RESEARCH PAPER

Reproductive development and phenotypic differences in garlic are associated with expression and splicing of LEAFY homologue gaLFY

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Abstract

Modern garlic (Allium sativum L.) cultivars are sterile and propagated only vegetatively. The recent discovery of fertile genotypes in Central Asia and the restoration of flowering and fertility by environmental manipulations open the way for in-depth florogenetic, genetic, and molecular research in garlic. In the present work, two bolting garlic accessions were employed: #3026, developing normal flowers and seeds, and #2509, in which flowers abort at the early stages of development. Morphological studies showed transition of the apical meristems from the vegetative to the reproductive stage and inflorescence initiation in both genotypes. Low temperatures promote transition of the apex and stem elongation, but have no effect on the phenotypic expression of the inflorescence development. The initial stages of reproductive development in non-flowering #2509 plants were followed by abortion of floral primordia at the differentiation stage. A search for genes involved in the control of flowering in garlic resulted in identification of the garlic LEAFY/FLO homologue, gaLFY. Further comparative analyses of gene expression revealed two gaLFY transcripts, differing in 64 nucleotides, with clear splicing borders. The short variant transcript was identified in both genotypes throughout all development stages, whereas the long variant appears in the flowering genotype #3026 only during reproductive development. The phenotypic differences in garlic, with regard to flowering, may be associated with the efficacy of the splicing process.

Key words: Allium sativum, alternative splicing, bulbs, fertility, flowering time control, LFY.

Introduction

Modern garlic (Allium sativum L.) cultivars are sterile and thus propagated only vegetatively. The lack of sexual propagation prohibits genetic studies, and severely impairs conventional breeding. Recently, fertility restoration in garlic has become a major goal of Allium researchers, and attention has been directed towards the morphological and physiological processes during florogenesis (Kamenetsky and Rabinowitch, 2001, 2002; Etoh and Simon, 2002; Simon and Jenderek, 2003). Garlic genotypes are categorized as non-bolting, semi-bolting, and bolting types (Takagi, 1990; Kamenetsky and Rabinowitch, 2001; Etoh and Simon, 2002; Simon and Jenderek, 2003). Garlic genotypes are categorized as non-bolting, semi-bolting, and bolting types (Takagi, 1990; Kamenetsky and Rabinowitch, 2001; Etoh and Simon, 2002; Kamenetsky et al., 2004a, b). In the former, the inflorescence is either not visible, or its initials abort at the early stages of differentiation. In the latter, scapes grow to their final size, but florogenesis is usually interrupted by development of topsets (bulb-like structures) in the inflorescence (Kamenetsky and Rabinowitch, 2001; Kamenetsky et al., 2004a, b). Environmental manipulations of temperature and photoperiod led to regulation of flower and/or topset development in the inflorescence, and to completion of floral development and seed

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Abbreviations: RT-PCR, reverse transcription–polymerase chain reaction; SEM, scanning electron microscopy.

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production (Kamenetsky et al., 2004b). Fertility restoration thus provided evidence that flowering in garlic is controlled by a number of genetic factors encoding a cascade of processes, some of which are differentially regulated by photoperiod and temperature, and that the garlic genome contains all genes required for the flowering and seed production (Kamenetsky et al., 2004b). A thorough understanding of the genetic coding of florogenesis and its interactions with the environment should improve our knowledge of flowering processes and facilitate fertility restoration in garlic.

Flowering regulation is a complicated process created by an intricate network of signalling pathways. Partial understanding of the genetics and molecular mechanisms underlying flower development was obtained by analysing genetic variations in model plants such as Arabidopsis thaliana, snapdragon (Antirrhinum majus), and corn (Zea mays), among others (Coen et al., 1990; Weigel and Nilsson, 1995; Bomblies et al., 2003; Sreekantan et al., 2004). These studies led to the identification of components within individual signalling pathways that affect flowering, and to their positioning within molecular hierarchies. The most important environmental factors controlling flowering in model species are photoperiod (Corbesier et al., 2004) and low temperatures (Michaels and Amasino, 2000). In addition, an autonomous pathway (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991) and flowering promotion by gibberellins (Wilson et al., 1992; Putterill, et al., 1995; Blazquez et al., 1998) were reported.

It was demonstrated in model plants that the transition from a vegetative to a reproductive meristem is controlled by floral meristem identity genes, followed by floral initiation, and that differentiation (organogenesis) is coded by floral homeotic genes, for example the Arabidopsis gene LEAFY (AtLFY) and its homologues in other plants (e.g. FLORICULA and FALSIFLORA) (Weigel and Meyerowitz, 1994; Simpson et al., 1999). In Arabidopsis, LFY is expressed constitutively throughout the annual life cycle; however, its expression is rather low during vegetative growth, and rises significantly in reproductive tissues (Weigel et al., 1992; Blazquez et al., 1997). In Antirrhinum, a decline in FLO mRNA levels coincided with an arrest of reproductive development and the initiation of vegetative organs in the inflorescence (Coen et al., 1990). In Titanotrichum oldhamii, which sequentially produces flowers and bulbils in the inflorescence, reverse transcription-polymerase chain reaction (RT-PCR) studies showed a rise and decrease in the expression of the LFY/FLO homologue GFLO during normal flower development and following bulbil formation, respectively (Wang et al., 2004). The roles of LFY/FLO homologues in flower development have been demonstrated in numerous dicots (Coen et al., 1990; Weigel and Nilsson, 1995; Sreekantan et al., 2004), while little is known about the function of these meristem identity genes in monocots.

Recently, LFY/FLO homologues were found in rice (Kyozuka et al., 1998) and in maize (Bomblies et al., 2003), and it was shown that these genes share conserved roles with dicots in flower and inflorescence pattern. LEAFY is a transcription factor that affects not only inflorescence initiation but also floral organ determination. In the latter situation, LFY regulates the downstream expression of at least three genes: the meristem and floral identity gene APETALA1 (AP1) which is expressed largely in petals and sepals; the floral organ-determining gene APETALA3 (AP3), required for the development of petals and stamens; and AGAMOUS (AG), which is required for the development of stamens and carpels (Parcy et al., 1998; Busch et al., 1999; Wagner et al., 1999, 2004; Lamb et al., 2002).

Alternative splicing has recently emerged as one of the most significant generators of functional complexity in higher plants (Kazan, 2003), as it generates multiple proteins with different functions from a single gene, and thus greatly enhances the coding potential of a genome. Multiple transcripts from a single gene can result from exon skipping, mutual exclusion of exons and retention of introns, and/or selection of an alternative 5' or 3' site. Alternative splicing can affect the stability and translatability at the RNA level and produce truncated or extended proteins with altered (increased, decreased, or loss of) activity, cellular localization, and/or regulation (Smith et al., 1989; Reddy, 2004). A few examples were already reported for flowering-related genes. Thus, FCA, a promoter of the floral transition in Arabidopsis, produces four transcripts (α, β, γ, and δ) by alternative processing (Macknight et al., 2002). Two major FLC (vernalization-associated gene, determined as a strong flowering antagonist) haplogroups (FLC^A and FLC^B) are associated with flowering time variation in Arabidopsis (Caicedo et al., 2004).

In the present work, two bolting garlic accessions were employed: #3026, developing normal flowers and seeds, and #2509, in which topsets develop in the inflorescence and flowers abort at the early stages of development. Tests were conducted to determine whether the LFY homologue is present in garlic and how it is expressed during plant development and floral initiation.

Materials and methods

Plant material and sampling procedures

Garlic bulbs, accessions #3026 and #2059, introduced into Israel from Kazakhstan in 1999, were obtained from the Field Gene Bank for Vegetatively Propagated Short-Day Allium spp., The Faculty of Agricultural, Food and Environmental Quality Sciences, Rehovot, Israel. Both plants produce mature bulbs under Israeli spring conditions. Each clone was propagated vegetatively from a single bulb, to guarantee genetic uniformity of the experimental stock. In June 2003, mature bulbs were harvested, cured, and stored under ambient conditions, in a roofed shed, until September. Healthy
looking intact bulbs were then transferred to controlled storage for 8 weeks, at either 4 °C or 20 °C in the dark. In November, bulbs were broken and cloves were planted at the experimental farm in Rehovot, using standard agricultural practices throughout.

Phenological data, including developmental stages, number of leaves, and floral scape length, were recorded throughout the experiment. Destructive morphological analyses were performed weekly, on five randomly selected plants per treatment. Apical meristems were sampled for RNA analysis (i) during ambient temperature, (ii) prior to bulb storage at 4 °C or 20 °C (September 2003); (iii) at the end of 8 weeks storage, prior to planting (November 2003); (iv) following sprouting of the fourth foliage leaf (15–20 December 2003); and (v) following transition to the reproductive stage (10–15 January 2004).

Morphological studies

Developmental morphology was studied under a stereoscope (Zeiss Stemi 2000-C), and by scanning electron microscopy (SEM; JSM-35C, JEOL Japan; Kamenetsky, 1994). Prior to microscopic observation, freshly harvested plants were carefully stripped of their leaves, and the spathe removed from the developing inflorescences. Samples for SEM analysis were fixed in a 5:5:90 (by vol.) mixture of glacial acetic acid:formalin (40%):ethanol (70%), and were dehydrated in a graded ethanol series (25, 50, 75, 95, 100%). Immediately thereafter, tissues were dried using liquid CO₂ in a Bio-Rad 750 critical-point dryer. Samples were then mounted on SEM stubs with double-sided tape and sputter-coated with ~10 nm of gold. SEM studies were performed with an accelerating potential of 15 kV.

Nucleic acid isolation

DNA from leaves was extracted using a Nucleon PhytoPure Genomic DNA Extraction Kit (Amersham Biosciences, Buckinghamshire, UK). An RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for total RNA extraction from the apical meristem, leaves, and roots.

PCR and cloning of PCR products for sequencing

The garlic homologue of LFY was amplified by standard PCR and RT-PCR procedures (Sambrook and Russell, 2001), using the following primers: forward, 5'-GAGCTCGACGACATGATG-3' (ELDDMM); and reverse, 5'-CTTGGGTGTTGATGTA-3' (NKPK). RT-PCR of garlic RNA was followed by nested PCR. The primers for RT-PCR were forward nested, 5'-ACCACCTCTTCC CACCTCTT-3'; and reverse nested, 5'-TTGCAATGCGCT GAACCT-3'. Primers for 18S rRNA were designed from Nicotiana tabacum (GB: AJ236016); forward, 5'-AGGAATTGACG GAAGGCAC-3'; reverse, 5'-GTGCGGCCAGAACATC TAAG-3'.

The amplified PCR products were analysed on an agarose gel, excised, and cloned into the vector pDrive (Qiagen) for sequencing. Alignment of the deduced amino acid sequences of the putative garlic DNA and cDNAs was performed using DNA MAN software.

Southern hybridization was performed according to Sambrook and Russell (2001), using the digoxigenin-labelled gaLFY clone as a probe (Roche Diagnostics GmbH, Germany).

RNase protection assays were performed according to Zeitoune et al. (1999).

Results

Effect of storage temperature on the development of two garlic genotypes

The two garlic clones #2509 and #3026 differ in their ability to undergo normal florogenesis (Table 1). In both genotypes, low storage temperatures significantly promote leaf elongation and transition from the vegetative to reproductive state (Table 1; Fig. 1A).

In cold-treated #3026 plants, initial inflorescence development occurred in ~85–90% of the plants. The

Table 1. Effect of storage temperature on vegetative and reproductive traits in garlic accessions #2509 and #3026 in Rehovot, Israel

<table>
<thead>
<tr>
<th>Growth parameters</th>
<th>#3026</th>
<th>#2509</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Storage</td>
<td>Storage</td>
</tr>
<tr>
<td></td>
<td>temperature</td>
<td>temperature</td>
</tr>
<tr>
<td>Mean leaf number*</td>
<td>7.3±1.35</td>
<td>8.1±1.21</td>
</tr>
<tr>
<td>Leaf length (cm)</td>
<td>55.02±9.4</td>
<td>44.6±8.81</td>
</tr>
<tr>
<td>Leaf number at scape appearance</td>
<td>6.57±0.78</td>
<td>7.7±1.02</td>
</tr>
<tr>
<td>Date of visible scape appearance; days after planting</td>
<td>11 March 2004; 116</td>
<td>11 March 2004; 116</td>
</tr>
<tr>
<td>Plants with visible scape (%)</td>
<td>91</td>
<td>84</td>
</tr>
<tr>
<td>Date of spathe break; days after planting</td>
<td>30 May 2004; 190</td>
<td>10 June 2004; 202</td>
</tr>
<tr>
<td>Mean final scape length (cm)</td>
<td>59.51±14.95</td>
<td>38.5±13.15</td>
</tr>
<tr>
<td>Inflorescence performance</td>
<td>Open, fully developed, mostly flowers</td>
<td>Open, aborted flower buds, developed topsets</td>
</tr>
</tbody>
</table>

* Records taken on the 13th week after planting.
transition from the vegetative (Fig. 1B) to the reproductive stage was followed by differentiation of flower primordia in the apical meristem (Fig. 1C), which thereafter developed into normal flowers (Fig. 1D). A small number of topsets were formed in the inflorescences, irrespective of storage treatment. In plants subjected to 4 °C, florogenesis proceeded to completion, thus resulting in the development of normal flowers by the end of May (Fig. 1E) and the production of fertile seeds. In comparison, bulb storage at 20 °C resulted in only 6% of the plants with visible scapes. However, elongation of those was arrested before the final size was reached (Table 1), and the developing inflorescences were aborted within the spathe. In 94% of the plants stored at 20 °C, scape was arrested in the early stages of elongation (Fig. 1F).

Initial inflorescence development occurred in 84% of the cold-treated #2509 plants. However, flowers were aborted at the early stages of their differentiation, and numerous topsets developed in the inflorescence (Fig. 1G, H). In plants stored at 20 °C, scape was arrested in the early stages of elongation.

**Gene identification and expression**

DNA segments from garlic accession #3026 were amplified by PCR, using primers from conserved LFY regions of homologous genes from various plants. The major PCR product (~700 bp) was isolated and cloned (Fig. 2), and sequence analysis of the translation product indicated 46.7% and 61% homology to AtLFY from Arabidopsis thaliana and FLORICAULA of Antirrhinum, respectively.
The gene fragment isolated from garlic was thus designated gaLFY and deposited in GenBank (accession no. AY4563104).

DNA from the two garlic genotypes was cleaved with the restriction enzymes EcoRI and HindIII. Southern blot analyses using the gaLFY clone as a probe indicated the presence of a single-copy LFY homologue in both garlic clones (Fig. 4).

Expression of gaLFY in developing garlic plants was followed by RT-PCR (Fig. 5). A single amplified transcript was observed in cDNA of garlic accession #2509, which under our experimental conditions does not produce fully developed flowers. However, amplification of cDNA from accession #3026 (producing fertile flowers and seeds after storage at 4°C) during the transition phase from the vegetative to reproductive state yielded two distinct bands (Fig. 5, lanes 5 and 7). The two bands were cloned, designated as ‘gaLFY short’ (gaLFYs) and ‘gaLFY long’ (gaLFYl), and their sequence analysis revealed 95% identity. The sequence of gaLFYl (570 bp) was identical to that of the corresponding sequence in garlic genomic DNA, whereas gaLFYs is shorter (506 bp) and it shows 89% identity to garlic genomic DNA, due to a 64 bp deletion between bases 161 and 225 (Fig. 6). The 64 bp deleted sequence was confirmed as an intron by the NetGene program, checking donor and acceptor sites, as well as the branch point, at a confidence level of 95%.

Spliced transcript gaLFYs appears in both genotypes during all developmental stages, whereas unspliced gaLFYl was shown only in #3026 in the transition phase from the vegetative to reproductive stage (four foliage leaves) and later at the stage of flower differentiation (eight foliage leaves) (Fig. 5).

RNA was further extracted from the inflorescences of both genotypes at the beginning of differentiation of the individual flowers, and RT-PCR was performed (Fig. 7). Both gaLFYl and gaLFYs were clearly visible in extracts from accession #3026. In #2509 plants, only the gaLFYs transcript was clearly visible. The more sensitive and size-dependent RNase protection assay demonstrated the presence of a considerable amount of both transcripts in accession #3026, in comparison with accession #2509, expressing predominantly gaLFYs (Fig. 8).

Discussion

The discovery of the fertile genotypes in Central Asia in the 1980s (Etoh, 1985; Etoh and Simon, 2002), the restoration of flowering and fertility in bolting plants by

![Fig. 2. PCR amplification of the LFY homologue of garlic. Left: garlic genomic DNA (accession #3026) was extracted and PCR-amplified with primers designed from conserved regions of the LFY homologue from various plant species. Asterisks indicate the major bands, which were extracted and sequenced. Right: size marker 100 bp.](image)

![Fig. 3. Alignment of LEAFY homologues from several plant species. Alignment of amino acid sequences showing the relationship between the translation product of gaLFY (accession #3026) and LEAFY homologous proteins of other plants. The GenBank accession numbers of the various sequences are: garlic (accession #3026) AY563104; Arabidopsis GI, 166776; Antirrhinum majus GI, 166430; Oryza GI, 6132108, and Zea GI, 28974117. Regions of sequence identity are shaded in black; conservative amino acid changes are shaded in grey.](image)
Morphological studies of two bolting genotypes showed transition of the apical meristems from the vegetative to the reproductive stage following the formation of a specific number of leaves. However, the initial stages of reproductive development in #2059 plants were followed by abortion of floral primordia already at the differentiation phase. In contrast, florogenesis in #3026 plants proceeded normally, and fertile flowers and seeds were produced. As with other geophytes (De Hertogh and Le Nard, 1993), florogenesis in garlic is promoted by low temperatures and a long photoperiod. Hence, scape elongation occurred only in a few (6%) of the #3026 plants treated at 20 °C, followed by inflorescence abortion (Table 1; Fig. 1; Kamenetsky et al., 2004b). Cold treatment promoted transition to the reproductive stage already at the physiological age of 6–7 leaves, whereas plants stored under non-inductive 20 °C underwent such a transition much later, at an older physiological age of 12–14 leaves, most probably when the autonomous pathway became operational, when the photoperiod became sufficiently long, or both (Table 1), as suggested for Arabidopsis (Simpson and Dean, 2002). It is therefore concluded that in both accessions, low temperatures promoted transition of the apex and stem elongation, but did not affect topset formation. These results indicate a strong genetic control and minimum or no effect of storage treatment on the phenotypic expression of flowering and topset differentiation.

A search for genes involved in the control of flowering in garlic resulted in identification of a fragment of a gene designated gaLFY, homologous to LFY and FLO (Fig. 3; Coen et al., 1990; Weigel et al., 1992). The temporal accumulation of LFY is often associated with determining florogenesis (Weigel and Nilsson, 1995; Blazquez et al., 1997), while mutations or down-regulation of LFY and its homologues lead to a reversion in flower determination and the development of vegetative shoots (Coen et al., 1990; Weigel et al., 1992; Molinero-Rosales et al., 1999; Wang et al., 2004). In garlic, Southern blot analysis revealed only one copy of gaLFY in the genome of garlic in both accessions (Fig. 4), but further comparative analyses of gene expression using RT–PCR revealed two gaLFY transcripts: gaLFY1 and gaLFYs. The two transcripts were amplified, and sequence analysis clearly indicated that gaLFY has an intron–exon border. LFY transcripts from Arabidopsis are known to undergo splicing (e.g. GenBank accession no. AF466800); however, to the best of our knowledge, this is the first evidence of alternative splicing of an LFY homologue.

Strong evidence is available to support the fact that alternative splicing plays a major role in the regulation of gene expression in plants, and that it has functional significance in the transition from the vegetative to the reproductive phase (Macknight et al., 1997; Eckardt, 2002; Kazan, 2003). Yet the nature and exact role of these

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**Fig. 4.** Southern blot analysis of DNA from garlic accessions #3026 and #2509. The probe was a 570 bp PCR product of the gaLFY clone. Lane 1: DNA isolated from garlic accession #3026 digested with EcoRI. Lane 2: DNA isolated from garlic accession #3026 digested with HindIII. Lane 3: DNA isolated from garlic accession #2509 digested with EcoRI. Lane 4: DNA isolated from garlic accession #2509 digested with HindIII.

**Fig. 5.** RT–PCR amplification of gaLFY from the accessions #3026 and #2509. In each lane, cDNA was amplified from RNA template. The amplification pattern of the 18S rRNA is shown as an internal control, indicating the levels of total RNA amplified in each experiment. Negative control (reactions without reverse transcriptase) did not yield bands (data not shown). RNA templates were extracted from the meristems at the following developmental stages. Lane 1: at the end of the previous growth period (June 2003). Lane 2: after storage at ambient temperatures (September 2003). Lane 3: at the end of an 8 week storage at 4 °C prior to planting (November 2003). Lane 4: at the end of an 8 week storage at 20 °C prior to planting (November 2003). Lane 5: plants emerging from cloves, stored at 4 °C, 17 December 2004, four foliage leaves. Lane 6: plants emerging from cloves, stored at 20 °C, 17 December 2004, four foliage leaves. Lane 7: plants developing from cloves stored at 4 °C, on 10 January 2004, eight foliage leaves. Lane 8: plants developing from cloves stored at 20 °C, on 10 January 2004, eight foliage leaves. Lane 9: genomic garlic DNA template.

environmental manipulations (Kamenetsky et al., 2004b), and the recent acquisitions of new variations in bolting and blooming (Table 1; Fig. 1; Etoh and Simon, 2002; Jenderek and Hannan, 2004; Kamenetsky et al., 2004a) open the way for in-depth florogenetic, genetic, and molecular research in garlic.
processes in floral regulation is not clear. For instance, *FCA* and *FLC*, known to be involved in the flowering process (Simpson *et al.*, 1999), were recently reported to be alternatively spliced (Eckardt, 2002; Caicedo *et al.*, 2004).

In garlic, spliced transcript *gaLFYs* is continuously present in all tissues of the plant, while the second unspliced transcript *gaLFYl* accumulates mainly during flower differentiation (Figs 5, 7). In both studied garlic accessions, splicing of *gaLFY* transcripts occurs throughout the vegetative phase. During reproductive development, splicing is partially inhibited and accumulation of the unspliced form (*gaLFYl*) was observed (Fig. 8). The two studied genotypes differ in the expression of the unspliced variant *gaLFYl*: it was strongly expressed in the flowering accession during both inflorescence initiation and flower differentiation, but not in the genotype #2509, in which differentiating flowers were aborted. It can be concluded that, of the two *gaLFY* transcripts, flower differentiation is associated with accumulation of the unspliced *gaLFYl* variant of the *LFY* homologue and presumably the phenotypic differences, with regard to flowering, are associated with the efficacy of the splicing process.

For most plant species, only one *FLO/LFY* homologue was reported to be involved in the regulation of florogenesis. Recently, however, two *LFY* homologues were isolated from apple floral buds (AFL1 and AFL2; Wada *et al.*, 2002), *Eucalyptus* (ELF1 and ELF2; Southerton...
et al., 1998), and maize (zf1 and zf2; Bomblies et al., 2003). In Eucalyptus, one gene is expressed in the developing floral organs in a pattern similar to LFY, while the other appears to be inactive (Southerton et al., 1998). Similarly to the two gaLFY transcripts, AFL1 is only expressed in floral buds of apple during transition from vegetative to reproductive growth, whereas AFL2 was detected in all tissues of the plant (Wada et al., 2002). gaLFY, on the other hand, seems to be a single-copy gene (Fig. 4), but two LFY products are generated by alternative splicing. Presumably, different plants apply different strategies in their evolutionary track, i.e. coding for the various LFY forms by multicopy genes or by alternative splicing.

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