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GRCD1, an *AGL2*-like MADS Box Gene, Participates in the C Function during Stamen Development in *Gerbera hybrida*

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Despite the differences in flower form, the underlying mechanism in determining the identity of floral organs is largely conserved among different angiosperms, but the details of how the functions of A, B, and C are specified varies greatly among plant species. Here, we report functional analysis of a Gerbera MADS box gene, *GRCD1*, which is orthologous to *AGL2*-like MADS box genes. Members of this group of genes are being reported in various species in growing numbers, but their functions remained largely unsettled. *GRCD1* expression is detected in all four whorls, but the strongest signal is seen in the developing stamen and carpel. Downregulating *GRCD1* expression by antisense transformation revealed that lack of GRCD1 caused homeotic changes in one whorl only: sterile staminodes, which normally develop in whorl 3 of marginal female florets, were changed into petals. This indicates that the *GRCD1* gene product is active in determining stamen identity. Transgenic downregulation of *GRCD1* causes a homeotic change similar to that in the downregulation of the Gerbera C function genes *GAGA1* and *GAGA2*, but one that is limited to whorl 3. Downregulation of *GRCD1* expression does not reduce expression of *GAGA1* or *GAGA2*, or vice versa; and in yeast two-hybrid analysis, GRCD1 is able to interact with GAGA1 and GAGA2. We propose that a heterodimer between the GRCD1 and GAGA1/2 gene products is needed to fulfill the C function in whorl 3 in Gerbera.

INTRODUCTION

The study of floral homeotic mutants in Arabidopsis and Antirrhinum has shown that despite their differences in flower form, the underlying mechanism of flower organ identity determination is largely conserved. The outcome of such studies is a simplified ABC model. Sepal development is determined by expression of the A function alone; the combination of the A and B functions or the B and C functions specifies petal or stamen development, respectively; and expression of the C function alone determines carpel formation (Coen and Meyerowitz, 1991). Most of the ABC function genes are shown to encode MADS-box transcription factors, which form homo- or heterodimers to perform their functions (see, e.g., Yanofsky et al., 1990; Jack et al., 1992; Goto and Meyerowitz, 1994). Plant MADS box genes (>200 known) have been divided into subgroups (clades) mainly by phylogenetic analyses of conserved parts of their predicted amino acid sequences (Doyle, 1994; Purugganan et al., 1995; Tandre et al., 1995; Theissen et al., 1996). Most clade members, although originating from different plant species, share similar expression patterns and have related developmental functions; the MADS box genes providing A, B, and C functions also fall into separate clades. However, the *AGAMOUS* (*AG*) clade, for example, comprises not only typical C function genes that show similar mutant or loss-of-function phenotypes—such as Arabidopsis *AGAMOUS*, Antirrhinum *PLENA* and *FARINELLI*, and Gerbera *GAGA1* and *GAGA2*—but also genes that determine ovule identity—such as Petunia *FBP7* and *FBP11* (Bowman et al., 1989; Schwarz-Sommer et al., 1990; Angenent et al., 1995; Davies et al., 1999; Yu et al., 1999).

Two models, which may act in combination, have been presented for Arabidopsis regarding how the different C function activities (stamen specification, carpel specification, and flower meristem determinacy) can be separated (Sieburth et al., 1995). For the single C function gene of Arabidopsis, *AGAMOUS*, the quantitative model predicts that the amount of gene product needed varies for each of those activities. On the basis of the phenotypes of the *ag-4* and *AG-MET205* mutations, the highest concentration of AG protein has been suggested to be required for determinacy, whereas mid-range amounts are needed for carpel specification, and the lowest concentration is sufficient for stamen specification. According to the protein–protein interaction model, the *AGAMOUS* gene product would make heterodimers with different partners in different whorls. This model is based on the finding that *ag-4* and *AG-MET205* mutations occur in the coiled-coil motif called the K box, which is thought to be important in protein–protein interactions (Sieburth et al., 1995; Fan et al., 1997). Yeast two-hybrid analysis and in

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vitro immunoprecipitation experiments in Arabidopsis suggest that in addition to the well-characterized ability to make homodimers, AG is also able to interact with four other MADS domain proteins—AGL2, AGL4, AGL6, and AGL9—all encoded by genes belonging to the *AGL2* clade. Fan et al. (1997) proposed (but did not demonstrate) that the interaction between AG and these proteins could contribute to the functional specificity of the AG protein. This is in line with the protein–protein interaction model for separation of the C function in Arabidopsis.

Here, we report direct mechanistic evidence from *Gerbera hybrida* bearing on the quantitative versus protein–protein interaction models. Gerbera, a readily transformable model system in the sunflower family (Asteraceae), has proven useful for shedding light on novel as well as incompletely understood plant gene functions (see, e.g., Eckermann et al., 1998; Kotilainen et al., 1999). Previous work on Gerbera indicated functions for several MADS box gene components of the ABC system (Yu et al., 1999). High sequence similarity, identical expression patterns, and similar transgene phenotypes for *GAGA1* and *GAGA2* (*AG*/*PLE* orthologs) imply that these C-class genes participate equally in C functions. For example, in transgenic lines with reduced *GAGA2* expression, petal-like organs formed in whorl 3. In whorl 4 of these transgenic lines, green organs with carpelloid- and sepaloid-like traits developed, and inside these altered whorl 4 organs were extra sepal and petal whorls (Yu et al., 1999).

Here, we report the isolation and characterization of *GRCD1* (*Gerbera Regulator of Capitulum Development 1*), a cDNA molecule representing a Gerbera member of the *AGL2* clade of MADS box genes. The *AGL2* lineage includes several genes that are expressed during flower development but whose precise functions in plant development remain obscure. Thus far, two functional analyses of *AGL2*-like genes have been published. Downregulation of petunia *FBP2* and tomato *TM5* genes disturbed normal development of flower organs in whorls 2 to 4 in petunia and tomato, respectively (Angenent et al., 1994; Pnueli et al., 1994a). During flower organ differentiation, *GRCD1* expression is detected in all whorls but is strongest in stamen and carpel primordia, suggesting that GRCD1 (gene product) could especially play a role in stamen and carpel development. Downregulation of *GRCD1* expression revealed that lack of GRCD1 caused a homeotic transformation in one whorl only: sterile staminodes, which normally develop in whorl 3 of outermost female ray flowers, changed into petals. In sexually perfect central disc florets, downregulation of *GRCD1* expression had only minor effects on stamen development, and fertile pollen was produced (although not released). Downregulation of *GRCD1* did not affect normal development of carpels in whorl 4. The transgenic phenotypes suggest that the *GRCD1* gene product is active in determining stamen identity during Gerbera flower development. Downregulation of the expression of the C function genes, *GAGA1* or *GAGA2*, did not markedly reduce *GRCD1* expression, and lack of GRCD1 did not reduce the expression of *GAGA1* or *GAGA2*. Furthermore, yeast two-hybrid analysis results showed that the gene product of *GRCD1* was able to make heterodimers with both the *GAGA1* and *GAGA2* gene products. These results indicate that GRCD1 has a role in the C function of whorl 3.

RESULTS

Isolation of a *GRCD1* cDNA by Differential Screening

During studies of region-specific control of gene expression along the longitudinal axis of Gerbera petals, differential screening and sequence comparisons showed that one of the cDNAs, the expression of which is strongest at early stages of petal development, belongs to the MADS box gene family. Given this expression pattern and the results of transgenic functional analysis (see below), we named this cDNA *GRCD1*.

DNA gel blot analysis with a highly specific probe (a 200-bp-long 3′ fragment of the *GRCD1* cDNA; 80% noncoding) recognized two bands at the stringency used in the RNA gel blot (data not shown). This indicates that the RNA gel blot analyses presented below correspond most probably to a transcript of a single locus, and the two bands found are the result of restriction site polymorphism in the heterozygous cultivar. However, the existence of a second very similar gene in the Gerbera genome cannot be ruled out.

GRCD1 Groups inside the *AGL2* Clade

The *GRCD1* cDNA is most similar to members of the *AGL2* group, for example, *AGL2*, *AGL4*, and *AGL9* of Arabidopsis; *DEFH49*, *DEFH72*, and *DEFH200* of Antirrhinum; and *FBP2* of petunia. To address the evolutionary relationships between *GRCD1* and other MADS box genes, a phylogenetic analysis of nucleotide sequences of the MADS and K box regions was performed (see Yu et al., 1999). The analysis revealed that *GRCD1* groups inside the *AGL2* clade and that it pairs with another Gerbera gene, *GRCD2* (*GRCD1* and *GRCD2* cDNAs have 64% identity at nucleotide level). Gene pairs or groups are typical of the *AGL2* clade, for example, in Antirrhinum (*DEFH200* and *DEFH72*) and in Arabidopsis (*AGL2* and *AGL4*) (Figure 1).

Spatiotemporal Expression Pattern of *GRCD1* during Flower Development

In situ hybridization analysis revealed that during flower development, *GRCD1* expression is first detected before any flower organ primordia emerge, thus coinciding with the onset of expression of Gerbera B- and C-class MADS box

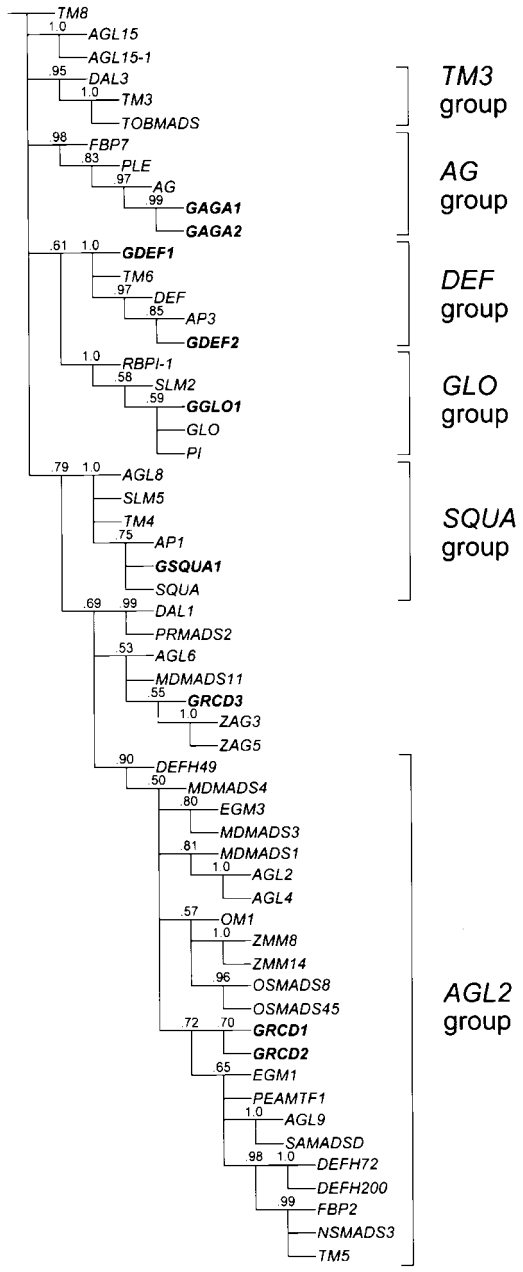

Figure 1. Phylogenetic Analysis of Selected MADS Box Genes.

Previously published plant MADS box genes were retrieved from the EMBL and GenBank databases.

genes. As with Gerbera B-class genes (Yu et al., 1999), induction of *GRCD1* expression was asymmetrical within the plug-like flower primordia (see Hill et al., 1998, regarding Arabidopsis) and proceeded centripetally in the capitulum (Figure 2A).

Later in flower development, when all flower organ primordia had been determined, *GRCD1* expression could be detected in all four whorls. As shown in Figure 2B, *GRCD1* was also expressed in all whorls at early phases of flower organ differentiation; but at later phases, *GRCD1* expression was strongest in stamen and carpel primordia, especially within carpel in the outer integument of the ovule. A weaker signal was also detected in differentiating petals (Figure 2C). This description of *GRCD1* expression applies to all floret types. In the sexually perfect disc florets, *GRCD1* signal in stamens was concentrated in anther connectives later in stamen development (data not shown).

The expression pattern of *GRCD1* during the late developmental stages of different plant organs was studied using RNA gel blot analysis. The expression of *GRCD1* was determined to be flower-specific; it was observed in all four flower organs—pappus bristles (simplified whorl 1 organs that lack vascular bundles), petals, stamens, and carpels (data not shown).

Downregulation of *GRCD1* Expression Changes Whorl 3 Identity from Stamens to Petals

Detailed expression analyses showed that *GRCD1* expression was concentrated in whorls 3 and 4 during the midstages of floral organ differentiation (Figure 2C), which might indicate that the GRCD1 protein plays a functional role in stamen and carpel development. To test this hypothesis, we generated transgenic plants in which *GRCD1* expression is downregulated by introducing a nearly full-length *GRCD1* cDNA (20 nucleotides missing in the 5′ end) in antisense orientation under the control of the cauliflower mosaic virus 35S promoter.

In the wild type, at early stages, the stamen primordia of different floret types are morphologically indistinguishable; the feminization of marginal florets occurs later in development, when stamen development in marginal ray and trans florets arrests. Anther tissues do not differentiate, and eventually they dry and wither, resulting in the formation of nonfunctional staminal rudiments or staminodes.

We obtained three independent antisense lines, t3, t7, and t8, which expressed much less *GRCD1* during capitula development (Figure 3A). Downregulation of *GRCD1* expression did not have an effect on normal carpel development in whorl 4 but did affect different floret types in whorl 3 differently. All three transgenic lines with reduced *GRCD1* expression had petals in place of stamen rudiments in whorl 3 of the marginal ray and trans florets (Figures 4 and 5). Typical petal characteristics, such as anthocyanin pigmentation, shape, and cuticular striation of epidermal cells, were seen in whorl 3 organs of these transgenic lines. As reported by Yu et al. (1999), arrest of whorl 3 organs depends on organ identity, not on whorl position. Similarly, the *GRCD1*-downregulated lines did not show any signs of developmental arrest in whorl 3 petal development. In accordance with the ABC model, the whorl 3 identity change from stamens to petals was also detected in marginal florets of transformant lines in which either of the two functionally similar Gerbera

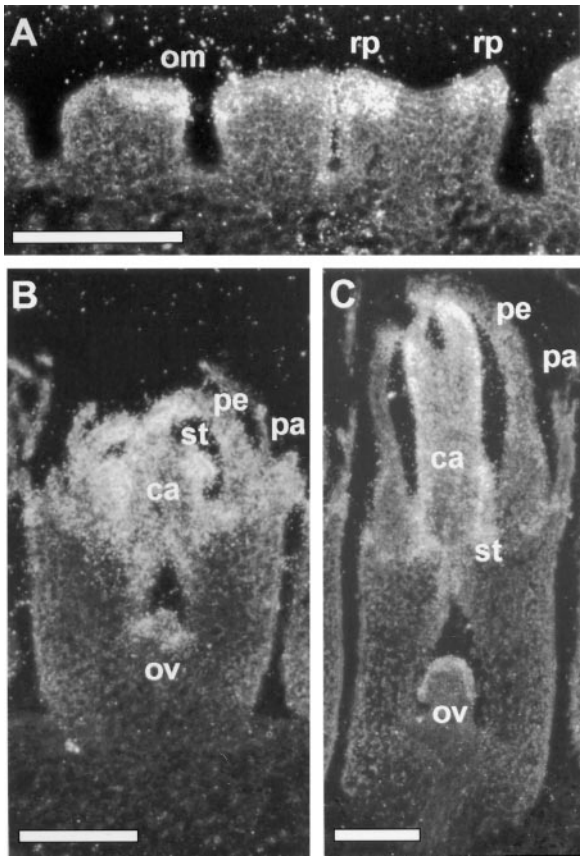

Figure 2. Analysis of *GRCD1* Expression during Early Stages of Flower Development Using in Situ Hybridization.

(A) Three flower primordia at progressive developmental stages. Onset of *GRCD1* expression takes place before flower organ determination, coinciding with the onset of expression of Gerbera B- and C-class MADS box genes (see Yu et al., 1999). *GRCD1* signal, like that of Gerbera B-class MADS box genes, was first detected in the outer margins of floret primordia, where ring primordia (origin of perianth) later develop.

(B) A single flower primordium at early differentiation stages of flower organ primordia development. *GRCD1* is expressed evenly in all four whorls and in the developing ovule.

(C) As the differentiation of flower organs progresses, *GRCD1* expression is focused on stamen and carpel primordia, particularly in the outer integuments of ovules.

ca, carpel; om, outer margin; ov, ovule; pa, pappus bristles; pe, petal; rp, ring primordium; st, stamen. Bars = 200 μm.

C-class MADS box genes, *GAGA1* or *GAGA2*, was downregulated (Figures 3 and 4C; Yu et al., 1999). The parallelism in downregulation phenotypes in whorl 3 among *GAGA1/2* and *GRCD1* suggests that GRCD1 participates in the normal C function for whorl 3.

In the central disc florets, downregulation of *GRCD1* expression had only slight effects on the development of fertile stamens in whorl 3. During normal flower development in Gerbera, stomata develop only on the abaxial surfaces of petals (Figure 5E). In the transformants, however, stomata also developed on the abaxial surface of anthers (Figure 5F). Pollen was not released from pollen sacs as it is in nontransformed control lines, indicating that normal anther development was disturbed. However, differentiation of tissues took place normally, and the pollen produced was fertile (data not shown). Thus, in the perfect disc florets, downregulation of *GRCD1* expression causes minor disturbances in whorl 3 development, which is keeping with the homeotic transformation of whorl 3 organs to petals seen in marginal florets.

RNA gel blot analysis of capitula of anti-*GRCD1* transformants showed that downregulation of *GRCD1* had no effect on the extent of expression or spatial distribution of *GAGA1* and *GAGA2*. These results, verified by in situ hybridization (data not shown), indicate that the gene product of *GRCD1* is not needed for the expression of the C-class genes and that *GRCD1* is directly involved in stamen development. Likewise, in anti-*GAGA1* or anti-*GAGA2* plants, which phenocopy the homeotic change in whorl 3 of ray florets (petals instead of staminodes) in anti-*GRCD1* plants, *GRCD1* expression was at the same amount as in nontransformed controls (Figure 3, and data not shown). This demonstrates that the gene products of *GAGA1* or *GAGA2* (Gerbera C-class genes) are not necessary for *GRCD1* expression.

Yeast Two-Hybrid Analysis Showed That GRCD1 Protein Can Pair with C Function Proteins GAGA1 and GAGA2

Earlier, *GAGA1* and *GAGA2* were shown to code in Gerbera for C function, which is needed for proper development of stamen and carpel (Yu et al., 1999). Transgenic downregulation of *GRCD1* phenocopies the downregulation of *GAGA1/2* in whorl 3, and because *GRCD1* does not seem to be upstream or downstream of *GAGA1* or *GAGA2* and because MADS proteins are known to form homo- and heterodimers to specify their functions, we hypothesized that perhaps GRCD1 participates in the C function by directly interacting with GAGA1 or GAGA2 in Gerbera. To study the putative protein–protein interactions among GRCD1, GAGA1, and GAGA2, we performed yeast two-hybrid analysis, finding that, indeed, GRCD1 made heterodimers with both GAGA1 and GAGA2 proteins. Interactions among GAGA1 and GAGA2 proteins were substantially weaker, and the GRCD1 protein did not homodimerize in this assay (Figure 6).

DISCUSSION

GRCD1 Participates in the C Function

GRCD1, a Gerbera *AGL2*-like gene, has flower-specific expression that was first detected in all four whorls and later

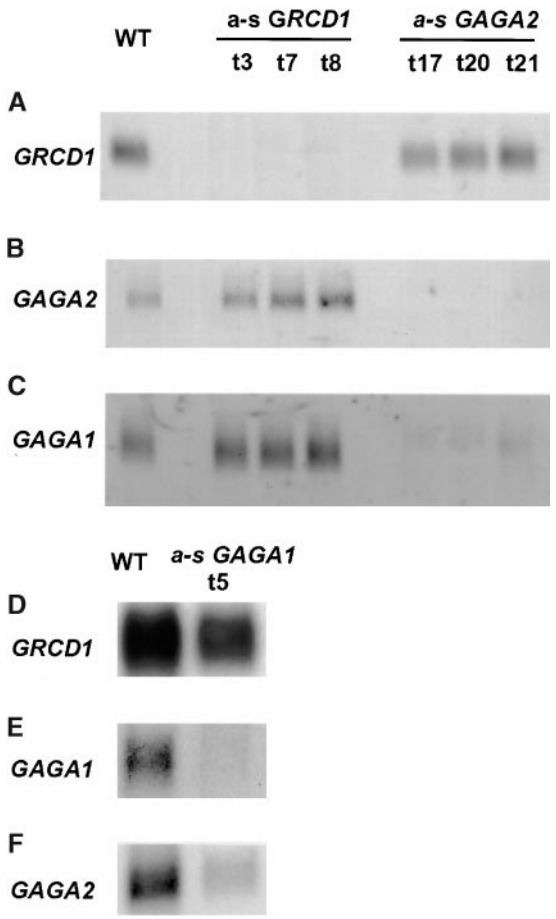

Figure 3. RNA Gel Blot Hybridization Analysis of Expression of *GRCD1* and Gerbera C-Class MADS Genes (*GAGA1* and *GAGA2*) in *GRCD1* Antisense Lines, in *GAGA2* Antisense Lines, in a *GAGA1* Antisense Line, and in a Nontransformed Control Line.

Capitula covering several early developmental stages (both at determination and differentiation stages of flower organ development) were examined. The probe for each blot is indicated at left.
(A) The expression of *GRCD1* was downregulated in all three antisense *GRCD1* lines (t3, t7, and t8), but downregulation of both *GAGA2* and *GAGA1* in the antisense *GAGA2* lines did not affect *GRCD1* expression.
(B) The expression of *GAGA2* was downregulated in three anti-*GAGA2* lines, but downregulation of *GRCD1* did not affect *GAGA2* expression.
(C) Because of high sequence similarity between *GAGA1* and *GAGA2*, the expression of *GAGA1* was also downregulated in anti-*GAGA2* lines (t17, t20, and t21), with the homeotic changes being most severe in whorls 3 and 4 (Yu et al., 1999). However, downregulation of *GRCD1* did not affect *GAGA1* expression.
(D) *GRCD1* was expressed in *GAGA1* antisense line t5.
(E) *GAGA1* expression was downregulated in *GAGA1* antisense line t5.
(F) *GAGA2* expression was markedly reduced in a *GAGA1* antisense line t5.
a-s, antisense; WT, wild type.

found to have its strongest expression in stamen and carpel primordia. To study the role of *GRCD1* in Gerbera flower development, we generated transgenic plants in which *GRCD1* expression was downregulated by introducing an antisense *GRCD1* cDNA into the Gerbera genome. Lack of the GRCD1 gene product especially affected stamen development in whorl 3. In marginal florets, a complete homeotic transformation of the sterile staminodes to petals took place, suggesting that GRCD1 is needed for determining stamen identity in Gerbera. In central disc florets, the stamens of anti-*GRCD1* lines remained fertile but had petal-like characteristics (see Results). Downregulation of *GRCD1* did not affect normal development of carpels in whorl 4.

As reported earlier (Yu et al., 1999), both expression patterns and analysis of transgenic plants reveal that Gerbera C-class MADS box genes *GAGA1* and *GAGA2* have similar functions. In Gerbera, downregulation of these C function genes caused whorl 4 organs to develop sepal-like characteristics. Inside these altered whorl 4 organs, extra whorl 1 and 2 organs were observed. Furthermore, and in accordance with the ABC model of flower development, downregulation of C function genes also caused a homeotic transformation of whorl 3 organs into petals in all flower types of Gerbera (Yu et al., 1999). As presented above, downregulation of *GRCD1* expression phenocopies the homeotic transformation of whorl 3 marginal florets that is obtained by downregulation of *GAGA1* or *GAGA2*. The similarity of GRCD1 function and GAGA1 and GAGA2 function in whorl 3 suggests that *GRCD1* participates in the C function in whorl 3.

AGL2-like MADS Box Genes Are Abundant among Different Plant Species

Phylogenetic analysis revealed that *GRCD1* is grouped into the *AGL2* clade, members of which are increasingly being discovered in seed plants. Gerbera *GRCD1* expression, like the expression of its characterized orthologs, varies spatially as flowers develop. The onset of *GRCD1* expression takes place simultaneously with that of B- and C-class MADS box genes. This is different, for example, from the expression of the Arabidopsis orthologs *AGL2*, *AGL4*, and *AGL9* and the Antirrhinum orthologs *DEFH72* and *DEFH200*, which precedes the accumulation of transcripts of B- and C-class MADS box genes, although the expression and accumulation overlap temporally to some extent (Flanagan and Ma, 1994; Savidge et al., 1995; Davies et al., 1996; Mandel and Yanofsky, 1998).

As with *GRCD1*, the expression of many angiosperm *AGL2* clade members is inflorescence specific, and all *AGL2*-like eudicot genes share similarities in their spatial expression patterns in flower development. For example, petunia *FBP2,* tomato *TM5*, pea *MTF1*, and Arabidopsis *AGL2*, *AGL4*, and *AGL9* are expressed mainly in petal, stamen, and carpel primordia, which permits these genes to participate

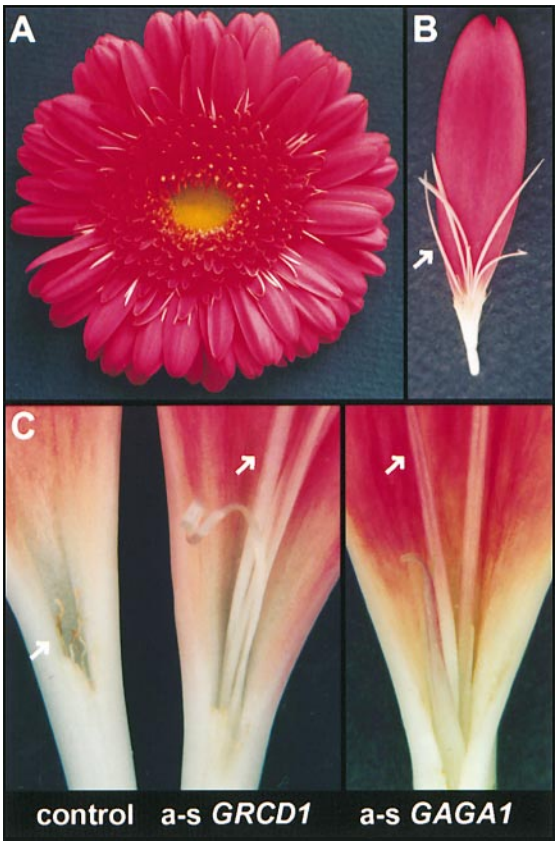

Figure 4. Downregulation of *GRCD1* Expression Caused Homeotic Transformation of Anther Rudiments into Petals in Whorl 3 in Ray Florets of the Anti-*GRCD1* Lines.

(A) Inflorescence of the anti-*GRCD1* line t3.
(B) An individual marginal ray floret of t3. Petals in whorl 3 were seen as light-colored, strip-like organs (arrow).
(C) The whorl 3 organs of marginal ray floret in a nontransformed control line, in *GRCD1* downregulation line t3, and in *GAGA1* downregulation line t5. The two petal rudiments were removed to uncover the whorl 3 organs (arrows). a-s, antisense.

in controlling the development of those organs (Pnueli et al., 1991; Angenent et al., 1992; Flanagan and Ma, 1994; Savidge et al., 1995; Buchner and Boutin, 1998; Mandel and Yanofsky, 1998).

Two functional analyses of *AGL2*-like MADS box genes have been reported. Cosuppression of *FBP2* in petunia resulted in developmental changes in whorls 2 to 4, causing the development of greenish and smaller than usual petals in whorl 2; stamens were replaced by green petaloid structures; and the size of the inner whorl was reduced, with no ovules or placenta forming, but new inflorescences developed in the axis of the carpels (Angenent et al., 1994). Similarly, organ-specific changes in antisense *TM5* tomato plants included evergreen, cauline petals; greenish sterile stamens; and defective carpels with sepaloid or petaloid characteristics (Pnueli et al., 1994a). Thus, downregulation of the expression of petunia *FBP2* and tomato *TM5* disturbed the normal development of petal, stamen, and carpel but did not cause complete homeotic transformation of any particular organ. The minor effect of *GRCD1* downregulation on stamen development in the perfect disc floret is in keeping with the partial downregulation phenotypes of petunia *FBP2* and tomato *TM5* floral organs.

Alternative Models for GRCD1 Function

GRCD1 and *GAGA1/2* are both needed for C function in whorl 3, given that a similar homeotic transformation of whorl 3 organs in the marginal ray florets from rudimentary anthers into true petals in all three antisense lines occurs (Figures 4 and 5; Yu et al., 1999). Three separate results suggest that the involvement of GRCD1 in the C function is direct and might occur through heterodimerization with GAGA1 and GAGA2. First, *GRCD1* expression correlates temporally and spatially with the expression patterns of C function genes, *GAGA1* and *GAGA2*, during stamen development (Figure 2 and Yu et al., 1999). Second, the expression analyses of antisense *GRCD1*, antisense *GAGA1*, and antisense *GAGA2* plants show that the gene product of *GRCD1* is not needed for upregulating the expression of *GAGA1* or *GAGA2* or vice versa (Figure 3). Third, GRCD1 can pair both with GAGA1 and GAGA2 proteins in yeast two-hybrid analyses. Interestingly, interactions among GAGA1 and GAGA2 proteins are much weaker than is their interaction with GRCD1 (Figure 6). This suggests that the GAGA1 and GAGA2 proteins could pair with different and alternative proteins in planta to perform the C function during flower development. Thus, we hypothesize that GRCD1 makes a heterodimer with GAGA1 and GAGA2 and participates in the C function specifically during stamen development.

Three different hypotheses of GRCD1 function during flower development can be drawn from the results presented. First, GRCD1 could participate in separation of the C function between whorls 3 and 4, thus supporting the protein–protein interaction model for separation of the C function in Gerbera (see above, Introduction). This would require that the GRCD1/GAGA heterodimers are active in whorl 3 only because of post-transcriptional regulation of *GRCD1* or because of other regulatory factors involved. Second, *GRCD1* function could be redundant with that of another gene, including a complete redundancy outside the stamen whorl. Good candidates would be the close ortholog *GRCD2* or a hypothetical duplicate locus of *GRCD1* (see Results). Because *GRCD2* is expressed in the antisense *GRCD1* lines (data not shown), it is possible that the ortholog might substitute for the *GRCD1* function partially in whorl 3 of disc florets and fully in whorl 4. Third, the *GRCD1* antisense plants may not be complete loss-of-function lines; that is, some re-

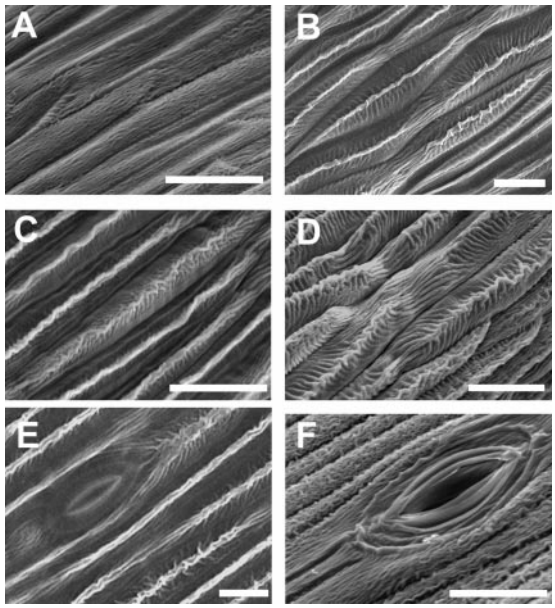

Figure 5. Scanning Electron Microscopy of the Whorl 2 and 3 Organs of Anti-*GRCD1*, Anti-*GAGA1*, and Nontransformed Lines.

(A) Surface of whorl 3 (stamen rudiment) in a nontransformed control line.
(B) Adaxial surface of whorl 2 (petal) in a nontransformed control line.
(C) Adaxial surface of whorl 3 organ in the anti-*GRCD1* line t3.
(D) Adaxial surface of whorl 3 organ in the anti-*GAGA1* line t5.
(E) Abaxial surface of whorl 2 organ (petal) in a nontransformed control line.
(F) Abaxial surface of whorl 3 organ in the anti-*GRCD1* line t5 showing stomatum of petal-like development. During wild-type flower development, stomata develop only on the abaxial surface of petal.
Bars = 20 μm.

sidual *GRCD1* activity might suffice for normal *GRCD1* function outside whorl 3. Bearing in mind the partial downregulation phenotypes of petunia *FBP2* and tomato *TM5* (see above), if either of the two latter hypotheses of *GRCD1* function is true, GRCD1 would also have a function in whorls other than stamen whorls.

In tomato, ectopic expression of the tomato C function gene *TAG1* changes the identity of petals in whorl 2 to sterile stamens (Pnueli et al., 1994b). Interestingly, Lifschitz (1996) mentions that in a double transformant in which *TAG1* was ectopically expressed and *TM5* (a tomato *AGL2*-like gene) was downregulated, the identity of whorl 2 reverted into petals. Because *TM5* is normally expressed in whorl 2, TM5 protein is required for the homeotic conversion of petals to stamens by the *TAG1* gene in lines expressing *TAG1* ectopically. Like Gerbera GRCD1 and GAGA1/2, TM5 and TAG1 are known to pair in yeast two-hybrid analysis (Lifschitz, 1996). These results suggest that as in Gerbera, a heterodimer between AGL2-like and C-class MADS box gene products could be needed to fulfill the C function in whorl 3 in tomato. Generalization of these findings in Gerbera and tomato to other angiosperms (e.g., Arabidopsis, Antirrhinum, rice, and maize) would lead to an advanced understanding of the generality of the ABC model, enhanced by including interactions with other regulatory functions within the large family of MADS box factors.

METHODS

Plant Material

Gerbera hybrida var Terra Regina used in this research was obtained from Terra Nigra BV, De Kwakel, Holland. Plants were grown under standard greenhouse conditions.

Isolation of Plant DNA and RNA

Plant DNA was isolated by the method of Dellaporta et al. (1983). Total RNA was isolated as described in Jones et al. (1985) or by using

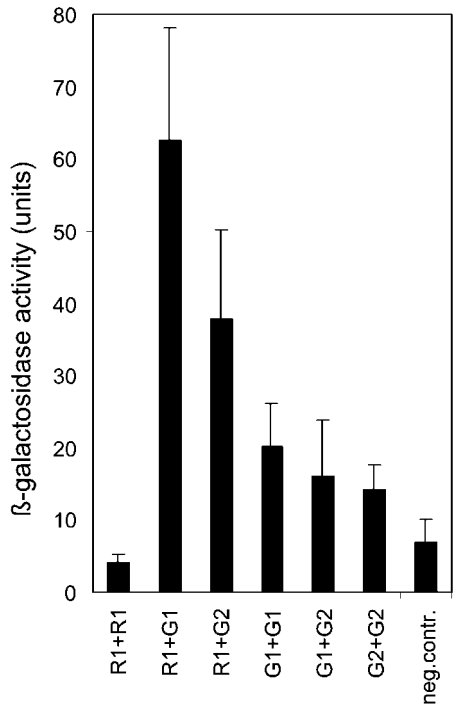

Figure 6. Interaction between GRCD1, GAGA1, and GAGA2 Proteins in the Yeast Two-Hybrid Assay.

LacZ reporter activity in yeast cells for the pairwise combinations of GRCD1 (R1), GAGA1 (G1), and GAGA2 (G2) is shown. The activity in the negative control (neg. contr.; containing vectors pLEXA and pB42AD) represents the background value in yeast cells. Error bars indicate SD.

the RNeasy Plant total RNA kit (Qiagen, Chatsworth, CA). Poly(A)$^+$ RNA was isolated by using oligo(dT) cellulose affinity chromatography (Sambrook et al., 1989).

Construction and Differential Screening of a Petal cDNA Library

Polyadenylated RNA (5 μg), extracted from the proximal part of ray floret petals at developmental stages 5 to 9 (Helariutta et al., 1993), was used to construct a cDNA library in the λ ZAPII vector (ZAP-cDNA synthesis kit; Stratagene, La Jolla, CA). From the nonamplified cDNA library, ~50,000 plaques were plated and transferred onto replica nylon membranes and then screened differentially with radiolabeled first-strand cDNA pools from the proximal part of the ray floret tube region and the distal part of the ligule (First-Strand cDNA synthesis kit; Amersham Corp.).

GRCD1 cDNA was isolated as a clone expressed at early stages of petal development. A nearly full-length (20 nucleotides missing from 5′ end) cDNA clone was isolated, subcloned into a pUC18 derivative, and sequenced using the AutoRead kit (Pharmacia, Uppsala, Sweden). The missing 5′ nucleotides were amplified by single-sided polymerase chain reaction (PCR) from genomic DNA, and the full-length cDNA was amplified once more by PCR from first-strand cDNA corresponding to RNA isolated from young capitula and sequenced. The *GRCD1* gene has been submitted to the EMBL database (accession number AJ400623).

RNA and DNA Gel Blot Analyses and in Situ Hybridization

To each lane was loaded 15 μg of total RNA or 10 μg of digested total genomic DNA. The electrophoresis and hybridizations were as described in Sambrook et al. (1989). The 200-bp-long 3′ fragment (of which 149 bp is from a noncoding region) served as the probe. Washing conditions of 0.2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at 58°C were applied in all RNA and DNA gel blot analyses. In situ hybridization was performed as described previously (Kotilainen et al., 1994) using ^{35}S-CTP–labeled antisense and sense (control) RNA probes. The probes were transcribed from the same fragment used in blot studies under the T7 promoter in vector pSP73 (SP6/T7 transcription kit; Roche Diagnostics, Mannheim, Germany).

Plant Transformation

Gerbera transformation was performed with Agrobacterium-mediated gene transfer basically as described previously (Elomaa et al., 1993, 1998). Three transformants with lowered *GRCD1* expression and changed phenotype as described in Results were obtained. Transformation was verified by DNA gel blot analysis, and the antisense effect was demonstrated by RNA gel blot and in situ analyses showing downregulation of *GRCD1* expression in young, developing capitula of the transformants. The analyses were performed on clones of the original transgenic plants (T_0).

Scanning Electron Microscopic Analysis

After the samples were fixed overnight in a buffer of 50% ethanol, 5% acetic acid, and 2% formaldehyde, they were transferred through ethanol series to 10% ethanol, critical-point–dried (Balzers CPD 020 critical point dryer; Bal-Tec, Balzers, Liechtenstein), and coated with platinum/palladium (agar sputter coater; Agar Scientific, Stansel, UK). Specimens were mounted on aluminum stubs by using graphite adhesive or tape and examined with a scanning electron microscope (digital scanning microscope model DSM 962; Karl Zeiss, Oberkochen, Germany) at the Institute of Biotechnology, Electron Microscopy Laboratory, University of Helsinki.

Phylogenetic Analyses

The parsimony jackknife approach to tree construction was used (Farris et al., 1996). The computer application Xac, written by J.S. Farris, was used to compute the parsimony jackknife support tree. The 1000 jackknife replicates were tested with branch-swapping and five random restarts per replicate. Previously published plant MADS box gene sequences were retrieved from the GenBank database: *DEF* (accession number X52023), *DEFH49* (X95467), *DEFH72* (X95468), *DEFH200* (X95469), *GLO* (X68831), *PLE* (S53900), and *SQUA* (X63701) from *Antirrhinum majus*; *AG* (X53579), *AGL2* (M55551), *AGL4* (M55552), *AGL6* (M55554), *AGL8* (U33473), *AGL9* (AF015552), *AGL15* (U22528), *AP1* (Z16421), *AP3* (D21125), and *PI* (D30807) from *Arabidopsis thaliana*; *OM1* (X69107) from *Aranda* × *Deborah*; *AGL15-1* (U22665) from *Brassica napus*; *EGM1* (AF029975) and *EGM3* (AF029977) from *Eucalyptus grandis*; *GAGA1* (AJ009722), *GAGA2* (AJ009723), *GDEF1* (AJ009724), *GDEF2* (AJ009725), *GGLO1* (AJ009726), and *GSQUA1* (AJ009727) from *Gerbera hybrida*; *MDMADS1* (U78947), *MDMADS3* (AF068722), *MDMADS4* (U78950), and *MDMADS11* (AJ000763) from *Malus domestica*; *TM3* (X60756), *TM4* (X60757), *TM5* (X60480), *TM6* (X60759), and *TM8* (X60760) from *Lycopersicon esculentum*; *NSMADS3* (AF068722) from *Nicotiana sylvestris*; *TOBMADS* (X76188) from *N. tabacum*; *OSMADS8* (U78892) and *OSMADS45* (U31994) from *Oryza sativa*; *FBP2* (M91666) and *FBP7* (X81651) from *Petunia hybrida*; *DAL1* (X80902) and *DAL3* (X79281) from *Picea abies*; *PRMADS2* (U42400) from *Pinus radiata*; *PEAMTF1* (AJ223318) from *Pisum sativum*; *RBPI-1* (AF052859) from *Rumex acetosa*; *SLM2* (X80489) and *SLM5* (X80492) from *Silene latifolia*; *SAMADSD* (Y08626) from *Sinapsis alba*; *ZAG3* (L46397), *ZAG5* (L46398), *ZMM8* (Y09303), and *ZMM14* (AJ005338) from *Zea mays*.

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed by using the MATCHMAKER LexA two-hybrid system (Clontech, Palo Alto, CA). The fusion protein plasmids were constructed as follows: the full coding sequences of genes *GRCD1*, *GAGA1*, and *GAGA2* were amplified by PCR with cDNA clones as templates. PCR fragments were purified (High Pure PCR product purification kit; Roche Diagnostics) and digested at their ends by restriction enzymes for subcloning into plasmids. Yeast transformation and selection of transformants were done according to the manufacturer's instructions. The transformants were grown on Gal/Raff induction plates (MATCHMAKER LexA two-hybrid system; Clontech, Palo Alto, CA), suspended into Z buffer (100 mM Na-PO_4, 10 mM KCl, 1 mM Mg SO_4, 50 mM B-mercaptoethanol, pH 7.0), and assayed for β-galactosidase activity with *o*-nitrophenyl β-D-galactopyranoside as substrate according to the manufacturer's protocol. β-Galactosidase was assayed in 7 to 12 individual transformants for each combination. β-Galactosidase activity was calculated according to Miller (1992).

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NOTE ADDED IN PROOF

While this manuscript was in press, Pelaz et al. (**Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E., and Yanovsky, M.F.** [2000]. B and C floral organ identity functions require SEPALLATA MADS-box genes. Nature **405,** 200–203) published that the MADS-box genes *SEP1/2/3* (formerly known as *AGL2/4/9*) are required for the B and C floral organ identity functions in Arabidopsis flower development.