears as one several redundant mechanisms driving BRI1 internalizing from the plasma membrane, whereas it is absolutely essential for BRI1 endosomal sorting (Fig. 7). The combination of both slower internalization and forced recycling caused by vacuolar targeting defects are therefore the basis of increased BRI1_{25KR} at the cell surface and increased spot density. This scenario is reminiscent of what is currently debated for EGFR in animals. Mutation of 15 ubiquitin conjugation sites found in the EGFR kinase domain or the Cbl E3 ligase-binding site does not affect EGFR internalization, although preventing lysosomal degradation (Huang et al., 2007). EGFR internalization from the cell surface is impaired only when 21 lysine residues in the kinase domain and C-terminal domain are mutated, although the three distal lysines in the C-terminal domain appeared to be acetylated *in vivo* (Goh et al., 2010). EGFR internalization is dependent on many redundant lysine residues and involves several redundant and cooperative mechanisms (Goh et al., 2010). Altogether, although artificial monoubiquitination of several cargos is sufficient for endocytosis, the presence of K63 polyubiquitin chains appears either critical for intracellular sorting or likely accelerates this process by increasing the avidity for Ub-binding proteins (Galan and Haguenaer-Tsapis, 1997; Blondel et al., 2004; Huang et al., 2006; Paiva et al., 2009; Lauwers et al., 2010; Huang et al., 2013). Similar observations likely applies to BRI1 Ub-mediated endocytosis.

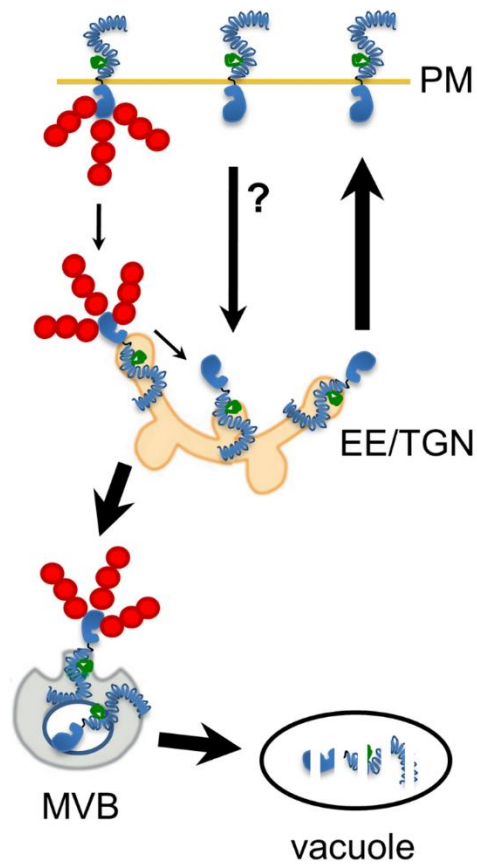


Figure 7. Model for the role of ubiquitination in BRI1 endocytosis. Ubiquitination of BRI1 acts at the cell surface, redundantly with other mechanisms, to mediate BRI1 internalization. Internalized BRI1 may be deubiquitinated and recycled to the plasma membrane, or sorted into late endosomal compartments for vacuolar delivery. Ubiquitination of BRI1 is essential for proper sorting and targeting to the vacuole.

Modification of BRI1 with Ub negatively regulates BRI1 function and BR signaling, as attested by the BR-hypersensitive phenotypes displayed by transgenic plants impaired in BRI1 ubiquitination. BRI1 ubiquitination is however largely independent of ligand binding, with only a transient increase in ubiquitination observed after a short term BL treatment. This is consistent with previous observations showing that BRI1 turnover is only mildly modulated by the presence or absence of BRs (Geldner et al., 2007). BRI1 ubiquitination however required BRI1 kinase activity and the presence of its co-receptor BAK1, both mechanisms being dependent on BRs. One explanation is that in the absence of steroids, BRI1 kinase shows basal kinase activity sufficient for triggering phosphorylation of important residues for ubiquitination in BRI1 but not involved in signaling. The requirement for BAK1 is puzzling considering the BR-dependency of BRI1/BAK1 interaction. However, recent evidence based on FRET-FLIM analyses indicates that significant proportion of BRI1 and BAK1 interact at the cell surface even in the absence of ligand (Bucherl et al., 2013). These different observations clarify how BRI1 ubiquitination may rely on hallmarks of BR receptor complex activation, although independently of ligand perception. The ability of BRI1 to signal from endosomes has been highly debated recently (Geldner et al., 2007). The increased plasma membrane localization of BRI1_{25KR}, associated to enhanced BR signaling, confirms recent evidence pointing to the initiation of BR signaling at the cell surface (Irani et al., 2012; Di Rubbo et al., 2013). BRI1 ubiquitination appears as a mechanism controlling the dynamics of BRI1 in the cell and thus impact on BR signaling, although in a BR-independent manner. Whether endogenous/exogenous cues may impact on BRI1 ubiquitination and thus affect BR-dependent growth will have to be investigated in the future.

Methods

Plant material and constructs

Wild-type (Col0), *bak1-3* (Chinchilla et al., 2007), *bri1* T-DNA knock-out (Jaillais et al., 2011) and the various transgenic plants generated in this study were grown at 21 °C with 16h light/8h dark cycles. The transgenic lines BRI1::BRI1-mCitrine, *bri1*/BRI1::BRI1-mCitrine, *bak1-3*/BRI1::BRI1-mCitrine and kinase-dead BRI1::BRI1_{K911R}-mCitrine express BRI1 under the control of its own promoter and were previously characterized (Jaillais et al., 2011; Belkhadir et al., 2012).

mCitrine-Ub and mCitrine-Ub_{7KR} fusions were cloned into pDONR-P2RP3 (Invitrogen). BRI1_{K866R} and BRI1_{25KR} were cloned in pDONR221 (Invitrogen). Final destination vectors were obtained by using three fragments recombination system using the pB7m34GW destination vectors (Karimi et al., 2007), and the entry vectors pDONR-P4P1r-BRI1prom (Jaillais et al., 2011), pDONR221-BRI1 or mutated BRI1 versions, and pDONR-P2rP3-mCitrine (Jaillais et al., 2011), mCitrine-Ub or mCitrine-Ub_{7KR}. The resulting constructs expressing BRI1 variants under the control of its own promoter were transformed into heterozygous *bri1* null mutant or wild-type plants. For all constructs, more than 50 independent T1 lines were isolated and between 3 to 6 representative mono-insertion lines were selected in T2. Independent lines homozygous for the transgene when transformed in wild-type background, or for both *bri1* mutation and the transgene when transformed in *bri1* mutants, were selected in T3. Confocal microscopy, phenotypic analysis and protein extraction were performed on representative mono-insertional homozygous T3 lines (Supplementary Table 1).

Chemical treatments

Inhibitors (Sigma-Aldrich) were used at the following concentrations: 2 µM ConcA (2 mM

DMSO stock), 50 μ M BFA (20 mM DMSO stock), 33 μ M TyrA23 (50 mM DMSO stock), 100 μ M CHX (100mM EtOH stock). BL (Chemiclones, 1 mM stock in DMSO) and BRZ (Chemiclones, 10 mM stock in DMSO) were used at concentrations indicated in the figure legends.

Hypocotyl and petiole length assays

Hypocotyl and petiole lengths from 15 seedlings were measured with ImageJ 1.48d on day 4 and 28, respectively. All dose-response hypocotyl elongation assays and petiole length experiments were performed in triplicates. For hypocotyl assays, imbibed seeds were stratify at 4°C for 4 days and care was taken that seed germination occurs simultaneously between the different genotypes.

Gene expression analyses

Total RNA was extracted from whole plants using the RNeasy RNA extraction kit (Qiagen). SuperScript reverse transcriptase (Life Technologies) was used to synthesize cDNA from RNA. Semi-quantitative PCR used the gene-specific primers are listed in Supplementary Table 2.

Immunoprecipitation and western blot analysis

Protein extraction and immunoprecipitation experiments were conducted using 12-day-old seedlings. For western blot analyses, total proteins were extracted from approximately 100 mg of starting material. For protein detection, the following antibodies were used: Monoclonal anti-GFP HRP-coupled (Miltenyi Biotec 130-091-833, 1/5000), anti-ubiquitin P4D1 (Millipore 05-944, 1/2500), anti K48 polyubiquitin Apu2 (Millipore 05-1307, 1/2000) and anti-K63 polyubiquitin Apu3 (Millipore 05-1308, 1/2000) (Newton et al., 2008), anti-

BRI1 (Belkhadir et al., 2010) (1/2000) and anti-BES1 (Yu et al., 2011) (1/5000). BRI1 protein stability was determined by blocking *de novo* BRI1 protein synthesis with 100 μ M CHX. Quantification of western blot was performed using the Densitometry plugin from Image J. Immunoprecipitation experiments were performed using 1 g of seedlings. Tissues were ground in liquid nitrogen and resuspended in 2 mL of ice-cold sucrose buffer (20 mM Tris, pH 8; 0.33M Sucrose; 1 mM EDTA, pH 8; protease inhibitor). Samples were then centrifuged for 10 min at 5,000 x g at 4 °C. Total proteins contained in the supernatant were centrifuged at 4 °C for 45 min at 20,000 x g to pellet microsomes. The pellet was resuspended in 1 mL of immunoprecipitation buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100) using a 2-mL potter-Elvehjem homogenizer and left on a rotating wheel for 30 min at 4 °C. Non-resuspended material was then pelleted for 10 min at 20,000 x g and 4 °C. The supernatant contained the fraction enriched in microsomal associated proteins. Immunoprecipitations were carried out on 1 mg of microsomal proteins using the μ MACS GFP isolation kit (Miltenyi Biotec). For quantification of BRI1 ubiquitinated pools, the ratio of normalized immunoprecipitation signal intensity obtained with anti-GFP and anti-Ub antibodies was determined using Image J. Immunoprecipitation and western blot analyses were performed in triplicates. Representative blots are shown in figures, and full scan blots in supplementary figure 7.

Mass Spectrometry

Immunopurified proteins were reduced with DTT, alkylated with iodoacetamide and digested with trypsin overnight. Salts and reagents were removed by reversed phase (C-18) cartridge clean-up. The salt-free peptides were dissolved in 0.1% formic acid and subjected to ESI-MS/MS analysis on a Thermo LTQ-Orbitrap XL instrument. A capillary column (inner diameter 75 μ m, packing length 10 cm of C-18 silica) with integrated spray tip was used with

a 300 nl/min 0.1% formic acid/acetonitrile gradient. Peptide precursor masses were determined with high accuracy by Fourier-transform MS in the Orbitrap followed by data dependent MS/MS of the top 5 precursor ions in each chromatographic time window. Data were analyzed using the Mascot algorithm (Matrix Science, London, (UK) on a local Mascot server (version 2.1.0) and searched against the latest Swiss Protein database allowing a variable isopeptide (Gly-Gly) modification on lysine residues.

Confocal microscopy

Plant samples were mounted in water and viewed on Leica TCS SP2 confocal laser scanning microscopes. Images were taken at the root tip from 7 day-old plants grown in the light, or in the upper part of 4-day-old etiolated hypocotyls. For imaging mCitrine fusion proteins, the 514-nm laser line was used. Laser intensity settings were kept constant in individual sets of experiments to allow for a comparison of expression and localization of reporter proteins. Quantification of total fluorescence intensity in roots, at the plasma membrane, in intracellular compartments, in BFA bodies or in the vacuole was performed using Image J.

TIRF microscopy and analysis of images

Arabidopsis plantlets were grown in MS medium for 4 days in the dark to obtain etiolated hypocotyls. Hypocotyls were imaged by TIRF on a Nikon Eclipse Ti microscope equipped with a Nikon APO TIRF 100x/1.49 oil immersion objective. The excitation wavelength used was 491 nm provided by a 100 mW diode laser Toptica AOTF and emission light was collected with an emission filter Chroma ET 525/50. Time-lapses were acquired during 2 minutes at 200 ms intervals and images were captured with a Photometrics[®] Evolve Delta Camera using the Metamorph Software version 7.7.9.0 (Molecular Devices[®], LLC) and the laser power at 40%.

The videos of 3 independent experiments were then analyzed using the ImageJ 1.48d software. To better visualize the videos, the Difference of Gaussian filter and the kymographs were obtained on the treated images using the MultiKymographs plugin (http://www.embl.de/eamnet/html/body_kymograph.html) of ImageJ. The time of residence was determined by the duration of tracks (n=350). To measure the density of BRI1 and BRI1_{25KR} at the plasma membrane, videos were treated with the Difference of Gaussian filter and the density of protein was quantified by the Find Maxima function within a region of same area using ROI manager. Each area measured was 40x41 pixels (0.160µm/pixel) in size.

Statistical analyses

Statistical analyses were performed with the software GraphPad Prism. Physiological plant parameters (hypocotyl and petiole length), western blot and immunoprecipitation quantification used the non-parametric Mann-Whitney test (two genotypes/conditions), or the non-parametric Kruskal-Wallis (three genotypes/conditions and more) test followed by a Dunn's or Dunnet's post-hoc test for multiple comparisons ($p < 0.05$).

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Author contributions

S.M., E.M.N.D., N.G. and G.V. designed the study; S.M., E.M.N.D, A.C., A.J. and W.F. performed experiments; F.P. and B.S.J gave technical support for model prediction and TIRF microscopy, respectively; Y.J. and J.C. provided reagents; Y.J., J.C. and N.G. gave conceptual advice; G.V wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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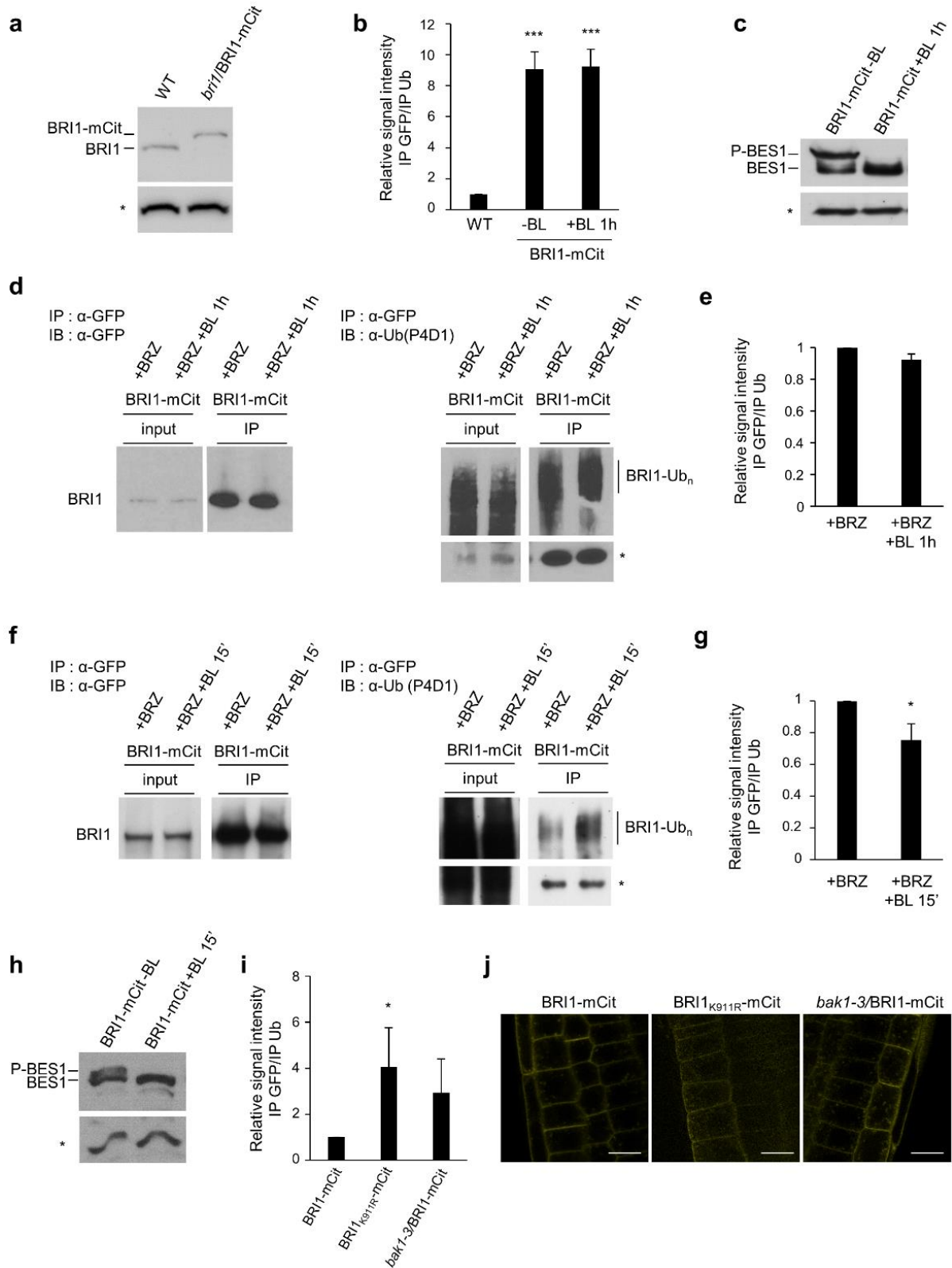
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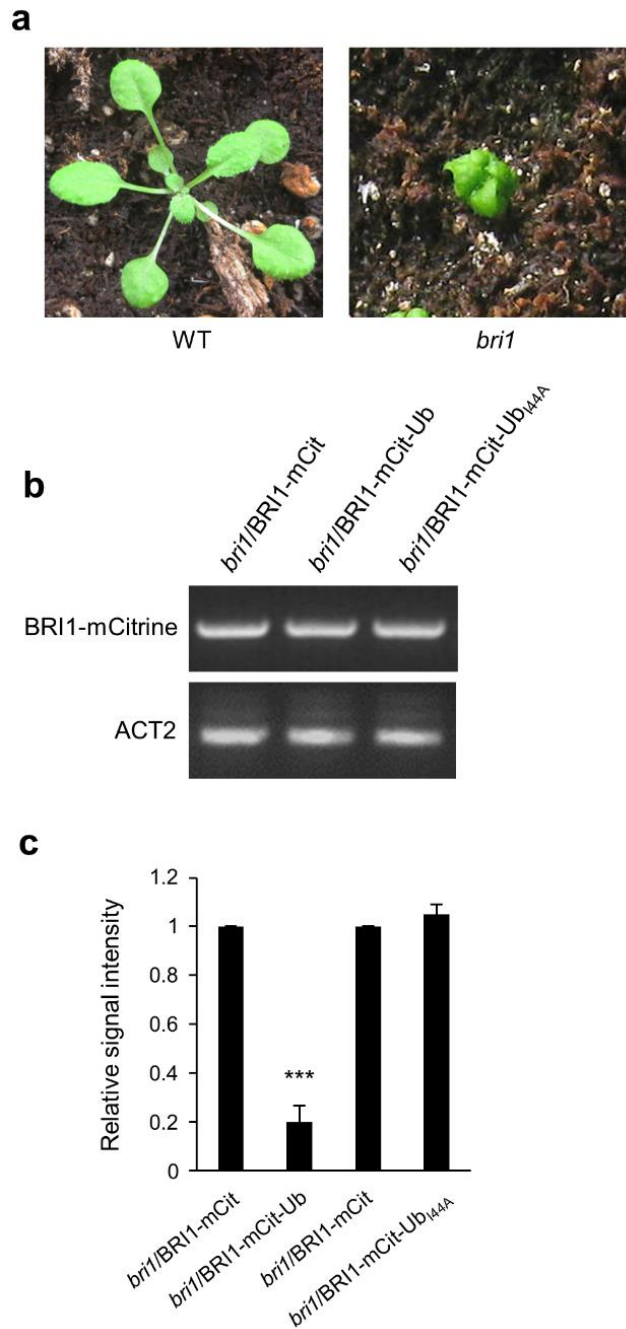
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Supplementary information

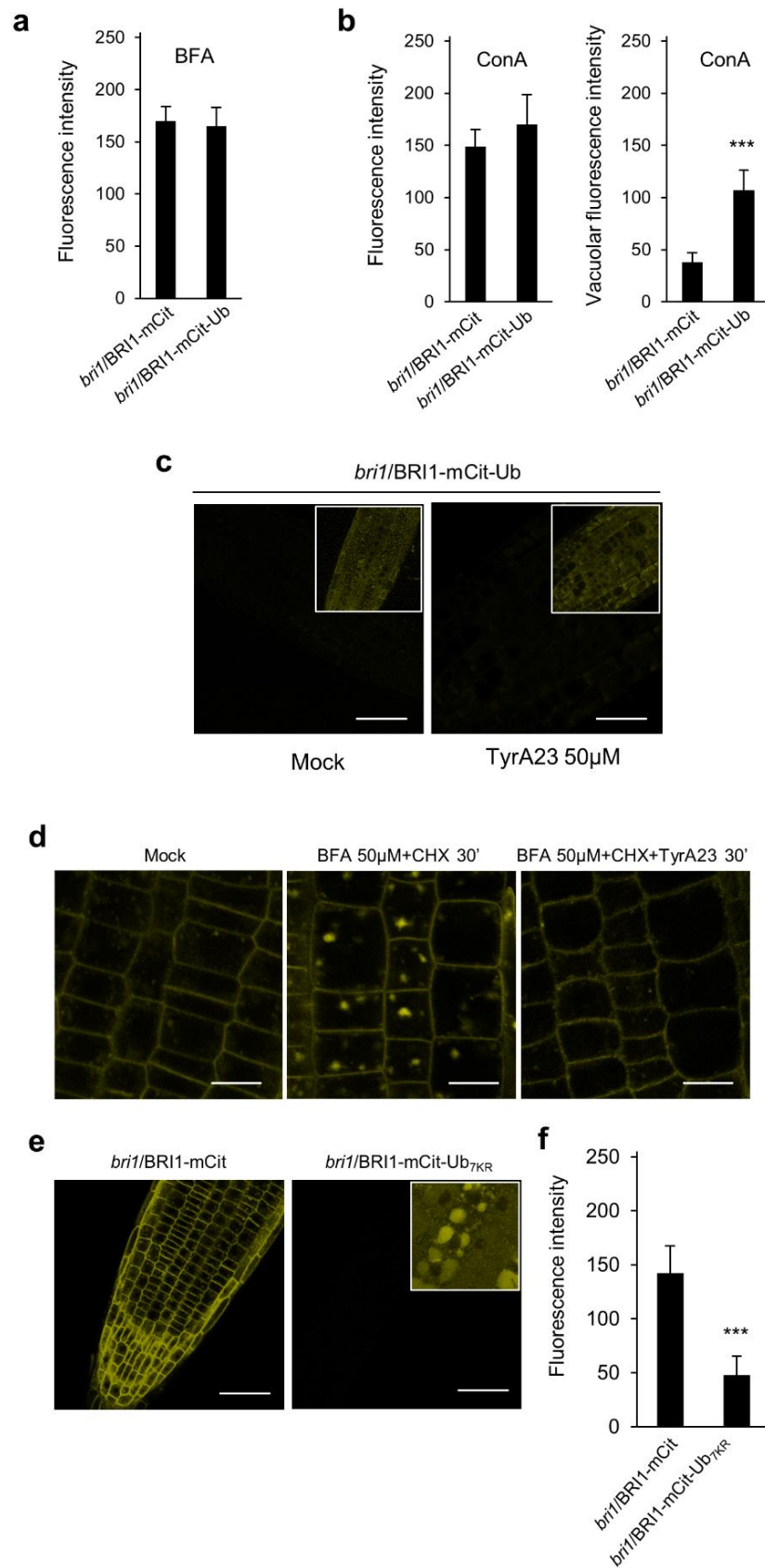


Supplementary figure 1. BRI1 ubiquitination is largely independent on ligand binding.

(a) Western blot analyses using anti-BRI1 antibodies on wild-type or *bri1*/BRI1-mCitrine protein extracts. The asterisks indicate non-specific signals used as loading control. (b) Quantification of BRI1 ubiquitination profiles in response to brassinolide (BL) treatment. Error bars represent standard deviation (n=3). The asterisks indicate statistical significance by Kruskal-Wallis one-way analysis of variance and Dunnett multiple testing ($P < 0.0001$). (c) Western blot analyses using anti-BES1 antibodies following mock (-BL) or exogenous application of 1 μ M BL for 1 hour (+BL). The asterisks indicate non-specific signals used as loading control. (d) *In vivo* ubiquitination analyses of BRI1 in BR-depleted plants. Immunoprecipitation was performed using anti-GFP antibodies on solubilized protein extracts from wild-type and BRI1-mCitrine plants grown on 2 μ M BRZ and treated with mock (+BRZ) or 1 μ M BL for 1 hour (+BRZ +BL 1h) and subjected to immunoblotting with anti-GFP (left) and anti-Ub P4D1 (right) antibodies. Asterisk indicates non-specific band used as loading control. (e) Quantification of BRI1 ubiquitination profiles using plants grown on grown on 2 μ M BRZ and treated with mock (+BRZ) or 1 μ M BL for 1 hour (+BRZ +BL 1h). Error bars represent standard deviation (n=3). The ubiquitination profile of brassinolide-treated plants is not statistically significant (Mann-Whitney, $P > 0.05$). (f) *In vivo* ubiquitination analyses of BRI1 in response to short-term BL treatment. Immunoprecipitation was performed using anti-GFP antibodies on solubilized protein extracts from wild-type and BRI1-mCitrine plants grown on 2 μ M BRZ and treated with mock (+BRZ) or 1 μ M BL for 15 minutes (+BRZ +BL 15') and subjected to immunoblotting with anti-GFP (left) and anti-Ub P4D1 (right) antibodies. Asterisk indicates non-specific band used as loading control. (g) Quantification of BRI1 ubiquitination profiles using plants grown on grown on 2 μ M BRZ and treated with mock (+BRZ) or short term 1 μ M BL (+BRZ +BL 15'). Error bars represent standard deviation (n=3). The ubiquitination profile of brassinolide-treated plants is statistically different from the control condition (Mann-Whitney, $P < 0.05$). (h) Western blot analyses using anti-BES1 antibodies following mock (-BL) or exogenous application of 1 μ M BL for 15 minutes (+BL 15'). The asterisk indicates non-specific signals used as loading control. (i) Quantification of BRI1 ubiquitination profiles in genetic backgrounds impaired in receptor complex activation. Error bars represent standard deviation (n=3). The asterisks indicate statistical significance by Kruskal-Wallis one-way analysis of variance and Dunnett multiple testing ($P < 0.05$). (j) Confocal microscopy analyses of representative mono-insertional transgenic lines expressing BRI1-mCitrine, kinase-dead BRI1_{K911R}-mCitrine and *bak1-3*/BRI1-mCitrine. Scale bar = 7 μ m.

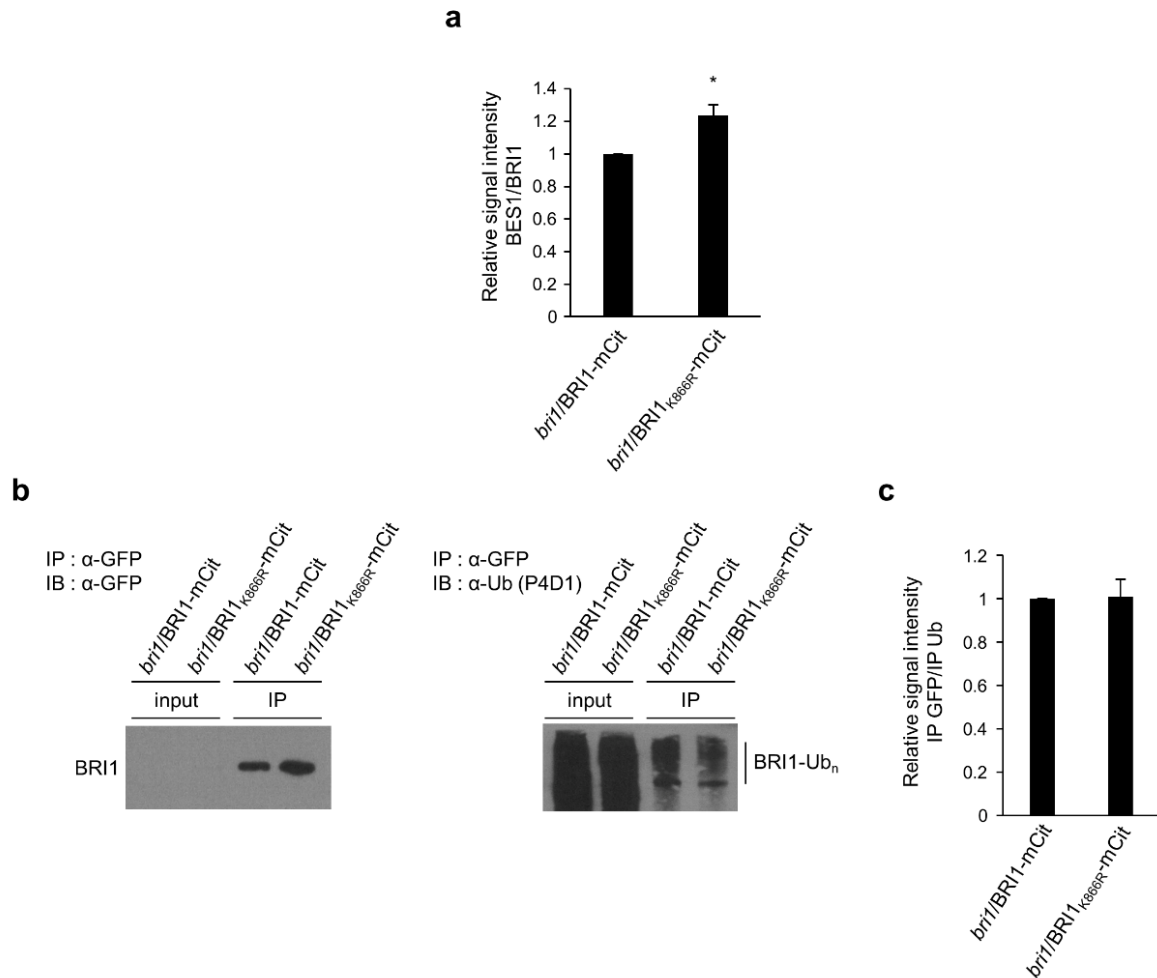


Supplementary figure 2. Characterization of transgenic lines expressing artificially ubiquitinated BRI1. (a) Phenotype of wild-type and *bri1* null mutant grown for 3 weeks in soil. (b) Analyses of transgene expression in representative mono-insertional homozygous *bri1/BRI1-mCitrine*, *bri1/BRI1-mCitrine-Ub* and *bri1/BRI1-mCitrine-Ub_{I44A}* lines. RT-PCR analyses were performed on total RNA extracted from the respective lines (25 cycles). Amplification of *ACT2* serves as a control. (c) Quantification of BRI1 protein levels in *bri1/BRI1-mCit*, *bri1/BRI1-mCit-Ub* and *bri1/BRI1-mCit-Ub_{I44A}*. Error bars represent standard deviation (n=3). The asterisks indicate statistical significance (Mann-Whitney, $P < 0.0001$).

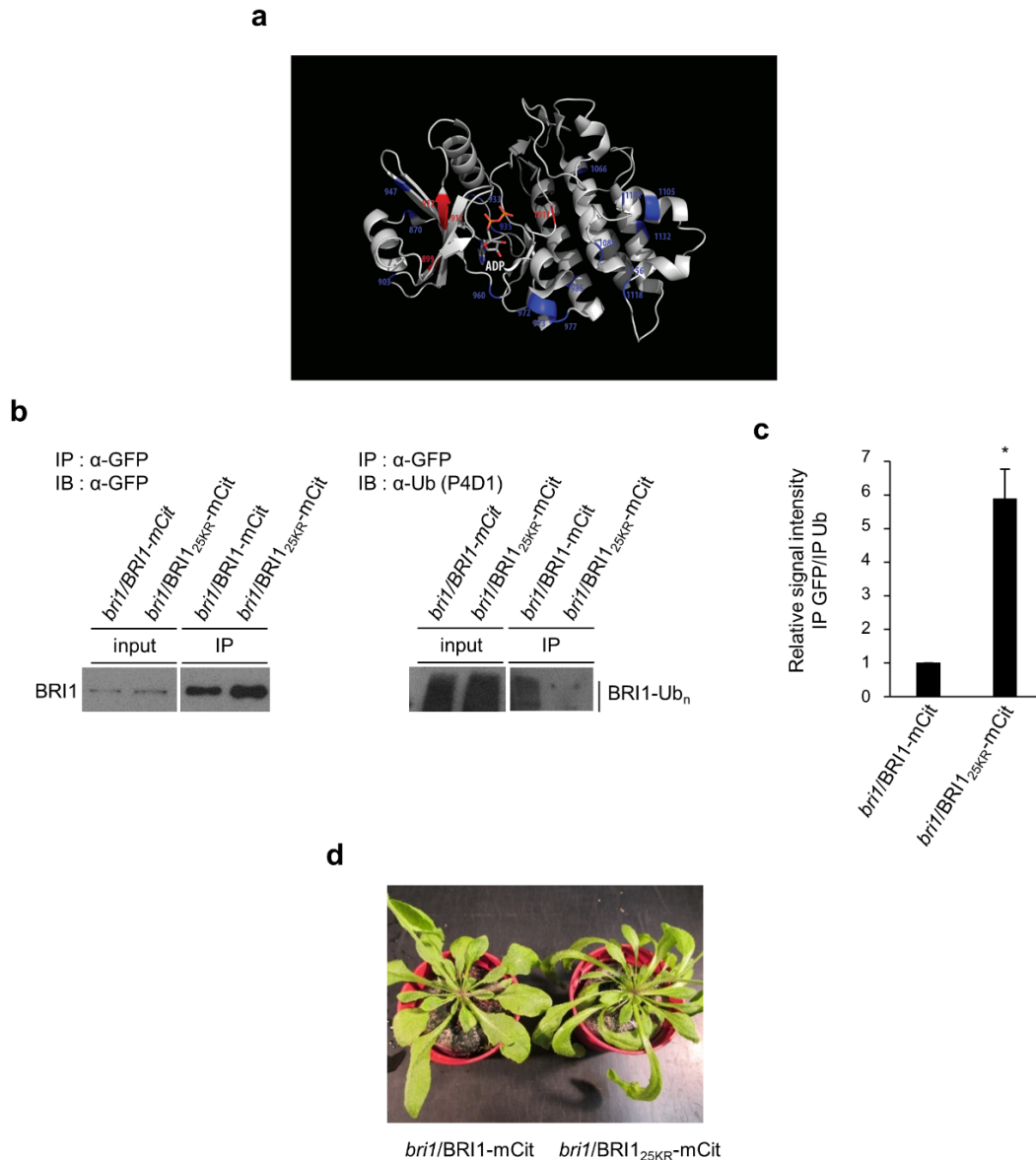


Supplementary figure 3. Artificial ubiquitination of BRI1 triggers BRI1 vacuolar

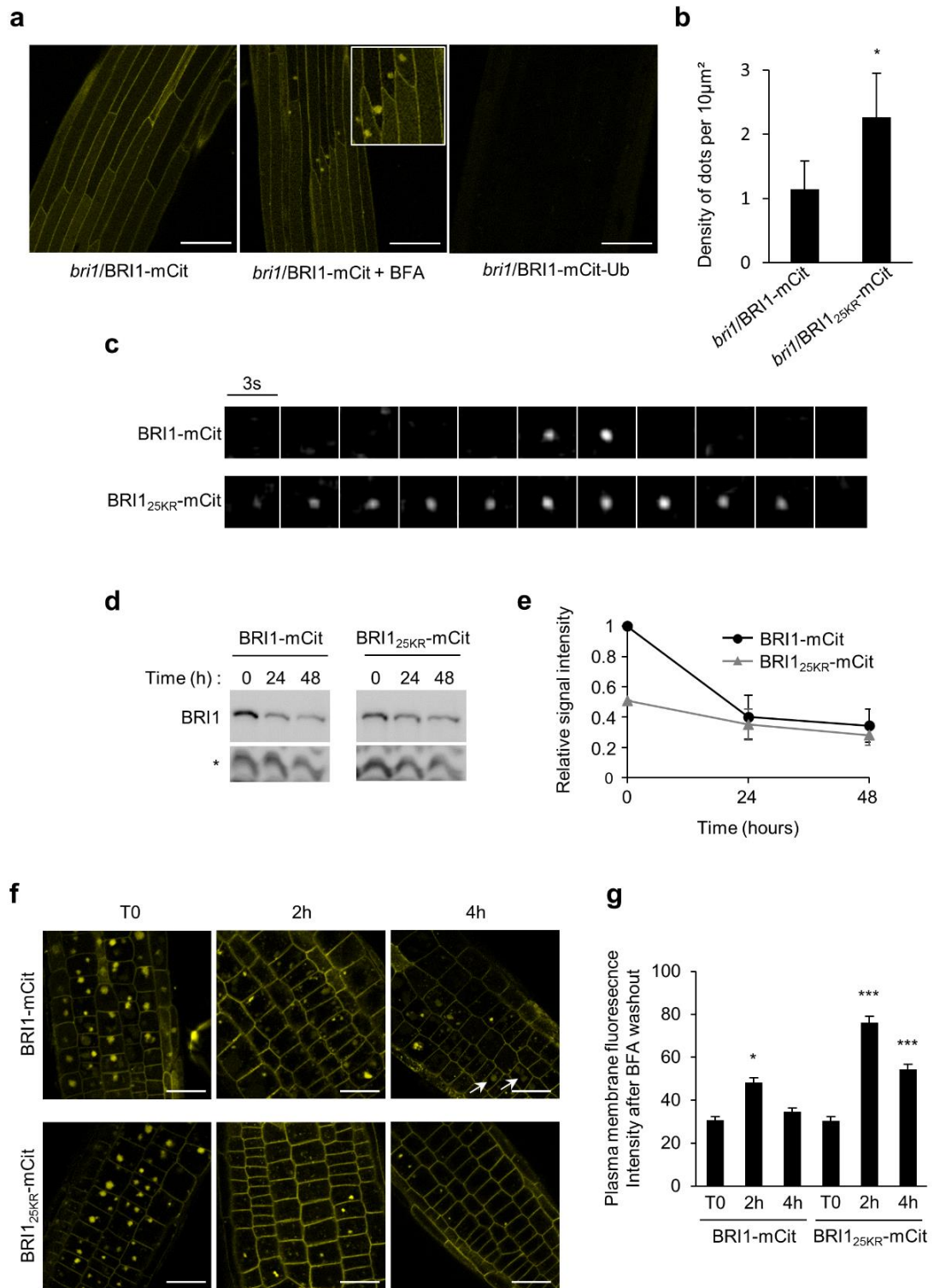
targeting. (a) Quantification of maximum fluorescence intensity of *bri1*/BRI1-mCitrine and *bri1*/BRI1-mCitrine-Ub plants treated with 50 μ M BFA for 1 hour. Error bars represent standard deviation (n=15). Fluorescence intensity is not statistically different between the two genotypes after BFA treatment (Mann-Whitney, $P > 0.05$). (b) Quantification of maximum fluorescence intensity (left) and maximum vacuolar fluorescence intensity (right) of *bri1*/BRI1-mCitrine and *bri1*/BRI1-mCitrine-Ub plants treated with 2 μ M ConA for 1 hour. Error bars represent standard deviation (n=15). The asterisks indicate a statistically significant difference with the control (Mann-Whitney, $P < 0.0001$). (c) Sensitivity of *bri1*/BRI1-mCitrine-Ub to TyrA23. Plants were exposed to TyrA23 (33 μ M) for 1 hour prior to imaging. Inset, High gain was used to observe possible plasma membrane localization of *bri1*/BRI1-mCitrine-Ub following TyrA23 treatment. Scale bars = 50 μ m (d) TyrA23 prevents accumulation of BRI1-mCitrine in BFA bodies. Plant were treated with mock or 100 μ M CHX for 30 minutes prior to treatment with 33 μ M TyrA23 and 50 μ M BFA for an additional 30 minutes. Scale bars = 5 μ m. (e) Confocal microscopy analyses of representative mono-insertional homozygous *bri1*/BRI1-mCitrine and *bri1*/BRI1-mCitrine-Ub_{7KR} plant roots. Similar confocal detection settings were used to compare the transgenic lines. Inset, ConA treatment allows visualization of BRI1-mCitrine-Ub_{7KR} vacuolar targeting. Scale bars = 50 μ m. (f) Quantification of maximum fluorescence intensity of *bri1*/BRI1-mCitrine and *bri1*/BRI1-mCitrine-Ub_{7KR} plants. Error bars represent standard deviation (n=15). The asterisk indicates a statistically significant difference between *bri1*/BRI1-mCit-Ub_{7KR} and the *bri1*/BRI1-mCit control (Mann-Whitney, $P < 0.0001$).



Supplementary figure 4. Characterization and ubiquitination profile of *bri1/BRI1_{K866R}-mCitrine*. (a) Quantification of dephosphorylated BES1 (BES1) protein levels relative to BRI1 proteins levels in representative mono-insertional homozygous *bri1/BRI1-mCit* and *bri1/BRI1_{K866R}-mCit*. Error bars represent standard deviation (n=3). The asterisk indicates a statistically significant difference in dephosphorylated BES1 levels between *bri1/BRI1-mCit* and *bri1/BRI1_{K866R}-mCit* (Mann-Whitney, $P < 0.05$). (b) Ubiquitination profile of *bri1/BRI1_{K866R}-mCitrine*. Immunoprecipitation was performed using anti-GFP antibodies on solubilized protein extracts from *bri1/BRI1-mCitrine* and *bri1/BRI1_{K866R}-mCitrine* plants and subjected to immunoblotting with anti-GFP (left) and anti-Ub P4D1 (right) antibodies. (c) Quantification of BRI1 ubiquitination profiles using *bri1/BRI1-mCit* and *bri1/BRI1_{K866R}-mCit*-expressing plants. Error bars represent standard deviation (n=3). The ubiquitination profile of *bri1/BRI1_{K866R}-mCit* plants is not statistically different from *bri1/BRI1-mCit* plants (Mann-Whitney, $P > 0.05$).

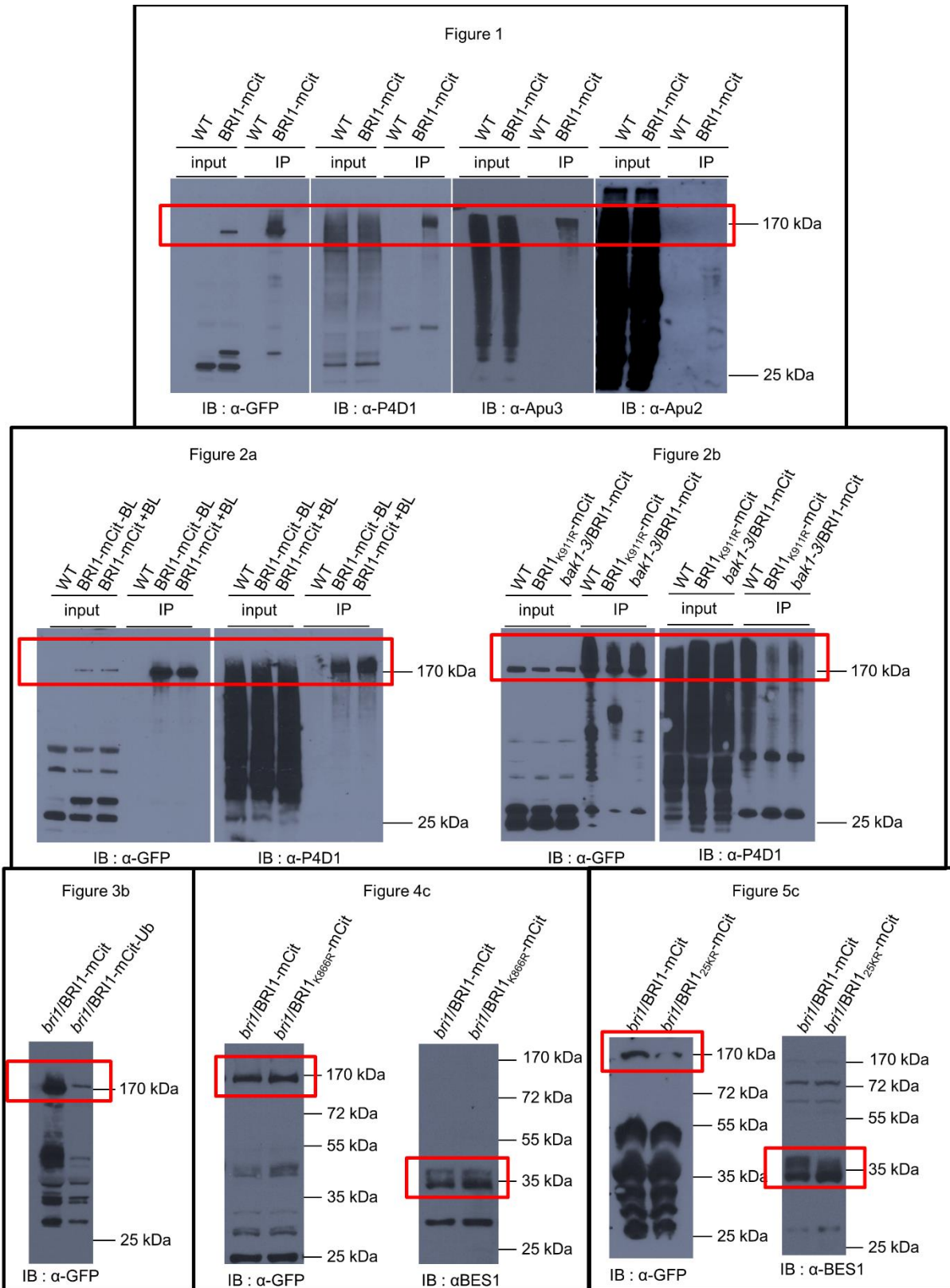


Supplementary figure 5. Identification of an ubiquitination-defective but functional BRI1. (a) Cartoon representation of the crystal structure of BRI1 kinase domain in complex with ADP (PDB code 4OAC, residues 865-1160). Lysine residues mutated to arginines in BRI1_{25KR} are shown in blue. (b) Ubiquitination profile of *bri1/BRI1_{25KR}-mCitrine*. Immunoprecipitation was performed using anti-GFP antibodies on solubilized protein extracts from representative mono-insertional homozygous *bri1/BRI1-mCitrine* and *bri1/BRI1_{25KR}-mCitrine* plants and subjected to immunoblotting with anti-GFP (left) and anti-Ub P4D1 (right) antibodies. (c) Quantification of BRI1 ubiquitination profiles using *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* plants. Error bars represent standard deviation (n=3). The asterisk indicates a statistically significant difference between *bri1/BRI1-mCit* and *bri1/BRI1_{25KR}-mCit* (Mann-Whitney, $P < 0.05$). (d) Phenotype of 6-week-old representative *bri1/BRI1-mCitrine* and *bri1/BRI1_{25KR}-mCitrine* mono-insertional homozygous plants.



Supplementary figure 6. Characterization of ubiquitination-defective BRI1 internalization and stability. (a) Characterization of BRI1-mCitrine trafficking in etiolated hypocotyl cells. 4-day-old etiolated *bri1/BRI1-mCitrine* hypocotyls were imaged by confocal microscopy after mock treatment (left panel) or after treatment with 50 μM BFA for 1 hour (middle panel). 4-day-old etiolated *bri1/BRI1-mCitrine-Ub* hypocotyl cells were imaged

using similar detection settings to compare with the control *bri1*/BRI1-mCitrine line. Scale bars = 20 μ m. **(b)** TIRF microscopy analyses of *bri1*/BRI1-mCitrine and *bri1*/BRI1_{25KR}-mCitrine plants. Density analysis of fluorescent spots at the plasma membrane of *bri1*/BRI1-mCitrine and *bri1*/BRI1_{25KR}-mCitrine cells. Error bars represent standard deviation (n=10). The asterisk indicates a statistically significant difference between *bri1*/BRI1-mCit and *bri1*/BRI1_{25KR}-mCit (Mann-Whitney, $P < 0.05$). **(c)** An example of real-time dynamic observation of BRI1-mCitrine and BRI1_{25KR}-mCitrine spots at the median of the distribution. The time scale is shown. **(d)** Protein stability of BRI1 and BRI1_{25KR}. Experiments were done in the presence of 100 μ M CHX and samples collected at indicated times. The asterisk indicates non-specific signals used as loading control. **(e)** Quantification of BRI1 protein stability in *bri1*/BRI1-mCitrine and *bri1*/BRI1_{25KR}-mCitrine plants. Error bars represent standard deviation (n=3). **(f)** BFA washout experiments using *bri1*/BRI1-mCitrine and *bri1*/BRI1_{25KR}-mCitrine lines. Plants were pretreated with 100 μ M CHX for 30 minutes, then treated with 50 μ M BFA and 100 μ M CHX for one hour prior to washout in 100 μ M CHX and confocal imaging. Scale bars = 10 μ m. **(g)** Quantification of plasma membrane fluorescent intensities after BFA washout over time. The asterisk indicates a statistically significant difference between *bri1*/BRI1-mCit and *bri1*/BRI1_{25KR}-mCit by Kruskal-Wallis one-way analysis of variance and Dunnett multiple testing ($P < 0.05$).



Supplementary figure 7. Full scans of blots.

Supplementary Table 1. Phenotypic and genetic analyses of mono-insertional transgenic lines generated.

<i>Transgenic plants</i>	<i>Generation</i>	<i>Genotype</i>	<i>Line</i>	<i>Phenotype</i>
			3k	Dwarf
<i>bri1/BRI1-mCit-Ub</i>	T3	<i>b/b T/T</i>	4e	Extremely dwarf
			12r	Extremely dwarf
			3a	Wild-type
<i>bri1/BRI1-mCit-Ub_{I44A}</i>	T3	<i>b/b T/T</i>	5c	Wild-type
			7f	Wild-type
			2a	Extremely dwarf
<i>bri1/BRI1-mCit-Ub_{7KR}</i>	T3	<i>b/b T/T</i>	14q	Extremely dwarf
			15c	Extremely dwarf
			8d	Wild-type
<i>bri1/BRI1_{K866R}-mCit</i>	T3	<i>b/b T/T</i>	10f	Wild-type
			14p	Wild-type
			5a	Long bending petioles
<i>bri1/BRI1_{25KR}-mCit</i>	T3	<i>b/b T/T</i>	57p	Long bending petioles
			61u	Long bending petioles

b, bri1 ; T, transgene

Supplementary Table 2. Primer list.

<i>Primer name</i>	<i>Sequence (5'-3')</i>
BRI1 RT F	ATAACAACCTTGTTGGATGG
mCitrine RT R	GAACTTGTGGCCGTTTACGTC
ACT2 RT F	GCCCAGAAGTCTTGTTCCAG
ACT2 RT R	TCATACTCGGCCTTGGAGAT

Chapter 3:

Steroid-dependent root responses to elevated ambient temperature

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Chapter 3

Steroid-dependent root responses to elevated ambient temperature

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Introduction

As sessile organisms, plants have to cope with and adjust to their fluctuating environment. Temperature elevation stimulates the growth of *Arabidopsis* aerial parts. This process is mediated by increased biosynthesis of the growth-promoting hormone auxin. How plant roots respond to elevated ambient temperature is however still elusive. Here we present strong evidence that temperature elevation impinges on brassinosteroid hormone signaling to alter root growth. We show that elevated temperature leads to increased root elongation, independently of auxin or factors known to drive temperature-mediated shoot growth. We further demonstrate that brassinosteroids negatively regulates root responses to elevated ambient temperature. Increased growth temperature specifically impacts on the level of the BR receptor BRI1 to downregulate BR signaling and mediate root elongation. Our results establish that BRI1 integrates temperature and BR signaling to regulate root growth upon long-term changes in environmental conditions and allow us to anticipate on the consequences of global warming.

Human activities releasing billions of tons per year of carbon dioxide (CO₂) and other heat-trapping gases in the atmosphere change earth's climate with an unprecedented pace. According to the World Meteorological Organization (WMO, <http://public.wmo.int>), the earth's lower atmosphere could rise of more than 4°C by the end of the 21st century as a consequence of global warming. This change in ambient temperature will impact all forms of terrestrial life, and especially plants that are rooted in soils and thus fixed to a specific location. Temperature has a large impact on numerous plant developmental processes, including germination, growth, flowering, and hormonal responses, as well as plant disease

resistance (Garrett et al., 2006; Wigge, 2013). The bHLH transcription factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) has been shown to be central for multiple responses to warmer temperature in *Arabidopsis*, including flowering, nastic leaf movements, and elongation of the embryonic stem named hypocotyl (Koini et al., 2009; Stavang et al., 2009; Franklin et al., 2011; Sun et al., 2012). Elevated temperature increases hypocotyl length through direct binding of PIF4 to the promoter of the *YUCCA8* (*YUC8*) and *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) auxin biosynthetic genes (Franklin et al., 2011; Sun et al., 2012).

Results

Influence of temperature on root growth

To evaluate the impact of elevated ambient temperature on roots, we grew *Arabidopsis* seedlings at 21 and 26°C and monitored root growth and development parameters. In our conditions, the primary root grew longer when plants were grown at 26°C compared to 21°C (Fig. 1a, b). The longer root phenotype of 26°C-grown plants is not explained by a higher rate of cell division at 26°C, as attested by the shorter meristem size (Fig. 1c, d), and the decreased expression of the *CYCBI;1* cyclin gene (Extended Data Fig. 1a, b). To investigate if the longer root phenotype displayed by plants grown at higher temperature resulted from increased root cell elongation, we measured the length of epidermal cells harboring a root hair bulge. These cells are indeed fully elongated and undergoing differentiation. A significant increase in differentiated root cell length was observed for plants grown at elevated temperature that explains the longer root phenotype of 26°C-grown plants (Fig. 1e).

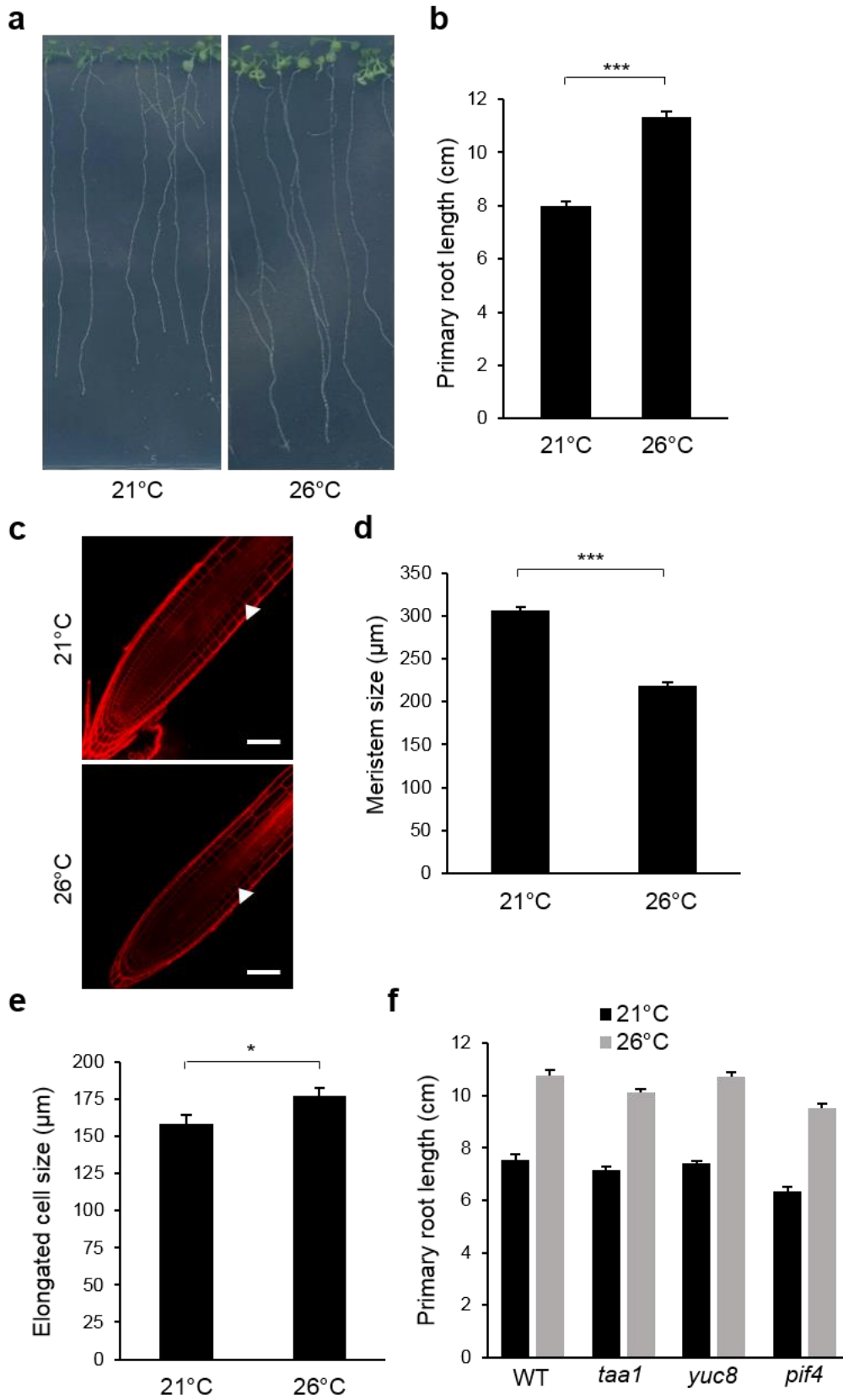


Figure 1. Elevated ambient temperature impacts on root elongation. **a**, Phenotype of 15-

day-old wild-type plants grown at 21 or 26°C. Representative pictures are shown. **b**, Primary root length of 15-day-old wild-type plants grown at 21 or 26°C (mean±s.d., n=25). **c**, Propidium iodide staining of wild-type plant roots grown as in **(a)**. Arrows represent the meristem boundary where cortical cells double their size. Scale bar, 50 μm. **d**, Quantification of meristem size, defined by the distance from the quiescent center to the meristem boundary, in plants grown at 21 or 26°C (mean±s.d., n=25). **e**, Quantification of differentiated root epidermal cell length (mean±s.d., n=25). **f**, Primary root length of 15-day-old wild-type plants (WT) and mutants impaired in hypocotyl temperature responses (mean±s.d., n=25). The difference between the different genotypes for a given growth temperature is not statistically significant.

In hypocotyls, PIF4 activates temperature-induced elongation through direct activation of the auxin biosynthetic genes *YUC8* and *TAA1* (Franklin et al., 2011; Sun et al., 2012). To determine whether root responses to elevated temperature involves the same machinery that in hypocotyls, we monitored primary root length of *pif4*, *yuc8*, and *taa1* mutants grown at 21 and 26°C. All three mutants showed longer roots at warmer temperature, similar to wild-type plants, indicating that root responses do require PIF4-, YUC8-, and TAA1-dependent auxin biosynthesis (Fig. 1f). Since *PIF4*, *YUC8* and *TAA1* are members of multigene families, our observation does not exclude the possible involvement of other family members. In the case of PIF4, it acts in concert with PIF5 in a number of biological processes including leaf senescence, phototropism, shade avoidance and hypocotyl growth in response to low blue light (Lorrain et al., 2008; Sun et al., 2013; Sakuraba et al., 2014; Pedmale et al., 2016). We therefore tested whether PIF5 was acting redundantly with PIF4 in the temperature-mediated root elongation by characterizing the *pif4pif5* double mutant, but failed to observe any difference with wild-type plants (Extended Data Fig. 1d).

Genome-wide root responses to elevated ambient temperature

To shed light on the pathways mediating root growth responses to elevated ambient temperature, we performed whole-genome mRNA-seq analysis comparing root transcriptomes from plants grown at 21 and 26°C. We found 2,681 genes upregulated (false discovery rate < 0.05 and induction fold >1.3) and 2,114 genes downregulated (false discovery rate < 0.05 and repression fold >1.3) by increased temperature growth, respectively (Supplementary Table 1). The top recurring gene ontology (GO) terms (ranked by false discovery rate < 0.05) among the upregulated genes at 26°C were related to biotic stress and defense responses against bacteria, fungi and herbivores (Fig. 2a and Supplementary Table 2). Elevated temperature therefore promotes root growth and likely primes root immune

responses against potential threats that multiply vigorously with warmth. GO terms associated with genes downregulated with increased temperature include oxidative stress and responses to metal ions (Fig. 2a). This is likely caused by a massive deregulation of genes involved in iron homeostasis with the nicotianamine synthase *NAS2* gene showing a 50-fold repression, and the iron uptake machinery genes *IRT1* and *FRO2* being downregulated 5- to 10-fold (Supplementary Table 3).

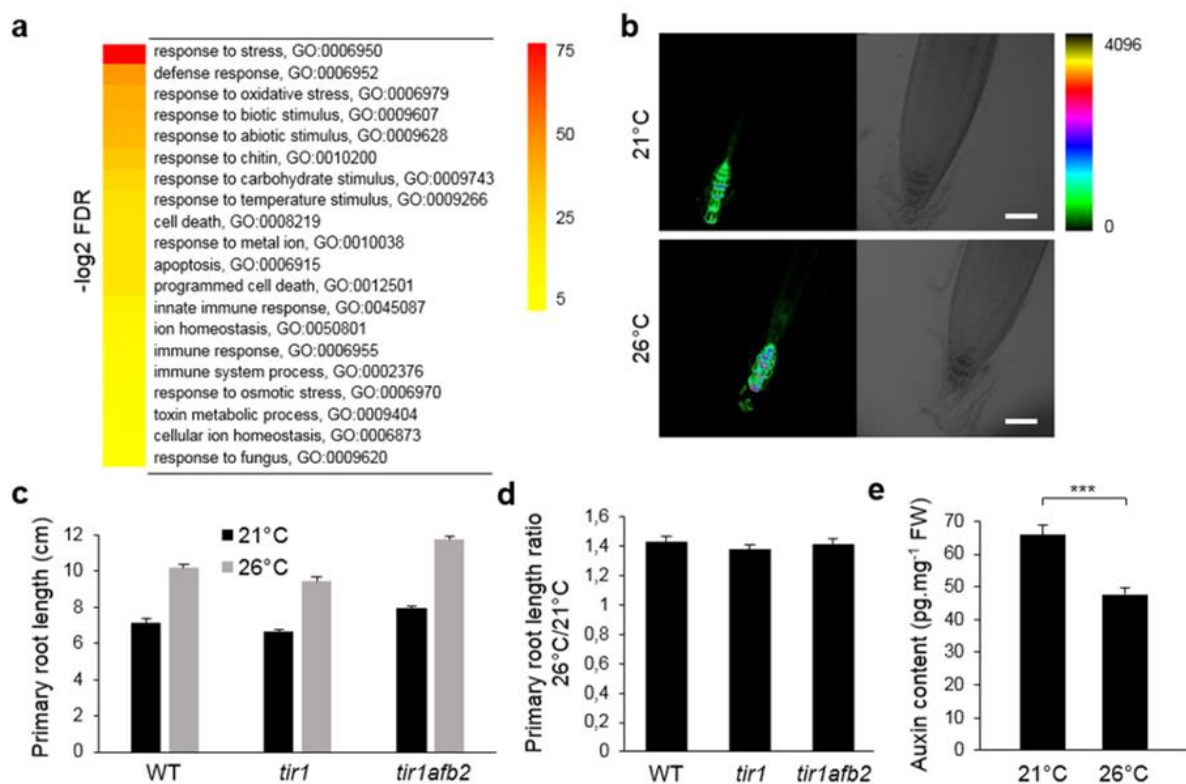


Figure 2. Elevated ambient temperature responses do not involve auxin. **a**, Top 20 (ranked by false discovery rate [FDR<0.05]) significantly enriched GO terms derived from genes that are regulated by increased temperature. The range of FDR is shown on the right. **b**, Influence of elevated growth temperature on auxin responses using the DR5::GFP synthetic auxin reporter. Similar confocal detection settings were used to compare the two growth conditions. The LUT is represented on the right. Representative images are shown. Scale bar, 50 μ m. **c**, Primary root length of wild-type (WT) and auxin perception mutants grown for 15 days at 21 or 26°C (mean \pm s.d., n=25). **d**, Free auxin (IAA) measurements in 15-day-old roots from wild-type plants grown at 21 or 26°C (mean \pm s.d., n=8).

Surprisingly, auxin was not found among enriched GO terms in our whole genome RNA-seq analysis. We therefore directly compared our list of root temperature-regulated genes with previously published auxin-responsive gene datasets (Nemhauser et al., 2006; Li et al., 2012). No overlap was observed with the 785 genes regulated by the auxin indole acetic acid (IAA) molecule (Extended Data Fig. 2a)(Nemhauser et al., 2006), and with the 335 known auxin-induced genes induced by shade conditions (Extended Data Fig. 2b)(Li et al., 2012), indicating that the genome-wide reprogramming of root gene expression upon high ambient temperature growth is independent of auxin responses. This is consistent with the minor effect of temperature on the DR5::GFP auxin response reporter line (Fig. 2b and Extended Data Fig. 2c). This conclusion is further supported by the wild-type primary root elongation response observed for the *tir1* and *tir1/afb2* auxin receptor mutants (Fig. 2c), although the same *tir1/afb2* double mutant show delayed root elongation to rapid temperature change from 22 to 29°C (Wang et al., 2016). The impact of higher order auxin perception mutants could be evaluated in our conditions since a large proportion of these plants arrested shortly after germination (Dharmasiri et al., 2005). Surprisingly, root auxin content was strongly decreased upon growth at elevated growth temperature (Fig. 2d), although auxin responses visualized by DR5::GFP appeared largely unaffected (Fig. 2b). The drop in auxin content is likely counterbalanced by the stabilization of the auxin receptors at higher temperature (Wang et al., 2016), explaining the lack of differential auxin response at 21 and 26°C. All together, these observations indicate that plants grown at elevated ambient temperature do not require auxin to further elongate their primary root.

Steroids drive root responses to warmth

To unravel the mechanisms leading to longer roots upon growth at elevated temperature, we analyzed further our whole-genome RNA-seq experiment. A large number of genes regulated

by elevated temperature in the root overlaps with root BR-regulated genes, or with genes directly bound by the BR signaling downstream transcription factors BRASSINAZOLE-RESISTANT-1 (BZR1) in whole seedlings (Extended Data Fig. 2d, e and Supplementary Table 4) (Sun et al., 2010; Chaiwanon and Wang, 2015). BRs impact root growth by modulating the elongation of differentiated cells and meristem size (Gonzalez-Garcia et al., 2011; Hacham et al., 2011; Fridman et al., 2014). As a result, the loss-of-function mutant for the BR-INSENSITIVE-1 (BRI1) gene encoding the BR receptor and the *bes1-D* constitutive BR response mutants both show shorter roots and shorter meristems (Gonzalez-Garcia et al., 2011; Hacham et al., 2011). To investigate the possible role of BRs in root responses to increased ambient temperature, we grew BR signaling mutants with altered responses to BRs at 21 and 26°C and scored their primary root length. *bri1* mutants showed shorter roots than wild-type plants at 21°C, but were clearly hypersensitive to elevated temperature as highlighted by the ~2-fold increase in root length at 26°C (Fig. 3a). This hypersensitivity is better visualized by the increase in the primary root length ratio of plants grown at 26°C over 21°C (Fig. 3b). Conversely, the constitutive *bes1-D* mutant failed to respond to increased temperature (Fig. 3a). This indicates that BR signaling negatively regulates root responses to elevated growth temperature. To evaluate the influence of warmer temperature on BR signaling, we used the phosphorylation state of the BES1 transcription factor as a readout (Vert et al., 2005). BES1 accumulates under phosphorylated (P-BES1) and unphosphorylated (BES1) forms, and exogenous BR application promotes the conversion of BES1 to its unphosphorylated form (Yin et al., 2002; Vert and Chory, 2006). Plants grown at 26°C reproducibly accumulated more phosphorylated BES1 than plants grown at 21°C (Fig. 3c), as evidenced by the increase in the P-BES1/BES1 ratio at elevated growth temperature (Fig. 3d). Similarly, the expression of BR feedback-regulated genes, such as *CPD* (Mathur et al., 1998), was higher at 26°C compared to 21°C, indicative of reduced BR signaling activity (Fig. 3e).

Taken together, these observations indicate that high temperature downregulates BR signaling to promote root elongation.

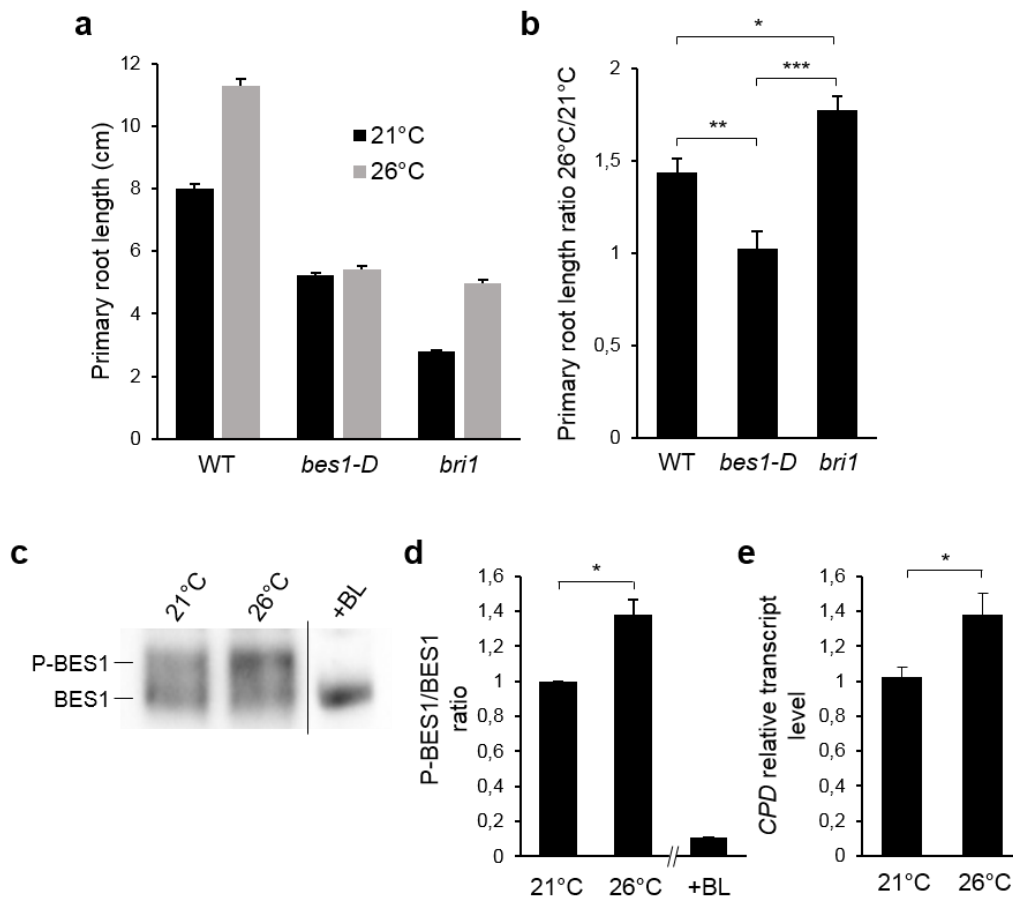


Figure 3. Brassinosteroids drive root responses to elevated ambient temperature. a, Primary root length of 15-day-old wild-type (WT) or brassinosteroid-related mutant plants grown at 21 or 26°C (mean \pm s.d., n=25). **b,** Ratio of primary root length from plants grown at 26°C/21°C as in (a). (mean \pm s.d., n=3) **c,** Western blot analyses monitoring the phosphorylation state of BES1 in wild-type plants grown at 21 or 26°C using anti-BES1 antibodies. A root protein extract from plants treated with brassinolide (BL) is used as a control. A representative blot is shown. **d,** Ratio of phosphorylated BES1 (P-BES1) over BES1 levels (mean \pm s.d., n=4). Quantitative RT-PCR monitoring the brassinosteroid feedback-regulated *CPD* gene expression in roots from wild-type plants grown at 21 or 26°C (mean \pm s.d., n=2).

Temperature impacts on BRI1 levels

The mechanisms by which temperature impinges on BR signaling may be diverse. No significant changes in mRNA accumulation was observed in our whole-genome RNA-seq experiment for the major factors involved in BR signaling (e.g. *BRI1*, *BAK1*, *BES1*, *BZR1*) (Extended Data Fig. 3). We therefore analyzed the levels of BRI1 protein in roots from plants grown at 21 and 26°C. To this purpose, we took advantage of two isogenic transgenic lines expressing a functional fusion of BRI1 to the Yellow Fluorescent Protein variant mCitrine (mCit) under the control of its own promoter, in the wild-type or *bri1* background (Jaillais et al., 2011). In these lines, BRI1-mCit accumulates to the levels of endogenous BRI1 protein (Martins et al., 2015). Interestingly, BRI1-mCit protein accumulated to lower levels upon growth at elevated ambient temperature (Fig. 4a), while *BRI1* mRNA levels monitored by quantitative RT-PCR remained unaffected (Fig. 4b). This points to the existence of a post-transcriptional regulation of *BRI1* gene expression by elevated temperature. Confocal microscopy observations using similar detection settings for BRI1-mCit plants grown at 21 and 26°C confirmed the decrease in BRI1-mCit protein levels at higher growth temperature (Fig. 4c). BRI1-mCit levels were however not affected in hypocotyls from seedlings grown at 26°C compared to 21°C, although the hypocotyl dramatically elongated with warmer temperature (Extended Data Fig. 4a). The influence of temperature on the levels of several plasma membrane proteins, including the Plasma membrane Intrinsic Protein 2;1 aquaporin (PIP2;1) and the PIN-FORMED2 auxin efflux carrier (PIN2), was monitored to determine if the observed effect on BRI1 accumulation were specific. Neither PIP2;1 nor PIN2 levels were affected by elevated growth temperature showing that temperature specifically impacts on BRI1 levels to regulate root growth (Extended Data Fig. 4b, c). The effect of temperature on BRI1-mCit accumulation was also monitored with time after plants were transferred from 21°C to 26°C. The decrease of BRI1-mCit protein was first observed 12 hours after plants

were transferred to elevated ambient temperature growth conditions and was maximum after 36 hours (Extended Data Fig. 4d, e), suggesting that this response is rather slow. Regardless, and consistent with the drop in BRI1 levels in the root, plants grown at 26°C were hyposensitive to the root growth inhibitory effect of exogenously applied brassinolide (BL) (Fig. 4e).

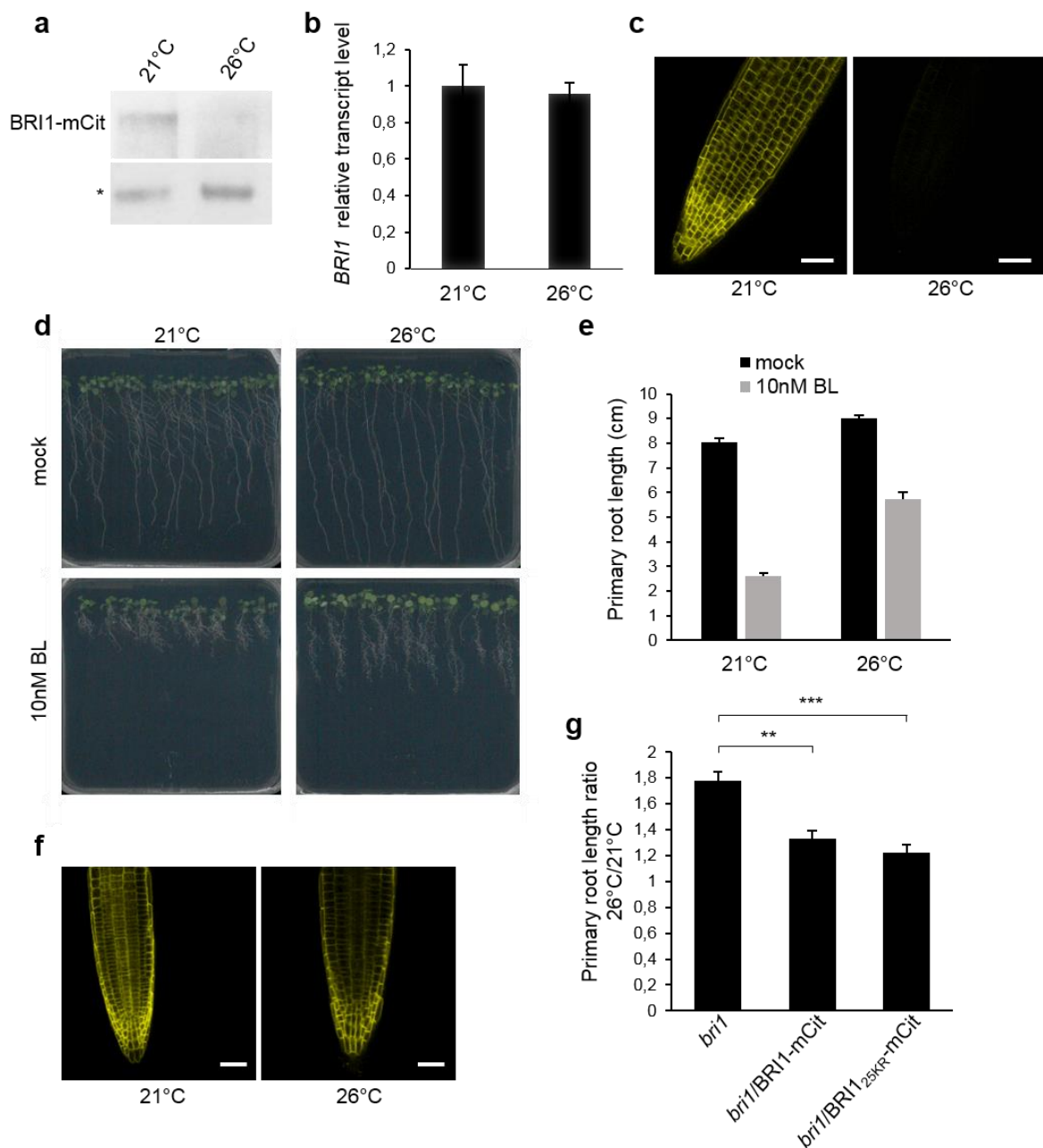


Figure 4. Elevated ambient temperature impinges on BRI1 levels and brassinosteroid

perception. a, Western blot analyses monitoring the accumulation of BRI1 protein in 15-day-old plants expressing a functional BRI1-mCitrine (mCit) fusion protein, under the control of the *BRI1* promoter, using anti-GFP antibodies. The asterisk represents a non-specific band used as loading control. A representative blot is shown. **b,** Quantitative RT-PCR monitoring *BRI1* gene expression in roots from wild-type plants grown at 21 or 26°C (mean \pm s.d., n=2). **c,** Influence of elevated ambient temperature on BRI1-mCit protein accumulation in roots. Similar confocal detection settings were used to compare plants grown at 21 and 26°C. Representative images are shown. Scale bar, 50 μ m. **d,** Phenotype of 15-day-old wild-type plants grown at 21 or 26°C and treated with mock or 10 nM brassinolide (BL). Representative images are shown. **e,** Primary root length of plants grown as in **(d)** (mean \pm s.d, n=25). **f,** Influence of increased growth temperature on BRI1-mCit and its non-ubiquitinatable BRI1_{25KR}-mCit variant. Similar confocal detection settings were used to compare the effect of temperature. Representative images are shown. Scale bar, 50 μ m. **g,** Ratio of primary root length from *bri1*/BRI1-mCit and *bri1*/BRI1_{25KR}-mCit plants grown at 21 and 26°C for 15 days (mean \pm s.d., n=3).

We recently demonstrated that BRI1 undergoes K63 polyubiquitin-dependent endocytosis and degradation (Martins et al., 2015). A functional but non-ubiquitinatable BRI1_{25KR} mutant version is more stable at the cell surface and fails to reach the vacuole due to defective intracellular sorting (Martins et al., 2015). To evaluate if BRI1 ubiquitination plays a role in the decrease of BRI1 protein levels and in the root responses to elevated ambient temperature, we subjected BRI1_{25KR}-mCit-expressing plants to growth at 21 or 26°C. As observed by confocal microscopy analyses and in contrast to the decrease in BRI1-mCit levels observed at 26°C (Fig. 4c), the non-ubiquitinatable BRI1 variant failed to respond to warmer growth conditions (Fig. 4f). This indicates that BRI1 ubiquitination mediates the drop in BRI1 levels at higher temperature. Consistently, transgenic *bri1* plants expressing BRI1_{25KR}-mCit under the control of its own promoter, which have been previously shown to be hypersensitive to BRs (Martins et al., 2015), displayed a slightly diminished response to elevated temperature than plants expressing its wild-type BRI1-mCit counterpart (Fig. 4g). The mild temperature response of the BR hypersensitive BRI1_{25KR}-mCit-expressing plants is explained by the lower accumulation of BRI1_{25KR}-mCit protein than BRI1-mCit in these specific lines (Martins et al., 2015). All together, these observations demonstrate that temperature impinges on BR signaling and BR-dependent root growth at the level of BRI1 ubiquitination.

Discussion

Here we demonstrated that root responses to elevated ambient temperature is associated with steroid-dependent reprogramming of root growth. The mechanisms identified in this study to translate the temperature input into growth changes is radically different from aerial parts where auxin is the major driver of growth. A recent study however, demonstrated that the auxin receptor TIR1 is stabilized in the root when plants are switched to higher growth temperature (Wang et al., 2016). Consistently, the *tir1afb2* auxin receptor mutant shows a

slower growth response following this change in growth conditions (Wang et al., 2016). Short-term changes in ambient temperature therefore likely influence root elongation through the rapid modulation of auxin responses, while steroid-dependent root growth control drives later stages of the response to temperature. The growth module PIF4-BES1/BZR1 enables hypocotyl cell elongation in response to heat (Jaillais and Vert, 2012; Oh et al., 2012). It is conceivable that temperature also crosstalks with BR signaling at the levels of BES1/BZR1 through interaction with a yet to be identified root-expressed PIF, providing more complexity in the mechanisms of ambient temperature control through BR signaling.

More importantly, our work offers the first glimpse on how climate change may impact plant underground organs. Warmth triggers the expression of a whole battery of defense mechanisms in the root against a wide variety of pathogens and herbivores. Setting up defense responses in the absence of threats is costly in energy, but may counteract the vigorous multiplication of bacteria, fungi and insects upon elevated temperature. Plant nutrition, and especially iron uptake, also appeared to be modulated by higher temperature. Considering the critical importance of iron for photosynthesis and its relatively low availability in soils, temperature changes in iron homeostasis may have significant impact on plant yield. Regardless, the rationale for plant roots growing deeper is unclear but may underlie the plant search for water often associated with warmth. Although the molecular basis of elevated temperature responses is only emerging, it becomes increasingly important to obtain a clear picture of what the agriculture of tomorrow may look like to anticipate negative effects of temperature on plant physiology.

Methods

Plant materials and growth conditions

The mutants and transgenic lines used in this study have been described previously: *pif4*, *pif4pif5* (Lorrain et al., 2008), *taa1* (Tao et al., 2008), *bri1* knock-out (Jaillais et al., 2011), *bes1-D* (Yin et al., 2002), *tir1* (Ruegger et al., 1998), *tir1afb2* (Dharmasiri et al., 2005), *yuc8* (Chen et al., 2014), *CYCB1;1::GUS*, *DR5::GUS*, *DR5::GFP* (Gonzalez-Garcia et al., 2011), *bri1/BRI1-mCit*, *BRI1-mCit* (Jaillais et al., 2011), *bri1/BRI1_{25KR}-mCit* and *BRI1_{25KR}-mCit* (Martins et al., 2015). Seeds were sterilized, stratified, and germinated on solid agar plates containing half-strength Linsmaier and Skoog medium without sucrose. After stratification, plates were incubated in growth chambers under long day conditions (16h light/8h dark, 90 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$) at 21 or 26°C for 15 days before phenotypic analyses were made.

Root growth parameters

Quantitative measurements of primary root length were performed on scanned images of seedlings using the Fiji image software (<http://fiji.sc/>). The root meristem size was determined using propidium iodide, as previously described (Alassimone et al., 2010). Meristem size was defined as the distance between the quiescent center and the meristem boundary where the cortical cell is twice the size of the immediately preceding cell. Cell divisions in root tips were visualized using plants expressing β -glucuronidase (GUS) under the control of the *CYCB1;1* promoter. GUS staining procedure was performed exactly as previously described (Gonzalez-Garcia et al., 2011). The size of fully elongated differentiated cells was determined by measuring the length of the first epidermal cells showing a root hair bulge.

For all root growth parameters, 25 plants seedlings were used per treatment or genotype. Experiments were done in triplicates.

Whole genome RNA profiling

Roots from plants grown at 21 or 26°C for 10 days were collected and total RNA extracted using the RNEasy kit and DNase treatment according to the supplier's instructions (www.qiagen.com). RNA-seq experiment were carried out at the POPS transcriptomic facility of the Institute of Plant Sciences - Paris-Saclay, thanks to IG-CNS Illumina HiSeq2000 privileged access to perform paired-end (PE) 100bp sequencing. RNA-seq libraries were prepared according to the TruSeq_RNA_SamplePrep_v2_Guide_15026495_C protocol (www.illumina.com). The RNA-seq samples were sequenced in paired-end, with a sizing of 260bp and a read length of 100 bases. Six samples per lane of HiSeq2000 were sequenced, using individual bar-coded adapters, and yielded approximately 25 millions of PE reads per sample.

Bioinformatic analyses

To facilitate comparisons, each RNA-Seq sample followed the same pipeline from trimming to count of transcript abundance. Read preprocessing criteria included trimming library adapters and performing quality control checks using FastQC (Version 0.10.1). The raw data (fastq) were trimmed for Phred Quality Score > 20, read length > 30 bases and sort by Trimming Modified homemade fastx_toolkit-0.0.13.2 software for rRNA sequences. Bowtie version 2 was used to align reads against the *Arabidopsis thaliana* transcriptome (Langmead and Salzberg, 2012). The 33602 mRNAs were extracted from TAIRv10 database with one isoform per gene to avoid multi-hits (Lamesch et al., 2012). The abundance of mRNAs was calculated by a local script which parses SAM files and counts only paired-end reads for which both reads map unambiguously to the same gene. According to these rules, around 98% of PE reads aligned to transcripts for each sample. Gene expression was analyzed using the EdgeR package (Version 1.12.0) (McCarthy et al., 2012), in the statistical software 'R'

(Version 2.15.0)(R Development Core Team, 2005). Gene ontology (GO) analyses were performed using the AgriGO portal (Du et al., 2010).

Data Deposition

RNA-seq data were deposited at Gene Expression Omnibus (accession #GSE XXXXX; www.ncbi.nlm.nih.gov/geo/) and at CATdb databases (Project: NGS_2015_05_HIGH_TEMPERATURE_ROOT; <http://urgv.evry.inra.fr/CATdb/>) according to the “Minimum Information About a Microarray Experiment” standards.

Auxin content

For quantification of free IAA, wild-type plants were grown at 21 or 26°C for 15 days. Roots were isolated, weighed, and measurements were performed as described previously (Ljung et al., 2005; Andersen et al., 2008), using eight biological replicates.

Western blot

Detection of BES1 and BRI1-mCit protein levels using anti-BES1 and anti-GFP antibodies, and was performed as previously described (Martins et al., 2015).

Quantitative real time PCR

Total RNA was extracted from roots using the GeneJet Plant RNA purification kit (ThermoFischer, www.thermofischer.com). First-strand cDNA was synthesized from 2 µg of total RNA using the RevertAid Reverse Transcriptase (ThermoFischer, www.thermofischer.com). Primer design was carried out using the Primer3 software (<http://frodo.wi.mit.edu/gate1.inist.fr/cgi-bin/primer3/>). Primer combinations showing a minimum amplification efficiency of 90% were used in real-time RT-PCR experiments

(Supplementary Table 5). For transcript normalization, we used the well-established At1g13320 reference gene encoding a protein phosphatase 2A subunit (Czechowski et al., 2005). Real-time RT-PCR amplifications were performed using the Light Cycler Fast Start DNA Master SYBR Green I kit on a Light Cycler apparatus according to manufacturer's instructions (Roche, www.roche.com). Cycling conditions were as follows: 95°C for 10 min, 40 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 15 sec. PCR amplification specificity was checked using a dissociation curve (55-95°C). A negative control without cDNA template was included for each primer combination. Experiments included two technical replicates performed on two independent cDNA synthesis derived from the same RNA, and two independent biological replicates. Ratios were done with constitutive controls for gene expression to normalize the data between different biological conditions.

Confocal microscopy

Plant samples were mounted in water and viewed on a Leica TCS SP2 confocal laser scanning microscope (<http://www.leica-microsystems.com/home/>). For imaging GFP and propidium iodide, the 488-laser line was used. Excitation of mCitrine used the 514-nm laser line. Laser intensity and detection settings were kept constant in individual sets of experiments to allow for direct comparison of protein levels. Quantification of total fluorescence intensity in roots was performed using the Leica Confocal Software.

Statistical analyses

Statistical significance of the biological parameters was assessed using t-test (two genotypes/conditions) or the one-way ANOVA (three genotypes/conditions and more) ($P < 0.05$). Quantification of western blots and auxin content experiments used the non-parametric Mann-Whitney (two genotypes/conditions) or Kruskal-Wallis (three

genotypes/conditions and more) tests ($P < 0.05$). Statistical analyses were performed with the software GraphPad Prism.

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Author Contributions

S.M. and G.V. designed the experimental strategy and analyzed data. A.C. performed q-PCR analyses. K.L. measured auxin content. S.H. performed the RNA-seq experiments. S.M. and G.V. wrote the manuscript. All authors commented on the manuscript.

Author information

We declare on competing financial interests

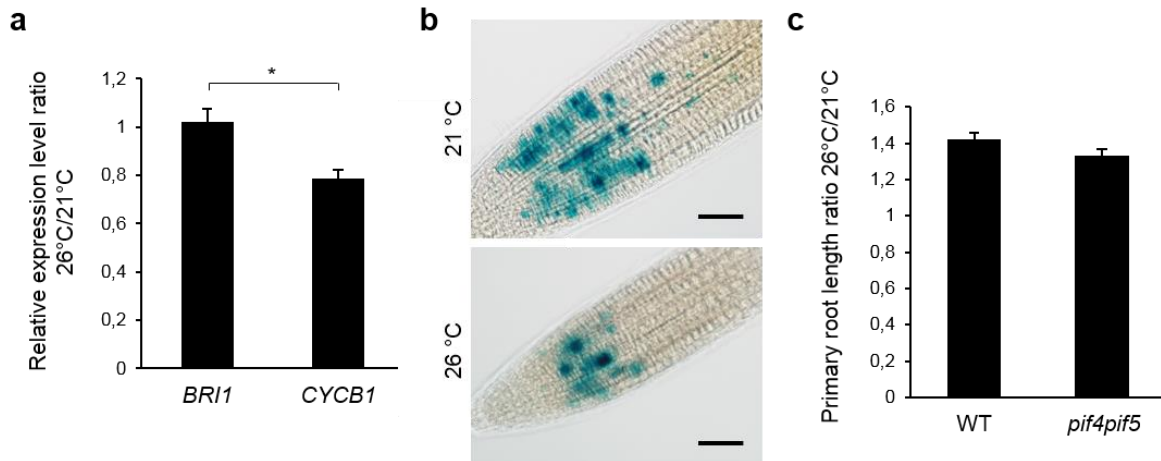
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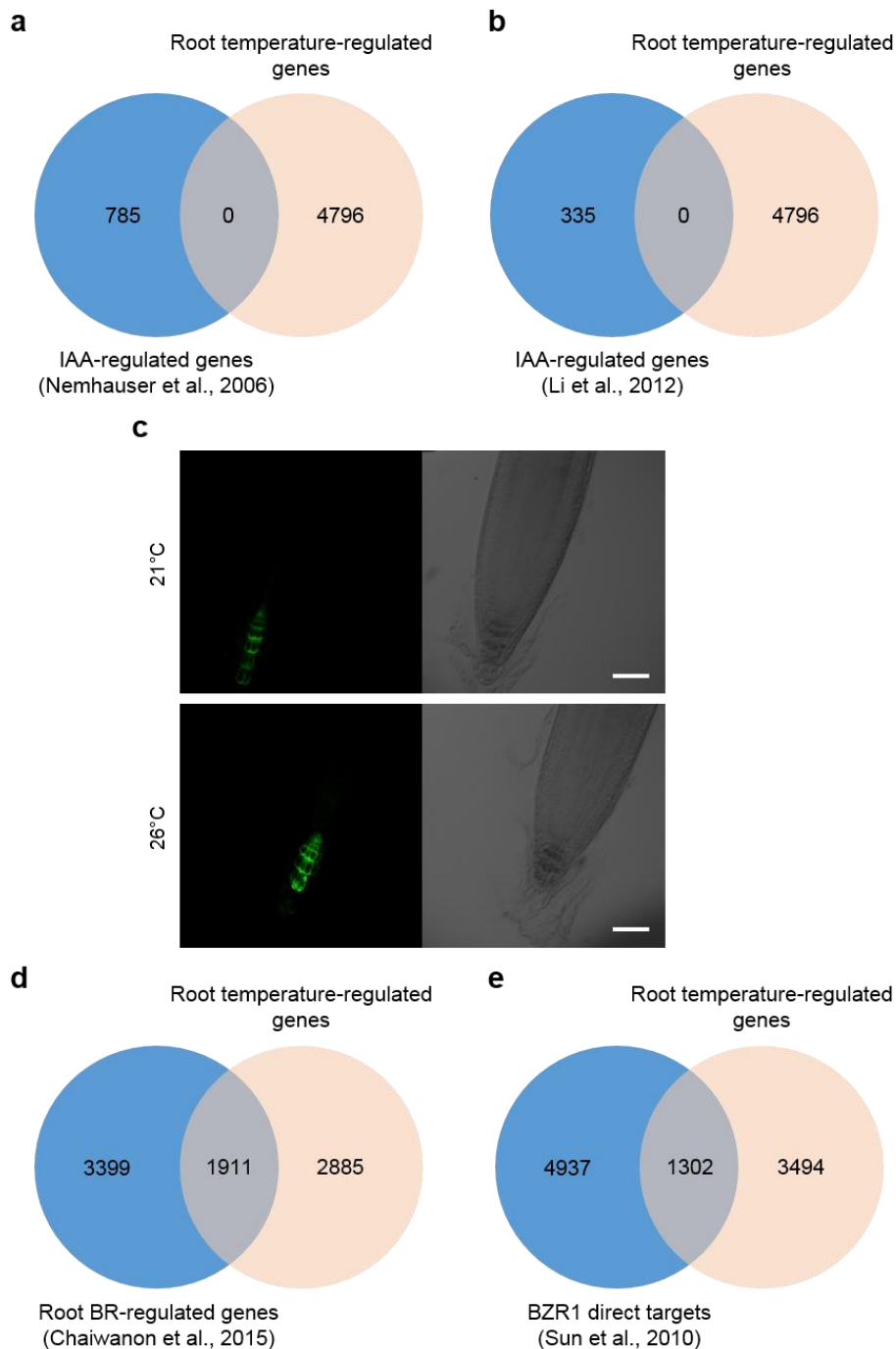
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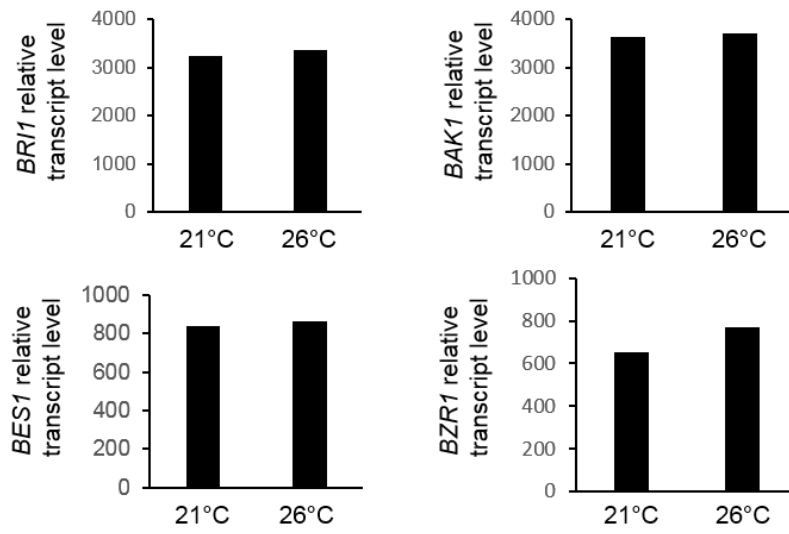
Extended Data



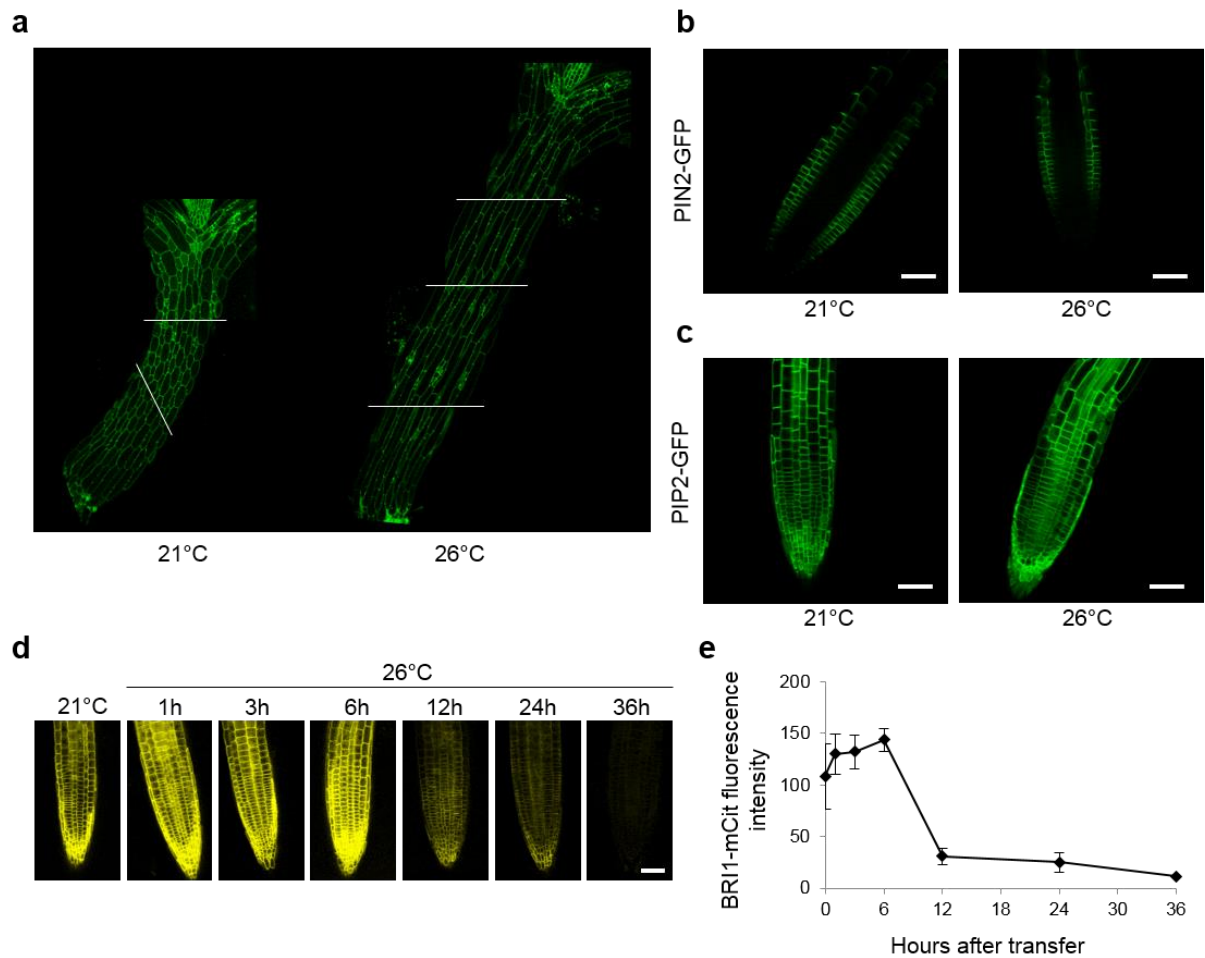
Extended Data Figure 1. **a**, Quantitative RT-PCR monitoring endogenous *BRI1* and *CYCB1;1* gene expression in roots from wild-type plants grown at 21 or 26°C (mean \pm s.d., n=2). **b**, Histochemical staining of the *CYCB1;1::GUS* cell cycle reporter line grown at 21 or 26°C. Representative images are shown. Scale bar, 50 μ m. **c**, Ratio of the primary root length from wild-type and *pif4pif5* double mutant plants grown at 26°C over 21°C (mean \pm s.d., n=25).



Extended Data Figure 2. **a, b**, Venn diagram representing the overlap between elevated ambient temperature-regulated genes and auxin-regulated genes (**a**), or genes regulated by the shade avoidance auxin-dependent process (**b**). **c**, Influence of elevated growth temperature on auxin responses monitored by the DR5::GFP synthetic auxin reporter. Similar confocal detection settings were used to compare the two growth conditions. Scale bar, 50 μm. **d, e**, Venn diagram representing the overlap between elevated ambient temperature-regulated genes and brassinosteroid-regulated genes in roots (**d**), or genes directly regulated by the binding of the BR-regulated downstream transcription factor BZR1 (**e**).



Extended Data Figure 3. Gene expression profiles of *BRI1*, *BAK1*, *BES1* and *BZR1* genes in roots from wild-type plants grown at 21 or 26°C (FDR<0.05). The data was extracted from the RNA-seq experiment.



Extended Data Figure 4. **a**, Reconstitution of BRI1-mCitrine fluorescence in hypocotyls from plants grown at 21 or 26°C. Similar detection settings were used to directly compare the effect of temperature on BRI1-mCit accumulation. **b**, **c**, Accumulation profile of PIN2-GFP (**b**) or PIP2;1-GFP (**c**) proteins in the primary root from plants grown at 21 or 26°C. Similar detection settings were used between the two growth temperatures. Representative images are shown. Scale bar, 50 μ m. **d**, Time-course analysis of BRI1-mCit levels in the root upon transfer from 21 to 26°C. Similar detection settings were used during the course of this experiment. Scale bar, 50 μ m. **e**, Quantification of BRI1-mCit levels upon transfer from 21 to 26°C growth conditions (mean \pm s.d., n=6).

Chapter 4:

General discussion

Chapter 4: General discussion

The role of ubiquitination in PM receptor trafficking and degradation by vacuole/lysosomes has been long studied in yeast and mammals. In plants, several recent reports have demonstrated the role of ubiquitination in PM transporters dynamics (Barberon et al., 2011; Kasai et al., 2011; Leitner et al., 2012; Lin et al., 2013). In the case of BOR1 and PIN2, ubiquitination appears to be associated with the intracellular sorting of the cargo. Non-ubiquitinatable forms of both PIN2 and BOR1 in which target residues have been mutated are still able to undergo internalization, as visualized by their accumulation in BFA compartments (Kasai et al., 2011; Leitner et al., 2012). In the case of IRT1, the non-ubiquitinatable IRT1_{K154,179R} mutant version accumulates at the cell surface, but whether this is the result of impaired internalization or forced recycling due to impaired targeting to the vacuole was not addressed in the original articles (Barberon et al., 2011; Barberon et al., 2014). Unpublished results from the lab indicates that, in contrast to PIN2 and BOR1, ubiquitination of IRT1 triggers internalization from PM (Dubeaux et al, Unpublished), similar to what has been described in yeast. In the case of the FLS2 receptor, ubiquitination has been linked to its degradation by the proteasome (Robatzek et al., 2006). The conclusions drawn for FLS2 mostly arose from the type of polyubiquitin chains (i.e. K48 polyubiquitin chains) that appear to decorate FLS2 and that have been associated with proteasome-mediated degradation. Although the implication of FLS2 ubiquitination in its internalization and sorting has not been reported so far, it is very likely that FLS2 is degraded by ubiquitin-mediated endocytosis like other receptors in yeast and mammals (Erpapazoglou et al., 2008; Lauwers et al., 2009; Huang et al., 2013), and like I demonstrated for BRI1 during my PhD (Martins et al., 2015). The main goals of my PhD was to study the role of ubiquitination of plant receptors and to study the interplay with signaling. For this, I used the BR hormone receptor, BRI1 as model

and examined how ubiquitination of BRI1 controls its trafficking (Chapter 2). I was also interested in understanding the implication of BRI1 ubiquitination in BR signaling (Chapter 2), and how ubiquitination of BRI1 has been coopted to crosstalk with other signaling pathway (Chapter 3).

4.1. Role of ubiquitination in BRI1 trafficking

4.1.1. Removing BRI1 from the plasma membrane: role of ubiquitination in internalization

To date, CME has been pointed as the main route to remove proteins from the plasma membrane in plants (Dhonukshe et al., 2007). However, the knowledge about the plant machinery involved in CME or in CIE remains restricted (Valencia et al., 2016). It is therefore not surprising that the mechanisms driving BRI1 internalization remain still unclear. Nevertheless, as mentioned before during my introduction, BRI1 undergoes CME (Di Rubbo et al., 2013; Gadeyne et al., 2014). BRI1 was indeed shown to interact with the subunit AP2A of the AP-2 complex (Di Rubbo et al., 2013). The TML protein from the plant TPLATE complex (TPC) seems to be important to mediate BRI1 endocytosis (Gadeyne et al., 2014). Accordingly, the downregulation TML reduce the accumulation of AP2A at the PM (Gadeyne et al., 2014). TPC and AP-2 have been reported to have overlapping but also distinct functions (Gadeyne et al., 2014). Therefore, more studies clarifying whether BRI1 internalization follows the three possible routes involving: AP2 alone, TPC alone, and a pathway involving a coordinated action of both AP2/TPC, is needed (Figure 1).

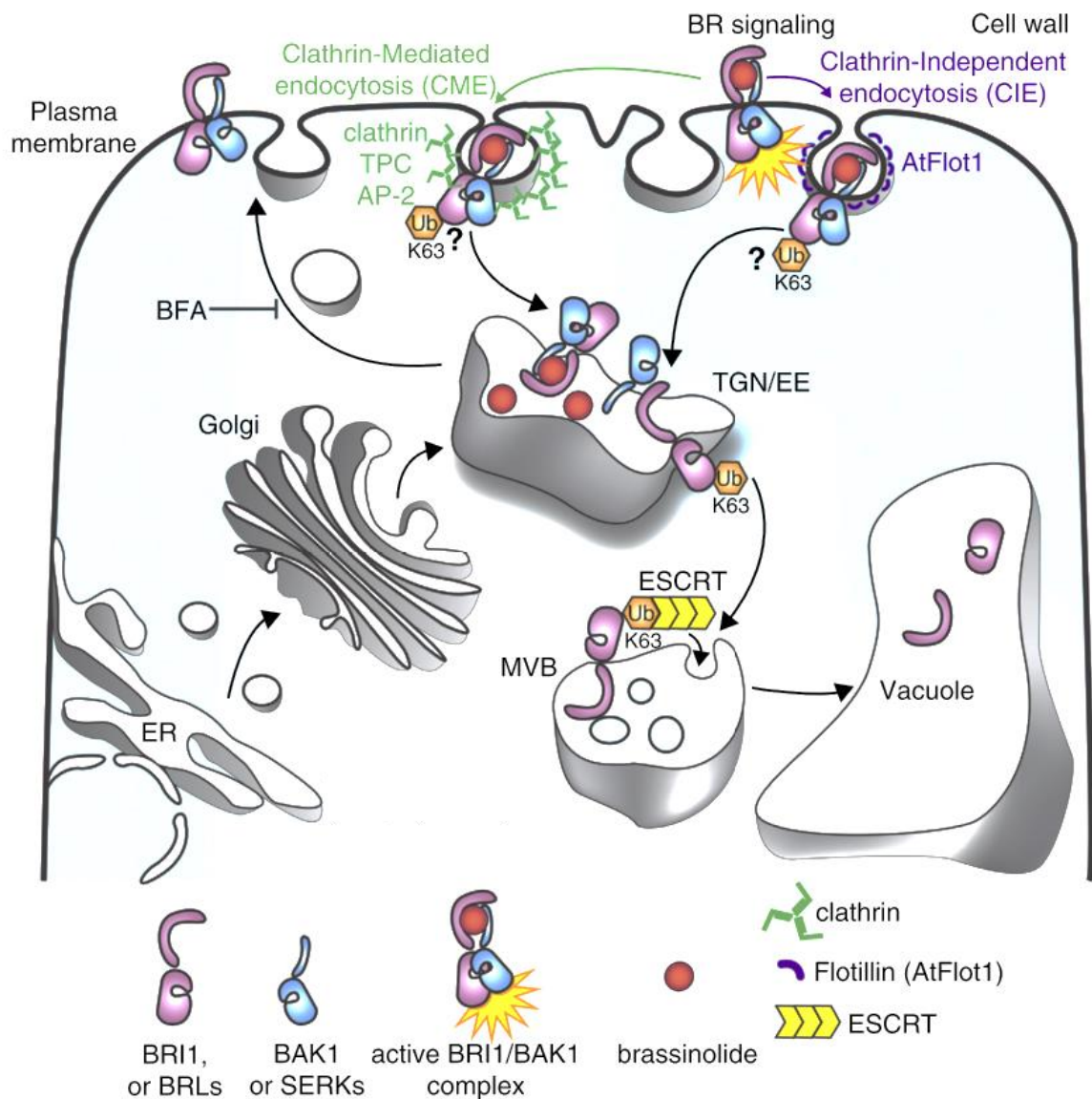


Figure 1. BRI1 trafficking to and from the cell surface (Adapted from (Jaillais and Vert, 2016)). ER, endoplasmic reticulum; TGN/EE, trans-Golgi network/early endosomes; MVB, multivesicular body; BRI1, Brassinosteroid-insensitive1; BAK1, BRI1-Associated-Kinase1; LRR, Leucine-Rich Repeat; ESCRT, Endosomal Sorting Ccomplex Required for Transport; TPC, TPLATE-Complex; AP-2, Adaptor Protein-2; Ub, K63-linked polyubiquitination. Question marks, ubiquitination may drive CME and/or CIE BRI1 endocytosis. Black arrows represent trafficking pathways, green and purple arrows indicate the recruitment of BRI1 into CME and CIE internalization pathways, respectively.

For instance, it would be interesting to perform TIRF imaging in order to evaluate the dynamics of the 3 proteins (AP2, TPC and BRI1) with different colors, and their possible co-

localization at single BRI1 endocytic events. Moreover, understanding the relative contribution of these pathways in BRI1 internalization as well as how BR binding or environmental conditions may impact on their respective role will be required to grasp the complexity and the roles of BRI1 internalization mechanisms.

During my introduction, I mentioned that tyrosine motifs are recognized by the μ -subunit of AP2 and are implicated in internalization by CME. In plants, the transporter BOR1 has been demonstrated to use several Tyr-based motifs for its polar delivery at the PM, and for its internalization and degradation in the vacuole upon high boron levels (Takano et al., 2010). It would be interesting to investigate the presence of tyrosine motifs in BRI1 and their possible roles in BRI1 dynamics. By analyzing the protein sequence of BRI1, 7 potential tyrosine motifs can be identified in the intracellular domain BRI1 (Figure 2).

mktfssffls vtlffsff slsfqaspsq slyreihqli sfkdvlpdkn llpdwssnkn pctfdgvter ddkvtsidls skplnvgsa vssllstg
leslflsnsh ingsvsgfkc sasltldls rmlsgpvtl ltlsgscgl kflnvssntl dfpgkvsagl klnslevldl sansisganv vgwvlsdgcg
elkhlaishn kisgdvdvsr cvnlefldvs snnfstgipf lgdcsalqhl disgnklsdgs fsraistete lklmnissnq fvgpipplpl kslqylslae
nkftgeipdf lsgacdtltg ldlsghfyg avppffgscs lleslalssn nfgelpmdt llkmrglkvl dlsfnfsgge lpsltnlsa sltldlssn
nfgspilpnl cqnkntlqe lylqngftg kipptlscs elvslhlsfn ylsgtipssl gsksklrdlk lwnlmegei pqelmyvktl etlildfndl
tgeipsglsn ctlnwisl nrrltgeipk wigrlenlai lksnnsfsg nipaelgder sliwldlntn lfnngtipaam fkqsgktaan fiagkryvvi
kndgmkkech gagnllefgg irseqlnrls trnncnitsr vygghtsptf dnngsmmfl dmsynmlsgyi pkeigsmpyl filnlghndi
sgsipdevgd lrgnlilds snkldgripq amsaltmlte idlsnnnlsg pipemgqfet fppakflnnp glcgyplprc dpsnadgyah
hqrshgrrpa slagsvamgl lfsfvcifgl lvgremrkr rkkkaelem yaeghngsgd rtannnwkl tgykealsin laafekpik lfadllqat
ngfhndslig sggfgdvka ilkdgsavai kklivhsqgg drefmaemet igkikhrlv pllgycvkd erllvymfk ygsledvlhd
pkkagvklw strkiaigs arglafhhn csphiihrdm kssnvllden learvsdfgm arlmsamdh lsvstlagtp gyvppyyqs
frcstkgdvy sygvlllell tgrptdspd fgdnnlvgwv kqhaklrld vfdpelmked paleiellqh lkvavacldd rawrrptmvq
vmamfkeiqq gsgidsqsti rsiedggfst iemvdsike vpegkl

▬ predicted signal peptide	▬ trans-membrane domain
— unassigned regions	▬ juxta-membrane region
▬ LRR (101 – 586; 680 – 750)	▬ kinase domain
▬ LRR 21 (methionine-rich repeat) (656 – 679)	▬ C-terminal extension
▬ island domain	▬ potential Tyr-based motives

Figure 2. Protein sequence of BRI1. The potential Tyr-based motifs Yxx ϕ (where ϕ is a bulky hydrophobic amino acid and x any aa) are highlighted in pink.

Introducing point mutations in the tyrosine of this motifs together or individually would allow to investigate the trafficking of BRI1. One should however pay attention: i) not to disrupt the BRI1 kinase activity doing so, and ii) not to abolish important phosphorylation events on tyrosine residues in BRI1. Visualization of BRI1 mutant version for the tyrosine residues by TIRF on CME related mutants can give insights about the role of such motifs in internalization of BRI1 via CME. Possibly, BRI1 impaired for its internalization would affect BR signaling in a similar manner as the non-ubiquitinatable BRI1.

More recently, CIE has been also shown to play a role in BRI1 internalization. BRI1 was indeed shown to co-localize with AtFlot1 flotillin, which is involved in the formation of PM microdomains (Wang et al., 2015). In agreement, the pace of BRI1 internalization is affected in plants weakened in CIE (Wang et al., 2015). BR levels trigger different types of internalization (Wang et al., 2015). It was suggested that in normal conditions and levels of BRs, BRI1 mostly undergoes CME. However, exogenous application of BRs enhances the co-localization with AtFlot1 and the contribution of CIE (Wang et al., 2015). It is possible that

high levels of BR can promote endocytosis and degradation of BRI1 in an ubiquitin-dependent manner, similar to what happens with EGFR. It is suggested that CME is responsible for EGFR internalization with little rate of degradation at normal levels of EGF, while high levels of ligand promote CIE and pronounced degradation (Sigismund et al., 2005; Sigismund et al., 2008; Sigismund et al., 2013).

In the Chapter 2 of my PhD thesis and in the corresponding published article, I demonstrated a role for ubiquitination in the internalization of BRI1. Our results showed that BRI1 is K63-polyubiquitinated *in vivo* and that it plays a dual role in BRI1 endocytosis. BRI1 ubiquitination is one of many redundant mechanisms driving BRI1 internalization from the cell surface under standard conditions. However, it is absolutely essential for its proper intracellular sorting and targeting to the vacuole. As such, BRI1 ubiquitination is important to control the levels and the distribution of the BR receptor in the cell, and any interference with BRI1 ubiquitination leads to altered BR signaling output.

Such dual role of ubiquitin in endocytosis is reminiscent of what has been uncovered for EGFR. Although EGFR was initially proposed to be internalized using a monoubiquitin-dependent mechanism (Haglund and Dikic, 2012), several conflicting results arose in the literature. EGFR was shown to be ubiquitinated on 5 lysine residues in its intracellular domain with K63 polyubiquitin chains (Huang et al., 2006). Biochemical analysis demonstrated that mutations in these 5 lysines decrease ubiquitination of EGFR by approximately 70%. An EGFR mutant version mutated in these 5 lysines was still able to undergo internalization but was impaired in its ability to reach lysosomes (Huang et al., 2006). Since significant ubiquitination still occurred after mutation of 5 lysines, it was not possible to completely rule out a role of ubiquitination in EGFR internalization. Mutation in 15 lysines in EGFR kinase domain showed that ubiquitination was still not essential for

internalization of EGFR while absolutely required for lysosomal targeting (Huang et al., 2007). The limitation of mutating ubiquitin target residues is that it does not allow to discriminate between the roles of different ubiquitination forms. A chimeric EGFR fused at its C-terminus with the AMSH (associated molecule with the SH3 domain of STAM) DUB (deubiquitinating enzymes), showing specificity towards K63 polyubiquitin chains, was generated in order to decipher between the role of mono- versus K63 polyubiquitination (Huang et al., 2013). As a result, the EGFR-AMSH fusion accumulates as monoubiquitinated. EGFR-AMSH was internalized at normal pace into early endosomes but was strongly impaired for its lysosomal degradation (Huang et al., 2013). The most recent evidence argue for several internalization pathway for EGFR, ubiquitination being one of them. Indeed, mutation in 15 or 21 lysines of EGFR unraveled four different mechanisms of internalization dependent of CME: these mechanisms involve ubiquitination of the receptor kinase domain, the clathrin adaptor protein (AP)-2 complex, interaction with the adaptor protein GRB2 and possible acetylation of C-terminal lysine residues (Goh et al., 2010). These mechanisms are dependent of different adaptors and can function in a cooperative way (Goh et al., 2010). Overall, it appears that ubiquitination of EGFR has a dual role on EGFR endocytosis. It plays a role at the cell surface, but is absolutely required for proper targeting to the lysosomes and degradation.

In plants, di-monoubiquitination of IRT1 iron transporter has been shown to be important IRT1 internalization and its steady state accumulation in early endosomes (Barberon et al., 2011). Recent evidence of the lab indicates that K63 polyubiquitination of IRT1, in response to metal excess, triggers its targeting to the vacuole (Dubeaux et al, Unpublished). Although we demonstrated that BRI1 was K63 polyubiquitinated, it is possible that a pool of BRI1 is monoubiquitinated. Monoubiquitinated BRI1 would undergo internalization to early endosomes, and extension of monoubiquitin to K63 polyubiquitin chains would target BRI1

to the vacuole. No E3 ubiquitin ligase acting on BRI1 was reported so far, but in this model it is conceivable that different E3s act on BRI1 along the endocytic pathway. Therefore, one can say that complexity of BRI1 internalization is still far to be fully understood.

The connection between BRI1 ubiquitination and CME or CIE internalization mechanisms still remains to be shown. Therefore, it would be interesting to decipher which pathway is used by ubiquitinated BRI1. Ubiquitination of BRI1 may also involve CIE, as reported for EGFR when challenged with high EGF concentrations (Sigismund et al., 2005). Challenging both wild type BRI1 and non-ubiquitinatable BRI1 in different mutants related to CME or CIE with BRs and analyze the dynamics of BRI1 can give insights about the role of ubiquitination in both CME and CIE. It is also possible that both CME and CIE mechanisms involve ubiquitination, although at different residues in the intracellular domain of BRI1. In that sense, the non-ubiquitinatable BRI1_{25KR} mutant version appear not useful since impaired in all ubiquitination sites.

4.1.2. Sorting BRI1 to degradation by the vacuole

As mentioned above, whether ubiquitination functions in the internalization or the sorting of PM proteins is still a subject of debate. We suggested a dual role for ubiquitination of BRI1 in internalization and sorting to degradation by the vacuole. Similarly, ubiquitination of EGFR has been proposed to have such a dual role on EGFR dynamics (Haglund and Dikic, 2012).

K63 polyubiquitination was shown to be required for degradation of EGFR (Huang et al., 2013). The degradation of EGFR is dependent of the recognition of the Cbl E3-ligase that is responsible for the ubiquitination of the receptor (de Melker et al., 2001). Recently, a PM protein-based interactome using a split-ubiquitin screen identified a potential interaction between BRI1 and an uncharacterized E3 ligase (Jones et al., 2014). Future work will be need to establish the possible role of such E3 ligase in the post-translational modification of BRI1

and in BR responses. Another important factor in the fate of PM proteins is the presence of DUBs. For instance, depletion of the DUB USP9X (ubiquitin-specific protease 9X) significantly delayed EGFR internalization and trafficking (Savio et al., 2016). AMSH3 was identified as a major DUB that hydrolyzes K48- and K63-linked ubiquitin chains *in vitro* and *in vivo* in Arabidopsis (Isono et al., 2010). AMSH3 is important for proper protein trafficking and vacuole biogenesis. It would be interesting to investigate the role of AMSH3 in BRI1 ubiquitination and trafficking to the vacuole.

The ESCRT complex has also been important for correct target to degradation by the vacuole/lysosomes (Herberth et al., 2012). Consistently, BRI1 vacuolar targeting is affected in the mutant for the ALIX (apoptosis-linked gene-2 interacting protein) ESCRT-III associated protein (Cardona-Lopez et al., 2015). It is likely that ubiquitinated BRI1 is recognized by the ESCRT for proper target to the MVB and degradation in the vacuole. The involvement of other ESCRT components in BRI1 trafficking still needs to be unravel. The CHMP1A (Charged Multivesicular Body Protein/Chromatin Modifying Protein 1A) and CHMP1B were identified as ESCRT members involved in the trafficking to the vacuole of PIN1, PIN2 and AUX1 (Spitzer et al., 2009). It would be possibly worth it to investigate the role of CHMP1A and CHMP1B in BRI1 trafficking to the vacuole, by expressing BRI1 on the double mutant of this proteins.

A forward genetic screen using BRI1-mCitrine and confocal imaging can also help the identification of different mechanisms involved in internalization and degradation of BRI1 by the vacuole. Mutants with defects on BRI1 trafficking can identify adaptors for the CME, proteins involved in CIE, E3-ligases, DUBs and ESCRT members, depending on the mutant localization. It would be expected to visualize BRI1 trapped at the PM, in different types of endosomes or even at the tonoplast. The use of markers to confirm the type of endosomes (Geldner et al., 2009) in the mutants should be appropriated.

4.1.3. Different conditions impact BRI1 internalization and degradation

We identified that elevated environmental temperatures triggers the decrease in BRI1 protein levels in roots, likely through ubiquitination. Although we observed stabilization of the non-ubiquitinatable BRI1 mutant version at higher temperatures compared to wild type BRI1, we failed to observe an increase of BRI1 ubiquitination pattern in the two temperature conditions. It is possible that different lysine residues are ubiquitinated in BRI1 at 21°C or 26°C. These different lysines may be implicated in the interaction with different adaptors of endocytosis and therefore associated with different dynamic behavior and pathways driving BRI1 internalization, in resemblance to what happens in EGFR (Goh et al., 2010). The non-ubiquitinatable BRI1, generated by mutating 25 lysines in the intracellular domain of BRI1, appears unsuited to address this issues. The identification of ubiquitin sites in BRI1 by mass spec only revealed a single lysine as in vivo target of ubiquitination under standard conditions. Increasing the resolution of mass spec analyses is therefore required before investigating BRI1 ubiquitination sites at both temperatures. Alternatively, the lack of difference in BRI1 ubiquitination at 21°C and 26°C may be explained by changes of levels of ubiquitinated BRI1, such as ESCRT components. Also, as previously mentioned, mutation in several lysines of EGFR unraveled interactions with different adaptors of endocytosis and therefore associated with different dynamic behavior and pathways driving EGFR internalization (Goh et al., 2010). It was also shown that lysines on the C-terminal of EGFR were subjected to a new posttranslational modification involved in protein internalization: acetylation (Goh et al., 2010). Similarly to what happens to EGFR, the mutation of the 25 lysines of BRI1 can also be acetylated or be necessary for interaction with other adaptors of endocytosis.

4.2. Impact of temperature in BR signaling and root growth

During the second part of my PhD, I uncovered a role for BRI1 ubiquitination in the integration of BR signaling and environmental growth conditions. Elevated ambient temperature indeed appears to impact on BRI1 levels *via* ubiquitin-mediated endocytosis, thereby impacting on steroid-dependent root growth.

4.2.1. Impact of temperature in plant growth: hypocotyls vs roots.

The effect of high ambient temperature has mainly been studied on the aerial part of *Arabidopsis* (Wigge, 2013; Choi and Oh, 2016; Quint et al., 2016). The PIF4 transcription factor, originally characterized for its role in light signaling (Huq and Quail, 2002), was shown to be required for temperature response in hypocotyls (Koini et al., 2009). Loss-of-function *pif4* mutants are completely unable to elongate their hypocotyls at elevated ambient temperature, highlighting the central role of PIF4 in temperature response (Koini et al., 2009). PIF4 is known to control hypocotyl elongation (Bours et al., 2015; Pedmale et al., 2016), flowering time (Thines et al., 2014) and integrate different hormone signaling pathways (Stavang et al., 2009; Oh et al., 2012; Sun et al., 2012). PIF4 was therefore defined as a hub for integrating endogenous and exogenous cues (Wigge, 2013; Choi and Oh, 2016; Quint et al., 2016).

PIF4 and BZR1 are known to act synergistically to promote cell elongation in hypocotyls in the dark or upon heat (Oh et al., 2012). Also, the up-regulation of auxin biosynthesis is required for the PIF4-mediated hypocotyl elongation in response to warm temperatures (Franklin et al., 2011; Sun et al., 2012). Indeed, PIF4 was shown to interact with the promoter region of the *YUCCA8* (*YUC8*) auxin biosynthetic gene. Plants mutated in *yuc8* display short hypocotyl phenotypes at high temperatures, similar to *pif4* (Stavang et al., 2009; Sun et al.,

2012). Moreover, overexpression of *PIF4* in *yuc8* mutants fails to recover elongation of hypocotyls, consistent with *YUC8* acting downstream of *PIF4*. In the same sense, auxin levels were shown to be higher in plants overexpressing *PIF4* (Sun et al., 2012). Consistently, plants transferred to higher temperatures show an increase in IAA levels (indole-3-acetic acid, or auxin) in aerial parts. This phenomenon is not observed in *pif4* mutants (Franklin et al., 2011). Another auxin biosynthetic gene was also shown to be involved in high-temperature-mediated hypocotyl response. The expression of the gene *TAA1* (*Tryptophan Aminotransferase of Arabidopsis 1*) was reduced in *pif4* mutant, but was increased in wild-type plants that were transferred from normal conditions to higher ambient temperature (Franklin et al., 2011). Although the role of *PIF4* and auxin in aerial part response to elevated ambient temperature is well established, whether similar mechanisms are also acting in roots upon elevated ambient temperatures is still elusive.

The first evidence of auxin-mediated response to temperature in roots was only published very recently (Wang et al., 2016). The authors demonstrated the involvement of *TIR1/AFB2* (Transport Inhibitor Response1/Auxin Response F-box2) receptors and the *HSP90* (Heat Shock Protein90) in root growth temperature-mediated response (Wang et al., 2016). Inhibitors of *HSP90* affect hypocotyl elongation in response to an increase of ambient temperature. Moreover, the expression of the auxin reporter *DR5::GUS* is inhibited in the presence of the inhibitor of *HSP90* showing that *HSP90* is necessary for plant response to an increase of ambient temperature by affecting auxin homeostasis. BiFC (bimolecular fluorescence complementation) and biochemical studies indicate that *HSP90* interact with the *TIR1* receptor *in vivo*. Moreover, *TIR1* and *AFB2* auxin receptors were shown to be stabilized at transcription and protein levels (Wang et al., 2016). It is hypothesized then, that *HSP90* is required to maintain *TIR1* levels stable, probably protecting it from proteasome-mediated pathway. My results showing that BRs impinge on temperature-mediated root growth raise

the question of the respective roles of auxin and BRs in elevated ambient temperature response. Auxin and BRs are known to have synergistic effect in hypocotyl elongation (Nemhauser et al., 2004). I therefore addressed if, in my own conditions, both hormones were acting on temperature responses.

As previously reported during this thesis, we first observed that elevated temperature only trigger a very modest increase in DR5::GFP auxin reporter in our conditions, contrasting with what has been shown for hypocotyls (Bours et al., 2015). Second, mutants impaired in hypocotyl response to temperature failed to show any root response. Indeed, *pif4*, *pif5*, *pif4pif5*, *yuc8* and *taal* mutants display wild-type-like root phenotypes in response to warmth, which clearly indicates a different mechanism in roots and shoots to temperature-response. More surprising is the fact that in our hands, the *tir1/afb2* auxin perception mutant respond normal to warmth, in contrast to what has been published (Wang et al., 2016). Furthermore, in our conditions, plants grown at elevated temperature show a sharp decrease in root auxin content. Since warmth has been shown to stabilize TIR1 (Wang et al., 2016), we hypothesize that this stabilization compensate for reduced auxin levels, thereby yielding globally unaffected auxin response as visualized by DR5 or RNAseq analysis.

To reconcile these contrasting results, we hypothesized that the conditions used between the two studies may yield different growth responses. We are growing our plants constantly at 21°C or 26°C (to mimic temperature elevation from global warming for example) while Wang and colleagues grow plants at 22°C and transfer them to 29°C, therefore investigating the consequences of temperature changes. The difference in the conclusions drawn might therefore underlie a role of BRs at low elevated temperatures (26°C), while auxin would drive responses at higher temperatures (29°C). Alternatively, it is conceivable that auxin conveys short term responses to changes in growth temperatures while BRs would mediate the long

term response. To gain further insight into the reasons for these observed differences, we are currently testing the response of BR-related genotypes using the 22°C/29°C transfer setup.

The fact that the auxin content in roots is lower at high temperature whereas the shoot auxin content strongly increases is surprising. Indeed, the auxin required in roots was believed to be produced mostly in shoots and transported to roots by PINs to create auxin maxima and trigger root growth (Petricka et al., 2012). Inactivation of the five root-expressed *YUC* genes ((*YUC3*, *YUC5*, *YUC7*, *YUC8* and *YUC9*; *yucQ* mutant) leads to the development of very short primary roots (Chen et al., 2014). Overexpression of *YUC* specifically in shoots of *yucQ* mutants was not enough to rescue the root phenotype of *yucQ*, while triggering auxin overproduction phenotypes in shoots (Chen et al., 2014). This suggests that the shoot-derived pool of auxin is not crucial for root auxin responses, and is consistent with the fact that although temperature yields massive biosynthesis of auxin in shoots, roots are deprived of auxin.

4.2.2 Root growth and brassinosteroids

We demonstrated using several complementary approaches that BRs negatively impact on root growth responses to elevated ambient temperatures. Consistently, *bes1-D* mutants are resistant to temperature whereas *bri1* mutants seem to be insensitive. As mentioned during the introduction of this thesis, BRs were first known to promote cell elongation in hypocotyls (Li et al., 1996). In roots, recent studies about BRs unraveled new roles for BRs not only in cell elongation but also in cell differentiation (Gonzalez-Garcia et al., 2011; Hacham et al., 2011). At low dose, BRs treatment leads to longer roots, while higher concentrations leads to shorter roots (Gonzalez-Garcia et al., 2011). Consistently, insensitive BR mutants and plants showing constitutive BR responses both show shorter roots. This suggests that BRs have an optimal range of action, and may either promote or inhibit growth depending on BR concentration of

BR signaling activity. We reported that BRI1 disappears from the PM and that BR signaling is downregulated at high ambient temperatures, specifically in roots. To explain that longer roots occur at 26°C, we have to consider that in our conditions of growth at 26°C, BR responses may be supraoptimal. Under this assumption, lowering BRI1 levels may therefore lower BR signaling while increasing root length. Determining BR levels at 21°C and 26°C, as well as precisely determining root responses to exogenously applied BL may help better understanding how BRs control root growth upon warmth.

4.2.3. Integration of different regulatory levels at high ambient temperature.

As I mentioned before, PIF4 integrates multiple signaling response pathways, including temperature and BRs. PIF4 and BZR1 interact with each other in response to temperature promoting hypocotyl elongation (Oh et al., 2012). It is unlikely that *pif4* plays the same role in root response to temperature due to the lack of phenotype of *pif4* mutants in high ambient temperature. This argues for a different mechanism for root responses to elevated temperature.

As mentioned above, the chaperone HSP90 seems to have an important role in root growth response to high ambient temperatures (Wang et al., 2016). Interestingly, in normal Arabidopsis growth conditions, HSP90 was shown to interact with BES1 independently of its phosphorylation status (Lachowiec et al., 2013). The use of a HSP90 inhibitor not only diminish the interaction between HSP90 with BES1, it also leads to shorter hypocotyls in seedling grown in the dark (Lachowiec et al., 2013). Work in Arabidopsis T87 cultured cells suggested that HSP90 interact with BES1 preferentially in its hyperphosphorylated state. It was also suggested that BL application to the cells induce the formation of HSP90 complexes. Treatment of transgenic cells expressing BES1 with HSP90 inhibitor showed higher levels of *CPD* genes (Shigeta et al., 2014), similar to what we observed in Chapter 2 at high

temperatures, and opposing to what happens to plants treated with BL. This suggests another mechanism of BR regulation at high temperature via HSP90. However, how HSP90 control BES1 activity and BR genomic responses remains unclear. It would be interesting to test HSP90 inhibitors in *bes1-D* mutant plants and analyze their response to temperature to further investigate the mechanisms involved in BR-mediated responses to temperature in roots. It is therefore, possible that temperature impinges on BR signaling not only at the level of BRI1 but also further downstream in the signaling pathway. Unravelling the contribution of these different regulatory levels, either in time or space (cell type) may help better understand how plant respond to temperature.

Conclusion

The main goal of my PhD was to use BRI1 receptor to study ubiquitin-mediated endocytosis and to evaluate the interplay between endocytosis and signaling. Indeed, we successfully showed that BRI1 K63-polyubiquitination is important to control BRI1 dynamics and identify a dual role for ubiquitination in BRI1 internalization and sorting to the vacuole. The intracellular dynamics of BRI1 were shown to be important for the BR responses in plants. In particular, the degradation of BRI1 and BR signaling was shown to be important in root growth response to the increase of ambient temperatures. The similarities between EGFR and BRI1 signaling can be viewed as a blueprint for unravel the interplay between endocytosis and signaling. The development of new high-resolution techniques in plants, such as TIRF can offer new insights about the role of ubiquitination on CME and CIE of BRI1, also in the context of temperature responses in roots.

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Annex 1:

Probing activation/deactivation of the BRASSINOSTEROID INSENSITIVE1 receptor kinase by immunoprecipitation

This annex is in press as a book chapter:

Sara Martins, Grégory Vert and Yvon Jaillais Probing activation/deactivation of the
BRASSINOSTEROID INSENSITIVE1 receptor kinase by immunoprecipitation. (*in press*)

Probing activation/deactivation of the BRASSINOSTEROID INSENSITIVE1 receptor kinase by immunoprecipitation

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Summary

Brassinosteroids are plant sterol-derived hormones that control plant growth and development. The BR receptor complex is encoded by the BRASSINOSTEROID INSENSITIVE1 (BRI1) and members of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE family. BR receptor complex activation and deactivation uses different post-translational modifications and recruitment of partner proteins. In this chapter, we describe optimized immunoprecipitation protocols and variants for biochemical analyses of BRI1 post-translational modification and protein-protein interaction.

Key words: Brassinosteroids, BRI1, Immunoprecipitation, Ubiquitination, Phosphorylation, Protein-protein interaction.

1. Introduction

Brassinosteroids (BRs) are plant steroid hormones that play important roles in plant growth and development (Vert et al., 2005; Jaillais and Chory, 2010; Belkhadir and Jaillais, 2015). Mutants with defects in BR biosynthesis or signaling display severe developmental defects such as reduced seed germination, extreme dwarfism, photomorphogenesis in the dark, altered distribution of stomata, delayed flowering and male sterility (Vert et al., 2005; Belkhadir and Jaillais, 2015). BRs are perceived at the plasma membrane by a pair of receptor kinases including BRASSINOSTEROID INSENSITIVE 1 (BRI1) and members of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family (SERK1 to SERK5, SERK3 being also called BRI1-ASSOCIATED KINASE 1 (BAK1)) (Li and Chory, 1997; He et al., 2000; Wang et al., 2001; Li et al., 2002; Nam and Li, 2002; Albrecht et al., 2008; Hothorn et al., 2011; Gou et al., 2012; Santiago et al., 2013). The perception of BRs by the BRI1/BAK1 receptor complex triggers transphosphorylation between BRI1 and BAK1 kinase domains in the cytoplasm (Wang et al., 2005; Wang et al., 2008). Downstream BR signaling involves a succession of phosphorylation and dephosphorylation events with kinases and phosphatases that culminates in the dephosphorylation of BES1 (bri1-EMS-SUPPRESSOR1) and BZR1 (BRASSINAZOLE RESISTANT1) transcription factors and BR-responsive gene transcription (Vert and Chory, 2006; Kim et al., 2009; Tang et al., 2011; Belkhadir and Jaillais, 2015). In the absence of ligand or upon deactivation of the BR receptor complex, BRI1 kinase domain is bound by the BRI1 KINASE INHIBITOR 1 that prevents interaction with BAK1 and spurious activation of the pathway (Wang and Chory, 2006; Jaillais et al., 2011).

Activation or deactivation of the receptor complex relies on post-translational modifications and interaction with positive and negative regulators. Over the past decade, extensive

knowledge was gained on the phosphorylation of BRI1 (Wang et al., 2005; Wang et al., 2008; Oh et al., 2009; Oh et al., 2012; Oh et al., 2012; Wu et al., 2012; Bojar et al., 2014; Belkhadir and Jaillais, 2015). Several phosphorylation sites have been mapped or more recently identified by mass spectrometry analyses after ligand binding. The timing of phosphorylation after addition of BRs together with detailed analyses of mutant variants of BRI1 impaired for specific phosphorylation sites unraveled sites playing a positive and negative role on BRI1 activity (Wang et al., 2005; Wang et al., 2008; Oh et al., 2009; Oh et al., 2012; Oh et al., 2012; Belkhadir and Jaillais, 2015). In addition, BRI1 was demonstrated to be post-translationally modified by ubiquitination, and more specifically K63 polyubiquitin chains (Martins et al., 2015). Although mostly independent of ligand binding to the receptor complex, BRI1 ubiquitination appears to be an important mechanism to downregulate BRI1 activity. Identification of an ubiquitination site by mass spectrometry coupled to model-based prediction allowed the generation of a non-ubiquitinatable BRI1 variant (Martins et al., 2015). Such ubiquitination-defective BRI1 show slower internalization from the cell surface and vacuolar degradation, leading to increased plasma membrane levels and BR hypersensitivity phenotypes.

The analysis of BRI1 activation and deactivation mechanisms is mostly based on protein-protein interaction detection (e.g. interaction with BAK1 and BKI1) and post-translational modifications (e.g. phosphorylation, ubiquitination) (Wang and Chory, 2006; Wang et al., 2008; Oh et al., 2009; Jaillais et al., 2011; Jaillais et al., 2011; Fabregas et al., 2013; Martins et al., 2015). Both approaches rely on prior purification of BRI1 protein by immunoprecipitation (Jaillais et al., 2011; Jaillais et al., 2011; Martins et al., 2015). Although antibodies recognizing endogenous BRI1 protein may be used, most studies aiming at immunoprecipitating BRI1 use anti-tag antibodies targeting functional tagged fusions of

BRI1, such as for example functional BRI1-mCitrine (Jaillais et al., 2011; Jaillais et al., 2011; Martins et al., 2015), BRI1-Flag (Wang et al., 2005; Wang et al., 2008) or BRI1-6xHA (Jaillais et al., 2011). Immunoblot using anti-tag antibodies after immunoprecipitation is used to confirm the enrichment in BRI1 compared to the total input of protein extracts. The subsequent detection of partner proteins or post-translational modifications uses specific antibodies (anti-phosphoserine/threonine, anti-phosphotyrosine, anti-ubiquitin or antibodies targeting a given protein (e.g. native or tagged versions of BAK1, BRI1 or BKI1) (Wang et al., 2008; Oh et al., 2009; Jaillais et al., 2011; Jaillais et al., 2011; Martins et al., 2015). Subtle differences in the immunoprecipitation protocols must be considered according to the goal of the experiment. While detection of reversible post-translational modifications requires the use of specific inhibitors preventing removal of the covalently attached modification, it also requires more stringent solubilization and wash conditions to avoid co-purification of other proteins also modified and that may migrate at a molecular weight close to BRI1. Detection of protein-protein interaction however uses milder conditions to allow detection of partner proteins. Below we provide detailed protocols to investigate the activation and deactivation mechanisms of BRI1.

2. Materials

Prepare all the solutions with ultrapure water (resistivity of 18.2 M Ω ·cm at 25°C). Keep the reagents at room temperature (unless indicated otherwise). Follow the regulation for waste disposals.

2.1. Proteins extraction from plants and immunoprecipitation

Keep the solutions and material at 4°C unless indicated otherwise.

1. Mortar and pestle at room temperature to grind tissues.

2. 1 M Tris-HCl (pH 8.0). In a glass beaker mix about 50 mL of water and 12.11 g of Tris-base. Once the mixture is homogenized, adjust the pH to 8.0 by adding Hydrochloric acid (HCl). Make up the volume to 100 mL and store at 4°C.
3. 0.5 M Ethylenediaminetetraacetic acid disodium salt (EDTA) (pH 8.0). In a glass beaker with 400 mL of water, mix 93.06 g of EDTA and stir with a magnetic stirrer. Adjust the pH to 8.0 with sodium hydroxide (NaOH). Make up the volume to 500 mL and autoclave the solution at 120°C.
4. Sucrose buffer: 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.33 M sucrose. In about 50 mL of water, add 2 mL of 1M Tris-HCl (pH 8.0), 200 µL of 0.5M EDTA (pH 8.0), and 11.3 g of sucrose. Dissolve the sucrose with the help of a magnetic stirrer and make up the volume to 100 mL with water. Autoclave the solution at 120°C or filter sterilize. Keep at 4°C.
5. Protease inhibitors cocktail inhibitors.
6. Phosphatase inhibitors cocktail.
7. 20 mM N-Ethylmaleimide (N-EM) solution in sucrose buffer: dissolve 25 mg of N-EM in 100 µL of ethanol 100% (*see Note 1*) and dilute this solution in 10 mL of sucrose buffer.
8. Sucrose buffer for ubiquitination studies: add 100 µL of protease inhibitors cocktail, and the 100 µL of 20 mM N-EM solution into 10 mL of freshly prepared sucrose buffer (*see Note 2*).
9. Sucrose buffer for phosphorylation studies: add 100 µL of protease inhibitors cocktail, and one tablet of phosphatase inhibitors cocktail into 10 mL of freshly prepared sucrose buffer (*see Note 2*).
10. Miracloth.
11. Sorval Legend Micro 21R Centrifuge (Thermo Fisher Scientific company, USA).
12. 1M Tris-HCl (pH 6.8). Proceed as for the preparation of 1M Tris-HCl (pH 8.0) described in step 1, but adjust the pH to 6.8 with HCl.

13. 4x Laemmli buffer: 2.8 mL of water, 2.4 mL of 1M Tris-HCl (pH 6.8), 0.8 g of SDS, 4 mL of 100% glycerol, 0.01% of bromophenol blue and 1 mL of β -mercaptoethanol (β -ME). Keep it at -20°C.
14. 2 mL tissue grinder potter.
15. Lysis buffer: 150 mM NaCl, 1% Triton[®] X-100, 50 mM Tris HCl (pH 8.0). μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
16. 20% SDS: Prepare 100 mL of 20% SDS by weighing 20 g SDS in a beaker. Add 70 mL of ultrapure water and mix it gently. Adjust final volume to 100 mL with ultrapure water.
17. Rotating wheel.
18. Heated block: Eppendorf ThermoMixer[®] (Eppendorf AG, Hamburg, Germany).
19. Anti-GFP MicroBeads (*see Note 3*). μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
20. Anti-HA MicroBeads (*see Note 3*). μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
21. μ Columns: MACS[®] Separation Columns (MACS Milteny Biotec GmbH company, Germany).
22. Place the μ MACS[™] Separator on the MACS MultiStand (MACS Milteny Biotec GmbH company, Germany).
23. Wash buffer 1: 150 mM NaCl, 1% Igepal CA-630 (formerly NP-40), 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl (pH 8.0). This buffer is supplied with the μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).
24. Wash buffer 2: 20 mM Tris HCl (pH 7.5). This buffer is provided with the μ MACS[™] Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany).

25. Elution buffer: 50 mM Tris HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol. This buffer is provided with the μ MACS™ Epitope Tag Protein Isolation Kits (MACS Milteny Biotec GmbH company, Germany)

2.2 Immunoblots.

1. Nitrocellulose membrane.
2. Orbital Shaker.
3. Petri dishes or a small container/dish where the nitrocellulose membrane can fit with some liquid.
4. Blotting tweezer to hold the nitrocellulose membrane.
5. 10X TBS: 20 mM Tris-HCl, 500 mM NaCl. In about 800 mL of water, dissolve 24 g of Tris-base and 88 g of NaCl. Once the mixture is homogenized, adjust the pH to 7.4 by adding Hydrochloric acid (HCl). Make up the volume to 1 L and store at room temperature.
6. Tween® 20.
7. Washing solution (TBS-T): 1X TBS, 0.1 % Tween® 20 (*see Note 4*). Dilute 100 mL of 10X TBS in 800 mL of water. Add 1 mL of Tween® 20 and make up the volume up to 1 L. Store at room temperature.
8. Bovine Serum Albumin (BSA).
9. Blocking solution: TBS-T, 1% BSA. Weight 1 g of BSA and mix gently with 90 ml of TBS-T in a beaker with the help of a stirrer (*see Note 5*). Make up the volume to 100 mL with TBS-T and store at 4°C.
10. Primary antibodies: Anti-GFP-HRP conjugated monoclonal antibody (MACS Milteny Biotec GmbH Company, Germany), Anti-HA-HRP conjugated monoclonal antibody (MACS Milteny Biotec GmbH Company, Germany), Ubiquitin P4D1 Mouse monoclonal antibody (Cell Signaling Technology®, USA), Lys63-Specific Anti-Ubiquitin rabbit monoclonal

(Apu3 clone, Merck Millipore, France), Anti-phosphothreonine antibody (Cell Signaling Technology, Beverly, MA, USA), Anti-phosphotyrosine HRP-coupled monoclonal antibody (4G10, Millipore), primary antibody recognizing a downstream BRI1 partner or a tagged downstream partner (e.g. BAK1-mCherry (Jaillais et al., 2011; Jaillais et al., 2011) or BKI1-mCitrine (Jaillais et al., 2011)).

11. Secondary antibodies: Goat Anti-Mouse IgG (H + L)-HRP Conjugate, Goat Anti-Rabbit IgG (H + L)-HRP Conjugate.

12. Super Signal[®] West Dura Extended Duration Substrate (Thermo Scientific Company, USA).

13. Transparent office file sheet protector.

14. Chemiluminescence Imaging System.

3. Methods

3.1. Protein extraction from plants and Immunoprecipitation

Pre-chill down the material to be used and keep everything on ice or in the cold room (*see Note 6*).

1. Grind tissues in liquid nitrogen (*see Note 7*) and transfer with a pre-chilled spatula to 2 mL of ice-cold sucrose buffer completed with the desired inhibitors according to the post-translational modification analyzed. It is also possible to use fresh tissues and grind it directly in ice-cold sucrose buffer (*see Note 7*).

2. Centrifuge for 10 minutes at 5,000 g at 4 °C to remove cellular debris and transfer the supernatant into 2 mL Eppendorf tubes.

3. Repeat step 2 until the supernatant is clean (*see Note 8*).

4. Pre-heat a heat block to 95°C.
5. Pipet 50 µL of the total protein fraction to use as input of the immunoprecipitation, and add 15 µL of 4X Laemmli buffer.
6. Boil the input for 3 minutes at 95°C. Keep the input on ice if you are planning to proceed with western blot the same day or freeze in liquid nitrogen and keep at -80°C for later use.
7. In order to obtain the membrane protein fraction, centrifuge for 45 minutes at 20,000 g at 4°C and keep the pellet (microsomal fraction) (*see Note 9*).
8. Add 1 mL of lysis buffer to the pellet (*see Note 10*) and resuspend carefully by pipetting up and down (*see Note 11*).
9. Transfer the lysate to ice-cold potters and homogenize (*see Note 12*). Transfer the samples to new 1.5 mL tubes.
10. Solubilize the microsomal fraction on a rotating wheel at 4°C for 30 minutes (Figure 1).
11. Centrifuge the samples for 10 minutes at 20,000 g at 4°C to pellet non-solubilized microsomes. Transfer the supernatant into a new 1.5 mL tube. This fraction corresponds to solubilized microsomal proteins and will be used for BRI1 immunoprecipitation.
12. Quantify the samples and use 1 mg of proteins of the supernatant to perform the immunoprecipitation of BRI1.
13. Add 50 µL of Anti-Tag MicroBeads to the supernatant and mix well (Figure 1).
14. Incubate on ice for 30 minutes to allow the formation of the BRI1-tag-antibody complex.
15. Place the µColumn on the µMACS™ Separator and apply 200 µL of the Lysis buffer to equilibrate the column (*see Note 10*).
16. Pipette 75 µL of Elution buffer for each sample into a new 1.5 mL tube and boil it on the pre-heated 95°C block.
17. Apply the samples lysate onto the center of the column and let it run through by gravity flow.

18. Wash the column with 200 μ L of the Wash buffer 1. Repeat 4 times.
19. Wash the column with 100 μ L of the Wash buffer 2.
20. Apply 20 μ L of Elution buffer kept at 95°C onto the center of the column and incubate at room temperature for 5 minutes (*see Note 13*).
21. Place a new 1.5 mL collecting tube under the column.
22. Apply 50 μ L of Elution buffer kept at 95°C onto the center of the column and collect the eluates (*see Note 14*). Freeze samples in liquid nitrogen and keep them at -80°C, or proceed directly with immunoblotting.

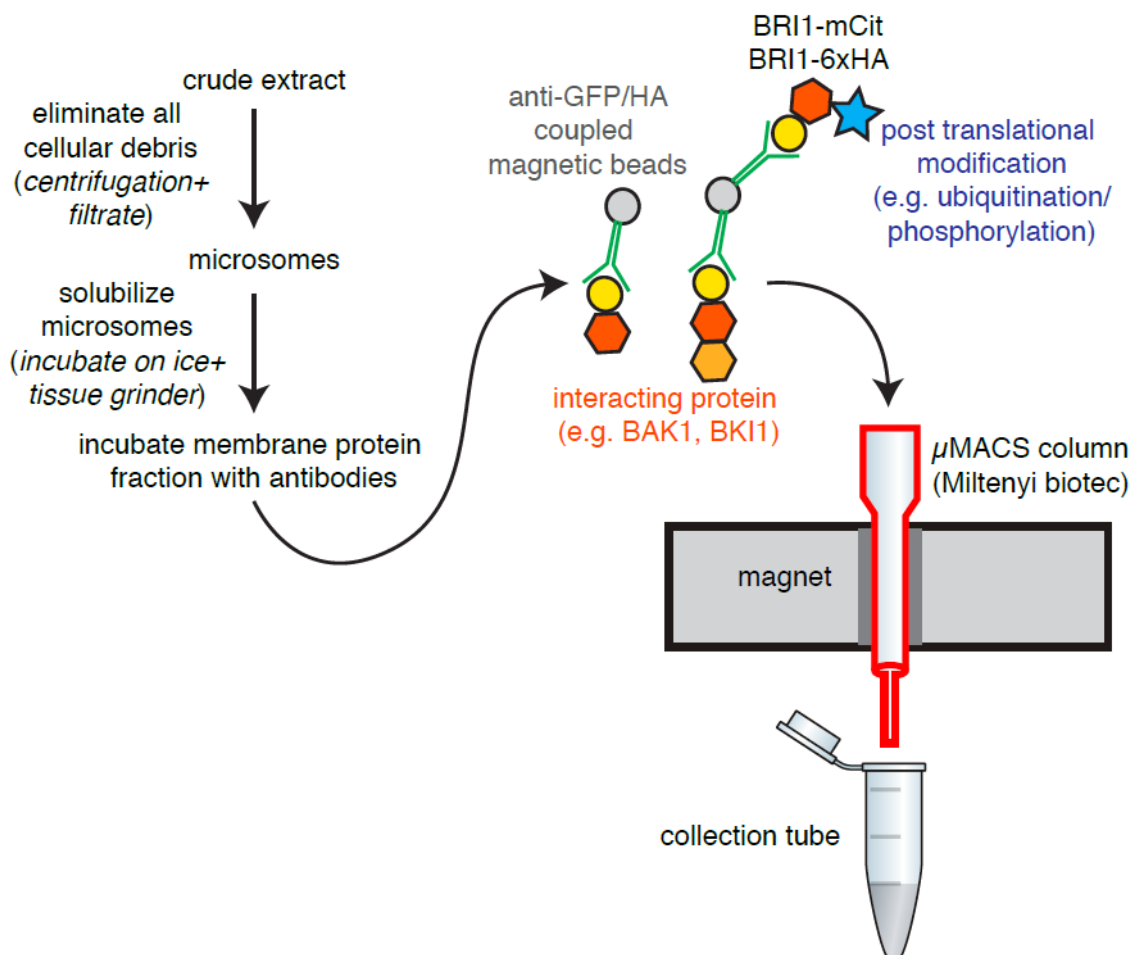


Figure 1: Protein extraction and purification scheme described in this protocol

3.2. Immunoblot.

Run the input and immunoprecipitated proteins in a standard SDS-PAGE, using a 7.5% separating gel (*see Note 15*). Transfer the proteins from the gel to a nitrocellulose membrane (*see Note 16*). It is important to handle the membrane with blotting tweezers and to keep it wet at all time. All procedures can be done at room temperature unless specified otherwise.

3.2.1. Detection of BRII-mCitrine or BRII-6xHA by western-blot

1. Place the nitrocellulose membrane in a petri dish and add the blocking solution until it covers the membrane. Shake gently for 1 hour at room temperature on the orbital shaker.
2. Prepare the solution containing primary antibodies by adding Anti-GFP-HRP or Anti-HA-HRP antibodies in new blocking solution. Use 1 μ L of antibody to 5 mL of blocking solution.
3. Replace the blocking solution with antibody solution. Incubate for 1 hour shaking gently on the orbital shaker (*see Note 17*).
4. Discard the antibody solution.
5. Wash the membrane 3 times for 10 minutes in washing solution under gentle agitation.
6. Discard the washing solution.
7. Apply the Super Signal[®] West Dura Extended Duration Substrate as indicated by the manufacturer and incubate for 5 minutes.
8. Place the membrane between a transparent office file sheet protector.
9. Proceed to signal detection using a chemiluminescence imaging system.

3.2.2. Detection of BRII ubiquitination, phosphorylation or co-immunoprecipitated proteins

1. Place the nitrocellulose membrane in a petri dish and add the blocking solution until it

covers the membrane and shake gently during 1 hour at room temperature on the orbital shaker (*see Note 17*).

2. Prepare the solution with the primary antibody in new blocking solution. Use the following dilutions: anti-P4D1 1:2,500; anti Apu3 1:2,000; anti-pThr 1:1,000, anti. Anti-pTyr 1:5,000.

Ee Figure 2 for an example of western blot with anti-GFP and anti-Ubiquitin antibodies.

3. Discard the blocking solution and add the antibody solution. Incubate for 1 hour and shake gently on the orbital shaker.

4. Discard the antibody solution.

5. Wash the membrane 3 times for 10 minutes in washing solution under gentle agitation.

6. Discard the washing solution.

7. Prepare the HRP-conjugated secondary antibody in blocking solution (dilution 1:20,000).

8. Wash the membrane 3 times for 10 minutes in the washing solution with gentle shaking.

9. Discard the washing solution.

10. Apply the Super Signal[®] West Dura Extended Duration Substrate according to the manufacturer instruction and incubate for 5 minutes.

11. Place the membrane between a transparent office file sheet protector.

12. Proceed with signal detection using a chemiluminescence imaging system.

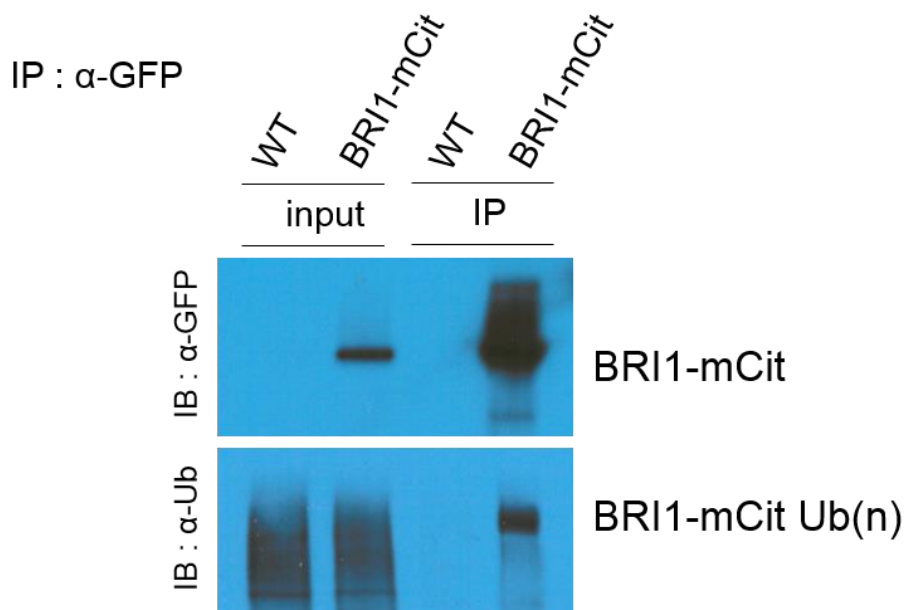


Figure 2: Example of western blots obtained following BRI1-mCitrine immunoprecipitation. Immunoprecipitation was performed using anti-GFP antibodies on solubilized protein extracts from wild-type and transgenic plants expressing BRI1-mCitrine and subjected to immunoblotting with anti-GFP and anti-ubiquitin antibodies. IP, immunoprecipitation; IB, immunoblotting

4. Notes

1. The N-EM does not dissolve in the sucrose buffer; therefore it is necessary to first dissolve it in 100% ethanol.
2. N-EM is an efficient deubiquitinating enzymes (DUBs) inhibitor, which prevents the potential deubiquitination of ubiquitinated proteins, including BRI1. Phosphatase inhibitors will prevent dephosphorylation of phosphorylated proteins. It is strongly advised to add the protease inhibitors cocktail, phosphatase inhibitor cocktail and N-EM extra-temporaneously to the sucrose buffer just before protein extraction and immunoprecipitation. The protease inhibitors cocktail is important to avoid protein degradation.

3. To perform the immunoprecipitation, we routinely use tagged form of BRI1, BRI1-6xHA (Jaillais et al., 2011) or BRI1-mCitrine (Jaillais et al., 2011; Jaillais et al., 2011; Martins et al., 2015), which can be recognized by the Anti-HA or Anti-GFP MicroBeads used in this protocol, respectively.
4. The TBS is recommended for proteins that are phosphorylated since it avoids a higher background on the nitrocellulose membrane during the Western-blot washes. The Tween® 20 is a detergent that will help to avoid non-specific binding to the membrane.
5. The BSA is a bovine protein that can be degraded at high temperatures mechanically, therefore it should be stored at 4°C. The BSA is important for blocking the membrane prior and during the incubation with the antibodies specific to the protein of interest in order to avoid non-specific binding of the antibody to the membrane.
6. Work and/or use a cold room (4°C). It's important that everything is cold at all time.
7. Keep the mortar and pestle at room temperature to avoid water condensation and the formation of ice crystals when in contact with the liquid nitrogen. Cool down the mortar and pestle with liquid nitrogen. Leave a small quantity of liquid nitrogen inside the mortar to help keep the tissue frozen while it is grinded. The best is to have at least 1g of tissue. Tissues must be kept in liquid nitrogen or at -80°C before being grinded. It is also possible to grind fresh tissue directly in the ice-cold sucrose buffer. This latter option is preferable when the plant material (e.g. seedlings) has been submitted to liquid treatments prior to extraction in order to avoid the formation of ice following incubation in liquid nitrogen.
8. These steps are critical since it is important to have a clean supernatant to avoid carrying over cellular debris to the membrane fraction. Usually, it takes 5 extra centrifugations at 4°C at 5,000 g for 10 minutes to obtain a clean supernatant. It is advisable to stay well clear of the pellet when pipetting the supernatant. Before the initial first two centrifugations, it is possible

to quickly filter out the bulk of the cellular debris by passing the solution through Miracloth.

It is important to always use ice-cold buffer and work at 4°C or on ice.

9. The supernatant resulting from the 20,000 g centrifugation corresponds to the fraction enriched in soluble proteins and the pellet contains the microsomal fraction, in which BRI1 is enriched.

10. The analysis of BRI1 post-translational modifications requires more stringent conditions, to strip off interacting proteins, by adding 0.1% SDS to the lysis buffer. In that case, the μ MACS column must be equilibrated with the SDS containing lysis buffer.

11. If the same sample is separated in two 2 mL tubes, pipette 500 μ L of lysis buffer into each tube. Resuspend the pellet carefully by pipetting up and down, and avoid pipetting the foam.

12. To avoid foaming while using the potter, we recommend to keep the pestle submerged in the sample.

13. The μ Column has a capacity of 20 μ L. When applied the first 20 μ L of Elution buffer is important to make sure that the Wash buffer 2 runs completely through the column to avoid residual salts and detergents from this Wash buffer on the eluate sample.

14. It is possible to improve the collection of the eluate sample, by placing parafilm on the top of the column and push it in order to create a little pressure and allow the eluate that is retained in the column to come out.

15. BRI1 is a 120 kDa protein, which reaches 160 kDa when coupled with GFP. For this reason we use a 7.5% polyacrylamide gels, but if low molecular weight interacting proteins are expected, it is also possible to use gels with higher polyacrylamide concentration.

16. In the case of BRI1, we recommend to use 20% methanol during the transfer.

17. It is important that the membrane is always in contact with the solution during the antibody incubation time. It is possible to adjust the speed of the agitation of the orbital shaker in order to optimize that.

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Title : Etude de l'endocytose dépendante de l'ubiquitine du récepteur aux hormones stéroïdes végétales BRI1 et sa régulation par la température

Mots clés : Brassinostéroïdes, endocytose, ubiquitination, BRI1, température

Résumé : Les brassinostéroïdes (BR) sont des hormones stéroïdes végétales qui jouent un rôle dans la croissance et le développement des plantes. Ils sont perçus à la surface cellulaire par le récepteur kinase BRI1 (BR insensitive 1) situé au niveau de la membrane plasmique. La voie de signalisation des BRs implique la *cis* et *trans*-phosphorylation de BRI1 et de son corécepteur BAK1 (BRI1 Associated Receptor Kinase 1) et aboutit à la déphosphorylation des facteurs de transcription BZR1 (Brassinazole Resistant 1) et BES1 (*bri1*-EMS-Suppressor 1) et l'activation des réponses génomiques aux BRs. Les mécanismes permettant la dé-activation du complexe de perception des BRs sont encore peu connus mais peuvent impliquer la déphosphorylation de BRI1, la fixation de régulateur négatif comme BKI1, et l'endocytose de BRI1. Mon travail de doctorat a consisté à étudier les mécanismes d'endocytoses et de dégradation de BRI1 et leurs régulations par les conditions environnementales.

L'ubiquitination est une modification post-traductionnelle impliquant l'attachement d'un polypeptide d'ubiquitine (Ub) sur une ou plusieurs lysines (K) d'une protéine cible. L'ubiquitine elle-même peut être sujette à l'ubiquitination, créant ainsi des chaînes de poly-ubiquitine (polyUb) qui peuvent adopter des topologies différentes selon le résidu K de l'ubiquitine impliqué dans la formation de la chaîne. Parmi ces chaînes de polyUb, la chaîne impliquant la lysine-63 (K63) est connue pour être impliquée dans la dégradation des protéines par endocytose chez les levures et les mammifères. Néanmoins, peu de choses sont connues sur l'endocytose dépendante de l'ubiquitine chez les plantes. Durant la première partie de ma thèse, j'ai démontré que BRI1 est modifié *in vivo* par des chaînes de polyUb K63 et j'ai pu identifier des sites putatifs d'ubiquitination dans BRI1.

En utilisant une forme artificiellement ubiquitinée de BRI1 ainsi qu'une forme non ubiquitinable de BRI1, j'ai montré que l'ubiquitination joue un rôle sur l'internalisation de BRI1 à la surface cellulaire et est essentielle pour son adressage à la vacuole. Par ailleurs, j'ai établi que la dynamique de la protéine BRI1 médiée par son ubiquitination joue un rôle important dans le contrôle des réponses des BR. La deuxième partie de ma thèse m'a permis de découvrir une connexion entre l'endocytose dépendante de l'ubiquitine de BRI1 et la réponse des plantes à une élévation de température. J'ai notamment montré que l'accumulation de la protéine BRI1 est réduite dans les racines lorsque les plantes sont cultivées à une température plus élevée (i.e. 26°C). Cependant, la forme non ubiquitinable de BRI1 ne répond pas à cette élévation de la température suggérant une implication de l'endocytose dépendant de l'ubiquitine dans de telles conditions. Cette déstabilisation de BRI1 observée à température plus élevée se traduit au niveau moléculaire par une inhibition de la voie de signalisation des BRs et une hypersensibilité à des traitements BRs exogènes. Les plantes répondent à une montée subite de la température par une augmentation de la longueur de l'hypocotyle, des pétioles et de la racine principale ; processus largement sous le contrôle de l'auxine. J'ai accumulé au cours de ma thèse des évidences génétiques et génomiques montrant que la déstabilisation de BRI1 et l'inhibition de la signalisation des BR à plus hautes températures contrôle l'élongation des racines indépendamment de l'auxine. En conclusion, les résultats obtenus indiquent que BRI1 intègre les informations de température et de signalisation des BRs pour ajuster la croissance des racines lors de variations des conditions environnementales.

Title : Ubiquitin-mediated endocytosis of the plant steroid hormone receptor BRI1 and regulation by elevated ambient temperature

Keywords : Brassinosteroids, endocytosis, ubiquitination, BRI1, temperature

Abstract : Brassinosteroids (BRs) are plant steroid hormones that play important roles on plant growth and development, and that are perceived at the plasma membrane (PM) by the receptor kinase BRI1 (BR insensitive 1). The perception of BRs by BRI1 triggers the *cis* and *trans*-phosphorylation of BRI1 and its co-receptor BAK1 (BRI1 Associated Receptor Kinase 1) initiating this way the BR signaling cascade that culminates with the dephosphorylation of the transcription factors BZR1 (Brassinazole Resistant 1) and BES1 (*bri1*-EMS-Suppressor 1), allowing these transcription factors to activate the transcription of thousands of BR-responsive genes. The mechanisms allowing the deactivation of the BR receptor complex are still elusive but may involve dephosphorylation, binding to negative regulators such as BKI1 and endocytosis. My PhD work focused on the endocytosis and the degradation of BRI1, and its regulation by environmental conditions.

Ubiquitination is a post-translational modification that consists of the attachment of ubiquitin (Ub) polypeptides to one or several lysine (K) of a target protein. Ubiquitin itself can undergo self-ubiquitination thereby creating polyubiquitin (polyUb) chains that can harbor different topologies depending on the lysine residue from ubiquitin involved in the chain formation. Among these, polyUb chains involving lysine-63 (K63) are known to be involved in the degradation of proteins by endocytosis in yeast and mammals, while very little is known in plants.

On a first part of my thesis, I demonstrated that

BRI1 is post-translationally modified by K63 poly-ubiquitin chains and I identified putative ubiquitination sites in BRI1. Using both artificial ubiquitination of BRI1 and the generation of an ubiquitination-defective BRI1, I showed that ubiquitination plays a role on the internalization of BRI1 from the cell-surface, and is essential for its vacuolar targeting. Furthermore, ubiquitin-mediated BRI1 protein dynamics was also shown to play an important role in the control of BR responses by stabilizing BRI1 at the PM.

The second part of the PhD uncovered a connection between BRI1 ubiquitin-mediated endocytosis and plant responses to elevated ambient temperature. I showed that BRI1 protein accumulation is lower at elevated temperature (i.e. 26°C) in roots, while the ubiquitin-defective form of BRI1 failed to respond, indicating a role of the BRI1 ubiquitin-mediated endocytosis in temperature responses. This temperature-mediated destabilization of BRI1 correlates with a downregulation of BR signaling and BR responses. Plants respond sudden rise in temperature by increasing their primary root length, a process that is under the control of auxin. Importantly, I accumulated genetic and genomic evidence that BRI1 destabilization and downregulation of BR signaling controls root elongation upon elevated ambient growth temperature, with little or no contribution of auxin. Altogether, our results establish that BRI1 integrates temperature and BR signaling to regulate root growth upon long-term changes in environmental conditions.