The AGCVIII kinase Dw2 modulates cell proliferation, endomembrane trafficking, and MLG/xylan cell wall localization in elongating stem internodes of *Sorghum bicolor*

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SUMMARY

Stems of bioenergy sorghum (*Sorghum bicolor* L. Moench.), a drought-tolerant C4 grass, contain up to 50 nodes and internodes of varying length that span 4–5 m and account for approximately 84% of harvested biomass. Stem internode growth impacts plant height and biomass accumulation and is regulated by brassinosteroid signaling, auxin transport, and gibberellin biosynthesis. In addition, an AGCVIII kinase (Dw2) regulates sorghum stem internode growth, but the underlying mechanism and signaling network are unknown. Here we provide evidence that mutation of *Dw2* reduces cell proliferation in internode intercalary meristems, inhibits endocytosis, and alters the distribution of heteroxylan and mixed linkage glucan in cell walls. Phosphoproteomic analysis showed that Dw2 signaling influences the phosphorylation of proteins involved in lipid signaling (PLD\alpha), endomembrane trafficking, hormone, light, and receptor signaling, and photosynthesis. Together, our results show that Dw2 modulates endomembrane function and cell division during sorghum internode growth, providing insight into the regulation of monocot stem development.

Keywords: *Sorghum bicolor*, stem growth, endomembrane system, endocytosis, cell wall heteroxylan, cell proliferation, mixed linkage glucan.

INTRODUCTION

The C4 grass *Sorghum bicolor* is an important drought- and heat-tolerant crop used for production of grain, forage, sugar, and biofuels (Rooney *et al.*, 2007; Mullet *et al.*, 2014). Grain sorghum was bred to have short stems to reduce lodging, increase the grain harvest index, and simplify mechanical harvesting. In contrast, bioenergy sorghum hybrids have 4–5 m long stems containing 40–50 internodes that account for approximately 84% of harvested biomass (Olson *et al.*, 2012). A subset of the bioenergy sorghum genotypes accumulate high levels of stem sucrose similar to sugarcane (McBee and Miller, 1982; Lingle, 1987). These ‘sweet’ sorghum genotypes have increased capacity to accumulate stem sugars and starch (McKinley *et al.*, 2018) due to their elongated stems and inactivation of NAC\_D, a gene that stimulates conversion of stem pith parenchyma to aerenchyma (Casto *et al.*, 2018; Xia *et al.*, 2018; Zhang, 2018).

The sequential production of phytomers containing nascent node–internode tissues by the shoot apical meristem (SAM) is a key early event in stem growth. Nascent internodes initially increase in size through balanced cell division and cell growth followed by a phase of rapid internode elongation (Kebrom *et al.*, 2017). Rapidly growing internodes contain a region of cell division in an intercalary meristem (IM) located at the base of the internode and a zone of cell elongation adjacent to and above the IM. Cells that have stopped elongating accumulate secondary cell walls in the upper portion of the internode (Kebrom *et al.*, 2017).

The length of sorghum internodes varies during plant development, in response to environmental factors, and
among genotypes. Insight into the biochemical basis of variation in internode length in sorghum has been obtained through analysis of *dwarfing* loci (*Dw*) used to shorten the stems of grain sorghum (Quinby, 1974). For example, *Dw3* encodes an ABCB1 auxin efflux transporter and inactivation of *Dw3* reduces internode length and cell elongation (Multani et al., 2003). The maize (*Zea mays*) homolog of *Dw3*, *BR2*, was shown to help export indole-3-acetic acid (IAA) from stem nodes, indicating that regulated auxin transport is required for normal internode elongation (Knöller et al., 2010). Positional cloning of *Dw1* identified a novel membrane protein with predicted plasma membrane (PM) localization (Hilley et al., 2016). *Dw1* was subsequently shown to stimulate internode cell proliferation by binding to and inhibiting the nuclear localization of Bin2, a negative regulator of brassinosteroid signaling (Yamaguchi, 2016; Hirano et al., 2017).

*Dw2*, the focus of the current study, was found to encode a member of the cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and protein kinase C (PKC) (AGCVIII) protein kinase family (Hilley et al., 2017). The closest *Arabidopsis thaliana* homolog of *Dw2* is Kinesin-like Calmodulin-Binding Protein Interacting Protein Kinase (KIPK) (Hilley et al., 2017). In *Arabidopsis*, KIPK interacts with KCBP, a plant-specific kinesin-like calmodulin-binding protein that regulates trichome development and the organization of cytoskeletal components (Day et al., 2000). AtKIPK also interacts with proline-rich extensin-like receptor-like kinases (PERK) that modulate growth (Humphrey et al., 2015). In *Arabidopsis*, KIPK mutants show only a very mild root phenotype and no stem growth phenotypes (Humphrey et al., 2015). Other members of the plant AGCVIII kinase family have been reported to regulate phototropism, gravitropism, and planar growth (Rademaker and Offringa, 2012).

In this work, we investigated the role of *S. bicolor Dw2* in regulating internode growth. We provide evidence that mutation of *Dw2* inhibits cell proliferation in elongating internodes, alters cell morphology in vascular bundles and root hairs, and causes a large number of changes in the phosphoproteome that could affect phospholipid signaling, vesicle trafficking, cytoskeletal functions, and cell proliferation. Mutation of *Dw2* also disrupts endocytosis and the localization of polysaccharides in cell walls that are trafficked to cell walls by the endomembrane system. Together these results provide insights into how *Dw2* regulates internode growth in C4 grass crops, a function not associated with homologs in dicot species.

RESULTS

Dw2 modifies stem and internode growth

The role of *Dw2* in stem and internode growth regulation was investigated using the near isogenic genotypes Dwarf Yellow Milo (DYM, *Dw2*) and Double Dwarf Yellow Milo (DDYM, *dw2*). DDYM encodes a *dw2* allele with a stop codon in the first exon, thereby producing a truncated protein of 190 amino acids lacking the kinase domain instead of the 809 amino acids in the full-length AGCVIII kinase (Hilley et al., 2017). DYM and DDYM have similar flowering times and produce the same number of stem internodes during development. However, DYM plants are taller than DDYM plants because DYM stem internodes are longer than DDYM internodes (Figure 1b).

Mutation of *Dw2* reduces the number of cells spanning the length of internodes

During vegetative growth, new phytomers comprised of nascent leaves, leaf sheaths, and internodes are produced by the SAM approximately every 3–4 days. The approximately four nascent internodes below the SAM contain relatively small non-elongated cells. Cell division occurring throughout nascent internodes increases cell number and size of these internodes prior to internode elongation (Figure 1a). In elongating internodes, cell division occurs in an IM located at the basal end of the internode. Cells displaced from the IM by cell proliferation stop dividing and enter a zone of elongation (ZoE) where an increase in cell length occurs. When internode cells reach full length, cells associated with vascular bundles subsequently develop lignified secondary cell walls in a region called the zone of maturation (ZoM). The number of fully elongated cells in the ZoM increases over time during internode growth until the activity of the IM ceases (Figure 1a).

The difference in DYM and DDYM internode lengths (Figure 1b) could be due to differences in cell number and/or cell length. Cells in internodes are stacked in vertical columns facilitating microscopic analysis and quantification of the number and length of cells that span the length of internodes. Analysis of fully elongated internodes showed

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**Figure 1.** Diagram of internode development in monocots; DYM2 phenotype. (a) Monocots grow stems in discriminate segments called internodes. Each internode is a part of a phytomer which contains the leaf blade and leaf sheath. At the base of elongating internodes is an area of active cell division, the zone of cell division (ZoD). Above the ZoD are areas of cell elongation (ZoE) and maturation (ZoM), where cell expansion and maturation/lignification occur, respectively. The apical meristem is located at the very tip of the growing stem and is where new phytomers are formed. Beneath the apical meristem are small undeveloped internodes of phytomers 1–3 that have yet to elongate. For this study, the apical meristem and Int(P1-4) were grouped together in what we define as the ‘apical dome’. (b) Mature DYM and DDYM internodes, which are wild-type and *dw2*, respectively. DYM internodes are longer. Scale bar is 5 cm. (c) Measure of maximum length of cells in a fully mature internode. There is no difference in maximum length of cells in DYM or DDYM. Error bars represent SD over three replicates. (d) Number of cells in the length of the internode, measured from nodal plane to nodal plane. DYM internodes have approximately 2.2× larger cells than DDYM internodes. Errors bars represent SD over three replicates.
Sorghum Dw2 regulates stem growth

(b) Phytomer

Leaf Blade + Leaf Sheath + Internode

*Phytomer 4 is defined by the first fully expanded leaf

(c) Graph showing average length of fully elongated cells

(d) Graph showing number of cells in length of internode

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that cells in the upper portion of fully elongated DYM and DDYM internodes were similar in length (Figure 1c) and that there were approximately 2.2 times more cells spanning DYM internodes than DDYM internodes (Figure 1d). Therefore, the difference in the overall length of the DYM and DDYM internodes is primarily due to a reduction in cell number in DDYM.

Mutation of Dw2 in DDYM could reduce the number of cells in the internode by inhibiting cell division prior to internode elongation and/or by reducing rates of cell division in the IM of elongating internodes. These alternatives were evaluated by quantifying the number of cells that span the length of internodes of phytomers 4–6 that correspond to internodes just prior to elongation (Int(P4)), those in an early stage of elongation (Int(P5)), and those at a late stage of elongation (Int(P6)). DYM and DDYM were found to have a similar number of cells in Int(P4) (Figure 2d); however, more cells accumulated in Int(P5) and Int(P6) in DYM compared to DDYM during the phase of internode elongation. The cells in the IM of Int(P6) were smaller in DYM compared to DDYM, consistent with more rapid rates of cell division in the IM of DYM. Fully elongated cells in the ZoM of Int(P6) of the two genotypes were similar in length (Figure 2c). Taken together, these results indicate that mutation of Dw2 reduces the extent of cell division in the IM during the phase of internode elongation.

Internode cell and vascular bundle morphology is altered in the Dw2 mutant

The morphology of internode cells of fully elongated Int(P6) of DYM and DDYM was examined to determine if mutation of Dw2 has an impact on cell shape and tissue-level organization in fully elongated internodes. Longitudinal sections and cross-sections of fully elongated DYM (Dw2) internodes (mid-internode) showed well-organized columns of cells and vascular bundles with typical monocot morphology (Figure 3a,b). In DDYM internodes, longitudinal sections revealed cells having more irregular...
shapes and sizes, and cell columns were less uniform compared to DYM (Figure 3c). In addition, cross-sections of DDYM internodes showed vascular bundles with irregular shapes and cell numbers sometimes containing more vessel elements compared to DYM stems (Figure 3d). Overall, approximately 35% of the vascular bundles in DDYM stems showed abnormal phenotypes. These results indicate that mutation of Dw2 affects cell and tissue morphology of fully elongated internodes, and that the morphology of vascular bundles located in or near the rind/epidermis was perturbed to the greatest extent.

**Dw2 is expressed in cells associated with vascular bundles and other stem cell types**

Dw2 expression in elongating and fully elongated internodes was previously reported (Hilley et al., 2017). In the current study, the distribution of Dw2 RNA among internode cells was examined using in situ hybridization (Figure 4). Dw2 RNA was detected in most internode cells of Int(P5) (Figure 4c). However, Dw2 expression was elevated in cells associated with nascent vascular bundles near the epidermis (Figure 4c) and in parenchyma cells associated

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with more fully developed vascular bundles located closer to the center of the stem (Figure 4f).

Phosphoproteomic analysis of DYM and DDYM internodes

To better understand how Dw2 influences internode growth, a phosphoproteomics approach was utilized to identify differences in protein phosphorylation between elongating internodes of DYM (Dw2) and DDYM (dw2). Tissue for phosphoproteomic analysis was collected from internodes of phytomer 5 (Int(P5)) that had just begun to elongate. Phosphoproteomic analysis identified 7138 phosphosites in 2065 proteins in this tissue. The relative phosphorylation of phosphosites within 206 of these proteins differed significantly between the two genotypes (q-value < 0.1) (Table S1). The extent of differential phosphorylation at specific phosphosites ranged from <2-fold to >32-fold. To distinguish between differential phosphorylation of a phosphosite and differences caused by variation in protein abundance, data on the proteome were collected from the same samples used for the phosphoproteomic analysis. High-pH reversed-phase chromatography was implemented as a prefractionation strategy and isobaric tagging was used for protein quantitation. This approach was used to quantify the relative abundance of 7390 proteins and showed that 22 proteins were significantly different in abundance in the two genotypes (q-value < 0.1). Only a few of the proteins that were differentially phosphorylated differed in relative abundance in internode 5 of the two genotypes. Dw2 was detected in DYM but not in DDYM, as expected, due to a mutation that truncates the protein. In DYM, a phosphosite in Dw2 (S628) was located near the insertion domain present in most AGCVIII kinases and a second phosphosite (S657) was located within the T-loop [SxS+FVGTxEYxAPE] activation segment (Rademacher and Offringa, 2012). Dw2 contains a C-terminal FxxF sequence motif present in other ACG kinases that mediates PDK1 binding and phosphorylation of active site amino acids (Rademacher and Offringa, 2012). SbPDK1 (Sobic.003G372200) is expressed in sorghum stem internodes; therefore, PDK1 is a potential regulator of Dw2 in this tissue. One other differentially phosphorylated protein, psbH, also showed a significant difference (1.4-fold) in abundance in internodes of the two genotypes (Table S2).

Overall, out of the 206 proteins that were differentially phosphorylated, 23 proteins contained phosphosites that were more highly phosphorylated in DYM compared to DDYM (Figure 5a). This subset of phosphoproteins was enriched in proteins involved in microtubule dynamics and maintenance (Figure 5b). The remaining 183 proteins showed higher phosphorylation in DDYM internodes, indicating that loss of Dw2 has a large indirect effect on protein phosphorylation. This subset of phosphoproteins was enriched in proteins involved in subcellular localization, cytoskeletal organization, exocytosis, and Golgi trafficking (Figure 5c).

Figure 4. Dw2 is expressed in pith and rind, but is concentrated around vascular bundles. (a,d) Negative control of RNAscope in situ hybridization of the bacterial gene dapB. (b,e) Positive control of in situ hybridization of a cytochrome P450 in DYM plants. (c,f) RNAscope in situ hybridization of Dw2 in Int(P5) of DYM plants. Signal (red spots) is present in pith of the internode (c), but is highly concentrated at the rind, surrounding VBs, and within VBs (f). X, xylem; P, phloem.
Proteins with higher phosphorylation in DYM Int(P5)

Proteins with higher levels of phosphorylation in internodes of DYM (Dw2) compared to DDYM (dw2) could be direct targets of Dw2. Therefore, proteins of similar abundance that were differentially phosphorylated in DYM were analyzed further to identify potential direct targets of the AGCVIII kinase (Table 1; Table S1). A phosphosite (S684) in phospholipase D (PLD\textsubscript{d}) was only detected in DYM although PLD\textsubscript{d} protein abundance was similar in internode 5 of the two genotypes (Table 1; Tables S1 and S2). Proteins involved in endomembrane function such as MPK3, a MAP kinase responsible for phosphorylating LIP5 to control multivesicular body (MVB) biogenesis (Wang et al., 2014), and Golgin Candidate 5 (GC5), a protein involved in vesicle tethering to the Golgi apparatus (Laijnhouwers et al., 2007; Kang and Staehelin, 2008), were more highly phosphorylated in DYM compared to DDYM (Table 1). SIL4, a protein phosphatase required for ER to Golgi trafficking in yeast that plays a role in cell cycle progression (Sutton et al., 1991; Bhandari et al., 2013), also had higher phosphorylation levels in DYM. In addition, a subunit of a vacuolar ATP synthase, the Rho-protein effector RIp5, and a homolog of ARK1, a kinesin involved in microtubule dynamics associated with root hair growth, showed differential phosphorylation in DYM (Eng and Wasteneys, 2014) (Table 1).

Two chloroplast-localized proteins, Lhcb2 and psbH, showed elevated levels of phosphorylation at threonine phosphosites in DYM compared to DDYM (Table 1). In Arabidopsis, Lhcb2 proteins are phosphorylated at an N-terminal threonine residue (T3) by the kinase State Transition 7 (STN7), which results in association of LHCCI trimers with PSI in blue light (Longoni et al., 2019). Phosphorylation of sorghum Lhcb2 on T109 could modulate state transitions or protein turnover. Phosphorylation of psbH and PSII core proteins such as D1 increases at high light and is generally attributed to the need for turnover and repair of damaged D1 to maintain PSII function (Fristedt et al., 2012; Levey et al., 2014).

Proteins with higher phosphorylation in DDYM Int(P5)

Proteins with higher relative phosphorylation in Int(P5) of DDYM compared to DYM were enriched in functions associated with the endomembrane, cytoskeleton, signaling, transport, and regulation (Table 2; Table S2). Numerous sorghum homologs of proteins involved in endomembrane system function were differentially phosphorylated in DDYM Int(P5), including Sec14, KEU, DELTA-ADR, and remorins, proteins involved in vesicle trafficking, cytokinesis, and endocytosis (Lam et al., 2001; Zwiewka et al., 2011; Wu et al., 2013; Fan et al., 2015) (Table 2). Proteins involved in ion transport such as BOR1 were differentially
Photosynthesis & light signaling  

Kinesins  

Endomembrane system & signal transduction  

(Takano et al., 2010; Kasai et al., 2011). The STRUBBELIG receptor (Sobic.001G500400) has three phosphosites with elevated phosphorylation in DDYM plants (Table 2). Family members of this receptor have been shown to regulate tissue morphogenesis and cell division planes in Arabidopsis (Chevalier et al., 2005, 2011; Christie et al., 2011). Proteins with homology to NRL5 were differentially phosphorylated in DDYM. Phosphorylation of proteins in the NRL gene family, such as NPH3, is known to modulate interaction with the AGC kinase phototropin 1 (Vaddepalli et al., 2011; Christie et al., 2013). The function of NRL5 is unknown, but these proteins may regulate Dw2 or be regulated by Dw2 signaling. Additionally, Root Hairless 1 (RHL1) shows relatively high differential phosphorylation in DDYM. RHL1 is a component of the DNA topoisomerase VI complex required for endoreduplication and ploidy-dependent cell growth (Sugimoto-Shirrasu et al., 2005). Furthermore, phosphorylation of a Sec14p-like family member at S291 was only detected in DDYM. The Sec14 family has many different biological roles, such as regulating membrane trafficking and phosphoinositol signaling (Huang et al., 2016). Sec14p-like proteins have been shown to localize to the cell plate to coordinate vesicle trafficking to the new cell wall of dividing cells (Peterman et al., 2004; Zhou et al., 2019).

**Phospholipase D inhibitor n-butanol phenocopies mutant plants**

The phosphorylation of PLD in DYM but not DDYM internode tissue indicated that PLD could be a direct phosphorylation substrate of Dw2. PLD plays a major role in lipid signaling by releasing phosphatidic acid (PA), a modulator of growth, the cytoskeleton, endocytosis, development, and defensive responses (Pleskot et al., 2013; Hong et al., 2016). Dw2 could modify cell proliferation in internodes by phosphorylating and activating PLD, increasing production of PA, which is required for several processes involved in cell proliferation (Wang, 2005). Further support for this hypothesis was obtained by treating DYM and DDYM internodes with n-butanol, an inhibitor of PLD (Munnik et al., 1995). Treatment of DYM with n-butanol reduced internode length by 50%, whereas treatment of the dw2 mutant DDYM had minimal impact on internode length (Figure 6a). In addition, n-butanol treatment of DYM increased the length of cells in the internode zone of cell division (ZoD), consistent with inhibition of cell proliferation, whereas treatment of DDYM with n-butanol did not alter the length of cells in the ZoD (Figure 6). These results indicate that PLD activity is necessary for normal cell proliferation during internode elongation. Analysis of RNA

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**Table 1** Phosphoproteomic data with greater phosphorylation in DYM (Dw2). Six replicates were used for each genotype

| Sorghum ID | Arabidopsis ID | Arabidopsis symbol | Fold change | Function | P-site |
|------------|----------------|--------------------|-------------|----------|--------|
| Dw2        | Sobic.006G067700 | AT2G36350 | KIPK | a | Signaling kinase, unknown specific function | S657, S628 |
| Endomembrane system & signal transduction | Sobic.002G285000 | AT4G39790 | PLD delta | a | Phospholipase D, PA signaling | S684 |
| | Sobic.009G298600 | AT1G79830 | GC5 | 7.0 | Golgin Candidate 5, vesicle tethering to Golgi apparatus | S873 |
| | Sobic.001G384000 | AT3G14172 | GPl-anchored Protein | 5.6 | Adhesin-like, unknown specific function | S740 |
| | Sobic.003G294400 | AT1G28280 | MPK3/MVQ1 | 4.1 | Oxidative stress signaling, cell proliferation, defense signaling | S228 |
| | Sobic.009G255700 | AT1G30470 | SIT4 | 2.6 | Phosphatase, cell cycle progression, required for ER to Golgi traffic (yeast) | S527 |
| | Sobic.006G203900 | AT4G23710 | VHA-G2 | 2.5 | Vascular ATP synthase subunit G2 | S8 |
| | Sobic.003G301200 | At5G60210 | RIP5 | 2.0 | ROP interactive partner 5, Rho protein effector | S115 |
| Kinesina | Sobic.001G500400 | AT3G54870 | KUNUC/ARK1 | 2.5 | Plus-end microtubule motor protein, microtubule catastrophe | S617 |
| Photosynthesis & light signaling | Sobic.002G380000 | AT2G05070 | LHCB2.2 | a | Light-harvesting complex II | T109 |
| | Sobic.003G168800 | ATCG00710 | psbH | 4.7 | Photosystem II, reaction center H | T5, T3 |

*No phosphorylation detected in DDYM.*
sequencing (RNA-seq) data previously collected from developing stems showed that PLD\(d\) is expressed in elongating internodes together with several other genes annotated as encoding PLDs (Sobic.001G34900, Sobic.003G050400, Sobic.010G185600, Sobic.001G320200) (Kebrom et al., 2017). Therefore, the specific role of PLD\(d\) in cell proliferation in the internode ZoD will require targeted mutation of this gene.

Dw2 signaling regulates endocytosis

Differential phosphorylation of proteins associated with the endomembrane system observed in DYM/DDYM could result in alteration of endomembrane functions that affect internode cell morphology and growth. FM4-64 is a lipophilic dye used to monitor endocytosis because it labels the PM and is internalized via endocytosis to endosomal compartments with subsequent distribution to the tonoplast (Sugimoto-Shirasu et al., 2005; Rigal et al., 2015). FM4-64 analysis of endocytosis is often carried out on roots and root hairs because cells are readily accessible and they lack chlorophyll. Additionally, root hairs were imaged because they require a complex coordination of vesicular trafficking and cytoskeleton organization to grow (Samaj et al., 2006). This analysis revealed that DYM root hairs were straight whereas DDYM root hairs were wavy or bent, a phenotype that could be linked to altered lipid signaling (Hirano et al., 2018) (Figure 7a). Exposure of emerging root hairs to FM4-64 for 15 min resulted in more dye internalization in DYM compared to DDYM (Figure 7b). FM4-64 uptake by root cells was examined next. Exposure of DYM root tissue to FM4-64 for 5 min resulted in the dye becoming associated with the PM (Figure 7c). After 30 min of treatment, the relative FM4-64 signal associated with internal membranes of the cytoplasm increased. In contrast to DYM, incubation of DDYM roots with FM4-64 resulted in less dye being associated with internal membranes after 30 min of treatment (Figure 7c (bottom row), d), indicating that uptake and trafficking of the dye occurs

Table 2 Phosphoproteomic data with greater phosphorylation in DDYM (dw2). Six replicates were used for each genotype

| Sorghum ID   | Arabidopsis ID | Arabidopsis symbol | Fold change | Function                                             | P-site |
|--------------|----------------|--------------------|-------------|------------------------------------------------------|--------|
| Sobic.010G187500 | AT1G75370       | Sec14p- like       | a           | Phosphatidylinositol/choline transfer, secretion     | S291   |
| Sobic.006G033200 | AT1G12360       | KEU                | 3.6         | SNARE-interacting protein KEULE, cytokinesis, vesicular trafficking | S590   |
| Sobic.007G059900 | AT1G34220       | Regulator of Vps4  | 2.5         | Regulator of Vps4 in MVB pathway                    | S400   |
| Sobic.010G231200 | AT1G3920        | Remorin Family    | 2.1         | Microdomain-associated endocytosis                  | S13    |
| Sobic.008G072200 | AT1G48760       | DELTA-ADR          | 2.0         | Clathrin adaptor complex, vesicle budding          | S182   |
| Sobic.002G359000 | AT5G67470       | FH6                | a           | Formin homolog, organization and polarity of actin  | S772, S150 |
| Sobic.009G163800 | AT2G39910       | ABCB1, SbDw3       | 4.9         | Auxin efflux                                       | S365   |
| Sobic.010G310000 | AT1G31000       | NRL5/NPY1          | 5.6         | Phototropism, ubiquitination                       | S372   |
| Sobic.004G187100 | AT5G64330       | RPT3               | 5.3         | Root phototropism protein, ubiquitination          | S23    |
| Sobic.003G074000 | AT3G50780       | BTB/POZ domain     | 3.9         | Phototropism, ubiquitination                       | S57    |
| Sobic.002G303300 | AT4G11610       | No symbol          | 6.1         | Calcium/lipid phosphoribosyltransferase, putative synaptotagmin | S207   |
| Sobic.008G132300 | AT2G47160       | BOR1               | 2.7, 2.5, 2.4, 2.2 | Boron transporter                                  | S672, S654, S668, S669 |
| Sobic.010G277900 | AT1G48380       | RHL1               | 27.7        | Root Hairless 1, DNA topoisomerase, endoreduplication | S98    |

*No phosphorylation detected in DYM.
more slowly in the mutant (Figure 7e). This was confirmed by quantification of the FM4-64 signal in the PM and the cytosol, which indicated a higher PM/cytosol ratio in DDYM compared to DYM after 30 min of treatment (Figure 7d). To extend this observation, Brefeldin A (BFA) was used to disrupt endocytosis. BFA inhibits endosomal cycling, which leads to the formation of endosomal aggregates called BFA bodies (Lam et al., 2009). Mutants with endocytic defects reduce the number of BFA bodies per cell (Stefano et al., 2018). After 1 h of BFA treatment, DYM root cells showed extensive accumulation of BFA bodies (approximately eight per cell, Figure 7e, left panel) white arrowheads, f). In contrast, DDYM root cells contained fewer BFA bodies (approximately two per cell Figure 6e, right panel), white arrowheads, f). Taken together, these data indicate endocytosis is reduced in the dw2 mutant.

**Mutation of Dw2 alters deposition of endomembrane trafficked cell wall polysaccharides**

Differences in endomembrane phosphoproteomics and activity observed in DYM and DDYM could affect secretion and deposition of cell wall polysaccharides such as heteroxylans (HXs) and mixed linkage glucans (MLGs) (Oikawa et al., 2013; Kim et al., 2018). In DYM, immunolocalization analyses showed that MLG is localized fairly uniformly in pith parenchyma cell walls located near vascular bundles in the ZoD of Int(P5) (Figure 8a; Figure S1). In contrast, in DDYM, MLG accumulated in the vertices where adjacent pith parenchyma cells meet and to a much reduced extent along the sides of adjacent cells (Figure 8b). Quantitation showed that the signal from MLG staining was differentially localized in cell vertices of

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**Figure 6.** The phospholipase D inhibitor n-butanol causes DYM to phenocopy DDYM. (a) Internodes from control and treated DYM and DDYM plants. (b) Treated DYM internodes are indistinguishable from DDYM internodes from either condition. Scale bar is 5 cm. (c) Images of cells from the zone of division (ZoD) of treated and control DYM and treated or control DDYM. (d) Treated DYM internodes have significantly longer cells in the ZoD than control DYM cells and are indistinguishable from DDYM internodes in either condition. Error bars are the SD of three replicates in the control and four replicates in the treated conditions.
DDYM compared to DYM (Figure 8c) (Oikawa et al., 2013; Kim et al., 2018).

HX is another component of plant cell walls that is delivered to the cell wall through the endomembrane system. In internodes of DYM, HX was localized in cell walls that surround pith parenchyma cells near vascular bundles (Figure 8d). In contrast, DDYM internode cell walls showed HX accumulated in a distinct ‘dashed-line’ pattern of deposition with higher abundance in the faces (or sides) of adjacent cells (Figure 8e,f).
Figure 8. Dw2 signaling regulates the localization of cell wall components trafficked by the endomembrane system. (a–c) Immunolocalization of mixed linkage glucan (MLG) in DYM (a) and DDYM (b) internode cells. MLG shows strong localization in the cellular vertices of elongating DDYM internodes (b,c). DYM plants show uniform MLG localization (a). Cell wall fluorescence intensity is normalized to DYM levels. The point in (c) is an outlier. (d–f) Heteroxylan (HX) immunolocalization in DYM (d) and DDYM (e) internode cells. In DDYM, HX shows strong localization at the planes where adjacent cells meet (faces) (e,f). DYM plants show uniform HX localization (d). Cell wall fluorescence is normalized to DYM levels. Both MLG and HX are polysaccharides that are trafficked through the endomembrane system. (g–i) Calcofluor White staining in DYM (g) and DDYM (h) internode cells. Calcofluor White preferentially stains cellulose and callose. Both DYM and DDYM cells show a uniform signal in their cell walls, with total fluorescence intensity reaching 90% of that in the DYM cells (i). Scale bar is 10 μm. Whiskers represent the minimum and maximum values, and the box indicates the interquartile range of 50 cells over three replicates. DDYM fluorescence intensity values are normalized to DYM values. ***P ≤ 0.001.
Cellulose is synthesized at the PM by cellulose synthases. Cellulose staining of DYM and DDYM cells in the ZoD by Calcofluor White, which preferentially stains cellulose and callose, showed that cellulose was uniformly distributed in cell walls of both genotypes (Figure 8g,h). There were slightly reduced levels of cellulose in the cell walls of DYMM internodes compared to DYM (Figure 8i). These results indicate that disruption of signaling propagated by Dw2 affects the deposition of cell wall polysaccharides that are synthesized in the Golgi and transported via vesicles to the cell wall and have less of an effect on polysaccharides synthesized at the PM.

**DISCUSSION**

The plant-specific AGCVIII kinase family regulates growth in response to light (phototropism), gravity (gravitropism), and developmental and morphogenic signals (Rademacher and Offringa, 2012; Barbosa and Schwechheimer, 2014). Dw2 is one of the 21 AGCVIII kinases encoded by the sorghum genome (Hilley et al., 2017). Ten of the sorghum AGCVIII kinases are expressed during stem internode development; four D6PKs, PHOT1, Dw2 (homolog of KIPK), KIPK-like, AGC1-3a, AGC1-3b, and AGC1-12 (Hilley et al., 2017; this study). D6PKs, PHOT1, and AGC1-12 regulate phototropic, gravitropic, and planar growth by phosphorylating PIN proteins, mediators of polar IAA transport. In the current study, Dw2 was found to regulate the extent of cell proliferation in elongating sorghum internodes. Phosphoproteomic analysis of DYM (Dw2) and DDYM (dw2) elongating internodes did not reveal differences in PIN phosphorylation, indicating Dw2’s mechanism of action differs from those of D6PK and PHOT. As discussed below, mutation of Dw2 caused downregulation of cell proliferation in elongating internodes, extensive changes in protein phosphorylation, inhibition of endomembrane activity, altered accumulation of cell wall polysaccharides, and altered vascular bundle morphology.

Mutation of Dw2 causes a significant decrease in the length of stem internodes, a trait that was selected for in grain sorghum breeding programs to reduce lodging (Hilley et al., 2017). The current study showed that mutation of Dw2 does not alter cell proliferation in nascent internodes located in the apical dome prior to their elongation, but inhibits cell proliferation in the IM of elongating internodes. This indicates that Dw2 has a specialized role in the control of cell proliferation in the IM during the elongation of internodes that occurs over a period of approximately 6-9 days. The regulation of cell proliferation in the IM of elongating internodes is important since the extent of cell proliferation has a significant impact on internode length, plant height, competition for light, and the amount of resources the plant allocates to stem growth (sink strength). The growth of grass stems occurs by the sequential production of phytomers containing internodes that elongate and differentiate during a specific phase of phytomer development. The rate of phytomer production is regulated during grass development and in response to environmental inputs. Likewise, the elongation of internodes is regulated so that it occurs following growth of the leaf blade and leaf sheath of the same phytomer. Once internodes begin elongating, polarized growth and differentiation occurs across the length of the internode that is sustained for 6-9 days by continued cell proliferation in the IM located at the base of the internode (Kebrom et al., 2017). Growth within zones of cell division and elongation requires coordination across tissues (epidermis to the center of the stem) and among different cell types. The unique developmental biology of grass stems may explain why mutations that affect internode growth in grasses often have minimal impact on stem growth in the dicot Arabidopsis. For example, mutation of KIPK, the closest Arabidopsis homolog of Dw2, does not affect stem growth in Arabidopsis (Humphrey et al., 2015; Kebrom et al., 2017). Similarly, mutation of the gene corresponding to sorghum ABCB1 (Dw3) has a significant impact on internode growth in grasses (Multani et al., 2003; Knöller et al., 2010), whereas mutation of AtABCB1 has only a minor impact on stem growth in Arabidopsis (Noh et al., 2001).

The main phenotype associated with mutation of Dw2 was shorter internode lengths due to reduced cell proliferation; however, several additional cellular phenotypes were observed that could contribute to our understanding of how Dw2 regulates cell proliferation and the identification of additional Dw2 regulatory functions. The cellular/subcellular phenotypes that distinguish DDYM from DYM include: (i) irregular internode cell shapes and vascular bundle morphology in fully elongated internodes, (ii) a wavy root hair morphology, (iii) inhibition of endocytosis/endomembrane activity measured by reduced rates of FM4-64 uptake, and (iv) modified accumulation and localization of HX and MLG in cell walls. We observed that Dw2 is expressed in most stem cell types, indicating that Dw2 could regulate cellular processes in many cell types and stages of internode development. For example, in situ analysis showed elevated expression of Dw2 in nascent vascular bundles of Int(P5) that persisted in parenchyma cells associated with vascular bundles in elongating internodes. The loss of Dw2 function in these vascular bundle cells could be responsible for the abnormal anatomy of vascular bundles in DDYM by altering endomembrane activity that normally promotes sympaptic movement of transcription factors such as SHORT-ROOT (SHR) that specify cell identity (Koizumi et al., 2011). Elevated expression of Dw2 in developing vascular bundles is interesting because PDK1, a regulator of AGC kinases, is also expressed in provascular tissues and Arabidopsis pkd1pkd2 mutants are dwarfed plants with abnormal vein morphology (Xiao and Offringa, 2020). Dw2 contains a
involved in cell wall biosynthesis, transmembrane transport, internode elongation and altered expression of genes (Radenacher and Offringa, 2012). PDK1-mediated activation of Dw2 therefore could stimulate endomembrane activity required for vascular tissue differentiation and cell proliferation. PDK1 activity is modulated by auxin-induced production of phosphoinositols (Xiao and Offringa, 2020). Mutation of the ABCB1 auxin efflux transporter in sorghum (dw3) (Multani et al., 2003) and maize (br2) (Knöller et al., 2010) causes inhibition of internode elongation, demonstrating the importance of auxin in C4 grass internode growth. Mutation of inositol polyphosphate 5-phosphatase in maize bv1 causes inhibition of internode elongation and altered expression of genes involved in cell wall biosynthesis, transmembrane transport, and cytoskeletal function (Avila et al., 2016). Mutation of Dw2 affects the expression of genes involved in these same pathways/processes. Taken together, these results indicate that lipid signaling plays an important role in C4 grass internode growth regulation and that Dw2 is an integral part of this pathway.

Phosphoproteomic and proteomic analyses of tissue from elongating internodes of DYM and DDYM were utilized to obtain insight into Dw2 targets and regulatory activity. Phosphoproteomic analysis identified 205 proteins containing phosphotyrosine that were differentially phosphorylated in DYM and DDYM tissue from Int(P5). The majority of these proteins (89%) showed higher phosphorylation in DDYM internodes, indicating these changes were indirect effects of loss of Dw2 function. This group included proteins involved in endomembrane function, transport, and hormone/light/calcium signaling, with an enrichment in localization and Golgi trafficking proteins. Of all differentially phosphorylated proteins, only 23 proteins showed higher phosphorylation in Int(P5) of DYM. These proteins could be direct targets of Dw2; however, many showed relatively low levels of differential phosphorylation. A few phosphoproteins were only detected in DYM or DDYM. For example, phosphorylation of Dw2 was only detected in DYM because Dw2 protein did not accumulate in DDYM, consistent with the mutation in Dw2 which creates a stop codon in the first exon leading to protein truncation (Noh et al., 2001; Hilley et al., 2017). PLDδ was present in the internodes of both genotypes but phosphorylation was only detected in DYM Int(P5), indicating that this protein could be a direct target of the Dw2 kinase. The treatment of DYM internodes with n-butanol, a PLD inhibitor, inhibited internode elongation to the same extent as observed in DDYM, suggesting that PLD signaling could play an important role in Dw2-regulated internode elongation. In animal systems, phosphorylation of PLD1 by RSK2 results in activation and synthesis of PA at locations of neurite cell proliferation and growth (Ammar et al., 2013). The authors proposed that mutations in RSK2 impairing PLD1 activity resulted in reduced vesicle trafficking and membrane synthesis required for growth.

Arabidopsis PLDδ was identified as a tubulin- and microtubule-binding protein that also interacts with actin, clathrin heavy chain, and a flotillin homolog (Ho et al., 2009). Clathrin heavy chain is involved in spindle organization and phragmoplasts in tobacco (Nicotiana benthamiana) cells (Tahara et al., 2007; Ho et al., 2009) and a tobacco homolog of PLDδ localizes to the mitotic spindle (Marc et al., 1996). Taken together, reduced activation of PLDδ in the IM of DDYM could contribute to lower cell proliferation. KIPK, the Arabidopsis homolog of Dw2, interacts with KCBP (Marc et al., 1996; Day et al., 2000) and PERKs (Humphrey et al., 2015). Interaction with KCBP is especially interesting because KCBP is a calcium-binding protein implicated in several aspects of cell division that interacts with microtubules and is localized to the cortical division zone (Vos et al., 2000; Vinogradova et al., 2009; Buschmann et al., 2015; Tian et al., 2015). If Dw2 also binds KCBP, then this could facilitate localization of Dw2 to cell division zones where regulation of PLDδ could affect vesicle trafficking and other processes involved in cell division. Two chloroplast-localized proteins, Lhc2b and psbH, showed high levels of phosphorylation in DYM compared to DDYM. The subcellular localization of Dw2 has not yet been characterized, but it is likely that elevated phosphorylation of these plastid-localized proteins is an indirect effect of mutation of Dw2. PA has been shown to activate the monogalactosyldiacylglycerol (MGDG) synthase MGD1, and MGDG is essential for chloroplast development (Dubots et al., 2010). Therefore, it is possible that mutation of Dw2 affects PLD-mediated PA synthesis, causing inhibition of chloroplast development and associated changes in Lhc2b and psbH phosphorylation. Alternatively, reduced internode growth in DDYM results in the internodes being wrapped in additional leaf sheaths, which attenuates and alters the spectral distribution of light reaching the internode surface. This could cause a delay in chloroplast development and alter light-mediated changes in phosphorylation involved in state transitions (Dubots et al., 2010; Longoni et al., 2019).

Lack of Dw2 in DDYM reduced endocytosis/endomembrane activity in seedling roots and emerging root hairs. Altered endomembrane activity in DDYM is likely responsible for altered deposition and localization of HX and MLG in stem pith parenchyma cells in the ZoD. In DYM, MLG was distributed uniformly in the apoplast-cell wall space. However, in DDYM, MLG was depleted from cell faces and accumulated in locations where several cells meet (edges or interstices). In contrast, HX accumulated along cell faces.
and was depleted in cell interstices, possibly because of high MLG accumulation in those regions. Cellulose, which is synthesized by a complex localized to the PM, was uniformly distributed around cells. The altered distribution of MLG and HX could be a consequence of depletion of these polysaccharides in cell walls (25–50%) which affected their detection (Martin-Tryon and Harmer, 2008; Xue et al., 2013). It is also possible that the altered distribution is a consequence of altered endomembrane activity and localized wall properties that affect the flow of cell wall polysaccharides to different regions of the cell wall. Prior studies have shown that endomembrane trafficking can specifically deliver materials/proteins to the apical and basal regions of cells and to cell facial and edge domains (Langowski et al., 2010; Kirchhelle et al., 2016). Based on these studies, it has been proposed that normal cell geometry requires maintenance of wall stiffness at geometric edges and that RAB-A5c, which is localized to unique domains of the trans-Golgi network, mediates endomembrane trafficking to edge domains. Mutation of RAB-A5c results in abnormal cell shapes. In this context it is interesting that mutation of CsIP, which encodes MLG synthase, results in altered cell wall stiffness (Kido et al., 2015). Moreover, lack of Dw2 activity in DDM results in internode cells that are less uniform in shape compared to DYM (Figure 3a,d). Therefore, it is possible that mutation of Dw2, which impairs endomembrane activity, directly or indirectly affects cell wall properties (i.e., stiffness), resulting in MLG accumulation at the cell edges located in interstices.

Sorghum grain breeders identified and utilized genotypes encoding inactive Dw2 alleles to reduce plant height and lodging (Quinby, 1974; Kido et al., 2015). During field assessment of the utility of dw2 genotypes for grain breeding, the genotypes were found to have significantly reduced stem length but also decreased panicle length and seed weight without reducing leaf number or altering flowering time (Graham and Lessman, 1966). The reduction in panicle length and grain yield was not due to a reduction in leaf area, although reduced leaf spacing along the stem could alter light interception. Reduced stem length could have limited the capacity to store carbohydrates and nitrogen used for grain filling with negative impact on seed weight. The current study also raises the possibility that reduced panicle length and lower grain yield could be due in part to reduced cell proliferation or altered endomembrane activity. Increases in the yield of grain sorghum have been very slow over the past 30 years (Pfeiffer et al., 2019), suggesting a limitation has been reached with the current dwarf hybrid crop genotypes that are used commercially. This study suggests that developing grain hybrids containing dominant alleles of Dw2 could be useful, while selecting for increased stalk strength similar to the development of bioenergy sorghum.

**EXPERIMENTAL PROCEDURES**

**Plant material and growth conditions**

DYM (Dw2) and DDYM (dw2) plants were grown in greenhouses or in growth chambers during 2017–2018. For height and cell morphology experiments, plants were grown in a long-day greenhouse. For all RNA-seq, phosphoproteomic, and immunolocalization experiments, plants were grown in long-day growth chambers (14 h/10 h day/night) to minimize biological variance. In all cases, plants were grown in five gallon pots and fertilized with osmocote initially and supplemented with Peter’s nutrient solution as needed. Images of plants were taken with Apple’s iPhone 8 and remained unprocessed other than cropping.

**Cell size, number, morphology, and staining**

Cell size and number calculations were performed by staining cells with Calcofluor White (1:20 in PBS) of various internodes and imaging on a Zeiss Axioplan 2 microscope. Sizes of cells were then measured using ImageJ and averaged using three biological replicates per target tissue. The length of each cell per micrometer of internode was used to estimate the number of cells in the total length of the internode.

For cell staining and morphology characterization, the center of mature internodes was harvested and stained with either 0.1% Toluidine Blue or Safranin O to visualize the cell wall. Images were obtained using a black and white microscope, which allowed for a greater contrast and better discrimination of cell wall and vascular bundle elements than with color images. For the abnormal vascular bundle phenotypes, total vascular bundle numbers were calculated in five images from mature internodes of both DYM and DDYM in three biological replicates and were marked as either ‘normal’ or ‘misshapen’. After summation, the numbers of misshapen vascular bundles were reported as a total percentage of all vascular bundles counted. Cellulose staining was achieved by staining cells with Calcofluor White (1:20 in PBS) for 2 min with a 5-minute destaining step and imaged using a Zeiss Axioplan 2 microscope. Three replicates were used for this experiment.

**In situ hybridization**

**Plant materials and paraffin embedding.** Plants were grown as previously described above. Int(P5) tissue samples were formalin-fixed in 10% NBF for 24 h at room temperature. Samples were then washed with 1× PBS, dehydrated through a series of graded ethanol washes, cleared with xylene, and embedded in paraffin wax according to established protocols (Karlgren et al., 2009). Embedded samples were stored at 4°C.

**Probe synthesis and mRNA in situ hybridization.** The sequences of Dw2 (Sobic.006G067700.2) and a Cytochrome P450 (positive control, Sobic.003G324800.1) were obtained from Phytozone and submitted to Advanced Cell Diagnostics, Inc. (ACD) for probe design. RNAscope RNA probes were synthesized by ACD using a custom probe design service. In general, several double Z probe pairs are produced that hybridize with high specificity to the target RNA. Binding of probes in pairs allows amplified signal generation, which is then observed as a dot of red chromogenic precipitate.

Embedded sorghum internodes were thinly sectioned (6 μm thick) using a Leica Microtome, and sections were placed on Fisherbrand Superfrost Plus slides (Cat. No. 12-550-15), put on a hot plate at 42°C for a few minutes, and then further dried.
overnight at room temperature. Slides were processed as previously described (Wang et al., 2012). Specifically, RNAscope 2.5 HD Detection Reagent – RED kit was used (Cat. No. 322360). Probed slides were sealed with EcoMount (Cat. No. EM897L). Images were obtained using bright field through a Zeiss Axios Imager.M2 microscope.

**HX and MLG immunolocalization**

Plant tissue was harvested and lightly vacuum-fixed in formalin-acetic acid-alcohol (3% paraformaldehyde, 5% acetic acid, and 50% ethanol by volume). Tissues were then embedded in 5% agarose blocks and hand sectioned either longitudinally or horizontally, depending on the type of imaging desired (Carraro and Peer, 2016). Primary antibodies used for HX (Kerafast, ELD017, Boston, MA, USA) or MLG (Biosupplies Australia, 400-3, Bundoora, Australia) staining were diluted to 1:500 in Starting Block Blocking solution. The supernatant from this step was further incubated with beads. Beads were collected by centrifugation and used to visualize the immunolocalization signal. Three replicates each were used for each experiment. Images were taken using a Zeiss Axioplan 2 epifluorescent microscope (HX) or an Olympus FV1000 Laser Confocal microscope (MLG) and processed (increasing contrast, subtracting background, etc.) using Zen Lite software or NIH’s ImageJ.

**Phosphoproteomics**

Isolation of phosphopeptides was performed as previously described (Humphrey et al., 2018) with modifications adapted to plant tissues. Frozen sorghum internodes were ground into fine powder using liquid nitrogen with a mortar and pestle. Ground tissues were suspended in cold sodium deoxycholate (SDC) lysis buffer (100 mM Tris-HCl, pH 8.5, 4% [w/v] sodium deoxycholate). The crude extracts were immediately heat-treated at 95°C for 5 min and then homogenized with sonication (60-sec pulses with 0.5 sec on/0.5 sec off at level 1.5). Protein (200 µg) was added to the sample and was applied for trypsin digestion and phosphopeptide enrichment as described (Humphrey et al., 2019) with the following modifications. TiO₂ beads (10 mg) (Tiansphere 5 micron; GL Sciences, Inc, Torrance, CA, USA) were re-suspended in bead suspension buffer and incubated with peptides at 40°C for 5 min in ThermoMixer R at 1400 rpm. Beads were collected by centrifugation and phosphopeptides were eluted, combined with the first, and further processed as one sample. Each sample was injected twice, onto a Q Exactive HF-X mass spectrometer using 90-min LC gradients as indicated. Six biological replicates were performed for each genotype.

**LC-MS/MS analysis**

Isolated phosphopeptides were re-suspended in 2% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) to 15 µL. Injections of 10 µL were automatically made using a Thermo (www.thermo.com) EASYnLC 1200 onto a Thermo Acclaim PepMap RSLC 99.9% water/0.1% formic acid, buffer B = 80% ACN/0.1% formic acid/19.9% water) at a constant flow rate of 300 nL/min. Column temperature was maintained at a constant temperature of 50°C using an integrated column oven (PRSO-V2, Sonation GmbH, Biberach, Germany). Eluted peptides were sprayed into a Thermo Scientific Q Exactive HF-X mass spectrometer (www.thermo.com) using a FlexSpray spray ion source. Survey scans were taken in the Orbitrap (60 000 resolution, determined at m/z 200) and the top 10 ions in each survey scan were then subjected to automatic higher-energy collision-induced dissociation (HCD) with fragment spectra acquired at 15 000 resolution.

Data files from the LC-MS/MS analysis were processed using MaxQuant (version 1.6.3.4) to identify proteins and calculate isobaric tag intensities using the Andromeda search engine using the default parameters and with an FDR of <0.01 at the protein and peptide levels. The Sorghum bicolor v3.1.1 proteome obtained from Phytozome containing 47 121 proteins was used for the search. Perseus (version 1.6.2.3) was used to compare the two genotypes. Phosphosites with location probabilities lower than 0.75 were removed, as were peptides from the reverse databases and probable contaminants. The intensities were log₂ transformed and phosphosites with less than three valid values in one of the two genotypes were removed. Missing values were imputed using a width of 0.3 and a downshift of 1.8. A two-sided Student t-test was conducted, and permutation-based multiple testing correction was applied as implemented in Perseus.

**Proteomics**

Samples were digested according to Kulak et al. (2014). Briefly, protein samples (100 µg) were re-suspended to 270 µL in 100 mM ammonium bicarbonate supplemented with 4% (w/v) DCC. Samples were reduced and alkylated by adding a solution of TCEP and iodoacetamide (10 and 40 mM, respectively, at pH 8) and incubated for 5 min at 45°C with shaking at 1400 rpm in an Eppendorf ThermoMixer R. The samples were allowed to cool to room temperature and trypsin/lysC enzyme mixture (Promega, V5071) in 100 mM ammonium bicarbonate was added at a 1:100 ratio (w/w, enzyme/protein). The mixture was then incubated at 37°C overnight in the ThermoMixer R with shaking at 1400 rpm. The final volume of each digest was approximately 300 µL. After digestion, SDC was removed by phase transfer and the peptides were acidified to 1% TFA. Peptides were then subjected to C18 solid-phase cleanup using StageTips (Rappisliber et al., 2007) to remove salts. Peptide eluates were dried by vacuum centrifugation and stored at −20°C. Five biological replicates were performed for each genotype.

**Isobaric peptide labeling**

Peptide samples were then re-suspended in 100 µL of 100 mM triethylammonium bicarbonate and labeled with TMT reagents from Thermo Scientific (www.thermo.com) according to the manufacturer’s instructions. Aliquots of 2 µL were taken from each labeled sample and reserved for testing the labeling/mixing efficiency by MS. Remaining labeled peptides were mixed 1:1 by volume and purified by solid-phase extraction using C18 StageTips. Eluted peptides were dried by vacuum centrifugation to approximately 2 µL and stored at −20°C.

**High-pH reversed-phase fractionation**

The combined peptide sample was re-suspended in 2% ACN/0.1% TFA to 50 µL and fractionated by high-pH reverse-phase chromatography. The entire sample was injected onto a Waters Acquity UPLC BEH 1.7 µm, 2.1 × 100 mm c18 column using a
Waters Acquity H-class UPLC. Bound peptides were washed using 0.1% TFA in water for 2 min and then separated over 60 min using a gradient of 1% B to 25% B at 49 min, raised to 60% B at 53 min, raised to 70% B at 55 min, and held at 70% B for the duration of the run (buffer A = 10 mM ammonium bicarbonate, pH 10, in water; buffer B = 10 mM ammonium bicarbonate, pH 10, in 90% ACN) at a constant flow rate of 0.3 ml/min. Column temperature was maintained at 50°C using an integrated column heater. Fractions were collected at 1 min intervals using a Gilson FC403B fraction collector and then concatenated into 12 total fractions post-run. Fractions were dried by vacuum centrifugation and frozen at –20°C.

LC-MS/MS analysis

Each fraction was re-suspended in 2% ACN/0.1% TFA to 20 µl and diluted 1:10 in the same buffer, on-plate. Injections of 5 µl were automatically made using a Thermo (www.thermo.com) EASYnLC 1200 onto a Thermo Acclaim PepMap RSLC 0.1 × 20 mm C18 trapping column and washed for approximately 5 min with buffer A. Bound peptides were then eluted over 95 min onto a Thermo Acclaim PepMap RSLC 0.075 × 500 mm resolving column with a gradient of 5% B to 8% B at 5 min, raised to 42% B at 83 min, raised to 90% B at 85 min, and held at 90% B for the duration of the run (buffer A = 99.9% water/0.1% formic acid, buffer B = 80% ACN/0.1% formic acid/19.9% water) at a constant flow rate of 300 nL/min. Column temperature was maintained at a constant temperature of 50°C using an integrated column oven (PRSO-V2, Sonation GmbH, Biberach, Germany).

Eluted peptides were sprayed into a Thermo Scientific Q Exactive HF-X mass spectrometer (www.thermo.com) using a FlexSpray spray ion source. Survey scans were taken in the Orbitrap (120 000 resolution, determined at m/z 200) and the top 15 ions in each survey scan were then subjected to automatic HCD with fragment spectra acquired at 45 000 resolution.

Data files from the LC-MS/MS analysis were processed using MaxQuant (version 1.6.3.4) to identify proteins and calculate isoformic tag intensities using the Andromeda search engine using the default parameters and with an FDR of <0.01 at the protein and peptide levels. The *Sorghum bicolor* v3.1.1. obtained from Phytozone containing 47 121 proteins was used for the search. Perseus (version 1.6.2.3) was used to compare the two genotypes. The intensities were log2 transformed and normalized by subtracting the mean intensity value for that sample from each value. A two-sided Student t-test was conducted, and permutation-based multiple testing correction was applied as implemented in Perseus. Enrichment analysis was performed using ThePlantRegMap’s Gene Ontology (GO) enrichment tool (Tian et al., 2020).

*n*-Butanol treatment

DYM and DDYM plants were grown for approximately 70 days, after which a small window was cut in the leaf sheath to expose the growing internode. Lanolin paste (control) and Lanolin paste +0.1% *n*-butanol were smeared onto the growing Int(P5). Exposed internodes were then covered with foil to prevent light damage and to promote continued growth. Plants were then allowed to grow for 8 days before imaging and quantification. Cells sizes were quantified as previously described above.

FM4-64 and Brefeldin A treatment

Growing seedlings of DYM and DDYM plants were placed in water containing 5 µM FM4-64 for 5 min. After two quick washes to remove excess dye, seedlings were mounted and imaged using an Olympus FV1000 laser confocal microscope at 5 and 30 min after staining as described in (Rigal et al., 2015; Stefano et al., 2018). For inhibitor treatment, seedlings were placed in 5 µM FM4-64 for 5 min, washed, and then placed in 3 µg/ml BFA solution and incubated for 1 h before imaging. All images are a projection of Z stack images. The fluorescent signal was quantified by taking the total fluorescence intensity of the PM and comparing it to the total fluorescence intensity within the PM. Five replicates were used for FM4-64 and three replicates were used for BFA + FM4-64. All image processing was performed in NIH’s ImageJ and all images used for comparisons were treated equally.

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AUTHOR CONTRIBUTIONS

JO and JM designed the experimental approach; JO carried out experiments, data analysis, and bioinformatics and constructed tables and figures; MF and CW conducted phosphoproteomic/proteomic experiments; BM helped with the bioinformatic analysis; SZD performed in situ analysis of RNA; FB and CW contributed to experimental design, data analysis, and discussion; JO and JM wrote the paper.

CONFLICT OF INTEREST

Federica Brandizzi, PhD, is a senior editor at *The Plant Journal*, but played no part in the approval or editorial process of this manuscript.

DATA AVAILABILITY STATEMENT

Proteomic and phosphoproteomic datasets are deposited in the PRIDE database with the accession number PXD020160. All other relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Sample collection atlas. Summary of tissue and cell type images collected for analysis. The experiment is listed in bold, the tissue or cell type is listed in italics, and the figure where data are presented is shown at the bottom line of each box.

Table S1. Phosphoproteomic data.

Table S2. Proteomic data.

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