A Maize Glutaredoxin Gene, Abphyll2, Regulates Shoot Meristem Size and Phyllotaxy

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Phyllotaxy describes the geometric arrangement of leaves and is important for plant productivity. Auxin is well known to regulate phyllotactic patterns via PIN1-dependent auxin polar transport, and studies of maize (Zea mays) aberrant phyllotaxy1 (abph1) mutants suggest the importance of auxin and cytokinin signaling for control of phyllotaxy. However, whether additional regulators control these patterns is poorly understood. Here, we report a new dominant maize mutant, Aberrant phyllotaxy2 (Abph2), in which the shoot meristems are enlarged and the phyllotactic pattern switches from alternate to decussate. Map-based cloning revealed that the Abph2 mutation was caused by transposition of a glutaredoxin gene, MALE STERILE CONVERTED ANther1 (MSCA1), which gained an altered expression pattern in Abph2 mutant embryos. msca1 loss-of-function mutants have reduced meristem size and revealed a novel function of glutaredoxins in meristem growth. In addition, MSCA1 interacts with a TGA transcription factor, FASCIATED EAR4, suggesting a novel regulatory module for regulating shoot meristem size.

INTRODUCTION

In the plant kingdom, all shoot organs are generated from stem cells sitting at the summit of the shoot apical meristem (SAM). Therefore, the activity and growth of the SAM determines the overall architecture of the plant. SAM size is dynamically controlled by complex and overlapping signaling networks, including the CLAVATA (CLV)-WUSCHEL (WUS) negative feedback loop, KNOX pathways, and in part through their effects on hormone signaling (Ha et al., 2010). In the CLV-WUS feedback loop, WUS expression in the organizing center promotes CLV3 expression in the stem cells (Mayer et al., 1998). CLV3, as a secreted peptide, is perceived by CLV1, CLV2, and RPK2/TOADSTOOL receptors, promoting CLV signaling, which in turn represses WUS expression (Brand et al., 2000; Clark et al., 1995, 1997; Schoof et al., 2000; Kinoshiba et al., 2010). This model explains the observations that clv mutants have enlarged SAMs as a consequence of ectopic WUS expression in more apical and lateral cells, and the stem cells in wus mutants are not maintained (Laux et al., 1996; Mayer et al., 1998; Fletcher et al., 1999). The KNOX genes (e.g., SHOOTMERISTEM-LESS in Arabidopsis thaliana and KNOTTED1 in maize [Zea mays]) are predominantly expressed throughout the SAM, except in incipient leaf primordia (Jackson et al., 1994; Long et al., 1996). Mutations in KNOX genes lead to smaller or terminated meristems (Long et al., 1996; Kerstetter et al., 1997; Vollbrecht et al., 2000), demonstrating their positive roles in meristem growth. KNOX genes promote the expression of cytokinin biosynthetic genes, leading to high cytokinin level in the SAM, which may in turn activate KNOX genes, forming an apparent positive feedback loop (Jasinski et al., 2005; Yanai et al., 2005; Kurakawa et al., 2007).

SAM growth also determines the diverse phyllotactic patterns found in different plant species. One of the prevalent phyllotaxies is alternate, with one leaf per node offset in opposite directions, as typically seen in monocots, such as maize, rice (Oryza sativa), and sorghum (Sorghum bicolor). In opposite and decussate phyllotaxy, two leaves form in pairs and subsequent leaf pairs are diverged by 90°. Other common phyllotaxies are spiral (as in Arabidopsis) and whorled. The establishment of these fascinating patterns has drawn attention of biologists and mathematicians for centuries. Auxin has been extensively studied as an important regulator of phyllotactic patternning and primordium initiation (Reinhardt et al., 2000, 2003; Benková et al., 2003; Kuhlmeier, 2007). High concentrations of auxin within the SAM are associated with regions of leaf initiation, whereas inhibition of PINFORMED1 (PIN1)-mediated polar auxin transport leads to naked “pin-shaped” SAMs and/or altered phyllotaxy (Okada et al., 1991; Scanlon, 2003). Studies of abph1 mutants in maize revealed the importance of an additional plant hormone, cytokinin, in regulating phyllotaxy, in part through its crosstalk with auxin (Jackson and Hake, 1999; Giuliani et al., 2004; Lee et al., 2009). ABPH1 encodes an A-type response regulator, which negatively regulates cytokinin signaling. Mutations in ABPH1 cause a striking change of normal alternate phyllotaxy to decussate and an enlarged SAM. SAM size to a certain degree affects...
phyllotactic patterns; however, it is not known whether other factors contribute to the mechanism of phyllotaxy establishment.

Glutaredoxins (GRXs) are small ubiquitous glutathione-dependent oxidoreductases, which modulate the activities of their target proteins by catalyzing the reduction of disulfide bonds in the presence of NADPH and glutathione reductase (Fernandes and Holmgren, 2004). Based on the conserved active site sequences, plant GRXs are divided into CPYC-, CGFC-, and CC-type classes. Plant GRX genes function in stress and pathogen responses (Nidamukong et al., 2007; Cheng, 2008; Guo et al., 2010) as well as jasmonic acid, gibberellic acid, and auxin signaling (Hou et al., 2008; Cheng et al., 2011; Zander et al., 2012). In Arabidopsis, two related CC-type GRX genes, ROXY1 and 2, were found to function in flower development (Xing et al., 2005, 2006; Xing and Zachgo, 2008). Loss-of-function mutation in ROXY1 leads to petal defects with normal fertility, and double roxy1 roxy2 mutants are male sterile and do not produce pollen, showing redundant functions in anther development. Related genes also control another development in grasses, as MALE STERILE CONVERTED ANther1 (MSCA1) in maize and MICROSPORELESS1 (MIL1) in rice, encode close homologs of ROXY (Hong et al., 2012; Kelliher and Walbot, 2012). ROXY proteins interact with TGA class bZIP transcription factors and are thought to posttranslationally modify their activities (Li et al., 2009, 2011; Murmu et al., 2010). For example, TGA9/10 and PERIANTHIA (PAN) are proposed to be modified by ROXY1 and 2 in the process of anther and petal development, respectively, in Arabidopsis. However, it is unknown whether GRXs function in other developmental processes.

Here, we characterize Abph2 and reveal a novel function of GRXs in shoot meristem size regulation. A dominant mutation, Abph2, leads to an increase in SAM size and a switch to decussate phyllotaxy, whereas loss of function causes a reduction in SAM size. The Abph2 mutation was caused by transposition of the MSCA1 GRX gene. We also show interactions between MSCA1 and FASCIATED EAR4 (FEA4), the maize ortholog of PAN, revealing a new regulatory module for control of meristem size and phyllotaxy.

RESULTS

Abph2 Mutants Develop Decussate Phyllotaxy and Enlarged Meristems

The dominant Abph2 mutant was generated by ionizing radiation in an unknown inbred genetic background. The mutants displayed an abnormal phyllotact pattern, by producing two leaves per node (decussate) starting at the 4th to 5th leaf stage, instead of one leaf per node (alternate) in the wild type (Figure 1A), and developed two opposite ears at a single node (Figure 1B). Both heterozygous and homozygous plants had decussate phenotypes, indicating that Abph2 was a dominant mutation. To understand the cause of Abph2 phenotypes, we visualized SAMs by scanning electron microscopy. Wild-type maize meristems develop one leaf primordium per node on the opposite side to the previous one, in an alternate phyllotaxy (Figure 1C). However, Abph2 plants produced two leaf primordia at the same time, exactly opposite to each other at each node, forming decussate phyllotaxy (Figure 1D). This change in phyllotaxy appeared to happen before leaf primordia were formed, as shown by two opposite PIN1-YFP (yellow fluorescent protein) maxima in Abph2 mutant meristems, compared with one maximum in the wild type (Figures 1E and 1F). Abph2 mutants also had dramatically enlarged SAMs; for example, in embryos 26 d after pollination (DAP), meristem width in Abph2 (185 ± 5 μm, n = 10) was nearly 2-fold that of the wild type (96 ± 12 μm, n = 17, P < 0.0001) (Figure 1J).

We introgressed Abph2 into various inbred lines, and the decussate phenotype was maintained in B73 and A619, whereas it became weaker in W22, W23, and Mo17 backgrounds. Interestingly, Abph2 showed stronger expressivity in A619, including twin shoot production (Figure 1G), as a result of SAM splitting (Figure 1H). The enlarged meristem state also continued after the reproductive transition, sometimes leading to a fasciated tassel apex (Figure 1I). In summary, a dominant mutation in Abph2 leads to meristem enlargement and altered phyllotaxy.

Abph2 Encodes a GRX

To map the Abph2 locus, we generated mapping populations with a total of ~730 mutant individuals, by crossing Abph2 to A619, B73, or W23 inbreds, followed by backcrossing. Using publicly available simple sequence repeat markers (http://www.maizegdb.org) and self-designed markers, we mapped Abph2 to a region of ~20 kb near the top of chromosome 7, with four predicted genes (Figure 2A). These genes were annotated as (a) uncharacterized, (b) ring-zinc finger protein, (c) tetratricopeptide repeat protein, and (d) CRINKLY4-like. However, direct sequencing and expression level comparisons of these genes in Abph2 mutants to those in wild-type siblings did not reveal any significant changes. We therefore constructed and screened a BAC library from Abph2 mutants in its original background using probes located in the 20-kb mapping interval. A positive BAC covering the entire interval was cloned and sequenced. Our analysis did not reveal any mutations in coding regions of the four annotated genes on this BAC, but we found a 4.5-kb fragment inserted within the 3’ untranslated region (UTR) of the uncharacterized gene (a) (Figure 2A, red triangle) that was not present at this position in the maize B73 reference genome. This 4.5-kb fragment contained the complete coding region of a GRX gene, as well as ~2.8-kb 5’ and ~1.2-kb 3’ sequences (Figure 2B; Supplemental Data Set 1). The GRX coding sequence was identical to a gene in the B73 reference genome that was located ~800 kb distal to the Abph2 locus (Figure 2A). This distal gene corresponded to the MSCA1 locus (Albertsen et al., 2011). Comparison of Abph2 and MSCA1 sequences showed that ~1.0 kb of the 5’ sequences and ~1.2 kb of the 3’ sequences were also highly conserved, with some nucleotide polymorphisms. However, the sequences (~1.8 kb) further 5’ within the 4.5-kb Abph2 insertion were not found in the B73 reference sequence but were related to transposons not present in the MSCA1 locus (Figure 2B). In summary, the Abph2 locus contains a transposed copy of the MSCA1 GRX gene, including its partial upstream and downstream sequences.

To establish if the transposed MSCA1 gene was responsible for the Abph2 phenotype, we performed a targeted EMS screen to “knock out” the dominant Abph2 allele. We pollinated wild-type ears with EMS-treated Abph2 pollen and scored the M1 progeny. Most of them were heterozygous for Abph2 and showed the
decussate phenotype. However, we also recovered putative knockouts with wild-type phenotypes, at a frequency of \( \frac{1}{1000} \). We sequenced the \( \text{Abph2} \) locus in these putative knockouts and found two different point mutations in different lines (Figure 2C). These mutations led to changes in two conserved amino acids, V65M and C75T, located in the \( \beta \)-sheet 1 region and \( \alpha \)-helix 1 region within the active site, respectively (Li et al., 2009). Both regions are conserved and are important for GRX protein functions (Rouhier et al., 2002; D’Ambrosio et al., 2003). These independent knockout alleles indicate that the transposed \( \text{MSCA1} \) gene was necessary for the \( \text{Abph2} \) phenotype.

We next asked if the transposed 4.5-kb region was sufficient to confer the \( \text{Abph2} \) phenotype. We fused the YFP in frame at the N terminus of the predicted \( \text{Abph2} \) GRX coding region and transformed this construct into wild-type maize. Among 13 independent transgenic events derived from embryogenic callus, the majority did not form organized shoots and did not survive, due to enlarged and disorganized SAMs (Supplemental Figures 1A and 1B). However, two events produced primary shoots with enlarged meristems (Figure 3B), and independent seedlings from these events developed decussate phyllotaxy (Figure 3C), which phenocopied \( \text{Abph2} \). In contrast, control transgenic plants harboring a nonrelated transgene had normal, alternate phyllotaxy (Figure 3A). We detected the YFP-tagged ABPH2 protein in nuclei of young leaf primordia, but not in the central region of the meristem (Figure 3D). We did not observe YFP-MSCA1 expression in the P0, which may be due to the limited number of samples available for imaging; however, we did observe YFP-MSCA1 expression in the P0 (Supplemental Figure 1C). In summary, a transgene containing the transposed \( \text{MSCA1} \) gene from the \( \text{Abph2} \) locus was sufficient to cause the enlarged meristem and decussate phyllotaxy phenotypes. Together with the results from the EMS revertants, our observations confirm that the transposed \( \text{MSCA1} \) GRX gene caused the \( \text{Abph2} \) phenotype. We refer to this locus as \( \text{Abph2} \), and not as an allele of \( \text{MSCA1} \), because it is at a different genomic locus and has a modified expression pattern (see below).

Altered Embryonic Expression of \( \text{Abph2} \) Correlates with the Enlarged Meristem Phenotype

We initially hypothesized that the additional transposed copy of the \( \text{MSCA1} \) gene in \( \text{Abph2} \) mutants might be the reason for the dominant mutant phenotype, for example, by increasing its expression. To test this, we crossed \( \text{Abph2} \) with \( \text{msca1-ref} \) mutants, which have

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a deletion of the MSCA1 locus. The F1 progeny had decussate phyllotaxy phenotypes, like Abph2 mutants, suggesting that a simple change in MSCA1 copy number is not the cause of the Abph2 phenotype. We also found that F2 populations segregating for msca1 and Abph2 had the expected number of abnormal phyllotaxy plants, again suggesting that Abph2 phenotypes were not due to a simple effect of gene dosage on gene expression. We obtained a few recombinants between Abph2 and msca1 (the two genes are closely linked) and found that Abph2 msca1 double mutants were decussate and male sterile, indicating that Abph2 could not complement the male sterile defect of msca1. Presumably this was a reflection of its altered expression pattern.

We next compared the expression pattern of MSCA1/Abph2 in the wild type and Abph2 mutants by in situ hybridization. MSCA1 and Abph2 have identical coding sequences, so the in situ hybridizations detected both transcripts. In wild-type seedlings, MSCA1 was expressed in incipient leaf primordia (P0; solid arrowhead, Figure 4A), marking the region where a new leaf will initiate, as well as in leaf vasculature. This pattern was largely unaltered in Abph2 mutants at this stage, except two incipient primordia were labeled, consistent with the decussate phyllotaxy (Figure 4B). RT-PCR and quantitative RT-PCR (qRT-PCR) analyses using shoot apices of 14-d-old seedlings did not reveal a significant change of MSCA1/Abph2 expression level between the wild type and Abph2 mutants (Supplemental Figure 2). We next investigated MSCA1 expression during embryo development because the Abph2 phenotype arises at the 4th to 5th leaf stage, and in maize, these leaves are initiated in the embryo. MSCA1/Abph2 expression was not detected in 10 DAP embryos but was first detected at -14 DAP, when approximately three leaf primordia had been initiated (Figure 4C). At this stage, no difference was observed in transcript accumulation patterns in Abph2 mutants (Figure 4D), which is consistent with a lack of phenotype at this stage. However, at ~18 DAP, when approximately four leaves had been initiated, MSCA1/Abph2 expression was more broadly distributed across the entire SAM apex of Abph2 mutants (Figure 4F), whereas it was still restricted to the incipient leaf primordium in the wild type (Figure 4E). This difference persisted in 30 DAP SAMs (Figures 4G and 4H). These observations strongly suggest that the broader expression of MSCA1/Abph2 in the Abph2 embryo is responsible for the enlarged SAM and phyllotaxy alterations.

Loss-of-function mutations in MSCA1 and its homologs in rice and Arabidopsis lead to male sterility and abnormal floral organs, demonstrating a conserved function of these CC-type GRX genes in flower development and fertility across monocots and dicots (Chaubal et al., 2003; Xing et al., 2005; Hong et al., 2012; Kelliher and Walbot, 2012). Consistent with this, we also found MSCA1

**Figure 2.** Map-Based Cloning of Abph2.

(A) Map representation of the Abph2 locus on chromosome 7. The numbers show the recombinants out of 730 F1 BC1 individuals. The transposed 4.5-kb fragment harboring Abph2 is shown by a red triangle. The MSCA1 gene located ~800 bp distal to the Abph2 locus is shown in green.

(B) Comparison of Abph2 and MSCA1 gene models. In the 4.5-kb transposed fragment, 1038 bp 5’ of the coding region, and 1209 bp 3’ are almost identical to MSCA1, but the upstream 1790 bp region is unique (shown in blue). Red lines represent nucleotide polymorphisms between the two genes.

(C) Part of the Abph2 sequence, aligned with homologs from Arabidopsis (ROXY1 and 2), sorghum (Sb02g003040), and rice (MIL1, Os07g05630). Two independent mutations leading to conserved amino acid changes found in EMS revertants are shown in red. The asterisks represent identical amino acids among the homologs.

**Figure 3.** Phenotypes of YFP-ABPH2 Transgenic Plants.

(A) A dissected control shoot tip showing normal alternate phyllotaxy, indicated by arrowheads.

(B) A dissected YFP-ABPH2 transgenic shoot, showing enlarged SAM (asterisk) and two leaf primordia (arrowheads) forming at the same time.

(C) A mature plant harboring YFP-ABPH2 transgene developed decussate phyllotaxy. The colored dots represent paired leaves from a single node.

(D) A confocal image showing nuclear localization of YFP-ABPH2 in young leaf primordia. Bar = 100 μm.
found a significant decrease in SAM size compared with segregating wild-type siblings (136.5 ± 8.3 μm, compared with 145.3 ± 7.8 μm; P < 0.001; Figures 5A to 5C). These data indicate that MSCA1 has a normal function to positively regulate SAM size. The decrease in SAM size of msca1-ref mutants was not dramatic, and this was likely due to a functional redundancy. Sequence analysis discovered two close homologs, GRMZM2G480903 and GRMZM2G470756. In situ hybridization using a gene-specific probe revealed that one of these two homologs had a very similar expression pattern to MSCA1, suggesting it has a similar function (Figures 5D and 5E; Supplemental Data Set 2).

Interaction between MSCA1 and a TGA Transcription Factor, FEA4

MSCA1 homologs in Arabidopsis, ROXY1 and 2, interact with TGA proteins, including TGA9/10 and PAN, and may be involved in posttranslational modification of their activities during flower development (Li et al., 2009; Murmu et al., 2010). We recently cloned a maize gene, FEA4, which encodes a TGA transcription factor, orthologous to PAN (Pautler et al., 2015). Loss-of-function mutations in FEA4 lead to strong fasciation in ear and tassel primordia, as well as enlarged vegetative shoot meristems, and altered phyllotaxy in certain inbred backgrounds, similar to Abph2. It is therefore tempting to hypothesize that FEA4 is a target of MSCA1 in the process of meristem size control. To test this hypothesis, we investigated the molecular interaction between MSCA1 and FEA4. By yeast two-hybrid assay, MSCA1 was found to interact with FEA4 (Figure 6A). Next, using a split YFP approach (Schütze et al., 2009), this interaction was confirmed in planta by coinfiltrating YN-MSCA1 and YC-FEA4 (Figure 6B). Strong YFP fluorescence, indicating an interaction, was found in nuclei. In contrast, YFP signal was not detected in a negative control, in which YN-MSCA1 and YC-ASYMMETRIC LEAVES1 (Byrne et al., 2000) were coinfiltrated (Figure 6C).

To search for molecular evidence to support the interaction between MSCA1 and FEA4, we performed in situ hybridization to ask whether their transcripts were colocalized in the SAM. We hybridized alternate sections using MSCA1 or FEA4 antisense probes. FEA4 expression domains were largely complementary to MSCA1 in the vegetative SAM (Figures 6D and 6E). However, regions of overlap were found, for example, below the P₀ domain in the SAM (Figures 6F and 6G), indicating that their physical interaction is likely to be biologically relevant.

Next, we constructed msca1 fea4 double mutants, to provide genetic support for their interaction. In an F2 segregating family, the SAM size of msca1-ref fea4 double mutants (177.1 ± 9.7 μm) was not significantly different from fea4 single mutants (186.4 ± 17.3 μm), though both classes had significantly larger meristems than wild-type siblings (163.2 ± 8.2 μm; in both cases, P < 0.0001) (Figure 6H). These results indicate that fea4 is epistatic to msca1 with respect to SAM size and support the idea that they interact in a pathway. We also analyzed Abph2 fea4 double mutants. As shown in Figure 6I, the double mutants showed a significant increase in meristem size (255.4 ± 43.7 μm) compared with single Abph2 (172.9 ± 16.0 μm, P < 0.05) or fea4 (147.1 ± 4.3 μm, P < 0.01) mutants, each of which showed a significantly enlarged meristem compared with wild-type siblings (131.1 ± 6.2 μm, P < 0.005 in Abph2 and P < 0.001 in fea4). The double mutants often produced leaves in a very irregular phyllotaxy and had dramatically

expression in anther primordia (Supplemental Figures 3A and 3B), in addition to its expression in the SAM.

MSCA1 Controls Shoot Meristem Size

Our embryo expression analyses suggested that misexpression of the transposed MSCA1 gene at the Abph2 locus was sufficient to cause defects in meristem size and phyllotaxy. To determine if MSCA1 was required for normal SAM development, we analyzed loss-of-function msca1-ref mutants. These mutants had normal phyllotaxy in the B73, W23, and Mo17 backgrounds; however, we found a significant decrease in SAM size compared with segregating wild-type siblings (136.5 ± 8.3 μm, compared with 145.3 ± 7.8 μm; P < 0.001; Figures 5A to 5C). These data indicate that MSCA1 has a normal function to positively regulate SAM size. The decrease in SAM size of msca1-ref mutants was not dramatic, and this was likely due to a functional redundancy. Sequence analysis discovered two close homologs, GRMZM2G480903 and GRMZM2G470756. In situ hybridization using a gene-specific probe revealed that one of these two homologs had a very similar expression pattern to MSCA1, suggesting it has a similar function (Figures 5D and 5E; Supplemental Data Set 2).

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Figure 4. MSCA1/Abph2 Is Misexpressed in Abph2 Mutant Embryos.

(A) and (B) During seedling stages, MSCA1/Abph2 was expressed in the P₀ domain of the SAM (solid arrowhead) and leaf vasculature (open arrowhead) in the wild type (A) and showed a similar expression pattern in two P₀ domains in Abph2 mutants (B).

(C) and (D) During embryonic stages, MSCA1/Abph2 was first detected in the P₀ domain (arrowhead) at 14 DAP in wild type, and was similar in Abph2 mutants (arrowhead points to P₀ domain).

(E) to (H) Starting from 18 DAP (E) and (F) and until 30 DAP (G) and (H), MSCA1/Abph2 expression spread across the entire SAM (caret) and also in P₀ domains (solid arrowheads) of Abph2 embryos (F) and (H), in comparison to the localized expression of MSCA1 in P₀ domains of wild-type embryos (arrowheads in (E) and (G)).

Bars = 100 μm.
reduced height (Figure 6J). These observations indicate that Abph2 is functional in the absence of FEA4, suggesting that it has additional targets. These presumably come under its regulation because of the expanded expression of Abph2 during embryo development.

**DISCUSSION**

Abph2 is a dominant mutant that develops an enlarged SAM and decussate phyllotaxy. In some genetic backgrounds, the phenotype is even more extreme, presumably due to the presence of modifiers, and extreme meristem enlargement leads to bifurcation or fasciation. Our map-based cloning revealed that Abph2 encodes a GRX gene. Plant GRXs function in defense against various stresses, hormone signaling, and flower development (Ndamukong et al., 2007; Cheng, 2008; Xing and Zachgo, 2008; Guo et al., 2010; Cheng et al., 2011; Zander et al., 2012). Remarkably, we found that Abph2 arose by a transposition of MSCA1, and this rearrangement was presumably caused by the ionizing radiation that induced the Abph2 mutation. MSCA1 shows high similarity to Arabidopsis ROXY1 and 2 (Ndamukong et al., 2007; Zander et al., 2012) and rice MIL1 (Cheng et al., 2011). These homologs function in floral organ development and fertility, demonstrating a conserved function across monocots and dicots. Here, however, we report that MSCA1 regulates meristem size and phyllotaxy. The enlarged SAM in the dominant Abph2 mutant, as well as the decreased SAM size in the loss-of-function msca1 mutant, demonstrates that MSCA1 normally functions to positively regulate meristem size. The modest reduction in SAM size in msca1 mutants can perhaps be explained by redundancy with two close homologs, and this idea is supported by the similar expression pattern of at least one of them. Our results suggest a conserved role for MSCA1 in anther and shoot meristem development, which might reflect a common ontogeny of these organs (Dawe and Freeling, 1990). It will be of interest to test whether MSCA1 function in the meristem responds to redox signals, as has been suggested for its anther function (Kelliher and Walbot, 2012).

Abph2 and MSCA1 share the same coding sequences and show polymorphism only in their noncoding regions, suggesting the two genes vary at the level of regulation. This was indeed the case, since MSCA1/Abph2 transcripts were detected throughout...
the shoot meristem in Abph2 mutant embryos, in comparison to a restricted expression domain of MSCA1 in the P0 of wild-type embryos. This was the only expression difference we observed in Abph2 mutants and started from the 4th to 5th leaf stage, corresponding to the stage at which the decussate phenotypes were first observed. These observations strongly suggest that the altered expression is responsible for the altered phyllotaxy arising in Abph2 mutants. Surprisingly, MSCA1/Abph2 expression during seedling development was indistinguishable between Abph2 and the wild type, except that it is expressed in both of the P0 domains in Abph2 mutants, as expected. Therefore, the altered expression conditioned by the different regulatory sequences around the Abph2 locus appears to be transient, occurring only in the embryo, and once the altered phyllotaxy is established, it may be maintained by mechanisms acting independently of MSCA1/Abph2 expression. To sum up, we hypothesize that the decussate leaf pattern of Abph2 is a consequence of a meristem size increase caused by Abph2 misexpression in the embryo, and once established, this altered phyllotaxy is maintained by an unknown mechanism.

GRXs are small redox enzymes that perform reversible thiol-disulfide exchange reactions between their active site cysteines and cysteines of their disulfide substrates, via a dithiol or monothiol mechanism (Fernandes and Holmgren, 2004). The dithiol mechanism depends on a conserved CXXC motif in the active site and can reduce both protein disulfides and GSH mixed disulfides. Monothiol GRXs typically containing a CXXS motif in the active site can only reduce GSH mixed disulfides (Vlamis-Gardikas and Holmgren, 2002; Lemaire, 2004). MSCA1 and ABPH2 contain
a CCMC motif in the active site, and the fact that a point mutation in the C-terminal cysteine of this motif fully abolishes the dominant phenotype of Abph2 suggests that this cysteine is critical for ABPH2 to exert its enzymatic function. This finding suggests that MSCA1 and ABPH2 reduce disulfides via a dithiol mechanism, in contrast to ROXY1, which may operate as a monothiol reductant (Xing et al., 2005). ROXY proteins in Arabidopsis interact with TGA transcription factors, including TGA8/10 and PAN (Xing and Zachgo, 2008; Li et al., 2009; Murmu et al., 2010), and are thought to posttranslationally modify their activities. We found that FE4A, the maize ortholog of PAN (Pautler et al., 2015), interacts with MSCA1, suggesting that it might be a target of MSCA1. Furthermore, overlapping expression domains of the FE4A and MSCA1 transcripts below the P0 provides the possibility for protein interaction and modification in vivo. Our genetic analysis provides further biological support for the significance of their physical interaction. First, msca1 and fea4 have opposite meristem size phenotypes, suggesting that FE4A activity is negatively regulated by MSCA1. Second, the SAM size of msca1-ref fea4 double mutants was not significantly different from fea4 single mutants, indicating that fea4 is epistatic to msca1; this is consistent with the hypothesis that these genes function in the same pathway, with FE4A acting downstream of MSCA1. Together with their physical interaction, our data suggest that FE4A activity is negatively regulated by MSCA1 and could define a novel pathway regulating meristem size, with the potential to control phyllotactic pattern. In contrast to the epistatic relationship of msca1-ref fea4 double mutants, Abph2 fea4 double mutants showed additive meristem size and phyllotaxy defects, suggesting that there are additional targets of Abph2, which come under its influence from its expanded expression domain in the embryo. These additional targets may act in parallel to FE4A.

The way in which MSCA1/Abph2 and FE4A regulate meristem size appears distinct from other characterized mechanisms. Notably, MSCA1 and FE4A are expressed in the meristem periphery and in leaf primordia, in contrast to CLV, WUS, and Knox factors that are exclusively or also expressed in the central region. Presumably, signals from the meristem periphery feed back to affect meristem development by a non-cell-autonomous signaling mechanism. How MSCA1/Abph2 regulates phyllotaxy also appears to be different, when compared with Abph1 (Lee et al., 2009) and other regulators of phyllotaxy defined in Arabidopsis (Pinon et al., 2013). abph1 mutants have drastically reduced PIN1 expression in the P0 domain, and this is one possible explanation for their change in phyllotaxy (Lee et al., 2009). However, Abph2 mutants appeared to have normal PIN1 expression levels in their two P0 domains, suggesting distinct mechanisms are at play.

In summary, our results reveal a distinct mechanism for the regulation of meristem size and one that can lead to altered phyllotaxy when perturbed. MSCA1 is a redox regulator, in a class that is proposed to post transcriptionally regulate transcript factor activities through catalytic modification of disulfide bridges and/or dimerization (discussed in Li et al., 2009). It will be of interest to characterize how MSCA1 modifies its putative target proteins and to establish whether it regulates meristem development by responding to redox signals in vivo.

**METHODS**

**Plant Growth and Molecular Mapping**

The msca1-ref seeds were kindly provided by Tim Fox, DuPont-Pioneer. Plants were grown in controlled greenhouse conditions at temperatures of 25 to 28°C under 16-h light conditions or in the field. For map-based cloning, three different F1 BC1 populations were created by crossing the Abph2 mutant with B73, A619, and W23, respectively, followed by backcrossing to the inbred and fine mapping using 730 F2 individuals (Bortili et al., 2008). To obtain revertants, Abph2 homozygous pollen was treated with EMS and pollinated onto A619 ears in a targeted EMS mutagenesis approach (Freele, and Walbot, 1994). In total ~10,000 M1 plants were screened. The putative revertants were self-pollinated, and plants homozygous for Abph2 revertants were confirmed to have a wild-type phyllotaxy and were subjected to sequencing.

**Meristem Measurements**

For vegetative shoot meristem measurements, ~2-mm longitudinal slices were cut from 2-week-old seedlings and fixed in ice-cold FAA (10% formalin, 45% ethanol, and 5% acetic acid) overnight, dehydrated through an ethanol series from 50 to 100% ethanol, and then passed into 50% (1:1 ratio for ethanol and methyl salicylate) and 100% methyl salicylate for 1 h each change. The cleared meristems were observed using Nomarski optics. The meristem size was determined by the width at the position just above the most recently initiated leaf primordium. Embryonic meristems for size measurement were dissected from ears at 26 DAP and fixed as above. Measuring and quantification were done using ImageJ. The measurements were repeated using at least three biological replicates.

**BAC Library Screening**

Young leaves from etiolated Abph2 plants were harvested. High molecular weight DNA was isolated according to Luo and Wing (2003), with some modifications. The resulting agarose-embedded high molecular weight DNA was partially digested with HinIII and size-selected twice on clamped homogeneous electrical field gels. DNA fragments, ranging from 150 to 250 kb, were electro-eluted and ligated into linearized and dephosphorylated pCC1BAC vector (Epicter) and electroporated into Epi3000 Escherichia coli cells (Epicerter) according to the manufacturer’s recommendations. A total of 73,728 recombinant colonies were picked automatically using a Qpix XT system. Positive colonies were picked manually using a Qpix XT system, grown individually, and gridded at high density on nylon filters. Clones were screened and identified by hybridization using immunological detection of digoxigenin-labeled PCR probes (Roche). Probe synthesis, wash, block, and detection were performed following the manufacturer’s recommendations. Primers to amplify the probes are listed in Supplemental Table 1. Positives BACs were retrieved and confirmed by PCR and sequenced.

**Transgenic Plants**

The YFP-Abph2 transgene was constructed by amplifying fragments of the entire 4.5-kb insertion from the positive BAC and fusing the YFP tag in-frame at the N terminus of the coding sequence (Gallavotti et al., 2008). All fragments were amplified using Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) and cloned into the ptF101 Gateway-compatible maize (Zea mays) binary vector using the MultiSite Gateway Three-Fragment System (Invitrogen). A sequence confirmed clone was transferred to Agrobacterium tumefaciens EHA101 and transformed into maize, as described previously (Mohanty et al., 2009). Primers used for YFP-Abph2 cloning are listed in Supplemental Table 1.

**Microscopy**

SAM images were taken under a Nikon SMZ1500 microscope to manually capture Z series, which were then merged by NIS elements to create
focused images. Scanning electron microscopy was performed on freshly dissected apices using a Hitachi S-3500N scanning electron microscope, as described (Taguchi-Shiobara et al., 2001). Confocal images were obtained from freshly dissected apices using a Carl Zeiss LSM 710 confocal microscope, using a 514-nm laser excitation and 520- to 560-nm emission for detection of the YFP.

In Situ Hybridization
Sample preparations and in situ hybridizations were performed as described previously (Greb et al., 2003) with 8% polyvinyl alcohol (molecular weight of 70,000 to 100,000; Sigma) added in the detection buffer. Tissues of the wild type and Abph2 mutants at similar developmental stages were collected and processed in parallel. The MSC1 probe included the entire coding sequences (516 bp) as well as 214-bp 3'UTR sequences. The probe against the MSC1 homolog GRMZM2G480035 used 350 bp of the 3' UTR sequences to avoid cross hybridization. These sequences were cloned into TOPO PCR II dual promoter system (Invitrogen), and full-length cDNA sequences of FEA4 were cloned into pDrive vector (Qiagen). The plasmids were linearized before the probe transcription.

Phylogenetic Tree
Full-length protein sequences were aligned using ClustalW 2.0 software (Larkin et al., 2007). The alignment file was then used to generate an unrooted tree with MEGA 5.0 (Tamura et al., 2011), applying the Bootstrap method and 1000 bootstrap replications.

Split YFP Assay
The split YFP assay was performed as described previously (Li et al., 2009). Full-length coding sequences of Abph2 and FEA4 were fused with N-YFP and C-YFP, respectively. The primers used to make the constructs are listed in Supplemental Table 1.

Yeast Two-Hybrid Assay
Full-length coding sequences of FEA4 and Abph2 were fused with GAL4-BD and GAL4-AD domains, respectively, in vectors pGBK7 and pGADT7 (Clontech). The primers used to make these constructs are listed in Supplemental Table 1. The yeast strain AH109 (Clontech) was co-transformed with appropriate GAL4 fusions as described previously (James et al., 1996).

RT-PCR Analysis
Total RNAs from fresh shoot apices of Abph2 and wild-type siblings were extracted and subjected for cDNA synthesis (Invitrogen) and RT- or qRT-PCR using standard methods and the primers listed in Supplemental Table 1. The amplification products were visualized by ethidium bromide for RT-PCR and by SYBR Green for qRT-PCR.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AY910752 (ROXY1), EU332351 (ROXY2), AK118286 (PAN), JN417767 (MIL1), and HJT17551.1 (MSC1).

Supplemental Data
Supplemental Figure 1. YFP-Abph2 Transgenic Plants.
Supplemental Figure 2. Expression Levels of MSC1/Abph2 in Vegetative Apices.

Supplemental Figure 3. MSC1 Expression in Anther Primordia.
Supplemental Table 1. Primers Used to Make the Constructs in This Study.
Supplemental Data Set 1. Abph2 Sequence.
Supplemental Data Set 2. Alignments Used to Generate the Phylogeny Presented in Figure 5D.

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AUTHOR CONTRIBUTIONS
F.Y., R.J., B.L., and D.J. designed the experiments. F.Y., H.T.B., R.J., B.L., and A.K. performed the experiments. V.L. and H.S. performed the BAC library screening. F.Y., M.P., H.T.B., R.J., B.L., and D.J. analyzed the data. F.Y. and D.J. wrote the article.

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A Maize Glutaredoxin Gene, \textit{Abphyl2}, Regulates Shoot Meristem Size and Phyllotaxy
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