SEASONAL TIME-COURSE OF EXPANSIN EXPRESSION IN FLOWERS AND GROWING GRAINS OF SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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ABSTRACT

Grain weight is a key component of yield and quality of sunflower. Taking into account that the ovaries of flowers became the pericarp of grains in grasses and dicots such as sunflower, it has been proposed that the maternal tissues impose a physical restriction to growing grains in these crops. The physiological processes supporting the hypothesis that the pericarp controls grain weight (GW) are only starting to be understood. Expansins (Expns.) are proteins that play a key role in plant cell growth by inducing the loosening of cell walls in plants, which determines the cellular growth expansion. The objective of the present study was to assess the seasonal time-course expression of Expn. genes in ovary, pericarp and embryo of flowers and growing grains of sunflower. Two sunflower genotypes contrasting in grain size and weight were sown in a split-plot design with three replicates at the Agricultural Research Station of the Universidad Austral de Chile in Valdivia, Chile. Ovaries and grains (divided in pericarp and embryo) were sampled at pre-anthesis and post-anthesis, respectively. Relative quantification of mRNA levels of Expns. was evaluated by qPCR. Final GW was different between genotypes (80 and 150 mg). Expression analysis by qPCR showed that specifics Expns. (Expn.3, 4 and 5) are associate better with grain growing dynamics in sunflower, and a variation in the expression of Expns. between genotypes contrasting in GW and in flower and grain tissues across the developmental stages.

Keywords: grain weight, expansin expression, yield, sunflower

INTRODUCTION

Expansins (Expns.) are proteins inducing cell wall extension (McQueen-Mason et al. 1992). They have been known as "factors that loosen the cell wall", allowing relaxation of the cell wall during cell expansion, playing a major role in growth. Expns. are involved in different physiological processes as the cell wall disassembly during fruit ripening (Brummel et al. 1999), organogenesis of leaves (Fleming et al., 1997), differentiation of vascular cells (Cho and Kende, 1998) and root system architecture (Ma et al., 2013; Marowa et al., 2016); however, there is little information about the role of Expansins in specific tissues like grains.

A recent study shows the evolutionary divergence between classes of Expns. especially α and β groups in grass and dicots plants, due to the difference in the composition of the cell wall,

highlighting that the β Expns. family has expanded considerably in grasses (Sampedro et al., 2015). This was important to consider when characterize Expns. in reproductive structures of sunflower.

In wheat, it has been estimated that there are at least 30 α and 65 β Expns. Generally, a multigene family suggests that different members may play unique developmental or tissue-specific roles. This may be the situation of the α Expn. group, which has shown organ or development stage specificity in wheat (Liu et al., 2007). The present study hypothesizes that some Expns. will be specifically involved in the pericarp extension, and that their expression could be related to grain growth dynamics in sunflower. Previous experiments found expression of 6 different Exp. cDNA sequences in pericarp tissues of wheat at 14 days after anthesis. Out of the 6, 4 matched known wheat Expn., whereas the other 2 were novel wheat Expns., similar to sequences described for *Festuca pratensis* and *Oryza sativa* (Calderini et al., 2006; Lizana et al., 2010).

A strong relationship between grain size dynamics, water content and Expn. expression was found in wheat grains during grain filling. Water content of grains was consistent with high abundance of Expns. gene transcripts (Lizana et al., 2010). Plant cell expansion is turgor-driven and regulated by cell wall mechanical properties and is related to Expns. proteins (Cosgrove, 2015), a key component in grain enlargement according to Lizana et al. (2010).

From the background outlined above, the expression of TaExpA6 transcript is consistent with grain elongation in wheat (Lizana et al., 2010). Therefore, the following questions arise:

i) Are there Expns. associated with grain growth in sunflower?
ii) Is the elongation of the pericarp and embryo driven by different Expns.?
iii) Is the timing of Expn. expression similar between the pericarp and embryo?

The objectives of this work was to identify the expression of Expns. And their time course in ovary, pericarp and embryo of sunflower during the growth of the reproductive organs.

The study of Expns. at both pre- and post-pollination and their relationship with the dynamics of grain growth will be key to a better understanding of processes controlling GW. It will also make it possible to establish the relationships among dry matter, water content, and Expns. expression in flowers and growing grains of sunflower.

MATERIALS AND METHODS

Plant material and field experiment

Two sunflower genotypes contrasting in GW and adapted to the south of Chile, Alybro (oilseed) and RHA280 (confectionery) were sown under field conditions at the Experimental Station of the Universidad Austral de Chile in Valdivia (39◦47´S, 73◦14´W). The genotypes were laid out in a randomized complete block design with three replicates. Sowing date were 20 of October as in previous evaluations in Valdivia. Plots consisted of seven rows, 0.70 m apart and 5 m long with a plant population density of 6 plants m^{-2}. Plots were fertilized at sowing with N, P and K based on soil analysis, ensuring that the crop was not affected by any nutrient limitation. Weeds, insects and diseases were prevented or controlled, and regular watering was supplied to complement rainfall throughout the experiment to avoid water stress.

Phenology, flower and grain sampling

Phenology of the crop was followed during the growing season according to the scale by Schneiter and Miller (1981). Individual plants were tagged at R3 (Schneiter and Miller, 1981) to evaluate the seasonal time-course of development, dry matter, water content and dimensions of

flowers and grains as in previous studies (Rondanini et al., 2009). Grains were split into pericarp and embryo at each sampling. Two capitula per replicate were harvested at 2 or 3 days intervals, florets and grains were sampled from two places in the capitulum at each sampling date (grains from peripheral position). Harvested flowers and grains were immediately processed (to measure fresh weight and dimensions) or preserved into cryotubes and quickly immersed in liquid nitrogen for molecular analysis. Samples were stored at -80°C until processing.

Grain weight and dimension measurements

Four flowers and grains from the peripheral position were weighed after harvest at each sampling date to determine fresh weight. In the case of grains, they were immediately separated into pericarp and embryo and each component weighed. In early phases of grain development the embryo included the aqueous endosperm (present at the early stages of embryo development and consumed during the embryo growth). Plant material were dried for 72 h at 60°C to determine dry weight.

Absolute water content (mg) of flowers and grains (pericarp and embryo) were calculated as the difference between fresh and dry weight, while water concentration (%WC, on a fresh weight basis) was estimated as the ratio between absolute water content and fresh weight, expressed as a percentage. Flower and grain dimensions (length, width, and height) were recorded quickly after sampling using an electronic caliper; this was measured in four grains.

Molecular analysis: In silico analysis and primer design

The first objective was to identify *in silico* the Expns. sequence genes expressed in grain of sunflower. Public databases of sunflower genome (https://www.heliagene.org/) enabled a search for putative Expns. using the BLAST tool with *Helianthus annuus* trascriptome and Zinnia elegans Exp. 3 mRNA sequence (GenBank: AF230333.1) because this is a related species of sunflower. The sequences obtained from BLAST were evaluated using the bioinformatic tool option "expression patterns" making it possible to consider Expns. putative expression patterns in plant organs. Expns. sequences expressed mainly in grain were chosen.

Selected sequences were aligned using the program Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to reveal the number of unique sequences. Sequences were searched against the non-redundant GenBank DNA and protein database using BLASTn and BLASTX (Altschul et al., 1990, 1997) and against the Uni Prot database resources using BLASTX. The best matches were used as the foundation for sequence identity-based annotations. Sequences were used in BLASTx searches to confirm that they correspond to Expn. Transcripts. In addition, nucleotide sequences were translated into protein with the ExPASy bioinformatic tool (http://web.expasy.org/translate/) to mark off the coding region for the design of specific primers. These primers were designed using the "PRIMIQUE" tool to detect different sequences of the gene family (Fredslund and Lange, 2007). Two primer pairs were chosen for the same sequence.

A bibliographic search were conducted for housekeeping genes of sunflower for use as an endogenous control to normalize the data for differences in input RNA and the efficiency of reverse transcription between the various samples. Primers reported by previous studies for elongation factor 1 (EF1), S19 protein, β-tubulin, actin, ubiquitin and 18S of sunflower grains were evaluated (Brunner et al., 2004; Layat et al., 2014; Meimoun et al., 2014; Oracz et al., 2008; Pramod et al., 2012).

RNA extraction and RT-PCR

Total RNAs were isolated with the RNeasy Plant Mini kit (Qiagen) according with the manufacturer's instructions. The kit provides a choice of lysis buffers depending on the amount and type of secondary metabolites in the tissue; therefore, the RNA extraction protocol should be standardized. The quality and concentration of RNA were measured by spectroscopy with Nanodrop (nd-1000, Thermo Fisher Scientific, USA).

The isolated RNA was pretreated with DNasaI. First-strand cDNA was synthesized from 250 ng RNA using the ImProm-II™ Reverse Transcription System. The oligo(dt)16-18 primer/template mix was thermally denatured at 70°C for 5 minutes and chilled on ice. A reverse transcription reaction mix was assembled on ice to contain nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor. 1u/µl of Recombinant RNasin® Ribonuclease Inhibitor was added. The template-primer combination was added to the reaction mix on ice. Following an initial annealing at 25°C for 5 minutes, the reaction was incubated at 42°C for up to one hour. The synthesized cDNA (20µl) was stored at -20°C. As a negative control, an RNA sample was replaced by water in this procedure.

Quantification of mRNA levels by using real time PCR (qPCR)

The PCR reaction was performed in a final volume of 25µL and containing 12,5µL Brilliant II SYBR Green PCR Master Mix (Stratagene, Agilent technologies), 1 µL 10 µM forward and reverse primers and 8,5 µL of sterile deionized water. After an initial DNA polymerase activation step at 95◦C for 10 min, the samples were subjected to 35 amplification cycles (95◦C for 15 s,60◦C for 15 s, and 72◦C for 15 s). No-template and no-transcriptase controls were included to detect genomic DNA contamination.

A melting curve was generated by incubating the reaction at 95°C for 15 s, 25°C for 1 s, and 70◦C for 15 s and then slowly increasing the temperature to 95 ◦C. The target gene expression was quantified with the method proposed by Livak (2001) by using the Agilent AriaMx software to calculate the transcript abundance relative to the calibrator, which becomes sample 1, and all other quantities are expressed as an n-fold difference relative to the calibrator.

After confirming the amplified specific products, a standard curve of each pair of primers was created with the product from the previous amplification. A dilution of 1: 1000 was prepared and then 7 serial dilutions were prepared by a factor of 10 starting from the 1:1000 dilution of the previously amplified product. This was achieve the efficiency of the primers.

Samples were subjected to service sequencing. Resulting sequencing chromatograms be viewed, evaluated and aligned. Sequence alignments and database searches were carried out using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and https://www.heliagene.org/.

Statistical analysis

Final grain weight was estimated using a bilinear model as in Calderini et al. (1999). The model was fitted using the iterative optimization technique of Table curve V 3.0 (Jandel, 1991). Data of final grain weight and variables from grain growth dynamics were assessed by ANOVA using the software STATISTICA v. 7.0 (Stat Soft, Inc., 2004). The LSD test (5%) was employed for differences among genotypes.

RESULTS AND DISCUSSION

Grain weight and dimensions were significantly different between genotypes ($P \leq 0.001$) as we expected. Grains of the confectionery genotype (RHA280) were havier in RHA280 (149 mg) than in Alybro (79 mg) as well as grain dimensions (Fig. 1a and b). These differences were evident as early as 3 days after antehsis (DAA) (Fig. 1). Moreover, a linear association ($r^2= 0.77$)

between final grain weight and ovary weight was found in this study (data not shown). These results agree with previous studies where positive relationships between grain weight and the weight/size of carpels at anthesis have been reported in sunflower (Cantagallo et al., 2004), sorghum (Yang et al., 2009) and wheat (Hasan et al., 2011). Therefore, the pre-anthesis period has proven critical to determining GW (Calderini et al., 1999; Ugarte et al., 2007). RHA280 had a mean of 465 filled grains and 184 empty grains per capitulum, and Alybro, 1497 filled grains and 80 empty grains per capitulum (data not shown).

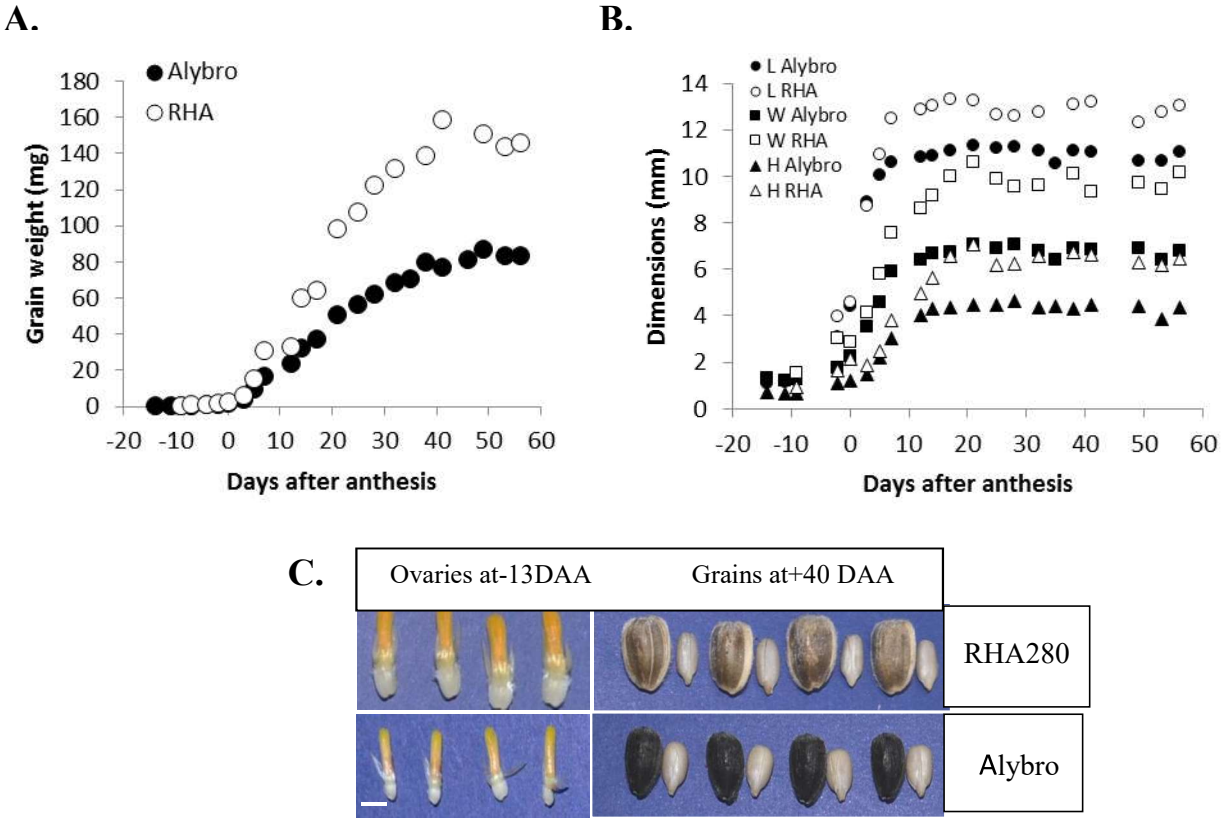

Figure 1. **Time-course of grain weight and grain dimensions of peripheral grains of two sunflowers genotypes (RHA280 and Alybro).** Dynamics of grain dry weight (A) and dimensions (B) of RHA280 (open symbols) and Alybro (closed symbols) are shown. The photos of ovaries (-13 DAA) and grains (+40 DAA) of both genotypes are also shown, Scale bar: 4 mm. (C). Grains were separated into pericarp and embryo. L: Length, W: width, H: high.

The expression of three putative Expns. Genes, named by us Expn. 3, Expn. 4 and Expn. 5 (accession in Heliagene database: Ha412T4l900C0S1, HaT13l007346, HaT13l009552, respectively) according to the name of the similar Expn. sequence with a maximum value of identity in the results of a BLAST, were chosen and evaluated by qPCR along the development of the reproductive organs in both genotypes.

Taking into account the dynamics showed in Figs. 1 and 2, it was found that some Expns. could be specifically involved in the grain tissues extension, suggesting their expression would control grain size of sunflower. For example, Expn. 4 follows a similar time course than the grain growth dynamic of genotypes (Fig. 2). Lizana et al., 2010 showed that the expression of few Expns. is associated with grain elongation when the grain is growing in wheat. The present is the first study showing parallelism between growing grains and Expns. expression in grain tissues of sunflower.

In addition to the time course of expression, specifics Expns. Seem to be controlling the extension of ovary, pericarp and embryo. Expn. 3 and Expn. 4 was found specific to maternal tissues (ovary and pericarp) and Expn. 3 was more abundant in the pericarp. On the other hand, Expn. 5 was found more abundant in the embryo (Fig. 2). The timing of Expn. 3 and Expn.4 in the pericarp showed a higher abundance at +7 DAA when in sunflower, the growth of the pericarp levels off soon after flowering, i.e., 8 days after anthesis at R5.1 (Rondanini et al., 2009; Lindström and Hernández, 2015). Interestingly, the confectionery genotype showed higher abundance of Expns. genes later than the oil genotype Alybro (Fig. 2), suggesting that Expns. isoforms control the growth in flowers and grains of sunflower. Furthermore, it has been hypostatized that the pericarp imposes a physical restriction to growing grains in grasses (Calderini et al., 1999; Ugarte et al., 2007; Yang et al., 2009), which might explain the importance of the pre-flowering phase for GW determination.

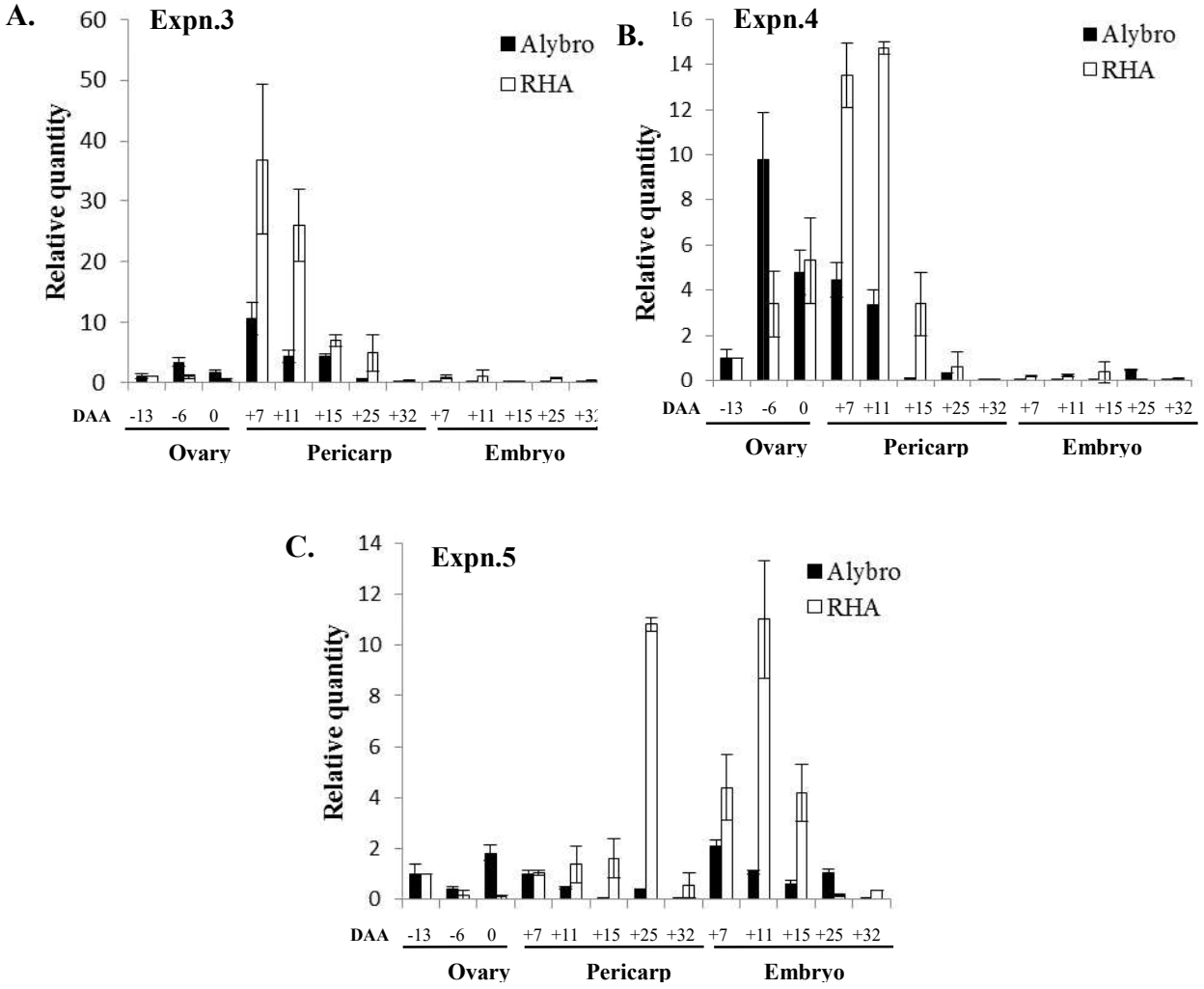

Figure 2. Seasonal time-course of expansin expression in ovary, pericarp and embryo of sunflower. A, B, C. Relative quantity analysis of three expansins genes, this target genes were quantified and normalized to β Tubulina using the Livak method (n=3).

In dicots, such as the model plant *Arabidopsis thaliana*, the control of GW by the growth of the inner integument during ovary and early phases of seed development has been demonstrated,

supporting the hypothesis that potential GW is controlled by the outer seed tissues (Adamski et al., 2009; Fang et al., 2012; Xia et al., 2013; Du et al., 2014). Remarkably, ovary mass at anthesis associate with final pericarp mass across both sunflower genotypes (see Table 2 in Lindström and Hernández, 2015). The present study support the hypothesis that the Expns. play an important role in the extension of grain maternal tissues. The importance of the pericarp on early grain development of wheat was stated many years ago (Rijven and Banbury, 1960), but the physiological processes through which the pericarp controls the final size of the grain is only now beginning to be understood (e.g., Garcia et al., 2005; Léon-Kloosterziel et al., 1994; Schruff et al., 2006; Song et al., 2007).

In sunflower and other crops, such as wheat, sorghum and coffee tree, maternal tissues (ovary/pericarp) have been proposed to control the potential grain size (Calderini and Reynolds, 2000; Yang et al., 2009; Budzinski et al., 2011; Lindström et al., 2006). In these crop species, the maternal tissues undergo rapid cell wall division and expansion, which in turn might determine the physical limit of the endosperm or embryo of the fruit (Budzinski et al., 2011). During the rapid cell wall expansion is where the Expns. proteins are acting.

In a previous study, transcripts of CaExpA2 were detected only in the pericarp of coffea fruit during the later stages of fruit maturation and ripening, highlighting the participation of these isoforms in the regulation of fruit size (Budzinski et al., 2011). Another study addressing fruit elongation showed two Expns. genes (ExpA4-a and ExpA5) inducing fruit length in cucumber, suggesting that the cell-wall related genes are required for fruit elongation in these species (Jiang et al., 2015). Studies by Harrison et al. (2001) found two Expns. in strawberry highly expressed during the process of rapid expansion of immature fruit, while four were expressed mainly during fruit ripening. In agreement, similar results were found in peach (Hayama et al. 2003), pear (Hiwasa et al. 2003) and banana (Asha et al. 2007), showing that high levels of mRNA coding for Expns. were observed in rapidly growing tissues, and multi-gene family of Expns. exhibited differential expression patterns for the different phases of fruit development. In the light of results shown in the present and previous studies, we propose Expn. 4 as a driver of grain growth in sunflower.

CONCLUSIONS

The analysis of results showed in this study supports different expression of Expns. genes between genotypes and across the developmental stages of flower and grain growth. The expression of Expns. variable in ovary, pericap and embryo tissues.

Expns. genes associated with grain dynamics would contribute to a better understanding of mechanisms controlling grain size in sunflower, especially Expn. 4, it is a good candidate for future research.

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