Targeted mutation of transcription factor genes alters metaxylem vessel size and number in rice roots

Jenna E. Reeger1 | Matthew Wheatley2 | Yinong Yang2 | Kathleen M. Brown3

Abstract

Root metaxylem vessels are responsible for axial water transport and contribute to hydraulic architecture. Variation in metaxylem vessel size and number can impact drought tolerance in crop plants, including rice, a crop that is particularly sensitive to drought. Identifying and validating candidate genes for metaxylem development would aid breeding efforts for improved varieties for drought tolerance. We identified three transcription factor candidate genes that potentially regulate metaxylem vessel size and number in rice based on orthologous annotations, published expression data, and available root and drought-related QTL data. Single gene knockout mutants were generated for each candidate using CRISPR-Cas9 genome editing. Root metaxylem vessel area and number were analyzed in 6-week-old knockout mutants and wild-type plants under well-watered and drought conditions in the greenhouse. Compared with wild type, LONESOME HIGHWAY (OsLHW) mutants had fewer, smaller metaxylem vessels in shallow roots and more, larger vessels in deep roots in drought conditions, indicating that OsLHW may be a repressor of drought-induced metaxylem plasticity. The AUXIN RESPONSE FACTOR 15 mutants showed fewer but larger metaxylem vessel area in well-watered conditions, but phenotypes were inconsistent under drought treatment. ORYZA SATIVA HOMEBOX 6 (OSH6) mutants had fewer, smaller metaxylem vessels in well-watered conditions with greater effects on xylem number than size. OSH6 mutants had larger shoots and more, deeper roots than the wild type in well-watered conditions, but there were no differences in performance under drought between mutants and wild type. Though these candidate gene mutants did not exhibit large phenotypic effects, the identification and investigation of candidate genes related to metaxylem traits in rice deepen our understanding of metaxylem development and are needed to facilitate incorporation of favorable alleles into breeding populations to improve drought stress tolerance.

KEYWORDS

candidate gene validation, CRISPR-Cas9, Metaxylem, rice root
1 INTRODUCTION

Rice is one of the most important cereal crops globally, and much of the world’s poor relies on rice as an income and food source (McLean et al., 2013). Rice production is severely affected by drought each year, where stress can reduce yields by up to 25% (Zhang et al., 2018), and climate change is expected to result in at least 30% more variable production worldwide (Ray et al., 2015). We therefore need rice varieties that maintain yields under abiotic stresses such as water limitation. Roots are a good target for drought tolerance traits because of their direct interaction with soil water uptake and transport. Root systems that optimize water acquisition are expected to perform better under drought conditions (Lynch, 2013). Much research has focused on root architectural traits such as rooting depth and angle, but variation in root anatomical traits may also have a significant impact on drought stress tolerance (Lynch, 2013). So far, very little is known about the genetic control of root anatomical traits in mature rice plants.

Root metaxylem traits may have important implications for drought tolerance in rice. The size and number of metaxylem vessels directly affect the capacity of a root to transport water axially from the root tip to the shoot, and axial hydraulic conductance can be estimated from these xylem parameters (Tyree & Ewers, 1991). Smaller metaxylem vessel diameter may be beneficial under drought due to reduced hydraulic conductance per root and lower risk of cavitation (introduction of an air embolism into the water column) and collapse (physical buckling of metaxylem walls). Reduced hydraulic conductance would conserve soil water resources and maintain moisture for the growing tip and surrounding rhizosphere to facilitate greater soil exploration (Comas et al., 2013; Lynch et al., 2014). Metaxylem vessels have increased risk of cavitation and collapse when soil water potential becomes very low, but smaller vessels are less prone to these effects (Guet et al., 2015; Sperry & Salindra, 1994). Studies have shown that smaller metaxylem vessels were associated with drought tolerance in the field in wheat seedlings (Richards & Saliendra, 1994). We identified novel rice xylem candidate genes for further study. Our search included genes that had annotations in Arabidopsis orthologs that were related to root vascular development, e.g., cell differentiation, cell patterning, auxin signaling, drought response, and cell wall development. We also used publicly available tissue-specific expression data from rice (Sato et al., 2013) to determine whether candidate genes were expressed in root stele tissue.

Candidate genes identified based on orthologous annotation and gene family identity must be validated to confirm their phenotypic effect before they are considered worthy of application in crop improvement programs. Targeted mutagenesis with CRISPR-Cas9 is an effective approach for loss-of-function analysis to determine the effect of one or more candidate genes on phenotypes of interest. CRISPR-Cas9 allows the introduction of small insertion–deletion mutations in one or a few genes of interest, leading to frame shifts and gene knockouts in rice with minimal off-target effects (Xie et al., 2015). In this study, we identified three candidate genes for...
metaxylem vessel formation and assessed the effect of single candidate gene knockouts on metaxylem vessel phenotypes under well-watered conditions and under drought stress treatment.

2 | METHODS

2.1 | Candidate gene identification

Candidate genes were identified based on the following criteria: (a) the gene had an annotation in rice or orthologous annotation in Arabidopsis or maize related to roots, anatomical development, drought tolerance, and/or cell wall formation/maintenance; (b) the candidate gene was expressed in root tissue according to RiceXPro (https://ricexpro.dna.affrc.go.jp; Sato et al., 2013); and/or (c) the gene was located in QTL identified for root or drought tolerance traits. Phylogenetic trees of rice genes with highest sequence similarity to each candidate gene and orthologous genes in Arabidopsis and Poplar were generated with the PhyloGenes webtool (Zhang et al., 2020).

2.2 | CRISPR-Cas9 construct and rice mutant generation

Three candidate genes for metaxylem vessel development, (Os02g0673500/ LOC_Os02g45170; Os01g0302500/ LOC_Os01g19694; Os05g0563400/ LOC_Os05g48870), were chosen for validation by CRISPR-Cas9 mutagenesis. Two guide-RNA spacer sequences (gRNAs) were designed to target two different sites for each of the candidate genes. These spacers were scrutinized for off-target activity and shown to be highly specific using the CRISPR-Plant database (Xie et al., 2014; Table S2). PTG (polycistronic tRNA-gRNA) synthetic genes were produced using golden gate assembly (Xie et al., 2015) and then ligated into pRGE32 (Addgene #63142). The vectors were transformed into Agrobacterium tumefaciens strain EHA105 by electroporation and subsequently into the callus of Kitaake rice cultivar (Xie et al., 2015). Hygromycin B selective media was used to screen for successfully transformed calli. All genotyping for individual plants (T0, T1, and T2) was confirmed by PCR amplification and Sanger sequencing. Two degenerate sequencing programs were used to resolve double peaks in heterozygous individuals (Dehairs et al., 2016; Liu et al., 2015). Biallelic mutations at one or both of the gRNA target sites that introduced frame-shift mutations in the coding regions were selected for subsequent phenotyping experiments. Off-target validation of CRISPR T1 mutants was assessed by sequencing the PCR product of potential off-target sites that share a high level of sequence identity with the gRNA spacers (Table S3).

2.3 | Rice plant phenotyping

T1 and T2 generation individuals were phenotyped for metaxylem vessel phenotypes to identify differences compared to wild-type lines in greenhouses at Penn State University, University Park, PA (40°48.7.4 N, 77°51.456.5° W). The T1 generation was compared to wild type under well-watered conditions only, and the T2 was evaluated under both drought and well-watered treatment. Metaxylem phenotypes were measured in deep and shallow roots separately to observe any effects of root class and local soil moisture on the phenotypes.

In the T1 experiment, six individuals from three T0 parents with a confirmed biallelic mutation as well as six individuals from one T0 parent with a confirmed lack of mutation as wild type (a total of 24 individuals) were grown in well-watered aerobic conditions. In the T2 experiment, four individuals from three T0 parents with a confirmed biallelic mutation as well as four individuals from one T0 parent with a confirmed lack of mutation were grown in well-watered aerobic treatment and a water deficit treatment (a total of 32 individuals). Individuals with different alleles for each knockout were used in these experiments, taking care to balance distribution of alleles between drought and well-watered treatments in the T2 experiment (Figures S4−S6). Plants were arranged in randomized complete block designs in plastic mesocosms (15 cm diameter × 1.2 m tall) lined with polyethylene liners. A medium of 40% v/v medium sand (0.3−0.5 mm, U.S. Silica Co.), 40% v/v vermiculite (Griffin Greenhouse Supplies), 5% v/v perlite (Griffin Greenhouse Supplies, Morgantown, PA, USA), 15% v/v sifted field soil (Hagerstown silt loam; 64% silt, 21% clay, 15% sand; fine, mixed, semi-active, medica Typic Hapludult), and slow-release fertilizer pellets at 3.2 g/L (Osmocote Plus, 15% N, 9% P, 12% K; 1% Mg, 6% S, 0.02% B, 0.05% Cu, 0.4% Fe, 0.06% Mn, 0.02% Mo, 0.05% Zn, 3- to 4-month release) was used. Seeds were de-hulled, sterilized with 10% (v/v) NaOCl in water, rinsed with deionized water, and planted directly into mesocosms at field capacity. Wire single-mesh baskets (14.3 cm diameter, 13 cm tall, Winco) were placed into the top 10 cm of the mesocosm to assess number of deep roots, and seeds were planted directly into the medium-filled baskets. Temperature was maintained at 25−28°C, and supplemental LED lights (200 μmol/m² s⁻¹ of PAR) were used to provide 14-hr day length when ambient light was less than 600 μmol/m² s⁻¹ of PAR. Plants were watered twice daily with deionized water for approximately 400 ml/day. Two weeks after emergence, irrigation was halted for the drought treatment. Both treatments were irrigated with 100-ml 1 mM CaCl₂ from the top of the pot weekly. Water content of the growth medium was measured biweekly using time-domain reflectrometry (TDR100, Campbell Scientific) probes with 10-cm probe length inserted 30.5 cm from the top and 30.5 cm from the bottom of one mesocosm per independent mutant or wild-type line per treatment (for a total of 20 mesocosms). Baseline measurements were taken in each mesocosm by initially bringing the medium to field capacity by flooding and draining for 2–4 hr before measurement, and subsequent measurements were scaled as a percentage of the field capacity measurement. At the end of the experiment, mesocosms from the drought treatment had an average of 81% less water content at the top and 30% less at the bottom compared to the well-watered treatment, and water content was 72% less in the top of mesocosms compared to the bottom in the drought treatment (Figure S10). No differences in water content were observed between mutant and wild-type plants.
Plants were sampled at 6 weeks after emergence. Gas-exchange measurements to determine stomatal conductance (mol H₂O m⁻² s⁻¹) and photosynthetic rate (μmol CO₂ m⁻² s⁻¹) were taken at the leaf midpoint on the first fully expanded leaf from 10 a.m.-12 p.m. the day before harvest (Licor 6400XT, Licor Biosciences). Tiller number was counted, and shoots were excised for shoot dry biomass determination. The root systems and medium contained within the plastic liner were laid horizontally and pulled from the pots, the liner was cut open to expose the roots and medium, and the remaining medium was gently washed from the root system with water. Clean root systems were preserved in 70% (v/v) ethanol in water for later sampling. While still in the wire mesh baskets, the number of deep roots was assessed by counting the number of roots with an angle of 50° or greater with respect to horizontal. Total crown root number was also counted. Root tissue at 10 and 20 cm from the root tip was collected from four to six deep crown roots (roots with a length greater than half the length of the total root system) and four to six shallow crown roots (roots with a length less than half the length of the total root system) per root system. Root segments were placed in plastic histocaps and dried in a critical point drier (Leica EM CPD 300, Leica Microsystems Inc, Buffalo Grove, IL, USA). High-resolution images of root transverse cross sections were taken with a laser ablation tomography system (Hall et al., 2019). Mipar® software (Mipar US) was used to assess root cross-sectional area (RXSA), stele area (TSA), cortical area (TCA), metaxylem vessel area (MXA), number of metaxylem vessels (MXV), and aerenchyma area (AA) in root images. Percent aerenchyma area (percAA) was calculated as AA/TCA × 100. Metaxylem traits were also evaluated as a ratio of stele area (MXA/TSA, MXV/TSA) and root cross-sectional area (MXA/RXSA, MXV/RXSA). Anatomy data reported in the figures represent four to six shallow or four to six deep roots for each of the six plants in generation T1 or four plants per treatment in generation T2, for a total of up to 36 (T1) or 24 (T2) data points represented in each mean.

### 2.4 Statistical analysis

Root anatomical phenotypes did not differ consistently between the 10- and 20-cm segments, so phenotypes were combined for analysis. Phenotypes of individuals with the same parent were grouped together so that any parental effects are not mixed between lineages or generations and since there were not enough individuals (n < 4) to group by each edit type for statistical testing. Root anatomical data were analyzed with pairwise t-tests to determine significant differences in root anatomical traits between independent line means and wild-type means. Percent differences in phenotype means were only reported when two or more independent lines differed significantly from wild type at the α = 0.1 level and were calculated as (\( \bar{x}_M - \bar{x}_T \)) / \( \bar{x}_T \) × 100, where M is the independent mutant line and T is wild type. Data were plotted using R v3.5.3 (R Core Team, 2016), package ggplot2 (Wickham, 2016).

### 3 Results

Three candidate genes were chosen for targeted mutagenesis based on their annotations relevant to vascular development and drought tolerance. CRISPR-Cas9 mutants of these genes were expected to exhibit altered root metaxylem vessel size (MXA) or number (MXV).

#### 3.1 Xylem candidate genes chosen based on orthologous annotation, expression patterns, and QTL data

One of the candidate genes is a basic helix-loop-helix (bHLH) DNA-binding protein or transcription factor, OsHLH or OsLHW (Os02g0673500/LOC_Os02g45170), that has two functional domains: a MYC N-terminal transcriptional activation domain and the bHLH DNA-binding domain (Heim et al., 2003; Kazan & Manners, 2013; Pires & Dolan, 2010). There are approximately 183 bHLH transcription factors in rice, and many have tissue-specific expression in response to drought in rice as well as in other species (Wang et al., 2011; Wei & Chen, 2018). OsLHW had moderate expression in root stele tissue in the RiceXPro tissue-specific expression database (Sato et al., 2013) and was located in a previously detected drought QTL qao2.1 for leaf osmotic adjustment (Zhang et al., 2001). The Arabidopsis ortholog, LONESOME HIGHWAY LIKE 3 (LHL3, AtbHLH157, AT1G64625; Figure S1), has DNA-binding transcription factor activity and is involved in responses to auxin stimulus. LHL3 is expressed in root meristems and vascular tissue and is involved in xylem differentiation associated with downstream auxin signaling (Ohashi-Ito et al., 2013). Mutants of LONESOME HIGHWAY (LHW) and LHL (LHL1, EMB1444, paralog to LHL3) in Arabidopsis exhibited only one to two xylem poles and one phloem pole, and double mutants ihl1 ihl3 exhibited an even stronger effect than either single mutant, indicating the roles of these genes in vascular differentiation (Ohashi-Ito & Bergmann, 2007; Ohashi-Ito et al., 2013). Overexpression of LHL3 resulted in more xylem vessels and more vascular cells in the stele, though xylem size was not quantified (Ohashi-Ito et al., 2013). These studies of the OsLHW ortholog in Arabidopsis provide support for the hypothesis that this gene affects xylem number in rice root tissue and that gene knockout may cause a reduction in xylem number.

The second candidate gene (Os05g0563400, LOC_Os05g48870) is auxin response factor 15 (ARF15 or OsETTIN1). ARFs bind to the promoters of auxin-responsive genes and upon dimerization with auxin/indole acetic acid (AUX/IAA) proteins, either activate or repress downstream gene transcription. Rice contains 25 ARFs, nine of which are activators and 19 of which are repressors, and OsARF15 is predicted to be a repressor (Wang et al., 2007). ARFs typically have three functional domains: an N-terminal DNA-binding domain, a middle activator or repressor domain, and a C-terminal AUX/IAA-binding domain (Guilfoyle & Hagen, 2007). ARF15 has moderate to high expression in root stele tissue at the elongation and maturation zones according to the RiceXPro database. ARF15 was
unregulated in response to drought in root tissue of two rice genotypes and was proposed to help maintain root growth under stress (Raveendran et al., 2018). The Arabidopsis ortholog, ETTIN (ETT) or ARF3 (Figure S2), has a known role in regulating drought stress response genes (Matsui et al., 2014) and organ polarity, though in floral structures (Sessions et al., 1997). Another member of the Arabidopsis ARF family, ARF5/MONOPTEROS (MP), regulates vascular development and embryo organization in the root meristem. The mp homozygous mutants do not produce a primary root, and in heterozygous mp mutants, which produce some axial roots, xylem and phloem vessels are not fully differentiated or connected (Hardtke & Berleth, 1998; Przemeck et al., 1996). ARF5 and ARF7 have also been shown to interact to regulate cell patterning and cell expansion in the embryo and stem meristematic tissue (Hardtke et al., 2004). Disruption of only the C-terminal AUX/IAA-binding domain has been shown to constitutively activate/repress auxin-response genes in Arabidopsis because of the lack of response to auxin levels (Tiwari et al., 2003). For these reasons, we hypothesize that ARF15 may play a role in vascular development in rice. Knockout mutants may exhibit underdeveloped xylem vessels or vessels of varying size, and C-terminal truncation may result in fewer and/or smaller xylem vessels.

The third candidate gene, Oryza sativa homeobox protein OSH6 (Os01g0302500/ LOC_Os01g19694), is a knotted1-type homeobox (KNOX) containing protein and transcription factor, OSH6. KNOX class genes are in the TALE superfamily, and there are 12 genes identified in this KNOX family in rice (Mukherjee et al., 2009). OSH6 is in KNOX subclass II, along with five other rice genes, OSH1, OSH6, OSH15, OSH43, and OSH71 (Sato et al., 2001; Sentoku et al., 1999).

Functional domains in KNOX genes include: a target gene suppressing KNAX1 domain, a homodimerizing KNAX2 domain which is essential for function, an ELK motif for nuclear localization and/or protein-protein interaction, and a DNA-binding homeodomain (Mukherjee et al., 2009; Nagasaki et al., 2001). KNOX genes are known to inhibit shoot meristem differentiation via increases in cytokinin and decreases in gibberellic acid (Tsuda et al., 2011). OSH6 had low expression in root stele tissue in the RiceXPro database but was located in the root fresh weight QTL rfw1b (Li et al., 2005). The orthologous Arabidopsis gene, KNOTTED1-LIKE HOMEOBOX GENE 6 (KNAT6, AT1G23380; Figure S3), is a class I member of the KNOX transcription factor family. KNAT6 is expressed in shoot meristems and is well-known for involvement in inflorescence architecture and abscission (Belles-Boix et al., 2006; Zhao et al., 2015), but KNAT6 is also expressed in vascular tissues at root meristems related to lateral root initiation (Dean et al., 2004). Two members of KNOX class II, KNAT3 and KNAT7, have been shown to negatively regulate secondary cell wall deposition in Arabidopsis. Single mutant knat7-1 or irx11 resulted in collapsed xylem vessel phenotype with thicker secondary cell walls (Li et al., 2012), but the double mutant knat3 knat7 displayed reduced cell wall thickness in xylem vessels in the stem (Qin et al., 2020). PagKNAT2/6b (POPTR_010G043500v3), the poplar ortholog to Arabidopsis KNAT2 and KNAT6 class I KNOX genes, represses xylem differentiation and cell wall thickness, and downregulation resulted in larger xylem size (Zhao et al., 2020). Though some differences exist in terms of tissue specificity and cell-wall component targets, transcriptional regulators of secondary cell wall formation play similar roles in Arabidopsis and rice (Rao & Dixon, 2018). We therefore hypothesize that OSH6 may affect cell wall deposition and therefore metaxylem vessel size in rice.

### 3.2 Root tissue-specific phenotypes and drought responses in mutant genotypes

We hypothesized that plants with smaller metaxylem vessels would perform better under drought stress than wild type. To reduce allometric effects of variance in root anatomical phenotypes due to variance in root and plant size, we focused on metaxylem vessel size and number scaled to stele (TSA) and root cross-sectional (RXSA) areas in deep (older) and shallow (younger) crown roots. Here, we report effects that were significantly different from wild type in at least two independent mutant lines for each root depth and treatment. Phenotypes are presented for sets of individuals from the same parent, designated as “lines”. We observed phenotypic differences between independent lines, but the differences were not clearly attributable to the nature of the mutation (heterozygous vs homozygous, size of deletions; Figures S4–S6). Photosynthetic rate and stomatal conductance were measured in all plants, but no significant differences were observed between mutants and wild-type plants (Table S1).

### 3.3 LHW mutant phenotypes differed between shallow and deep roots

The candidate gene encoding a bHLH DNA-binding protein (LHW, Os02g0673500, LOC_Os02g45170) was hypothesized to affect metaxylem vessel number. Two guide RNAs were designed to target sequences upstream of the bHLH DNA-binding domain (Figure 1, Table S2), which resulted in indel edits creating premature stop codons and a 19-residue deletion upstream of the bHLH domain (Figure S4). Each of these edits resulted in interrupted function of the target gene. Based on the DNA sequence analysis of predicted off-target sites, no off-target editing was observed in these independent lines (Table S3).

The LHW knockout mutants showed altered metaxylem phenotypes compared to wild-type plants under well-watered and drought treatments (Figure 2). In well-watered plants, shallow roots of T1 mutant lines had less total MXA/TSA and greater MXV/TSA compared to wild type, and deep roots had less MXV/TSA (Figure S7), but these phenotypes were not observed in well-watered plants in the T2 generation. Mutations in LHW reduced TSA and RXSA only in shallow roots of T2 plants (Figure S7). In the T2 generation, significant phenotypic differences were only observed under drought treatment (Figure 3). In deep roots of drought-treated T2 plants, all three independent lines had significantly greater (15%–23%) total MXA/RXSA (Figure 3). Like shallow roots in T2, shallow roots of
drought-treated T2 plants had 10%-23% less total MXA/TSA than wild type (Figure 3). Metaxylem vessel number (MXV) was also affected by these mutations. MXV/TSA was 14%-25% greater in deep roots and 13%-18% less in shallow roots of mutant plants under drought. Two of the lines that exhibited greater total MXA/RXSA in deep roots (lhw-5, lhw-6) also had 12%-23% less median MXA under drought (Figure S8). Shoot dry biomass, crown root number, and deep root number were not different between mutants and control in either generation or treatment.

3.4 | ARF15 mutants showed greater relative median MXA and less MXV in well-watered treatment

The ARF15 gene (Os05g0563400, LOC_Os05g48870) encodes an auxin response factor. CRISPR-Cas9 genome editing of the AUX/IAA binding domain region generated four mutations introducing either premature stop codons or deleting 28-30 residues within this domain (Figure 1 and Figure S5). Disruption of the AUX/IAA domain is likely to cause ARF15 to repress ARF response genes constitutively without sensitivity to auxin (Guilfoyle & Hagen, 2007), so we expected fewer and/or smaller metaxylem vessels. No off-target editing was observed in potential off-target sites in these independent lines (Table S3).

The ARF15 knockout mutants showed altered metaxylem phenotypes compared to wild type in well-watered conditions (Figures 2 and 4). Shallow roots had greater median MXA/RXSA in well-watered treatments across both generations (Figure 4). In T1 lines, median MXA/RXSA was 27%-36% greater than in wild type, while T2 lines had 17%-25% greater median MXA/RXSA in well-watered conditions. Unscaled median MXA in shallow roots was also 13% greater in one T1 line (arf15-2) and 14%-21% greater in two T2 lines (arf15-4,5) of the ARF15 mutants (Figure 4). However, ARF15 mutants did not have consistent MXA phenotypes in deep roots, with the exception of having less median MXA in two lines and less total MXA/TSA in three lines under drought stress (not shown). Metaxylem vessel number of mutants was less than wild type in both deep and shallow roots of well-watered T2 plants (Figure 4).

3.5 | OSH6 mutants showed less relative MXA and MXV under well-watered treatment but greater median MXA than wild type under drought

The OSH6 gene (Os01g0302500, LOC_Os01g19694) encodes a knotted1-type homeobox domain containing protein. CRISPR-Cas9-mediated mutagenesis was targeted to the middle of the KNOX2 domain and the C-terminal end of the ELK motif (Figure 1). A one base-pair deletion in the KNOX2 domain sequence in every mutant allele induced a frame-shift mutation that ultimately generated a premature stop codon before the ELK motif (Figure S6). Since the KNOX2 domain is essential for protein function (Mukherjee
**Figure 2** Representative root cross-sectional images of wild type (WT, left) and mutants (right). Treatment (well-watered, drought) and root depth (shallow, deep) are indicated per row. Scale bars represent 0.15-mm lengths.

- Smaller, fewer metaxylem vessels in shallow roots under drought.
- Greater number of metaxylem vessels increases scaled metaxylem vessel area.
- Fewer, larger metaxylem vessels.
- Fewer metaxylem vessels.
- Greater median metaxylem vessel area under drought.
et al., 2009; Nagasaki et al., 2001), these mutant genotypes suggest that OSH6 function was disrupted in all individuals. No off-target editing was observed in potential off-target sites in these independent lines (Table S3).

The OSH6 knockout mutants often had fewer metaxylem vessels compared to the wild-type control plants in well-watered conditions (Figure 2). Deep roots of T₁ and T₂ mutants had 14%–21% less total MXA/RXSA, and shallow roots of T₂ had 7%–13% less total MXA/RXSA (Figure 5). This decrease in total MXA/RXSA resulted from reduced MXV/RXSA in OSH6 mutants; i.e., mutants had fewer vessels rather than smaller vessels. MXV/RXSA was reduced by 22%–32% in deep roots of two T₁ (osh6-1,2) and two T₂ (osh6-4,5) mutant lines and by 25%–28% in shallow roots of all three T₂ mutant lines (Figure 5). When not scaled by RXSA and TSA, median MXA was 11%–16% greater, and MXV was 19%–24% less in deep roots of the two T₂ lines that showed less total MXA/RXSA and MXV/RXSA (osh6-4,5, Figure S9). Median and total MXA were 13%–18% less in shallow roots of two T₁ lines where TSA was also 12%–14% less (osh6-1,2, Figure S9).

In a separate experiment comparing T₂ plants under well-watered and drought conditions, well-watered plants had phenotypes similar to those in the T₁/T₂ experiment described above (compare Figures 5 and 6). Under drought, shallow roots of mutants showed 10%–17% greater median MXA/RXSA in two lines (osh6-4,6) but 8% less in one line (osh6-5) and 10%–15% less total MXA/TSA in two lines compared to the drought control (osh6-4,5, Figure 6). The two
lines with greater median MXA/RXSA in shallow roots (osh6-4,6) had 8%–16% less MXV, while RXSA and TSA did not differ from control under drought (not shown).

In both experiments, shoot biomass was 41%–90% greater in all T2 generation OSH6 mutants compared to wild type when plants were well watered (Figure 7). In well-watered plants, T1 mutant lines had 27%–59% greater crown root number (Figure 7) and 82%–106% greater number of deep roots (Figure 8) than wild-type plants. However, despite having more crown roots (Figure 7), only one line of T2 generation mutants had greater deep root numbers (osh6-6, Figure 8). Under drought, there were no significant effects of OSH6 mutations on shoot biomass, but crown root number was 38%–45% greater in two T2 mutant lines compared to wild type (osh6-4,6, Figure 7). Under drought, deep root number was greater than wild type only in osh6-6 (Figure 8).

4 | DISCUSSION

In order to test the effect of three candidate genes for metaxylem vessel development, we took the approach of targeted mutagenesis in rice using CRISPR-Cas9. This system allowed us to generate knockout mutants for the candidate genes to assess whether metaxylem vessel area and number differed between the mutants and wild-type plants. Shallow and deep (presumably younger and older, respectively) crown roots of T1 and T2 generation plants were phenotyped for metaxylem traits, and in a second, separate experiment, phenotypes were assessed in T2 generation plants exposed to well-watered and drought treatments, since drought alters metaxylem traits in rice (Hazman & Brown, 2018; Henry et al., 2012; Kadam et al., 2017). Targeted mutagenesis of these candidate genes resulted in significant effects on metaxylem vessel area and number,
and these effects varied between root classes, generations, and drought treatments.

The OsLHW gene was expected to affect metaxylem vessel traits in rice root tissue based on location of expression in the root stele (Sato et al., 2013) and the influence of its ortholog on xylem vessel number in Arabidopsis (Ohashi-Ito et al., 2013; Růžička et al., 2015). The OsLHW mutants showed greater differences in metaxylem vessel phenotypes from wild type under drought stress than in well-watered conditions. Drought-treated mutants had fewer vessels per stele area in shallow roots but more vessels per stele area in deep roots (Figure 3). In the deep roots, this led to greater total metaxylem area across the section. Only vessel number, not size, was responsible for this difference in total metaxylem area, since median individual metaxylem area was less in two lines (lhw-5,6) and not different in the third (lhw-4, Figure S8). In both treatments, shallow roots are younger and tended to be thicker, while deep roots are older and thinner (Figure S8). Since anatomical features tend to scale with root thickness (Hazman & Brown, 2018; Kadam et al., 2017), we would expect there to be more, larger vessels in the shallow roots and fewer, smaller vessels in the deep roots, but the mutant lines showed the opposite phenotype.

With LHW disrupted, there were differences in xylem number in shallow and deep roots in response to drought that we would predict to be adaptive. Drought was imposed in such a way that more moisture was available at depth (Figure S10). Fewer smaller vessels in shallow roots would reduce axial hydraulic conductance to conserve water in the drying soil, keep the growing root tip more hydrated, and prevent vessel cavitation, while in deep roots, more and larger metaxylem vessels would be able to transport more water from the deep wet soil to the shoots. The LHW candidate gene may closely regulate metaxylem vessel number and area and prevent plasticity in response to stress, so disruption of this gene may be beneficial for root hydraulic responses to drought.

**FIGURE 5** OSH6 knockout affects root anatomical phenotypes in mutants in the T₁ and T₂ generations. Root anatomical phenotypes of independent mutant lines carrying alleles listed in Figure S6, are compared to the wild-type (WT) control. Significance levels (p < .1 yellow, 0.05 green, .01 blue, .001 orange) are shown for pairwise t tests between mutants and wild type within treatments.
OsARF15 (OsETTN1) was expected to influence metaxylem vessel size and number in rice root tissue. Orthologous ARF family members in Arabidopsis are drought response regulators (Matsui et al., 2014), and auxin and ARFs are known participants in root organ development, including vascular tissue differentiation (Aloni et al., 2006; Guilfoyle & Hagen, 2007). Our mutants were likely to have disrupted AUX/IAA binding domains (Figure S5). Since ARF15 is predicted to be an auxin response repressor (Wang et al., 2007), we expected knockout mutants to constitutively repress specific ARF-response genes (Tiwari et al., 2003) leading to decreases in metaxylem vessel size and/or number. The ARF15 mutants generally showed greater median metaxylem vessel area in well-watered conditions (Figure 4). In two of the three lines (arf15-5,6), metaxylem vessel number was less in the well-watered treatment; i.e., mutants had fewer but larger metaxylem vessels compared to wild type. There were few phenotypic differences under drought stress, so no conclusions can be made about their adaptive value under that condition.

Metaxylem phenotypes in ARF15 mutants were generally less consistent than those of the other mutants, which could be due to the redundant function of many ARF family members in rice. For example, OsARF2 has very high sequence similarity with OsARF15 (Wang et al., 2007; Figure S2), so knockout of both OsARF15 and OsARF2 may be necessary to see clear phenotypic effects.

Of the candidate genes tested, mutation of OSH6 had the greatest effect on xylem phenotypes and was the only one to consistently and significantly affect overall plant growth. Shoot biomass, tiller number, crown root number, and root depth were all greater than wild type in the well-watered treatment (Figure 7), indicating that the occurrence of fewer and smaller metaxylem vessels in mutants was not simply due to these anatomical phenotypes scaling with plant size. The effects of OSH6 mutants on plant growth are consistent with the occurrence of this gene within a root fresh weight QTL (Li et al., 2005). It is possible that OSH6 influences overall growth as well as the specific root metaxylem phenotypes that we measured via its likely effect on hormone signaling. Orthologous KNOX genes in Arabidopsis and maize have been implicated in controlling ratios of cytokinin to gibberellin in meristematic tissue and influencing polar auxin transport (Hay & Tsiantis, 2010).
Mature metaxylem vessels are composed almost exclusively of rigid secondary cell wall tissue, so genes that affect secondary cell wall formation may also impact xylem morphology. Arabidopsis and popular orthologs of OSH6, KNAT6, and KNAT2/6b, respectively, are negative regulators of secondary cell-wall deposition (Li et al., 2012; Qin et al., 2020; Zhao et al., 2020), but the function of KNAT6 orthologs in monocot roots has not been investigated. Differential regulation of secondary cell wall tissue via mutation of OSH6 may affect metaxylem vessel size or number in rice roots, and we might expect that xylem vessels in knockout mutants would be collapsed and deformed or have thicker walls. The OSH6 mutants had fewer metaxylem vessels in the well-watered treatment, whether or not the number was scaled to cross-sectional area (Figures 5 and 6). Vessels did not appear collapsed or deformed in any images of nodal root tissues, as we might have expected from disruption of secondary cell wall formation. There were also no observable differences in vessel thickness or fluorescence associated with lignin deposition in root cross-sectional images taken with laser ablation tomography. The disruption in the regulation of secondary cell wall formation may have negatively influenced the formation of metaxylem vessels in these mutants. The size of individual vessels was usually not significantly affected by the OSH6 mutants when scaled to RXSA, and when not scaled, vessels were sometimes larger than wild type (Figure S9).

Smaller metaxylem vessels, resulting in reduced axial hydraulic conductance, has been shown to improve drought tolerance in other crops such as wheat (Richards & Passioura, 1989) and maize (Klein et al., 2020), so we hypothesized that rice mutants with fewer or smaller vessels would benefit under drought. While some mutants displayed differences in metaxylem traits compared to drought-treated wild-type plants, we were unable to detect significant differences in shoot biomass between mutants and wild type under drought, though there was a trend toward greater biomass in drought-treated OSH6 mutant plants (Figure 7) that may have revealed a significant effect with greater replication.

Root traits are often quantitatively controlled by many genes (Gowda et al., 2011), so it is not surprising that disruption of only one
Gene would produce small alterations to the phenotype. CRISPR-Cas9 mutagenesis will be most successful for genes that have a large effect, which may not exist for complex traits such as xylem size and number. Further work is needed to identify and validate large-effect drought tolerance genes to expedite breeding efforts for more drought tolerant rice varieties.

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CONFLICT OF INTEREST
The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTIONS
J.E.R. and K.M.B. conceived and designed the research with assistance from Y.Y.; J.E.R. performed the experiments with technical assistance from M.W.; J.E.R. and K.M.B. analyzed data; M.W. and Y.Y. contributed to data interpretation; J.E.R. and K.M.B. wrote the manuscript with input from M.W. and Y.Y.

REFERENCES
Aloni, R., Aloni, E., Langhans, M., & Ulrich, C. I. (2006). Role of cytokinin and auxin in shaping root architecture: Regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. *Annals of Botany*, 97, 883–893. https://doi.org/10.1093/aob/mcl027

Belles-Boix, E., Hamant, O., Witiak, S. M., Morin, H., Traas, J., & Pautot, V. (2006). KNAT6: An Arabidopsis homeobox gene involved in meristem activity and organ separation. *The Plant Cell*, 18, 1900–1907.

Berleth, T., Mattsson, J., & Hardtke, C. S. (2000). Vascular continuity and auxin signals. *Trends in Plant Science*, 5, 387–393. https://doi.org/10.1016/S1360-1385(00)01725-8

Bishop, A., Helf, H., El-Shewk, S., Weijers, D., Scheres, B., Friml, J., Benkova, E., Mähönen, A. P., & Helariutta, Y. (2011). A mutually inhibitory interaction between auxin and cytokinin specifies vascular pattern in roots. *Current Biology*, 21, 917–926. https://doi.org/10.1016/j.cub.2011.04.017

Bollhöner, B., Prestele, J., & Tuominen, H. (2012). Xylem cell death: Emerging understanding of regulation and function. *Journal of Experimental Botany*, 63, 1081–1094. https://doi.org/10.1093/jxb/err438

Carlsbecker, A., Lee, J.-Y., Roberts, C. J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M. A., Vatén, A., Thitamadee, S., Campilho, A., Sebastian, J., Bowman, J. L., Helariutta, Y., & Benfey, P. N. (2010). Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature*, 465, 316–321. https://doi.org/10.1038/nature08977

ORCID
Jenna E. Reeger https://orcid.org/0000-0002-9292-8433
Yinong Yang https://orcid.org/0000-0002-2698-1754
Kathleen M. Brown https://orcid.org/0000-0002-4960-5292

FIGURE 8 Number of deep roots is affected in OSH6 knockout mutants in the T1 and T2 generations. Root anatomical phenotypes of independent mutant lines carry alleles listed in Figure S6 and are compared to wild type (WT). Significance levels (p < .1 yellow, .05 green, .01 blue, .001 orange) are shown for pairwise t tests between mutants and wild type within treatments.
system. Plant Physiology, 153, 906–914. https://doi.org/10.1104/pp.110.154013
Yamaguchi, M., Kubo, M., Fukuda, H., & Demura, T. (2008). Vascular-related NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. The Plant Journal, 55, 652–664. https://doi.org/10.1111/j.1365-313X.2008.03533.x
Yamaguchi, M., Mitsuda, N., Ohtani, M., Ohme-Takagi, M., Kato, K., & Demura, T. (2011). VASCULAR-RELATED NAC-DOMAIN 7 directly regulates the expression of a broad range of genes for xylem vessel formation. The Plant Journal, 66, 579–590.
Zhang, J., Zhang, S., Cheng, M., Jiang, H., Zhang, X., Peng, C., Lu, X., Zhang, M., & Jin, J. (2018). Effect of drought on agronomic traits of rice and wheat: A meta-analysis. International Journal of Environmental Research and Public Health, 15, 1-14. https://doi.org/10.3390/ijerph15050839
Zhang, J., Zheng, H. G., Aarti, A., Pantuwan, G., Nguyen, T. T., Tripathy, J. N., Sarial, A. K., Robin, S., Babu, R. C., Nguyen, B. D., Sarkarung, S., Blum, A., & Nguyen, H. T. (2001). Locating genomic regions associated with components of drought resistance in rice: Comparative mapping within and across species. Theoretical and Applied Genetics, 103, 19–29. https://doi.org/10.1007/s001220000534
Zhao, M., Yang, S., Chen, C. Y., Li, C., Shan, W., Lu, W., Cui, Y., Liu, X., & Wu, K. (2015). Arabidopsis BREVIPEDICELLUS interacts with the SWI2/SNF2 chromatin remodeling ATPase BRAHMA to regulate KNAT2 and KNAT6 expression in control of inflorescence architecture. PLoS Genetics, 11, 1–21. https://doi.org/10.1371/journal.pgen.1005125
Zhao, Y., Song, X., Zhou, H., Wei, K., Jiang, C., Wang, J., Cao, Y., Tang, F., Zhao, S., & Lu, M. Z. (2020). KNAT2/6b, a class I KNOX gene, impedes xylem differentiation by regulating NAC domain transcription factors in poplar. New Phytologist, 225, 1531–1544.
Zhong, R., Lee, C., Zhou, J., McCarthy, R. L., & Ye, Z. H. (2008). A battery of transcription factors involved in the regulation of secondary cell wall biosynthesis in Arabidopsis. The Plant Cell, 20, 2763–2782.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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