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The gladiolus *GgEXPA1* is a GA-responsive alpha-expansin gene expressed ubiquitously during expansion of all floral tissues and leaves but repressed during organ senescence

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ABSTRACT

The final shape and size of the flower is genetically and developmentally controlled by tight regulation of cell number and cell size with cell expansion playing an important role The gladiolus expansin gene, *GgEXPA1*, was expressed prominently during phases of active tepal expansion and cell elongation in stamen filaments, gynoecium styles and expanding leaves but not in tissues where expansion had ceased and senescence had been initiated. Within tepals, differential expression between the proximal and distal portions that differ in cell elongation was observed. The expression of the gene was responsive to GA and inhibited by the GA biosynthesis inhibitor, paclobutrazol. The promoter of *GgEXPA1* showed strong expansion-responsive GUS expression in young agro-infiltrated gladiolus tepals and in etiolated hypocotyls and light grown expanding cotyledonary leaves of transgenic Arabidopsis seedlings. Inhibition of hypocotyl elongation by paclobutrazol blocked the expression of the promoter-driven reporter gene indicating GA responsiveness of the promoter. *GgEXPA1* provides an interesting example of a single expansin gene being involved in expansion processes in different plant tissues such as tepals, stamens, pistils and leaves that are both spatially as well as temporally distinct in their development. The studies provide a basis for GA mediated expansion of floral organs via expansins prior to anthesis.

1. Introduction

The development of a flower is a complex process that begins with initiation of floral primordia, differentiation of the floral organs, increase in floral size, bloom and finally senescence. The shape and size of the floral organs is genetically and developmentally controlled through regulation of cell number (by division), cell size (by expansion) and influenced by growth rate, anisotropy and direction (Meyerowitz, 1997; Mizukami, 2001; Rolland-Lagan et al., 2003; Weiss et al., 2005; Anastasiou and Lenhard, 2007). While early development in most flowers is characterized by cell division, cell wall expansion plays an important role in determining the final shape of flower (Kotilainen et al., 1999; Reale et al., 2002; Yamada et al., 2009a). Stamens and pistils expand longitudinally while petals expand in all directions. Cell wall expansion requires not only cellulose biosynthesis and deposition but also changes in the cytoskeleton and the matrix of the cellulose/hemicellulose/pectin network which allow flexibility for wall expansion (Fagard et al., 2000; Martin et al., 2001; Pagant et al., 2002; Smith, 2003). Prominent amongst the wall proteins that provide flexibility for cell expansion are expansins that are believed to cause wall loosening in a non-hydrolytic, turgor driven manner by disrupting hydrogen bonds that link cellulose and hemicellulose microfibrils. Their action causes slippage between the cellulose–hemicellulose polymers followed by water absorption and expansion of the cell wall (McQueen-Mason and Cosgrove, 1995). Expansins belong to a multi-gene family in all plants studied so far and are conserved in structure but display diversity in function (Sampedro and Cosgrove, 2005). Thus expansins have been shown to play an important role in several plant processes that require wall modification such as cell expansion, hypoxic stem elongation in rice, leaf and root initiation and other non-expansion processes such as wall softening in fruit ripening and organ abscission (Rose et al., 1997; Fleming et al., 1997; Brummell et al., 1999; Cho and Cosgrove, 2002; Choi et al., 2003; Belfield et al., 2005; Sane et al., 2007; Asha et al., 2007). Individual members of the large expansin family in each plant are believed to provide specificity of function in each tissue and may be governed differentially by hormones especially gibberellins. The expansion of petals has previously been reported to be due to accu-

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mulation of GA in the petals (Murakami, 1973, 1975; Koning, 1984). GA is also known to affect stamen filament length and pistil length in flowers (Murakami, 1973, 1975; Pharis and King, 1985). We are interested in studying genes that are actively involved in floral organ growth and expansion and the role of hormones like GA in their regulation during expansion. In this paper, we describe the characterization of a new expansin gene from gladiolus that is closely associated not only with tepal growth and expansion but also with stamen, pistil and leaf elongation and functions in a GA-responsive manner thus providing a basis for GA mediated expansion of floral organs via expansins prior to anthesis.

2. Materials and methods

2.1. Plant material

Flowers of gladiolus (*Gladiolus grandiflorus* cv Snow Princess) grown in the field were chosen for study. The spikes, containing about 12–14 flowers in different stages of growth and senescence, were cut with a sharp blade and immediately placed in water. Flowers on the spike were marked as previously described (Azeez et al., 2007). The fully open flowers were designated as 0 stage. Flowers above this stage in different stages of opening were marked as −1, −2 and −3 in an ascending order with −3 indicating the bud stage. Flowers below the 0 stage were marked progressively as +1, +2 and +3 in a descending order and represented flowers in different stages of senescence with +3 indicating completely wilted flowers.

2.2. Estimation of tepal area and stamen/pistil length

For estimation of tepal area, at least five complete flowers of each stage were chosen. Individual tepals were spread on a graph paper and area of each tepal was measured and summed up to obtain the entire tepal area. Stamens and pistils of the same flowers were chosen for study of expansion in length. For stamens, the length of the filament was measured while for pistils, the length of the style was measured. All data were expressed as mean length (in cm) ± SD for stamen filaments and pistil styles or mean area (in mm^2) ± SD for tepal.

2.3. Treatment of flowers with GA and paclobutrazol

To study hormonal regulation of expansin expression, flowers from three stages viz. −2, 0 and +2 were cut, and placed individually in beakers containing either water (as control), GA3 (10 μM, Duchefa, Netherlands) or the GA biosynthesis inhibitor, paclobutrazol (20 μM, Duchefa, Netherlands) for 24 h. Flower tepals were ground in liquid nitrogen and RNA extracted for reverse transcription and semi-quantitative RT-PCR. To study the effect of GA3 and paclobutrazol on flower growth, buds at −3 stage were placed in a beaker containing either water, GA or paclobutrazol for 4 days. Whole tepal area was measured after 4 days when flowers opened. Studies were carried out on three independent flowers for each treatment.

2.4. Isolation of RNA

Floral parts (tepals, stamens and pistils) from flowers belonging to different stages of growth and senescence were ground in liquid nitrogen to a fine powder. RNA was isolated from these tissues as described by Asif et al. (2000). RNA was also isolated from the basal and distal portions of the tepals of fully open flower (stage 0) and from five different stages of leaves starting with the very young (Y ~ 12 cm), growing (G1 ~ 25 cm and G2 ~ 35 cm), mature (M ~50 cm) to senescing (S ~ 58 cm) leaves.

2.5. Cloning of expansin cDNA

DNA free RNA from 0 stage flower was reverse transcribed using MuMLV Revertaid reverse transcriptase (MBI Fermentas) and primed with the 3′ RACE adapter primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′) from Invitrogen. This was used as cDNA template for further amplifications of expansin. To amplify the expansin gene fragment, degenerate primers for expansin (forward primer ExpF1 5′-GCHTCDGGMACHATGGDTGGDGCDTGTGG-3′ and reverse primers, ExpR1 5′-CCRTTKAYBGTRAAYCKDATHCCTCC-3′ and ExpR2 5′-AAGTGNKKNHGDGGDGGRTTRCACC-3′) were designed from conserved regions as described by Sane et al. (2005). The F1R1 pair was used to amplify a product of 374 nt while the F1AP pair was used to amplify a 979 nucleotide fragment which was subsequently cloned in pBluescript IISK (Stratagene, USA). Amplified fragments were sequenced on an automated DNA sequencer (ABI 373A from Applied Biosystems Inc., USA) using the thermosequenase dye terminator cycle sequencing kit from Amersham-Pharmacia. The 5′ end of the gene was obtained from a Gladiolus genome walker library created using the genome walker kit (Clontech, Palo Alto, USA). Gene specific reverse primers Gex-R1 (5′-CAG TAA CGA CAA TTG AAC CCG AAA GAC-3′) and Gex-R2 (5′-GGT CGT CGT TGC ACT TCA TCT CAT AG-3′) were used in combination with the genome walker adapter primers GWAP1 (5′-GTA ATA CGA CTC ACT ATA GGG C-3′) and GWAP2 (5′-ACT ATA GGG CAC GCG TGG T-3′) as described in the genome walker manual. A fragment of approximately 1.8 kb, containing 474 nucleotides of the gene (inclusive of a 200 nt intron) towards the 5′ end and 1.3 kb sequence upstream of the initiation codon, was cloned and sequenced. Based on the sequence, a forward primer containing the initiation codon, GExpOEF 5′-GGA TCC ATG TAT TCC CTT TCC AAA ATC-3′ was synthesized and used in combination with the 3′AP primer to obtain the cDNA sequence containing the complete open reading frame of 762 nt besides a 3′ UTR of 329 nt (Accession number FJ042664).

2.6. Northern analyses

Total RNA (30 μg) was resolved on a 1.2% denaturing formaldehyde-agarose gel as described (Sambrook et al., 1989) and modified in the Qiagen Oligotex handbook, 2002. RNA was transferred to nylon membranes (Hybond N, Amersham-Pharmacia Biotech, Uppsala, Sweden) by vacuum transfer using a vacugene apparatus (Pharmacia) and crosslinked by baking for 2 h. Radio-labeling of probes for northern blots was performed by random priming using α-^{32}PdCTP and the 3′ variable region (213–762 nt + the 329 nt 3′ UTR) of the expansin gene. Hybridization and washings of blots were performed as described by Sambrook et al. (1989). Signals obtained on the blots were quantified on a phosphorimager (Molecular Imager FX, BioRad) using the software QuantityOne-4.2.3 version.

2.7. Semi-quantitative RT-PCR

Equal amounts of DNA free RNA (5 μg) from the basal and distal portions of the tepals were reverse transcribed using the MuMLV Revertaid reverse transcriptase (MBI Fermentas). The primer GExpOEF was used in combination with the reverse primer GExpSM-R 5′-TGC TGC AGT GTT GGT TCC ATA CCC TT-3′ to amplify a fragment of 210 nt. Actin was used an internal control using the primers F1 5′-ATG ACA TGG AGA AGA TCT GGC ATCA-3′ and R1 5′-AGC CTG GAT GGC AAC ATA CAT AGC-3′ to amplify a fragment of 179 nt. Reactions were run on a PerkinElmer 9700 PCR machine at an initial denaturation of 94 °C (2 min) followed by 32 cycles of 94 °C (10 s), 55 °C (10 s) and 72 °C (20 s) and a final extension at 72 °C for 5 min. The gladiolus *GgDAD1* (*DEFENDER AGAINST APOPTOTIC DEATH1*), a marker