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Relative expression of CYP and Arabinogalactan genes in soybean seed coats[1]

Expressão relativa dos genes CYP e Arabinogalactana em tegumentos de sementes de soja

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ABSTRACT - The aim of this study was to evaluate the relative expression of two candidate genes, CYP (cyclophilin) and Arabinogalactan, possibly involved with seed quality, in contrasting soybean seed coats. The following genotypes were used: BMX Potência RR and CD 202, both with yellow coats, and TP and IAC, with black coats. The relative expression of the genes was evaluated by qPCR in seven development phases of the coats of the four genotypes at 25; 30; 35; 40; 45; 50 and 55 days after anthesis. The design was completely randomized, with three replicates. The data were submitted to analysis of variance and the means were compared via the Scott Knott test, at 5% probability. The CYP gene showed greater expression in the intermediate stages of development of the coats of the genotypes CD 202 and IAC, yellow coat and black coat, respectively. The A rabinogalactan gene presented greater expression in the final stages of coats development of BMX Potência RR.

Key words: Contrasting genotypes. Candidate genes. Cyclophilin. qPCR.

RESUMO - O objetivo do trabalho foi avaliar a expressão relativa de dois genes candidatos, CYP (ciclofilina) e Arabinogalactana, possivelmente envolvidos com a qualidade das sementes, em tegumentos contrastantes de sementes de soja. Foram utilizados os genótipos BMX Potência RR e CD 202, ambos de tegumentos amarelos, e os genótipos TP e IAC, de tegumentos pretos. A expressão relativa dos genes foi avaliada pela técnica qPCR, em sete fases de desenvolvimento dos tegumentos dos quatro genótipos, aos 25; 30; 35; 40; 45; 50 e 55 dias após a antese. O delineamento foi o inteiramente casualizado, com três repetições. Os dados foram submetidos à análise de variância e as médias comparadas pelo teste de Scott Knott, ao nível de 5% de probabilidade. O gene CYP apresentou maior expressão nas fases intermediárias de desenvolvimento dos tegumentos dos genótipos CD 202 e IAC, tegumento amarelo e tegumento preto, respectivamente. O gene Arabinogalactana apresentou maior expressão nas fases finais de desenvolvimento dos tegumentos de BMX Potência RR, de tegumento amarelo.

Palavras-chave: Genótipos contrastantes. Genes candidatos. Ciclofilina. qPCR.

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INTRODUCTION

In seeds, such as those of soy, there is genetic variability for coat characteristics (DELLAGOSTIN *et al*., 2011). There are also differences in the physiological quality of seeds among soybean genotypes, which have been evidenced due to differences in the color of the coat, mainly between yellow and black genotypes (MERTZ *et al*., 2009) but also brown genotypes (SANTOS *et al*., 2007), in which seeds with a darker-colored coat have presented superior quality.

This is due to the correlation between dark color and other attributes, such as anatomical differences that may confer greater resistance to physical damage and delay the entry of water (MERTZ *et al*., 2009), or the presence of secondary metabolites that offer protection against pathogens (MOÏSE *et al*., 2005).

Although studies have pointed to better performance in seeds of a genotype with a darker coat (MERTZ *et al*., 2009; SANTOS *et al*., 2007), it is believed that a number of other factors are involved in seed quality as a function of this outer tissue, since Giurizatto *et al*. (2003) observed that some cultivars with yellow coat presented physiological quality superior to black coat lines. This reinforces the need for studies at the level of gene expression in order to investigate the genes possibly involved with favorable coat characteristics, even among genotypes with similar color in the aforementioned tissue. These attributes can be incorporated into cultivars of high productivity, aiming for improvements in the physiological quality of the seed, with the aid of molecular biology tools combined with bioinformatics, in the characterization of genes involved in the control of this attribute and its physiological responses in seeds (HENNING *et al*., 2009; MOÏSE *et al*., 2005).

In the present study, the expression of two genes in seed coats was evidenced. They are believed to code for the proteins CYP and ARABINOGALACTAN. Cyclophilins belong to a group of proteins present in all species, tissues and cells that have been investigated to date. Among the functions highlighted are protein folding, mRNA processing, protein degradation, and signal transduction, which may be crucial during development and the ability of plants to respond to stress (KUMARI *et al*., 2013; ROMANO *et al*., 2005; ROMANO; HORTON; GRAY, 2004; WANG; HEITMAN, 2005).

Arabinogalactan proteins are an extremely diverse class of cellular surface glycoproteins, widely distributed in the plant kingdom. It is suggested they act in many biological processes of cellular multiplication, programmed cellular death, development and regeneration of the cell wall, pathogen-plant interaction and mediation between cell wall, plasma membrane and cytoplasm (ELLIS *et al*., 2010; SEIFERT; ROBERTS, 2007). Despite all the advances in the study of these proteins, recent reviews highlight the need to deepen the understanding of their functions (ELLIS *et al*., 2010; KUMARI *et al*., 2013; SEIFERT; ROBERTS, 2007; WANG; HEITMAN, 2005). Moreover, their expression in soybean seed coats has not yet been reported and discussed, even in Miernyk and Johnston's study (2013).

The aim of this study was to evaluate the relative expression of two candidate genes, CYP (cyclophilin) and ARABINOGALACTAN, possibly involved in the quality of seeds, using the qPCR technique, in contrasting soybean seed coats at different stages of development.

MATERIAL AND METHODS

Four soybean genotypes contrasting for coat characteristics were used, cultivars CD 202 (conventional) and BMX Potência RR (transgenic), both with yellow coat, and the TP and IAC lines, both with black coat. The experiment consisted, firstly, of the multiplication of the plant material in a greenhouse in the municipality of Capão do Leão/RS, in the harvest of 2012/2013.

From anthesis, flower marking was carried out so that all the seeds sampled were in the same development phase. Seven collections of pods were performed at five-day intervals (25; 30; 35; 40; 45; 50; 55 days after anthesis) for each contrasting genotype.

Immediately after each collection, the coats were separated from the seeds with the help of sterilized slides, taking care to keep the plant tissue free of impurities. After separating the seeds, the coats of each genotype were stored in an ultra low temperature freezer, at -80 °C, until the RNA obtaining procedure.

RNA was extracted using Concert Plant RNA Reagent (Invitrogen™). The extraction was carried out at the same time, considering each treatment. After extraction, the RNA samples were treated with DNase and had their purity and integrity measured by absorbance (260/280 nm) and 1% agarose gel electrophoresis analysis. RNA extraction and cDNA synthesis were carried out using three biological replicates, with each replicate consisting of a mixture of seed coats at each stage evaluated.

Single strand cDNAs were synthesized by reverse transcription from 2 μg of total RNA in a final volume of 20 μl using the enzyme SuperScript III (Invitrogen TM), according to the manufacturer's recommendations. To evaluate the quality of the cDNA, a semi-quantitative

PCR was performed using Master Mix Go Taq, cDNA of each sample, water and ß-actin. The purity and integrity of the cDNA were also measured to ensure the quality of the material used.

The selection of CYP (cyclophilin), access AF456323.1, and ARABINOGALACTAN, access XM_ 003548689.1, was based on the extensive review on seed and coat formation performed by Moïse *et al*. (2005) and also in the available literature.

Five normalizing genes were preliminarily tested, ACT11, SKIP16, UKN1, UKN2 and ß ACTIN. For the evaluation of their stability, eight random cDNA samples were used, opting for normalizers ACT11 and SKIP16, which presented lower expression variation among the samples.

For the design of the primer pair of the CYP genes (sense 5'AGTAGTGTCATTTACGCTTCTCTTCTCTGCAG 3' and antisense: 5'TGGAAGGACGA GCCCTTGTAGTG 3') and ARABINOGALACTAN (sense: 5'CCATGTTGCGATGGTAG CAACTG 3' and antisense: 5'GTGACCAACCACTACTCAAGCT GAGTTG 3'), a search was performed through the EST sequences of the proteins corresponding to the genes together with the database of the National Center for Biotechnology Information (NCBI).

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

After relative quantification, the quality of the amplified product was verified by means of dissociation curves at the end of the qPCR, by the progressive increase of temperature in the reaction. In this manner, the calculation related to the emission of the fluorescence was carried out, analyzing the analytical specificity of the primers by means of the denaturation of the generated PCR product.

Efficiency for each gene was determined via curve with serial dilutions of 1:3; 1:30; 1:300; 1:3000. After the dilutions, it was possible to calculate the slope efficiency of each curve following the formula: $E = 10^{(-1/slope)}|-1$ (ZHAO; FERNALD, 2005), obtaining for the CYP gene a slope (S) value of -3.616 and Efficiency (E) of 0.890378 and for ARABINOGALACTAN a slope (S) value of -3.127 and Efficiency (E) of 1.0883.

The quantitative analysis of real-time gene expression of target genes was performed on a LightCycler 480 Instrument II (96) (Roche Applied Science®) using SYBR® Green.

Samples were allocated to 96-well optical plates (Roche Applied Science®) and coated with optical adhesives (Roche Applied Science®). At the end of the reaction trials, the Cp (Crossing point) of the increase of the fluorescence that occurred during the reaction cycles was obtained. The optical data were later analyzed using Light Cycler® 480 Gene Scanning Software.

The relative expression of the two candidate genes was calculated based on the amplification efficiency (E) and the PCR cycle, where the fluorescence increase above the basal signal was detected (PFAFFL, 2001). After obtaining the relative expression values of the target genes they were normalized from the values verified in the control, with the development phase of 25 days after the anthesis for the BMX Potência RR genotype being adopted for this purpose.

The expression results were submitted to analysis of variance and, later, compared by means test (Scott-Knott), at 5% of probability.

RESULTS AND DISCUSSION

The analysis of variance indicated that there was interaction between contrasting genotypes and development stages of the soybean seed coats (days after anthesis) for the two evaluated genes (Table 1).

Figure 1 presents the results of CYP gene expression in coats at different stages of development of the BMX Potência RR, CD 202, TP and IAC genotypes. In general, it is possible to verify that the contrast between the genotypes in gene expression varies considerably depending on the development stage of the coats. At 25 days after anthesis (DAA), it was not possible to verify significant difference in CYP gene expression; at 30 DAA the gene was expressed higher in CD 202 genotype coats in detriment to the others, which did not differ among themselves; at 35 DAA the expression of the gene was similar to that verified at 25 DAA, with no difference between the evaluated genotypes; at 40 DAA, two genotypes showed higher gene expression, CD 202 and IAC, which did not differ significantly among themselves; finally, from 45 DAA to 55 DAA there was no difference in expression between the genotypes for the candidate gene evaluated.

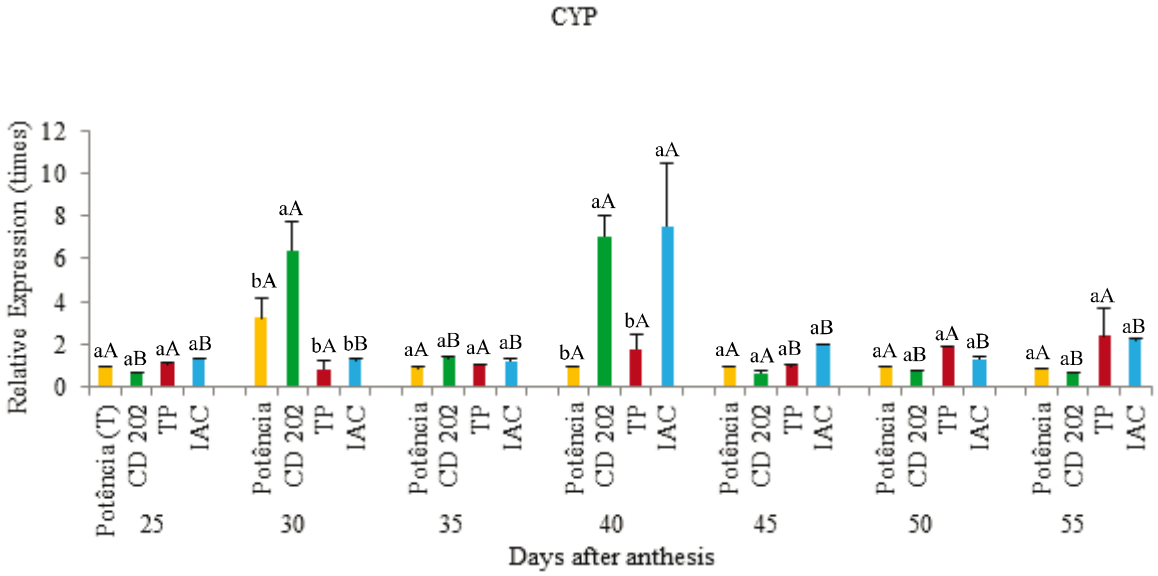
Among the coat development phases, for BMX Potência RR, the highest expression of the CYP gene occurred at 30 DAA, but no difference was observed between the other phases; in CD 202, the highest levels of expression were verified at 30 and 40 DAA, not differing from each other, just as the other phases also did not differ significantly among themselves.

Table 1 - Summary of variance analysis for the relative expression of CYP and ARABINOGALACTAN genes in contrasting coats of four soybean genotypes

SOURCES OF VARIANCE	G.L.	MEAN SQUARE	
		CYP	ARABINOGALACTAN
Genotypes (F1)	3	5,10^{ns}	108,77*
Coat collection times (F2)	6	12,97*	50,87*
Interaction F1xF2	18	5,94**	64,91*
Residue	28	2,78	3,10
Treatments	27	7,41	66,66

G.L. –Degrees of freedom. *Significant at 1% probability via F test. **Significant at 5% probability via F test ns - not significant

Figure 1 - Relative expression of the CYP (Cyclophilin) gene in soybean seed coats collected in seven stages of development after anthesis, in four contrasting genotypes for the coat characteristics. [1]Means followed by distinct letters, lowercase between genotypes, within each collection period and, capitals within each genotype and between collection periods, differ from one another by Scott Knott test at 5% probability



For the black coat genotype, TP, it was not possible to verify distinct expression between the coat development phases, presenting low accumulation of relative transcripts. Finally, for the other black colored genotype, IAC, the highest accumulation of CYP gene transcripts occurred at 40 DAA, which was significantly higher than the other phases, which did not differ among themselves (Figure 1).

The structure, biosynthesis and, mainly, the already verified or probable functions of cyclophilins (CYP) have been described in detail in recent literature reviews by Kumari *et al*. (2013) and Wang and Heitman (2005). These molecules belong to a group of proteins known as immunophilins, which until now have been found in all organisms in which they have been investigated, in all cells and in the various constituents of these cells, such as cytosol, nucleus, mitochondria, secretory pathway and chloroplast. Furthermore, in Arabidopsis more than 29 different cyclophilins have been observed (KUMARI *et al*., 2013; ROMANO; HORTON; GRAY, 2004; WANG; HEITMAN, 2005).

The abundance and diversity of plant isoforms suggests that they are important proteins involved in a wide variety of cellular processes, such as protein folding, mRNA processing, protein degradation, signal transduction and, therefore, can be crucial during development and stress response capacities (ROMANO; HORTON; GRAY, 2004).

Notably, the most recognized role of cyclophilins is to act in the folding of proteins, such as Peptidyl prolyl cis-trans isomerase (PPIase) (BARIK, 2006; KUMARI *et al*., 2013; ROMANO *et al*., 2005). Folding allows proteins to assume their functional configuration, and many of these require other proteins to speed up the process because the cellular environment is often not in favor of these modifications (ROTHMAN; SCHEKMAN, 2011). Due to their apparently auxiliary function, the presence of folding proteins might not be indispensable during plant development, especially under normal conditions (KUMARI *et al*., 2013). However, their elevated presence in all living cells suggests the opposite. In this sense, Miernyk and Johnston (2013) also mentioned that it seems unlikely that soybean seed coat proteome would cover a complexity of proteins that needed assistance from others to make their configuration functional, or that such unfavorable conditions could occur in this tissue to the point of compromising the process autonomously. Unexpectedly for these same authors, a large amount of proteins folders was found in soybean seed coat, and in all stages of development studied, but mainly in the intermediate phase. This is consistent with the results of the present study, in which CD 202 and IAC showed greater CYP gene expression at 40 days after anthesis, and emphasizes the importance of this type of protein in soybean seed coat.

According to Miernyk and Johnston (2013), it was observed that in the early stages of soybean seed coat development there was a relatively higher expression of proteins associated with protein synthesis, which justifies the greater expression of the Cyclophilin gene in this study, in the initial and intermediate phases of development, activating these new proteins produced in the coat.

The function of protection against pathogens in the seed has been attributed to the coat (MOÏSE *et al*., 2005). From all classes of proteins evidenced in soybean seed coats, the secondary metabolites have been given this antimicrobial characteristic, including flavonoids, isoflavonoids, and anthocyanidins, whose presence in coats correlates with dark color (MIERNYK; JOHNSTON, 2013; MOÏSE, 2005). However, in addition to these proteins, cyclophilins have also been shown to act against pathogens in fabaceae seed coats (YE; NG, 2000; ZHU *et al*., 2011). However, because they are not related to the color of the coat, they are important in cultivars with yellow coat, less favored by secondary metabolites in this tissue.

The selection for cultivars with higher content of certain proteins in the coats that favor tolerance to infections without altering other characteristics in the seed, such as color, is important, because, since the great majority of present cultivars are made up of yellow coat, the adoption of cultivars with dark coloring by farmers could be slow. In addition, there would be great difficulty, in the improvement, in inserting only some characteristics of cultivars with dark coat, avoiding other characteristics that are undesirable, such as seed dormancy. It is not clear if the role of cyclophilins against biotic stresses, as now mentioned, or abiotics, occurs directly or occurs due to the role of auxiliary protein, giving others, a direct function in the response to active stresses (KUMARI *et al*., 2013), justifying further research to highlight such attribution.

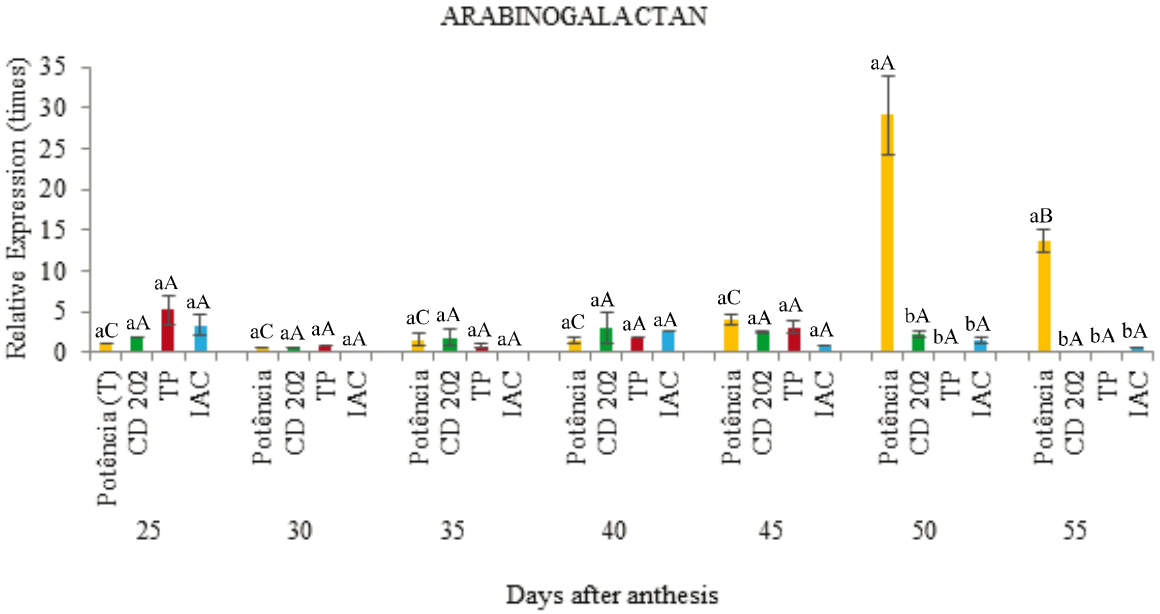
The results of ARABINOGALACTAN gene expression in the seed coats of soybean genotypes under evaluation showed that it did not differ between the genotypes studied at 25, 30, 35, 40 and 45 days after anthesis (DAA), however, at 50 and 55 DAA, superior expression of ARABINOGALACTAN was observed in the BMX Potência RR coats, in comparison to the other genotypes, which did not differ among themselves (Figure 2).

Between the development phases of the coats, there was only a difference for BMX Potência RR, in which the highest expression occurred at 50 DAA, being superior and differing from that of 55 DAA, which then differed from the other phases evaluated, which did not express differences among themselves. For CD 202, TP and IAC, there was no difference in expression of the ARABINOGALACTAN gene as a function of the development phases of coats (Figure 2).

In the large and recent literature reviews by Ellis *et al*. (2010) and Seifert and Roberts (2007) the following were described: the structure, biosynthesis and, mainly, the probable or already evidenced functions of arabinogalactan proteins. In general, it is an extremely diverse class of cell surface glycoproteins widely distributed in the plant kingdom. It is suggested that they act in many biological processes of cell multiplication and survival, development and regeneration of the cell wall, establishment of patterns, growth, pathogen-plant interaction, mediation between cell wall, plasma membrane and cytoplasm among a number of other actions (ELLIS *et al*., 2010; SEIFERT; ROBERTS, 2007).

The role of arabinogalactans in increasing cell wall extensibility was reported by Lamport, Kieliszewski and Showalter (2006). After physiological maturity, moisture gains and losses cause the coat to be extended and contracted, which can cause wrinkling of the coat or, in more harmful cases, the cleft. Mertz *et al*. (2009) observed that coats of CD 202 soybean cultivar seeds showed cracks, probably due to fluctuations in air moisture, even with the plants having been grown under

Figure 2 - Relative expression of the ARABINOGALACTAN gene in soybean seed coats collected in seven stages of development after anthesis in four contrasting genotypes for coat characteristics. ¹Means followed by distinct, lowercase letters between genotypes, within each collection period, and capitals within each genotype and between collection periods differ from one another via Scott Knott test 5% probability level



greenhouse conditions. This cultivar is considered as having a highly fragile coat (MERTZ *et al*., 2009) and in the present study did not present a greater expression of the ARABINOGALACTAN gene, which in a way allows to suggest its function of providing extensibility to the cell. To a certain extent, Miernyk and Johnston (2013) observed that in the final stages of development of soybean seed coat there was a greater expression of proteins associated with the stress response, which they attributed as a reaction to the relatively rapid dehydration period that the coat, like all seeds, goes through, on the way to complete maturation.

Arabinogalactan proteins are involved in programmed cell death (ELLIS *et al*., 2010). In the case of Arabidopsis, it was found that the cells of some of the coat layers undergo programmed death (NAKAUNE *et al*., 2005), since the growth of seed cotyledons tends to compress the cells of the coat as they grow. This phenomenon is still in the initial stage of elucidation and there are still no reports on its relation with arabinogalactan proteins, but it is emphasized that there is a considerable similarity between brassica seed coats, such as Arabidopsis, and fabaceae seed coats, such as soybean (MOÏSE *et al*., 2005).

The fact that the greater expression of this gene, which may exert an important function in seed coats, occurred in the genotype with yellow coat (BMX Potência RR), reinforces what was previously discussed- that the genetic variability for coat characteristics can allow for characteristics of interest to be evidenced for this attribute in genotypes with yellow coat, as well as those with black coat, which have already demonstrated a number of characteristics of interest for the genetic improvement of seed quality attributes (MERTZ *et al*., 2009). This is reinforced by the results of Giurizatto *et al*. (2003), which showed that some genotypes with yellow coat were superior to those of black coat in terms of physiological quality.

Both proteins, which probably result from the expression of the genes studied, still require much research to better elucidate their functions in plant metabolism. The authors of recent literature reviews on cyclophilins (KUMARI *et al*., 2013; ROMANO *et al*., 2005) and arabinogalactans (ELLIS *et al*., 2010; SEIFERT; ROBERTS, 2007) reinforce that this knowledge is only in the initial stage. While so few of the functions of these proteins are understood in the general scope, even less is understood about their action in soybean seed coat, as studies on this subject are still in the initial stage of development. This leads to the suggestion of more advanced studies, especially in contrasting genotypes for this characteristic.

CONCLUSIONS

1. The CYP gene showed greater expression in the intermediate stages of development of the coats of CD 202 and IAC genotypes, yellow coat and black coat, respectively;
2. The ARABINOGALACTAN gene presented greater expression in the final stages of development of coats of the BMX Potência RR genotype, with yellow coat.

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