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Article Addendum

Genetic interactions between the *miRNA164-CUC2* regulatory module and *BREVIPEDICELLUS* in Arabidopsis developmental patterning

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The proper regulation of enlargement and patterning of plant lateral organs is essential for plant functionality. In an earlier work, we characterized the role of a microRNA (miRNA)-transcription factor regulatory module, *miRNA164-CUC2,* in the enlargement and patterning of multiple lateral organs in Arabidopsis. This regulatory module genetically interacts with another transcription factor, *CRC*, in fruit development patterning. Here, we characterize the genetic interaction of this module with a homeodomain transcription factor, *BREVIPEDICELLUS (BP),* that has been shown to play roles in leaf development patterning.

Plant development is an intricate process. Multiple lateral organs are generated that carry out specialized functions. These lateral organs must be properly patterned and correctly sized relative to the entire plant to fulfill their functions. Intensive work over the last several decades has made significant progress in uncovering players that function in these processes. However, our understanding of the regulatory mechanisms that direct these complex developmental processes are far from complete.

In a screen for lateral organ development mutants, we uncovered a central regulatory module that functions as a positive regulator of lateral organ enlargement and patterning.[1] This regulatory module consists of a NAC-domain transcription factor, *CUC2*, and its known targeting miRNA, *miRNA164*. The module was isolated as a single point mutation in the miRNA target site of *CUC2* from an Arabidopsis mutant screen. As predicted, this mutation results in a dominant allele of *CUC2*, and thus was named *cuc2-1D*. The *cuc2-1D* allele enabled us to uncover and characterize a previously unknown role this module plays in regulating the size of plant lateral organs. A disruption of miRNA targeting of *CUC2*, due to the mutant miRNA target site, results in overaccumulation of *CUC2* transcript and enlarged vegetative and reproductive lateral organs. This increased size is due to a disruption of a cell proliferation checkpoint; cellular proliferation continues beyond its usual developmental timeframe in expanding lateral organs, resulting in larger organs.

In addition to the role of *miRNA164* and *CUC2* as a regulatory module in lateral organ enlargement, this module also regulates aspects of both vegetative and floral lateral organ patterning. Silique patterning is disrupted, resulting in reduced seedset. Leaf patterning is also disrupted; the leaves display a lobed leaf margin. Work using a triple loss-of-function mutant line of all three *miRNA164* loci and transgenic *cuc2* alleles with mutant miRNA target sites have also shown similar patterning phenotypes.[2,3] Recent work has also uncovered a role for NAC-domain transcription factors in the regulation of leaflet formation in species having compound leaves.[4,5] In our earlier work, we characterized a genetic interaction with another transcription factor, *CRC* in flower developmental patterning. Here we report on the genetic interaction with another transcription shown to play a role in leaf patterning.

Several of the class 1 *KNOTTED*-like members from *Arabidopsis thaliana* (*KNAT*) homeodomain proteins have been shown to play roles in shoot apical meristem maintenance and organ patterning and development. *BP* (*KNAT1*) has been shown to play a role in multiple developmental processes. *BP* was first described as a genetic marker in Arabidopsis,[6] and was later cloned as *KNAT1*.[7] Loss of function alleles of *bp* show several flowering phenotypes including downward pointing siliques.[7] In addition, overexpression of *BP* in Arabidopsis results in deeply lobed leaves, similar to an overexpression phenotype observed in maize.[8] Other *KNAT* members *SHOOT-MERISTEMLESS (STM)* and *KNAT6* play important roles in plant development and genetically interact with *CUC* genes.[9-11] Furthermore, during flowering, *BP* plays a role in restricting *KNAT6* and *KNAT2* expression to properly guide floral development.[12] The transcription factor, *ASYMMETRIC LEAVES1* and gradients in the hormone auxin repress *BP* and thus promote

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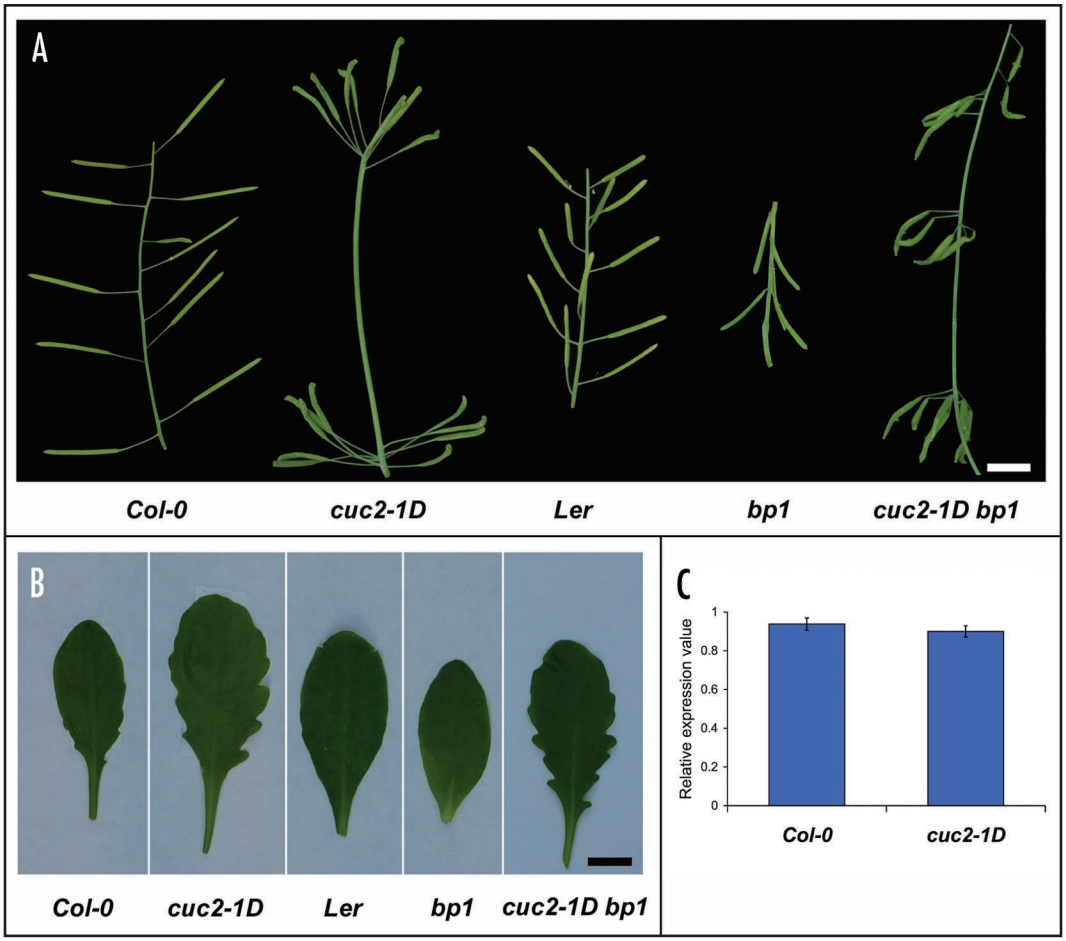

Figure 1. Genetic interactions between *miRNA164-CUC2* and *BP*. (A) *cuc2-1D* displays a clustered silique spacing on the flowering stalk relative to the background Col-0. *bp1* displays downward orientated siliques on the flowering stalk relative to the background Ler. In the double mutant, *cuc2-1D bp1*, the plants display an additive phenotype with clustered and downward orientated siliques. (B) *cuc2-1D* displays a lobed leaf phenotype relative to Col-0, while *bp1* has a smooth leaf margin. In the double mutant, *cuc2-1D bp1*, the lobed leaf phenotype is retained. (C) qPCR was used to track *BP* transcript levels in *cuc2-1D* plants. No significant differences were seen in *cuc2-1D* relative to the background Col-0. The average and SE of three biological replicates are shown. qPCR was completed as in (Larue et al. 2009) except that the following primers were used for *BP*: forward 5'-CTA ACA AGG CCC ATT CAG GA-3' and reverse 5'-TGT CAC TCT TCC CAT CAG GA-3'. Scale bars in (A and B): 1 cm.

leaf fate.[13] In the gain of function mutant *jagged lateral organs-D, BP* and *STM* were upregulated and bulk auxin transport reduced resulting in strongly lobed leaves.[14] *BP* is also regulated by the *BEL1-LIKE HOMEODOMAIN (BLH)* members *SAWTOOTH1 (BLH2/SAW1)* and *SAWTOOTH2 (BLH4/SAW2)*.[15] In loss of function *saw1 saw2* double mutants, *BP* is ectopically expressed and the leaves display a serrated margin. Thus, *BP* clearly plays a role in the development of lobed leaves and genetically interacts with multiple other transcription factors. Additionally, since *CUC* genes have been shown to interact with other *KNAT* members, we investigated the genetic interactions between the *miRNA164-CUC2* regulatory module and *BP* during leaf development.

To investigate the genetic interactions between the *miRNA164-CUC2* regulatory module and *BP*, we characterized the segregating F2 population from a cross using *cuc2-1D* and *bp1*. *bp1* is a loss of function mutant in a Landsberg *erecta* (L*er*) background.[6,7] However, *er* loss of function mutants where observed to suppress both the leaf enlargement and leaf lobation phenotype in *cuc2-1D*.[1] Therefore, we selected for plants in which *ER* was present at least as a heterozygote. In this population, we isolated plants which were homozygous *cuc2-1D* and *bp1*. In the flowering stalks, these double mutant plants displayed an additive phenotype; the silique patterning was disrupted and the arrangement along the inflorescence stalk was clustered as in *cuc2-1D* (Fig. 1A). The siliques were also downward orientated as in *bp1*. The leaves displayed the typical lobed margin found in *cuc2-1D* single mutants (Fig. 1B). These data suggest *miRNA164-CUC2* and *BP* either act independently of each other or *miRNA164-CUC2* acts downstream of *BP*.

It is possible that *cuc2-1D* increases the expression or results in misexpression of *BP*, resulting in a lobed leaf phenotype. However, in the *bp1* loss of function line, other unknown factors may still be modulated by *cuc2-1D* and these could be sufficient for the lobed leaf phenotype. Therefore as a further check, we also determined if *cuc2-1D* modulates the expression of *BP* using quantitative reverse transcription polymerase chain reaction (qPCR). However, there

was no significant change in *BP* expression levels of *cuc2-1D* relative to Col-0 plants (Fig. 1C). Additionally, RNA in situ analysis did not detect any misexpression of *BP* in developing leaves of *cuc2-1D* (data not shown). This suggests the *miRNA164-CUC2* regulatory module does not directly modulate *BP* expression to affect the lobed leaf phenotype.

In conclusion, we propose two possible genetic interactions between *miRNA164-CUC2* and *BP*. The first is these two important developmental regulators act independently. This would be unique from that shown with some of the genetic interactions with *CUC2* and other *KNAT* members in Arabidopsis.[9-11] In this case, *cuc2-1D* must act through alternative unknown players to direct leaf lobation. The second possible interaction is that *miRNA164-CUC2* acts downstream of *BP*. Thus, *cuc2-1D* bypasses the need for *BP* in the leaf lobation phenotype. If this were true, we would expect a modulation of the downward orientated silique phenotype found in *bp1*. Although we observed no change of this phenotype in the double mutant, we can not preclude that the silique orientation phenotype and the leaf phenotype are independent with respect to *miRNA164-CUC2*. Thus, much additional work remains to fully characterize the role of *miRNA164-CUC2* in leaf development. Additional mutant screening in a *cuc2-1D* background will be helpful in allowing us to begin to uncover additional players in these important developmental processes. Future work to dissect the complex regulatory pathways that direct plant developmental patterning and enlargement will likely yield more critical pieces in this puzzle.

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