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Functional diversification of duplicated CYC2 clade genes in regulation of inflorescence development in *Gerbera hybrida* (Asteraceae)

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SUMMARY

The complex inflorescences (capitula) of Asteraceae consist of different types of flowers. In *Gerbera hybrida* (gerbera), the peripheral ray flowers are bilaterally symmetrical and lack functional stamens while the central disc flowers are more radially symmetrical and hermaphroditic. Proteins of the CYC2 subclade of the CYC/TB1 like TCP domain transcription factors have been recruited several times independently for parallel evolution of bilaterally symmetrical flowers in various angiosperm plant lineages, and have also been shown to regulate flower type identity in Asteraceae. The CYC2 subclade genes in gerbera show largely overlapping gene expression patterns. At the level of single flowers, their expression domain in petals show a spatial shift from the dorsal pattern known so far in species with bilaterally symmetrical flowers, suggesting that this expression change may have evolved after the origin of Asteraceae. Functional analysis indicates that GhCYC2, GhCYC3 and GhCYC4 mediate positional information at the inflorescence proximal-distal axis, leading to differentiation of ray flowers, but that they also regulate ray flower petal growth by affecting cell proliferation until the final size and shape of the petals is reached. Moreover, our data shows functional diversification for the *GhCYC5* gene. Ectopic activation of GhCYC5 increases flower density in the inflorescence, suggesting that GhCYC5 may promote flower initiation rate during expansion of the capitulum. Our data thus indicates that modification of the ancestral network of TCP factors has, through gene duplications, led to the establishment of new expression domains and to functional diversification.

INTRODUCTION

Asteraceae, the sunflower family, is among the largest families of plants including approximately 10% of all angiosperm species. Typical for the Asteraceae is a highly compressed inflorescence, the capitulum, which superficially resembles a solitary flower but is composed of tens to hundreds of flowers. In homogamous (unisexual) capitula all flowers are similar whereas heterogamous (hermaphroditic) capitula harbour showy, marginal ray flowers that differ morphologically and functionally from the centrally located, less conspicuous disc flowers. The ray flowers with their large corolla ligules are bilaterally symmetrical (zygomorphic/monosymmetric) and sterile or female, while the disc flowers are radially symmetrical (actinomorphic/polysymmetric) and develop pollen producing stamens in addition to carpels. The presence of the showy marginal flowers in heterogamous heads has been shown to be associated with pollination specialization, outcrossing rate, genetic diversity and fitness, and is the likely reason for the evolutionary success and rapid tribal radiation of this large plant family (Marshall and Abbott, 1984; Stuessy *et al*., 1986; Sun and Ganders, 1990, Endress, 1999; Cubas, 2004; Sargent, 2004; Andersson, 2008). Classical genetic studies have suggested that the presence or absence of ray flowers is under simple genetic control involving one or two major genes and an unidentified number of modifier genes (reviewed in Gillies *et al*., 2002).

Studies in the model species gerbera (*Gerbera hybrida*), groundsel (*Senecio vulgaris*) and sunflower (*Helianthus annuus*) have brought insight into the molecular control of flower type identity in Asteraceae by implicating the involvement of CYCLOIDEA/TEOSINTE BRANCHED1-like (CYC/TB1) TCP domain transcription factors in the establishment and generation of flower-type

heterogeneity (Broholm *et al*., 2008; Kim *et al*., 2008; Fambrini *et al*., 2011; Chapman *et al*., 2012; Tähtiharju *et al*., 2012). Members of the CYC/TB1 subfamily of TCP proteins are considered as general developmental regulators of reproductive and vegetative axillary structures, i.e., flowers and lateral shoots (Martín-Trillo and Cubas, 2010). Phylogenetic analysis has revealed that in core eudicots the CYC/TB1 (or ECE) clade has experienced gene duplications and can be divided into three subclades: CYC1, CYC2 and CYC3 (Howarth and Donoghue, 2006). Early studies in *Antirrhinum majus* showed that the CYC2 clade genes *CYCLOIDEA* (*CYC*) and its paralog *DICHOTOMA* (*DICH*) function partially redundantly in establishing the identity of the dorsal domain of the flower and to generate asymmetry (Luo *et al*., 1996; Luo *et al*., 1999). Afterward, numerous studies across rosids and asterids have indicated that CYC2 clade genes have been recruited several times independently for the common genetic function of regulating bilateral symmetry of single flowers (reviewed by Busch and Zachgo, 2009; Rosin and Kramer, 2009; Martin-Tríllo and Cubas, 2010; Preston *et al*., 2011). In Asteraceae, the CYC/TB1-like gene family has expanded, and both in gerbera and sunflower, ten gene family members have been identified (Chapman *et al*., 2008; Tähtiharju *et al*., 2012). In particular, the duplicated CYC2 clade genes have been shown to be associated with complex capitulum structure and to regulate flower type differentiation.

Our previous studies in gerbera have focused on characterization of a single CYC2 clade gene, *GhCYC2* (Broholm *et al*., 2008). *GhCYC2* expression followed the radial organization of the inflorescence being upregulated in the marginal ray flowers while no expression was detected in the centremost disc flowers. Broholm *et al*. (2008) demonstrated that ectopic expression of *GhCYC2*

in transgenic gerbera converted disc flowers into ray-like with elongated petals and disrupted stamen development. Kim *et al*. (2008) showed that the *RAY* locus, which controls the radiate condition of *Senecio vulgaris* flower heads, corresponds to two closely linked CYC2 clade genes that also show specific upregulation in the marginal ray flower primordia. In sunflower, *chrysanthemoides* (*chry*) or *double* (*dbl*) mutants bear flowers that are solely of ray identity (Fambrini *et al*., 2003; Berti *et al*., 2005). In contrast, in the *tubular ray flower* (*turf*) and *tubular-rayed* (*tub*) mutants, marginal flowers are almost radially symmetrical and develop male and female reproductive organs as do wild type disc flowers (Berti *et al*., 2005; Chapman *et al*., 2012). These mutants show altered expression pattern of the CYC2 clade gene *HaCYC2c* (Fambrini *et al*., 2011; Chapman *et al*., 2012). The *turf* mutation is caused by an insertion of a transposable element in the *HaCYC2c* TCP motif that leads to a premature stop codon (Fambrini *et al*., 2011; Chapman *et al*., 2012), causing reduced expression and radialization of the normally zygomorphic ray flowers (Chapman *et al*., 2012). In the *dbl* mutant, due to an insertion in the promoter region, *HaCYC2c* is expressed throughout the capitulum, converting disc flowers into ray-like flowers (Chapman *et al*., 2012).

Our previous study (Broholm *et al*., 2008) demonstrated that although overexpression of *GhCYC2* converted disc flowers into ray-like, suppression of *GhCYC2* expression was not sufficient to cause loss of ray flower identity. This indicates that there must be additional genes involved in the regulation of flower type differentiation. Comparative analysis of the gene family in gerbera and sunflower showed that all six CYC2 clade genes in gerbera and five in sunflower are upregulated during early stages of ray flower primordia (Tähtiharju *et al*.,

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2012). The expression analysis also suggested that CYC2 clade genes may have more specialized functions at the level of single flowers, including late functions in reproductive organs (Tähtiharju *et al*. 2012). Pairwise protein-protein interactions assays further indicated that most CYC/TB1-like proteins in gerbera and sunflower show competence to form higher-order protein complexes (Tähtiharju *et al*., 2012). In this study we extended the analysis of the gerbera CYC2 clade genes to localize their expression at the level of single flowers. Functional studies in *Arabidopsis* background indicate that the CYC2 clade proteins have highly conserved biochemical functions. Still, ectopic expression in gerbera shows evidence for both functional redundancy and diversification among the gene family members, suggesting that functional specificity for CYC2 clade proteins is obtained by formation of context specific protein complexes involving CYC2 proteins and their co-regulators that may target different downstream genes.

RESULTS

Gerbera CYC2 clade genes show highly overlapping expression patterns

Our earlier results indicated that the CYC2 clade genes share strikingly similar expression patterns during ray and disc flower primordia development. Quantitative real-time PCR (qPCR) for excised floral primordia at early developmental stages indicated that all CYC2 clade genes, with the exception of *GhCYC7*, are predominantly expressed in developing ray flower primordia and are lacking from the centremost, most strongly actinomorphic disc flowers (Tähtiharju *et al*. 2012). In order to localize the expression domains in more detail at organ and tissue level, we performed *in situ* hybridization analysis.

Our previous qPCR data indicated that *GhCYC3* is the only CYC2 clade gene that is upregulated in ray flower primordia and is absent from both trans and disc flower primordia (Tähtiharju *et al*., 2012). *In situ* hybridization confirmed the abundant expression in ray flowers but we also detected expression in petals and carpels of outermost disc flowers during later developmental stages (Figure 1). In contrast to the previously characterized *GhCYC2* gene, *GhCYC3*expression was absent from the rudimentary stamens in ray flowers but showed expression both in the ventral ligule (formed of three fused petals) as well as in the two dorsal petals (Figure 1). *GhCYC4* showed an identical expression pattern with *GhCYC3* in ray flowers (Figure 1). The expression of *GhCYC9*, which is the recently duplicated paralog of *GhCYC4* (Tähtiharju *et al*., 2012), was below the detection level by *in situ* analysis (Figure S1a, b). *GhCYC5* and *GhCYC7* expression was detected in all whorls of organs, but the latter showed relatively weak expression in inflorescences of 12 mm in diameter (Figure 1). The expression of *GhCYC7* was more prominent at an earlier developmental stage, in inflorescences of 3-4 mm in diameter, where its expression was ubiquitously localized in the undifferentiated inflorescence meristem and young developing flower primordia (Figure S1c, d). In the outermost disc flowers, all CYC2 clade genes were expressed in petals and carpels, and *GhCYC5* was additionally expressed in pappus bristles (whorl 1) (Figure 1). In conclusion, with the exception of *GhCYC2* being absent from dorsal petals (Broholm *et al*., 2008), the CYC2 clade genes were redundantly expressed in all five petals as well as in carpels in both ray and disc flowers. The ray flower stamen primordia lacked *GhCYC3* and *GhCYC4* expression, while *GhCYC2*, *GhCYC5* and *GhCYC7* expression was present.

We also investigated temporal expression of the CYC2 clade genes during later stages of ray flower petal development. Analysis of pooled ray flower petal samples from stages 2, 4, 6, and 8 (stages described in Helariutta *et al*., 1993; Kotilainen *et al*., 1999; Laitinen *et al*. 2007) revealed that the CYC2 clade genes *GhCYC2, 3, 4* and *5* showed highest expression levels while *GhCYC9* showed only very low signal and *GhCYC7* was not expressed (Figure 2a) and were excluded from further analysis. Analysis of ray flower petal samples at developmental stages 1-11 showed that the expression of *GhCYC3* peaks at stage 1 and then gradually decreases until stage 9 when the inflorescence is already fully open and the petals have achieved their final size and shape (Figure 2b). The other CYC2 clade genes, *GhCYC2, GhCYC4* and *GhCYC5*, are expressed at lower levels in petals. *GhCYC2* and *GhCYC4* are expressed quite uniformly throughout the development, while the expression of *GhCYC5* peaks at the late stage 9.

Heterologous expression in *Arabidopsis* indicates conserved biochemical function for the gerbera CYC2 clade proteins

For functional analysis, all six gerbera CYC2 clade genes were ectopically expressed in *Arabidopsis thaliana*. For comparison, we also generated plants that ectopically expressed the orthologous *Arabidopsis* gene *TCP1*. At the seedling stage (7 days after germination [DAG]) many of the transgenic *35S::TCP1* lines were clearly smaller in size than the wild type seedlings (Figure S2). The *35S::TCP1* lines did not show root phenotypes. In contrast, constitutive expression of the gerbera CYC2 clade genes did not affect seedling size, but did result in shorter root length for all genes except *GhCYC2* (Figure S2).

The T1 lines could be grouped based on the severity of the phenotype into strong and intermediate lines, as well as lines with no clear phenotype (similar phenotypic categories were described for *35S::TCP1* in Busch and Zachgo, 2007). Constitutive expression of all the gerbera CYC2 clade genes, except *GhCYC2*, led to phenotypes similar to the intermediate lines of *35S::TCP1* (Figure 3). The overall plant size was smaller than in wild type and they produced flowers that had a smaller petal size. Furthermore, they produced short siliques with fewer seeds than the wild type. Constitutive expression of *GhCYC4* and *GhCYC7* also led to phenotypes very similar to those of the strongest *35S::TCP1* lines (Figure S3). These plants were dwarfed and their first flowers failed to open, lacked mature floral organs, and produced only few if any seeds. In the 50 *35S::GhCYC2* T1 lines studied, and the four T2 lines selected for phenotypic verification, we did not see additional phenotypic changes other than some lines producing short siliques and thereby fewer seeds. Thus, reduced reproductive success was common to all the transgenic *Arabidopsis* lines showing constitutive expression of the gerbera CYC2 clade genes. We can conclude that when tested in a heterologous system, the gerbera CYC2 clade genes (excluding *GhCYC2*) are capable of causing similar phenotypes as the orthologous *Arabidopsis TCP1* gene, and thus, their biochemical function is conserved in this context.

Ectopic activation of GhCYC3 and GhCYC4 functions indicate redundancy in regulation of flower type identity in gerbera

We observed that the constitutive expression of CYC genes in gerbera caused severe growth defects (e.g. Broholm *et al*., 2008) and difficulties in regeneration

of transgenic shoots. Therefore we performed functional analyses in gerbera by using an inducible system to transiently activate GhCYC function. The dexamethasone (DEX) inducible rat glucocorticoid receptor (GR) fusion system is suitable for nuclear transcription factors. All gerbera CYC2 clade proteins have a nuclear localization signal in their amino acid sequences, and are predicted to be targeted into nucleus, except GhCYC5, for which TargetP analysis suggested localization in the chloroplast. We tested the subcellular localization of the gerbera proteins using transient expression of *GhCYC*:*GFP* fusions in onion epidermal cells and confirmed that all of them (including GhCYC5) are localized in the nuclei (Supporting information Experimental procedures, Figure S4).

For functional analyses in gerbera, we focused on *GhCYC3*, *GhCYC4* and *GhCYC5* that showed highest expression levels in young inflorescences as well as specific expression patterns during ray flower ligule development. We produced 6 to 16 transgenic lines constitutively expressing *GhCYC3:GR*, *GhCYC4:GR* or *GhCYC5:GR* fusion constructs, respectively (Figure S5). Induction of ectopic activity of GhCYC3 and GhCYC4 in gerbera inflorescences altered the morphology of disc flowers to resemble ray flowers. Disc flower ligule length increased significantly (Tables 1, 2), leading to pronounced bilateral symmetry, and stamen development was arrested (Figure 4). In *35S::GhCYC3:GR* lines, the ligule lengths of ray and trans flowers were also significantly altered being shorter in rays, and longer in trans flowers when compared to the DEX-treated wild-type Terra Regina (Table 1). In *35S::GhCYC4:GR* lines, ray flower ligule length was unaltered but trans flower ligules were longer (Table 2). Furthermore, ectopic expression of *GhCYC3* and

GhCYC4 significantly promoted the growth of the two dorsal petals (marked with arrows in Figure 4). In conclusion, the ectopic activation of GhCYC3 and GhCYC4 function led to similar but more extreme phenotypes as previously reported for lines with constitutive *GhCYC2* expression (Broholm *et al*., 2008). To better compare the phenotypes, we generated inducible *35S::GhCYC2:GR* lines. The induced ectopic GhCYC2 function affected disc flower petals and stamens in a similar manner as described for the overexpression lines in Broholm *et al*. (2008) (Figure S6). Thus, the more complete conversion, compared to GhCYC2, of disc flower morphology upon activation of GhCYC3 and GhCYC4 was not due to the use of different methods for ectopic activation. Despite numerous attempts, we were not able to produce RNAi silencing lines for the CYC2 clade genes.

To study whether the larger ligule size of the *35S::GhCYC3:GR* and *35S::GhCYC4:GR* lines was achieved by affecting cell proliferation or cell size, we measured the size of epidermal cells in SEM images of disc flower ventral ligules from the DEX-treated lines. Ligule cell length was not significantly altered in the *35S::GhCYC3:GR* or *35S::GhCYC4:GR* plants in comparison to the wild type (Figure 5). Thus, the larger ligule size is likely to result from increased cell number.

Crested gerbera cultivar shows upregulation of *GhCYC3* expression

Due to its resemblance with the transgenic phenotypes, a crested gerbera cultivar obtained from a breeder's collection was further investigated for CYC2 clade gene expression (Figure 6). This cultivar produces only flowers with ray identity. All CYC2 clade genes were expressed in the outermost ray flower primordia of

the crested cultivar similarly to the wild type variety Regina. However, unlike in the wild type, only *GhCYC3* was highly upregulated in the centermost flower primordia of the crested cultivar.

GhCYC5 shows divergent function in regulating the flower density of the inflorescence

The induced activation of GhCYC5 function did not cause any striking visual changes at the inflorescence level (Figure 7a, b). However, closer analysis of the transgenic lines indicated changes in the number of flowers in the capitula (Table S1). As the capitulum area may vary according to the season or growth conditions and in individual inflorescences of the same plant, we calculated flower density, i.e. the number of flowers per capitulum unit area (mm^2). By analysing five independent transgenic lines in comparison to the DEX-treated wild-type Terra Regina we observed a statistically significant increase in flower density (Figure 7, Table S1). We did not detect any morphological alterations, including organ identity changes, in the phenotypes of individual flower types. We also followed ligule development and found that, in contrast to activation of GhCYC2, GhCYC3 or GhCYC4 functions, the lengths of the ligules were not changed in any of the flower types (Table S2).

DISCUSSION

The expanded CYC2 clade gene family in Asteraceae has been shown to be associated with the development of the complex inflorescence architecture (Broholm *et al*., 2008; Kim *et al*., 2008; Chapman *et al*., 2012; Tähtiharju *et al*., 2012). In this paper, we extended characterization of the individual CYC2 clade

gene family members in gerbera. Our data indicates a ventralized pattern for CYC2 clade gene expression in the petal whorl that may have evolved after the origin of Asteraceae. Still, the largely overlapping expression domains at the level of individual flowers suggest that functional specificity for the CYC2 clade genes may be obtained through context specific protein complexes that activate different downstream targets. Functional analysis in transgenic gerbera further indicated that in addition to *GhCYC2* (Broholm *et al.*, 2008), *GhCYC3* and *GhCYC4* show redundant functions in regulation of ray flower identity and promoting ray flower petal development. *GhCYC5* instead shows a diverged function in regulating the rate of flower initiation in the inflorescence.

Gerbera CYC2 clade expression is shifted from the ancient dorsal domain

Typically, the CYC2 clade genes involved in regulating floral zygomorphy in core eudicots show asymmetric expression patterns localizing to the dorsal domain of the flower, as shown, e.g., in *Antirrhinum majus* (Luo *et al.*, 1996; 1999) and *Iberis amara* (Busch and Zachgo, 2007). Broader functional domains of duplicated CYC gene paralogs in dorsal and lateral petals have been observed in Fabales, in *Lotus japonicus* and *Pisum sativum* (Feng *et al.*, 2006; Wang *et al.*, 2008), and in *Primulina heterotricha*, Gesneriaceae (Gao *et al.*, 2008; Yang *et al.*, 2012). The single CYC2 clade gene of *Arabidopsis*, *TCP1*, also shows expression in dorsal side of the meristem of actinomorphic flowers, however only transiently in the earliest stages of flower meristem development (Cubas *et al.*, 2001).

Dorsal-specific gene expression has likely evolved after the duplication event within the CYC/TB1 clade that gave rise to the CYC2 subclade (Preston

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and Hileman, 2009), but at a time predating the divergence of *Antirrhinum* (asterids) and *Arabidopsis* (rosids) (Cubas *et al*., 2001). Our data, however, indicates that none of the gerbera CYC2-clade genes behave in this dorsal-specific manner. In contrast, *GhCYC2* expression is excluded from the dorsal petals and has shifted to the ventral domain (Broholm *et al*., 2008), while the expression of *GhCYC3*, *GhCYC4*, *GhCYC5* and *GhCYC7* is detected both in the two rudimentary dorsal petals and the fused ventral ligule, i.e., in all five members of the petal whorl. So far, ventralized pattern of *CYC* gene expression in the petal whorl has not been reported in any other plant lineage, indicating that it may have evolved specifically after the origin of Asteraceae. However, *CYC* genes also control stamen number, and Song *et al*. (2009) showed evidence that in *Opithandra* (Gesneriaceae), expression of a *CYC* gene is associated with the abortion of the ventral stamen. Still, in *Veronica* and *Gratiola* (Plantaginaceae), *CYC* gene expression and stamen reduction in the ventral domain of the flower are not correlated suggesting a different genetic mechanism (Preston *et al*., 2009).

Ectopic expression of *GhCYC* genes in *Arabidopsis* causes reduced growth during both vegetative and reproductive development

Ectopic expression of the gerbera CYC2 clade genes in *A. thaliana* indicated that *GhCYC3, GhCYC4, GhCYC5*, *GhCYC7* and *GhCYC9* all affect growth in a similar way as the endogenous *Arabidopsis* homolog *TCP1*. Intriguingly, although the gerbera CYC2 clade genes show subfunctionalization in gerbera and generally promote growth in reproductive tissues (*see below*), their effect on growth in the heterologous *Arabidopsis* background is repressive. We observed

defects in vegetative growth and reproductive success. In addition, we detected reduction in petal growth, similarly to the *35S::TCP1* lines, as previously reported by Busch and Zachgo (2007). Interestingly, the ectopic activation of the *Antirrhinum majus CYC* in *Arabidopsis* led to an opposite effect on petal growth, i.e. enlarged petals due to enhanced cell expansion (Costa *et al*., 2005). This suggests that the growth effect caused by the rosid genes (*Arabidopsis thaliana* and *Iberis amara*; Busch and Zachgo, 2007) and asterid CYC2 clade genes (from gerbera) represent the more ancestral functions of the CYC2 clade genes, while the growth effect caused by the asterid *Antirrhinum CYC* represents a more recent innovation. However, the ectopic expression of all of these genes causes reduced vegetative growth, indicating that at least in vegetative organs their function is conserved. Notably, also in gerbera, the ectopic expression of *GhCYC2* led to strongly reduced vegetative growth (Broholm *et al.,* 2008), an effect that we deliberately avoided in this study by performing the DEX treatments only on early inflorescence primordia and not on vegetative tissues.

Although the *35S::GhCYC2* lines did not produce phenotypes similar to the other transgenic *Arabidopsis* lines, its ectopic expression in gerbera did produce an inflorescence phenotype similar to *35S::GhCYC3:GR* and *35S::GhCYC3:GR* lines (Broholm *et al.*, 2008). It is possible that GhCYC2 function is truly different in heterologous *Arabidopsis* background but we cannot exclude the possibility that we would detect a similar phenotype with other CYC2 clade genes by generating more lines or that the phenotype would be visible in some other growth conditions. Markedly, the *35S::GhCYC5* phenotypes in *Arabidopsis* are similar to the phenotypes caused by the other CYC2 clade genes, but in gerbera show clear functional differentiation. One possible explanation is that

GhCYC5 has diverged protein interaction capacity (*see below*) and/or target genes that are not present in *Arabidopsis*.

Gerbera CYC2 clade genes show redundant functions in regulating ray flower identity

The gerbera CYC2 clade genes showed partially overlapping expression domains during early stages of flower primordia development. Ectopic activation of GhCYC2, GhCYC3 and GhCYC4 caused very similar phenotypes in transgenic gerbera (Broholm *et al*., 2008; this study). All gene activities converted disc flowers into ray-like by promoting ligule growth through enhanced cell proliferation and suppressed stamen development. However, the phenotypic changes caused by GhCYC4 activation were more pronounced. In trans flowers, both GhCYC3 and GhCYC4 promoted ligule growth whereas GhCYC2 did not (Broholm et al., 2008). In ray flowers instead, both GhCYC2 and GhCYC3 activation led to reduced petal length. Ectopic activation of GhCYC3 and GhCYC4 also promoted the growth of the dorsal petals. Interestingly, *GhCYC3* and *GhCYC4* are expressed in the dorsal petals of wild type ray flowers which, however, remain rudimentary.

Altogether, these observations indicate that the growth effects of these genes may vary based on the site and timing of their expression. Opposite growth effects have also previously been reported for CYC/TB1-like transcription factors. For example, the *Antirrhinum* CYC suppresses the growth of the dorsalmost stamen and while initially suppressing the growth of the dorsal petals it later on promotes their growth (Luo *et al*., 1996). Tähtiharju *et al*. (2012) showed that the gerbera CYC2 clade proteins have the ability to interact in yeast

two hybrid assays. Therefore, we postulate that the functional specificity in given tissues (or in various flower types) is connected with formation of context specific protein complexes involving CYC2 proteins and their co-regulators that may target different downstream genes. CYC2 clade proteins have also been shown to autoregulate themselves and to cross regulate each other leading to formation of autoregulatory loops that may trigger threshold dependent genetic switches (Yang *et al*., 2012). The presence of autoregulatory connections between the gerbera CYC2 clade genes may also affect the differential growth effects and will form an interesting target for future studies.

CYC2 clade genes show functional diversification in regulation of stamen development and late petal development in ray flowers

Both in gerbera and sunflower, CYC2 clade genes are predominantly expressed in ray flowers, in which the stamen development is disrupted and their expression is absent from the actinomorphic disc flowers with functional stamens (Tähtiharju *et al*., 2012). *In situ* hybridization analysis indicated that *GhCYC3* and *GhCYC4* expression is absent from the rudimentary stamens of wild type ray flowers while *GhCYC5, GhCYC7* and *GhCYC2* (Broholm *et al*., 2008) are present suggesting functional diversification among the gene family members. Still, ectopic activation of GhCYC2, GhCYC3 and GhCYC4 led to suppression of stamen development in the modified disc flowers suggesting that these proteins may share common downstream targets. However, we cannot exclude the possibility that the ectopic expression of *GhCYC3* and *GhCYC4* might activate GhCYC2 through an autoregulatory mechanism.

Temporal expression analysis during ray flower ligule development indicated that CYC2 clade genes regulate the late stages of ligule growth. *GhCYC3* showed highest expression levels in petals at stages 1-4 but was maintained throughout ligule development until stage 8-9. The expression of other CYC2 clade genes was lower and relatively uniform throughout development. *GhCYC3* expression level correlated with our previous biometric studies showing that petal growth is tightly controlled and invariable; the ligule expands at a constant rate both longitudinally and laterally until stage 9 when its growth ceases and the petals have reached their final size and shape (Kotilainen *et al.*, 1999). Based on the expression dynamics of genes annotated in the functional class "cell growth and structure", microarray analysis of gerbera petal organogenesis indicated that stage 4 can be considered as a transition stage between cell division and elongation, while from stage 6, organ growth is caused by cell expansion (Laitinen *et al.*, 2007). The highest expression of *GhCYC3* thus correlates with the cell division phase. This also correlates well with our conclusion that larger petal size after induction of ectopic *GhCYC3* and *GhCYC4* expression is likely due to increased cell proliferation and not to enlarged cell size. A recent study exploring natural variation of petal size and shape in three *Arabidopsis thaliana* ecotypes identified *ERECTA* as a major locus determining petal shape. The allelic variation in this locus was associated especially with petal cell proliferation and not cell size (Abraham et al., 2013). Further studies are required to assess the possible regulatory links between TCP factors and, e.g., ERECTA-like receptor kinases, as well as to identify the direct target genes required to define the final size and shape of petals. We also showed that *GhCYC3* expression was upregulated in the centremost flowers of gerbera

crested cultivars, further supporting the major role of this gene in promoting petal growth and flower type identity.

GhCYC5 affects the rate of flower initiation

Ectopic activation of GhCYC5 uncovered a diverged function for a CYC2 clade gene. We observed a statistically significant increase in number of flowers per unit area of the capitulum. In Asteraceae, the number of seed producing flowers is an important component of final yield (López Pereira *et al*., 1999; Cantagallo and Hall, 2002). Studies in sunflower have indicated that flower initiation and meristem tissue expansion are strongly co-ordinated and responsive to various environmental signals (Dosio *et al*., 2006, 2011). Our previous studies in gerbera give similar indications. Transgenic lines with suppressed *GRCD2* (a *SEPALLATA*-like gene) expression retain an undifferentiated, expanding region in the centre of the inflorescence that permits continuous initiation of new disc flower primordia (Uimari *et al*., 2004). In sunflower, the final number of flowers in a capitulum is determined by the initial meristem area before flower initiation and the rate of tissue expansion in the meristem during flower initiation while the duration of the expansion phase remain stable under different environmental conditions (Dosio *et al*., 2006). Our data suggests that in gerbera, GhCYC5 affects the rate of flower initiation in a given time frame leading to increased flower density. Interestingly, the organization of the capitulum was not affected, and their appearance did not differ from the wild type.

The phenotypic changes caused by ectopic expression of GhCYC5 in *Arabidopsis* were similar to the other gerbera CYC2 clade genes indicating differentiation of the regulatory network in Asteraceae. The yeast two hybrid

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assays by Tähtiharju *et al*. (2012) indicated that GhCYC5 is exceptional compared to the other CYC2 clade proteins as it did not show ability to interact with the other CYC-like proteins nor to homodimerize. The early stages of meristem patterning and flower initiation in Asteraceae are poorly understood and, in fact, functional analyses of putative meristem identity determinants are entirely lacking. Thus,more detailed analyses using lines in which *GhCYC5* is silenced as well as analyses of GhCYC5 target genes and its interactome are needed to discover the mechanisms of GhCYC5 function and possible connections with other key regulators involved in flower initiation.

EXPERIMENTAL PROCEDURES

In situ **expression analysis**

For *in situ* analysis, the treatment of the plant material and sectioning was done as in Elomaa *et al*. (2003). Gene specific probes for the CYC2 clade genes *GhCYC3*, *GhCYC4*, *GhCYC5*, *GhCYC7* and *GhCYC9* corresponding to their 3'UTR sequences were amplified with primers shown in Table S3. All probes were amplified with primers containing a few extra nucleotides and a T7 overhang (CAtaatacgactcactataggg) in the 5' end. When included in the forward primer, the T7 recognition site gave the sense probe whereas the in the reverse primer, an antisense probe was obtained. The gel-purified PCR products (200-300 bp) were used as templates for *in vitro* transcription with T7 polymerase and labelled using the DIG RNA Labelling Kit (Roche) according to the manufacturer's instructions. Hybridizations were performed essentially as described in Ruonala *et al*. (2008). For the post-hybridization washes, an InsituPro Vsi 3.0 (Intavis AG) device was used as in Hofer *et al*. (2012). The

colour reaction to visualize the hybridized probe was done for approximately 24 hours at room temperature. Sections were photographed using AxioImagerZ1 (Zeiss, Munich, Germany) equipped with AxioCam MRc (Zeiss).

Quantitative real-time PCR analyses

Ray flower petal samples corresponding to inflorescence developmental stages 1-11 (Helariutta *et al.* 1993, Laitinen *et al.* 2007) were collected from the variety Terra Regina (Terra Nigra B.V., The Netherlands). During stages 1-3 the petals are covered by involucral bracts. At stage 5, anthocyanin pigmentation becomes visible. The opening of the inflorescence occurs between stages 7 and 8 and at stage 9, the petals have reached their final length and width. After stage 11 the petals start to wilt (Kotilainen *et al.*, 1999). For the crested cultivar CH02.663 (Terra Nigra B.V., The Netherlands) and the radiate cultivar Terra Regina, marginal and central flower primordia samples corresponded to primordia stages 4 and 6 (Laitinen *et al.*, 2006), and samples were pooled from 4-6 inflorescences. Total RNA was isolated using the Trizol reagent (Life Technologies/Gibco-BRL) following the manufacturer's instructions. The RNA was treated with RNase free DNase and cleaned up with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). RNA concentrations were equalized within a sample set and the RNA integrity was analyzed by gel-electrophoresis. The cDNA synthesis, real-time RT-PCR and the evaluation of product specificity was carried out as previously described (Tähtiharju *et al.* 2012). The qPCR primers were the same as used previously (Tähtiharju *et al.* 2012, Table S3). The PCR efficiencies were analyzed for all primer combinations to make sure that they were close to 100% (Table S3). Relative expression levels were calculated using

the $2^{-\Delta Ct}$ method (Pfaffl 2001). The expression levels were normalized to *GhACTIN* expression levels. Two biological replicate samples each with three technical replicates were used for the real-time RT-PCR.

Inducible transgene constructs

The GhCYC ORFs were fused to the rat glucocorticoid receptor (GR) encoding sequence (Lloyd *et al.* 1994) and expressed under CaMV35S promoter. The forward primers used to amplify *GhCYC* open reading frames introduced a restriction site immediately before the respective start codons and the reverse primers removed the GhCYC stop codons and created a restriction site at the C terminus. The PCR fragments were digested with and cloned into the BamH1 restriction site of the plasmid GR-pBluescript (pRS020; obtained from the Arabidopsis Biological Resource Center; Sablowski and Meyerowitz, 1998; Lloyd *et al*., 1994) to create in-frame translational fusions at the C-terminus of GhCYCs with the rat glucocorticoid hormone-binding domain. The fusion fragments (*GhCYC:GR*) were further cloned into the plasmid vector pHTT603 Sma1 site. The vector pHTT603 is similar to pHTT602 (Elomaa and Teeri, 2001) but contains the multiple cloning site in reverse orientation. The *35S::GhCYC:GR* constructs in pHTT603 were conjugated into *Agrobacterium tumefaciens* strain C58C1 (van Larebeke *et al*., 1974) with the disarmed Ti plasmid pGV2260 (Deblaere *et al*., 1985) using triparental mating (van Haute *et al*., 1983).

Plant transformation

The ORFs for gerbera CYC2-clade genes and the *Arabidopsis* ortholog *TCP1* were cloned into the Gateway^TM entry vector pDONR221 (Tähtiharju *et al.*, 2012) and then under the CaMV35S promoter in pK7WG2D (Karimi *et al.*, 2002), following Invitrogen's instructions. The *Arabidopsis* ecotype Columbia was transformed by floral dipping method (Clough and Bent, 1998). Kanamycin-resistant transformants (T1 generation) were first selected on MS plates (50 µg/ml kanamycin) and then transplanted on soil and grown in long day conditions. We screened for 27 – 63 T1 plants per construct (52 T1 plants for *35S::TCP1*, 50 for *35S::GhCYC2*, 60 for *35S::GhCYC3*, 27 for *35S::GhCYC4*, 62 for *35S::GhCYC5*, 38 for *35S::GhCYC7*, and 43 for *35S::GhCYC9*). Phenotypes were first screened in T1 generation, and 4-6 representative lines were chosen for characterization in T2 generation to confirm the heritability of the phenotypes. All the Figures shown are taken from representative T2 plants. Transgene expression was verified with RT-PCR from young leaf tissue of representative T2 lines.

Gerbera transformation was conducted as described in Elomaa and Teeri (2001). Transgene expression was verified with RT-PCR from capitula of 10-16 mm in diameter. For RT-PCR total RNA was isolated using the Trizol reagent (Life Technologies/Gibco-BRL) following the manufacturer's instructions. 5 µg of total RNA was used for the first strand cDNA synthesis according to Invitrogen's Superscript III RT protocol. 2 µl of cDNA was used as template for RT-PCR that was conducted with a gene specific forward primer for *GhCYC3*, *GhCYC4* or *GhCYC5* and a reverse primer GER297 (CGAAGTGTCTTGTGAGACTCC) that anneals to the GR sequence. The PCR

conditions were as following: 94°C 30 sec, 60 °C 60 sec, and 72°C 45 sec for 35 cycles.

Dexamethasone inductions and analysis of the induced plants

To activate the CYC protein function in transgenic gerbera lines, we used 10 μM dexamethasone (DEX) solution with 0,015% Silwet. The induction was done by pipetting the DEX solution on top of very young inflorescences located in the middle of the rosette between the emerging young leaves (3 ml/plant). The DEX treatment was done for five times, once a day, for all the other lines except for *35S::GhCYC4:GR*, in which the five day treatment stopped all growth and led to senescence of the plants. Thus, we used a three day DEX treatment for *35S::GhCYC4:GR* lines.

From the DEX-induced plants (GhCYC2, 3, 4 and 5) and DEX-treated wild type control, the lengths of the ligules (excluding the basal tube) were measured using five independent inflorescences per line. From a fully open inflorescence (stage 9, Helariutta *et al.*, 1993) all ray flowers were measured as well as more than 50 trans and disc flowers from the same inflorescence section. Stamen phenotypes were documented using light microscopy (Zeiss SteREO discovery V20) equipped with AxioCam ICc3 (Zeiss, Germany). Statistical pairwise test was done with SPSS using Dunnett's test or one way ANOVA.

For the DEX-treated *35S::GhCYC5:GR* we also calculated the number of flowers in the inflorescences, capitulum area (mm^2) and density of flowers (n/mm^2) from ten biological replicates per line (stage 9). The diameter of the capitulum was measured as an average of five measurements when all flowers and bracts were removed. The surface area was calculated with a formula of a

circle or, when perpendicular diameter measurements differed more than 1.5 mm, with a formula of an ellipse. Statistical pairwise test was done using Dunnett's test.

Scanning electron microscopy and cell measurements

Scanning electron microscopy (SEM) was used to measure cell sizes in disc flower ligules of the DEX-treated *35S::GhCYC3:GR* and *35S::GhCYC4:GR* lines in comparison to DEX-treated wild type. We analysed cell sizes from seven individual disc flower ligules from two inflorescences of a *35S::GhCYC3:GR* line (TR12), two inflorescences of a *35S::GhCYC4:GR* line (TR7), and three wild type inflorescences. SEM samples were prepared as described in Uimari *et al*. (2004). Samples were examined using the FEI Quanta 250 Field Emission Gun Scanning Electron Microscope at the Electron Microscopy Unit of the Institute of Biotechnology at the University of Helsinki. Images were taken from adaxial side of disc flower ligules, at the same distance from the tip of the ligule in relation to the ligule length (for wild type ligules, at the distance of 1.6 mm from the tip, and for the *35S::GhCYC3:GR* and *35S::GhCYC4:GR* lines, 2.8 mm from the tip of the ligule). Two images were taken from each sample, and the lengths of 20 cells per image were measured by using ImageJ 1.47 (http://imagej.nih.gov/ij/ index.html).

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SUPPORTING INFORMATION

Figure S1. *In situ* hybridization of *GhCYC9* and *GhCYC7* in developing inflorescence of gerbera.

Figure S2. Vegetative phenotypes of transgenic *Arabidopsis* seedlings ectopically expressing *TCP1* and gerbera CYC2 clade genes (7 days after germination).

Figure S3. Transgenic *Arabidopsis* with strong phenotypes.

Figure S4. Nuclear localization of gerbera CYC2 clade proteins.

Figure S5. Verification of *GhCYC:GR* expression in transgenic gerbera lines.

Figure S6. Phenotype of the transgenic *35S::GhCYC2:GR* line.

Table S1. Number of flowers, capitulum area (mm^2) and density of flowers (n/mm^2) in wild type and transgenic lines with activated GhCYC5 function.

Table S2. Length of the ventral ligules (mm) of different flower types in wild type and transgenic lines with activated GhCYC5 function.

Table S3. Primer sequences used in this study.

Experimental procedures. Subcellular localization of CYC2 clade proteins.

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TABLES

Table 1. Length of the ventral ligules (mm) of different flower types in wild type and transgenic gerbera lines with activated GhCYC3 function.

Line	Ray flower	Trans flower	Disc flower
wild type	47.3 ± 1.7	17.8 ± 2.0	11.3 ± 0.6
35S::GhCYC3:GR TR11	37.8 ± 2.8*	26.4 ± 1.7*	16.5 ± 1.2*
35S::GhCYC3:GR TR12	38.8 ± 3.0*	27.1 ± 1.6*	15.7 ± 2.4*

The lengths of the ventral ligules were measured from two independent transgenic lines in comparison to wild type Regina after DEX-treatment. Differences were tested by pairwise comparison with Dunnett-test using five biological replicates. Statistical significance is indicated with * ($p < 0.001$).

Table 2. Length of the ventral ligules (mm) of different flower types in wild type and transgenic gerbera lines with activated GhCYC4 function.

Line	Ray flower	Trans flower	Disc flower
wild type 1	45.4 ± 2.5	17.9 ± 0.9	11.7 ±0.8
35S::GhCYC4:GR TR7	42.2 ± 2.3	29.3 ± 1,7*	19.4 ± 3.6*
wild type 2	47.7 ± 1.5	16.4 ± 1.8	11.3 ± 0.3
35S::GhCYC4:GR TR5	44.2 ± 4.3	35.0 ± 3.0*	20.4 ±1.6*

The lengths of the ventral ligules were measured from two independent transgenic lines in comparison to DEX-treated wild type Regina. Differences were tested by pairwise comparison with one way ANOVA using five biological replicates. Statistically significant differences are indicated with * (for TR7 $p < 0.002$ and TR5 $p < 0.001$).

FIGURE LEGENDS

Figure 1

In situ expression analysis of CYC2 clade gene family members. The panels in left show the expression patterns in the marginal ray flowers (RF) and panels in right in the outermost disc flowers (DF) of gerbera inflorescences (diameter 12 mm). Abbreviations: dPe = dorsal petal, vLi = ventral ligule, ruSt = rudimentary stamen, Ca = carpel, St = stamen, Pe = petal, Pa = pappus bristles. Scale bars 100 μm.

Figure 2

(a) Relative gene expression of gerbera CYC2 clade genes in a pooled ray flower petal samples. The expression levels were calculated relative to their expression in a root sample. (b) Expression of *GhCYC2*, *GhCYC3*, *GhCYC4* and *GhCYC5* during ray flower petal development (stages 1-11). Bars show the mean expression of two biological replicates. Expression levels of *GhACTIN* were used for normalization.

Figure 3

Ectopic expression of the gerbera CYC2 clade genes in *Arabidopsis* affects vegetative growth and petal size. In comparison to the wild type Columbia (a), ectopic expression of *GhCYC3* (d)*, GhCYC4* (e)*, GhCYC5* (f)*, GhCYC7* (g) and *GhCYC9* (h) but not *GhCYC2* (c), led to reduced vegetative growth and smaller petal size, similarly to the control *35S::TCP1* lines (b). Scale bars 1 cm.

Figure 4

The effect of activated GhCYC3 and GhCYC4 function on inflorescences, flower types and stamen development in transgenic gerbera. (a) Wild type Terra Regina inflorescence. (b) Inflorescence of a transgenic *35S::GhCYC3:GR* gerbera line. (c) Inflorescence of a transgenic *35S::GhCYC4:GR* gerbera line. (d) Ray, trans and disc flowers of the wild type. (e) Ray, trans and disc flowers of the transgenic *35S::GhCYC3:GR* gerbera line. (f) Ray, trans and disc flowers of the transgenic *35S::GhCYC4:GR* gerbera line. (g) Stamen of the wild type. (h) Disrupted stamen of the transgenic *35S::GhCYC3:GR* gerbera line. (i) Disrupted stamen of the transgenic *35S::GhCYC4:GR* gerbera lines. Scale bars 1 cm (a, b, c, d, e, f), 1 mm (g, h, i). The arrows in (e) and (f) point out to the enlarged dorsal petals that are rudimentary in the wild type (d).

Figure 5

Epidermal cell lengths of disc flower ligules in wild type and transgenic gerbera lines. (a) *35S::GhCYC3:GR* line and (b) *35S::GhCYC4:GR* line in comparison to wild type Terra Regina after DEX-treatment. The error bars represent the standard error of mean. The student's t-test showed that differences in cell numbers are not statistically significant (p-values for *35S::GhCYC3:GR*- and *35S::GhCYC4:GR*-lines 0.119 and 0.120, respectively).
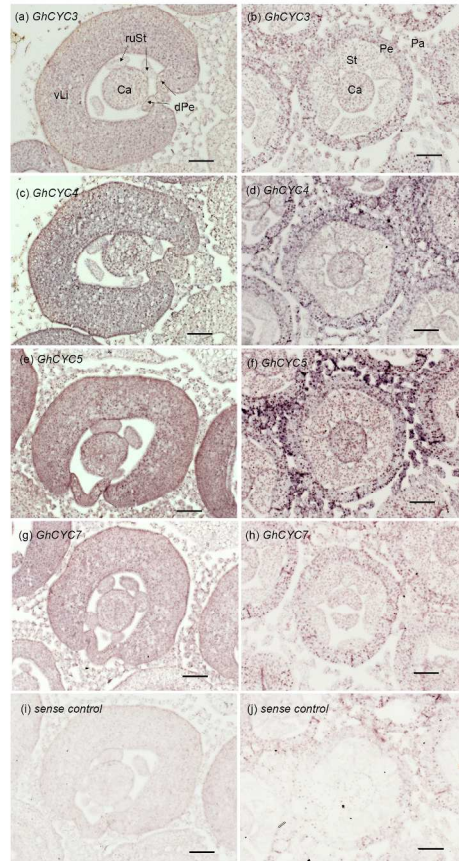
Figure 6

Expression of *GhCYC* genes in marginal and central flower primordia of the crested cultivar CH02.663 (b) in comparison to the radiate cultivar Terra Regina (a). (c) qPCR indicates that *GhCYC3* is upregulated in centremost flower primordia in the crested (Cre) cultivar. Two different developmental stages (4, 6)
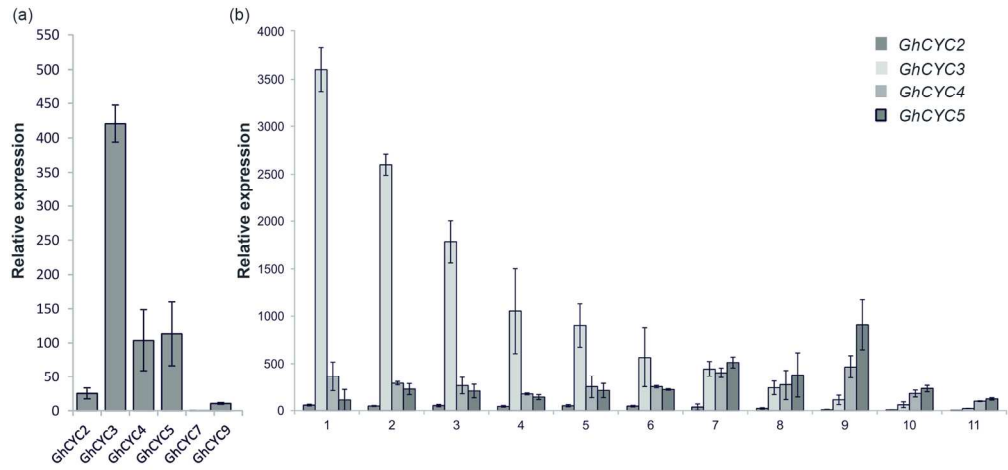
were included in the analysis. RF = ray flower primordia, CF = central flower primordia. Mean fold changes were calculated relative to the expression in Terra Regina separately at each developmental stage, for each gene and flower primordia type. Error bars represent standard deviations of three technical replicates. Fold changes were not analysed for *GhCYC2*, *GhCYC4* and *GhCYC5* in the central flowers since the expression levels were very low (Cp values >29).
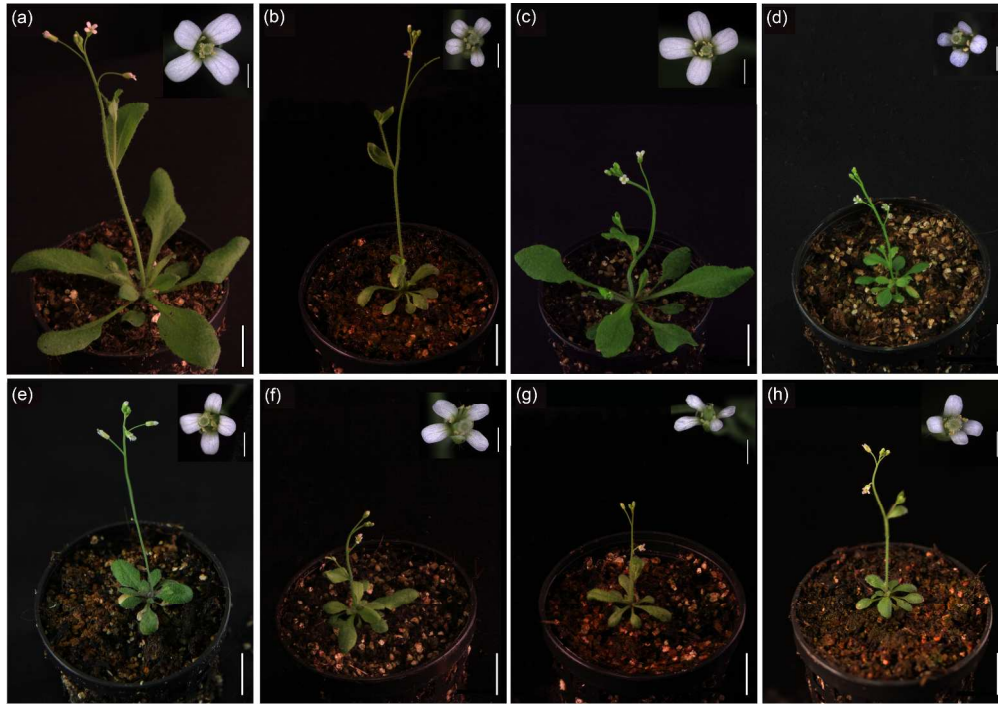
Figure 7

The effects of activated GhCYC5 function. (a) Wild type Terra Regina inflorescence. (b) Inflorescence of a transgenic *35S::GhCYC5:GR* gerbera line. (c) The density of flowers in an inflorescence indicated as a number of flowers per square millimeter (n/mm^2). The density of flowers was measured from five independent transgenic lines. Differences between the DEX-treated wild type Terra Regina and transgenic lines were tested by pairwise comparison with Dunnett-test using ten biological replicates. Statistically significant differences are marked with * (p< 0.001). Scale bars 1 cm.
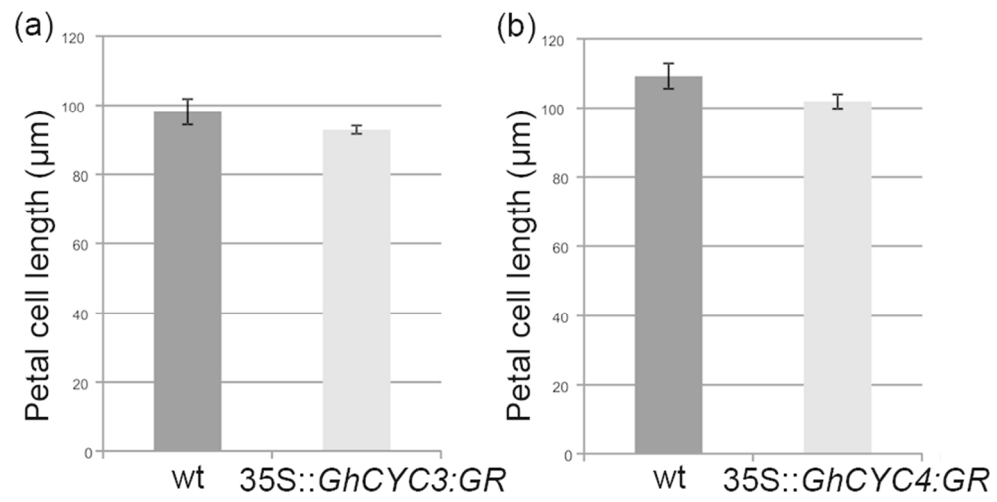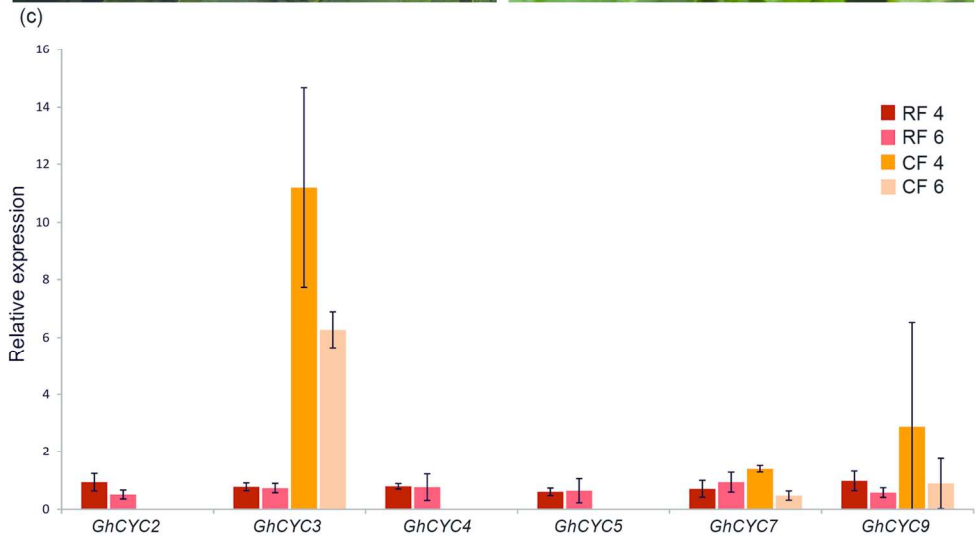
169x79mm (299 x 299 DPI)

152x152mm (300 x 300 DPI)

(a) Petal cell length (µm) — wt, 35S::*GhCYC3:GR*

(b) Petal cell length (µm) — wt, 35S::*GhCYC4:GR*

86x43mm (299 x 299 DPI)

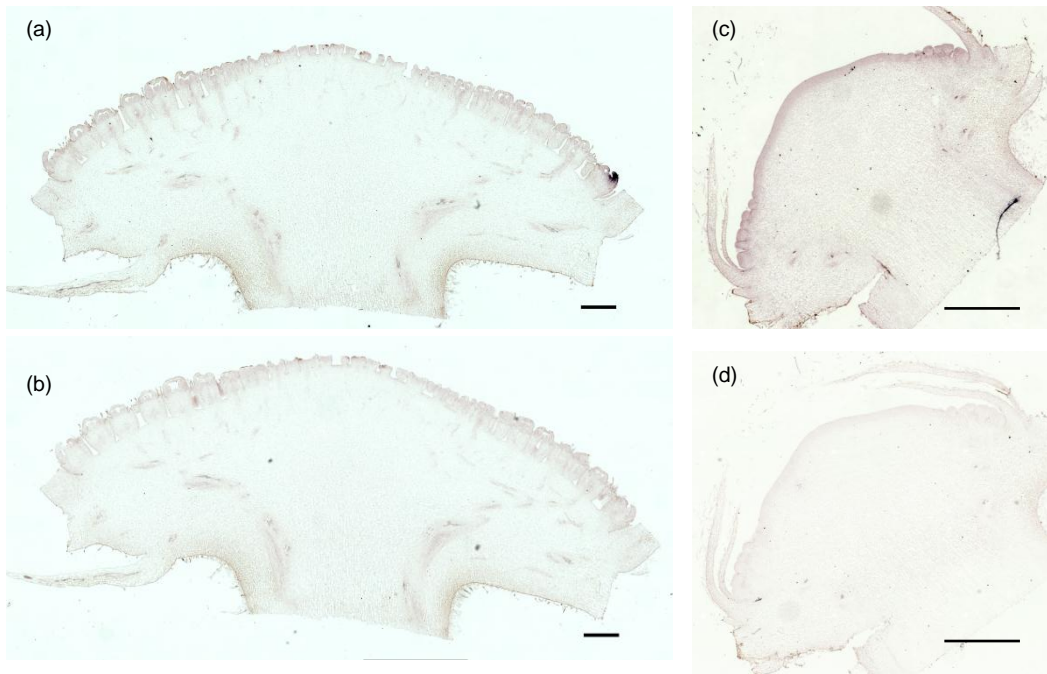138x138mm (299 x 299 DPI)

168x50mm (300 x 300 DPI)

Figure S1
In situ **hybridization of** *GhCYC9* **and** *GhCYC7* **in developing inflorescences of gerbera.** (a) *GhCYC9* expression in the capitulum of 12 mm in diameter is below detection level and comparable to *GhCYC9* sense control (b). (c) *GhCYC7* is uniformly expressed in the undifferentiated inflorescence meristem as well as in young emerging flower primordia in a capitulum of 3-4 mm in diameter. (d) Negative control hybridized with a sense *GhCYC7* probe. Scale bars 1 mm.
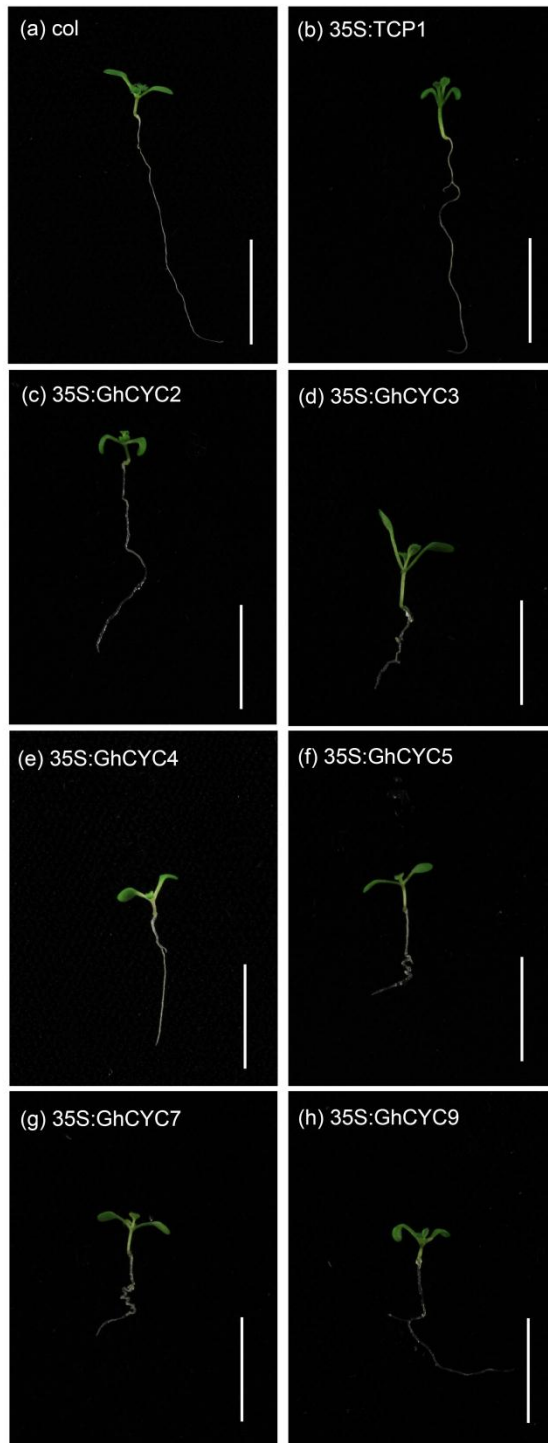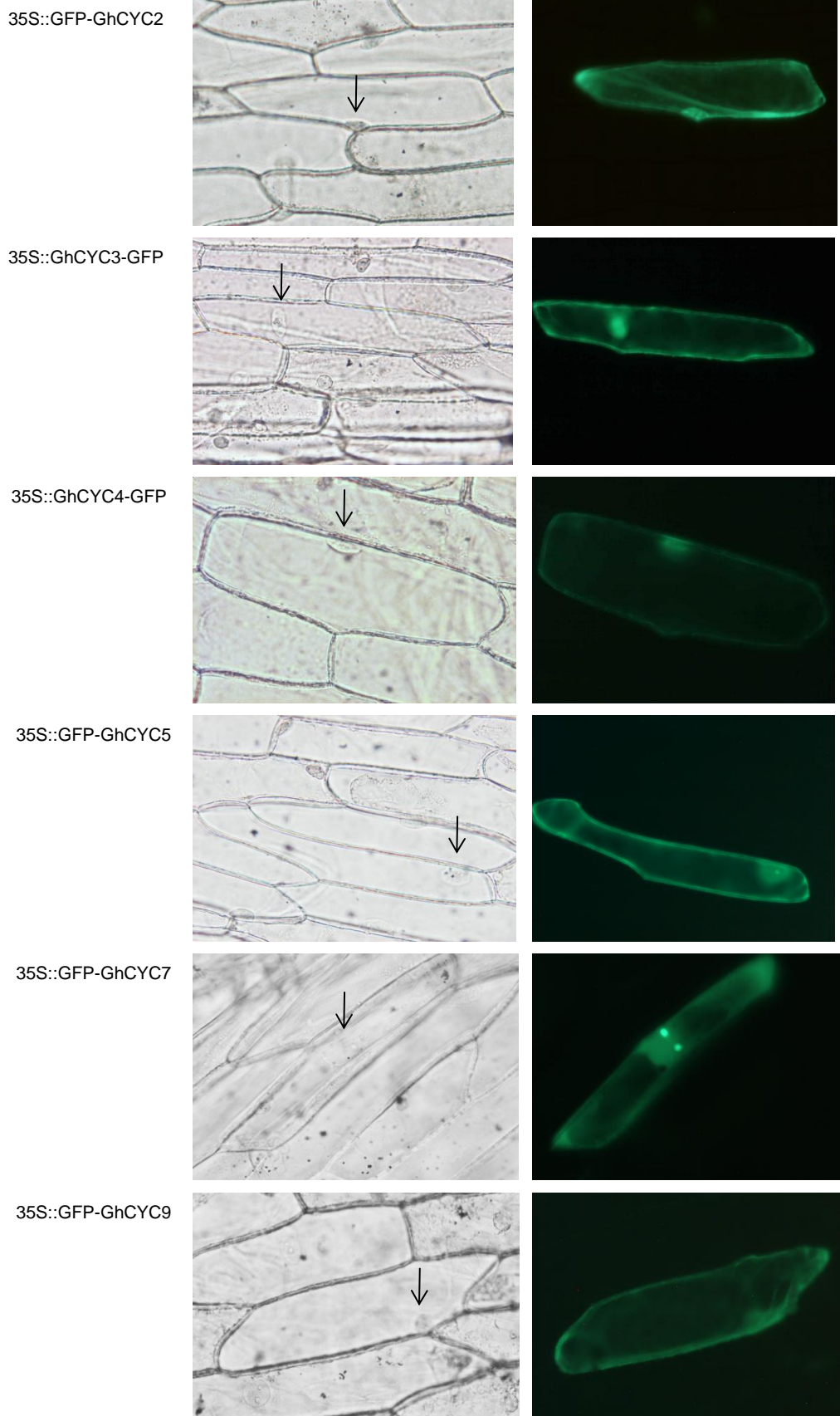
Figure S2
Vegetative phenotypes of the *Arabidopsis* seedlings ectopically expressing *TCP1* and the gerbera CYC2 clade genes (7 DAG). Wild type Columbia (a) and the transgenic *Arabidopsis* lines that ectopically express *TCP1* (b), and the gerbera CYC2 clade genes *GhCYC2* (c), *GhCYC3* (d)*, GhCYC4* (e)*, GhCYC5* (f)*, GhCYC7* (g), and *GhCYC9* (h) photographed 7 days after germination (DAG). Scale bars 1 cm.

Figure S3
The transgenic *Arabidopsis* with strong floral phenotypes. Wild type Columbia
(a), and the transgenic *Arabidopsis* lines ectopically expressing *TCP1* (b), *GhCYC4*
(c), and *GhCYC7* (c) that show the strongest phenotypes. These plants were dwarf
and their first flowers failed to open, petal and stamen development was severely
disrupted and carpels were unable to produce normal siliques with viable seeds.
Scale bars 1 mm.

35S::GFP-GhCYC2

35S::GhCYC3-GFP

35S::GhCYC4-GFP

35S::GFP-GhCYC5

35S::GFP-GhCYC7

35S::GFP-GhCYC9

Figure S4
Nuclear localization of gerbera GhCYC2 clade proteins. *GhCYC* ORFs were fused with GFP reporter and the constructs were delivered by particle bombardment into onion epidermal cells. Arrows are indicating the location of the nuclei.
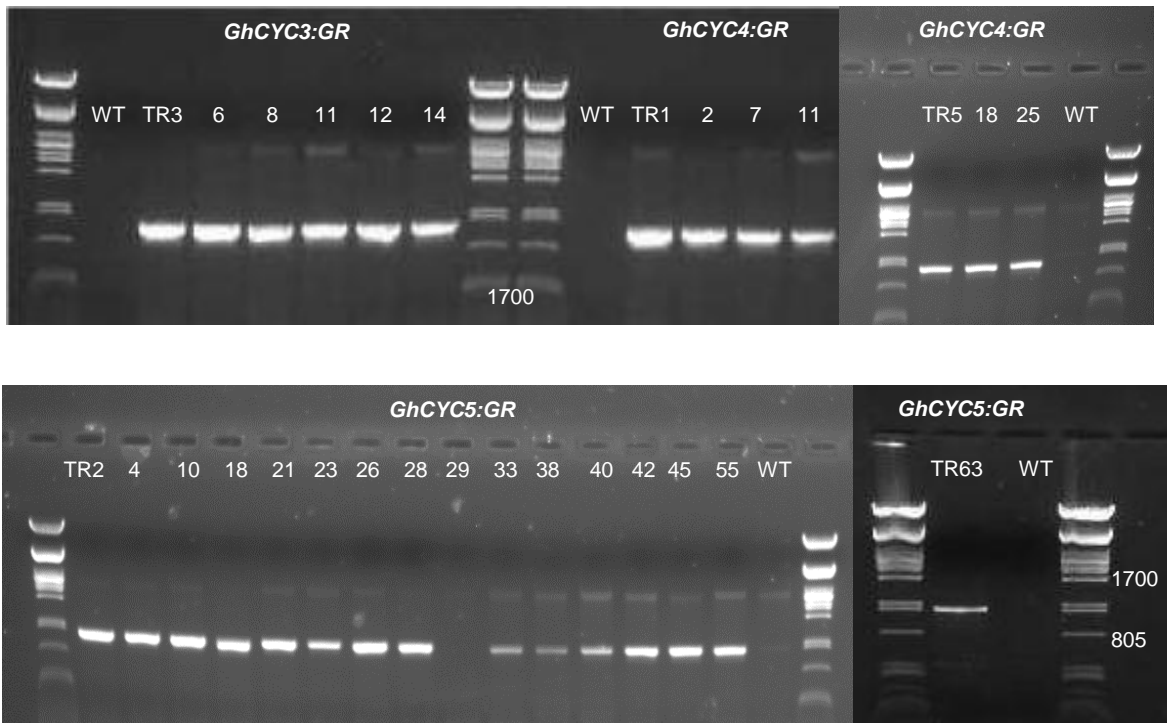
Figure S5
Verification of *GhCYC:GR* expression in independent primary transgenic gerbera lines by RT-PCR. WT represents non-transgenic Terra Regina.

Figure S6
Phenotype of the transgenic *35S*::*GhCYC2:GR* line. (a) Wild typeTerra
Regina inflorescence. (b) Inflorescence of a transgenic *35S*::*GhCYC2:GR*
gerbera line. (c) Ray, trans and disc flowers of the wild type. (d) Ray, trans and
disc flowers of the transgenic *35S*::*GhCYC2:GR* gerbera line. Scale bars 1cm.

Table S1. Number of flowers, capitulum area (mm^2) and density of flowers (n/mm^2) in wild type and transgenic gerbera lines with activated GhCYC5 function.

Line	Number of flowers	Capitulum area	Density
wild type	745 ± 93	265 ± 34	2.8 ± 0.3
35S::GhCYC5:GR TR2	612 ± 70	147 ± 13	4.2 ± 0.4
35S::GhCYC5:GR TR4	647 ± 95	149 ± 25	4.4 ± 0.7
35S::GhCYC5:GR TR21	816 ± 152	215 ± 46	3.8 ± 0.4
35S::GhCYC5:GR TR55	802 ± 78	218 ± 26	3.7 ± 0.5
35S::GhCYC5:GR TR63	878 ± 145	234 ± 37	3.8 ± 0.4

Number of flowers, capitulum area and density of the flowers were measured and calculated from five independent transgenic lines in comparison to DEX-treated wild type Regina using ten biological replicates.

Table S2. Length of the ventral ligules (mm) of different flower types in wild type and transgenic lines with activated GhCYC5 function.

Line	Ray flower	Trans flower	Disc flower
wild type	38.3 ± 2.7	17.7 ± 1.4	9.7 ± 1.0
35S::GhCYC5:GR TR2	35.9 ± 2.9	19.1 ± 1.7	9.8 ± 0.4
35S::GhCYC5:GR TR4	36.3 ± 1.6	20.6 ± 0.8	9.7 ± 0.6
35S::GhCYC5:GR TR10	37.4 ± 1.6	17.9 ± 1.0	9.9 ± 0.9

The lack of significant differences was tested using five biological replicates using pairwise comparison with Dunnett-test ($p>0.05$).

Table S3. Primer sequences used to amplify the gene specific probes for *in situ* hybridization analysis and primers used for qPCR expression analysis.

Name	Sequence	Additional information
Forward and reverse primers for *in situ* hybridization		Probe length
GhCYC3	GCAGTGTTTAGATCCCCAAGA	
	TAATGGCCTTGAACGTACCAC	257 (3'UTR)
GhCYC4	TCCAATCAAGCTGTTGGAGT	
	TTTCACCATCCCTACCAGGA	242 (3'UTR)
GhCYC5	AGGCTCGAATGCAATGATTT	
	CTCCAGTTAATTCCCCCAAA	217 (3'UTR)
GhCYC7	AAAAGCAGGCTCGAATCCAT	
	CAAGCACTCAAAGGCATACAA	215 (3'UTR)
GhCYC9	GACCAGTCAAGGGCTGAAGC	
	AGAAAGCTGGGTCCTCTTATATCTT	224 (CDS+3'UTR)
qPCR oligos		Primer efficiences
GhCYC2		
GER43	GGAAAGAAAACGACACGAAAACAC	
GER44	ACAGGACAGCGACATTCATTAAG	90,8%
GhCYC3		
GER69	AGGGATAGGAGGGTGAGATTGTC	
GER70	TCCGATCCTTCCTTCATGGTTTCC	96,8%
GhCYC4		
GER71	GACCTCAAAGAAAGATGGGCATAG	
GER72	CTTAATCGCTGTCTTGGACTTGG	104,6%
GhCYC5		
GER73	CGGCGATTAAGGAATTGGTTGAAG	
GER74	GCTCTTGCTCTTGCCTCTGC	102,3%
GhCYC7		
GER67	CGGTTCGTCTTCGTCGCTTTCC	
GER68	CCTCGCCCTCGCCTCTGC	96,6%
GhCYC9		
GER378	GAAGAAAGATGGGCGTAGCAAG	
GER379	TAGCGAAGAGCCAATCAAGGG	98,5%
GhACTIN		
GER37	AGGAAATCACTGCTCTTGCG	
GER38	AACAAACTCAACCCTCCAAACC	97,6%

Supporting information Experimental procedures

Subcellular localization of CYC2 clade proteins

The full open reading frame (ORF) of CYC2 clade genes were amplified and recombined (as described in Tähtiharju *et al*. 2012) into the pK7WGF2.0 vector (Karimi *et al*. 2002), resulting in an in-frame N-terminal fusion proteins of GFP gene and the respective GhCYC ORF under the control of the CaMV35S promoter. Similarly, the full ORFs of GhCYCs without the stop codon were recombined into the pK7FWG2.0 vector resulting in an in-frame C-terminal fusion of GhCYC with GFP (Karimi *et al*. 2002). The constructs were transformed into onion (*Allium cepa*) epidermal cells by particle bombardment using the Biolistic® PDS-1000/He Particle Delivery System (BioRad) essentially as described by Albert von Arnim (CSH Protocols 2007;doi:10.1101/pdb.prot4689) except that gold particles (1µm) were used instead of tungsten. Onion peels were mounted in water and GFP imaging was performed on an inverted epifluorescence microscope (Leitz Laborlux S, Ernst Leitz Wetzlar GmbH, Germany).