Published November 28, 2016

RESEARCH

Storage-Associated Genes and Reserves Accumulation in Soybean Cultivars Differing in Physiological Strategies for Attaining High Seed Protein Concentration

Florencia Poeta,* Ana C. Ochogavía, Hugo R. Permingeat, and José L. Rotundo

ABSTRACT

High seed protein concentration (HP) in soybeans [*Glycine max* (L.) Merr.] is attained by increases in protein content in large seed genotypes (HP large seed) or by reductions in oil and carbohydrates contents in small seed genotypes (HP small seed). We hypothesized that these alternative strategies impact seed development, component accumulation, and gene expression differently. We compared a standard protein commercial genotype with two HP genotypes having contrasting seed size. The HP large seed genotype exhibited the fastest rate and longest period of seed growth and reserves accumulation compared with the HP small genotype. Seed development of these contrasting genotypes was normalized using a moisture depletion framework. Expression levels of some of the genes involved in protein and oil synthesis were lower in the HP small seed genotype compared with the other genotypes. No difference in gene expression was observed between the commercial and the HP large seed genotypes, suggesting a role for assimilate supply controlling high protein concentration based on this strategy. Our results indicate that seed development and gene expression are not necessarily associated with high seed protein concentration per se; a better understanding of seed composition requires acknowledging the contrasting strategies, in terms of seed size, to attain high seed protein concentration.

F. Poeta, A.C. Ochogavía, and J.L. Rotundo, IICAR, UNR, CONICET (Instituto de Investigaciones en Ciencias Agrarias, Univ. Nacional de Rosario, Consejo Nacional de Investigaciones Científicas y Técnicas), Facultad de Ciencias Agrarias, Campo Experimental, J.F. Villarino, Zavalla, Argentina; H.R. Permingeat, Laboratorio de Biología Molecular, Facultad de Ciencias Agrarias, Univ. Nacional de Rosario, S2125ZAA Zavalla, Argentina. Received 19 July 2016. Accepted 13 Oct. 2016. *Corresponding author (florencia.poeta@unr.edu.ar). Assigned to Editor Shawn M. Kaeppler.

Abbreviations: *AAP*, amino acid permease gene; *ACC*, acetyl-CoA carboxylase gene; *ADPGP*, ADP-glucose pyrophosphorylase gene; *DGAT*, diacylglycerol acyl transferase gene; HP, high seed protein concentration; PCR, polymerase chain reaction; *PEPC*, phosphoenolpyruvate carboxylase gene; *SUT*, sucrose transporter gene; SWC, seed water concentration.

Soybean [*Glycine max* (L.) Merr.] seeds are the most used and preferred protein source for animal feeding. The international market demands high protein seeds (~38%, dry base) to achieve the required high quality meal for profitable marketing. Given the importance of this trait, developing soybean varieties with superior protein concentration has become a high research priority (Wilson, 2004). However, it is well-documented that improvements in protein concentration are associated with lower oil concentration (Wilson, 2004) and seed yield (Brim and Burton, 1979; Carter, 1982; Wilcox and Zhang, 1997; Cober and Voldeng, 2000; Wilcox and Shibles, 2001). It has been also documented that protein concentration is a quantitative character greatly affected by genotypic and environmental effects (Yaklich et al., 2002; Dardanelli et al., 2006; Naeve and Huerd, 2008; Rotundo and Westgate, 2009; Medic et al., 2014; Rotundo et al., 2016). Due to the complexity of this trait, a comprehensive approach involving both seed physiology and molecular studies is needed to better understand the processes determining soybean seed protein concentration.

Published in Crop Sci. 57:1–10 (2017).
doi: 10.2135/cropsci2016.07.0611

The final chemical composition of a soybean seed results from the numerical ratio between each component content (mg seed^{-1} of protein, oil, and carbohydrate) and total seed weight (Ishii et al., 2010). At the same time, the content per seed of each chemical constituent depends on the component accumulation rate (mg seed^{-1} d^{-1}) and the filling duration (days) (Swank et al., 1987). In line with these concepts, we identified high protein soybean genotypes in a previous study that achieve high seed protein concentration through two alternative strategies based on contrasting seed size (Poeta et al., 2016). High seed protein concentration (HP) can be attained by more-than-proportional increases in seed protein content in large seed genotypes (from now, HP large seed) or, alternatively, by more-than-proportional reductions in oil and carbohydrates contents in small seed genotypes (from now, HP small seed). This previous work has demonstrated that the same trait (for instance, seed protein concentration) can be attained via contrasting strategies associated with seed size. Furthermore, our previous results showed that these different alternatives have contrasting impacts on whole-crop physiology. The HP large seed genotypes had longer filling periods, faster seed growth rates, and greater assimilates per seed compared with the HP small seed genotypes; HP small seed genotypes had a higher leaf area index at the beginning of seed filling and faster leaf senescence but very low levels of assimilate supply compared with the HP large seed genotypes. Currently, there is no information about the possible impact of these contrasting strategies on other processes related to protein, oil, and residual (i.e., carbohydrate plus ash) accumulation at the physiological or molecular level.

Several studies in different plant species have been performed to understand the molecular basis of seed protein deposition. It has been suggested the importance of some key enzymes involved in carbohydrates and protein metabolism for increasing seed protein concentration. In pea (*Pisum sativum* L.) cotyledons, it has been demonstrated that the overexpression of a sucrose transporter stimulated storage protein accumulation by increasing intracellular sucrose levels, leading to the activation of storage protein genes (*St SUT1*; Rosche et al., 2002). In *Vicia narbonensis* L., seed protein concentration has been increased by overexpression of phosphoenolpyruvate carboxylase (*PEPC*) (Rolletschek et al., 2004). This enzyme has a main function in the carbohydrate synthesis pathway and it was demonstrated that its increase led to a higher incorporation of carbon into proteins (Rolletschek et al., 2004; Radchuk et al., 2007). Likewise, the overexpression of amino acid permease (*VfAAP1*; Miranda et al., 2001) resulted in raised seed protein concentrations due to higher amino acid availability (Rolletschek et al., 2005). The antisense inhibition of ADP-Glucose pyrophosphorylase (*ADPGP*) increased amino acid availability for protein biosynthesis, leading to higher seed protein concentration (Rolletschek et al., 2002). In these previous works, authors only consider protein concentration increases related to increased protein contents (HP large seed strategy). In the present work, we evaluated the hypothesis that the molecular basis of high seed protein concentration trait depends on the physiological strategy that determines it (HP large or small seed).

Oil is the other valuable constituent of soybean seed. Several expression studies have evaluated the role of acetyl Co-A carboxylase (*ACC*) and diacylglycerol acyl transferase enzymes (*DGAT*) in seed oil accumulation (e.g., Ohlrogge and Jaworski, 1997; Hills, 2004). Lardizabal et al. (2008) reported that the overexpression of *DGAT* increments seed oil content in transgenic soybean. In *Arabidopsis*, the expression of extra transcript copies of *DAGT* using an embryo-specific promoter increases seed oil content (Jako et al., 2001). Moreover, Roesler et al. (1997) found increments in fatty acid content by overexpressing the cytosolic homomeric of *ACC* in plastids. Considering this background, it might be hypothesized that high seed protein concentration in HP small seed genotypes is associated with a reduced expression of some of these genes.

The physiological strategies described previously (Poeta et al., 2016) provide a useful framework to investigate whether contrasting molecular and/or developmental patterns exist, even though both strategies seeks to increase the same trait (i.e., seed protein concentration). Here we compared a standard protein cultivar versus two HP genotypes having the proposed contrasting physiological strategies. The specific objectives were (i) to describe seed growth and normalize seed development using a moisture depletion framework in genotypes of contrasting seed size and composition; (ii) to asses differences in the rate and duration of protein, oil, and residual deposition among the same group of genotypes; and (iii) to evaluate, across genotypes, the expression of six genes encoding enzymes involved in seed reserve accumulation. No intention was made to present a complete gene expression profile, but instead to focus on the expression of previously reported genes involved in seed composition. The genes evaluated were: amino acid permease (*AAP*), phosphoenolpyruvate carboxylase (*PEPC*), sucrose transporter (*SUT*), ADP-glucose pyrophosphorylase (*ADPGP*), acetyl-CoA carboxylase (*ACC*), and the diacylglycerol acyl transferase gene (*DGAT*). The characterization of transcript levels of seed reserve accumulation genes, as well as the assessment of other seed development processes, contributes to the understanding of physiological and molecular processes that lead the seed protein accumulation.

MATERIALS AND METHODS

Plant Material

Three soybean genotypes were evaluated: a commercial cultivar ('DM3100') with high yield (4374 kg ha^{-1}) but standard

protein concentration (35%), content (53 mg protein seed^{-1}) and seed size (152.8 mg seed^{-1}) (from now, commercial); a large seed genotype ('PI538376'; 196.2 mg seed^{-1}) with high protein concentration (42.1%) and content (103.7 mg protein seed^{-1}) (HP large seed); and a small seed genotype ('PI518757'; 74.2 mg seed^{-1}) with high protein concentration (41.8%) associated with reduced oil and carbohydrates contents (HP small seed). These genotypes were selected at random from nine genotypes evaluated for growth and developmental traits in Poeta et al. (2016).

Plant Culture

Experiments were conducted at the Campo Experimental Villarino, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario in Zavalla, Argentina. The soil type was a silt clay loam Vertic Argiudoll, Roldán series. Planting dates were 1 Nov. 2011 and 13 Nov. 2012, with a stand density of 34 plants m^{-2}. Plots were four rows, 6 m long with 0.52 m between rows. Seeds were inoculated before sowing. Weeds were chemically controlled at planting and hand removed during crop growth. Pests and diseases were prevented by spraying recommended fungicides and insecticides.

Tissue Collection

Pods from 10 consecutive plants from the two central rows were sampled three times per week from R5 (beginning seed) to R8 (harvest maturity) (Fehr and Caviness, 1977), totaling 8 to 10 sampling dates per experimental unit. Since soybean seeds from different canopy positions differ in size and developmental stage at any point in time, all pods from the three central nodes of the main stem were sampled (Escalante and Wilcox, 1993; Poeta et al., 2014). After sampling, pods were immediately placed in plastic hermetic bags and stored on ice for transport to the laboratory.

Pod samples were divided into two subsamples: a first subsample of seeds was excised from pods in a moisture-saturated humid box to avoid dehydration. This subsample was also used for seed weight and chemical determinations. The second subsample was excised from pods at room conditions and immediately frozen in liquid nitrogen until RNA extractions for gene expression assays. Each sample was composed of 15 to 30 seeds per sampling date.

Determination of Seed Developmental Traits and Seed Reserve Accumulation

Seed fresh weight was estimated from the first subsample by weighing the fresh sample. Seed dry weight (SDW, mg seed^{-1}) was estimated after drying this sample at 65°C for 96 h. Seed water concentration (SWC, %) was calculated as the difference between seed fresh weight minus dry weight, divided the fresh weight and multiplied by 100.

Protein concentration (%, mg protein mg^{-1} tissue × 100) was determined as nitrogen concentration multiplied by 6.25 using the Kjeldahl method in a 0.1-g subsample (AOAC, 1990). Oil concentration (%, mg oil mg^{-1} tissue × 100) was determined gravimetrically after extraction with petroleum ether in a 0.15-g subsample. Protein and oil contents (mg protein seed^{-1} oil) were estimated as the product between individual seed dry weight and component concentration. Residual content (mg residual seed^{-1}) was calculated as the difference between total seed dry weight and protein plus oil contents (Hanson et al., 1961). Concentration and component contents were expressed on a dry matter basis.

Variation in SWC during seed development was modeled as quadratic seed moisture decay from R5 to R8:

$$\text{SWC (mg mg}^{-1} \times 100) = a + bx^2 \qquad [1]$$

where y is SWC, a is the intercept (SWC at R5), b is the desiccation rate, and x is days after R5. The linear term in the quadratic function was considered zero to properly model the decay in water concentration assuming maximum SWC at R5 (days after R5 = 0).

The rate and duration of seed dry weight (SDW) and component content (protein, oil, and residual) accumulation were modeled using a bilinear function with a plateau (Gambín et al., 2006; Rotundo et al., 2011):

$$\text{SDW or content (mg seed}^{-1}) = a + bx \text{ for } x < c \text{ (linear function)} \qquad [2]$$

$$\text{SDW or content (mg seed}^{-1}) = a + bc \text{ for } x > c \text{ (plateau function)}$$

where x is days after R5 (days), a is the y-intercept (mg seed^{-1}), b is the linear rate of dry weight or component content accumulation (mg seed^{-1} d^{-1}), and c is x at maximum seed weight or component content. The duration of seed dry weight or component accumulation (days) was calculated as $c - (a/b)$.

Relative RT-qPCR Analysis

Since the seed developmental stage of evaluated genotypes may differ for a single calendar day, SWC was used to normalize seed development (e.g., Swank et al., 1987; Borrás and Westgate, 2006; Poeta et al., 2014). Subsamples having ~70% SWC (equivalent to 50% of seed-fill duration, regardless of genotype and year; Fig. 1) were used for gene expression analyses. This subsample was selected after analyzing the seed development pattern of each genotype and evaluation year.

Seed total RNA was extracted using the SV Total RNA System Kit (Promega Corporation) according to the manufacturer instructions. The RNA was quantified spectrophotometrically, and integrity was verified through 1.5% agarose gel. It was stored at −80°C. Reverse transcription was performed using 1.5 μg of total RNA with the iScript complementary DNA synthesis kit for RT-PCR™ (reverse transcription polymerase chain reaction, BIO-RAD) according to the manufacturer instructions. Complementary DNA was diluted to a final concentration of 10 ng μl^{-1} in nuclease-free water.

Soybean genes sequences *ACC*, *AAP*, *DGAT*, *PEPC*, *SUT*, *ADPGP*, and *β-tubulin* were gathered from NCBI GenBank. Primers were designed using the Primer 3 (Rozen and Skaletsky, 2000) and Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). To determine primer pair properties like melting temperature, guanine-cytosine content, primer loops, primer dimmers and primer–primer compatibility, Oligo Analyzer 1.1.2 software was used. The primer pairs are listed in Table 1.

Real-time PCR analysis was performed using the Rotor-Gene Q (Qiagen®) thermal cycler. The reaction contained 1× SYBR Green PCR Master Mix (Mezcla Real®, Biodynamics, Argentina), 0.6 μM of the forward and

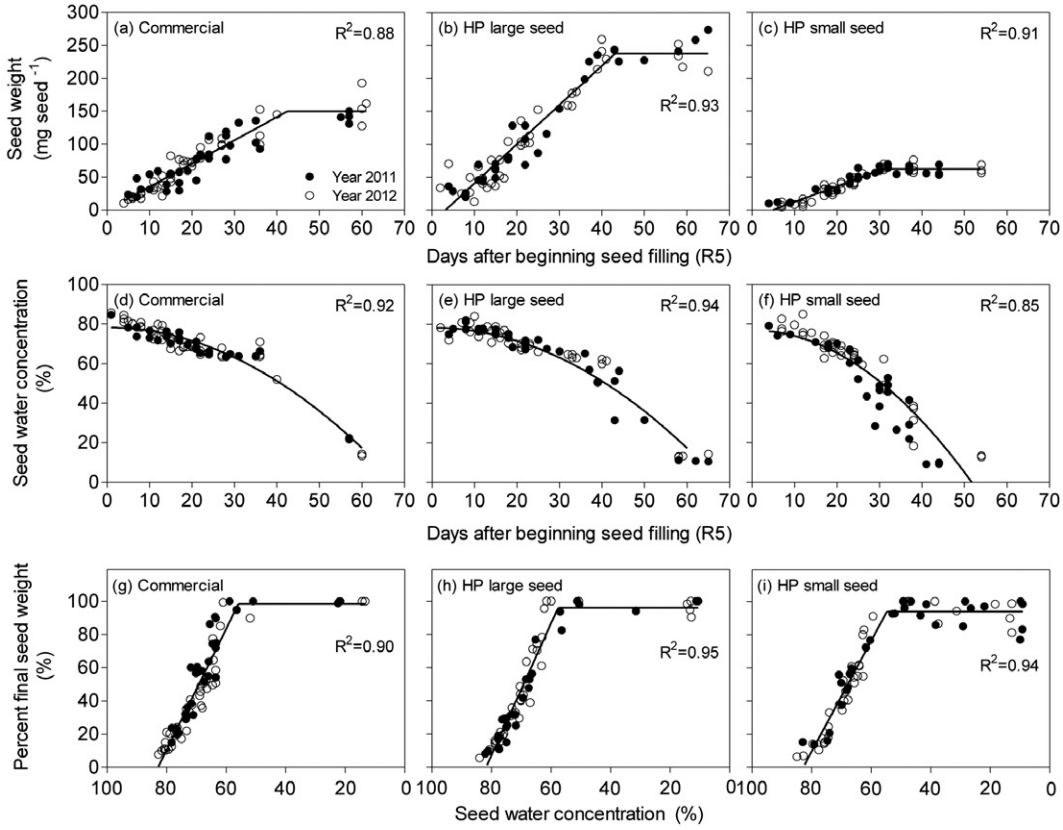

Fig. 1. Relationship between seed dry weight and days (a–c), water concentration and days (d–f), and relative final seed weight and seed moisture (g–i) for a commercial genotype and two genotypes expressing contrasting physiological strategies for attaining high seed protein concentration (HP large seed and HP small seed), evaluated in two growing seasons (2011–2012 and 2012–2013).

Table 1. Oligonucleotide primers used for relative quantitative polymerase chain reaction (PCR).

Gene symbol	Target gene	Gene product function	Accession	Primer sequence (5′–3′)
ACC	Acetyl-CoA Carboxylase gene	Fatty acid synthesis (e.g., Ohlrogge and Jaworski, 1997)	AF163149.1	Forward: ACGAGGAAGAGTTGGCAATG Reverse: CGTTCACCCTGTTAGCGAGT
DGAT	Diacylglycerol acyltransferase gene	Triacylglycerol synthesis (e.g., Settlage et al., 1998)	AY652765.1	Forward: CCATGCTTAAGGCGTGGTAT Reverse: ACAGCAGAAACCAGGAATGC
AAP	Amino acid transporter gene	Amino acid uptake and interorgan transport (e.g., Okumoto et al., 2002)	AY029352	Forward: GCCCTTATTTGCATTTGTGG Reverse: GCGGTGACAAAATTGCTCTT
SUT	Sucrose transporter gene	Sucrose transport (e.g., Rosche et al., 2002)	XM_006589399.1	Forward: GACAGTAGGGGAAGGGAAGG Reverse: AGTTCAACATCAGCCCCAAC
ADPGP	ADP-glucose pyrophosphorylase gene	Starch synthesis (e.g., Weber et al., 1995)	BG370154.1	Forward: ACCGCTGGGAGAGTTTATCAAGGC Reverse: ACAGCGGTGATTGCTCATAGCCG
PEPC	Phosphoenolpyruvate carboxylase gene	Carbon skeletons synthesis (e.g., Turpin et al., 1990)	AB097087.1	Forward: GTGAATATGCCCCTGGTTTG Reverse: CCAGCAGCAATACCCTTCAT
β-Tubulin	β-tubulin gene	Subunit protein of microtubules (e.g., Ludueña, 1997)	M21296.1	Forward: TGCCAGTCCAACCTTTTCAT Reverse: CACTGAGCTATGGACCCAAGT

reverse primers and 3 μL of complementary DNA in a total volume of 15 μL. No-template controls were also included. Quantitative PCR cycle conditions were 15 s at 94°C for denaturation, 30 s at 60°C for annealing, and 20 s at 72°C for extension. Gene identity was confirmed through 2.5% gel electrophoresis and each melting profile (Bustin et al., 2010). A checklist outlining the RNA to quantitative PCR quality and methodology based on Bustin (2002) is detailed in Supplemental Table S1. All PCR amplifications were performed in triplicate. Quantification cycle (Cq) and efficiency (E) for each amplicon were obtained from the Comparative Quantitation software supplied by Corbett Research for Rotor Gene. *β-tubulin* was selected as a reference gene (Cq for tubulin showed a standard deviation of 1.3 under all conditions). This gene was used several times as a reference gene in soybean quantitative PCR experiments and was proved to be stable (Wang et al., 2006, 2007). The transcript levels of the targets genes were normalized against the *β-tubulin* gene in the same messenger RNA sample (Bennett et al., 2015). Normalized expression value was calculated for each gene according to Simon's formula (Simon, 2003). Relative expression values were calculated for each genotype

independently and expressed with respect to the lowest normalized expression value.

Experimental Design and Statistical Analysis

A 2-yr field experiment was arranged in a randomized complete block design with four replications. Seed development and reserve accumulation were assessed in the four replications ($n = 8$). Gene expression analyses were assayed in three field replications during the 2 yr ($n = 6$). Each field replication also had three technical repetitions for gene expression. Variables were tested using ANOVA including year, block nested within year, and genotype (commercial, HP large seed, and HP small seed) as main factors. The analysis was performed using a GLM procedure from SAS software (SAS Institute, 1999). Significant differences were considered at the 0.05 probability level. Multiple comparisons between means were performed using a Fisher's LSD test corrected by Bonferroni's method.

The *β-tubulin* expression stability was evaluated by coefficient of variability (CV) inspection and ANOVA. Normality of the empirical distribution of data was assessed by the Shapiro–Wilk (W) test. Homogeneity of variance was evaluated by the relationship between studentized and predicted residuals. Variable transformation was not necessary.

RESULTS

Seed Growth and Seed Development Normalization

Genotypes had different rate and duration of seed dry weight accumulation (Fig. 1a–1c). The HP small seed genotype had the slowest seed-filling rate compared with the other genotypes ($P < 0.05$); it was 2.4 mg seed^{-1} d^{-1} for the HP small seed genotype, 6.0 mg seed^{-1} d^{-1} for the HP large seed genotype, and 3.5 mg seed^{-1} d^{-1} for the commercial genotype. The duration of seed-filling rate was the shortest for the HP small seed genotype compared with the other genotypes ($P < 0.05$). The effective seed-filling period was 26, 40, and 42 d for the HP small seed, HP large seed, and commercial genotypes, respectively.

The desiccation rate also differed among genotypes (Fig. 1d–1f). The HP small seed genotype had the fastest desiccation rate (−0.029% d^{-1}) compared with the other genotypes ($P < 0.05$); the HP large seed and the commercial genotypes were not significantly different from one another (average −0.018% d^{-1}).

In spite of contrasting patterns of seed growth and moisture depletion dynamics, it was possible to normalize seed development across these very dissimilar genotypes (Fig. 1g–1i). The parameter c in Eq. [2], which is the SWC at physiological maturity, was 56% and did not differ across genotypes ($P > 0.05$). Also, no significant differences were obtained for the rate of seed weight increase (percent of final weight) among genotypes ($P > 0.05$), and the mean value for the three genotypes was 3.65 (parameter b in Eq. [2]).

Developmental Traits Related to Seed Component Accumulation

Major seed storage components can be analyzed independently in terms of rate and duration of accumulation (Poeta et al., 2014). Variation in protein content (mg seed^{-1}) at maturity was closely and linearly related with the rate of

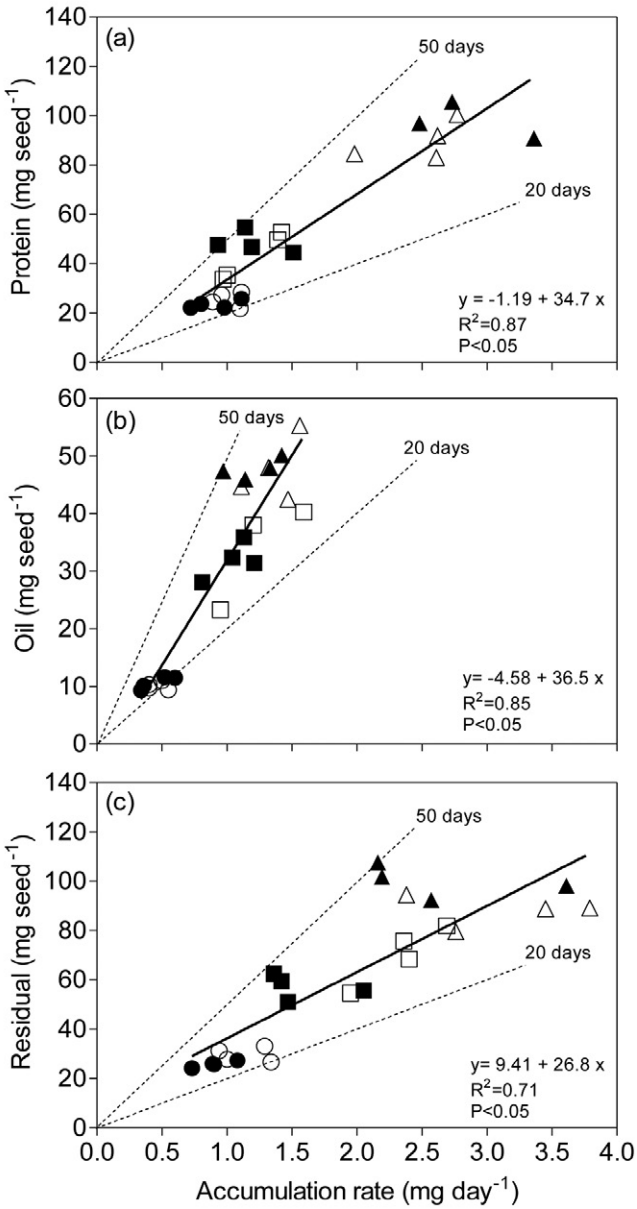

Fig. 2. Relationship between seed (a) protein, (b) oil, and (c) residual contents versus rates of component accumulation for a commercial genotype and two genotypes expressing contrasting physiological strategies for attaining high seed protein concentration (HP large seed and HP small seed) evaluated in two growing seasons (2011–2012 and 2012–2013). Closed symbols are year 2011–2012, open symbols are year 2012–2013. Squares are the commercial genotype, triangles are the HP large seed genotype, and circles are the HP small seed genotype. Dashed lines represent equal seed component durations.

accumulation among genotypes, but not with duration ($R^2 = 0.87$, $P < 0.05$) (Fig. 2a). There was no relationship between rate of accumulation and protein concentration ($P > 0.05$, Fig. 2a); the HP large seed genotype showed the highest rate of protein accumulation (~2.65 mg d^{-1}), while the HP small seed and commercial genotypes had the lowest (~1 mg d^{-1}) and did not differ statistically ($P > 0.05$). As expected from the seed growth analysis, both HP large seed and commercial genotypes had a longer period of protein accumulation (36 and 39 d, respectively) compared with the HP small seed (26 d, $P < 0.05$).

Seed oil content was also positively associated with the oil accumulation rate ($R^2 = 0.85$, $P < 0.05$) and not with the accumulation period (Fig. 2 b). The rate of seed oil accumulation was not correlated with seed protein concentration; the HP large seed and the commercial genotypes had the fastest rate of oil accumulation (1.13 and 1.29 mg oil d^{-1}, respectively) compared with the HP small seed one (0.46 mg oil d^{-1}) ($P < 0.05$, Fig. 2b).

Variation in residual content at maturity across genotypes and years was closely and linearly related ($R^2 = 0.71$, $P < 0.0001$) with the rate (Fig. 2c). The duration, however, did not explain any difference in residual content ($P > 0.05$). There was no association between the rate of residual accumulation and seed protein concentration; the commercial genotype had an intermediate value of 1.96 mg residual d^{-1}, while the HP small and large genotypes had 1.01 and 2.86 mg residual d^{-1}, respectively (Fig. 2c, $P < 0.05$).

Transcriptional Levels of Genes

Expression level of studied genes was assessed by quantitative PCR using *β-Tubulin* as a reference gene. Expression stability was confirmed by a low coefficient of variability (CV = 4.8) and nonsignificant differences detected by ANOVA ($P > 0.05$) among samples.

Relative expression of genes involved in seed reserve accumulation was compared among genotypes expressing contrasting strategies for increased seed protein concentration. For *AAP*, *DGAT*, and *PEPC* genes, there was a significant interaction between year and strategy. However, this interaction explained a low proportion of total variation in transcript levels of *AAP* and *DGAT* (11 and 7% of sum of squares, respectively) and 38% in transcript levels of *PEPC*. A complete description of these interactions is available in Supplemental Table S2. Due to this relatively low variation explained by the interaction between expression and year, and for the sake of simplicity, we focused on strategy main effects.

Transcript relative expression of *AAP*, *PEPC*, *DGAT*, and *ACC* genes showed a similar behavior at the evaluated seed development stage (70% moisture), with reduced expression in the HP small seed genotype compared with the other genotypes (Fig. 3a–3d). The highest expression ratio was observed for the *AAP* gene, in which HP large

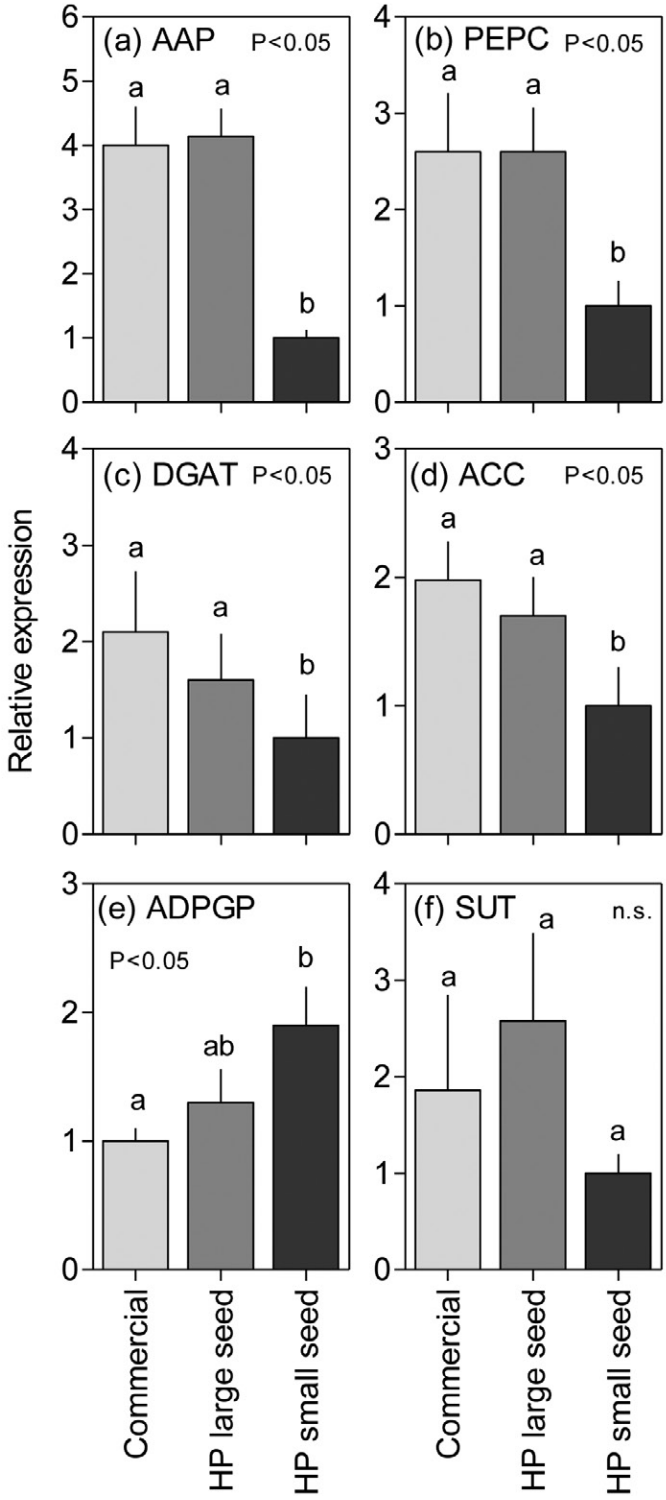

Fig. 3. Relative expression levels of amino acid permease (*AAP*), phosphoenolpyruvate carboxylase (*PEPC*), sucrose transporter (*SUT*), ADP-glucose pyrophosphorylase (*ADPGP*), acetyl-CoA carboxylase (*ACC*), and diacylglycerol acyl transferase (*DGAT*) genes for a commercial genotype and two genotypes expressing contrasting physiological strategies for attaining high seed protein concentration (HP large seed and HP small seed) evaluated in two growing seasons (2011–2012 and 2012–2013) at the same developmental stage (70% seed moisture). Mean values with different letter are significantly different at the 0.05 probability level (Fisher's LSD multiple comparison corrected by Bonferroni's method). Vertical bars indicate the SE of the mean.

seed and commercial genotype expression was four times higher than HP small seed expression (Fig. 3a). The transcription level of *PEPC* for HP large seed and commercial genotypes was 2.6 times higher compared with the HP small seed genotype (Fig. 3b). The expression levels of *DGAT* and *ACC* genes were doubled when comparing HP large with small seed genotypes (Fig. 3c–3d).

Conversely, *ADPGP* transcript levels were the highest in the HP small seed genotype and lowest in the commercial genotype (Fig. 3e); the commercial cultivar was not different for *ADPGP* expression compared with the HP large genotype ($P > 0.05$). The expression level of *SUT* was not significantly different among any of the evaluated genotypes (Fig. 3f).

DISCUSSION

We present results of an experiment aimed to understand seed development, component accumulation, and gene expression of some key enzymes in soybean genotypes differing in seed composition. These genotypes were grown in replicated field trials with realistic agronomic management conditions, taking into account environmental repetition, as attained by conducting the experiment in two different years. Findings from this experiment support the concept that high protein concentration in soybean seeds can impact seed development and transcript levels differently, depending on the strategy that determines this trait. Many studies assessed the molecular physiology (e.g., Golombek et al., 2001; Miranda et al., 2001; Rolletschek et al., 2004; Götz et al., 2007) and developmental processes (e.g., Rotundo et al., 2011; Poeta et al., 2014) controlling seed protein deposition in legumes; however, none of them have considered the physiological strategies proposed in our previous work (Poeta et al., 2016) and evaluated here. Understanding how seed components are accumulated during seed development and which major genes (associated with protein and lipid accumulation) are involved in this process is critical to identify specific targets for seed quality breeding.

As it was reported in previous studies, increasing protein concentration could be achieved by a more-than-proportional increase in protein content (mg seed^{-1}) or by a more-than-proportional reduction in the contents of the other seed components (Rotundo and Westgate, 2009; Ishii et al., 2010). Change in seed component contents has been determined by different combinations of rate and duration of component accumulation (Munier-Jolain et al., 1998; Egli and Bruening, 2007; Rotundo and Westgate, 2009). In the current study, we found that contrasting developmental patterns related to rate and duration of reserve deposition emerged from the strategies assessed. These results are in line with the idea that the accumulation of the different seed chemical components is controlled independently (Jenner et al., 1991; Poeta et al., 2014). For example, the HP large seed genotype (associated with increased seed protein concentration based on increased protein content per se) exhibited the fastest rate and longest duration of accumulation for all seed constituents. These results are in line with Rotundo and Westgate (2009) and Rotundo et al. (2011), in which they compare commercial cultivars against a set of HP large seed genotypes. The HP small seed genotype (associated with increased seed protein concentration based on reduced oil and carbohydrate contents) showed an opposite pattern; it had the slowest rate and shortest duration for all seed component accumulation. The commercial cultivar was similar to the HP small seed genotype for rate of protein accumulation but was more alike to the HP large seed genotype for the rate of oil accumulation. This is a clear indication that seed developmental processes that determine seed composition are not associated with seed protein concentration per se, as was previously suggested (Sinclair and de Wit 1975; Salado-Navarro et al., 1985). In this sense, a better understanding of the developmental processes determining seed composition requires acknowledging the existence of contrasting strategies in terms of seed size to attain high seed protein concentration.

Using a seed moisture depletion framework, it was possible to normalize seed development of genotypes having contrasting seed composition and size. This type of procedure was demonstrated to be adequate across genotypes and environments in many species (Swank et al., 1987; Calderini et al., 2000; Borrás and Westgate, 2006). Here, we propose that this is a critical step for correctly comparing expression levels during seed development. Using days instead of seed moisture to refer to seed development would have been misleading, as the genotypes to be compared differed in seed-filling duration.

When comparing the relative expression of genes at 70% seed moisture content (which is equivalent to 50% final maximum seed size across genotypes), we discovered that it is possible to have the same seed protein concentration level in genotypes having contrasting transcriptional patterns. Specifically, we found that high expression levels of some genes involved directly (*APP*) or indirectly (*PEPC*) in protein synthesis were detected in high seed protein concentration genotypes. On the other hand, we found that high protein concentrations in the HP small seed genotype were related to low transcript levels of oil synthesis genes (*DGAT* and *ACC*). Our results are in concordance with Lardizabal et al. (2008) and Roesler et al. (1997) findings, in which high oil contents were achieved by overexpressing *DGAT* or *ACC*, respectively. Further information about post-transcriptional and post-translational regulatory

mechanisms is needed to understand the molecular mechanisms involved in the seed reserve accumulation processes (Hills, 2004).

Based on previous works involving the overexpression of *PEPC* (Rolletschek et al., 2004; Radchuk et al., 2007), *SUT* (Rosche et al., 2002), and *AAP* (Miranda et al., 2001; Rolletschek et al., 2005) genes in legume species, it was expected to observe more expression in the HP large seed compared with the commercial genotype for some of the putative genes. However, this expectation is based on the preconception that seed composition is an embryo-determined character. Current evidence from previous work on soybeans shows that there is a strong maternal control for the expression of seed protein concentration, as determined by the HP large seed strategy (Rotundo et al., 2009, 2011). This maternal control occurs at the level of the amount of assimilates (sucrose and amino acids) reaching the seed apoplast (Hanson, 1986, 1991). Indeed, commercial cultivars having standard protein concentration can be "converted" into high protein genotypes by increasing the amount of assimilates via manipulative depodding treatments (Rotundo et al., 2009, 2011). Because there was no difference in gene expression between the HP large seed and commercial cultivars, a possible explanation would be that, for these strategies, the supply of seed assimilates were the important feature and not the level of expression of genes under study.

This work provides a novel insight into the study of seed protein concentration, emphasizing (i) the importance of analyzing seed constitute accumulation independently during seed filling, (ii) the existence of different physiological strategies to attain high protein concentration, (iii) the use of seed moisture to normalize seed development for gene expression analysis, and (iv) the concept that high seed protein concentration can be achieved via different patterns of expression of some seed storage genes. Next step includes a more exhaustive screening of genotypes and genes to extrapolate these results to other cultivars.

Conflict of Interest

The authors declare there to be no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

Acknowledgments

This research was supported by Agencia Nacional de Investigaciones (PICT-2011-1292), CONICET (PIP-202), and Fundación Ciencias Agrarias-UNR (PICT 2012). F. Poeta held a graduate scholarship from CONICET, the Argentine National Research Council. J.L. Rotundo and A.C. Ochogavía are members of CONICET. H.R. Permingeat is member of University of Rosario (UNR).

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