SOURCE-SINK RATIO EFFECTS ON THE EXPRESSION OF GENES ASSOCIATED WITH GRAIN GROWTH IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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ABSTRACT

Grain size is the result of the coordinated growth of the embryo, endosperm and maternal tissues. Understanding the clues of the development and growth of these tissues is essential for increasing grain weight, a key component of sunflower yield and quality. This research was aimed at evaluating the effect of pre-anthesis shading (source-sink ratio reduction) on grain growth and the expression of genes associated with grain size between R3 and physiological maturity in sunflower. Two sunflower genotypes contrasting in grain weight were sown in a split plot design with three replicates. Shading treatments (nets intercepting 80% of incident radiation) were set over the plots from R3 to R5 stage. Ovaries and grains (the last divided in pericarp and embryo) were sampled from R3 to R9 stage. RNA was extracted from ovary and grain tissues. The time-course of the expression of putative orthologous genes for sunflower of HaGW2 (RING-type E3 ubiquitin ligase-like) and HaAP2 (EREBP-like), were assessed by qPCR. Grain weight was affected ($P< 0.05$) by both genotype and shading treatments. The lower source-sink ratio decreased final grain weight. Interestingly, the expression of HaGW2 and HaAP2 genes was affected by the genotypes and the source-sink ratio in flowers and grains tissues across the developmental stages. Results presented here suggest that HaGW2 and HaAP2 genes act in the pericarp and might be involved in driving the growth of grains in this crop.

Key words: Sunflower, grain weight, grain size, genetic control, AP2, GW2.

INTRODUCTION

Grain weight in crop plants is an important agronomic trait and a key component of sunflower yield. Grain development requires a double fertilization event generating two products within the embryo sac: the embryo and the endosperm (Lopes and Larkins, 1993). The embryo is surrounded by the endosperm, which, in turn, is enclosed within the maternal seed coat. The grain size is regulated by the coordinated growth of the embryo, endosperm, and maternal tissues (Fang et al., 2012; Xia et al., 2013).

In recent years, the knowledge about grain development improved considerably, and some genetic and molecular mechanisms are now known, mainly in model plants (Sundaresan, 2005; Sun et al., 2010; Li and Li, 2015; Orozco-Arroyo et al., 2015). However, in grain crops like sunflower this knowledge is still partial. Grain size is affected by the

maternal and/or zygotic tissues. It is known that in various crops the grain weight and grain size has a polygenetic control (Zhang et al., 2012; Kesavan et al., 2013). In *Arabidopsis* APETALA 2 (AP2) encodes a member of the AP2/EREBP (ethylene responsive element binding protein) may restrict grain growth by limiting cell proliferation in the integuments (Jofuku et al., 2005; Ohto et al., 2005). Ap2 mutant grains exhibit delayed cellularization of the endosperm resulting in larger embryo sacs and bigger embryos that show increased cell number and size and this larger grain trait was passed through the maternal sporophyte and endosperm genome (Jofuku et al., 2005; Ohto et al., 2005, 2009).

The role of the ubiquitin pathway on the grain size determination has been widely investigated over the last years in *Arabidopsis* (Li and Li, 2014). Several members involved in this pathway have been identified. DA1 and DA1-related (DAR) encode for plant-specific ubiquitin receptor protein. DA1 protein might act antagonistically with native DA1 or DAR, and would be negatively regulating cell proliferation in maternal grain tissues (Li et al., 2008). DA2 and enhancer of DA1 (EOD1) encode protein with E3 ubiquitin ligase activity and are also negative regulators of grain size (Xia et al., 2013). In rice GW2 (RING-type E3 ubiquitin ligase) functions as a negative regulator of grain width and weight, the loss of function of GW2 leads to increased cell number, a widers pikelet hull and an accelerated grain milk-filling rate, which increases grain width, grain weight and yield (Song et al., 2007). In recent years, extensive studies of GW2 have been carried out in wheat (Su et al., 2011; Yang et al., 2012; Zhang et al., 2013; Qin et al., 2014; Simmonds et al., 2014, 2016; Hong et al., 2014). Most of them reported that GW2 is a negative regulator of grain size and weight, except the study accomplished by Bednarek et al. (2012), where the authors reported the positive effect of GW2.

In the last decades, many key regulators of grain size have been identified; however, it is still limited the knowledge on genetic control of grain size and weight in crops, and this knowledge is virtually lacking in sunflower. The present research was aimed at evaluating the effect of pre-anthesis shading (source-sink ratio reduction) on flower and grain growth as well as the expression of genes associated with grain size between R3 and physiological maturity in sunflower the dynamics of grain dry matter accumulation and grain dimensions were analyzed in parallel with the expression of AP2 and GW2 genes.

MATERIALS AND METHODS

Field site description, treatments and experimental conditions

A field experiment was conducted at the Agricultural Experimental Station (EEAA) of Universidad Austral de Chile in Valdivia (39°47´S, 73°14´W, 19 m ASL), Chile in the 2014-2015 growing season. Two genotypes contrasting in grain weight and with similar phenology were arranged in a split plot design with three replicates, where the source-sink manipulation treatment was assigned to main plots and genotypes to subplots. Plant density was 6 plants m^{-2} and the dimension of plots (experimental units) was 9 rows at 0,70 m apart. 20 plants were sown per row. The treatments were the outcome of combining (i) two genotypes and (ii) two source-sink rates (control without shading and shading treatment by nets intercepting 80% of incident radiation, imposed during the pre-anthesis period, from R3 to R5). Genotypes were Alybro from Panam Seeds of small size grain (oilseed) and RHA-280 of big size grain (confectionery), from NCRPIS, USDA-ARS.

Sampling and grain measurements

Phenology was recorded by using the scale proposed by Schneiter and Miller (1981). Flowers and grains (the last separated in pericarp and embryo) were sampled from R3 to R9. Samples were individually removed, and were immediately frozen in liquid nitrogen. Frozen samples were stored at -80 °C until analysis. Dry matter, water content and dimensions

(length, width, and height) of flowers and grains were evaluated every three days. Final grain weight, dimensions, and the timing of physiological maturity were estimated using a linear model as in Calderini et al. (1999). The model was fitted using the iterative optimization technique of TableCurve 2D V5.01 (Systat Software Inc., Chicago, IL, USA).

Search and isolation of candidate gene sequences

To find sequences of genes AP2 and GW2 in sunflower we conducted a search in the sunflower transcriptome database Heliagene (https://www.heliagene.org/) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/), based on orthologous genes from *Arabidopsis* and rice. Using the "ExpressionPatterns" tool available in Heliagene, we chose candidate gene sequences, which had greater expression in grain tissue. Sequences were analyzed by blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Putative proteins were predicted by ExPASy (http://web.expasy.org/translate/). A protein analysis was conducted by InterPro (https://www.ebi.ac.uk/interpro/) and Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd) to verify the structure of the protein, domains and conserved sites and ensure that the sequences corresponding to the putative genes.

Real-time (qPCR) expression analysis

Total RNA was extracted from flower ovaries, pericarps and embryos from control and shaded plants using the kit NucleoSpin® RNA Plant (Macherey-Nagel). It was treated with DNase I (Invitrogen). First strand cDNA synthesis was performed using an Affinity Script cDNA Synthesis Kit Reverse Transcription System (Agilent Technologies) following the manufacturer's instructions. Three biological replicates for each sampling date were used. Specific 5´-3´ primers for AP2, GW2 and β-tubulin (as internal control) genes from Heliagene database, were designed using Primique (Fredslund and Lange, 2007) (http://cgi-www.daimi.au.dk/cgi-chili/primique/front.py), with high stringency to avoid amplification of non-specific PCR products. All primers were synthesized by Macrogen Inc. (South Korea) (http://www.macrogen.com/). Primer pair sequences were: AP2: AGGATGGGCCAATTTTTAGG (forward), ATGGCAGCCTTATCATACGC (reverse), GW2: GAAGCCATCTGGTTGTCGTT (forward), TGGATGCTAAGAGGCGAACT (reverse), and β-tubulin: GGCGTCTACCTTCATTGGT (forward), TCCATCTCATCCATTCCTTC (reverse) (Meimoun et al., 2014). The amplicon sizes were 92 bp for the AP2 gene, and 115 bp for GW2 gene.

The amplification reactions were performed using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions in an AriaMx real-time PCR system (Agilent Technologies Inc., Santa Clara, CA, USA). PCR conditions were: 95°C for 10 min; 35 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s. No template control (NTC) and no reverse transcriptase control (no-RT) were included for detecting gDNA contamination. A dilution series was built to estimate the amplification efficiency using a cDNA mix as template prepared from control ovaries samples (-14 to 0 days after anthesis). Each reaction was performed in triplicate, and a negative water control was included in each run. Fluorescence was measured at the end of each annealing step. The amplification efficiency was estimated through a melting curve and amplification products were visualized on agarose gels (1.5%, w/v). The relative expression levels were first normalized against the β-tubulin gene and using non-shading samples from day one as calibrator, with a nominal value of 1. The method described by Livak and Schmittgen (2001) was used to make all calculations.

Statistical analysis

The recorded data were assessed by two-way analysis of variance. LSDs were calculated using STATISTICA v7.0 (StatSoft Inc.) and used for mean separation (α= 0.05).

RESULTS AND DISCUSSION

The final grain weight of peripheral grain position measured at physiological maturity stage showed a wide range (58.2 – 148.8 mg) of values (Table 1). Both grain weight and dimensions (length, width and heigh) were significantly affected by genotype ($p \leq 0.001$) and the source-sink ($p \leq 0.001$) treatments (Table 1). Previous studies demonstrated that grain weight and grain number are sensitive to shading during pre-flowering in sunflower (Cantagallo and Hall, 2002; Alkio et al., 2003; Cantagallo et al., 2004; Lindström et al., 2006). Our results confirm the high sensitivity of sunflower under strong source shortage at the immediately pre-anthesis stage. These results support that grain traits are set at pre-anthesis, therefore, the size of the ovary would determine the potential weight of grains (Cantagallo et al., 2004; Rondanini et al., 2009) like in wheat (Hassan et al., 2011). The dynamic of dry weight of peripheral ovaries and grains from R3 to maturity is shown in Fig. 1 a, b.

Peripheral grain weight showed a positive relationship with grain length ($r^2 = 0.92$; $P < 0.05$), grain width ($r^2 = 0.98$; $P < 0.05$) and grain height ($r^2 = 1$; $P < .0.001$). Taking into account the close associations shown by the final grain weight and dimensions, especially the height and width, these would seem to be crucial for grain weight determination. In fact, studies by Lizana et al., 2010; Hasan et al., 2011 in wheat show that length grain is very important in final grain weight determination. Unlike wheat where length grain shows better associaciations with grain weight, in sunflower it seems the height and width would be more important in grain weight determination, probably because they are different species, and different architecture grains.

Table 1. Final grain weight, grain dimensions (length, width and height) of peripheral grain position in Alybro and RHA-280 genotypes measured at in physiological maturity.

Genotype	Treatment	Length	Width	Height	Final grain weight (mg)
RHA-280	Control	13,01 a	9,80 a	6,49 a	148,78 a
	S-S	12,30 b	8,40 b	5,21 b	106,97 b
Alybro	Control	10,96 c	6,78 c	4,35 c	79,20 c
	S-S	9,62 d	5,54 d	3,72 d	58,15d
Genotype		***	***	***	***
Treatment		***	***	***	***
Genotype x treatment		***	ns	***	ns

Different letters indicate LSD test differences ($p < 0.05$). S-S: source-sink treatment. *** Mean significant at 0,001 probability level; ns: non-significant.

The putative sequences of AP2 (accession: HaT13l014337) and GW2 (accession: HaT13l009504) were obtained from Heliagene database (https://www.heliagene.org/). Both two sequences in sunflower were named HaAP2 and HaGW2 respectively. These sequences encoding proteins similar to *Arabidopsis* and grape vine (*Vitis vinifera* L.) respectively. The complete coding sequence of HaAP2 shares 100% identity with APETALA 2 (AP2) of *Arabidopsis* (NCBI accession: NP_195410) (Mayer et al., 1999), and the analyses of the protein sequence predict an AP2-like ethylene-responsive transcription factor, DNA-binding domain and AP2/ERF domain. Regarding HaGW2, the sequence shares 61% identity with RING-type E3 ubiquitin ligase (VvGW2) from grape vine (GenBank accession: AII80417.1). The protein analyses of HaGW2 predict a Zinc finger, RING-type domain. These results

support that the sequences evaluated in this study correspond to the HaAP2 and HaGW2 putative sunflower genes.

To investigate the source-sink manipulation effect on HaAP2 and HaGW2 candidate genes and grain development, we evaluate genes expression at eight times from R3 (14 days before anthesis) to R9. (32 days after anthesis), i.e. three times in pre-anthesis, and five times in post-anthesis (Fig. 1). Our results show a differential expression of the two genes between genotypes and among grain tissues.

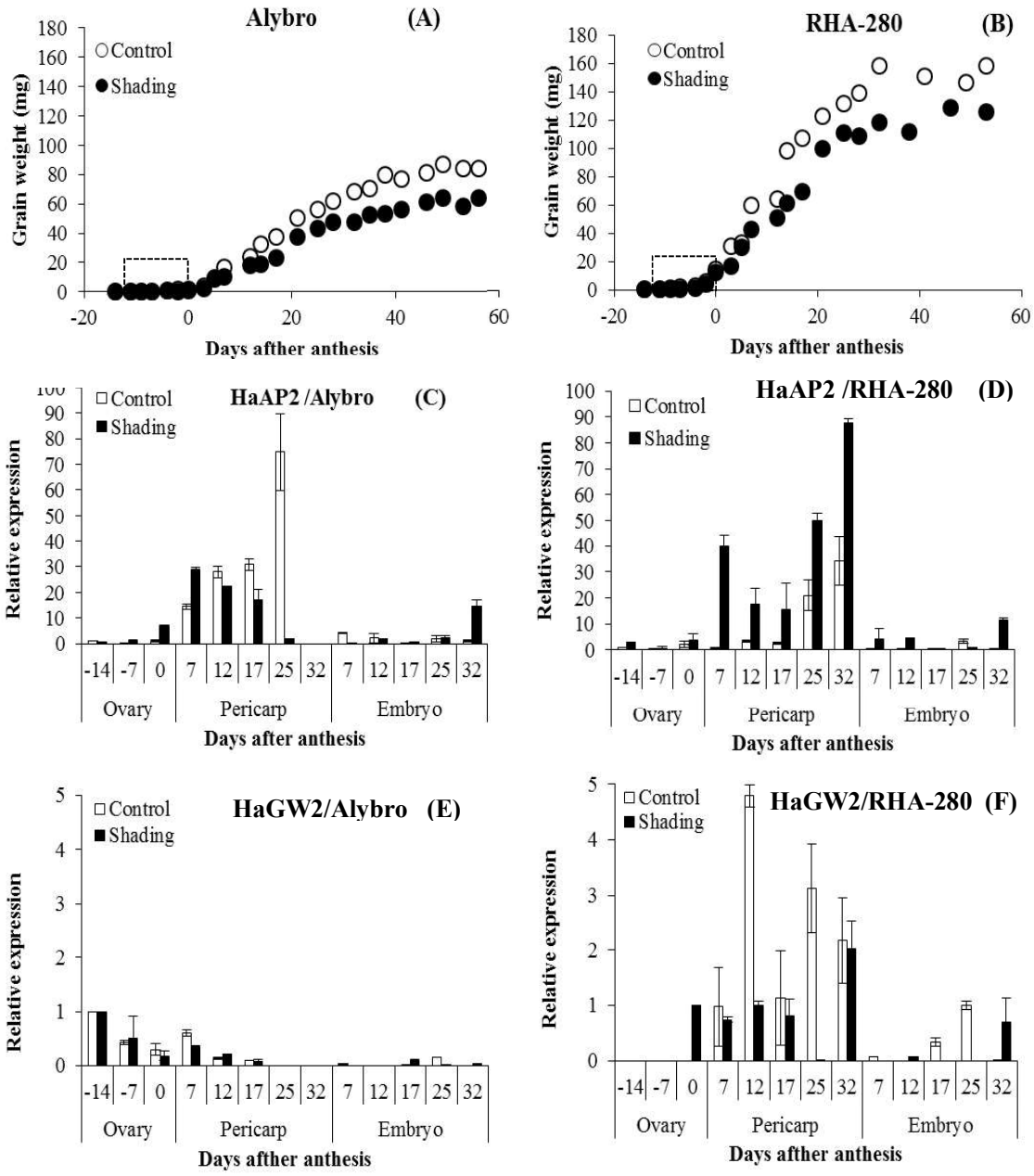

Figure 1. Relative expression of HaAP2 and HaGW2 genes in growing grains of contrasting grains weight genotypes of sunflower before and after anthesis under source-sink manipulation. Time-course of grain dry weight in Alybro (a) (small grains) and

RHA-280 (b) (large grains). Bar dashed line indicates the shading period. Relative expression of HaAP2 in Alybro (c) and RHA-280 (d). Relative expression of HaGW2 in Alybro (e) and RHA-280 (f). Bars on the graphs indicate the standard error. Open symbols indicate the control plants and filled symbols indicate shaded plants.

In control plants the HaAP2 gene showed higher expression in the genotype Alybro (small grain) than in genotype RHA-280 (large grains) (Fig. 1c, d). The expression of HaAP2 in both genotypes was mainly detected in the pericarp tissues, in Alybro from 7 to 25 days after anthesis, while in RHA-280 a longer expression was found (until 32 days after anthesis).

Shaded plants and control plants of Alybro showed similar expression pattern of HaAP2 in pericarp tissues, however, the relative expression in shade plants was lower compared to controls plants between 12 and 25 days after anthesis (Fig. 1c). Surprisingly, higher expression levels in shade plants were found in RHA-280 compared to controls, mainly in the pericarp tissues (Fig. 1d). This higher expression under shading of the large grain genotype parallels with the lower grain weight dynamic and lower final grain weight (Fig. 1d Tabla 1.). These results suggest that this gene may be acting in the pericarp maternal tissues, downregulating the growth of grains. Our results agree with previous studies of AP2, where that the authors suggest that AP2 negatively regulate the size of *Arabidopsis* grains. AP2 may restrict grain growth by limiting cell proliferation and cell expansion in the integuments (Jofuku et al., 2005; Ohto et al., 2005, 2009). In addition, AP2 is required for ovule and seed coat development (Leon-Kloosterziel et al., 1994; Modrusan et al., 1994; Jofuku, 1994).

HaGW2 gene showed low expression in control plants in Alybro genotype (small grain) (Fig. 1e), whereas in RHA-280 genotype (large grain) a higher expression was observed (Fig 1f). The expression of this gene also was mainly detected in the pericarp tissues in RHA-280 and Alybro, although with lower expression in the last genotype, suggesting that the HaGW2 gene might positively affect grain size and weight. The greatest expression of HaGW2 found in control plants of RHA-280 was observed early after anthesis (from 7 to 12 days after anthesis) in agreement with the linear growth phase of the ovule and ovary (Lindström and Hernández, 2015). The expression of GW2 in the pericarp decreased at 12 days after anthesis, when these tissues reached the final size and weight. In shaded plants of Alybro, the HaGW2 gene expression was similar to the controls (Fig. 1e), however, in RHA-280 a lower expression profile was observed in the reduced source-sink treatment compared to the controls (Fig. 1f). Similarly, the expression was tissue-specific in the pericarp. The dry weight of grains under shading was lower than controls during grain filling (Table 1, Fig. 1b), supporting upregulation of this gene in the growth of the pericarp tissue in sunflower.

In plants, the ubiquitin pathway has recently been shown to play important roles in seed size control (Li and Li, 2014), In *Arabidopsis* was reported that DA1, an ubiquitin receptor with two ubiquitin-inter- acting motifs (UIMs), and a single zinc-binding LIM domain (Li et al., 2008), acts as maternal control of seed size. DA1 regulates seed growth by limiting cell proliferation in the maternal integuments of developing ovules and seeds (Li et al., 2008; Xia et al., 2013). In the present study, we found evidence that the HaGW2 gene, a putative RING-type E3 ubiquitin ligase, would be acting in maternal tissues of sunflower, because primarily observed expression in pericarp tissue, but different to most studies that have examined the ubiquitin pathway as a negative regulator of seed size in cereals (Song et al., 2007; Li et al., 2008; Hong et al., 2014; Simmonds et al., 2016), we observe evidence of upregulation. The HaGW2 gene seems to have a positive effect on grain weight and size in sunflower, in wheat, where TaGW2 has been extensively studied in recent years, there are controversial results about the regulation of this gene on grain size (Bednarek et al., 2012). Therefore, it is likely that HaGW2 upregulates the weight and size of sunflower grain.

CONCLUSIONS

A clear association was found in this study between grain growth dynamics and the expression of HaAP2 and HaGW2 putative genes. The expression, tissue-specific pericarp suggests that these genes would be acting by maternal tissues. The present study provides clear evidences on the genetic control of grain growth and could be helpful to improve the knowledge f of grain weight and grain size determination in sunflower.

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