Studies on Expression of Genes involved in Somatic Embryogenesis and Storage Protein Accumulation in Saffron Crocus (*Crocus sativus* L.)

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Abstract

To initiate the evaluation of gene expression patterns in saffron, 80 random chosen clones from a cDNA library prepared from corms were immobilized in amino-silane coated slides and hybridised with labelled cDNA from *in vitro* embryos at different developmental stages. Constitutive genes showed similar expression patterns at all stages, while developmentally regulated genes changed their expression. We detected strongly regulated expression of a xyloglucan endotransglycosylase (a cell wall loosening activity enzyme involved in cell growth), formaldehyde dehydrogenase and an abscisic stress ripening protein. Some unidentified genes also showed high hybridisation signal. In addition, the expression pattern of the major storage protein, a mannose-binding lectin, was studied by means of RT-PCR. The expression level is higher during summer, when the corm is at a dormant stage, and this lectin accumulates in big storage vacuoles. Once the corm sprouts and blooms, the expression of this protein decreases to minimum levels.

INTRODUCTION

Saffron, *Crocus sativus*, is a sterile triploid plant that is vegetatively propagated by means of corms. The formation of somatic embryos is a previous step in the micropropagation protocol. Somatic embryogenesis is a process from which a somatic cell generates a differentiated dipolar embryo. This is the ideal system to carry on studies about differentiation in plants (Komamine et al., 1992). However, the mechanisms underlying pattern formation during embryogenesis are still unknown. To analyse differential expression during embryo development, cDNA microarrays can be used. DNA microarray hybridisation is a powerful technology in molecular biology that allows analysis of changes in expression patterns of a large number of genes in parallel. The results show the cellular mechanisms of metabolic adaptations to a wide variety of growth and developmental conditions or to analyse tissue specific gene expression (Maguire et al., 2002). Here, we report the first approach of microarray technology in somatic embryos of saffron.

Little is known about gene expression in the corm, and crucial processes like storage accumulation, dormancy or sprouting are not still fully understood. The corm is an organ that accumulates reserves. The major component of this reserves are carbohydrates (Chrungoo et al., 1983; Farooq et al., 1985), but the corm also produces and accumulates storage proteins that serve as a source of amino acids for sprouting (Chrispeels and Raikhel, 1991; Conlan et al., 1995). The major storage protein of the corm has been previously identified as a mannose-binding lectin (Oda and Tatsumi, 1993; Escribano et el., 2000), a well-characterized family of storage proteins from monocots (Van Damme et al., 2000). The expression of the main storage protein is analysed, as the corm is a storage organ, and one of the critical processes in this organ is accumulation of

reserves.

MATERIAL AND METHODS

Saffron cDNA Clones

We used 80 random chosen clones from a cDNA library prepared in a lambda-derived vector (Stratagene) from saffron corms at a stage of maximum enlargement, collected in February (Álvarez-Ortí, 2003). The sequences of the clones were previously analysed for sequence similarity against public protein and nucleic acid non-redundant databases. The top scoring genes were used to assign the putative identification of the clones.

Amplification of cDNA Inserts

cDNA clones were PCR amplified from the vector using T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-GTAATACGACTCACTATAGGGC-3') vector-specific primers. Clone inserts were amplified in 100 µl reaction volumes as described by Hedge et al. (2000). Five microlitters of each PCR reaction was electrophoresed in 1% agarose gels to confirm amplification quality and quantity. PCR products were isopropanol precipitated and stored dry for subsequent microarray fabrication. In general, the total quantity of each PCR product was around 2 µg. The average size of cDNA insert was around 500 bp.

cDNA Microarray Preparation

PCR products were resuspended in 4 % SSC and transferred to 96-well arraying plates. The PCR products were printed onto amino-silane coated glass microscope slides. Post-printing slide procedures were performed as described in Hedge et al. (2000).

Plant Material and RNA Isolation

For the study of microarrays, we used embryos at different developmental stage obtained at the Laboratory of Biotechnology in the Instituto de Desarrollo Regional (Albacete, Spain) as starting material for RNA extraction for probe preparation. Embryos were at stage E1 (appearance of embryogenic nodules), E2 (monopolar embryos) and E3 (dipolar embryos). Total RNA was isolated in a single-step method using Trizol (Gibco BRL).

For expression analysis of the major storage protein, corms were collected during one year at 15 days intervals form a small field located in Tarazona de la Mancha (Albacete, Spain). RNA was isolated in the same way as described above.

Preparation of Fluorescent Probes

Total RNA samples were reverse-transcribed in the presence of nucleotides labelled with either Alexa Fluor 532, 568 or 594 dyes (Molecular Probes). RT-PCR was carried out with the LabelStar Array Kit (Qiagen), following the instructions provided by the manufacturer for labelling reaction and cleanup of the labelled probes, using an oligo (dT) as primer for the reverse transcription reaction.

Microarray Hybridisation and Scanning

The probe mixture was denatured at 95ºC for 5 min and cooled on ice. Then SSC, formamide and SDS were added to give a final hybridisation volume of 20 µl (4 x SSC, 50 % formamide, 0.2 % SDS). The labelled probe was applied to a microarray slide and covered with a plastic coverslip. The slide was placed in a hybridisation chamber, at 72ºC for 5 min, and then at 37ºC for 18-20 h. The array was then removed from the hybridisation chamber and washed with 0.2 SSC, 0.05 % SDS at room temperature until the coverslip falls off. The slides were then washed in 0.2 x SSC for 2 min. and dried by centrifugation. Slides were scanned and analysed with QuantArray software, and array images were processed with Photoshop software.

Expression Analysis (RT-PCR)

To evaluate the expression of the major storage protein of the corm, RNA extracted from corms at different developmental stages was transformed into cDNA using the kit Ready-To-Go ^{TM} You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc.), with an oligo(dT) as a primer. cDNA was used as template for PCR amplification with specific oligos Cslec5' (5'-ATGGCAAAGTCCCTAGTCCTCTCCT-3') and Cslec3' (5'-TCGAGACTCACACTAGTACAAGGCG-3') in 25 µl reactions containing 1xPCR buffer, 2.5 mM $MgCl_2$, 250 nM dNTPs, 1 unit of Taq Polymerase (Promega), 25 pmol of each oligo and 100 ng of cDNA. Reactions were carried out in 30 cycles of 94 ºC, 1 min; 60 ºC, 1 min; 72 ºC, 1 min with a previous denaturation step at 94 ºC, 5 min and a final extension step at 72 ºC, 10 min. PCR products where electrophoresed in 2 % agarose gels containing ethidium bromide.

In Situ Hybridisation

Saffron corms collected in July were fixed in paraformaldehyde fixative for 2 h with two vacuum infiltrations. Paraffin embedding and cutting of sections were made according to Schwarzacher and Heslop-Harrison (2000). Slides were incubated for 1 h at room temperature with rabbit antiserum to the *C. sativus* lectin (1:1000 dilution) in blocking buffer containing 5% (w/v) skim milk and 0.05 % Tween 20 in PBS (Escribano et al., 2000). Slides were washed twice in PBS, incubated for 30 min with fluorescent antibodies against rabbit IgG (1:2000 dilution) in blocking buffer and then washed for 10 min in 50mM Tris-HCl pH 7.5, 150 mM NaCl. Hybridisation signal was observed directly under fluorescent microscopy.

RESULTS

Hybridisation Results

cDNA microarrays containing 80 saffron ESTs (Table 1) were hybridised with fluorescently labelled in vitro probes prepared from somatic embryos at a different developmental stage (E1, E2, E3), obtained in vitro. For each DNA spot, the fluorescence signal intensity of the labelled probes reflects the relative abundance of the corresponding transcript in each RNA sample. Hybridisation signal was reproducible for two copies of the same cDNA microarray, and all spots on the array generated signals above local background values. When we analysed E1, E2, and E3 together, the expression pattern was quite similar. High hybridisation signal was observed for a chitinase, some ribosomal proteins, xyloglucan endotransglycosylase, formaldehyde dehydrogenase, ornithine carbamoiltransferase, 4-coumarate-CoA ligase, an early light induced protein, an abscisic stress ripening protein, and some unidentified genes (Figure 1). When compared two by two, some differences were observed (Figure 2). Hybridisation signal was significantly higher for xyloglucan endotransglycosylase, formaldehyde dehydrogenase and an unidentified gene in E3 than in E1; while differences between E2 and E3 were only detected for an abscisic stress ripening protein, being more expressed in E3 than in E2. The rest of cDNAs included in the arrays did not show differential expression.

Expression Pattern of the Major Storage Protein

We analysed the expression pattern of the major storage protein of the corm by means of RT-PCR, performed with cDNA prepared from total RNA from corms collected every month during a year, and oligos specifically designed to bind both ends of the gene which codes for this protein. When PCR products were run on an agarose gel, we obtained one band of the expected size (around 850 bp). The expression is higher during summer months when the corm is at a dormant stage. Once the corm sprouts, expression level decrease to the minimum and when the flower appears, no expression is observed (Figure 3). The accumulation of this protein in the dormant corm was analysed with antibodies and fluorescent microscopy, and hybridisation signal show that lectin is accumulated in great storage vacuoles in parenchyma cells of the corm (Figure 4).

DISCUSSION

80 random chosen cDNA clones from an expression library constructed from saffron corms were printed in aminosilane coated slides and hybridised with fluorescently labelled cDNA from somatic embryos at different developmental stage obtained in vitro.

The three stages showed high hybridisation signal for a chitinase, two ribosomal proteins, 4-coumarate-CoA ligase, an early light induced protein and ornithine carbamoyltransferase, an enzyme of the urea cycle that catalyses the production of citrulline from ornithine. Citrulline is a nitrogen storage molecule that has been found related to drought stress resistance (Yokota et al., 2002). In addition, high hybridisation signal was found for some novel sequences, which did not show significant similarities to any known sequences in current public databases. Other cDNA microarrays have revealed novel plant sequences involved in nutrient responses, seed formation, circadian/diurnal regulation, and anther/pistil differentiation (Wang et al., 2000; Girke et al., 2000; Schaffer et al., 2001; Endo et al., 2002). Our work supports the idea that cDNA microarrays provide an efficient approach to assess the function of novel sequences by observing their expression patterns (Maguire et al. 2002).

Some differences were found when stages were analysed two by two, suggesting differential expression through somatic embryo development. Higher hybridisation signal was observed for a xyloglucan endotransglycosylase in stage E3 (dipolar embryos) when compared to E1 (embryogenic nodules). Proteins with cell wall loosening activity have been associated to growing reproductive structures (Loopstra et al., 1998). Signal was also higher in E3 for a formaldehyde dehydrogenase (a detoxifying enzyme) and an unidentified gene. When stages E3 and E2 were compared, only one gene out of 80 showed significant difference in hybridisation signal, corresponding to an abscisic stress ripening protein. Similar studies have been done during development of embryos in soybean (Thibaud-Nissen et al., 2003), and at the beginning of gymnosperm embryogenesis (van Zyl et al., 2003), showing the utility of cDNA microarrays in the searching for developmentally regulated genes. However, mostly clones analysed in our work did not show differences in the hybridisation signal, suggesting they are constitutively expressed. Future studies are to be made including a higher number of ESTs from the rest of organs of the plant and with development related genes. These analyses will build up complete picture of gene expression and regulation in saffron. The relatively small number of arbitrarily chosen genes analysed here provisionally identified several with developmentally regulate expression, showing the utility of boutique microarrays in the characterization of gene expression during development.

The major storage protein of the saffron corm is a mannose-binding lectin. Here, we report the expression pattern of this lectin analysed by means of RT-PCR. The results obtained support the storage role of this protein, as expression is higher during summer months prior to sprouting, and decreases once the corm sprouts. To protect storage proteins from premature degradation, they are included in protein bodies or storage vacuoles derived from the endoplasmic reticulum (Muntz, 1998; Herman and Larkins, 1999). This is concordant with the results obtained here from the hybridisation with fluorescent antibodies against this lectin, which show hybridisation signal in big vacuoles in parenchyma cells. In addition, in the sequence of this lectin an amino-terminal peptide acts as a signal peptide, which directs this protein through the endoplasmic reticulum membrane to the secretory pathway (data not shown). In spite of the corm is at a dormant stage during summer months, with decreased cell activity, it continues producing and accumulating storage proteins for sprouting when environmental conditions are favourable.

ACKNOWLEDGEMENTS

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Tables

Table 1. Putative identification of cDNA clones included in microarrays

	A	B	C	D	E
1	**CsaC3003** Gamma thionin	**CsaC3004** Chitinase	**CsaC3009** Unknown (zinc	**CsaC3010** Elongation factor	**CsaC3011** Unknown
2	**CsaC3013** Unknown	**CsaC3014** Pectin methylesterase	**CsaC3016** ATP synthase	**CsaC3017** Unknown	**CsaC3018** Apoptosis
3	**CsaC3020** Thiol protease	**CsaC3021** Heat shock protein	**CsaC3022** B12D protein	**CsaC3023** UDP-Glc	**CsaC3027** Gibberellin-
4	**CsaC3035** Unknown	**CsaC3037** Unknown	**CsaC3038** Fructokinase	**CsaC3040** DnaJ protein	**CsaC3041** Cation
5	**CsaC3044** Receptor-like	**CsaC3046** Metallothionein	**CsaC3047** Starch	**CsaC3048** Unknown	**CsaC3052** Unknown
6	**CsaC3053** Sucrose synthase	**CsaC3055** Extensin homolog	**CsaC3056** Unknown	**CsaC3058** Unknown	**CsaC3060** Unknown
7	**CsaC3062** Reverse	**CsaC3065** Formaldehyde	**CsaC3066** Putative	**CsaC3067** Ran protein/TC4	**CsaC3069** Microsatellite
8	**CsaC3074** Unknown	**CsaC3079** Unknown	**CsaC3080** Dioxygenase	**CsaC3081** Unknown	**CsaC3084** Unknown
9	**CsaC3086** Inorganic	**CsaC3092** Seed allergenic	**CsaC3095** Profilin	**CsaC3097** Sec 61	**CsaC3098** Polyprotein
10	**CsaC3100** Unknown	**CsaC3104** Unknown	**CsaC3105** Unknown	**CsaC3107** Unknown	**CsaC3115** Unknown
11	**CsaC3116** No similarity	**CsaC3117** Unknown	**CsaC3118** TCP-1	**CsaC3122** Transposon TNT 1-	**CsaC3123** Sec 1
12	**CsaC3126** Ribosomal	**CsaC3128** TCTP	**CsaC3129** Glioxalase	**CsaC3130** Putative protein	**CsaC3135** 4-coumarate-
13	**CsaC3138** Unknown	**CsaC3141** Ribosomal protein	**CsaC3145** Ornithine	**CsaC3146** Sec 61	**CsaC3149** Unknown
14	**CsaC3156** No similarity	**CsaC3170** 14-3-3 protein	**CsaC3197** Scarecrow	**CsaC3227** ER-33	**CsaC3230** Expansin
15	**CsaC3237** SINA	**CsaC3279** Xyloglucan	**CsaC3322** LEA	**CsaC3368** ELIP	**CsaC3378** Calmodulin
16	**CsaC3384** RAS-related	**CsaC3387** Catalase	**CsaC3427** Coat protein	**CsaC3436** GTP-binding	**CsaC3485** Abscisic

Figures

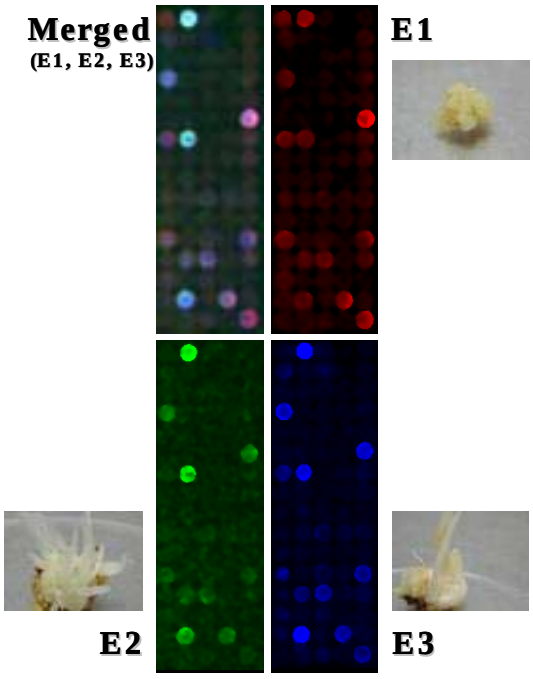

Fig. 1. 80 cDNA clones from a saffron corm library were printed in aminosilane-coated slides and hybridised with labelled cDNA probes from somatic embryos of saffron at different developmental stage. Top left: merged image. Top right: hybridisation with cDNA from E1 stage (embryogenic nodules) labelled with Alexa Fluor 594 (red signal). Bottom left: hybridisation with cDNA from E2 stage (monopolar embryos) labelled with Alexa Fluor 568 (green signal). Bottom right: hybridisation with cDNA from E3 stage (dipolar embryos) labelled with Alexa Fluor 532 (blue signal). Hybridisation was performed for 20 hours at 37 ºC.

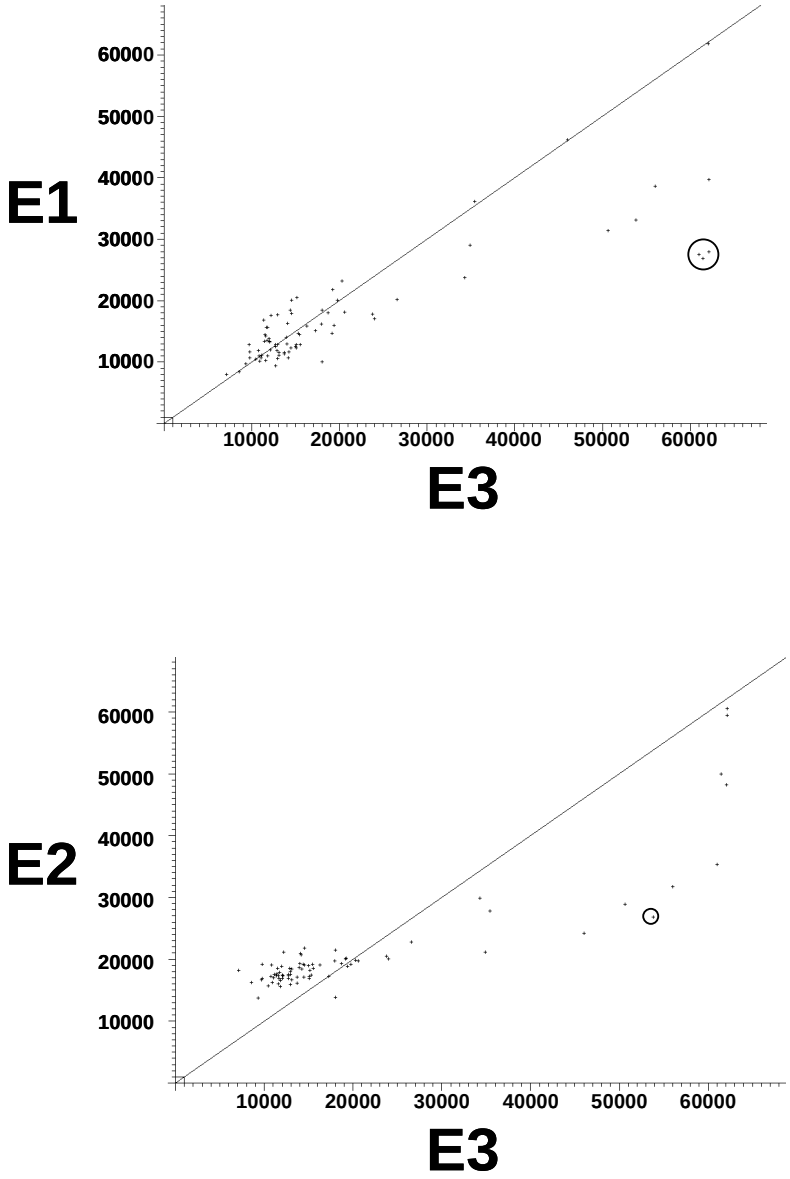

Fig. 2. Scatter plot of signal values for all clones on the microarray. Signal intensities for each clone are shown. Top: comparison between stages E1 and E3. Bottom: comparison between stages E2 and E3. The diagonal lines represent a ratio of 1.0 (co-expression). Encircled points indicate significant differences. In the top, the three points correspond to the signal for a xyloglucan endotransglycosilase, formaldehyde dehydrogenase and an unidentified gene. In the bottom, the encircled point corresponds to the signal for an abscisic stress ripening protein.

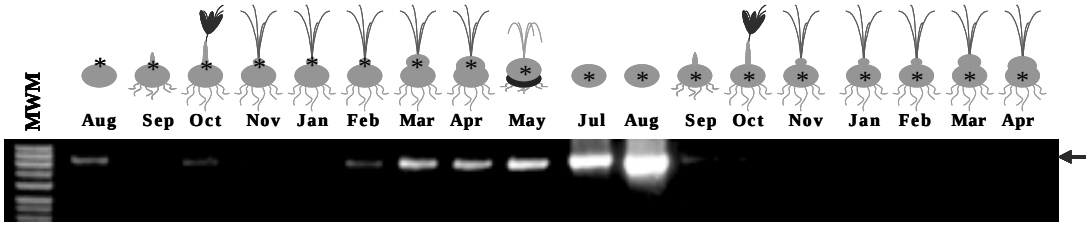

Fig. 3. RT-PCR analysis showing expression of the major storage protein of the saffron corm through development. Arrow indicates the band of the expected size (around 850 bp). Asterisks indicate the material from which RNA was extracted for RT-PCR.

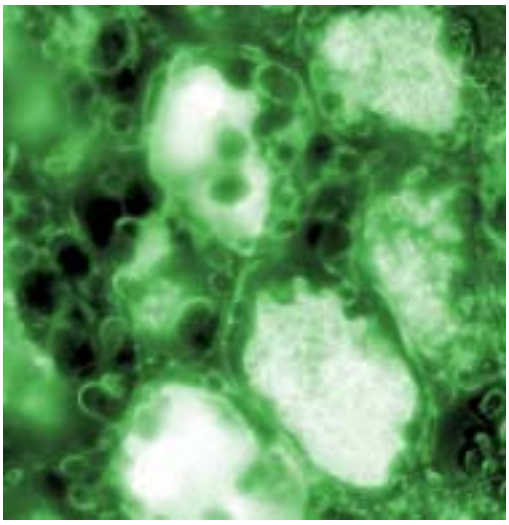

Fig. 4. In situ hybridisation with antibodies against the main storage protein of the corm showing accumulation in big storage vacuoles of parenchyma cells of dormant corm collected in July.