The mechanism of Arabidopsis shoot gravitropism mediated by LAZY1-BRXL4 interaction

By

Ximing Che

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The dissertation is approved by the following members of the Final Oral Committee:

Edgar P. Spalding, Professor, Botany
Marisa S. Otegui, Professor, Botany
Hiroshi Maeda, Professor, Botany
Simon Gilroy, Professor, Botany
Patrick H. Masson, Professor, Genetics

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Abstract

Gravitropism is a fundamental process that plants evolved to be successful on land. It guides growth to shape plant shoot and root architecture. *LAZY1* is an important player required for gravity response mechanism in Arabidopsis, but its specific role remains obscure. Mutations in *LAZY1* impair stem gravitropism and cause wide inflorescence branch angles. The LAZY1 protein resides at the plasma membrane and the nucleus LAZY1 contains an EAR motif associated with transcription repression and a nuclear localization signal. The plasma membrane pool is necessary and sufficient for setting branch angles. To investigate how LAZY1 performs its gravity related functions, we identified *BRXL4* as a LAZY1 interacting protein. The BRXL4-LAZY1 interaction occurred at the plasma membrane in plant cells, and not in the nucleus. Mutations in the C terminus of LAZY1 prevented the interaction. *BRXL4* is mainly expressed in the petiole of cotyledons and in the abaxial side of the cauline leaves. Opposite to *lazy1*, *brxl4* mutants displayed faster gravitropism and more upright branches. Overexpressing *BRXL4* produced strong *lazy1* phenotypes. These phenotypes are consistent with *BRXL4* reducing *LAZY1* expression or the amount of LAZY1 at the plasma membrane. LAZY1 mRNA is 3-fold more abundant in *brxl4* mutants and almost undetectable in *BRXL4* overexpressors. Furthermore, plasma membrane LAZY1 was higher and nuclear LAZY1 lower in *brxl4* mutants compared to wild type. LAZY1-GFP in *pro35S* lines formed bright spots in the nucleus and accumulated in the cytoplasm. To explain these results, we propose that BRXL4-LAZY1 interaction at the plasma membrane exposes the LAZY1 nuclear localization sequence, resulting in transport of LAZY1 to the nucleus where it suppresses its own transcription. Thus, BRXL4 modulates LAZY1 function to set architectural features such as branch angles.

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Chapter I

Introduction

I. Gravitropism overview

Plants use environmental signals such as gravity, light, or nutrients to modify their genetically programmed architecture. The modifications may increase fitness when the plant populations are experiencing various environmental stresses. Elucidating how environmental cues modify genetically programmed processes will reveal basic rules of plant life and enable new ways to modify plants for practical purposes. Among all the cues that shape plant architecture, gravity is one of the most profound and fundamental signals that guides the direction of plant growth, by a process known as gravitropism. Shoots generally grow upwards to get more light energy for photosynthesis, while roots grow downwards to better uptake water and nutrient (Morita. 2010). Lateral organs are typically not as close to vertical as the main plant axis. In adult *Arabidopsis thaliana* plants, depending on the light conditions, branches emerge from the inflorescence axis at an upright angle of 30-40°. Lateral roots emerge at an average downward angle of 30-40°. If the entire plant is tilted, the primary inflorescence stem, its branches, and the lateral roots will all reorient via gravitropism to achieve the preferred gravity set-point angle (GSA), a concept proposed by Digby and Firn (1995). Understanding how gravity signaling modifies growth is a major topic in plant biology and the central theme of this dissertation.

Modifications of plant architecture are prevalent and important in agricultural contexts. One of the traits that has been repeatedly adjusted by human is the angle of branches. Upright shoot gravitropism of trees and crops is indispensable for managing plantations and increasing yield, because these changes can allow more plants to be grown per unit of area and make harvesting more efficient (Dardick *et al*. 2013). Manipulating root gravitropism to create more robust root

system helps to overcome drought stress in the field (Uga *et al*.2013). There is also increasing space science research that aims to optimize the growth condition of plants in space, which only has microgravity and is overall stressful for plants to grow (Link *et al*., 2014; Medina *et al.,* 2021). Therefore, a better understanding how gravity signaling modifies plant architecture and growth is likely to improve the yields of agricultural products both on earth and in space.

Gravitropism is a complex and coordinated process under delicate genetic control. There are two types of gravitropic responses, negative gravitropism directs growth away from the Earth against gravity vector, which is typical of shoots whereas the opposite, positive gravitropism, is typical of roots (Dong *et al*., 2013). In order to achieve the preferred GSA, gravity needs to be perceived and sensed first, and then the gravity signal is translated to a physiological signal transmitted from the point of perception to a spatially distinct response region. In the end, the gravitropic response usually entails an asymmetric bending of the organ (Su *et al*., 2017). The curvature is produced by differential elongation of cells from two different sides and this differential elongation is very finely regulated by auxin distribution, a pivotal plant hormone involved in many growth and behavioral processes in plant life cycles and are essential for plant body development.

There has been intensive research focusing on plant gravitropism sensing mechanism, primarily in root tissue, which appears to be universal among all land plants. Removal of root cap physically or genetically was proved to be sufficient to abolish the gravitropic response in roots, but not necessarily root growth. (Barlow. 1974; Blancaflor *et al*., 1998). The root cap is a renewable tissue that is located at the tip of the root. It is made of central columella, lateral and tip cells. It is believed that one of the primary functions of the root cap is to protect

meristematic tissue, however, in the context of gravitropism, columella cells in the root cap that contain sedimentary amyloplasts is the most important and decisive site to sense gravity.

There is less research addressing the shoot gravitropism, because shoots do not share the same structure as roots; for example, there are no columella cells or a "shoot cap," and the specific treatment of shoot tissues to remove certain cells is significantly more difficult. But the fact that shoot endodermis also contains sedimentary amyloplasts as well as some genetic evidence suggest the essential function of endodermis in shoot gravitropism (Fukaki *et al*. 1998). *Shoot Gravitropism1* (*SGR1)* and *SGR7* genes were the very first genes identified that cause defects in the formation of the endodermis both in roots and in hypocotyls (Di Laurenzio *et al*. 1996). *sgr* mutants exhibit almost no gravitropic responses in the shoot while display normal gravitropism in root, despite abnormal development of endodermis in root. It is consistent with the fact that gravity sensing site in root is the columella cells not the endodermis (Fukaki *et al*. 1998).

It is logical that statholiths evolved to be the gravity sensing site because large starch containing amyloplasts would respond to gravity directly, that is, move towards the direction of gravity due to their higher density (Wayne and Staves. 1997). Displacement of the amyloplasts is transduced into biochemical signals, and there is a comprehensive intracellular trafficking machinery receives the signals and ultimately results in a trans-organ auxin gradient that achieves different growth rates. There are some nuances about the starch-statolith model, for instance, it appears that amyloplasts are not solely dependent on gravity to move, a dynamic actin-filament network also plays an indispensable role. Disturbed actin-filament network impairs gravity perception and in turn results in reduced gravitropic responses (Zheng *et al*. 2014; Morita. 2010).

What the intracellular trafficking machinery is and how it transduces the gravity signal still largely remain unknown. It is well characterized that there is a peripheral arrangement of endoplasmic reticulum (ER) in gravity sensing cells both in root and shoot (Morita 2010). One proposed secondary messenger, Ca^{2+}, is stored in the peripheral ER and can be released once interacting with sedimentary amyloplasts in statocytes cells. Statocytes were observed to display elevated levels of Ca^{2+} and upon gravistimulation, levels of inositol 1,4,5-trisphosphate (IP3), an activator of Ca^{2+} release, fluctuates at the bottom half of oat coleoptiles and Arabidopsis inflorescence stem (Perera *et al*. 2006).

Though many mysteries about the signal transduction phase remain to be uncovered, it is well understood that gravitropism is ultimately achieved by reoriented auxin transporters that redistribute auxin. One of the known players is PIN-FORMED3 (PIN3), an auxin efflux protein. In Arabidopsis root and hypocotyl, PIN3 will move to the new lowest side of the cell when subject to reorientation. This redistributes the normal flow of the hormone auxin to the lower side (Taniguchi *et al*. 2017). Other auxin transporters are also regulated and help to generate a gradient of auxin along the root or shoot during gravitropism.

Auxin usually promotes plant cell division and growth at lower concentrations; however, it drastically suppresses growth at higher concentrations. Notably, roots are significantly more sensitive to excessive auxin molecules than shoots. When plants are rotated 90 degrees and stimulated a gravitropic response, auxin will be redistributed and accumulated at the lower side of roots and shoots. The accumulated auxin will suppress root cell expansion at the lower side yet promote shoot cell expansion at the lower side to introduce tropism. Auxin controls cell growth and expansion by regulating the activity of plasma membrane pumps resulting in cell

wall acidification and cytoplasmic alkalinization. The establishment of asymmetry in the wall pH during gravitropic stimulation was demonstrated in a rigorous measurement experiment by Fasano *et al.*, 2001 and was comprehensively summarized in the review article by Du *et al.*, 2020. By growing Arabidopsis seedlings on growth media containing a novel cell wall pH sensor (a cellulose binding domain peptide–Oregon green conjugate) and a cytoplasmic pH sensor (plants expressing pH-sensitive green fluorescent protein) to monitor pH dynamics, this pH adjustment, acting as a secondary messenger, is thought to activate some biochemical mechanisms which in turn translates into asymmetrical wall relaxation and extension in the corresponding cells.

II. *LAZY* Gene Family in Arabidopsis Gravitropism

Despite much progress having been made in uncovering the molecular mechanism of Arabidopsis gravitropism, many unclear processes still remain in the model, especially in the early stage from gravity perception to signal transduction (Su *et al.*, 2017). Therefore, it would be pivotal to identify new molecular players involved in the processes right after the gravity sensing step but before the signal transduction event.

In the past decade, Spalding lab has published numerous studies which uncovered that *LAZY* family genes are key players in gravity signaling pathway that control the GSA of Arabidopsis roots and shoots (Yoshihara *et al*., 2013; Yoshihara and Spalding. 2017). The first *LAZY* gene, *LAZY1*, was firstly identified in rice and the rice *oslazy1* mutant displayed a lazy prostrate

growth phenotype due to its gravitropic defect (Yoshihara and Lino, 2007). Homologous Arabidopsis *LAZY1* also functions in the inflorescence stem and hypocotyl gravitropism. Arabidopsis genome contains six *LAZY* genes, which are characterized by regions of conserved sequences I at the N-terminus to the family-defining region V at the C-terminus (Yoshihara *et al*. 2013; Yoshihara and Spalding. 2017). The LAZY proteins have no known or predictable biochemical function according to genomic annotation data. Current genomic data also suggests that *LAZY* genes are only present in land plant, and a recent study identifies *LAZY1* in weed was under selection during the vavilovian mimicry process (Ye *et al*. 2019). Those results coincide with *LAZY1's* function in shaping plant architecture.

Arabidopsis *lazy1* mutant showed major gravitropism defects, including increased branch angle (80° from 45° of wild type) and reduced tropism of etiolated hypocotyl and inflorescence stem responding to 90 degrees rotation. However, there is no obvious branch angle phenotype for other *lazy* mutants, which implies *LAZY1's* predominant function in branch angle determination. *LAZYs* do share redundancy that *lazy1, 2, 3, 4* quadruple mutant appears not agravitropic but instead tend to grow in the wrong direction, which is defined as reverse gravitropism (Ge and Chen, 2016; Yoshihara and Spalding, 2017; Ge and Chen, 2019). Interestingly, *lazy1,2,3,4* showed no trouble in phototropism when exposed to unilateral blue light, which indicates phototropic signaling does not require LAZY proteins. (Yoshihara *et al*. 2013; Yoshihara and Spalding. 2017). Phototropism shares the auxin redistribution process across a growing organ with gravitropism (Spalding. 2013). These results make *LAZY* family especially valuable for gravitropism studies because they seem to function in a gravity-specific signaling mechanism upstream of the auxin distribution mechanism, and possibly also upstream of gravitropic signal

transduction stage. Furthermore, the sedimentation of amyloplasts in the *lazy* mutants was all normal (Taniguchi *et al*., 2017). Thus, LAZY proteins appear to be an integral component of the earliest stages of a gravity signaling mechanism.

Despite *lazy1*'s striking phenotype, *LAZY1's* specific molecular function largely remains still obscure except that it bridges gravity sensing and auxin redistribution. There are few known functional domains in LAZY1 protein, with exceptions of one nuclear localization signal (NLS) as well as an EAR motif in region V. The EAR motif is the most predominant form of transcriptional repression motif so far identified in plants. TOPLESS (TPL) and AtSAP18 are examples of interacting proteins of the EAR motif that serve as co-repressors (Kagale and Rozwadowski, 2011). Paradoxically, the relevance of nuclear LAZY1 to its branch GSA function is currently unknown, and LAZY1 functions at the plasma membrane. Mutating the NLS of LAZY1, which removes nuclear LAZY1, does not disrupt its branch angle-setting function (Yoshihara et al., 2013). Conversely, mutated Region I of LAZY1 prevent plasma membrane association, permit nuclear accumulation, and completely nullify function with respect to setting branch angle (Yoshihara and Spalding, 2020). Thus, LAZY1 at the plasma membrane appears to be necessary for branch GSA while we have limited knowledge on how exactly LAZY1 is associated to the plasma membrane. One study demonstrated that LAZY1 is a peripheral membrane protein through its C-terminus and LAZY1 can be solubilized by a non-ionic detergent or at a high pH condition (Sasaki and Yamamoto. 2015). OsLAZY1 appears to rely on its nuclear localization to regulate tiller angle in rice, which differs from its homolog in Arabidopsis (Li et al. 2019), but this observation can be interpreted in different way, which will be elaborated later in this dissertation.

Although little is known about LAZY1's regulators or regulatees within the gravitropic signaling process, there has been emerging knowledge related to the functional regions of LAZY1. The predicted LAZY proteins are moderate in size, ranging from 19 to 41 kDa in Arabidopsis. Dardick et al. (2013) showed that region II of LAZY genes contains a GφL(A/T)GT motif, which places LAZY genes within a larger family named IGT, to which the Tiller Angle Control 1 (TAC1) gene belongs. Importantly, region V is what distinguishes *LAZY* family from other genes, such as TAC1, which contains region II and serves an opposite role as LAZY1 in controlling branch angle (Yu et al. 2007; Dardick et al. 2013). An Arabidopsis *tac1 lazy1* double mutant presents lazy1-like wide branch angles, showing *lazy1* is epistatic to *tac1* (Hollender et al., 2020). Furthermore, region V seems to be the protein-protein interaction site of LAZY1, for instance, ZmLAZY1 interacts with a protein kinase catalytic domain (PKC) at the region V (Dong et al. 2013), and same as the interaction between OsLAZY1 and OsBRXL4 (Li et al. 2019). Furutani et al. (2020) reported that region V of LAZY proteins interacts with the BRX domain within a protein called RLD. Besides region V, the importance of other regions of LAZY1 was also thoroughly investigated by site-directed mutagenesis of key conservative amino acids residues. Regions III and IV seem less important and have little contributions to function or localization. Mutations in Region I, II, or V would completely disrupt normal *LAZY1* GSA function. Region I is required for LAZY1 to reside at the plasma membrane, which is necessary for its function. Region II is particularly special because its two conservative amino acid substitutions (L92A/I94A) had dramatic effect of switching shoot gravity responses from upward bending to positive downward bending, that is, reverse gravitropism. However, the corresponding mechanism as how it drastically reverses the

gravitropic response through only two amino acid substitutions is unclear, nor do we know if wild-type LAZY1 protein can rescued the reverse gravitropism (Yoshihara and Spalding. 2017).

As illustrated above, *LAZY1* is the starting point of this dissertation project given its significant role in gravitropism and mysterious functional mechanism due to unavailable annotation information. However, oftentimes interactors of unknown proteins can help us infer their function and further uncover the molecular mechanisms. Therefore, a Yeast Two Hybrid (Y2H) screen was performed by me. A protein named BRXL4 appeared to be the most interesting and promising interacting candidate.

Chapter II

BRXL4-LAZY1 interaction at the plasma membrane controls Arabidopsis branch angle and gravitropism

Ximing Che, Bessie L. Splitt, Magnus T. Eckholm, Nathan D. Miller, and Edgar P. Spalding

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Research contributions:

X.C. participated in experimental design and data interpretation, performed confocal imaging, image analysis, Y2H assay, and qPCR, and generated transgenic plants and identified mutants.

B.L.S. performed confocal imaging on *HSP18:LAZY1-GFP* and measured hypocotyl gravitropic angles

M.T.E contributed to constructs generation, planting, mutant identifications.

N.D.M developed the program to analyze stem gravitropism data and the software to measure fluorescent intensity.

E.P.S participated in experimental design, data interpretation, and model building.

Abstract

Gravitropism guides growth to shape plant architecture above and below ground. Mutations in *LAZY1* impair stem gravitropism and cause less upright inflorescence branches (wider angles). The LAZY1 protein resides at the plasma membrane and in the nucleus. The plasma membrane pool is necessary and sufficient for setting branch angles. The presence of a nuclear localization signal and a motif associated with transcription repression indicate the nuclear pool of LAZY1 has a yet-unknown purpose. To investigate how LAZY1 functions, we screened for LAZY1-interacting proteins in yeast. We identified BRXL4, a shoot-specific protein related to BREVIS RADIX. The BRXL4-LAZY1 interaction occurred at the plasma membrane in plant cells, and not in the nucleus. Mutations in the C-terminus of LAZY1 prevented the interaction. Opposite to *lazy1*, *brxl4* mutants displayed faster gravitropism and more upright branches. Overexpressing *BRXL4* produced strong *lazy1* phenotypes. These phenotypes are consistent with BRXL4 reducing *LAZY1* expression or the amount of LAZY1 at the plasma membrane. Experiments indicated that both are true. *LAZY1* mRNA was 3-fold more abundant in *brxl4* mutants and almost undetectable in *BRXL4* overexpressors. Plasma membrane LAZY1 was higher and nuclear LAZY1 lower in *brxl4* mutants compared to wild type. To explain these results, we propose that BRXL4 interaction with LAZY1 at the plasma membrane promotes LAZY1 transport from its site of gravity signaling to the nucleus where it suppresses its own transcription. This explanation of how BRXL4 negatively regulates LAZY1 suggests ways to modify shoot system architecture for practical purposes.

Introduction

Plants use gravitropism to align the growth of roots and shoots, and their branches, at angles that are genetically set yet subject to modification by environmental variables such as light, water, and nutrient gradients (Roychoudhry *et al.*, 2017). Many molecular, cellular, and physiological events associated with gravitropism have been described (Morita, 2010, Su *et al.*, 2017), but an end-to-end understanding of the complex and coordinated mechanism has not yet been achieved. Elucidating the mechanism would lead to a better understanding of how gravity-guided plant architecture affects resource acquisition and interplant competition below and above ground.

Gravitropism requires that an organ registers the direction of the gravity vector and then adjusts growth to align the organ with its gravitropic set-point angle (GSA). Cells containing dense amyloplasts called statoliths may register the direction of the gravity vector by determining which face of the cell the statoliths sediment on (Su *et al.*, 2017). Intracellular trafficking machinery then directs more auxin-transporting PIN proteins to the plasma membrane at this lowest face (Kleine-Vehn *et al.*, 2010, Nakamura *et al.*, 2019). Redirecting auxin produces a gradient that causes cells on opposite sides of the organ to elongate at different rates (Spalding, 2013). The resulting differential growth reorients the organ.

The present study concerns the role of a LAZY protein in the gravitropism mechanism. The first member of the *LAZY* gene family was identified by genetically mapping a mutation in rice responsible for wide tiller angles (Jones and Adair, 1938, Li *et al.*, 2007, Yoshihara and Iino,

2007). The maize *lazy* mutant (van Overbeek, 1936) was subsequently shown to be a transposon-disrupted allele of a homologous gene (Dong *et al.*, 2013, Howard *et al.*, 2014). The Arabidopsis genome contains five genes that possess all five regions of conserved sequence (regions I-V) typical of *LAZY* genes (Yoshihara *et al.*, 2013). A sixth Arabidopsis gene consists of only region V. Mutant analysis showed that, of the five complete *LAZY* genes in Arabidopsis, *LAZY1* contributes the most to shoot system architecture (Yoshihara and Spalding, 2017). Branches emerge from the primary inflorescence stem of the wild type at an upright angle of approximately 45° degrees but the branches of *lazy1* are almost horizontal (approximately 80°), due to impaired gravitropism rather than a stiffness defect (Yoshihara and Spalding, 2020).

If gravitropism consists of a detection phase, an auxin redistribution phase, and a growth control phase, LAZY proteins appear to act only in the first or to connect the first to the second (Jiao *et al.*, 2021). Briefly, the evidence is that statoliths sediment normally in a *lazy1* mutant (Godbole *et al.*, 1999, Taniguchi *et al.*, 2017), a LAZY protein and an interacting protein redistribute in columella cells after gravistimulation (Godbole *et al.*, 1999), an auxin gradient does not form following gravistimulation in various *lazy* mutants (Overbeek, 1938, Godbole *et al.*, 1999, Yoshihara and Iino, 2007, Yoshihara and Spalding, 2017, Yoshihara and Spalding, 2020), but applying auxin to one side of a *lazy1* stem produces a normal differential growth response (Yoshihara and Spalding, 2020).

In Arabidopsis, *LAZY1* is expressed at low levels throughout the shoot system (Yoshihara *et al.*, 2013). Its protein is present at the plasma membrane and in the nucleus (Yoshihara *et al.*, 2013,

Howard *et al.*, 2014). To obtain this result, it was necessary to insert GFP within a non-conserved region of LAZY1, because terminal fusions disrupted function, and it was necessary to drive expression with a heat shock promoter in transgenic Arabidopsis seedlings, or the strong 35S promoter in *Nicotiana benthamiana* leaves (Yoshihara *et al.*, 2013, Yoshihara and Spalding, 2020). Experiments conducted in these systems showed that a mutation in region I that prevents LAZY1 from associating with the plasma membrane also prevents rescue of the *lazy1* branch angle defect (Yoshihara *et al.*, 2013, Yoshihara and Spalding, 2020). Mutating a predicted nuclear localization signal (NLS) between regions III and IV prevents LAZY1 from accumulating in the nucleus but does not interfere with its GSA function (Yoshihara *et al.*, 2013). *LAZY2* lacks an NLS and the protein is detected only at the plasma membrane. Expressing *LAZY2* under the control of the *LAZY1* promoter in a *lazy1* mutant completely rescued the branch angle phenotype (Yoshihara and Spalding, 2020). Thus, LAZY1 acts at the plasma membrane in a gravity-response process that sets branch angles. A study of rice LAZY1 reached the opposite conclusion, that nuclear OsLAZY1 was responsible for setting tiller angle and that the plasma membrane-associated pool was ineffective (Li *et al.*, 2019).

LAZY1 contains an EAR motif in the C-terminal region V. EAR motifs are associated with a major mechanism for repressing transcription (Kagale and Rozwadowski, 2011, Yang *et al.*, 2018). Mutations in region V including amino acid substitutions within the EAR motif prevent LAZY1 from establishing a wild-type branch angle (Yoshihara and Spalding, 2020). Nuclear localization and an EAR motif in a conserved and necessary region V indicate that LAZY1 regulates transcription. However, LAZY1 can rescue the branch angle phenotype of a *lazy1* mutant even

when mutations in the NLS prevent the protein from accumulating in the nucleus (Yoshihara *et al.*, 2013). These observations appear to contradict each other. The present study resolves the apparent contradiction by identifying a region V-interacting protein that promotes a shift of LAZY1 from the plasma membrane where it functions in gravity signaling to the nucleus where it may repress its own expression.

Results

LAZY1 interactor screen identifies BRXL4

Identifying a LAZY1 interacting protein may produce testable hypotheses about how LAZY1 functions in setting shoot architecture. Therefore, we screened an Arabidopsis cDNA library with the yeast two-hybrid (Y2H) method. Table 2.S1 presents an annotated list of 16 putative LAZY1 interactors that we isolated more than once in the screen. One of the genes in this list, *BRX-like 4* (*BRXL4*; At5g20540) is the subject of the present study. The originally isolated clone was a gene fragment. Figure 2.1a shows that a full-length *BRXL4* cDNA produced a strong interaction with LAZY1 when presented as bait or prey.

BRXL4 physically interacts with LAZY1 at the plasma membrane in planta

Like LAZY1, BRXL4 is located at the plasma membrane and nucleus when transiently expressed in *Nicotiana benthamiana* leaves (Figure 2.1b,c), but bimolecular fluorescence complementation (BiFC) mediated by BRXL4-LAZY1 interaction is apparently restricted to the plasma membrane (Fig. 1d,e). The BiFC result was the same regardless of which protein carried the nYFP or cYFP half of the yellow fluorescent reporter (Figure 2.1d,e). These results confirm

that the BRXL4-LAZY1 interaction in yeast also occurs in plant cells, and they indicate that the interaction is conditional. The plasma membrane and nucleus may differ with respect to factors that affect the conformation or accessibility of a LAZY1 or BRXL4 binding site. Neither nYFP-LAZY1 nor nYFP-BRXL4 interacts with free cYFP, a standard control test (Figure 2.S1).

BRXL4 is one of five Arabidopsis genes in a family founded by the *BREVIS RADIX* (*BRX*) gene (Mouchel *et al.*, 2004, Briggs *et al.*, 2006). Like the *LAZY* family, *BRXL* genes occur only in plants. BRXL proteins have no known or predicted enzymatic function, but they all have two large BRX domains (Figure 2.2a) that have been shown to facilitate protein-protein interactions (Briggs *et al.*, 2006) and to associate with membranes (Koh *et al.*, 2021). Proteins may associate with membranes via basic and hydrophobic (BH) domains (Barbosa *et al.*, 2016). A software program (Brzeska *et al.*, 2010; https://hpcwebapps.cit.nih.gov/bhsearch/) that successfully predicted membrane-associating BH domains in Arabidopsis proteins (Reuter *et al.*, 2021) predicted two BH domains in BRXL4, one in each of the BRX domains (Figure 2.2a). The same analysis of the LAZY1 sequence predicted a BH domain in region I (Figure 2.2b) that included the residues previously found to be necessary for localizing LAZY1 to the plasma membrane (Yoshihara *et al.*, 2013). However, this BH domain cannot fully explain LAZY1 membrane association because region I alone does not target GFP to the plasma membrane (Yoshihara and Spalding, 2020). A second predicted BH domain within the NLS region and a third within region IV (Figure 2.2b) may augment LAZY1 membrane association. Figure 2.S3 shows the full BH score profiles for the BRXL4 and LAZY1 sequences.

Previous LAZY1 structure-function studies identified conserved amino acids in region I, II, and V that disrupt LAZY1 GSA function when changed to alanine (Yoshihara and Spalding, 2020). Figure 2.2b shows that the disabling mutations in region I, which disrupt association with the plasma membrane, or region II did not affect the LAZY1-BRXL4 interaction, while the mutations in each of the three different locations in region V greatly diminished the LAZY1-BRXL4 interaction. These results are evidence of a functionally relevant interaction between BRXL4 and region V of LAZY1. A previous study used rice LAZY1 (OsLAZY1) as a Y2H bait and identified OsBRXL4 as an interactor (Li *et al.*, 2019). Deleting the BRX domain of OsBRXL4 prevented the interaction.

BRXL4 **negatively regulates branch angle in Arabidopsis**

If the physical interaction between BRXL4 and LAZY1 at the plasma membrane where LAZY1 functions to control lateral branch GSA is functionally important, then a *brxl4* knockout mutant should display a branch angle phenotype. Two different lines with T-DNA inserted in the fifth exon of *BRXL4* were obtained. Both *brxl4-1* and *brxl4-2* mutants displayed an upright inflorescence architecture with branch angles being approximately 39° compared to approximately 48° for wild type (Figure 2.3a-c,h). This phenotype is opposite to *lazy1*, which produces branch angles that are approximately 30° wider than wild type, approximately horizontal (Figure 2.3d,h). If *BRXL4* is a negative regulator of branch angle as the mutant phenotypes indicate, a *BRXL4* overexpressor should show wider branch angles and an inflorescence architecture like that of *lazy1*. We generated a *BRXL4* overexpressor by transforming a wild-type plant with a copy of *BRXL4* under its native promoter (*proBRXL4*), and

another version based on the strong, constitutive 35S promoter (*pro35S*). The *proBRXL4* plant displayed horizontal (approximately 90°) *lazy1*-like branch angles (Figure 2.3e,h). The *pro35S* line displayed even greater (approximately 120°) branch angles (Figure 2.3f,h). Figure 2.S2 shows additional examples of these shoot architecture phenotypes.

The results in Figure 2.3 show that *brxl4* and *lazy1* have opposite phenotypes, which presents an opportunity to determine which phenotype prevails in a double mutant, which can establish the order of action between the interacting proteins. The *brxl4 lazy1* double mutant exhibited *lazy1*-like wide branch angles rather than more upright, *brxl4*-like branch angles (Figure 2.3g,h). If modeled as a linear signaling pathway, BRXL4 therefore acts negatively upstream of LAZY1.

The expression patterns of *BRXL4* and *LAZY1* would be expected to overlap in the organs displaying altered GSA if the protein interaction is mechanistically relevant. We expressed a *proBRXL4*:*GFP-NLS* reporter gene in a wild type plant. GFP signals indicative of BRXL4 promoter activity were evident in mature shoots in the node region including newly emerging axillary buds (Figure 2.4a-c). In etiolated seedling, expression was evident in the hypocotyl (Figure 2.4d). Expression was noted at the base of flowers and siliques (Figure 2.4e,f). These sites of expression are very similar to those reported for *LAZY1* (Yoshihara *et al.*, 2013, Hollender *et al.*, 2020) supporting the conclusion that BRXL4 and LAZY1 proteins act together to determine branch angle.

***BRXL4* negatively regulates gravitropism**

The wide branch angle of *lazy1* is associated with defective gravitropism (Yoshihara *et al.*, 2013)(13). If *BRXL4* negatively regulates *LAZY1* then *brxl4* mutants should respond more strongly in a gravitropism assay. We tested this hypothesis using excised inflorescence stems as described previously (Yoshihara and Spalding, 2020) except in this case we developed custom software to automate the image analysis. The assay demonstrated clear hypergravitropism in both *brxl4* alleles, impaired gravitropism in the *proBRXL4* overexpressing line, and almost no gravitropism in the *pro35S* line (Figure 2.5a,b). One hour after being placed horizontal, *brxl4* inflorescence stems achieved an angle of 90°, the wild type had reached 45°, and *proBRXL4* barely exceeded 10° (Figure 2.5a,b). The *pro35S* line had not yet begun to respond 1 h after 90° rotation.

Gravitropism of etiolated hypocotyls was also tested. Images were automatically collected in complete darkness using infrared illumination. Like the inflorescence stem result, both *brxl4-1* and *brxl4-2* displayed faster and greater gravitropism than wild type, achieving approximately 65° at the end of 6 h, while the wild type more slowly reached a final angle of 45° (Figure 2.5c,d). Hypocotyl gravitropism in *proBRXL4* was greatly diminished, and the *pro35S* line was essentially agravitropic (Figure 2.5c,d). We conclude that the steeper branch angle phenotype

of *brxl4* mutants (Figure 2.3) is a consequence of enhanced gravitropism (Figure 2.5) due to disabled negative regulation of *LAZY1*.

***BRXL4* negatively regulates *LAZY1* expression**

A negative effect of *BRXL4* on *LAZY1* expression could explain the negative regulation evidenced by the mutant phenotypes. Consistent with this possibility, Figure 2.6a shows that the two *brxl4* alleles, which expressed little or only trace amounts of *BRXL4* mRNA (Figure 2.6b), expressed approximately 3-fold more *LAZY1* mRNA, compared to the wild type, in inflorescence stems and etiolated hypocotyls. Furthermore, overexpressing *BRXL4* reduced *LAZY1* mRNA. The *proBRXL4* plant, which overexpressed *BRXL4* by 3.5-fold (Figure 2.6b) displayed approximately 50% of the wild-type level of *LAZY1* mRNA in inflorescence stems (Figure 2.6a). Overexpressing *BRXL4* approximately 80-fold in hypocotyls and 40-fold in inflorescence stems with the 35S promoter (Figure 2.6b) reduced *LAZY1* mRNA to essentially undetectable levels (Figure 2.6a). These results demonstrate a dosage-dependent negative effect of *BRXL4* on *LAZY1* expression.

BRXL4 shifts LAZY1 to nucleus

The protein interaction results indicate that the regulatory effect of BRXL4 on LAZY1 occurs at the plasma membrane and not the nucleus (Figure 2.1d,e). The mechanism appears to include an effect on *LAZY1* transcription or mRNA accumulation (Figure 2.6a,b). These two conclusions appear to be inconsistent with each other. Experiments designed to determine the effect of

BRXL4 on LAZY1 subcellular localization provided an explanation. As in previous studies (Yoshihara *et al.*, 2013, Yoshihara and Spalding, 2020), we used a heat-shock method to express LAZY1-GFP and a custom image analysis method for quantifying the amount of signal at the plasma membrane and in the nucleus. Figure 2.7a,b shows representative confocal microscope images of LAZY1-GFP after induction of expression in *lazy1* and *brxl4-1* hypocotyl cells. Figure 2.7c shows an example of the automated plasma membrane sampling method. The results in Figure 2.7d show that loss of BRXL4 (*brxl4*) increases the amount of LAZY1 at the plasma membrane, where evidence indicates it functions in gravity signaling, and decreases the nuclear pool. Thus, BRXL4 affects the subcellular distribution of LAZY1.

LAZY1-GFP is expressed in the cytoplasm and forms abundant bright bodies in pro35S plants

Figure 2.7b, d shows that loss of *BRXL4 (brxl4)* increases the amount of LAZY1 at the plasma membrane and decreases the nuclear LAZY1, and we concluded BRXL4 serves to shift LAZY1 from the plasma membrane to the nucleus. It is logical to examine what the localization of LAZY1-GFP would be in a *BRXL4* overexpressor background.

We used the same heat-shock method to express LAZY1-GFP in *pro35S* plants. Figure 2.S4. shows representative confocal microscope images of LAZY1-GFP after induction of expression in *lazy1* (WT context), *brxl4*-1, and *pro35S* hypocotyl cells. I obtained this line of pro35S plants after a replacement of the laser of the confocal microscope, thus the fluorescent signals are considerably stronger compared to those in Figure 2.7, which was collected before the replacement.

In a *BRXL4* overexpressor environment, LAZY1-GFP seems to be localized to the cytoplasm and form abundant bright bodies in addition to its usual subcellular localization in the nucleus and possibly the plasma membrane. The images are generally cloudier than either WT or brxl4 background, apparently due to LAZY1-GFP in the cytoplasm.

Discussion

The small family of Arabidopsis *BRXL* genes founded by *BREVIS RADIX* is defined primarily by two similar sequences of 55 amino acids (BRX domains) separated by a variable sequence of at least 100 amino acids (Briggs *et al.*, 2006). BRX domains occur in proteins outside of this family, and they can function in protein-protein interactions. Most relevant to the present study, a BRX domain in the RLD protein was recently shown to interact with region V of LAZY4 (Furutani *et al.*, 2020). Deleting entire BRX domains from a rice BRXL protein (OsBRXL4) prevented its interaction with region V of OsLAZY1 (Li *et al.*, 2019), consistent with the effects of region V mutations on BRXL4-LAZY1 interaction (Figure 2.2b). BRX domains also associate proteins with membranes. An engineered fluorescent protein consisting only of two BRX domains and the intervening linker localized to the plasma membrane in root cells (Koh *et al.*, 2021). Our finding of BH domains within BRX domains could explain the plasma membrane localization of intact BRXL4 (Figure 2.1b). We propose an explanation for the *brxl4* phenotypes presented here that includes a role for a BRX domain-region V interaction, peripheral membrane associations mediated by BH domains, and transcription repression expected from the EAR domain of LAZY1.

The model in Figure 2.8 shows the BH domains of LAZY1 interacting with the plasma membrane while the protein performs its GSA signaling function in a wild-type plant. When associated with the plasma membrane, the NLS is blocked or otherwise inoperative, and the EAR motif is irrelevant. We propose that when BRXL4 interacts with region V of LAZY1 at the plasma membrane (Figure 2.1d,e), the NLS in LAZY1 becomes effective, which results in LAZY1 moving to the nucleus. This explains why *brxl4* has more LAZY1 at the plasma membrane and less in the nucleus than wild type (Figure 2.7). More LAZY1 at the membrane functioning in gravity signaling is consistent with more upright branches and hypergravitropism in *brxl4* (Figure 2.3). Less LAZY1 in a *brxl4* nucleus (Figure 2.7d) yet approximately 3-fold more *LAZY1* mRNA (Figure 2.6a) is consistent with nuclear LAZY1 repressing *LAZY1* transcription (Figure 2.8). LAZY1 does not contain recognizable DNA-binding motifs but its EAR motif may bind to transcriptional regulators such as TPL (Kagale and Rozwadowski, 2011) to repress LAZY1 expression. A protein like TPL replacing BRXL4 would explain why BRXL4 is present in the nucleus but apparently not interacting with LAZY1 (Figure 2.1). Overexpressing *BRXL4* shuts off *LAZY1* expression (Figure 2.6) and produces essentially agravitropic shoots (Figures 3 and 5). More BRXL4 shifting more LAZY1 to the nucleus (Figure 2.7) where it does not function in gravity signaling could explain these results, but nuclear BRXL4 may play a role in repressing *LAZY1* expression beyond facilitating the latter's nuclear enrichment. Studies of the related BRX protein conclude that auxin induces its movement from the plasma membrane to the nucleus in roots (Scacchi *et al.*, 2009, Koh *et al.*, 2021), where it influences auxin gradients and the differentiation of root protophloem cells (Marhava *et al.*, 2018, Marhava *et al.*, 2020).

The model in Figure 2.8, which provides a consistent explanation for the results in Figures 1-7, may prompt new hypotheses or provide a framework for interpreting new findings. Experiments are needed to test the validity of the BRXL4-dependent LAZY1 nuclear transport suggestion, and the proposed LAZY1-dependent *LAZY1* expression-repression. Unfortunately, the Y2H screen did not identify any plausible components to pursue (Table 2.S1).

The model in Figure 2.8 differs significantly from the conclusions of a previous study of rice LAZY1 (OsLAZY1), even though some of the results are consistent. Li et al. (Li *et al.*, 2019) screened for OsLAZY1 interactors and found OsBRXL4. Their *OsBRXL4* overexpressing lines appeared *lazy1*-like (wider tiller angle) and RNAi knockdown lines displayed narrower tiller angles, consistent with the Arabidopsis phenotype data in Figure 2.3. However, Li et al. concluded that the rice BRXL4 protein reduces, rather than promotes, nuclear localization of OsLAZY1 and they concluded that gravity signaling requires nuclear OsLAZY1, not the plasma membrane pool. Some step in the rice mechanism may be reversed compared to Arabidopsis. Either LAZY1 or BRXL4 may function oppositely in rice. However, the phenotypes of the Arabidopsis and rice *lazy1* mutants indicate that the proteins do not oppositely regulate GSA. A phylogenetic analysis showed that monocot and dicot *BRXL* genes cluster separately, which may indicate major functional differences (Beuchat *et al.*, 2010). However, rice *BRXL* genes could rescue the root growth phenotype of the Arabidopsis *brx* mutant, indicating some degree of common function (Beuchat *et al.*, 2010). Instead of a fundamental difference in the BRXL4-LAZY1 GSA mechanism (Wang *et al.*, 2021), differences in experimental methodologies may explain the opposite conclusions.

In the present study (Figure 2.1b-e) and in previous studies (Yoshihara *et al.*, 2013, Yoshihara and Spalding, 2020), we found LAZY1 residing at the plasma membrane and/or nucleus (depending on the experiment) in *Nicotiana benthamiana* leaves and Arabidopsis hypocotyls, with typically no signal in the cytoplasm. Our GFP-tagged LAZY1 typically rescues the branch angle phenotype of *lazy1* mutants, indicating the tagged protein is functional. To obtain this functional GFP-tagged LAZY1, we had to insert the GFP within the gene body, between Lys308 and Ala309 (Yoshihara *et al.*, 2013). Terminal tags did not function. Li et al. (Li *et al.*, 2019) fused mCherry to the C-terminus of OsLAZY1 and did not demonstrate that the tagged protein could rescue the rice *lazy1* mutant. When expressed in rice protoplasts, the OsLAZY1-mCherry reporter was evident in the cytoplasm and nucleus. The conclusion that OsBRXL4 acts to reduce OsLAZY1 nuclear localization is largely based on a reduction in the visually scored presence or absence of OsLAZY1-mCherry in the nucleus of protoplasts transfected with different amounts of *OsBRXL4* plasmid (Li *et al.*, 2019). Our assay quantified the change in LAZY1 abundance at the plasma membrane and nucleus caused by a *brxl4* mutation (Figure 2.7d).

The alteration of LAZY1 localization in *brxl4* was very significant and striking, and it connects the *brxl4* hypergravitropic phenotype and the overexpression of LAZY1 in *brxl4* mutants (Figure 2.7). Performing the experiment in the *BRXL4* OE context is also important and potentially able to complete the picture from the opposite direction. Therefore, I examined LAZY1-GFP subcellular localization in the *BRXL4* OE lines driven by the heat-shock promoter. I indeed observed strong LAZY1-GFP signal in the nucleus in *pro35S*. However, there are two caveats that prevent me from incorporating the results into Figure 2.7. 1) Strong LAZY1-GFP signals in the cytoplasm of

BRXL4 OE plants makes it difficult to selectively measure the plasma membrane pool with the same image analysis method. 2) The microscope's laser was replaced after the results in Figure 2.7 were obtained so it would not be possible to directly compare the plasma membrane and nuclear signal strengths even if they could be measured with the same method. That is why I report these new results in the Figure 2. S4 instead. The large amount of LAZY1-GFP in the cytoplasm of *pro35S* plants may reflect LAZY1 in the process of being transported to the nucleus and/or the result of a mechanism for exporting excess LAZY1 protein from the nucleus. It also makes sense because overexpression of BRXL4 should prevent LAZY1 being anchored at the plasma membrane. Repeating more trials of *HSP:LAZY1-GFP/pro35S* would be necessary to generate a more convincing conclusion. Choosing a later growth stage may show more consistently bright nuclei because the images in Figure 2. S4 may reflect a snapshot of a dynamic system that is still settling.

Regarding architectural phenotypes, Yoshihara et al. measured branch angles in more than 100 transgenic plants (individual T1 plants) transformed with NLS-mutated *LAZY1*, and a similar number of control plants transformed with wild-type *LAZY1*, to demonstrate that rescue of the *lazy1* phenotype did not require LAZY1 in the nucleus (Yoshihara *et al.*, 2013, Yoshihara and Spalding, 2020). Statistically analyzing the distribution of the branch angles showed that even the wild-type *LAZY1* gene would not always rescue the phenotype, possibly because of insertion position effects on transgene expression (Yoshihara and Spalding, 2020). Pictures showing wide tiller angles in two transgenic lines support the conclusion that NLS-mutated OsLAZY1 cannot rescue the tiller angle phenotype (Li *et al.*, 2019). A quantitative analysis of many more lines

may show that an NLS-mutated *OsLAZY1* usually can rescue the tiller angle phenotype as found in Arabidopsis.

If opposite conclusions about the role of BRXL4 and the functions of nuclear LAZY1 are better explained by differences in experimental details than by opposite mechanisms operating in Arabidopsis and rice (Wang *et al.*, 2021), then the results reported here may establish a general framework for setting branch angles in all terrestrial plants. Downstream of gravity vector-detection and upstream of auxin redistribution, the physical and molecular-genetic interplay between BRXL4 and LAZY1 may constitute a middle stage in a process that determines land plant shoot architecture. Other proteins surely play roles in this stage. Future work should investigate if the model in Figure 2.8 can accommodate a role for *TILLER ANGLE CONTROL 1* (*TAC1*), a gene that belongs to the IGT superfamily, of which the *LAZYs* are a subgroup (Waite and Dardick, 2021). A *tac1* mutant displays more erect branches, much like *brxl4*, and functions to integrate effects of photosynthesis on shoot architecture (Waite and Dardick, 2018).

In addition to positioning leaves in ways that determine canopy photosynthesis, shoot architecture can influence reproductive success. Wender et al. (Wender *et al.*, 2005) reported that branch angle is one of the genetically-determined variables that explains Arabidopsis seed dispersion patterns, especially when plant density is significant. Thus, the BRXL4-LAZY1 stage of the GSA setting mechanism may affect ecological competitiveness. Selection may have acted on this stage of the process to produce the great variety of branch angles observable in Nature more so than the vector detection or differential growth stages. The BRXL4-LAZY1 stage may be

where natural selection mediates the tradeoffs between energy harvesting, density tolerance, reproduction strategies, and various contingencies. Some evidence supports this suggestion. A recent study of tiller angle in the barnyard grass weed (*Echinochloa crus-galli*) indicated that selection has acted on this stage of the GSA-setting mechanism (Ye *et al.*, 2019). A rice-like form of the weed (narrow tiller angles) is found in Chinese rice paddies, apparently because farmers notice and remove individuals with wider tiller angles that are typical of non-paddy environments. Genome sequencing identified *LAZY1* as one of the genes responsible for this human-assisted, inadvertent selection of a crop mimicking phenotype (Ye *et al.*, 2019). Intentional selection of crop species including trees has produced a great variety of branch GSAs, with more upright branches often desired because they allow higher density plantings (Dardick *et al.*, 2017, Waite and Dardick, 2021). Thus, the results presented here may help to produce a better understanding of shoot architecture evolution and show how shoot architecture could be engineered for practical purposes.

Experimental Procedures

Yeast two-hybrid library screen and targeted validation assays

A GAL4-based yeast two-hybrid system was used to screen for plant protein-protein interactions in yeast. A library of Arabidopsis shoot apex–derived cDNA in pEXP-AD502 vectors was kindly provided by Scott Michaels (Indiana University, Bloomington, IN). AH109 yeast cells containing pDEST32-LAZY1 were transformed with the cDNA library, and then spread on synthetic dropout media lacking Trp, Leu, and His (SDIII), and supplemented with 20 mM 3-

amino-1, 2, 4-triazol (3-AT) to suppress auto-activation. After incubating the plates at 30°C for 4 d, yeast plasmids were extracted from many vigorously growing colonies using Zymoprep Yeast Plasmid Miniprep (www.zymoresearch.com). The cDNA portion of the pEXP-AD502 plasmid was amplified by PCR, sequenced, and subjected to BLAST analysis to identify putative LAZY1 interactors.

For targeted Y2H assays, AH109 yeast cells were co-transformed with the bait (pDEST32) and prey (pDEST22) plasmids containing the indicated inserts, then spread on media lacking Trp and Leu (SDII). After growing for 3 d at 30° C, five individual colonies were picked and transferred to SDII liquid media. After growth overnight, the cultures were adjusted to an OD_{600} of 1.0 by addition of liquid medium and then diluted 10-fold and 100-fold to produce a series of samples that were spotted on plates containing SDIII + 20 mM 3-AT media. Some targets were point mutations of *LAZY1* created with the Phusion Site-Directed Mutagenesis Kit (www.thermofisher.com).

BiFC in *Nicotiana benthamiana* leaves

The *BRXL4* and *LAZY1* cDNA sequences were cloned into the pDOE8 BiFC vector (Gookin and Assmann, 2014) using the SanDI cutting site in MCS3 and the PacI cutting site in MCS1. Leaves of *N. benthamiana* were infiltrated with the GV3101 strain of *Agrobacterium tumefaciens* containing the completed pDOE8 plasmid to transiently express the resulting BRXL4 and LAZY1 fusion proteins. To determine if and where BRXL4 interacted with LAZY1, a Zeiss 710 laser-

scanning confocal microscope was used to collect mVenus fluorescence (520 nm-585 nm) excited by a 488 nm laser 2 d after infiltration.

Arabidopsis mutants and transgenic plants

The Columbia ecotype (Col-0) of *Arabidopsis thaliana* was the wild type used in this study. The *lazy1* T-DNA insertion mutant was previously described by Yoshihara et al. (Yoshihara *et al.*, 2013). The Arabidopsis Biological Resources Center supplied the *brxl4-1* (SALK_147349c) and *brxl4-2* (SALK_022411c) T-DNA insertion mutants. The gene-specific DNA primers used to check their genotypes with PCR are shown in Table 2.S2.

To overexpress *BRXL4*, a genomic fragment of *BRXL4* was amplified with an Age I site at the 5′ end and a BamH I site at 3′ end. A 2.5kb of the BRXL4 promoter region was also amplified with Sac I site at the 5′ end and an Age I site at 3′ end. These two amplified fragments were inserted between the Sac I/BamH I sites of the binary vector pEGAD (Cutler *et al.*, 2000) using GeneArt Seamless Cloning (www. thermofisher.com) to generate a native promoter overexpression construct. Also, the *BRXL4* fragment was inserted between the Age I/BamH I sites of pEGAD to generate a 35S-driven overexpression construct. The Sac I/Age I sites of pEGAD were used to insert the *BRXL4* promoter fragment to generate pBRXL4-eGFP-NLS. The SV40 NLS was inserted in the EcoRI and BamHI sites after the eGFP. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998).

Phenotype measurements

To measure inflorescence branch angles, seedlings were first grown for 10-14 days on agar plates under continuous light in an incubator and then transplanted to soil and grown for 3-5 weeks in a growth chamber maintained at 22°C and illuminated with approximately 90 µmol $m^{-2}s^{-1}$ of white light on a 16 h light/8 h dark cycle. Inflorescences were harvested and trimmed to produce a 4 cm section containing the most basal node and 2 cm of its branch. Digital images of these sections were collected with a flatbed scanner, and the branch angle measured as previously described (Yoshihara *et al.*, 2013).

Etiolated hypocotyl gravitropism was measured as previously described (Yoshihara and Iino, 2007), except seedlings used in this study grew for 3 d rather than 2 d. Gravitropism of excised and trimmed primary inflorescence stems was also measured using infrared light and computer-controlled cameras as described (Yoshihara and Spalding, 2020), except that instead of manually measuring the angle of the tip at each 10-min time point, an image analysis algorithm was developed in the MATLAB computer programming language to automate the measurement. The analysis algorithm determined the midline of each stem present in each frame of an experimental time series even if one crossed another during the bending response. A straight line was fit to the most apical portion of the midline. Its angle relative to the horizon was recorded as the tip angle for that frame and plotted versus time.

Effect of BRXL4 on LAZY1 levels and subcellular redistribution

We used a temperature-inducible *pHSP18.2:AtLAZY1-eGFP* reporter gene (Yoshihara *et al.*, 2013) to determine the effect of a *brxl4* mutation on the subcellular distribution of LAZY1. Etiolated 3 d-old wildtype or *brxl4-1* seedlings growing on agar were placed in an incubator maintained at 37°C for 5 h to induce expression of the tagged LAZY1. GFP fluorescence was examined using a Zeiss 710 confocal microscope fitted with a C-Apochromat 40X water immersion lens. Laser (488 nm) intensity, pinhole size, and detector gain were the same for all trials. A trial consisted of one field of view collected from one hypocotyl. A custom image analysis procedure written in the MATLAB computer programming language quantified the GFP signal (500 nm-560 nm) at the plasma membrane. A Gaussian filter (21x21 pixels, SD=4) applied to the 12-bit GFP channel increased the signal to noise ratio. The Otsu threshold value (Otsu, 1979) was used to create a binary mask, the mask was skeletonized, and then recursively de-spurred to produce a single pixel-wide representation of the plasma membranes in the captured focal plane. Branch points were removed to produce segments of plasma membrane. The longest segments (top quintile for length) were subjected to a 3-pixel dilation. The intensity values of the pixels within these segments were averaged to measure the amount of LAZY1-GFP at the plasma membranes. To sample nuclear LAZY1-GFP, a human clicked on each visible nucleus to create a circular sample patch with a radius of 7 pixels. The average GFP channel value within this patch was recorded.

Measuring mRNA by RT-qPCR

GeneJET Plant RNA Purification Kit (www.thermofisher.com) was used to extract total RNA from either 3 d-old etiolated seedlings or 4-week-old inflorescence stems. Treatment with TURBO

DNase (www.thermofisher.com) removed genomic DNA from the RNA extracts. RT-qPCR was conducted using Luna Universal One-Step RT-qPCR Kit (www.neb.com) and a Mx3000P QPCR system (www.agilent.com) with the comparative $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Equal amounts of RNA (1500 ng) were used in the qPCR reactions. Arabidopsis UBQ10 mRNA served as an internal control, and the LAZY1 or BRXL4 expression levels of all genotypes were normalized to wild type. Three biological replicates per genotype and three technical replicates per sample were performed. The gene-specific primers used are listed in Table 2.S2.

Data Statement

The figures display all the data that this study used to reach its conclusions. The corresponding author (spalding@wisc.edu) will provide the values displayed in the plots upon request.

Acknowledgements

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Figures and legends

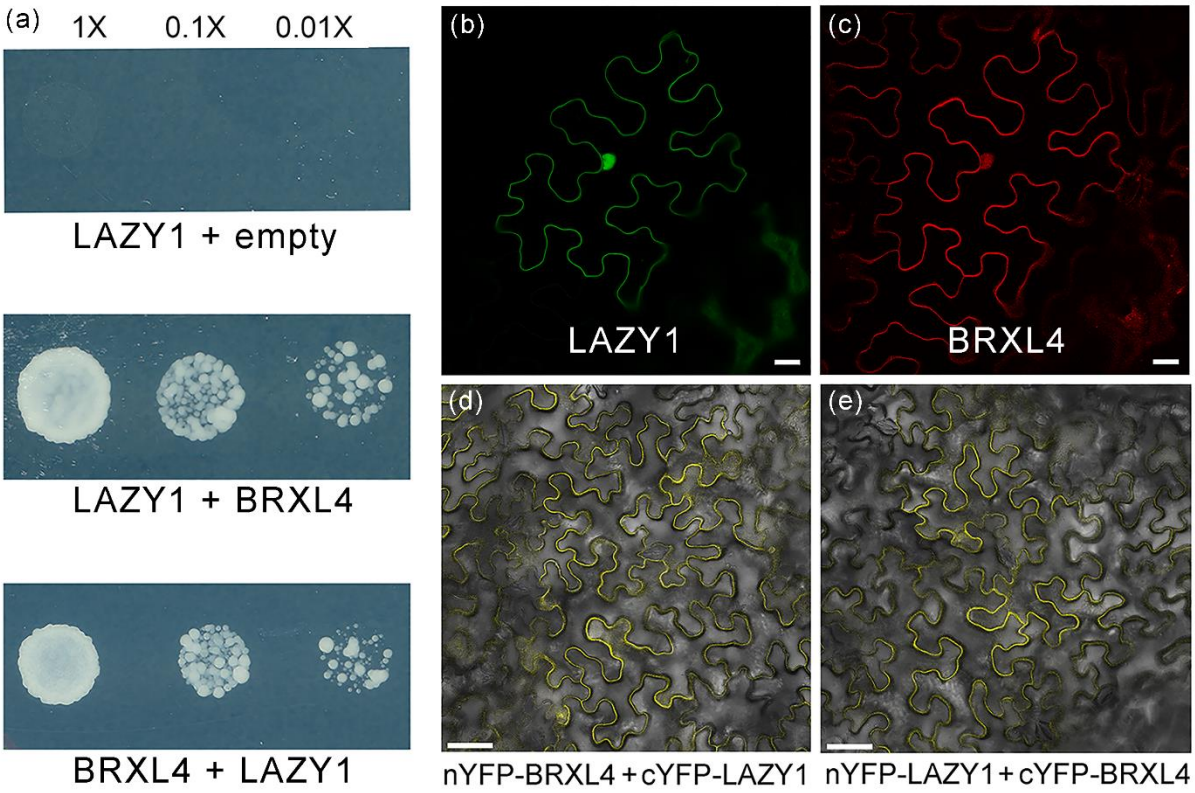

Figure 2.1. BRXL4 physically interacts with LAZY1 in yeast and in plant cells. (a) A dilution series of yeast grown for 3 d on selective media also containing 20 mM 3-AT, an inhibitor of autoactivation. The displayed result is representative of 5 independent trials. Cells containing LAZY1 presented as bait with an empty prey vector (control) did not grow. Cells with LAZY1 bait and BRXL4 prey grew, indicating interaction, as did cells with BRXL4 bait and LAZY1 prey. (b) Subcellular localization of LAZY1 tagged with GFP and (c) BRXL4 tagged with RFP co-expressed in *Nicotiana benthamiana* leaf epidermal cells. Both proteins appear at the plasma membrane and in the nucleus. Scale bar = 20 µm. (d) Bimolecular fluorescence complementation assay in *Nicotiana benthamiana* leaf epidermal cells. LAZY1 interacts with BRXL4 to functionally join the amino-terminal half of YFP (nYFP) to the carboxy terminal half (cYFP), at the plasma membrane

but not the nucleus, indicate by yellow fluorescence signal. (e) The interaction was also evident when the molecules were presented in the other the possible configuration. Scale bar = 50 µm.

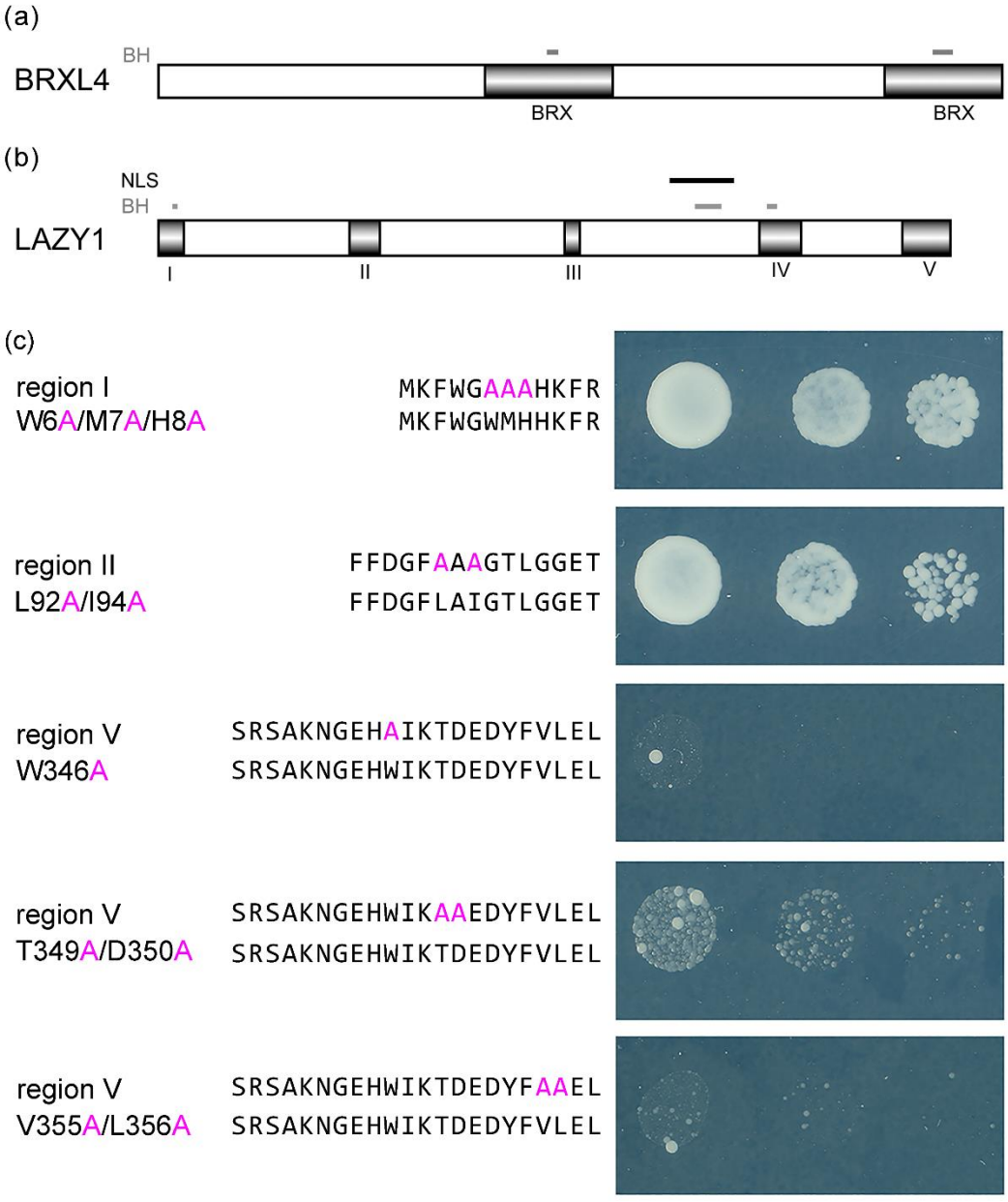

Figure 2.2. Structural features of BRXL4 and LAZY1 and their relevance to physical interaction. (a) BRXL4 is a 384 amino-acid protein containing two BRX domains. A BH domain is predicted within each BRX domain. (b) LAZY1 is a 358 amino-acid protein with five regions of substantial sequence conservation (I-V), a nuclear localization signal (NLS) and three predicted BH domains. The BH domain in region I contains the residues known to be required for associating LAZY1 with the plasma membrane. Another BH domain lies within the NLS. (c) Highly conserved residues in LAZY1 were replaced with alanine. Mutations in regions I and II did not affect BRXL4-LAZY1 interaction in a yeast two-hybrid assay performed as in Figure 2.1, but three different alterations in region V greatly diminished the interaction.

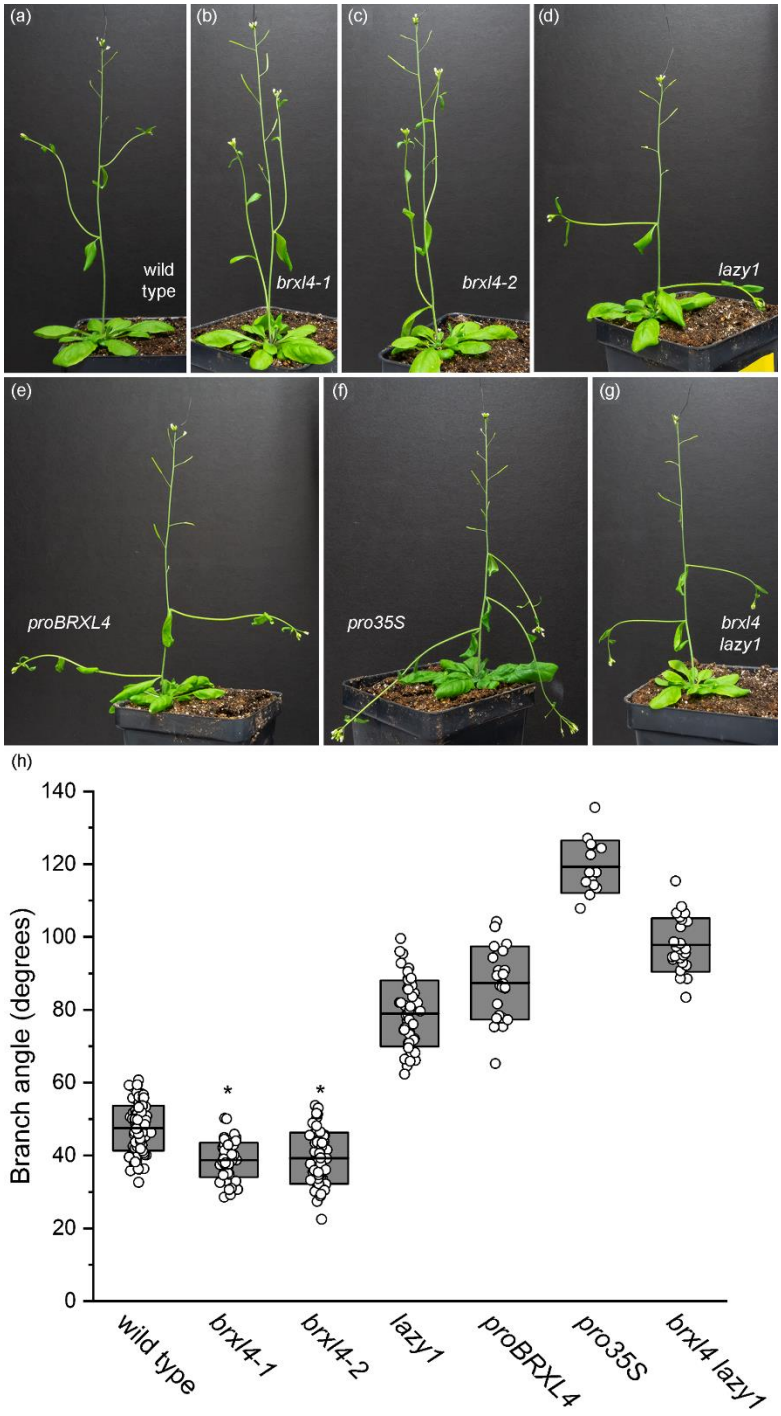

Figure 2.3. Shoot architecture phenotypes associated with *BRXL4* mutation and overexpression. (a) Wild type, (b) *brxl4-1*, (c) *brxl4-2*, (d) *lazy1,* (e) wild-type plant transformed with *BRXL4* controlled by the *BRXL4* promoter, (f) wild-type plant transformed with *BRXL4* controlled by the

strong *35S* promoter, and (g) *brxl4 lazy1* double mutant. A thin wire connected to the shoot apex was gently lifted with a micromanipulator to straighten the main inflorescence stem so branch angles could be clearly seen in each photographs. (h) Angle of the first-formed branch for the genotypes shown in (a-g). The *brxl4* alleles have a significantly smaller angle than wild type, * = $p < 0.05$ as determined by Student's t-test. Box size denotes the standard error of the mean.

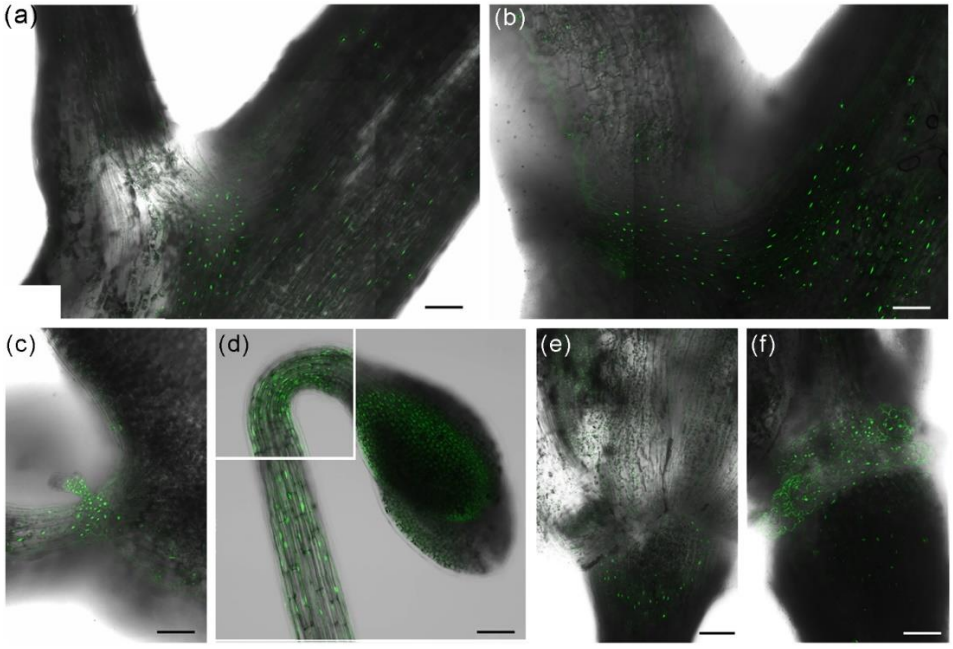

Figure 2.4. *BRXL4* expression pattern visualized by expressing a nuclear-localized version of GFP controlled by the *BRXL4* promoter. Expression is evident in the nodes of the inflorescence stem and branch bases (a, b), in axillary buds (c), throughout etiolated hypocotyls (d), at the base of flowers (e), and siliques (f). Some pictures were prepared by overlapping separate frames. Scale bar = 200 µm except (a), which = 400 µm.

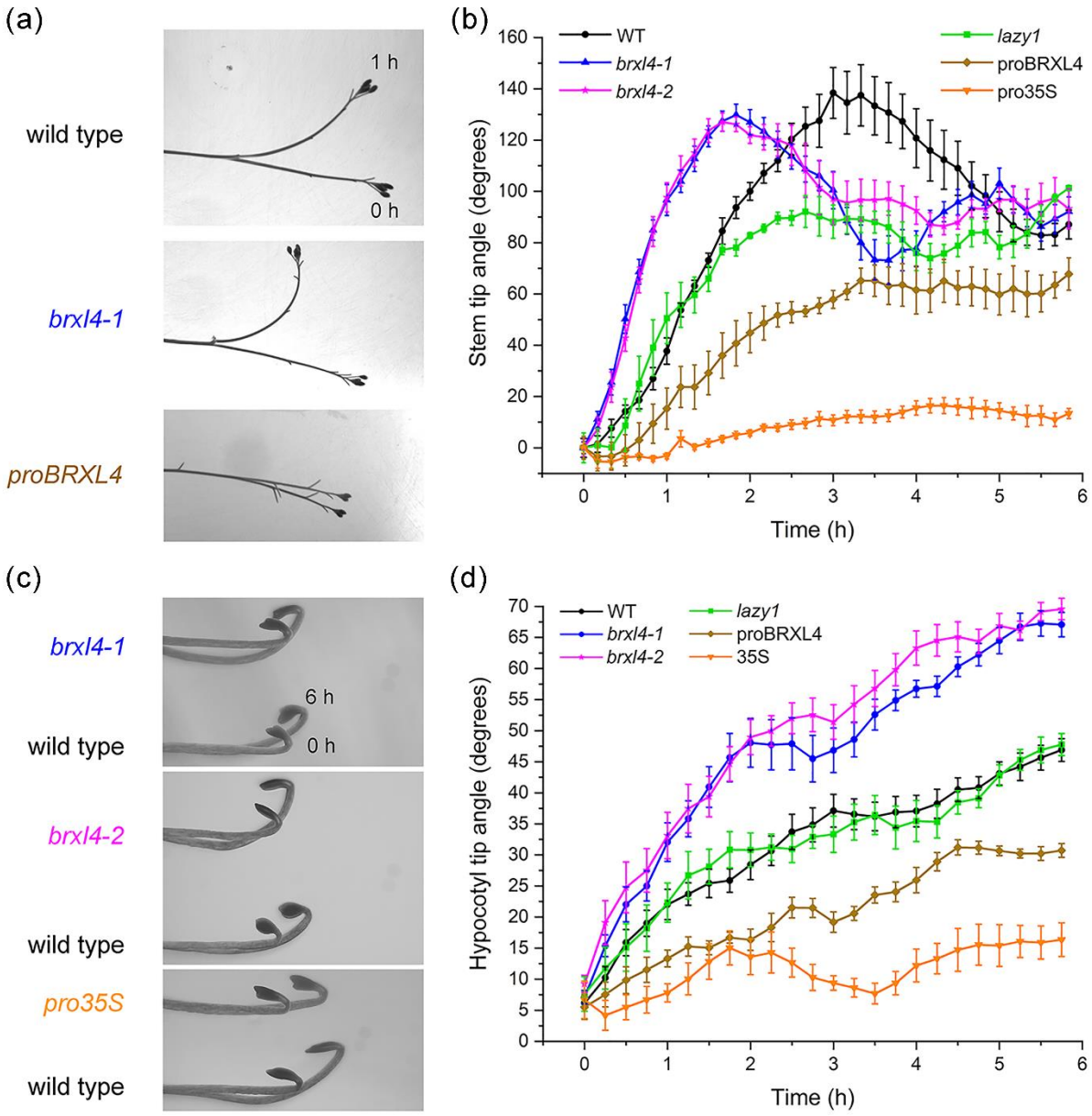

Figure 2.5. Gravitropism phenotypes of *brxl4* mutants and *BRXL4* overexpressors compared with *lazy1* and double mutants. (a) Inflorescence gravitropism examples. Excised primary inflorescence stems are shown when rotated horizontally and 1 h later. Hypergravitropism of *brxl4* and weak gravitropism of *proBRXL4* are evident. (b) Time course of inflorescence gravitropism measured by automated image analysis shows faster, hypergravitropism of two *brxl4* alleles and suppressed gravitropism in plants overexpressing *BRXL4*. Error bars denote

SEM. (c) Etiolated hypocotyl gravitropism examples. Intact seedlings are shown when rotated horizontally and 6 h later. (d) Time course analysis shows faster, hypergravitropism of two *brxl4* alleles and suppressed gravitropism in plants overexpressing *BRXL4*. Error bars denote SEM.

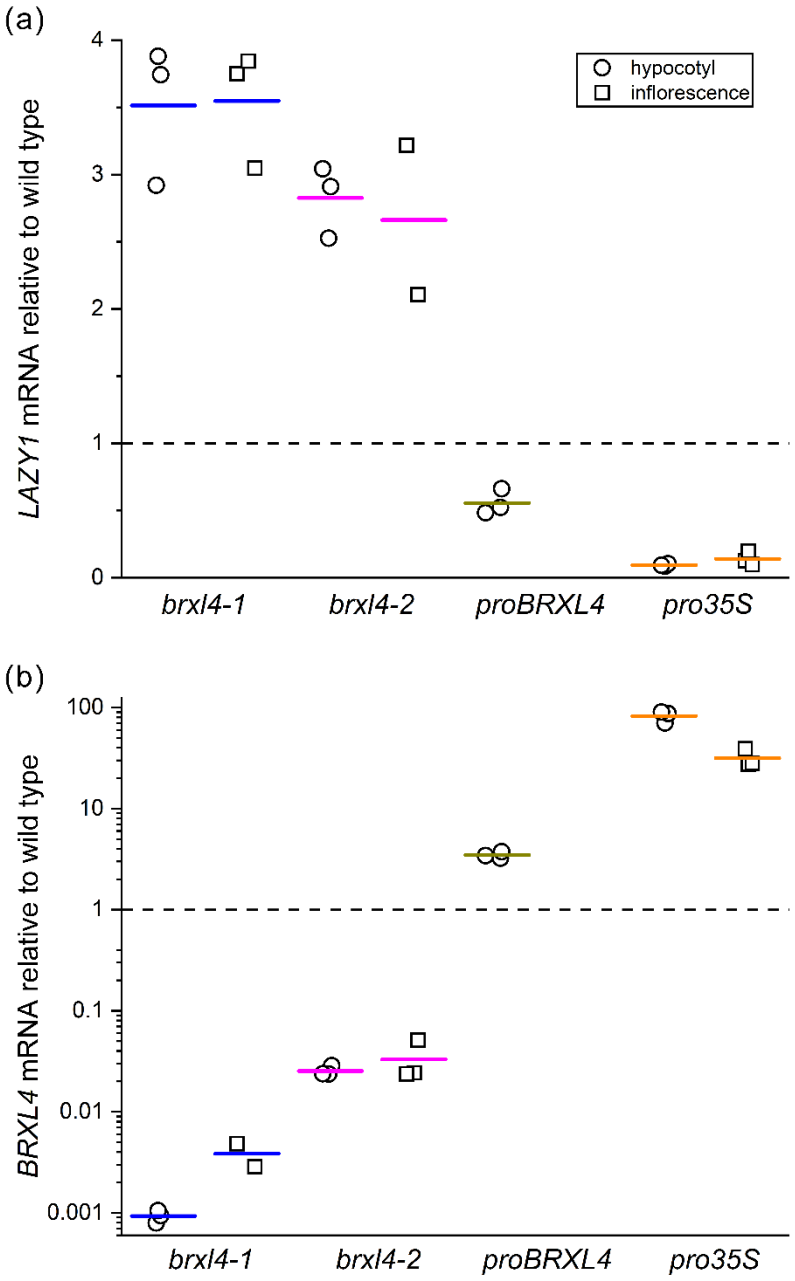

Figure 2.6. *LAZY1* expression is inversely related to *BRXL4* expression. Expression of *LAZY1* as a function of *BRXL4* expression. (a) *LAZY1* mRNA level is approximately 3-fold higher in *brxl4* than in the wild type, in hypocotyls and inflorescence stems. Expressing *BRXL4* with its native promoter (*proBRXL4*) in the wild type reduced *LAZY1* expression by approximately 50%, and to nearly undetectable levels when the 35S promoter (*pro35S*) controlled expression. *LAZY1* mRNA was not measured in proBRXL4 inflorescence stems. (b) No or very low *BRXL4* expression in the brxl4 alleles used here, while *proBRXL4* increased *BRXL4* mRNA approximately 3-fold, and the *pro35S*:*BRXL4* construct increased *BRXL4* mRNA approximately 80-fold in hypocotyls. Note the logarithmic scale of the y-axis. Each symbol represents an independent experiment, i.e. separate biological replicate.

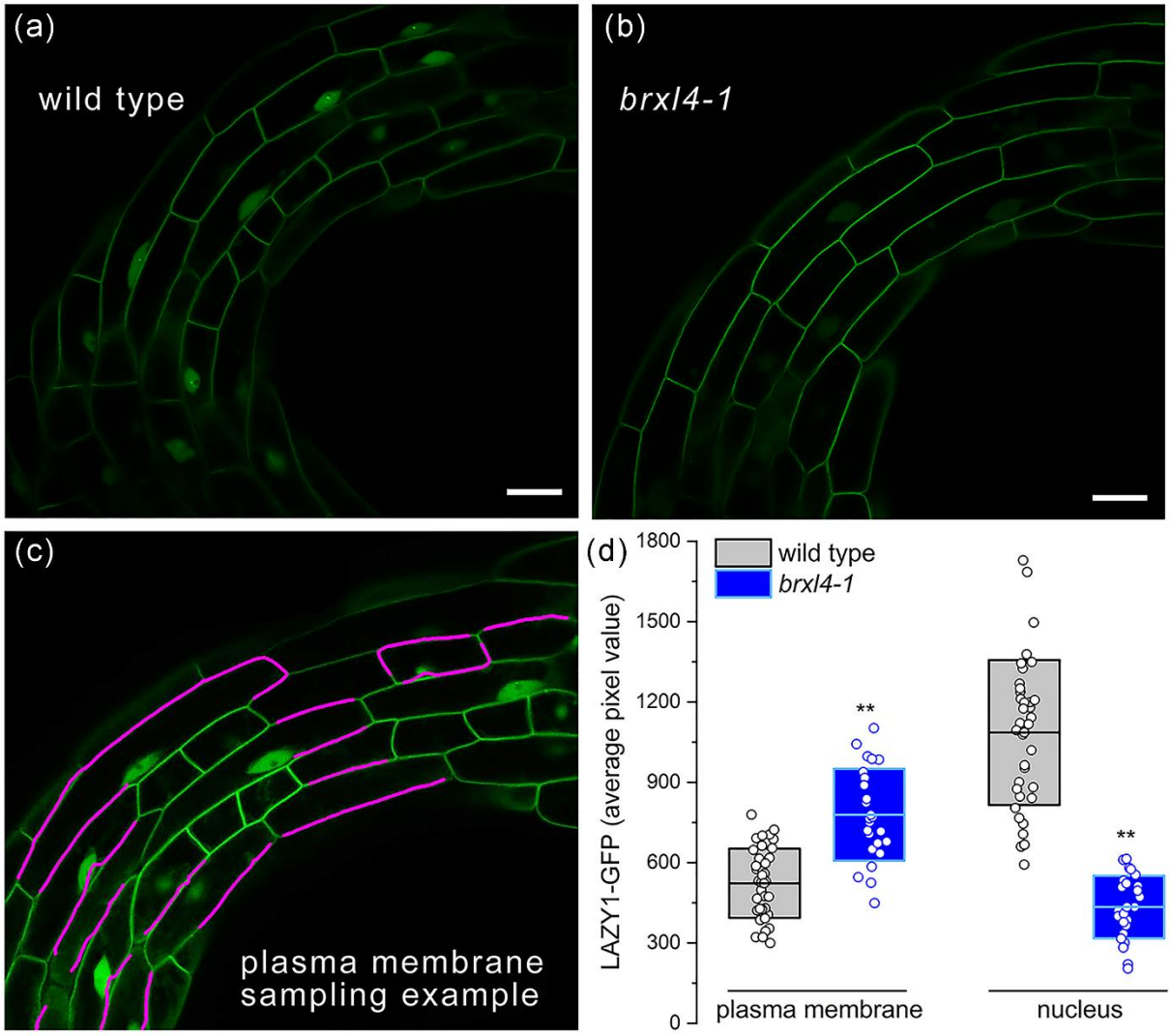

Figure 2.7. BRXL4 affects LAZY1 subcellular distribution. (a) LAZY1-GFP was present at the plasma membrane and in the nucleus when expressed in a *lazy1* mutant (wild type context) controlled by a heat-shock promoter to produce measurable signals. (b) LAZY1-GFP was primarily at the plasma membrane and secondarily in the nucleus when expressed in a *brxl4-1* mutant. Scale bar = 20 µm. (c) Magenta lines show the regions of an example image that the custom analysis method automatically sampled to determine the GFP level at the plasma membrane. (d) The average GFP channel value for plasma membrane or nuclear pixels in the

indicated genotypes. Each symbol represents the average pixel value sampled from the plasma membranes or nuclei in one image, and each image represents a separate hypocotyl. Box size denotes SEM. ** indicates that the *brxl4* values are significantly different from the wild type at a p = 0.01 as determined by a Student's t-test.

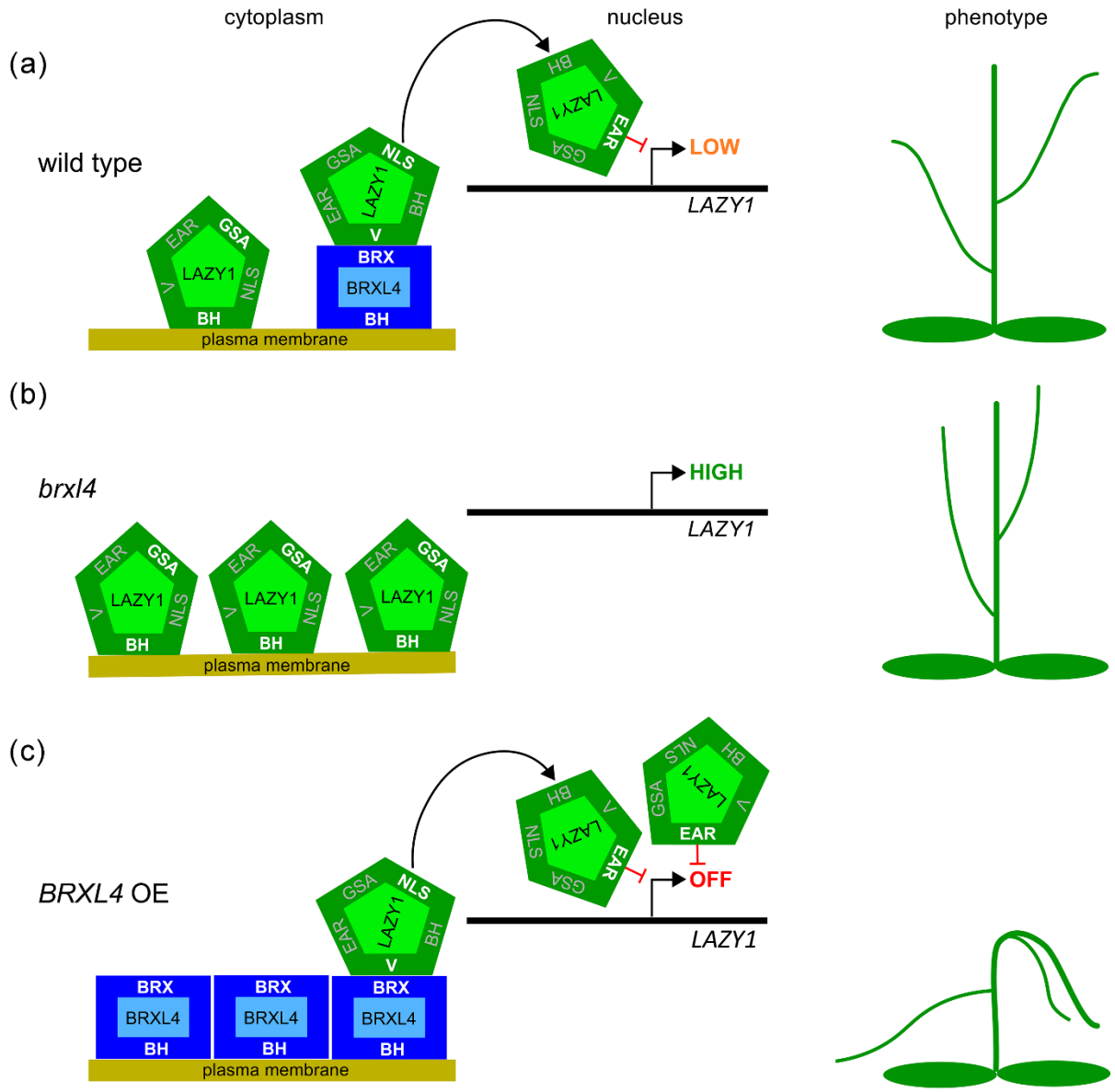

Figure 2.8. BRXL4 may negatively regulate LAZY1 by shifting it from the plasma membrane, where it functions in gravity signaling, to the nucleus, where it may suppress its own expression. (a) According to this model, the basic-hydrophobic (BH) domains of LAZY1 interact with the plasma membrane. LAZY1-mediated gravity signaling sets the gravity setpoint angle (GSA) of branches. The transcription-repressing EAR domain, the nuclear localization sequence (NLS), and the BRX-interacting region V (V) are inoperative in this situation and are therefore shown in gray. BRXL4 associates with the plasma membrane via the BH domain and with region V of LAZY1 via the BRX domain, which enables the NLS to promotes transport of LAZY1 to the nucleus where the EAR domain suppresses expression of *LAZY1*. (b) In a *brxl4* mutant, LAZY1 remains at the plasma membrane with its GSA function active and its NLS function suppressed. The resulting *LAZY1* overexpression produces hyperactive gravitropism and steeper branch angles. (c) *BRXL4* overexpression shifts more LAZY1 from the plasma membrane to the nucleus where its EAR domain suppresses its own expression, resulting in weak gravitropism and wide branch angles.

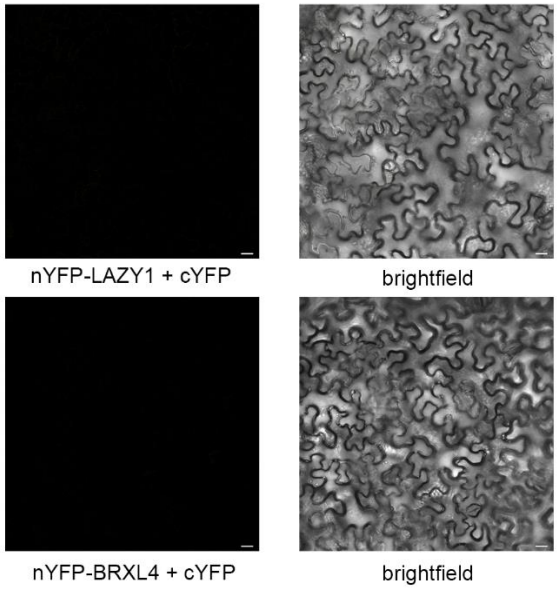

Figure 2.S1. Control experiments show that the n-terminal portion of YFP attached to either LAZY1 or BRXL4 does not interact with the c-terminus of YFP to create a functional fluorescent YFP protein. The left panels would show fluorescence corresponding to the cells in the right panel if the fluorescence complementation shown in Fig. 1B was not due to BRXL4-LAZY1 interaction. Scale bar = 20 µm

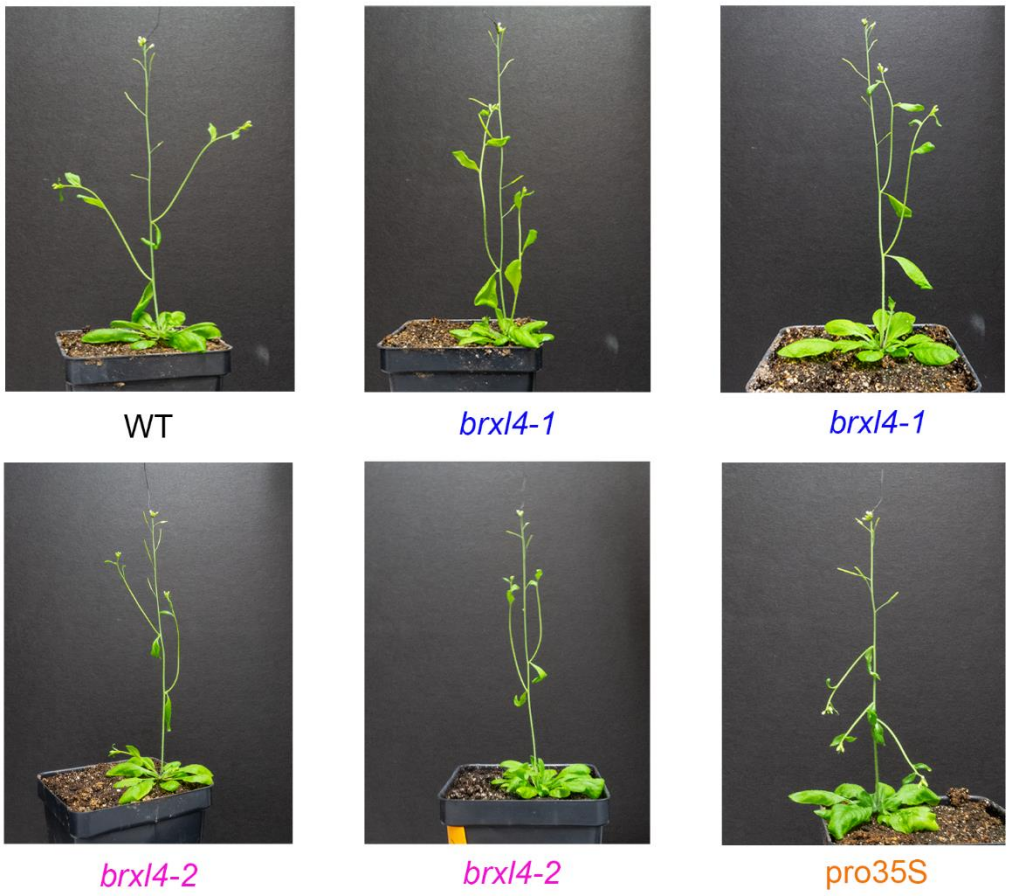

Figure 2.S2. Additional examples of the *brxl4* mutants and the *pro35S*:*BRXL4* overexpressor.

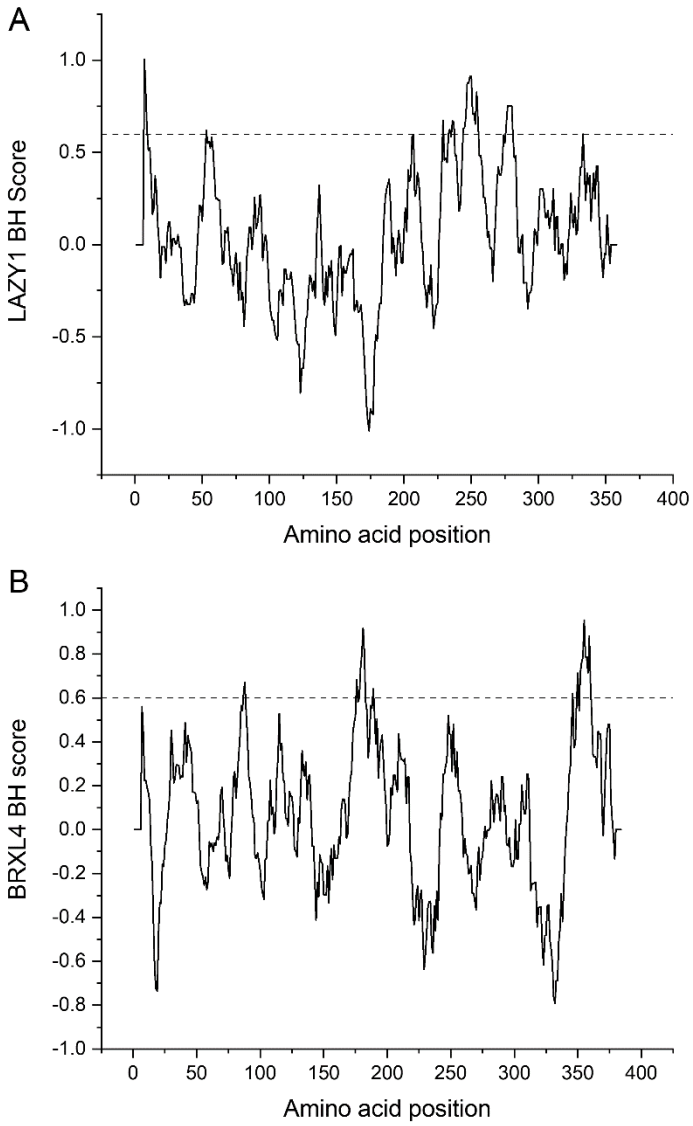

Figure 2.S3. Basic Hydrophobic (BH) score profiles for LAZY1 and BRXL4 proteins. Peaks above the horizontal dashed line indicate locations of predicted BH domains that can associate proteins with membranes. The relationships between the predicted BH domains and the recognized domains of LAZY1 and BRXL4 are shown in Figure 2.2. The plot was generated with the software described in Brzeska et al. (2010) and available as a Web service here: https://hpcwebapps.cit.nih.gov/bhsearch/.

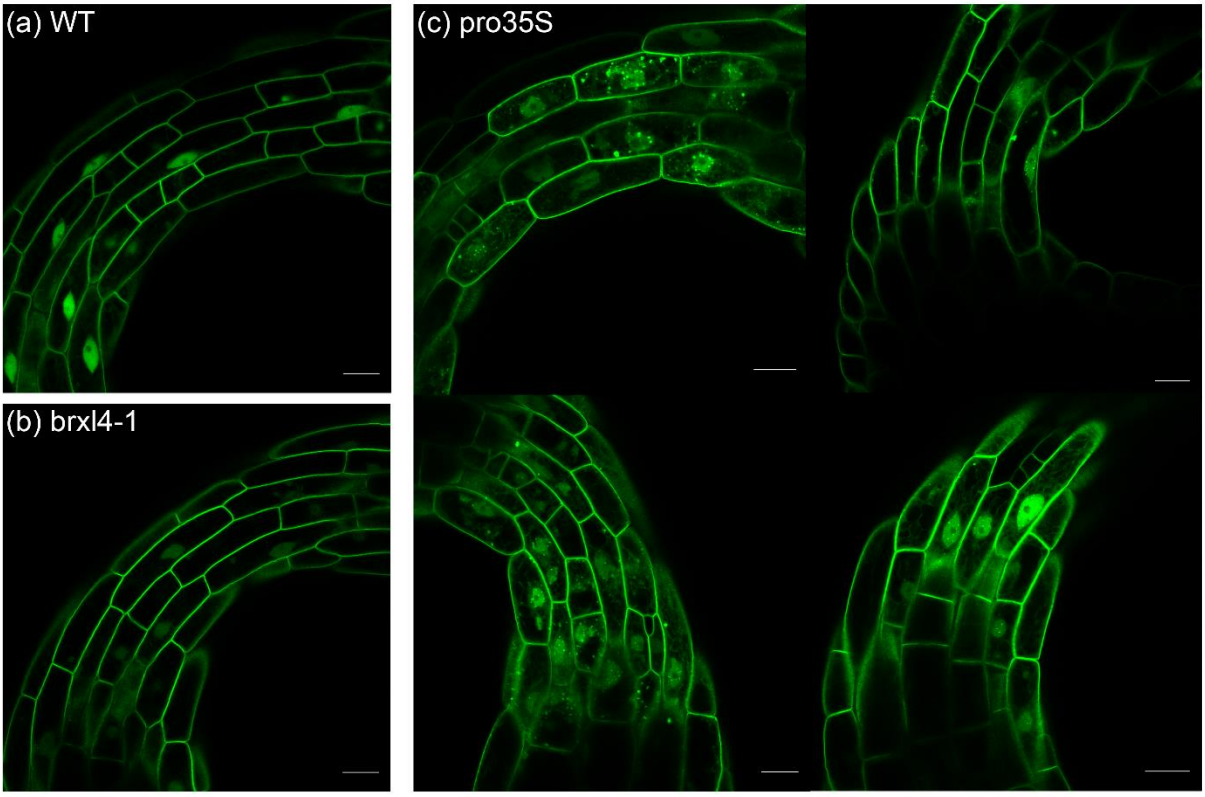

Figure 2.S4. LAZY1-GFP subcellular distribution in Arabidopsis hypocotyl. (a) LAZY1-GFP was present at the plasma membrane and in the nucleus when expressed in a lazy1 mutant (wild type context) controlled by a heat-shock promoter to produce measurable signals. (b) LAZY1-GFP was primarily at the plasma membrane and secondarily in the nucleus when expressed in a *brxl4-1* mutant. (c) LAZY1-GFP is localized to the cytoplasm and forms abundant inclusion bodies in pro35S plants. Scale bar = 20 µm.

Gene identifier	**Name**	**Function**
At5g20540	*BRXL4*	shoot-specific member of the *BREVIS RADIX-LIKE* family
At1g32060*	*PRK*	phosphoribulokinase, Calvin cycle enzyme
At5g17920*	*METS1*	methionine synthase; chromatin silencing via methylation
At4g13930*	*SHM4*	serine hydroxymethyltransferase, associated with many processes
At3g09840*	*CDC48*	cell division cycle AAA-ATPase associated with many processes
At3g01500*	*βCA1*	beta family carbonic anhydrase, many non-photosynthesis functions
At5g20290*	*RP8SA*	cytoplasmic small ribosomal protein, now called heS8A
At3g25520	*RPL5A*	cytoplasmic large ribosomal protein, now called huL18A
At1g07320	*PRPL4*	plastid ribosomal protein, embryo defective mutant (*EMB2784*)
At1g32990	*PRPL11*	plastid ribosomal protein
At3g63490	*PRPL1*	plastid ribosomal protein, embryo defective mutant (*EMB3126*)
At5g66570	*PSBO-1*	photosystem II extrinsic protein, functions in O_2 evolution
At1g06680	*OEE2*	oxygen-evolving enhancer, functions in photosystem II
At1g29930	*LHCB1.3*	light harvesting complex II subunit, functions with photosystem II
At2g34420	*LHCB1.5*	light harvesting complex II subunit, functions with photosystem II
At1g29910	*LHCB1.2*	light harvesting complex II subunit, functions with photosystem II
At3g54890	*LHCA1*	light harvesting complex I component, functions with photosystem I
At5g01530	*LHCB4.1*	light harvesting complex II subunit, functions with photosystem II

At5g62690	*TUB2*	tubulin beta chain
At2g21250		NAD(P)-linked oxidoreductase superfamily protein
At2g36830	*TIP1;1*	aquaporin-like protein, transports water across tonoplast
At3g26520	*TIP1;2*	aquaporin-like protein, transports water across tonoplast
At2g24790	*COL3*	Constans-like transcription factor that functions in light signaling
At1g20620	*CAT3*	annotated as a catalase, appears to function in nitrous oxide signaling
At5g63310	*NDPK2*	Nucleoside-diphosphate kinase, functions in light and ROS signaling

*gravity stimulation changed protein abundance in inflorescence stems as reported by Schenck *et al*.

Table 2.S1. Genes isolated more than once in the Y2H screen for LAZY1 interactors

Genotyping primers		
Mutant	Forward primer	Reverse primer
brxl4-1	5’ AGTCCCCTAGCCTCTGTGTTC 3’	5’ TCATGTATCCTTGCCCTGTTC 3’
brxl4-2	5’ TTGAGCAAAGGAGACAACATC 3’	5’ GGGTTTTTGATTAGCCGAGAC 3’
lazy1	5’ ATACTCGCTAGGGAGAGCTGG 3’	5’ TCTAAGTGAGCAAGGAGCCAG 3’
qPCR primers		
Gene	Forward primer	Reverse primer
LAZY1	5’ AAGATGCTTCTACTGGCAACAGC 3’	5’ ACCTCTTGGCTATCAAGGGATGG 3’
BRXL4	5’ GAGCTTCGAAGAGTCAGATTCAGC 3’	5’ ACCATAATCTCGCGTGCATCTCC 3’
UBQ10	5’ CACACTCCACTTGGTCTTGCGT 3’	5’ TGGTCTTTCCGGTGAGAGTCTTCA 3’

Table 2.S2. Primers used to determine the genotype of T-DNA mutants and to measure mRNA levels by quantitative RT-PCR.

Chapter III

BRXL4 negatively controls root gravitropism in Arabidopsis as well as LAZY1-GFP nuclear localization in *Nicotiana*

In this Chapter III, I present some additional logical experiments related to *BRXL4*, the LAZY1 regulator, to further examine the function and mechanism of *BRXL4* in regulating *LAZY1*, branch angle and shoot gravitropism in Arabidopsis, and subcellular localization in *Nicotiana*. Edgar Spalding and I participated in experimental design, data analysis, and result discussion. Bessie Splitt and Ashely Henry helped with root gravitropism data acquisition and processing.

Abstract

BRXL4 (BREVIS RADIX LIKE4) is a novel shoot regulator from *BRX* family. BRXL4-LAZY1 interaction at the plasma membrane controls Arabidopsis branch angle and shoot gravitropism. A GUS reporter (*proBRXL4:GUS*) revealed expression overlapping with *LAZY1* expression patterns, as it is mainly expressed in the petiole of cotyledons, the hypocotyl-root junction in seedlings, and in the abaxial side of the cauline leaves. Cauline leaf removal, the potential site of action, could occasionally rescue the branch angle phenotype of *proBRXL4:BRXL4* overexpressors. Consistent with the hypergravitropic shoot phenotypes of *brxl4* mutants, a *brxl4* mutation can fully reverse the dominant negative effect that the LAZY1 Region II variant LAZY1$^{L92A/I94A}$ plays on branch angle in wild type. Furthermore, *BRXL4* also negatively regulates root gravitropism in Arabidopsis. Complementing the fact that plasma membrane LAZY1 was higher and nuclear LAZY1 lower in *brxl4* mutants compared to wild type, LAZY1-GFP in *pro35S* lines of *BRXL4* formed bright spots in the nucleus and accumulated in the cytoplasm. Conversely, when transiently overexpressed in *Nicotiana benthamiana*, untagged BRXL4 or RFP-BRXL4 reduced the nuclear localization of LAZY1 or LAZY1 region I variant.

Introduction

In Chapter II, I conducted a Y2H screening to identify a LAZY1 interactor, BRXL4, and demonstrated that *BRXL4* negatively regulates Arabidopsis branch angles and LAZY1 expression by affecting LAZY1 subcellular distribution. Li *et al*. (2019) working with rice also demonstrated the interaction between OsLAZY1 and OsBRXL4 and that *OsBRXL4* negatively regulates rice tiller angles.

BRXL4 belongs to the *BRX* family. The *BRX* genes only exist in land plant, same as *LAZYs*. There are five *BRX* genes in Arabidopsis genome and all members contain two characteristic *BRX* domains required for *BRX* activity. *BRX* stands for "short root" in Latin and it was originally identified as a novel regulator of root. However, analyses of *brxl1*, *brxl2*, *brxl3*, and *brxl4* mutants supported that *BRX* is the only member with a dominating role in root development (Briggs *et al*., 2006). Though *BRXL1* and *BRXL* genes in other organisms, but not *BRXL4*, can apparently rescue *brx* mutant phenotype (Beuchat *et al*., 2010).

BRX is primarily located to the plasma membrane (PM) but displays a dynamic auxin-responsive PM-to-nucleus movement, which indicates the potential for distinct functions in different cellular compartments (Scacchi 2009; Marhava *et al*., 2018). When a nuclear export signal (NES) is deleted from BRX, it is only present in nucleus. In addition, mutation of the two cysteines in the N-terminus of BRX completely disrupted its plasma membrane localization and polarization (Rowe *et al*. 2019). However, a recent discovery diminished the functional importance of the nuclear localization of BRX regarding its known phloem functions. No biological role can be

firmly attributed to the nuclear localization of BRX and that Scacchi *et al*. 2009 proposed central function as a transcriptional regulator needs to be rediscussed (Koh *et al*., 2021).

Interestingly, *BRXL4*, although non-redundant with regards to *BRX* activity in the root, is primarily expressed in the shoot tissue, and it appears to be the only *BRX* gene that regulates shoot gravitropism (Beuchat *et al*., 2010). Overexpression of OsBRXL4 leads to a prostrate growth phenotype, whereas *OsBRXL4* RNAi plants displayed a compact phenotype (Li *et al*. 2019).

Similar to the situation of *LAZY1*, we do not know much about the molecular mechanism of BRXL4 function. There is little information about the structural functions of BRXL4 protein or interacting proteins of BRXL4. Nevertheless, there are a few studies addressing the function and the regulatory role of *BRX* on root growth (Koh et al., 2021; Scacchi et al., 2009). Given that *BRXL4* appears to be the predominantly expressed *BRX* gene in shoots, though the absolute expression level is still low, those *BRX* associated roles may be applicable to *BRXL4* in shoot (Beuchat et al., 2010). *BRX* was reported to enhance *PIN3* expression to promote root meristem growth (Scacchi et al, 2009). It is intriguing because asymmetric PIN3 reorientation in root and hypocotyl contributes to the redistribution of auxin induced by gravity stimuli, and *LAZY* genes fail to create an asymmetric distribution of PIN3 in root, subsequently asymmetric auxin concentration (Taniguchi et al. 2017). It is possible that BRXL4 participates in the regulation of LAZY1 on PIN3 in Arabidopsis hypocotyl.

Results

GUS reporter assay revealed proBRXL4:GUS expression pattern to overlap with proLAZY1:GUS

In Chapter II, I examined tissue-specific *BRXL4* gene expression patterns in Arabidopsis plants throughout development using a *proBRXL4*:*GFP-NLS* reporter system. The *BRXL4* pattern largely overlapped with the *LAZY1* expression patterns. The GFP signal, indicating *BRXL4* promoter activity, was primarily detected in the node region including newly emerging axillary buds as well as at the base of flowers and siliques (Figure 2.4). In this chapter, a complementary GUS reporter assay is presented. I generated an Arabidopsis line containing the *BRXL4* promoter driving GUS (*proBRXL4:GUS*) and histochemically stained for GUS activity. In one-week old seedlings, I found that the *BRXL4* promoter had strong expression in the petiole of cotyledons and the shoot-root junction. Strong staining was also visible in the upper root but not the root tips (Figure 3.1a–c). In contrast, the *LAZY1* promoter has minimal activity in the root and does not control root gravitropism (Yoshihara *et al.*, 2013, Hollender *et al.*, 2020). Almost no staining was observed in *proBRXL4:GUS* cotyledons, same as the *LAZY1* promoter activity.

In adult plants, staining for *BRXL4* expression was mostly observed in the abaxial side of the cauline leaves (Figure 3.1d–f) as well as the base of flower (Figure 3.1e). Minimal staining was observed in the lateral branches or the apical region of the shoot. This result is somewhat similar to the *proBRXL4:GFP-NLS* expression pattern, though the GFP signal appeared more prevalent all over the node region and strong in newly emerging axillary buds (Figure 2.4). Generally, the *LAZY1* promoter activity largely overlapped with the *BRXL4* promoter GUS staining result in the inflorescence stem and flower tissue, except that *proLAZY1:GUS* displayed

strong expression in the shoot apex (Hollender *et al.*, 2020). Overlapping *BRXL4* and *LAZY1* expression patterns are consistent with the finding that LAZY1 and BRXL4 proteins interact to control Arabidopsis branch angle and shoot gravitropism.

Cauline leaf removal could occasionally rescue the branch angle phenotype of proBRXL4:BRXL4 overexpressors

Based on the evidence that *BRXL4* is strongly expressed in the lower (abaxial) side of cauline leaves (Figure 3.1), I wanted to test if cauline leaves are necessary for setting branch angle. I removed the cauline leaves of *proBRXL4:BRXL4* overexpressors (*proBRXL4*) to determine if removal of the putative site of action could offset the overexpression of *BRXL4* driven by its native promoter. Figure 3.2 displays two of the *proBRXL4* line that presented a rescued branch angle phenotype after the removal of the corresponding cauline leaves. The cauline leaf was removed when the auxiliary bud just emerged from the main inflorescence stem, and the cauline leaves were removed by hand tearing. Normally, when cauline leaves are untouched, the branch angle of *proBRXL4* line is approximately 90° (Figure 2.3), as what is also shown in Figure 3.2. For the two branches that had no cauline leaves attached developing from auxiliary buds, they appeared to be much more upright than other branches with untouched cauline leaves. One straightforward explanation is that the removal of cauline leaves greatly reduced the *BRXL4* expression in the node region, based on the GUS staining result indicating that *BRXL4* promoter activity is evident at the bottom of the cauline leaves. Without the overexpression of *BRXL4*, *LAZY1* would not be suppressed, hence the branches were able to develop wild-type-like

angles. Notably, this branch angle phenotype rescue did only happen infrequently, possibly due to individual variances as well as the inconsistent timing of the cauline leave removal.

LAZY1 $^{L92A/I94A}$ has a dominant negative effect on branch angle in wild-type plants but not in brxl4 *mutants*

Yoshihara and Spalding (2020) reported that *proLAZY1:LAZY1$^{L92A/I94A}$/atlazy1* plants display a "weeping" inflorescence phenotype, that is, the shoot gravity responses shifted from negative (upward bending) to positive (downward bending). To test whether the LAZY1 region II variant, LAZY1$^{L92A/I94A}$, would disrupt normal LAZY1 function when expressed in wild type (WT) background, moreover, whether a *brxl4* mutation would apply a unique effect.

Multiple transgenic *proLAZY1:LAZY1$^{L92A/I94A}$*/WT plants were generated, and the shoot architecture was assessed. It is surprising that a large majority of the T1 plants showed horizontal branch angle, and the branches all appeared to possess reversed gravitropic response (downward bending) (Figure 3.3a). The phenotype is clearly more extreme than *lazy1* mutant which displays negative gravitropism (Figure 2.5), and it is also similar to *proLAZY1:LAZY1$^{L92A/I94A}$/lazy1* plants, in terms of the reversed gravitropic response of the branches. It is evident that LAZY1 region II variant has a dominant negative effect that it may override the normal LAZY1 function to cause the reversed gravitropism. I have not examined the inflorescence stem gravitropism of *proLAZY1:LAZY1$^{L92A/I94A}$*/WT.

In order to test if a *brxl4* mutation could alleviate the reversed gravitropism caused by LAZY1$^{L92A/I94A}$, *proLAZY1:LAZY1$^{L92A/I94A}$* was expressed in both *brxl4-1* and *brxl4-2* mutants.

Consistent with the enhancing effect of *brxl4* mutation in gravitropism, *proLAZY1:LAZY1*$^{L92A/I94A}$/*brxl4* showed a branch GSA closer to wild type compared to *proLAZY1:LAZY1*$^{L92A/I94A}$/WT (Figure 3.3b,c). No quantitative data were collected but *proLAZY1:LAZY1*$^{L92A/I94A}$/*brxl4* plants do not look as compact as *brxl4* mutants. It is striking that the absence of BRXL4 protein would offset the dominant negative effect of LAZY1$^{L92A/I94A}$ and brings the plants back to the wild-type plant shoot architecture. It is also notable that the branches of *proLAZY1:LAZY1*$^{L92A/I94A}$/*brxl4* appear to droop more than wild type.

BRXL4 negatively regulates root gravitropism in Arabidopsis

In Chapter II, the negative role *BRXL4* plays in shoot gravitropism was investigated and characterized. *BRXL4* is also expressed in root tissue (Figure 3.1a-c), but it is believed to be less influential in roots because BRXL4 cannot rescue the *brx* root phenotype (Beuchat et al. 2010). Nevertheless, I was interested to know if *BRXL4* also negatively regulates root gravitropism just like shoot gravitropism.

Wild-type, *brxl4* mutants, and *pro35S* plants were grown on vertical ½ MS media plates for one week (n=6), and then rotated 90° to induce gravitropism. The plates were scanned after 6 h. *pro35S* roots were completely agravitropic, similar to their shoots. The initial angle of their roots (their GSA) was extremely variable (Figure 3.4b). *brxl4* mutants seemed to show a more robust or enhanced root gravitropism (Figure 3.4a, c, d).

In order to rigorously examine the root gravitropism phenotype in the aforementioned genotypes, a high-throughput computing (HTC) image analysis pipeline described in Moore *et al*.

(2013) was used to quantify root gravitropism. Ashley Henry helped me set up and learn the image acquisition process, and HTC automatically measured the root tip angles generated within the total data collection time span of 8 h. Consistent with the variable root GSA of *pro35S* plants, their roots did not react to a gravitropic stimulus and stayed almost horizontal the entire time. *brxl4* mutants responded indistinguishably from wild type at early time points but displayed an enhanced root gravitropism after 4 h (Figure 3.5), which is different from the stem gravitropism reactions that *brxl4* mutants showed faster growth from the very beginning (Figure 2.5). None of the genotypes plateaued at 90°, which is not typical for Arabidopsis seedling roots. Some uncontrolled variables such as temperature may be the reason. However, it is evident that *BRXL4* negatively regulates root gravitropism in Arabidopsis.

Overexpression of untagged BRXL4 or RFP-BRXL4 reduces the nuclear localization of LAZY1 or LAZY1 region I variant in Nicotiana benthamiana

To investigate the BRXL4 effect on subcellular localization of LAZY1, a GFP-tagged LAZY1 was transiently over-expressed with or without untagged BRXL4 in *Nicotiana benthamiana* leaves by the infiltration method. The *Nicotiana* grew for 2 days after infiltration before being examined by confocal fluorescence microscopy. Compared to the obvious nuclear signal of LAZY1-GFP (Figure 3.6a), overexpression of untagged BRXL4 seemed to reduce the nuclear signal of LAZY1 (Figure 3.6c). LAZY1-I is referred to the LAZY1 region I variant, which has the mutation within a predicted BH domain (Figure 2.2b) and is only localized to the nucleus with minimal affinity to the plasma membrane (Yoshihara and Spalding. 2020). Figure 3.6b repeated the same

observation, but overexpression of untagged BRXL4 also made LAZY1-I-GFP localized mostly to the plasma membrane (Figure 3.6d), similar to the normal LAZY1 situation. Furthermore, I introduced the marker, rm-pk, which was adopted as the plasma and nuclear membrane marker from prior studies (Yoshihara *et al*. 2013). It is clear that rm-pk marker highlights the plasma membrane and the edge of nucleus (nuclear envelope), and the nuclear localization of LAZY1-GFP is evident (Figure 3.7a,b). Once the untagged BRXL4 was co-transformed, the nuclear LAZY1 was absent, as shown by the hollow red circle (Figure 3.7c,d). The confocal images shown are representatives of greater than 30 examples.

For these two trials, there was no corresponding fluorescent marker of BRXL4 to validate the presence of its overexpression, but it should not be a major concern because most of the observed *Nicotiana* cells showed the absence of nuclear signal of LAZY1-GFP and the only difference was the introduction of untagged BRXL4. Therefore, it is highly likely that overexpression of BRXL4 made a difference. Nevertheless, I performed a third trial that included UBQ:RFP-BRXL4 in the system. Consistent with the effect of untagged BRXL4, RFP-BRXL4, when co-overexpressed with LAZY1-GFP, reduced the nuclear signal of LAZY1-GFP (Figure 3.8a,b). It also made the mutated LAZY1-I-GFP version appear identical to the native LAZY1-GFP localization rather than only expressed in the nucleus (Figure 3.8c,d). In summary, Overexpression of untagged BRXL4 or RFP-BRXL4 reduces the nuclear localization of LAZY1 or LAZY1-I in *Nicotiana benthamiana*.

Figures and legends

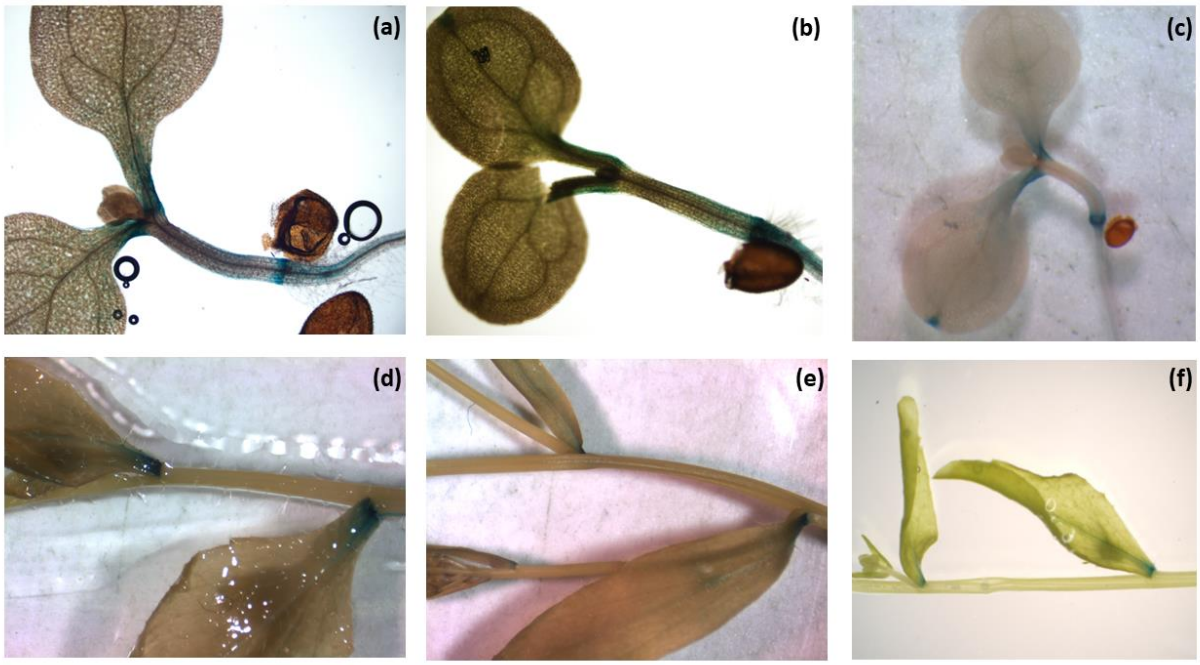

Figure 3.1. *BRXL4* expression pattern visualized via GUS driven by the *BRXL4* promoter. Expression is evident in the petiole of cotyledons and shoot-root junction (a-c), in the base of cauline leaves (d-f), at the base of flowers (e).

Figure 3.2 Cauline leave removal rescued the branch angle phenotype of *proBRXL4* plant, two examples are shown

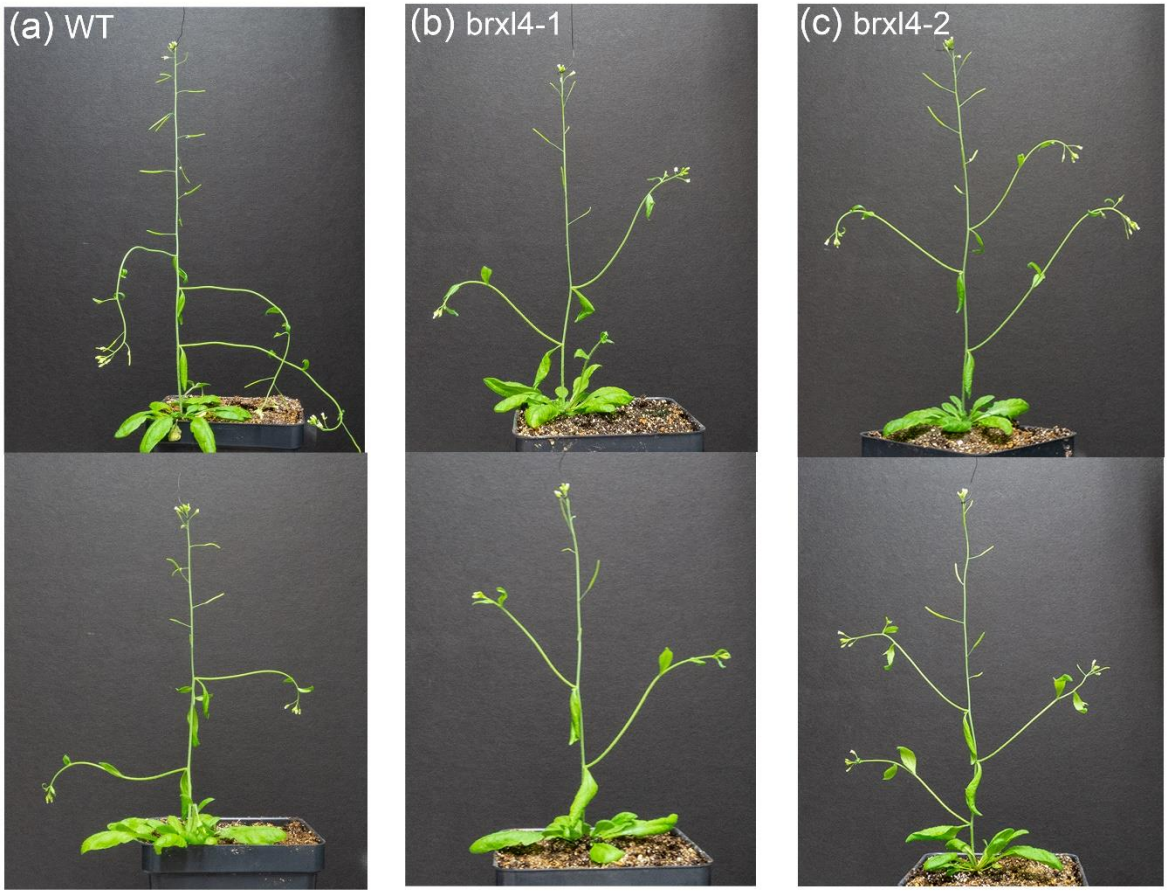

Figure 3.3. Shoot architecture phenotypes of LAZY1$^{L92A/I94A}$ expressed in different genetic backgrounds. (a) wild-type plant transformed with *proLAZY1:LAZY1*$^{L92A/I94A}$, (b) *brxl4-1* plant transformed with *proLAZY1:LAZY1*$^{L92A/I94A}$, and (c) brxl4-2 transformed with *proLAZY1:LAZY1*$^{L92A/I94A}$. A thin wire connected to the shoot apex was gently lifted with a micromanipulator to straighten the main inflorescence stem so branch angles could be clearly seen in each photograph.

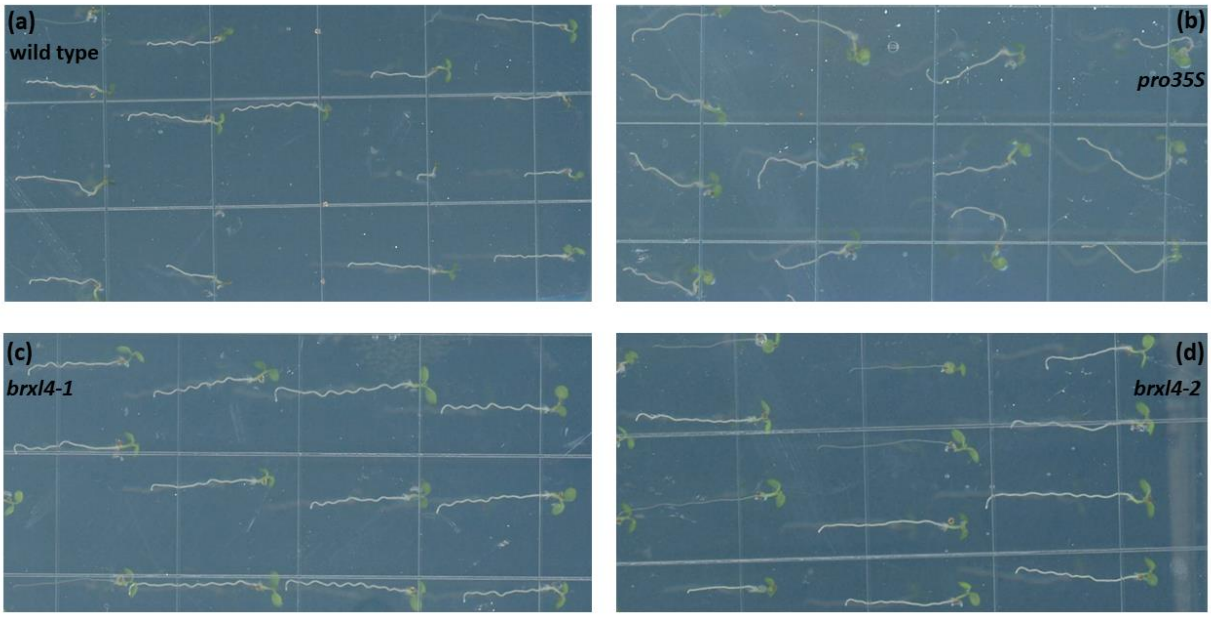

Figure 3.4. Root gravitropism associated with *BRXL4* mutation and overexpression. (a) Wild type, (b) *pro35S,* (c) *brxl4-1*, (d) *brxl4-2*. Plants were horizontally grown in ½ MS media for one week and rotated 90° to receive gravitropic stimulus. The plates were scanned after 6 h.

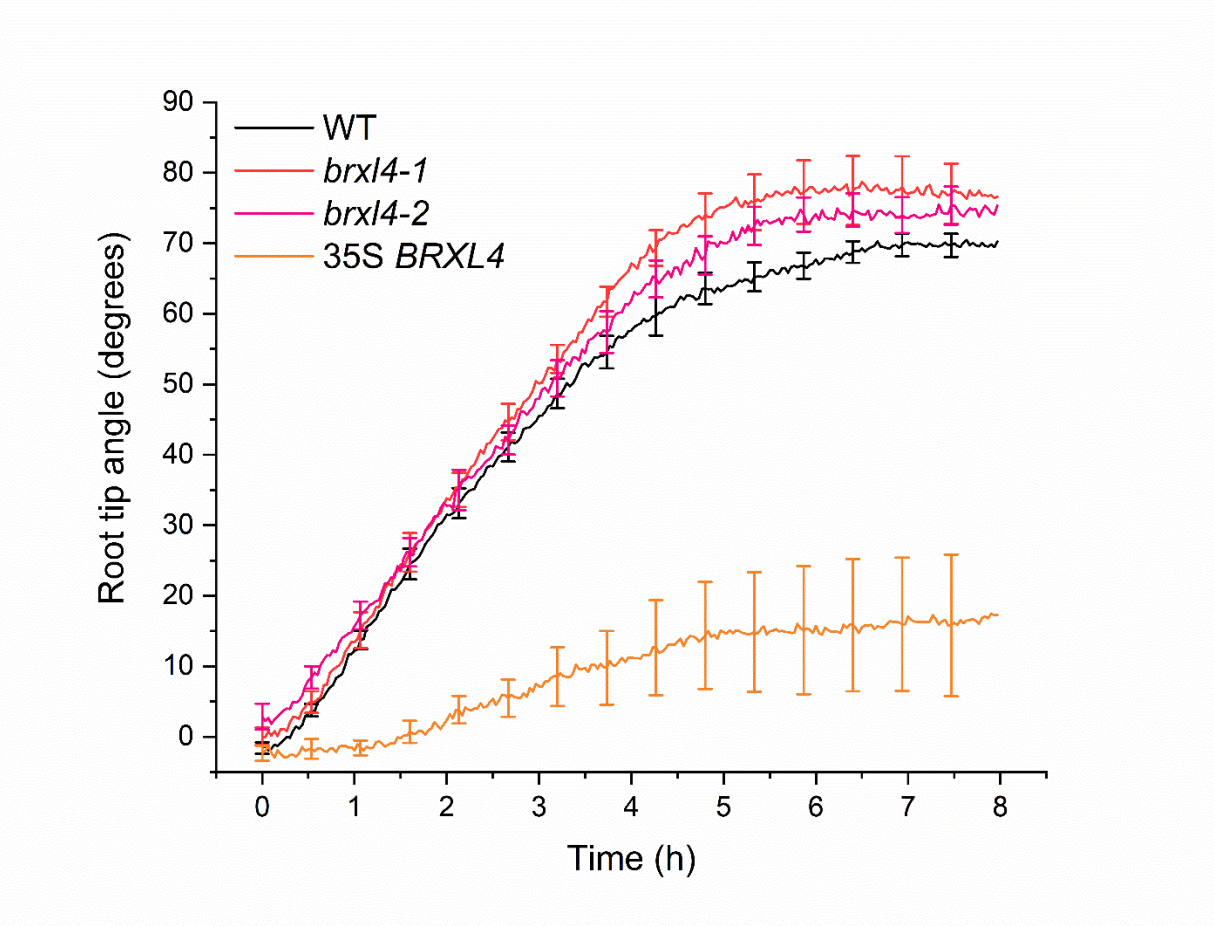

Figure 3.5. Time course of root gravitropism measured by automated image analysis shows faster, hypergravitropism of two *brxl4* alleles and suppressed root gravitropism in plants overexpressing BRXL4. Error bars denote SEM. n=6.

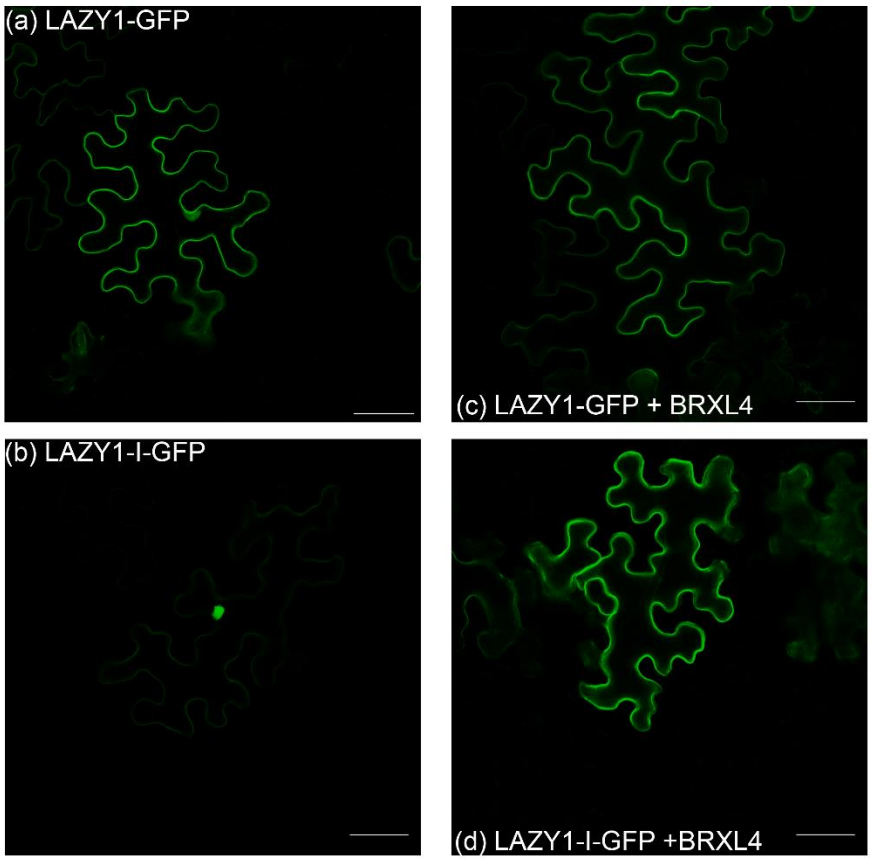

Figure 3.6. Untagged BRXL4 affects LAZY1-GFP and LAZY1-I-GFP subcellular distribution in *Nicotiana benthamiana*. (a) LAZY1-GFP is localized to the plasma membrane and the nucleus. (b) LAZY1-GFP is mostly localized to the plasma membrane with untagged BRXL4 overexpressed. (c) LAZY1-I-GFP is mostly localized to the nucleus. (d) LAZY1-I-GFP appears to be like native LAZY1. Scale bar = 20 µm.

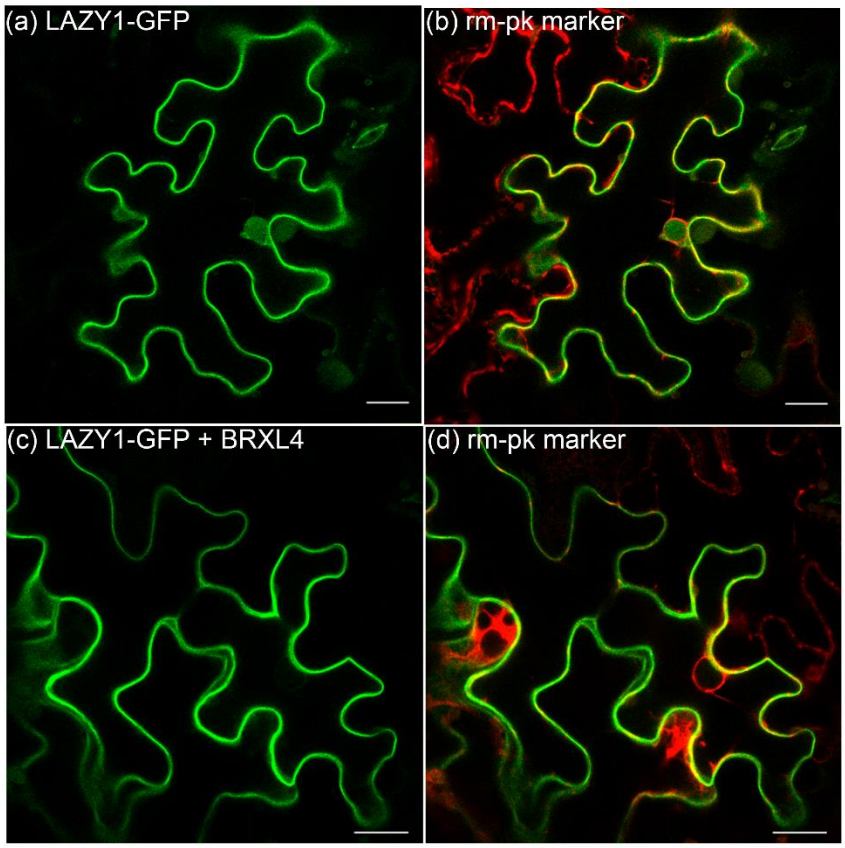

Figure 3.7. Untagged BRXL4 affects LAZY1-GFP subcellular distribution with rm-pk marker in *Nicotiana benthamiana*. rm-pk (red fluorescence) serves as the plasma and nuclear membrane marker.

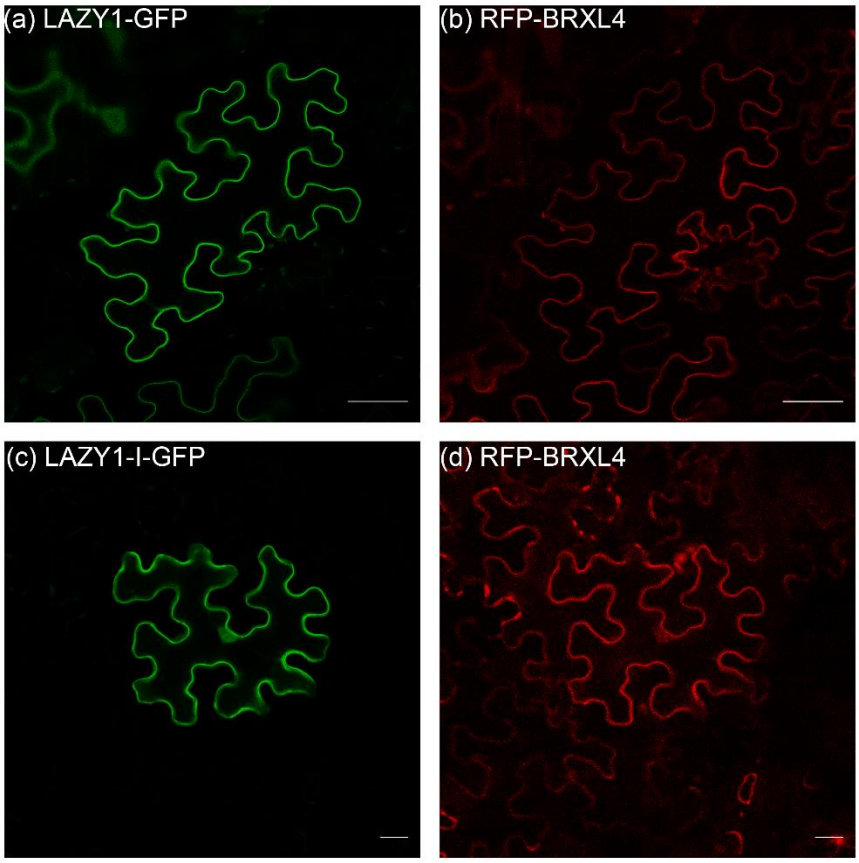

Figure 3.8. RFP-BRXL4 affects LAZY1-GFP and LAZY1-I-GFP subcellular distribution in *Nicotiana benthamiana*. UBQ:RFP-BRXL4 was co-transformed with 35S:LAZY1-GFP or 35S:LAZY1-I-GFP.

Discussion

BRXL4 belongs to the *BRX* family originally identified as players in regulation root growth and development, but *BRXL4* has no apparent effect on any root related phenotype of Arabidopsis (Mouchel *et al*. 2004). *BRXL4* cannot rescue the *brx* mutant phenotype, different from *BRXL1* and *BRXL* genes in other organisms (Beuchat et al., 2010). In fact, there is apparently no published indication of any *BRXL4* function in Arabidopsis as of today. The eFP browser gene expression tool shows that *BRXL4,* though expressed at a low level across different tissues*,* is the most abundantly expressed *BRXL* family gene in the hypocotyl and first internode of inflorescence stem among all *BRX* genes, suggesting its potentially unique role in shoot related biological processes.

Chapter II revealed BRXL4's novel regulatory role of negatively controlling branch angle and shoot gravitropism in Arabidopsis, and its discovery was derived from the interacting protein screening of LAZY1, a vital positive regulator of shoot gravitropism. The previous chapter discussed the structural features of BRXL4 and LAZY1 and their relevance to physical interaction, shoot architecture and gravitropism phenotypes associated with *BRXL4* mutation and overexpression, and the hypothetical model that BRXL4 may negatively regulate LAZY1 by affecting its subcellular localization. This Chapter contains the logical experiments that I designed to further explore the function and mechanism of *BRXL4* in regulating *LAZY1* and branch angle in Arabidopsis. A lot of the intriguing results do not fully make sense at this point and this dissertation certainly does not answer all questions. However, it reveals this important interaction between LAZY1 and BRXL4 in Arabidopsis as well as *brxl4* phenotypes that were not

previously known, and all the results shed some lights in future directions and raise many valuable questions at the same time.

The expression patterns of *BRXL4* and *LAZY1* are very similar at the tissue level that neither of them shows strong expression in the branch (Figure 2.4, 3.1; Hollender *et al*., 2020; Yoshihara *et al*., 2013). Furthermore, high *proBRXL4* expression in the cauline leaves and not in bending region of lateral branch suggests branch GSA may be set early on based off the young branch curvature and remembered throughout the growth, possibly by the early regulated equilibrium between gravitropic and anti-gravitropic offset components or epigenetics mechanism. The cauline leaf removal experiment serves as supporting evidence of the GUS staining results (Figures 3.1, 3.2). It appears that the removal of the putative site of action would sometimes completely alleviate the *BRXL4* overexpression effect in *proBRXL4* plants, i.e., increasing the branch angle to 90°. However, another side of the story is that large majority of the leave removal did not generate any visible difference. There are two major caveats: 1) the timing of the removal could have made fundamental differences, as we are still unsure of how and what developmental stage BRXL4 affects the branch angle without being visible in the branch. It is conceivable that the majority of leaf removal attempts were too late to stop the *BRXL4* OE impairment in branch angle, while it would have been too difficult to perform the removal when it was appropriately early. 2) BRXL4 does not seem to be only expressed in the cauline leaves (Figure 2.4) and there was no way to determine if the leaf removal was complete and thorough. I did attempt some burning-based removal trials which did not generate anything exciting. Nevertheless, results from Figure 3.2 still substantially support the GUS staining results and further solidify *BRXL4*'s function in controlling branch angle in Arabidopsis. It would be

helpful for completing the story to explore BRXL4's interactors and trace the ones that serve as the bridges to bring the GSA regulating info from the cauline leaves to the branches during the development. It would also be interesting to see what epigenetic regulations is involved in the process by analyzing the *BRXL4* OE lines genome from the branch tissues. If it can be confirmed that *BRXL4* activity in the cauline leaf plays a major role in setting branch GSA, then it is acting non-cell autonomously. Such a result would imply that BRXL4 participates in producing a mobile signal that sets branch angle.

It was previously not known if LAZY1$^{L92A/I94A}$ would disrupt normal LAZY1 function when expressed in the WT background, because the previous demonstration of its reversed gravitropism trait was made in a *lazy1* mutant background. The *brxl4* mutation's apparent rescuing ability of this dominant negative effect (Figure 3.3) further increases the complexity of the mystery. We know very little about the nature of region II in LAZY1, where the two amino acids mutation resides. It does not affect LAZY1's subcellular localization (Yoshihara and Spalding. 2020), nor does it contribute to the LAZY1-BRXL4 interaction (Figure 2.2). BRXL4 must be present for the LAZY1$^{L92A/I94A}$ variant to disturb the normal LAZY1 function. A simple explanation is that the absence of BRXL4 would promote most LAZY1 and presumably LAZY1$^{L92A/I94A}$ to stay on the plasma membrane, according to the model (Figure 2.8). The overexpressed LAZY1 in *brxl4* and the predominant plasma membrane localization makes it harder for the LAZY1$^{L92A/I94A}$ to disrupt LAZY1's GSA function. In the future, it would be pivotal to investigate the shoot gravitropic responses for the *proLAZY1:LAZY1*$^{L92A/I94A}$*/brxl4* and the *proLAZY1:LAZY1*$^{L92A/I94A}$/WT plants and see if they are consistent with the branch angle phenotype. Furthermore, PIN proteins and auxin levels in different tissues could be explored.

As previously mentioned in the Introduction and Discussion, there was no proof that *BRXL4* functions in any capacity in the root tissue. Therefore, the root gravitropism results in Figures 3.4 and 3.5 are also an important addition to the *BRXL4's* functions in Arabidopsis. *brxl4* mutants showed more robust stem gravitropism from the beginning compared to the wild type, but only showed the difference in root gravitropism after 4 h, it indicates that there may be other *BRXLs* in root that regulate root gravitropism. Admittedly, my dissertation project was mostly focusing on the shoot gravitropism aspect, and I did not spend much time exploring root related phenotypes, and some more biological replicates (n=6 is the bare minimum) would be needed for future experiments and conclusions. And the phenotypes would be presumably more significant if a higher order mutant of other *brxls* were to be examined, because their mutants have root growth phenotype, and they were more expressed in the root tissues compared to shoot tissues. Moreover, wavy assays could also be performed with *brxl4* and *BRXL4* OE lines, similar to the study of Mochizuki *et al*. 2005, because I did observe some distinct wavy patterns from *brxl4* mutants and *BRXL4* OE lines, which I did not pursue. Also, how the direction of root growth changes in response to obstacles and gravity could provide insightful cues to understand how *BRXL4* regulates root growth.

The *Nicotiana* system is optimized for subcellular localization investigation because of its sizable epidermal cells and accessible transformation technique, whereas it is definitely not the ideal system to generate any functional analysis of Arabidopsis proteins in a heterologous system, for obvious reasons such as homologous tobacco genes would complicate the situations. The results regarding BRXL4's effect on LAZY1 localization in *Nicotiana* across different trials were all very consistent. Whether the untagged or RFP tagged BRXL4 is overexpressed with LAZY1-

GFP or LAZY1-I-GFP in *Nicotiana*, the nuclear GFP signal is usually greatly diminished (Figures 3.6-3.8). We already know that LAZY1 and BRXL4 only stably interact at the plasma membrane in *Nicotiana* (Figure 2.1), therefore, a straightforward interpretation is that the overexpressed BRXL4 would attract LAZY1 or LAZY1-I to interact and be preferentially localized to the plasma membrane instead of the nucleus. The interaction affinity is apparently strong enough that it can override the inability of LAZY1-I to anchor to the plasma membrane. We have to cautiously interpret the results from *Nicotiana* context, and many caveats need to be considered. These seemingly opposite observations do not necessarily invalidate our model in Figure 2.8. The NLS mediated transporting mechanism might be different in *Nicotiana* or some other related players are different which influence how Arabidopsis BRXL4 interacts and unfolds LAZY1 at the *Nicotiana* plasma membrane. Future experiments should be focused on the native Arabidopsis environment, like the quantitative studies I conducted in Figures 2.7.

We hypothesized the model in Figure 2.8 based on all the evidence we generated so far as well as some reasonable speculations. The model is consistent with the experimental results and many follow-up tests should be planned in the future to further validate the accuracy of our model. I would like to discuss some sensible future experiments in the end.

1) A central piece of our model is the putative LAZY1 self-suppression in the nucleus, which follows logically from the presence of an EAR motif and the results in Figures 2.6, 2.7. We could detect the direct effect of LAZY1 on its own transcriptional activity using dual-luciferase transcriptional activity assay in Arabidopsis wild-type protoplasts, though LAZY1 may affect the expression of one or more regulators of LAZY1 expression in indirect manner. If LAZY1 indeed inhibits its own expression, we should further detect

whether the inhibition effects alter in the protoplasts of *brxl4* mutant and *BRXL4* OE plants compared with that of wild-type plants, respectively. Another possible route is to pursue a Chromatin Immunoprecipitation Sequencing (ChIP-Seq) experiment to examine what DNA sequences are attached to the LAZY1 complex extracted from plants. A positive result would directly support evidence of a LAZY1 complex interacting with the *LAZY1* promoter.

2) The crystal structure of LAZY1 protein could be assessed using *E. Coli* expression system. The crystal structure would potentially help us elucidate the functional domains of LAZY1, such as the Region V interaction sites or the special two amino acids mutation in Region II. I would also be interested to see if the NLS of LAZY1 is indeed embedded and supposedly needs BRXL4 to unfold and be transported into the nucleus.
3) Y2H is a very useful and facile tool to screen interactors *in vitro*, and that was how BRXL4 was found using LAZY1 as the bait. The limitation of Y2H is also apparent that yeast cells lack some fundamental biological processes that plants cells possess, which could skew or miss important biologically relevant interactions. Screening interactors *in vivo* using either pull-down or Co-Immunoprecipitation (Co-IP) is a common technique to provide complementary search for important protein interactors. In fact, this work has already been started and *35S:Strep-LAZY1* transgenic plants were generated to purify Strep-tagged LAZY1 protein using MagStrep XT beads (IBA). The beads contain Strep-Tactin®XT which has a strong affinity with Strep-tag. The information regarding this experiment will be presented in the Appendix.

4) Structure-function studies on BRXL4 would be essential to complete the whole picture of BRXL4-LAZY1 interaction. Deletion or point mutations in BRX domain is expected to be able to disrupt the interaction but it would also be possible if other motif of BRXL4 actually serves as the interaction site. It is unclear how BRXL4 is localized to the plasma membrane being a soluble protein (no transmembrane domains), and mutations within the BH domains within BRXL4 would be a logical exploration to see if they are necessary for its plasma membrane localization.
5) The starting point of my dissertation project was LAZY1 with unknown structural function, and I attempted to search LAZY1's interactors hoping to infer LAZY1's molecular function. Despite being an influential interactor, BRXL4 currently still remains relatively mysterious. One natural follow-up would be to screen for BRXL4 interactors. hopefully it will identify interactors with well-recognized functions. It may help to uncover how BRXL4 affects the LAZY1 subcellular localizations or how BRXL4 affects branch angle/development being only in the adjacent tissue.
6) We now know that PIN3 contributes to the redistribution of auxin in Arabidopsis hypocotyl because PIN3 will move to the new lowest side of the cell when subject to reorientation. *lazy1, 2, 3* mutant fails to create the polarized localization of PIN3-GFP within the columella cells (Taniguchi *et al*. 2017). It indicates that *LAZY1* controls the hypocotyl gravitropism through regulating PIN3. Moreover, *BRX* was reported to enhance *PIN3* expression to promote root meristem growth (Scacchi *et al*, 2010). Collectively, it seems possible that BRXL4-LAZY1 interaction regulate PIN3 in hypocotyl.

One of the concerns of this experimental design is its feasibility. Most of studies that are related to visualizing PIN3 localization were conducted in roots, where imaging is easier. However, Rakusová *et al* (2016) showed very clear and bright images for their studies of PIN3 on hypocotyl validating the feasibility of observing PIN3 in Arabidopsis hypocotyl associated with some modifications on LAZY1 and/or BRXL4.

RT-qPCR should be used to test if PIN3 expression is altered in *brxl4* and *BRXL4* OE plants at the transcriptional level. Transforming *ProPIN3:PIN3-GFP* as well as *DR5rev:GFP* (Ottenschläger *et al*. 2003) to *brxl4* and *BRXL4* OE background will enable us to investigate if knock-out or overexpression of BRXL4 will influence the PIN3 expression level and the localization of PIN3 after gravitropic stimulation. The auxin response reporter *DR5rev:GFP* could indicate the auxin concentration within different tissues serving as additional evidence.

Experimental Procedures

RFP-BRXL4 and LAZY1-GFP localization in *Nicotiana benthamiana* leaves

The *BRXL4* cDNA sequences were cloned into the pUBN-RFP-DEST vector. *35S*:*LAZY1-GFP* construct has the *eGFP* insertion inside the *LAZY1* non-conservative sequence, same as the one reported in Yoshihara *et al* (2013). Leaves of *N. benthamiana* were infiltrated with the GV3101 strain of *Agrobacterium tumefaciens* containing the completed constructs to transiently express the resulting BRXL4 and LAZY1 fusion proteins. To determine the RFP-BRXL4 and LAZY1-GFP localization, a Zeiss 710 laser-scanning confocal microscope was used to collect *mRFP*

fluorescence (570nm-640nm) excited by a 561nm argon laser and *eGFP* fluorescence (500nm-560nm) excited by a 488nm argon laser, respectively, 2 d after infiltration.

Root gravitropism measurement

The same HTC image analysis pipeline described in Moore *et al*. (2013) was adopted in this study. After 3 d of stratification, wild type, *brxl4-1*, *brxl4-2*, and *pro35S* were grown for 3 d in min media (1mM KCl, 1mM $CaCl_2$, 5mM 4-morpholineneethanesulfonic acid, pH5.7, 1% agar), the primary root was 2-8 mm in length. Images were acquired every 2 min under the total span of 8 h, and the root tip angle measurement was generated automatically by the same program. All genotypes have a sample number (n) of 6.

GUS staining assay

Tissues (7-d-old seedlings or 3-week-old stems) were submerged in staining solution (100 mM sodium phosphate buffer, pH 7.0, 0.1% (v/v) Triton X-100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 1 mM X-gluc), and incubated at 37°C for 24 h. Chlorophyll in the seedings and the stem was removed in 70% (v/v) ethanol at 37°C for 3h and 24h, respectively.

Appendix

Future researchers may wish to continue my efforts to isolate proteins that interact with an epitope-tagged version of LAZY1 that I overexpressed in Arabidopsis plants. This Appendix summarizes the materials available for future research and the progress I made.

Figure A1. *35S:Strep-LAZY1/lazy1* rescued *lazy1* branch angle phenotype and display curled leaves. (a) Wild type, (b) *lazy1*, (c) *lazy1* plants transformed with *35S:Strep-LAZY1,* (d) Closer look at the curled leaves phenotype. It indicates that the N-terminal Strep tag did not interfere with the native LAZY1 function. Note that the same curled leave phenotype was observed by Dr. Yoshihara in our lab and reported in the *35S:LAZY1* plants (Hollender *et al*. 2020)

Arabidopsis Protein extraction protocol: all steps are conducted at 4°C.

1. 1-3g of Arabidopsis seedlings (WT and *35S:Strep-LAZY1/lazy1*) are ground in liquid Nitrogen. The frozen powder is transferred into a 50mL Falcon tube, then add 1-2 ml of protein extraction buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, protein inhibitor cocktail and 0.5% Nonidet P-40]. A piece of cloth is used to collect crude extract and screen out the big tissues.

2. Crude extract is centrifuged at 15,000x g to remove starch and cell debris to get the soluble portion of the protein extract (Solu). 4X SDS is added to the Solu and then 10µl is used to run SDS-PAGE.

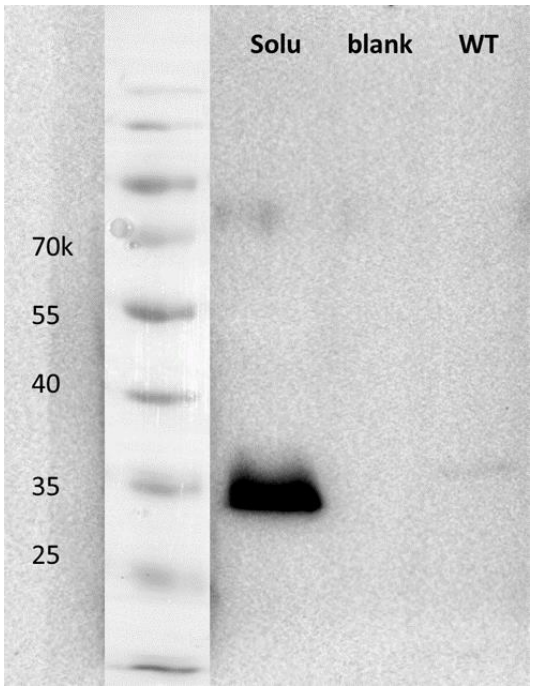

Figure A2. Western blot incubated with Strep antibody, HRP conjugated (GenScript, A00875). Soluble protein extract of *35S:Strep-LAZY1/lazy1* (Solu) generated a band at around 35kD. Wild type has no visible signal. Though the right size of LAZY1 protein should be about 40kD.

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