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Characterization of *SQUAMOSA*-like genes in *Gerbera hybrida*, including one involved in reproductive transition

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Abstract

Background: The flowering process in plants proceeds through the induction of an inflorescence meristem triggered by several pathways. Many of the genes associated with both the flowering process and floral architecture encode transcription factors of the MADS domain family. *Gerbera*, a member of the sunflower family, Asteraceae, bears compressed inflorescence heads (capitula) with three different flower types characterized by differences in both sexuality and floral symmetry. To understand how such a complex inflorescence structure is achieved at the molecular level, we have characterized the array of *Gerbera* MADS box genes. The high number of *SQUAMOSA*-like genes in *Gerbera* compared to other model species raised the question as to whether they may relate to *Gerbera*'s complex inflorescence structure and whether or not a homeotic A function is present.

Results: In this paper we describe six *Gerbera* genes related to the *SQUAMOSA/APETALA1/FRUITFULL* genes of snapdragon and *Arabidopsis*. Based on phylogenetic analysis of the entire gene lineage, our data indicates that *GSQUA1* and *GSQUA3* are members of the *SQUA/AP1* clade, while *GSQUA2*, *GSQUA4*, *GSQUA5* and *GSQUA6* are co-orthologs of the *Arabidopsis FUL* gene. *GSQUA1/GSQUA3* and *GSQUA4/GSQUA5/GSQUA6*, respectively, represent several gene duplication events unknown in the model systems that may be specific to either *Gerbera* or Asteraceae. *GSQUA* genes showed specific expression profiles. *GSQUA1*, *GSQUA2*, and *GSQUA5* were inflorescence abundant, while *GSQUA3*, *GSQUA4*, and *GSQUA6* expression was also detected in vegetative organs. Overexpression of *GSQUA2* in *Gerbera* led to accelerated flowering, dwarfism and vegetative abnormalities, all new and specific phenomena observed in transgenic *Gerbera* plants with modified MADS box gene expression.

Conclusions: Based on expression patterns, none of the *Gerbera SQUA*-like genes are likely to control flower organ identity in the sense of the floral A function. However, our data shows that the *FUL*-like gene *GSQUA2* plays a vital role in meristem transition. The roles of other *GSQUA*-genes in *Gerbera* floral development are intriguing, but require still further study.

Background

Arabidopsis thaliana has been the principal model plant for molecular developmental studies of flowers for two decades. Several traits of *Arabidopsis* contribute to its attractiveness as a model system. However, not all phenomena in angiosperm flower development are present in *Arabidopsis*, and some processes are in fact specific to *Arabidopsis* or its close relatives (reviewed in [1]). Thus, extrapolating floral developmental paradigms from *Ar-*

bidopsis to other flowering plants is not always straightforward [1-3]. To obtain a broader understanding of floral development, studies on species representing a broad taxonomic distribution are necessary. Our research interest has focused on floral development in *Gerbera hybrida*, a model species of the sunflower family (Asteraceae). *Gerbera* inflorescences consist of hundreds of flowers, which can be divided into three different types based on their size, sex, and position in the inflorescence. We have previously shown that many basic principles of floral development apply to *Gerbera* [4], but that in addition, *Gerbera* has special features of its own [5,6]. For example, the B and C functions of the ABC model of flower devel-

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opment [7] are applicable to *Gerbera*, but the A function has remained elusive.

Based on the ABC model, A function genes are involved in determining sepal and petal identity by repressing C function in whorls one and two [7]. *Arabidopsis* has two A class genes *APETALA1* and *APETALA2* (*API*, *AP2*) [8-12]. *API* is a MADS box gene, as are the majority of the ABC function genes [12], while *AP2* is a member of the *AP2/ERF* ethylene response family. Both *API* and *AP2* act as A function genes, but they also have several other functions (reviewed in [1]). *API* has been shown to fulfil a dual function in specifying *Arabidopsis* sepal and petal identity as well as affecting floral meristem development [9,13]. *API* acts closely together and partially redundantly with other inflorescence architecture genes, *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) [14]. Despite attempts to establish similar functions for related genes in other plant species, success has been limited. For example, the *Antirrhinum* *SQUAMOSA* (*SQUA*) gene plays a role in inflorescence meristem development but does not affect floral organ identity [15]. A similar function has been shown for the related gene *Antirrhinum* *DEFH28*, which is not involved in determination of sepal and petal identity [16]. Several plant species appear to have genes closely related to *API*, but apparently none have similar functions in specifying sepal and petal identity [17-22]. The pea (*Pisum sativum*) gene *PEAM4* seems to be the closest to *API* in function and has been suggested to be a functional homologue of *API* [23] based on similar expression pattern and floral phenotype. However, several authors [1,24-26] have been inclined to suggest that the entire concept of an A function might be specific to *Arabidopsis* and perhaps other Brassicaceae.

In addition to previously characterized *Gerbera* MADS box genes [4-6], we have recently identified several *Gerbera* genes similar to *API*, *FUL* [9,11] and *SQUA* [15]. *API* and *SQUA* are often described as A function genes, but only *API* has characteristics of a homeotic selector gene. *API* and *SQUA* do, however, play strong roles in defining floral meristem identity, together with the genes *LEAFY* in *Arabidopsis* and *FLORICAULA* in snapdragon [27,28].

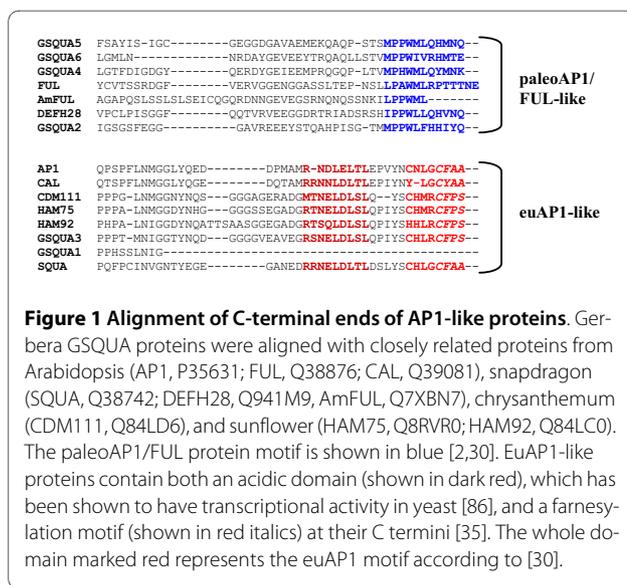
Here, we analyze the expression and phylogenetic position of six *Gerbera* genes, *Gerbera SQUAMOSA-LIKE1-6* (*GSQUA1-6*), which are closely related to *API*, *SQUA*, and *FUL*. Our data indicate that none of the *GSQUA* genes are, by themselves, likely to play a role in defining floral organ identity in the sense of the A function of the floral ABC model [7]. However, *GSQUA2* does function as a strong positive regulator of meristem transition in *Gerbera*. Overexpression of *GSQUA2* in transgenic *Gerbera* results in an early flowering dwarf phenotype, which displays abnormal vegetative architecture.

Results

Isolation and phylogenetic analysis of the *Gerbera hybrida* *GSQUA* genes

GSQUA1 was isolated earlier by low stringency screening of an inflorescence cDNA library using a spruce MADS box gene probe, and was so named based of its sequence similarity to *SQUA* of *Antirrhinum* [4,15]. PCR amplification using a degenerate MADS-box specific primer yielded three additional partial sequences of *Gerbera SQUA*-like genes: *GQUA2*, *GSQUA3*, and *GSQUA4*. Two more *SQUA*-like genes, *GSQUA5* and *GSQUA6*, were identified from a *Gerbera* EST collection [29]. Full length cDNA sequences were recovered using 5' and 3' RACE for all *GSQUA* genes except for *GSQUA4*.

In *Arabidopsis*, the A function/meristem-identity gene *API* and the fruit function/meristem-identity gene *FUL* share a high degree of sequence similarity despite their partially different functions [9,11,14]. The C termini of plant MADS domain proteins are variable, but within closely related groups, conserved protein motifs can be recognized. Both *API*- and *FUL*-like proteins are characterized by such motifs, the euAPI-motif for the former, and the paleoAPI- or *FUL*-motif for the latter [2,30]. Alignment of the predicted amino acid sequence of *GSQUA2* with similar sequences from other plant species showed that *GSQUA2* contains a protein motif similar but not identical to the paleoAPI/*FUL*-motif. The same motif was also recognizable in *GSQUA4*, *GSQUA5* and *GSQUA6*. In contrast, *GSQUA3* possessed a euAPI-motif (CFPS) that is divergent from the consensus motif (CaaX) [2,30], while still containing several conserved amino acids (Figure 1). In the previously isolated *GSQUA1* protein [4] a euAPI-motif was not evident, but phylogenetic analysis (Additional files 1 and 2) nevertheless suggested a close relationship between *GSQUA1* and



GSQUA3. The deduced *Gerbera* GSQUA amino acid sequence alignments and the corresponding protein motifs are shown in Figure 1.

Phylogenetic analysis suggested that *GSQUA1* and *GSQUA3* are close paralogs, together co-orthologous to *API* (and *SQUA*). Similarly, *GSQUA4*, *GSQUA5* and *GSQUA6* are co-orthologs of *FULL*, and *GSQUA2* is phylogenetically close to the snapdragon gene *DEFH28*. Although interrelationships among the *API/SQUA*, *DEFH28*, and *FULL* clades are not well supported in the phylogenetic analysis, the conserved C terminal motifs suggest that *GSQUA2/DEFH28/AmFULL* are *FULL*-like. The full maximum likelihood tree, based on our sequences added to the alignment of [2], is shown in Additional file 2. An alignment of *GSQUA* DNA sequences is shown in Additional file 1.

RNA gel blots and *in situ* hybridization of GSQUA genes

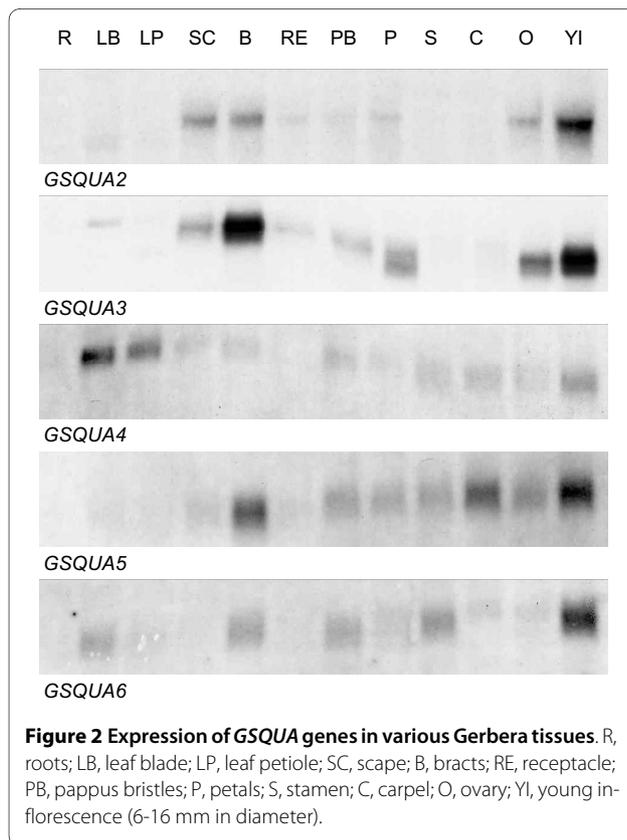
Figure 2 summarizes the expression patterns of *GSQUA2-6* at RNA gel blot level. Based on previous studies, *GSQUA1* expression was in the young inflorescence, scape and bracts [4]. In addition to *GSQUA1*, the expression of *GSQUA2*, and *GSQUA5* was restricted to floral tissues and no expression was detected in vegetative organs. Interestingly, *GSQUA3*, *GSQUA4* and *GSQUA6* also showed expression in leaves, in addition to expression in floral and inflorescence-derived organs. None of

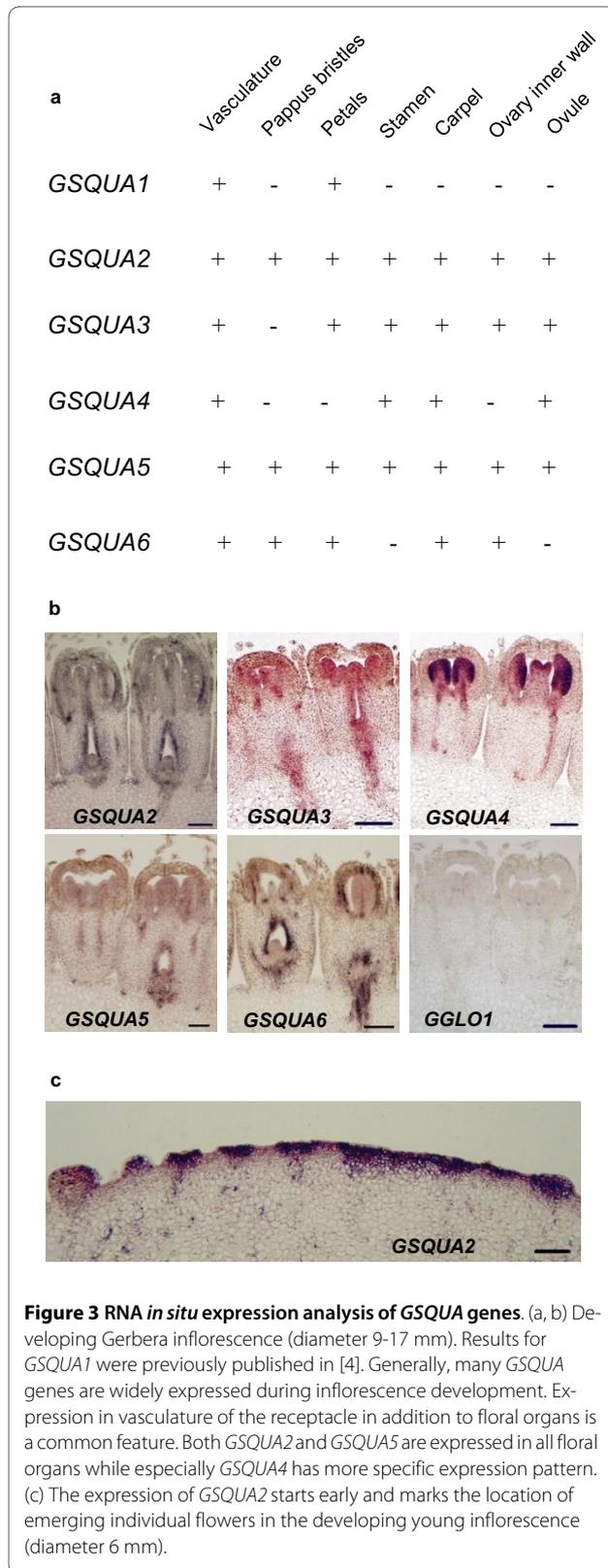
the studied *GSQUA* genes were expressed in *Gerbera* roots. At the level of single (ray) flowers at relatively late developmental stages, *GSQUA2* and *GSQUA3* transcripts were most abundant in whorls one and two, while *GSQUA4*, *GSQUA5*, and *GSQUA6* were expressed in all floral whorls (Figure 2). Different developmental stages of *Gerbera* ray flower petals (see [31]) were screened by RNA gel blot hybridization to ascertain whether expression levels of *GSQUA* genes varied over time. The expression levels of *GSQUA3* and *GSQUA5* did not vary during ray flower petal development, whereas the expression of *GSQUA4* was barely detected during ray flower petal development, and both *GSQUA2* and *GSQUA6* showed differential expression. *GSQUA2* expression was stronger during early stages (1,2,3) and faded noticeably toward later developmental stages (4,5,6,7,8,9,10,11). *GSQUA6* expression displayed a pattern opposite to that of *GSQUA2*; its expression grew stronger toward later developmental stages (8,9,10,11) (Additional file 3).

To localize *GSQUA* expression during the early stages of inflorescence development, a more detailed RNA *in situ* hybridization analysis of young, developing *Gerbera* inflorescences (diameter 6-17 mm) was performed (Figure 3). In general, *GSQUA* genes studied here showed a wide range of expression patterns. In fact, the vasculature of the capitulum receptacle was the only common location where all of the *GSQUA* genes were expressed. In contrast to other *GSQUA* genes, *GSQUA1* was entirely restricted to the vasculature of the capitulum receptacle and petals [4]. While *GSQUA2* and *GSQUA5* were found to be expressed in all parts of the inflorescence, *GSQUA3* and *GSQUA6* displayed a slightly narrower expression pattern at the inflorescence level. *GSQUA4* was expressed only in the reproductive organs in addition to the vasculature (Figure 3a, b,). Figure 3b shows examples of developing individual ray flowers, while the summary in Figure 3a is based on larger number of *in situ* hybridizations. *GSQUA2* expression was also seen in the receptacle between the emerging individual flowers (inflorescence size 6 mm, visible also in inflorescence size 14 mm) and petal expression was localized to the adaxial surfaces (Figure 3b). The location of emerging flowers in the developing inflorescence was marked by strong *GSQUA2* expression even before clear anatomical differentiation was visible at the center of the capitulum (inflorescence diameter 6 mm) (Figure 3c).

Phenotypic changes in *GSQUA2* overexpression lines

For functional analysis, we were only able to obtain clear and consistent phenotypes by overexpressing *GSQUA2*. Transformation of *Gerbera* with *GSQUA2* under the 35S promoter yielded five lines strongly overexpressing *GSQUA2* and one line with weaker overexpression, which correlated with milder phenotypic changes (Additional





file 4). Compared to the non-transformed Gerbera cultivar 'Terra Regina', all strong overexpression lines showed altered vegetative growth very early in development. The posture of the plants was upright, with leaves curving adaxially. The normal growth habit that leads to a tight rosette-like arrangement of leaves in Gerbera [32] was loosened, with the segments/vegetative axis of the stem strongly elongated. Inflorescences started to form after only two months in the greenhouse whereas the wild type cultivar 'Terra Regina' typically reaches the flowering stage after 6 months (Figure 4). Root formation of the overexpression plants was poor. The plants were susceptible to molds in greenhouse conditions and they typically died after forming only a few inflorescences. Transformants grown in more controlled and contamination-free growth chamber conditions survived for longer periods of time. The general appearance of overexpression lines of GSQUA2 was unstable due to their aberrant architecture, and they required support to remain upright. One milder phenotype was also observed (TR3). This line was not as dramatically dwarfed, but was clearly smaller and more delicate in structure, both vegetatively and inflorescence-wise, as compared to non-transgenic plants. RNA gel blot analysis showed strong expression for GSQUA2 in the inflorescence, but overexpression in leaves was weaker compared to overexpression lines showing the dwarfed phenotype (Additional file 4).

The number of flowers in the inflorescence of GSQUA2 overexpression lines was reduced compared to wild type. Non-transformed Gerbera 'Terra Regina' inflorescences, grown side by side with the transformants in the greenhouse, contained on average about 900 individual flow-

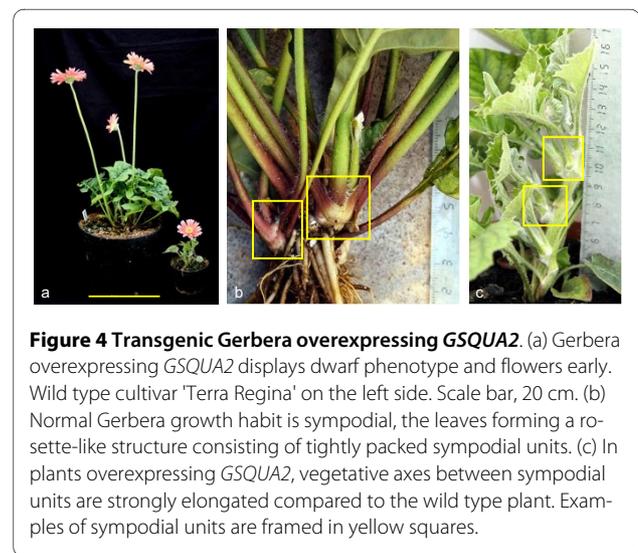


Table 1: The number of individual flowers in wild type *Gerbera* 'Terra Regina' inflorescence vs. *GSQUA2* overexpression lines

Inflorescence	Number of flowers/inflorescence	Average
wt 'Terra Regina'	882, 830, 965, 1001, 859	907,4
CaMV 35S :: <i>GSQUA2</i>	534, 457, 535, 371, 202	419,8*

* Significant at $P < 0.001$ (t-test)

Inflorescences upregulated for *GSQUA2* contain less than 50% of flowers of wild type *Gerbera*.

ers. The *GSQUA2* overexpression lines produced on average only 420 flowers in their inflorescences (Table 1).

Inspection of *GSQUA2* overexpression lines with stereomicroscopy or scanning electron microscopy showed no homeotic changes in floral organs of any flower type (data not shown). However, petals of all flower types were shorter compared to the wild type petals, which is congruent with dwarfism and the overall smaller size of the inflorescence. Additionally, inflorescence color differed from wild type in being paler. Despite three transgenic lines producing antisense RNA for *GSQUA2*, no silencing of the endogenous *GSQUA2* was observed.

Discussion

The *GSQUA* subfamily of *MADS* box genes contains at least six members in *Gerbera*

In addition to the previously published *Gerbera SQUA*-like genes, *GSQUA1* [4], *GSQUA5* and *GSQUA6* [29], we isolated three new sequences, *GSQUA2*, *GSQUA3* and *GSQUA4*. The number of *GSQUA* genes is large compared to most other plant species and it is tempting to relate this diversity to the complex structure of the *Gerbera* inflorescence [33]. *Arabidopsis API* and *FUL*, which function in sepal and petal, fruit, and meristem development [9,11,14], are closely related to *GSQUAs* at the sequence level. The relationship of the two *Arabidopsis* proteins has been further analyzed by [2] and [30], and they described conserved C-terminal protein motifs (euAP and paleoAP/*FUL*) in a number of *API*- and *FUL* like sequences. Identification of these motifs facilitates the classification of related proteins, since phylogenetic analysis of *API*- and *FUL*-like sequences is not always unambiguous. The paleoAP/*FUL*-like protein sequences have a hydrophobic motif (L/MPPWML), which is not found in euAP1-like sequences. EuAP1-like sequences in turn have two conserved motifs, a transcription activation domain RRNaLaLT/NLa (where 'a' stands for an acidic amino acid [2]) and a farnesylation signal CaaX (where C is Cys, 'a' is an aliphatic amino acid, and X is Cys, Met, Ser, Ala, or Glu [34]) that terminates the protein. A farnesylation motif generally directs proteins to a membrane [34], but the role of farnesylation in plant proteins might be more diverse [35-37]. In the case of transcription factors, this function could be part of post-

transcriptional regulation, or necessary for protein complex formation [36]. AP1 has been shown to be farnesylated *in planta*, but membrane localization was not observed [36]. Not all euAP1-like proteins possess this farnesylation signal, however, and thus it may not be an essential part of the protein function [1,23].

Based on the presence of conserved C terminal protein motifs, *GSQUA3* can be classified as belonging to the euAP1-like proteins, while *GSQUA2*, *GSQUA4*, *GSQUA5* and *GSQUA6* harbor a paleoAP1/*FUL*-like protein motif at the C terminus of their amino acid sequence (Figure 1). *GSQUA1* does not possess a recognizable protein motif of either type at its C terminal end, but phylogenetic analysis places it close to *GSQUA3* (Figure 2). In fact, the *GSQUA1* sequence terminates 16 amino acids before the expected euAP1 protein motif. Furthermore, the *GSQUA3* protein sequence contains the transcriptional activation domain RSNELDSL, but no strong transcriptional activation was seen in yeast assays [38]. The motif differs slightly from the consensus motif RRNaLaLT/NLa [2], the second arginine being replaced by serine in *GSQUA3* and threonine or asparagine being substituted for serine. The functional relevance of these changes is not clear. Despite the close sequence similarity in the C terminal domain of *GSQUA3* to related proteins such as AP1 and SQUA [9,11,15], the farnesylation domain of *GSQUA3* (CFPS) differs from the most common version of the motif, CFAA/T [35], which is found in many plant SQUA-like proteins [2]. EuAP1-protein motifs similar to *Gerbera GSQUA3* are also present in related protein sequences of other species in Asteraceae, including sunflower (*Helianthus annuus*) and Chrysanthemum (*Dendrathera grandiflorum*) (HAM75, HAM92, CDM111) [21,39]. Still, these Asteraceae specific variants are within the definition of the farnesylation motif CaaX [35]. The current definition of the consensus motif is possibly too narrow, and as more plant species are studied in detail, the farnesylation consensus motif may require redefinition.

A detailed phylogenetic analysis of *GSQUA2*, *GSQUA3*, *GSQUA4*, *GSQUA5* and *GSQUA6* produced results in line with the relationships suggested by analysis of C terminal protein motifs. The maximum likelihood tree suggests that *GSQUA2* may be orthologous to the

snapdragon gene *DEFH28*, which is involved in the regulation of floral meristem identity and fruit development [16]. Both of these *DEFH28* functions are similar to *FUL* of Arabidopsis, and the authors concluded that *DEFH28* most likely represents the ortholog of *FUL*. However, this interpretation was later challenged by [2] based on the discovery of *AmFUL*, which, according to phylogenetic and protein motif analysis more likely represents the snapdragon gene orthologous to *FUL*. Unfortunately, *AmFUL* has not been further characterized. *GSQUA2* does share the early flowering function of *DEFH28*, however. A potential role of *GSQUA2* in fruit development was not studied in this work.

Previous and recent studies on *FUL*-like genes further distinguish two groups [1,2,40]. *FUL* and *AmFUL* belong to the *euFUL* group [41,2], while *AGL79* and *DEFH28* belong to *euFULII* group [16,40]. Based on the phylogenetic analysis *GSQUA4*, *GSQUA5*, and *GSQUA6* genes are closer to the *euFUL* group, while *GSQUA2* belongs to the *euFULII* group.

GSQUA1 [4] and *GSQUA3* appear to be recent paralogs and are co-orthologous to *SQUA* of snapdragon [15]. Similarly, *GSQUA4*, *GSQUA5* and *GSQUA6* are coorthologous to *FUL* of Arabidopsis [14,41].

The expression patterns for *GSQUA* genes do not support a homeotic A function

All *GSQUA* genes, despite being closely related, exhibit different expression patterns at the vegetative and floral organ levels. However, none of the *GSQUA* genes investigated share the expression pattern of Arabidopsis *API* or snapdragon *SQUA* in the sense that they would be particularly abundant in floral whorls 1 and 2 (sepals and petals) in early stages of development. In general, at earlier developmental stages, expression domains of *GSQUAs* are widespread at the inflorescence level, with the exception of *GSQUA4*, which is expressed in reproductive organs and in the vasculature of the capitulum receptacle (Figure 3a, b). Only later in floral development *GSQUA2* and *GSQUA3* are weakly expressed in sepals and petals (Figure 2). The expression in vasculature is common among all *GSQUA* genes studied here. Expression in vasculature is also known for *FUL* [41] and *AmFUL* [2], but vascular expression is not a uniform trait for *euAPI*-, *euFUL*- or *euFULII*-like genes. This expression pattern may reflect a function in developing vascular bundles, but the phenomenon has not been extensively discussed previously and its functional significance for *GSQUA* genes remains unclear.

The broad expression pattern of *GSQUA2* during early stages of ray flower development resembles what has been previously reported for *FUL* and other *FUL*-like genes, and contrasts with the expression of *API*, which is confined to the first two whorls [10]. *FUL*-like genes are

commonly expressed in the carpel [21,42-45], meristems [13,41] and vegetative tissues, including bracts [18,21,43,46]. Expression has also been observed in the inflorescence [18,19,21,47,48], floral meristems [19,49], stamens [17,45], and perianth organs [17,42,43,45]. For some species, expression has been visible in all floral whorls [45,50]. The expression pattern for Arabidopsis *FUL* is biphasic, which is in accordance with its early (floral meristem identity) and late (silique development) functions in reproductive development [14,41].

The functional role of *FUL* in fruit development was first detected in Arabidopsis mutant lines lacking *FUL* expression. Gerbera does not bear a fruit similar to Arabidopsis; its ovary position is inferior as opposed to superior in Arabidopsis and the fruits (achenes) are indehiscent. Thus the late function for *GSQUA2* might be entirely different (like *DEFH28* in snapdragon; [16]) or lacking completely. The most dramatic phenotypic effects in 35S::*FUL* lines are cell type changes in valve margins and the outer replum, which lead to developmental failure of the dehiscence zone and eventually to indehiscent fruit [14]. Interestingly, *GSQUA2* expresses strongly in ovary inner walls and the ovule (Figure 3), so despite the fact that no homeotic changes in *GSQUA2* overexpression lines were visible in ovaries and ovules at the relatively late developmental stage 8, a role for *GSQUA2* in Gerbera fruit development, possibly at the level of cell differentiation, cannot be ruled out.

GSQUA2 is involved in meristem transition

Among the several related *GSQUA* genes of Gerbera, only *GSQUA2* lent itself to further functional characterization based on transgenic Gerbera lines overexpressing the gene. Several transgenic lines both for *GSQUA3* and *GSQUA5* were generated and analyzed for overexpression and downregulation, but no consistent floral phenotypes were observed. Both genes, *GSQUA2* and *FUL*, seem to share the same function of meristem identity determination in early floral development, but the inflorescence abundance of *GSQUA2* expression distinguishes it from *FUL*, as *FUL* is expressed also in vegetative parts of Arabidopsis [13]. However, when *GSQUA2* is ectopically expressed throughout Gerbera tissues, dramatic vegetative changes such as dwarfism and vegetative axis elongation appear. Gerbera growth habit is sympodial with very short, leafy lateral shoots forming the sympodia. Typically, the sympodial rhizome forms 7-24 leaves before the first inflorescence is formed by the apical meristem. Two inflorescences are formed per one vegetative shoot, the second inflorescence being formed in the axil of the uppermost leaf primordium. The vegetative axis continues to develop in the axil of the second leaf primordium. The fully-formed axis grows 2-8 leaves before forming a terminal inflorescence, a lateral inflorescence,

and again a vegetative shoot, the growth cycle being iterative [32]. The vegetative axis between lateral shoots is very short and the lateral shoots form a tightly packed entity. However, in plants overexpressing *GSQUA2*, the vegetative axis between lateral shoots is strongly elongated compared to wild type *Gerberas* (Figure 4). The poor root formation of the overexpression lines may be to ectopic expression of *GSQUA2* under the 35S promoter, which interferes with the normal root development and is thus not necessarily informative of the gene's normal function.

Overexpression lines of *GSQUA2* flower substantially earlier than wild type plants, which suggests this gene to be involved in floral meristem transition. The strong localized expression of *GSQUA2* in emerging flower primordia at the early stages of flower development also supports this hypothesis (Figure 3c). Despite of the strong expression in overexpression lines, only minor morphological changes, such as reduced petal size and color, were detected at the level of individual flowers. At the inflorescence level, however, a considerably reduced number of flowers was observed, since the overexpression lines for *GSQUA2* contained only half the number of flowers in their inflorescences as non-transgenic *Gerbera*. A similar phenomenon was reported with birch *BpMADS4* overexpression lines [51], and may relate to accelerated development, including accelerated consumption of the inflorescence meristem.

In wheat and ryegrass, the *API*-like MADS-box gene *VRNI* is expressed in vegetative tissues and has been suggested to control the transition to flowering [52,53]. Based on the vegetative expression pattern, *GSQUA3*, *GSQUA4* and *GSQUA6* are *Gerbera* candidates for this kind of function, but at least for *GSQUA3* we have data that its ectopic expression does not cause early flowering.

In *Arabidopsis*, accelerated flowering is regularly observed when different MADS-box genes are overexpressed, including those not directly related to flowering time [54-63]. In *Gerbera*, all overexpression lines with MADS box genes other than *GSQUA2* have retained their normal vegetative size and flowering time, although many have displayed homeotic or meristem identity changes in the inflorescences [4-6].

GSQUA proteins interact with other *Gerbera* MADS domain proteins

API/*SQUA*-like MADS domain proteins have been suggested to function as mediators of higher order complex formation, acting as 'bridge proteins' and facilitating the formation of protein quartets [64,65]. However, based on pairwise assays [38], *GSQUA* proteins seem unlikely to function as interaction mediators in *Gerbera*, since their interaction capacity appears to be limited [38]. This feature distinguishes all *GSQUA* proteins from the closely

related *Petunia* protein FBP29. FBP29 is capable of interacting with several MADS domain proteins of different functional classes [43]. Moreover, other *FUL*-like proteins from *Petunia*, PFG and FBP26, show more extensive interaction capacity than the studied *GSQUA* proteins [42,43]. Also *Arabidopsis* *FUL* was shown to be active in multiple protein-protein interactions [66]. *GSQUA2* was found to interact with three other *Gerbera* MADS domain proteins in a screen of fourteen proteins, whereas *GSQUA1* and *GSQUA3* proteins interacted with only two other proteins, all partners being members of the *SEP*-like *GRCD* family of *Gerbera* proteins. *GSQUA5* remained inactive in pairwise assay showing no interaction with any tested *Gerbera* proteins. The most interesting *GSQUA2* specific partner is *GRCD2*, a pleiotropically active *Gerbera* *SEP*-like protein with functions in carpel identity, meristem identity and inflorescence determinacy [6]. Interestingly, when *GSQUA2* and *GRCD2* were combined in yeast, a strong autoactivation function emerged - separately, neither of the proteins show transcriptional activation. This function of the *GSQUA2*/*GRCD2* dimer could reflect its importance in *Gerbera* floral development. Both *GSQUA2* and *GRCD2* are co-expressed in young inflorescences and their expression patterns are overlapping [6], rendering the interaction feasible also *in planta*.

When assaying for higher order complex formation, *GSQUA* proteins showed greater activity. Together with the *Gerbera* B function dimer *GGLO1*/*GDEF2*, and when combined with a *Gerbera* *SEP*-like *GRCD* protein and with a C function *GAGA* protein, all *GSQUA* proteins showed activity [38]. While *GSQUA* proteins did not interact with each other in the pairwise assays, addition of a *GRCD* protein made some complexes with two *GSQUA* proteins stable in yeast.

Even as interaction of *GSQUAs* with E function proteins (*GRCD4* and *GRCD5*, pairwise) or with B function proteins (*GGLO1*/*GDEF2*, threesome) can be seen as consistent with a homeotic A function for *GSQUAs*, interaction with C function proteins (*GAGA1* and *GAGA2*, threesome with *GRCDs*) is not. In *Arabidopsis*, expression of *API* (with homeotic A function) is excluded in cells where the C-function gene *AGAMOUS* (*AG*) is expressed [67]. *API* alone does not repress the C function in whorls one and two, but rather acts together with the non-MADS proteins *LEUNIG* and *SEUSS* [68,69] in a complex including other MADS domain proteins, *AGL24* and *SVP* [68]. However the *AG* gene has functions beyond the floral homeotic one in *Arabidopsis*. *AG* is known to control the meristematic state of flower primordia and to downregulate the meristem organizing gene *WUSCHEL* together with unknown factors [70,71] which in *Petunia* are MADS domain proteins [72].

It is tempting to relate the large number of *SQUA*-like genes in *Gerbera* to the complex structure of the inflorescence in Asteraceae. At least some interactions for homologous *Chrysanthemum* MADS domain proteins are similar to the *Gerbera* proteins. CDM41, which is closely related to *GSQUA* proteins, interacts with *Chrysanthemum* CDM44, which is homologous to *SEP3* of *Arabidopsis* [21]. This interaction is similar to *GSQUA*'s interaction with *GRCD4* and *GRCD5*. In yeast three-hybrid assay, CDM41 combined with the *Chrysanthemum* B protein heterodimer (CDM86 and CDM115), and the complex was active, as are *Gerbera* complexes with a *GSQUA* protein and the B protein dimer. Sunflower (*Helianthus annuus*) also contains several genes closely related to *API* and *FUL* [39]. Obviously duplication of this lineage of genes has also taken place in sunflower. Perhaps gene duplication and divergence in the *SQUA/ API/ FUL* gene lineage has participated, together with the unique diversity in *TCP* family transcription factors [73] to help shape the complex Asteraceae inflorescence.

Conclusions

Gerbera has an array of *SQUA*-like genes, which can be classified either as euAPI-like, or as FUL-like [2,30]. However, none of these genes appear to act as an A function gene in the sense of the classical ABC model [7]. Based on these results, *Gerbera* can be added to the growing list of plant species that lack the A function comparable to *Arabidopsis*. *GSQUA2* is intimately involved in the regulation of meristem transition in *Gerbera* as overexpression of *GSQUA2* led to accelerated flowering. The role of *GSQUA1*, *GSQUA3*, *GSQUA4*, *GSQUA5*, and *GSQUA6* in the floral development of *Gerbera* requires further study. The complex inflorescence structure and the high number of *Gerbera GSQUA*-like genes lead to a temptation to associate these two phenomena, but verifying this hypothesis requires more research.

Methods

Identification of *Gerbera GSQUA* genes

GSQUA2, *GSQUA3* and *GSQUA4* were identified using reverse transcription PCR with inflorescence mRNA as a template. The 5' primer E0364 (GCG GAG CTC GAG TTA AGA GRA TAG ARA ACA, where R = A/G) was designed based on previously published alignment of the MADS domain from several plant species, including *Gerbera* [4,74]. The 5' end of the primer contained two restriction enzyme recognition sites (for *SacI* and *XhoI*) to aid cloning. For the 3' end, an anchored oligo-d(T) primer (G ACC ACG CGT ATC GAT GTC GAC TTT TTT TTT TTT TV, V = G/C/A) (Boehringer Mannheim 5'/3' RACE kit 1734792) was used. This primer contained three restriction enzyme cut sites (*MluI*, *Clal*, *Sall*) at its 5' end. The cDNA was synthesized from *Ger-*

bera inflorescence mRNA (pooled RNA sample, inflorescence sizes 10-13 mm in diameter) (Boehringer Mannheim kit 1483188). Taq DNA polymerase (Promega), 50 pmols of both primers and *Gerbera* inflorescence cDNA were used in a standard PCR reaction with 30 cycles. In an agarose gel, the result of the PCR showed several clear-cut bands of DNA. Four bands (estimated sizes 820 bp, 780 bp, 700 bp, and 550 bp) were isolated from the gel, ligated into the pBluescriptII SK + vector and sequenced. The largest fragment contained nearly full length sequences for *GSQUA2*, *GSQUA3* and *GSQUA4*. *GSQUA5* and *GSQUA6* were identified in the *Gerbera* EST collection previously described [29]. *GSQUA5* was recovered as a full-length cDNA from the EST collection, but *GSQUA6* was about 100 nucleotides short at the 5' end of the gene.

Isolation of full length sequences

Amplification with the E0364 primer left MADS box genes short of sequences encoding the amino acids in the N terminus of the protein. The missing sequences were amplified by the 5' RACE method [75] (5'/3' RACE kit, Boehringer Mannheim, cat. no. 1734792). Gene specific 5' RACE primers were designed from the intervening region between the MADS and the K boxes to ensure sufficient specificity. New cDNA was synthesized from *Gerbera* inflorescence mRNA (pooled RNA sample, inflorescence sizes 10-13 mm) (Boehringer Mannheim kit cat. no. 1483188). For each reaction, a band of approximate size of 500 bp was isolated from an agarose gel and ligated into the pGEM-T Easy vector (Promega). The missing 3' sequences of *GSQUA3* and *GSQUA4* were amplified using the same RACE kit. Finally, each full-length cDNA sequence was reamplified using gene specific 5' and 3' primers, ligated into the vector pBluescriptII SK + and verified by sequencing. Full-length sequences were obtained by 5' and 3' RACE methods for all *GSQUA* genes, except for *GSQUA4*, which lacks nucleotides encoding presumably about eight N terminal amino acids.

Phylogeny reconstruction

For phylogenetic positioning of the *GSQUA* nucleotide sequences, we added them to the large data set used in [2]. The original data was kindly transmitted by A. Litt, and sequence abbreviations used by [2] apply to the present tree as well. The new alignment including *Gerbera SQUA*-like genes was made by hand, using the inferred amino acid sequences as a guide. The original *GSQUA1* sequence in the [2] data matrix was deleted to avoid double representation. Phylogenetic analysis on the nucleotide data was performed using the maximum likelihood method, via the PHYML program [76], web interface [77]. 100 bootstrap resampling replicates were done to

estimate support for the clades [78]. The options used with the PHYML web interface were the HKY molecular evolutionary model [79], transition/transversion ratio preset to 4, estimated proportion of invariant sites = 0.065, empirical nucleotide frequencies [$f(A) = 0.32198$, $f(C) = 0.21318$, $f(G) = 0.24109$, $f(T) = 0.22375$], 4 substitution rate categories, estimated gamma distribution parameter = 1.095, starting tree constructed using BIONJ [80], tree topology optimization using NNI and SPR tree rearrangement algorithms to search tree space, and branch length and rate parameter optimization.

RNA gel blots

RNAs from different plant organs and from different stages of petal development (stages 1-11, see [31]) were isolated using Trizol (Invitrogen, cat. no. 11596-018) and quantified by spectrophotometer. Equal amounts (10 µg) of RNA were run in a 0.8% agarose gel as described by [31]. The rRNA bands were visualized by EtBr staining to record even loading of the gel. The RNA was blotted on Hybond-N membrane (Amersham Biosciences) and hybridized in the UltraHyb hybridization buffer (Ambion). For *GSQUA2*, a gene specific probe (260 or 320 bp) from the 3' UTR was used. The probe was labeled with [^{32}P] dCTP and hybridized at + 42°C for 16 h. The membranes were washed with 1 x SSC, 0.1% SDS at + 42°C for 20 minutes. Subsequent washes were performed at + 65°C in the same buffer for 15 minutes, 1-2 times depending on the desired level of final activity. Films were exposed at -80°C. For *GSQUA3*, *GSQUA5*, and *GSQUA6*, full length probes (889 bp, 948 bp, and 812 bp) were used in hybridization due to unspecific hybridization patterns produced with shorter 3' probes. For *GSQUA4*, a longer probe of 450 bp was used due to problems with specificity. For RNA blots hybridized with longer probes, more stringent washing conditions with 0.2 x SSC, 0.1% SDS at + 65°C were applied, leading to increased specificity judged by simpler band patterns.

In situ hybridization

In situ hybridization analysis was performed as described in [81] and [82]. *GSQUA2*, *GSQUA3*, *GSQUA4*, *GSQUA5* and *GSQUA6* gene specific *antisense* probes (250 bp, 385 bp, 300 bp, 187 bp and 235 bp from the 3' UTR) were prepared and quantitated using the DIG RNA labeling kit (Boehringer Mannheim cat. no. 11175025910) according to the manufacturer's instructions. Paraffin sections (10 µm thick) were mounted in 50% glycerol after hybridization. A 217 bp fragment of Gerbera *GGLO1* from the 3' UTR [4] was used as a *sense* control in *in situ* hybridization.

Plant material and transformation

Gerbera hybrida var. 'Terra Regina' was obtained from the commercial producer Terra Nigra, De Kwakel, the Netherlands. In the greenhouse, day length followed the natural day length during the summer season and was set to ten hours during the winter - day length is, however, not critical for *Gerbera* growth and flowering. The temperature was +16... + 18°C during nighttime and + 18... + 20°C during daytime. The plants were drip-irrigated and fertilized with NPK fertilizer (Kukka-Superex NPK 11-3-26, Kekkilä, Finland). The relative humidity was set for 65%. In growth chambers, temperatures were + 18°C at night and + 20°C during day, and the day length was set to 10 hours. For functional analysis, the full length *GSQUA* sequences were cloned under the CaMV 35S promoter in both *sense* and *antisense* orientation as described in [83]. *Gerbera* transformation was performed using an *Agrobacterium*-mediated gene transfer method as previously described [84,85].

Additional material

Additional file 1 *GSQUA* nucleotide sequence alignment. Nucleotide sequence alignment of the *Gerbera GSQUA* genes with other *APETALA1* and *FRUITFULL* like genes.

Additional file 2 Phylogenetic tree of *SQUA*-like genes. Phylogenetic analysis on the nucleotide data was performed using the maximum likelihood method.

Additional file 3 Expression of *GSQUAs* during ray flower petal development. RNA gel blots showing the expression of *GSQUA2*, *GSQUA3*, *GSQUA4*, *GSQUA5*, and *GSQUA6* at different stages of *Gerbera* ray flower development.

Additional file 4 Transgenic lines overexpressing *GSQUA2*. RNA gel blots showing *GSQUA2* overexpression in transgenic lines.

Authors' contributions

SR designed the experiments, carried out the experiments, analyzed the results and drafted the manuscript. YPN performed the RNA gel blot and *in situ* analyses for *GSQUA5* and *GSQUA6*. SKB carried out the light microscopic and SEM analysis of the transformant lines for *GSQUA2*. VAA did the phylogenetic analysis, participated in the interpretation of the results and helped to draft the manuscript. PE participated in the design of the experiments, analysis of the results and helped to draft the manuscript. THT supervised the study, contributed to the design of the experiment, analysis of the results and helped to draft the manuscript. All authors read and approved the final manuscript.

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