 http://dx.doi.org/10.1590/2317-1545v36n3977

SGlu2 gene expression in coats of soybean seeds[1]

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ABSTRACT – Glucanases can act in plant defense against biotic factors. Despite its importance, research to study the expression of genes encoding glucanases in soybean seed coats is limited. The aim of this study was to assess the relative expression of the SGlu2 gene (β-1.3-Glucanase 2), possibly involved in defense against biotic factors, in coats of seeds of four soybean genotypes. Two genotypes of black seed coats, IAC and TP, and two of yellow seed coats, BMX Potência RR and CD 202 were used. Seeds were multiplied in a greenhouse at Embrapa Clima Temperado – ETB, and the gene expression assay was performed at the Laboratório de Sementes e Biotecnologia, UFPel. Seed coat gene expression was assessed by qPCR technique in four development stages: 40, 45, 50 and 55 days after anthesis. The SGlu2 gene shows more expression in the BMX Potência RR genotype compared to other genotypes. The gene expression in the seed coat is constant in different development stages of CD 202 cultivar and IAC and TP strains, except at 45 DAA (days after application) for this latter genotype.

Index terms: *Glycine max*, seed development stages, qPCR technique.

Expressão do gene SGlu2 em tegumentos contrastantes de sementes de soja

RESUMO – As glucanases podem atuar na defesa das plantas contra fatores bióticos. Apesar da sua importância, pesquisas visando estudar a expressão de genes que codificam glucanases em tegumento de sementes de soja são limitadas. O objetivo do trabalho foi avaliar a expressão relativa do gene SGlu2 (β-1,3-Glucanase 2), possivelmente envolvido na defesa contra fatores bióticos, em tegumento de sementes de quatro genótipos de soja. Foram utilizados dois genótipos de tegumentos pretos, IAC e TP, e dois de tegumentos amarelos, BMX Potência RR e CD 202. As sementes foram multiplicadas em casa de vegetação na Embrapa Clima Temperado – ETB, sendo o ensaio de expressão gênica realizado no Laboratório de Sementes e Biotecnologia da UFPel. A expressão do gene no tegumento das sementes foi avaliada pela técnica qPCR, em quatro fases de desenvolvimento: 40, 45, 50 e 55 dias após a antese. O gene SGlu2 apresenta maior expressão no genótipo BMX Potência RR em relação aos demais genótipos. A expressão do gene no tegumento das sementes é constante nas diferentes fases de desenvolvimento da cultivar CD 202 e das linhagens IAC e TP, com exceção aos 45 DAA para este último genótipo.

Termos para indexação: *Glycine max*, fases de desenvolvimento de semente, qPCR.

Introduction

Coats perform many important functions during the development of soybean seeds, such as temporary storage and supply of nutrients to an embryo being formed, modulation of interactions among the internal structures of the seed and the external environment, process control of germination and dormancy, among others (Marcos-Filho, 2005; Moise et al., 2005; Weber, 2005).

As a structure protection, seed coats play a unique role against biotic and abiotic factors that may compromise the physiological, physical and sanitary qualities of the seeds, especially in the final stages of development and after physiological maturity, which involves the expression of many genes (Moise et al., 2005; Senda et al., 2004). This expression is variable among genotypes, among tissues and among stages of development of each tissue and also suffers direct influence of external factors.

Among the expressed gene families in plant tissues that can act to protect these ones against biotic and abiotic factors are the β-1.3-glucanases, which have, among others, the function of defense against invading pathogens, particularly in synergism with chitinases, degrading the homopolymer β -1.4-N-acetylglucosamine, an abundant cell wall component of many pathogens (Bishop et al., 2005; Jin et al., 1999).

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Despite the recognized importance of glucanases for vegetables, there are few studies related to its expression in seed coats of soybean seeds. Based on this, the application of specific molecular biology techniques can contribute to the development of superior constitutions, reflecting the quality of the seeds produced, especially when the research emphasizes the study of soybean genotypes with contrasting seed coats, which have a higher genetic variability (Liu et al., 2007; Mertz et al., 2010; Ranathunge et al., 2010; Tuteja et al., 2004).

Therefore, this study aimed to assess the relative expression of the SGlu2 gene, possibly involved in the defense against biotic and abiotic factors, by means of the qPCR technique, in seeds of four soybean genotypes with contrasting seed as to the color of the coats and at different stages development.

Materials and Methods

Four genotypes of soybeans, TP and IAC strains, both with seeds featuring black coats and CD 202 (conventional) and BMX Potência RR (GM) cultivars were used, both with seeds of yellow coat.

The multiplication of the plant material was performed in a greenhouse at Embrapa Clima Temperado – Estação Terras Baixas (CPACT/ETB), located in the Brazilian city of Capão do Leão/RS, in harvest 2012/2013.

From the anthesis, the marking of flowers was performed, so that all the seeds sampled were at the same stage of development. Four samples of vegetables were collected every five days (40, 45, 50 and 55 days after anthesis), for each genotype.

Immediately after each collection at Laboratório de Sementes e Biotecnologia, Universidade Federal de Pelotas, the coat of about fifteen seeds was removed with the aid of sterile blades, and care was taken to keep the plant tissue free of impurities. Once separated from the seeds, the coats of each genotype were stored in Ultrafreezer at -80 °C until the procedure for obtaining RNA.

RNA was extracted using *Concert Plant RNA Reagent* (Invitrogen™). The extraction was performed at the same time for all treatments (sampling dates of coats and contrasting genotypes). After extraction, RNA samples were treated with DNase and their purity and integrity were assessed by analysis of absorbance (260/280 nm) and electrophoresis in 1% agarose gel. RNA extraction and cDNA synthesis were performed using three biological replicates and each replicate consisted of a mixture of coats of seeds in each stage assessed.

Single-stranded cDNAs were synthesized by reverse transcription using the *SuperScript III*® (Invitrogen™) enzyme, Single-stranded cDNAs, according to the manufacturer's instructions. To assess the quality of the cDNA, semi-quantitative PCR reaction was performed using *Master mix Go Taq*, cDNA of each sample, water and ß-actin. Purity and integrity of the cDNA were also measured to ensure the quality of the material used.

For the design of the primer pair for amplification of the gene SGlu2 (β-1.3-Glucanase 2), access AF034107.1, (sense: 5' CGGCGTGTGTTATGGAAGACTTGG 3' and antisense: 5' CTGAAAC GTATCTGAATCTGACATTGTTGGCATAG 3') was carried out by a search of the EST sequences of proteins corresponding to genes at the *National Center for Biotechnology Information* (NCBI) database.

Primers were designed with the aid of the program Vector NTI Advance 11.0 (Invitrogen™, 2008), observing the parameters of the annealing temperature, primer size, percentage of GC (40-60%), size of the amplicon, absence of dimerization and absence of secondary annealing sites.

Five normalizing genes were tested preliminarily, ACT11, SKIP16, UKN1, UKN2 and ß-ACTIN, opting by normalizers ACT11 and SKIP16, which showed lower variation of expression.

After the relative quantitation, the quality of the amplified product was found by means of the dissociation curve at the end of qPCR (Bustin et al., 2009) by the gradual increase of the reaction temperature. Thus, the calculation related to the fluorescence emission was carried out, assessing the analytical specificity of the primers by denaturing the PCR product generated.

The efficiency of the SGlu2 gene was determined by performing a curve with serial dilutions of 1:3; 1:30; 1:300; 1:3000. After the dilutions it was possible to perform the calculation of efficiency through the slope of each curve, following the formula: $E = |10^{(-1/slope)}|-1$ (Zhao and Fernald, 2005), obtaining the value of *slope* (S) of -3.687 and Efficiency (E) of 0.867339.

Quantitative analysis of the gene expression in real-time of the target gene was performed in the equipment LightCycler 480 Instrument II (96) (*Roche Applied Science*®) using *SYBR® Green*.

At the end of the tests of reaction was obtained the C_q (*Quantification Cycle*) of the increase in fluorescence occurring during the reaction cycles. The optical data were subsequently analyzed using the program Light Cycler® 480 *Gene Scanning Software.*

The relative gene expression was calculated based on the amplification efficiency (E) and in the PCR cycle, in which was found the increase in fluorescence above the baseline signal (Pfaffl, 2001). After obtaining the values of relative expression of the SGlu2 gene, this one was normalized to the values observed in control, adopting for such a development stage of 40 days after anthesis (first stage of collection) for the

IAC genotype, because it is a rustic material, which has not undergone any process of artificial selection or improvement, and it may present some features of interest.

The gene expression results were submitted to analysis of variance and then compared by a test of means, applying Scott-Knott, at 5% probability.

Results and Discussion

The analysis of variance indicated an interaction among genotypes with seeds showing different colors of coats and stages of their development, for the assessed gene (Table 1). Thus, the average expression data were compared among genotypes for each stage of development of the coats and among the developmental stages of the coats, for each genotype (Figure 1).

Table 1. Summary of analysis of variance for the relative expression of the SGlu2 gene in contrasting coats of four soybean genotypes.

Sources of variation	DL	Medium square
		SGlu2
Genotypes (F1)	3	6821.69779*
Coat collection times (F2)	3	567.79720*
Interaction F1 x F2	9	487.13848*
Treatments	15	1770.18208
Residue	16	37.10850

DL – Degrees of liberty. *Significant at the 1% level of probability by F test.

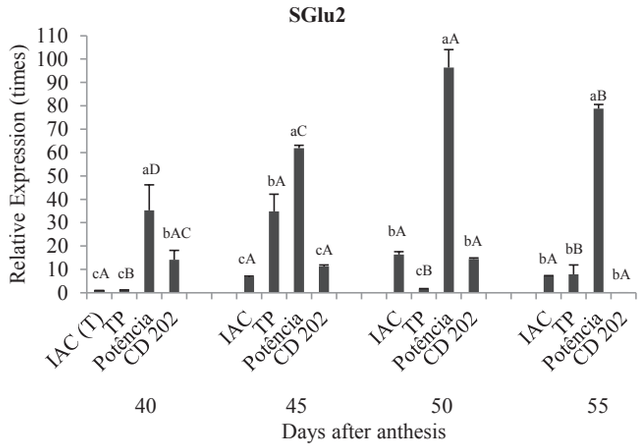

Figure 1. Relative expression of the SGlu2 gene in soybean seed coats collected in four stages of development after anthesis in four contrasting genotypes for the characteristics of coats.

[1]Means followed by different letters, smaller lower case among genotypes, within each collection time, and larger upper case within each genotype and among sampling times, differ by the Scott-Knott test at 5% probability.

After 40 days after anthesis (DAA), the highest transcript accumulation was observed in the coat of cultivar BMX Potência RR, followed by another cultivar with a yellow coat, CD 202. The lowest expression was observed in the genotypes of black coat, IAC and TP, which did not differ (Figure 1).

At 45 DAA, the expression of the gene under study remained higher in cultivar BMX Potência RR. However, cultivar CD 202 decreased in expression, together with the IAC strain, with no differences among them. Intermediate value of expression occurred in the TP strain (Figure 1).

After fifty days of development of the seeds, the lowest SGlu2 gene expression took place in the strain of TP black coat. Intermediate values were observed in the coat of the seeds of cultivar CD 202 and IAC strain, which did not differ. The higher expression of the gene remained seen in cultivar BMX Potência RR, as at 55 DAA. However, in this last stage of development, gene expression did not differ among the other genotypes assessed (Figure 1).

In the individual comparison of genotypes, the expression of the SGlu2 gene in IAC did not vary among the different stages of seed development, showing no significant difference (Figure 1).

For the TP black coat strain, the highest gene expression took place at 45 DAA, differing from the other coat development stages, which did not differ (Figure 1).

Regarding the BMX Potência RR, the highest accumulation of transcripts of the SGlu2 gene took place at 50 DAA, followed by 55 DAA, both differing. As for the lowest expression, it was observed at 40 DAA and with an intermediate value among this one and 55 DAA, the expression that took place at 45 DAA (Figure 1).

A situation similar to the one seen for the IAC genotype took place at the CD 202 genotype, in which there was not a significant difference regarding the gene expression among the different development stages of the coats (Figure 1).

Although the literature does not provide information on the SGlu2 gene expression in soybean seeds coats, it is known, based on studies performed in other plant tissues, in tobacco, that it is related to class III of proteins related to the pathogenesis, PR-Q, showing a higher transcript accumulation in defense systems against attack by pathogens (Payne et al., 1990); therefore, it is of interest to the seed area to identify genotypes that have a higher expression of these genes in the coats, indicating the possibility of them having higher resistance against biotic factors that might affect the seed quality yield.

In a study seeking to analyze and map the gene family that encodes β-1.3-glucanases in soybeans, Jin et al. (1999) found that pathogen invasion is not a prerequisite for the expression of genes of this family, such as for SGlu2, and

may be an intrinsic characteristic of the genotype, which is in agreement to what was observed by Memelink et al. (1990). This finding is important since the design of the primers of this gene was based on access from work developed by the authors. They also show that this particular gene showed a higher accumulation of mRNAs in young roots and hypocotyl of soybeans, being probably located in the extracellular spaces of tissues.

Although the results from Jin et al. (1999) and Memelink et al. (1990) indicate a role for preventive or proactive defense (regardless of microbial attack), providing some measure of protection against infection by pathogens during the crucial stages of the life cycle in which the tissues of plants are more susceptible, it is not possible to rule out the role of the β-1.3-glucanases in plant defense post-infection of microorganisms (Knogge et al., 1987).

In order to better assess the role of β-1.3-glucanase in *Arabidopsis thaliana* under infection of plant parasitic nematodes, Hamamouch et al. (2012) found that in mutants that overexpressed the genes encoding these pathogenesis-related enzymes there was a lower susceptibility of the plants to *Heterodera schachtii*, homologous to *Heterodera glycines* nematodes, which attack soybean plants. As for mutants in which there was a silencing of these genes, there was an increased susceptibility of *Arabidopsis* plants to attack by nematodes of the species *Heterodera schachtii*, once again proving the important role played by genes encoding glucanases enzymes to the defense system of plants under attack by pathogen microorganisms.

However, it is noteworthy that the expression of genes encoding glucanase enzymes, giving greater protection of plants to attack by microorganisms, is something very complex and dynamic, which deserves to be analyzed with discretion, since there is a constant evolution in the pathogen/host relationship regarding this system. The phytopathogenic microorganisms of the genus *Phytophthora* are an example, which, to infect plants, have glucanases inhibitory of proteins encoded, thus allowing infection to occur in the host (Damasceno et al., 2008).

Besides being related to the pathogenesis, another attribution has been given to glucanases, in this case to β-1.3-glucanase 1. According to Leubner-Metzger (2005), in seeds of *Nicotiana tabacum*, the genes encoding these enzymes have been playing a key role after the maturity of the seeds, under a minimum threshold of moisture, giving these a break of dormancy imposed by the seed coat, which is an important line of research in the area of seeds with such characteristics, and should be further investigated.

Conclusions

In comparing the genotypes, the SGlu2 gene has a higher expression in the BMX Potência RR coats, at all stages of development studied.

The highest expression of SGlu2 gene occurs 50 days after anthesis in BMX Potência RR genotype, and in the other genotypes, the expression is constant at different stages of development of the coats, except for TP, at 45 days after anthesis.

Acknowledgments

To CNPq, FAPERGS and CAPES for granting the scholarship and other financial aid.

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