Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness

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In plants, members of microRNA (miRNA) families are often predicted to target the same or overlapping sets of genes. It has thus been hypothesized that these miRNAs may act in a functionally redundant manner. This hypothesis is tested here by studying the effects of elimination of all three members of the MIR164 family from Arabidopsis. It was found that a loss of miR164 activity leads to a severe disruption of shoot development, in contrast to the effect of mutation in any single MIR164 gene. This indicates that these miRNAs are indeed functionally redundant. Differences in the expression patterns of the individual MIR164 genes imply, however, that redundancy among them is not complete, and that these miRNAs show functional specialization. Furthermore, the results of molecular and genetic analyses of miR164-mediated target regulation indicate that miR164 miRNAs function to control the transcript levels, as well as the expression patterns, of their targets, suggesting that they might contribute to developmental robustness. For two of the miR164 targets, namely CUP-SHAPED COTYLEDON1 (CUC1) and CUC2, we provide evidence for their involvement in the regulation of growth and show that their derepression in miR164 loss-of-function mutants is likely to account for most of the mutant phenotype.

KEY WORDS: MicroRNA, miR164, Organogenesis, Developmental robustness, Phyllotaxis, CUP-SHAPED COTYLEDON

INTRODUCTION

MicroRNAs (miRNAs) are short (~21 nucleotide) non-translated RNAs that are generated by the enzymatic processing of stem-loop regions of longer precursor RNAs (Bartel, 2004; Valencia-Sanchez et al., 2006; Vaucheret, 2006). miRNAs are present in both plants and animals, and they regulate gene expression in a sequence-specific manner by targeting mRNAs for cleavage or translational repression. Animal miRNAs typically act by inhibiting translation of their targets, with which they usually share relatively low sequence complementarity (Kim et al., 2005). As a result, a given animal miRNA might target many different genes, and a large fraction of the animal transcriptome has been proposed to be directly influenced by miRNA control (Farh et al., 2005; Stark et al., 2005). By contrast, plant miRNAs use cleavage as the preferential mechanism for target gene regulation, they tend to exhibit a high degree of complementarity to their targets, and appear to have fewer target genes per miRNA (Llave et al., 2002; Schwab et al., 2005). The existence of perfect or nearly perfect complementarity between plant miRNAs and mRNAs has greatly facilitated the identification of putative targets for many of the characterized miRNAs (Rhoades et al., 2002). In Arabidopsis, 165 miRNA loci have been identified so far, which are grouped into 93 different miRNA families (Lu et al., 2006; Rajagopalan et al., 2006; Reinhart et al., 2002) (miRBase, release 9.0, http://microrna.sanger.ac.uk/). At least 70 of these miRNA loci, comprising 21 gene families, have been predicted or demonstrated to target genes encoding transcription factors. This prevalence of regulatory genes points to a central role for miRNAs in the control of gene regulatory networks in plants. Consistent with this notion, it has been shown that miRNAs are required in many developmental processes in plants, including organ polarity determination, meristem function, floral patterning, vascular development, lateral root development and hormone response (Baulcombe, 2004; Chen, 2005; Mallory and Vaucheret, 2006). The participation of miRNAs in these processes has been established primarily through their overexpression, or by generating plants that express miRNA-resistant versions of their target gene(s). By contrast, mutants have been isolated for only a few miRNAs (Aukerman and Sakai, 2003; Baker et al., 2005; Guo et al., 2005; Kim et al., 2005; Palatnik et al., 2003; Williams et al., 2005), and thus far only a single loss-of-function mutant for a plant miRNA has been identified in a forward genetic screen: the Arabidopsis early extra petals1 mutant (eep1), in which miR164a function is disrupted (Baker et al., 2005).

The scarcity of identified loss-of-function mutants and phenotypes might be attributed to the fact that many miRNAs belong to multigene families, which are predicted to target the same (or overlapping) sets of genes, opening the possibility of substantial functional redundancy among miRNAs in plants. Although for Caenorhabditis elegans it has been shown that some members of the let-7 family can have redundant functions (Abbott et al., 2005), evidence for redundancy among plant miRNAs has only been circumstantial. For example, loss of a single miRNA of a multigene family did not result in an aberrant phenotype in tissues where the miRNA was expressed (Mallory et al., 2004).

The Arabidopsis MIR164 family comprises three members (miR164a, miR164b and miR164c) and negatively regulates, through miRNA cleavage, several genes that encode NAC-like transcription factors (Baker et al., 2005; Guo et al., 2005; Kasschau et al., 2003; Laufs et al., 2004; Mallory et al., 2004; Park et al., 2002). These genes include CUP-SHAPED COTYLEDON1 (CUC1) and CUC2, which are expressed in, and are necessary for, the formation of boundaries between meristems and emerging organ primordia (Aida et al., 1999; Heisler et al., 2005; Takada et al., 2001). Failure to establish organ boundaries leads to severe developmental consequences, and in loss-of-function cuc1 cuc2 double-mutant
seedslings the two cotyledons fail to separate and the seedling meristem arrests (Aida et al., 1997). Expression of miRNA cleavage-resistant versions of CUC1 and CUC2 in Arabidopsis has revealed that miR164-mediated repression of CUC1 and CUC2 is necessary for proper control of organ number (Baker et al., 2005; Mallory et al., 2004) and for organ boundary formation (Laufs et al., 2004). Analysis of eep1 mutants has shown that miR164c functions to prevent extra petals in early-arising flowers by repressing CUC1 and CUC2 (Baker et al., 2005). The role of the MIR164 family is not limited to flower development, however, as both miR164a and miR164b have been reported to prevent lateral root initiation by repressing the miR164 target NAC1 (Guo et al., 2005). In addition, ectopic expression of miR164-resistant versions of CUC1 and CUC2, respectively, was shown to lead to abnormal vegetative development (Laufs et al., 2004; Mallory et al., 2004; Nikovics et al., 2006). Taken together, these results suggest that miR164 miRNAs may act throughout plant development.

Here, we report on the elimination of the activity of the entire MIR164 family, and its consequences for development, demonstrating that all miR164 miRNAs function redundantly during Arabidopsis shoot development, and uncovering new functions for these genes, including the regulation of phyllostaxy (the arrangement of organs along the stem) and developmental robustness.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana plants were grown as described previously (Baker et al., 2005). miR164a-d (this study) (Nikovics et al., 2006) and miR164b-1 (Baker et al., 2005; Mallory et al., 2004) were isolated in the accession Columbia (Col-0). eep1, here referred to as mir164c-1, was isolated in Landsberg erecta (Ler). Although miR164c-1 mutants transcribe reduced amounts of pri-miR164c, they fail to effectively repress CUC1 and CUC2 function and thus may represent functionally null mutants (Baker et al., 2005). The miR164c-2 allele (Col-0 background) represents a d5pm transposon insertion (d5pM_3.25571) 85 bp 3’ of the mature miR164c miRNA. Disruption of the predicted pre-miR164c stem loop in this allele does not cause any obvious mutant phenotype. In addition, miR164a-b-1 c-2 triply homozygous mutant plants fail to show any increase in severity of their phenotype as compared with the respective single mutants (data not shown). The miR164a-4 allele, isolated from the GABI-Kat collection (Rosso et al., 2003), was crossed to plants doubly homozygous for mir164b-1 and mir164c-1 (Baker et al., 2005). Progeny of three independent crosses (n=246) were genotyped at all three MIR164 loci (see below). Twelve plants were homozygous for all three mir164 mutant alleles. mir164a-4 b-1 c-1 triply homozygous plants always showed a novel phenotype, referred to as the mir164abc mutant phenotype. Floral organ counts were performed on 15 mir164abc triple mutants and 15 mir164abcAbbcC’ wild-type control plants, in both cases of a mixed Ler/Col background. The mir164abc triple mutant was backcrossed once to Col-0 and to Ler wild-type plants to assess potential contributions of either background to the phenotype.

Plasmid constructs

The pCUC1::CUC1-GFP and pCUC1::CUC1m-GFP constructs have been described previously (Baker et al., 2005). pCUC2::CUC2-GFP was cloned by recombining DNA fragments essentially as described for pCUC2::CUC2-VENUS-N7 (Heisler et al., 2005), except that mGFP5 was used as an alternative green fluorescent protein (GFP) instead of VENUS-PS389. The promoter fragment was introduced into the NcoI site of plantid plasmid pPD35 (Heisler et al., 2005) and the resulting plasmid was sequenced for the correct orientation of the insert. pCUC2::VENUS-N7 has been previously described (Heisler et al., 2005). 1.4 kb EcoRI-Sfl fragment that contained the endogenous CUC1 regulatory sequences (Baker et al., 2005) was blunt-ended using T4 DNA polymerase; this promoter fragment was subsequently introduced into the Smal site of plantid plasmid pPD35 (Heisler et al., 2005) (kindly provided by Dr P. Das) and the resulting plasmid was tested for the correct orientation of the insert. The Ndel-XhoI digested PCR fragment was cloned into the corresponding restriction sites of pPD35 to generate pMIR164a::3XVENUS-N7. The fragment corresponding to 2584 bp of miR164a 5’ upstream sequence was cloned analogously with primers PS240/PS241 and with XhoI and BamHI to generate pMIR164b::3XVENUS-N7, pMIR164c::3XVENUS-N7 was made by cutting pMIR164c::GUS (Baker et al., 2005) with XhoI and BamHI and ligating into the pPD35 vector. The NoI cassette from each pPD35 subclone was shuttled into the pMILBAR (Eshed et al., 1999) binary vector as previously described (Baker et al., 2005).

All plasmid constructs were sequenced in order to detect potential PCR-introduced point mutations and subsequently transformed into plants by Agrobacterium-mediated floral dip infiltration (Clough and Bent, 1998).

Microscopy

Protocols for light microscopy (LM) and SEM were as previously described (Baker et al., 2005). Confocal laser scanning microscopy (CLSM) imaging of live plants was performed using a ZEISS LSM 510 Meta using either a 63× 0.95 W or a 40× 0.05 W Achromat water objective as described (Heisler et al., 2005; Reddy et al., 2004). FM4-64 dye (Molecular Probes) was used as a plasma membrane marker. Specimens of the VENUS-PS397 and FM4-64 combination were excited with an argon laser that was attenuated to 10% at 514 nm. Single tracking line-scale mode was used in combination with a NFT 635 VIS main dichroic short-pass filter. Each scan represents the mean of two scans. The emission was split by a 545 nm secondary dichroic filter and sent through a 530-600 nm band pass filter for detection of VENUS, and a 650 nm long-pass filter for FM4-64 signal, respectively. A single tracking line-scale was used for GFP/FM4-64 co-visualization. GFP/FM4-64 specimens were excited using the 488 nm laser line together with a NFT 635 VIS main dichroic short-pass filter in combination with a 545 nm secondary dichroic to split the emission. GFP and FM4-64 were detected using a 505-530 nm band pass and a 650 nm long-pass filter, respectively.

Genotyping

Presence of the mir164a-d T-DNA insertion was confirmed by PCR amplification across the junction between the left border of the T-DNA and the genomic DNA by using the primer pair PS321/PS322 (Table 1). The PCR product was sequenced with primer PS323 to confirm the presence of the genomic DNA by using the primer combination PS272/PS273 (Table 1). All plasmid constructs were sequenced in order to detect potential PCR-introduced point mutations and subsequently transformed into plants by Agrobacterium-mediated floral dip infiltration (Clough and Bent, 1998).

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<td>PS240</td>
<td>5’-CCGGCTCAGGAGGAACGTGTTAGTGATG-3’</td>
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<tr>
<td>PS241</td>
<td>5’-CGGAGATCTCTGCGCATCACACCTTCC-3’</td>
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<tr>
<td>PS272</td>
<td>5’-CCCCATTGACGTTAGCTTAGAC-3’</td>
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<tr>
<td>PS273</td>
<td>5’-GGGAATTCTAGAGCTGACCCCTTCTATGG-3’</td>
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<tr>
<td>PS278</td>
<td>5’-AGGATTGTTGAAAATTAGGGCAGA-3’</td>
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<tr>
<td>PS279</td>
<td>5’-CCATTGGAGCTGAACTGAGCAC-3’</td>
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<tr>
<td>PS283</td>
<td>5’-ATTTGACATCATCCTACTTGC-3’</td>
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<tr>
<td>PS285</td>
<td>5’-TACGAATAAGGACGTCATTACCTAGAGTA-3’</td>
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<td>PS286</td>
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<td>5’-AAGACCGTTTGAGACTCCTG-3’</td>
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Gene expression analysis

RNA blot analysis was performed essentially as described (Chen, 2004; Reinhart et al., 2002; Williams et al., 2005) with the following modifications. RNA (15 μg) enriched for small RNAs was isolated from Arabidopsis plants using the mirVana RNA Isolation Kit (Ambion, cat. #1560) in combination with Plant RNA Isolation Aid (Ambion, cat. #9690) according to the manufacturer's instructions. Blots were hybridized using a [γ-32P]-ATP end-labeled locked nucleic acid ath-MIR164a oligonucleotide (Exiqon A/S, Denmark, cat. #30024). A mixture of 0.1 μM PS388/PS389 oligonucleotides were used as 20- and 30-nt size standards, respectively. As a loading control, blots were stripped and re-probed with a [γ-32P]-ATP end-labeled DNA oligonucleotide (5'TTCGCGAGGGCAATCTTCGCGCAGG-3') complementary to U6 RNA (Mallory et al., 2004). ImageJ 1.34s software (http://rsb.info.nih.gov/ij) was used for quantification of mir164. Quantitative reverse transcriptase-mediated polymerase chain reaction (qRT-PCR) analyses were performed as described (Baker et al., 2005). Transcript levels were normalized to the level of the non-target gene TUB4 (At5g44340). The primer pairs used to detect transcripts of TUB4, CUC1, CUC2, NAC1, At5g07560 and At5g61430 were described previously (Mallory et al., 2004). Primer pair PS396/PS397 (Table 1) was used to detect At5g3910. RNA in situ hybridization analyses for CUC1 and CUC2 were performed as described (Baker et al., 2005). For the miRNA in situ hybridizations, we followed the protocol of Valoczi and co-workers as originally described (Valoczi et al., 2006). The locked nucleic acid (LNA) miRNA oligos were end-labelled using the DIG Oligonucleotide 3'-End Labelling Kit, 2nd Generation, from Roche (cat. #03 353 575). A total of five washes were performed at 50°C with 2× SSC-50% formamide. An RNaseA digest was included after the third wash to remove non-specific background signal. Standard blocking and washing steps in combination with anti-DIG antibody (Roche) were used for the immunological detection (Long and Barton, 1998). Western Blue Reagent (Promega) in combination with levamisole was used for the detection reaction. Slides were mounted in Glycerol in TE.

RESULTS

Analysis of mir164abc triple-mutant plants reveals functional redundancy among MIR164 miRNAs

To assess the potential for functional redundancy among the three members of the MIR164 family of miRNAs, we constructed triple-mutant plants carrying loss-of-function mutations in MIR164a, MIR164b, and MIR164c. A mutant allele for MIR164a was isolated from the GABI-Kat collection of transferred DNA (T-DNA) insertion lines (Rosso et al., 2003). This allele was named mir164a-4, as three other mir164a alleles had been described previously (Guo et al., 2005). mir164a-4 is likely to represent a null allele because the T-DNA is inserted 29 bp 3' of the last nucleotide of the processed mir164a sequence and thus disrupts the predicted stem-loop structure of the mir164a precursor, which is essential for miRNA biogenesis (see Fig. S1 in the supplementary material) (Nikovics et al., 2006; Parizotto et al., 2004). Plants homozygous for mir164a-4 have been reported to show deepened serration of the leaf margins (see Fig. S2 in the supplementary material) (Nikovics et al., 2006). Shoots of the previously described mir164b-1 mutant also displayed an essentially normal phenotype (Mallory et al., 2004), whereas mir164c-1 mutant plants formed extra petals in early-arching flowers, but otherwise largely resembled wild-type plants (Baker et al., 2005). We used these single-mutant lines to generate plants homozygous for mir164a-4, mir164b-1 and mir164c-1 (henceforth referred to as mir164abc triple mutants). The results of RNA blot analyses revealed that in mir164abc triple mutants, mir164-type 21 nt miRNAs are severely reduced in abundance, if not completely abolished, compared with wild-type plants (Fig. 1A). mir164abc triple mutants were indistinguishable from wild-type plants during vegetative development, except for rosette leaves that appeared slightly more serrated in the triple mutant (see Fig. S2 in the supplementary material) (Nikovics et al., 2006). Severe phenotypic alterations were observed, however, after the switch from vegetative to reproductive development. Arabidopsis wild-type flowers are composed of an almost invariant number of floral organs, with four sepals, four petals, six stamens, and two fused carpels arranged in four concentric circles or whorls (Fig. 1B). By contrast, most flowers of the mir164abc triple mutant had an increased number of sepals and petals, but slightly fewer stamens than Ler wild-type and mir164aAbBcC (Ler/Col) control plants (Fig. 1C-G, and data not shown). Organ numbers in mir164abc triple mutants were highly variable, which is reflected in relatively large standard deviations of the organ counts for flowers at different positions along the stem (Fig. 1F,G). In addition to the organ-number defects, individual organs varied in size and carpels typically failed to fuse in the triple mutants, resulting in a severe reduction in fertility (Fig. 1C-E).

Thus, loss of mir164a and mir164b function substantially enhances the floral defects of mir164c plants, as floral organs are affected in all four whorls and in all flowers independent of their time of initiation (Fig. 1E-G). These miRNAs might therefore control flower development in a redundant manner.

Control of phyllotaxis by mir164 miRNAs

In addition to the flower defects described above, the phyllotaxis of mir164abc triple-mutant plants was severely disrupted (Fig. 2, and see Fig. S3 in the supplementary material). In wild-type plants, flower primordia are successively initiated on the flanks of the inflorescence meristem, so that an incipient primordium is initiated in a position that is furthest away from the two preceding primordia (Reinhart, 2005). This leads to a spiral arrangement, in which developing flowers are positioned both radially and vertically at regular intervals along the stem (Fig. 2E). By contrast, mir164abc triple-mutant plants displayed a highly unequal and distorted arrangement of flowers, both with respect to the angle formed by two consecutive flowers and their distance from each other along the axis of the stem (Fig. 2C-D). The average internode distance (the distance between two flowers) of the mixed Ler/Col wild-type control was 8.7±3.6 mm (s.d., n=149), and 8.6±8.6 mm (s.d., n=150) for mir164abc triple-mutant plants (Fig. 2K). Thus, the mean internode length was almost unaffected in the mir164abc mutant. However, whereas in the wild-type control 87% (129/149) of all flowers remained within the standard deviation of the mean value for the internode distance of the wild type (categories 5 mm to 12 mm), this was true for only 32% (48/150) of mir164abc triple-mutant flowers. Notably, in mir164abc mutants, 35% of all flowers were separated by a distance of 1 mm or less, compared with fewer than 2% (2/149) in the control (Fig. 2J,K).

The initial positioning of flower primordia, however, was normal in mir164abc triple mutants when compared with the wild type (Fig. 2A,B), indicating that the disruption of the phyllotaxis in mir164abc mutants occurs after flowers have been initiated. Thus, the positioning of flowers in Arabidopsis appears to be dependent not only on their initiation pattern at the shoot apex, but also on mechanisms that actively retain their initial arrangement during flower maturation and growth.
In order to identify the cause of the phyllotaxis defects in mir164abc mutants, stem segments were examined by scanning electron microscopy at a position basal to the meristem, where flowers are at an advanced stage of development. Whereas mature flowers were separated by uniformly elongated and rectangular epidermal cells in the wild type (Fig. 2F,H), cells between the clustered flowers of mir164abc triple-mutant plants appeared smaller and more variable in shape (Fig. 2G,I). In addition, successive mir164abc flowers were often radially separated by only five or six cells (Fig. 2I), which is similar to the number of cells found between two neighboring floral primordia at the time of initiation (Heisler et al., 2005; Reddy et al., 2004). This suggests that in mir164abc plants, cell division activities are repressed in the internodes that separate individual flowers.

**MIR164 precursors are expressed in partially overlapping domains**

In order to determine how the mir164abc mutant phenotype correlates with MIR164 expression, we analyzed the expression patterns for all three members of the MIR164 family. To enable the detection of expression patterns of individual family members, we used green fluorescent protein (GFP)-based transcriptional reporters. For the construction of the reporters we used the 5′ regulatory regions upstream of the individual miRNA precursor sequences that had been previously reported to be sufficient for compensating for a loss of the individual miRNA precursor sequences in a single nucleotide, different miR164 miRNAs cannot be distinguished on an RNA blot; thus, signals are derived from all three miR164 miRNAs. The experiment was repeated twice with the same result. The antisense MIR164 oligonucleotide probe hybridizes to two distinct RNA size classes, of 21 and ~24 nt, in agreement with previous reports (Dunoyer et al., 2004; Valoczi et al., 2006). It has been proposed that the 21 nt form of miR164 is the functional entity sufficient to guide target cleavage, for which the ~24 nt form, which has distinct requirements for its biogenesis, appears to be dispensable (Dunoyer et al., 2004).

**Fig. 1. Floral phenotype of mutant plants impaired in miR164 biogenesis.** (A) Quantification of miR164 abundance in mir164 mutants. RNA blot analysis of the small RNA fraction isolated from wild-type Col-0, mir164a-4, mir164b-1, mir164c-1, mir164c-2, and mir164a-4 b-1 c-1 triple-mutant inflorescences hybridized with probes complementary to miR164a (upper blot) and U6 small RNA (middle blot), respectively. The ethidium bromide-stained agarose gel is shown beneath (numbers indicate fold-change of miR164 accumulation with respect to Col-0 wt, which was set to 1). As miR164a and mir164b differ from mir164c in a single nucleotide, different miR164 miRNAs cannot be distinguished on an RNA blot; thus, signals are derived from all three miR164 miRNAs. The experiment was repeated twice with the same result. The antisense MIR164 oligonucleotide probe hybridizes to two distinct RNA size classes, of 21 and ~24 nt, in agreement with previous reports (Dunoyer et al., 2004; Valoczi et al., 2006). It has been proposed that the 21 nt form of miR164 is the functional entity sufficient to guide target cleavage, for which the ~24 nt form, which has distinct requirements for its biogenesis, appears to be dispensable (Dunoyer et al., 2004). (B-D) Results of SEM analysis. (B) Mature (stage 13) wild-type flower of accession Ler. Flower stages were defined according to Smyth et al. (Smyth et al., 1990). (C) Stage 12 and (D) stage 13 flowers of mir164abc triple-mutant plants show variable organ numbers and unfused carpels. Sepals have been removed for better visibility of the inner organs. Scale bars: 200 μm in B; 100 μm in C,D. Abbreviations: pe, petals; ca, carpels; st, stamens. (E) A mir164abc triple-mutant inflorescence. (F,G) Charts representing organ counts from mir164abc triple-mutant (black) and mir164aaAbBcC plants (gray), which served as the wild-type control to assess the potential influence of the mixed Ler/Col-0 background on the phenotypic changes. The average floral organ number (‘Organ count’) is plotted against each flower position along the stem (‘Flower’). Numbers indicate the position of the flower along the stem from the oldest (1) to the youngest (25). Error bars represent s.d. in (F) sepal and (G) petal number. Stamen number was reduced with respect to Col-0 and slightly reduced with respect to the wild-type control. Notably, variability in stamen number, but not in sepal and petal number, increased in the mixed Ler/Col-0 background, when compared with the Col-0 background (data not shown). Carpel number is only weakly affected in mir164abc mutants.
was detected in leaves (Nikovics et al., 2006), and it was observed in the boundaries between the inflorescence meristem and floral primordia, in young floral buds (stages 2–4; stages according to Smyth et al. (Smyth et al., 1990)), as well as in the adaxial domains of older flowers (Fig. 3A). Thus, the expression patterns of MIR164a and MIR164c are partially overlapping and are consistent with the regions of the plant affected in mir164abc triple-mutants. By contrast, GFP expression in the MIR164b reporter line appeared to be excluded from meristems and was strongest in abaxial epidermal cells of sepals (Fig. 3B). The RNA blot analysis (Fig. 1A) suggests that the miR164b locus contributes substantially to the overall population of miR164 RNA molecules in the inflorescence. However, it is unknown whether the three miR164 miRNAs are equally well processed, or how the efficiency of processing varies among cells, which may explain the apparent discrepancy between the results obtained in the RNA blot analysis and those obtained through the use of the transcriptional reporter. Alternatively, additional regulatory sequences that could affect the degree of miR164b accumulation may not have included in the reporter construct.

In addition to expression during the reproductive phase of development, we also detected GFP expression in the MIR164a and the MIR164c reporter lines in certain vegetative tissues (see Fig. S5 in the supplementary material). In summary, the results of our expression analyses suggest that the different members of the MIR164 family are expressed in distinct patterns during plant development, and that their expression patterns overlap only partially.

Mature miR164 miRNAs are essentially identical in sequence and are predicted to target the same set of transcripts. However, the availability of the triple mir164abc mutant, as well as of the various double mutants, allowed the use of in situ hybridization to infer characteristics of the expression patterns of the individual MIR164 family members. DIG-labeled LNA oligo probes were used to detect miR164 miRNA accumulation patterns on tissue sections of inflorescences. In Ler wild-type plants, combined signal of all three miR164 miRNAs was detected in vegetative leaves, in inflorescence meristems, in young flower primordia as well as in floral organ primordia. Strong signal was also detected in the locules of the anthers (Fig. 3E,F). The miR164 expression pattern in A. italiana thus resembled the pattern of miR164 expression in N. benthamiana (Valoczi et al., 2006). There was no signal above background in the Scramble-miR control (Fig. 3G,H). The miR164 expression pattern in A. italiana as well as in N. benthamiana was not detected in the miR164abc triple-mutant background (Fig. 3O,P). This signal might represent processed miR164 originating from leaky expression of one or more of the three mir164 mutant loci. Alternatively, the probe might hybridize to another RNA fragment, for instance to the ~24 nt band that was detected on the RNA blot (Fig. 1A), miR164c accumulation, as detected in mir164a-4 b-1 double-mutant plants (Fig. 3L), was found in the expected tissue but did not accumulate to levels significantly above the level of miR164 signal observed in mir164abc triple mutants. miR164b on the other hand, as detected in mir164a-4 c-1 double mutants, reached a level of expression that was comparable to miR164 accumulation in the wild type (Fig. 3K,L). Accumulation of miR164a, when examined in mir164b-1 c-1 double-mutant plants was comparable to the result obtained for miR164c, with the difference that the expression in leaves remained strong in mir164b-1 c-1 double mutants (Fig. 3M,N).
 Altogether, the in situ hybridization data are consistent with the results obtained from RNA blot experiments, which indicate that miR164b miRNAs contribute substantially to the miR164 miRNA pool in shoots.

**miR164 miRNAs regulate the abundance of all predicted target transcripts**

The miR164 miRNAs were all predicted previously to target six members of the transcription factor-encoding NAC family, including CUC1, CUC2 and NAC1 (Rhoades et al., 2002). Cleavage products of the target transcripts that are consistent with miR164 miRNA-dependent degradation were detected in wild-type plants (Guo et al., 2005; Laufs et al., 2003; Mallory et al., 2004). Analysis of mir164 single mutants showed that CUC1 and CUC2 transcript levels, but not those of the other predicted targets, are elevated in shoot apices of miR164 plants as compared with the wild type (Baker et al., 2005), and that NAC1 transcripts are enriched in roots of miR164a and mir164b mutants (Guo et al., 2005). Furthermore, expression of miR164 from the constitutive 35S promoter led to a reduction in transcript levels of the predicted targets (Guo et al., 2005; Laufs et al., 2004), indicating that these transcripts can be under miRNA-dependent regulation when miR164 miRNAs are ectopically expressed. However, the individual miR164 miRNAs are not expressed ubiquitously, but rather in specific patterns (Fig. 3). Thus, it is possible that certain targets, at least in some tissues, are not regulated by miRNAs because their expression domains and those of the MIR164 genes do not overlap.

To test whether all of the predicted targets are subjected to miRNA-dependent regulation and whether there are tissue-specific differences in the degree to which individual transcripts are controlled by miR164 miRNAs, their transcript levels were measured in inflorescences, rosette leaves and seedlings of mir164abc triple-mutant plants by qRT-PCR. All of the predicted targets accumulated in mir164abc mutants to higher levels than in wild-type plants (Fig. 4A–C), confirming that they are indeed regulated by the endogenous miR164 miRNAs. Moreover, the extent to which transcripts of the targets accumulated in the different tissue samples varied substantially, indicating tissue-specific effects of miR164 miRNAs on target gene expression. These differences might be due to variable degrees of overlap between regions of target gene and miRNA expression in the tissues tested.

**Regulation of CUC gene expression by miR164 miRNAs**

It has been proposed that miRNAs control development by selectively clearing cells of miRNAs that encode cell fate determinants, thereby promoting rapid cell fate transitions and differentiation of cell lineages (Rhoades et al., 2002). In accordance with this idea, the plant miRNAs miR171, miR172 and miR165/166 and their respective target miRNAs were found in adjacent, but non-overlapping domains (Chen, 2004; Juarez et al., 2004; Kidner and Martienssen, 2004; Parizotto et al., 2004; Williams et al., 2005). For miR164 miRNAs, however, a different mechanism for the control of target gene expression has been proposed. In miR164c single mutants, transcripts of CUC1 and CUC2 were found to be elevated when compared with the wild type, but remained restricted to cells in boundary regions (Baker et al., 2005), implying that miR164c does not act by clearing CUC1 and CUC2 miRNAs from non-boundary cells, but rather by regulating transcript abundance in a pre-existing pattern.

In addition to miR164c, miR164a and miR164b are also likely to be involved in regulating CUC1 and CUC2 expression, as inactivation of miR164a and miR164b leads to an enhancement of

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**Fig. 3. Expression patterns of miR164 miRNAs in wild-type plants.** (A–D) Confocal images of inflorescences. Each transgenic plant expresses the GFP variant 3xVENUS-N7 (green/yellow) under control of the individual miRNA regulatory sequences (as indicated). In A, B, FM4-64 dye was used to stain plasma membranes (red); in C, D, organ outlines are highlighted by red chlorophyll autofluorescence. T1 plants were examined and representative expression patterns are shown. The number of plants showing depicted expression pattern (n) with respect to total sample size (ntot), indicated as ratio (ntot/n0), was 7/7 (A), 2*/20 (B) and 5/5 (C, D). *No expression was detected in 18 out of 20 transgenic lines harboring pMIR164b:3xVENUS-N7. (E–P) In situ hybridization analysis of miR164 miRNA distribution (E,F,P) using DIG-labeled LNA-ath-miR164a antisense oligos, in Ler wild-type (E,F), in mir164a-4 b-1 double-mutant (I,J), in mir164a-4 c-1 double-mutant (K,L), in mir164ab-1 c-1 double-mutant (M,N) and in mir164abc triple-mutant (O,P) plants. The inset in K shows mir164 accumulation in developing flowers. (G,H) No signal above background was detected when DIG-labeled Scramble-miR LNA-oligo was used as a control probe on Ler wild-type tissue. Scale bars: 100 μm.
the defects shown by mir164c plants (Figs 1 and 2). The possibility of additional effects of mir164-dependent regulation on CUC1 and CUC2 expression was tested by examination of the distribution of wild-type CUC1 and CUC2 and of mirRNA cleavage-resistant versions of these genes (CUC1m and CUC2m), translationally fused to GFP and expressed from their own promoters, in wild-type plants. CUC1-GFP and CUC2-GFP fusion proteins were detected by CSLM in narrow columns of cells that separate flower primordia and the inflorescence meristem (Fig. 5A,B), in agreement with the reported boundary-specific expression of the corresponding genes (Aida et al., 1999; Takada et al., 2001). By contrast, the mirNA-resistant versions CUC1m-GFP and CUC2m-GFP accumulated in boundaries to much higher levels than the wild-type proteins, as well as weakly in the center of meristems (Fig. 5, compare C,D with A,B, respectively). These differences in protein accumulation strongly suggest that mir164 miRNAs function by dampening the transcript levels of CUC1 and CUC2, so that the initially strong expression in boundary regions is greatly reduced, whereas the weak expression in meristems is repressed below the detection limit of CSLM. These results also imply that miRNA-dependent regulation is not required per se for the expression of CUC1 and CUC2 in boundaries, and thus that the establishment of the expression patterns for these genes is largely under transcriptional control. In agreement with this idea, transcriptional reporters for CUC1 and CUC2 showed strong expression in the expected pattern (Fig. 5E,F).

To further investigate the role of mir164 miRNAs in controlling target transcript abundance, expression of CUC1 and CUC2 was examined by in situ hybridization in tissues that are affected in

Fig. 4. Loss of mir164 miRNA-mediated regulation quantitatively affects target gene expression. (A-C) Bar charts showing the results of relative qRT-PCR experiments, in which the target transcript abundance was assessed for different tissue types (as indicated) of wild-type and mir164abc triple-mutant plants. Results were normalized using TUB4 transcript levels. The transcript abundance of all predicted mir164 targets is higher in the mir164abc triple mutant (m) as compared with the wild type (wt) in all tissues tested. Fold-change differences in transcript levels between the wild type and the mir164abc triple mutant are shown. Bars represent the s.e. of the measurements.

Fig. 5. Effect of mir164 miRNA regulation on target gene expression. Representative confocal images of inflorescences of primary transformants. FM4-64 dye was used to stain plasma membranes (red). (A-D) Effects of mir164-mediated regulation on CUC1 and CUC2 expression. Consequences of permitted (A,B) and abolished (C,D) mir164-mediated regulation for translational fusions of CUC1 (A,C) and CUC2 (B,D) to GFP. The same confocal microscopy settings have been used for the images shown. Arrowheads (A,B) mark cells that weakly express GFP in boundaries between the inflorescence meristem and flower primordia. (E,F) Transcriptional reporters for CUC1 (E) and CUC2 (F) expressing the GFP variant 3xVENUS-N7 (green). Number of plants showing depicted expression pattern (x) with respect to total sample size (ntot), indicated as ratio (x/ntot), was 7/9 for pCUC1::CUC1-GFP (A), 6/6 for pCUC1::CUC1m-GFP (C), 10*/20 (*no expression detected in others) for pCUC2::CUC2-GFP (B), 4/4 for pCUC2::CUC2m-GFP (D), 7/8 for pCUC1::3xVENUS-N7 (E) and 6/7 for pCUC2::3xVENUS-N7 (F). Scale bars: 100 μm.
mir164abc triple-mutant plants. Analysis of transverse sections of flowers revealed that CUC2 expression is strongly upregulated in carpel margin tissue in mir164abc plants (Fig. 6B, arrow), as compared with the wild type (Fig. 6A), whereas expression levels in stamens appeared to be unaffected. These tissue-specific differences in CUC2 transcript accumulation correlate with the expression pattern of MIR164c, which is strongly expressed in carpel margin tissue but not detected in stamen primordia (Fig. 3D). These results, together with the observation that the carpel fusion defects of mir164c and mir164abc plants were not observed in CUC1 expressing plants (Baker et al., 2005) (this study), suggest that overexpression of CUC2, and not of CUC1, is responsible for this aspect of the mutant phenotype. For CUC1, elevated levels of transcript accumulation were seen in inflorescences of mir164abc triple-mutant plants within the normal domain of expression (Fig. 6D,F) as compared with wild type (Fig. 6C,E). In addition, expression was observed in apparently random patches of cells within and especially in between floral primordia (Fig. 6D,F), where cell division activities are often reduced in the triple mutant (Fig. 2). Thus, certain phenotypic alterations of mir164abc plants, such as the carpel fusion defect and the reduced growth between flower primordia, are tightly correlated with ectopic target transcript accumulation.

**Fig. 6. Target mRNA accumulation in mir164abc triple mutants.** (A-F) In situ localization of CUC2 (A,B) and CUC1 (C-F) transcripts in transverse sections of stage 9 flowers (A,B) and of inflorescences (C-F). Tissue of wild-type (A,C,E) and mir164abc mutant (B,D,F) plants was processed equivalently and was present on the same microscope slide (A,B), or was hybridized in the same slide sandwich (C,D), to allow a direct comparison of the signals obtained. Arrows (A,B) point to regions of elevated CUC2 expression in partially fused carpels (ca) of mir164abc mutants as compared with the wild type. By contrast, CUC2 expression in stamens (st) appeared to be unaffected. (C,D and their enlargements E,F) Randomly located foci of high CUC1 expression were sometimes observed within primordia (asterisks) and between primordia (arrowheads) of mir164abc mutant plants. Scale bars: 20 μm.

**CUC1 and CUC2 function as growth inhibitors**

The ectopic expression of CUC1 in regions that show reduced cell proliferation in the miR164abc triple mutant (Fig. 6) raised the possibility that CUC transcription factors may function by inhibiting cell division activities. To test this, we re-examined transgenic plants expressing miR164 cleavage-resistant versions of CUC1 and CUC2 under the control of the strong, constitutive 35S promoter (Baker et al., 2005). These plants form flowers with misshapen sepal, petals and stamens, which are significantly reduced in size compared with the organs of wild-type flowers (Baker et al., 2005). To determine whether these growth defects are a consequence of reduced cell division rates, a decrease in cell elongation, or both, we examined the abaxial epidermis of sepal of 35S::CUC1m-GFP plants (Fig. 7C), which were about fourfold shorter than those of the wild type (Fig. 7E). Compared with wild-type sepal (Fig. 7A) and those of mir164abc triple mutants (Fig. 7B), the average size of the epidermal cells was not significantly changed in the CUC1-overexpressor lines (Fig. 7D,F,G). Thus, the dramatically reduced length of sepal of the transgenic plants is not caused by an inhibition of cell elongation, and therefore must be due to a reduction in cell number, suggesting that CUC genes function by repressing cell division. The elevated levels of CUC activity in between floral primordia, where growth is severely reduced in mir164abc triple-mutant plants, might lead to a suppression of growth between neighboring primordia, thus keeping primordia together while the stem continues to grow. Similarly, the expansion of the CUC expression domain in mir164abc triple mutants into meristems (Figs 5 and 6) might interfere with primordium formation in the flower and hence cause organ-number defects.

**Fig. 7. CUC1 acts as a growth antagonist.** (A-C) SEM images of the (abaxial) sepal epidermis of wild-type Ler (A), mir164abc mutant (B), and 35S::CUC1m-GFP transgenic plants (C). The sepal of mir164abc mutants were typically narrower than, but otherwise indistinguishable from, wild-type sepal (compare B with A). (D-G) The bar chart in D depicts the average number of epidermal cells touching a 100 μm by 100 μm square projected onto the central abaxial region of sepal of wild-type (G) and 35S::CUC1m-GFP transgenic plants (F). The average cell number per 0.01mm2 was 31.8±8.8 (s.d., ntot=10) for 35S::CUC1m-GFP transgenic plants and 28.1±2.5 (s.d., ntot=11) for wild-type sepal. The bar chart in G depicts the average sepal length of 35S::CUC1m-GFP and wild-type plants. The average sepal length was 360±101 μm (s.d., ntot=10) for 35S::CUC1m-GFP plants and 1529±89 μm (s.d., ntot=9) for wild-type plants. Scale bars: 100 μm.
DISCUSSION

Functional redundancy and specialization among miR164 miRNAs

In this study, we have analyzed the potential for functional redundancy among plant miRNAs by studying the effects that inactivation of the entire MIR164 family has on shoot development. We found that miR164abc triple-mutant plants are characterized by severe defects in flower development and phyllotaxis that are not observed in plants in which only individual MIR164 genes are disrupted. These results indicate that miR164 miRNAs control shoot development in a redundant manner. However, the degree to which individual miR164 miRNAs contribute to the regulation of different developmental processes varies. Whereas mir164a and mir164b single mutants exhibit no obvious defects in shoot development, mir164c plants have floral defects that are similar to, but weaker than, those of the mir164abc triple mutant. Thus, mir164c contributes to a larger extent to the control of flower development than its two sister miRNAs. In the control of phyllotaxis, however, all three miRNAs appear to function in an equal manner, as none of the mir164 single mutants exhibits any discernable alteration in the arrangement of flowers. Because the miR164 miRNAs are essentially identical in sequence and have the same target specificities, it is likely that the differences in expression patterns that we have detected in our analysis for the individual family members (Fig. 3), account for their functional diversification.

Functional redundancy is often found in plants, as well as in animals, among protein-coding genes that originated from gene or genome duplications. Although gene duplicates are thought to be often lost over time, retaining duplicated genes can be beneficial for an organism because they might buffer fundamental developmental processes from the detrimental effects of random mutations (Chapman et al., 2006). It is also possible that duplicated genes functionally diverge over time and undergo functional specialization (subfunctionalization), or acquire functions other than that of the progenitor gene (neofunctionalization). These processes are often a result of mutations in the regulatory regions of the gene duplicates that can lead to distinct spatial and/or temporal expression patterns. It has been proposed that miRNAs evolved from their targets by inverted duplication (Allen et al., 2004), and recent evidence suggests that large-scale segmental duplications may play a key role in the establishment of miRNA families in plants, including the MIR164 family (Maher et al., 2006). Thus, miRNA genes might evolve similarly to protein-coding genes. The finding that the miR164 miRNAs, though largely functionally redundant, contribute differently to certain aspects of development is therefore in agreement with functional diversification through subfunctionalization, an idea that is further supported by the distinct, but partially overlapping expression patterns of the individual MIR164 miRNA genes.

miR164 miRNAs contribute to the robustness of development

The absence of miR164 miRNAs leads to phenotypic alterations that are correlated with elevated and/or ectopic target transcript accumulation. This suggests that the role of miR164 miRNAs in development is to prevent fluctuations in target gene expression and, thus, to increase the precision of the developmental programs underlying organogenesis and to protect them from the intrinsic stochasticity of biochemical processes such as transcription and translation. miR164 miRNAs appear to control development by dampening transcript accumulation of their targets, where their expression patterns and those of the targets overlap. Furthermore, CUC1 and CUC2 expression domains are enlarged in mir164abc mutant inflorescence meristems (Fig. 6), indicating that the miR164 miRNAs can spatially limit target mRNA accumulation in addition to reducing the levels of target transcripts. These seemingly different effects are likely to be a consequence of spatial differences in target transcript accumulation. Where target gene expression is high, the pool of miR164 miRNAs might not suffice to efficiently clear the target transcripts from cells. By contrast, the level of miR164 miRNAs may be high enough to completely eliminate target transcripts where they are expressed at comparatively low levels. This mode of action would be consistent with findings that showed an miRNA-dependent reduction, but not an elimination, of highly expressed transcripts in mammalian tissues (Farh et al., 2005; Sood et al., 2006), as well as with the results of a recent study that reported miR168 and its target AGO1 as being co-expressed in Arabidopsis (Vacheret et al., 2006). Thus, dampening of gene expression is a mechanism of miRNA-target interaction that is likely to be found in both plants and animals.

The ability of miRNAs to reduce fluctuations in transcript abundance suggests that miRNAs may be involved in buffering developmental processes. In the absence of the miR164 miRNAs, the domain of CUC expression is less precise and can expand seemingly at random from boundary regions into peripheral regions of the inflorescence meristem and also into flower primordia (Fig. 6). This indicates that transcriptional control per se lacks the accuracy to prevent fluctuations in the CUC expression domains. The variability in flower positioning in mir164abc mutants correlates with local alterations in the CUC expression pattern and can be explained by the lack of precision in the control of the CUC expression domain. These observations are in agreement with a role in stabilizing developmental processes, a function that has also been proposed for animal miRNAs (Hornstein and Shomron, 2006).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/6/1051/DC1

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