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Article

Expression of AGP12 and AGP17 during Megagametophyte development in *Ricinus communis*

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Abstract

The development of the female gametophyte of angiosperms is controlled by multiple genetic, hormonal, positional, and epigenetic factors. Arabinogalactan-proteins are a group of proteins that act both structurally and signaling during development, particularly in the embryo sac. From 2018 to 2020, the development of the female gametophyte was analyzed in *Ricinus communis* plants established in the experimental field of the National Technological Institute of Mexico-Technological Institute of Roque. *Ricinus communis* is a species that has gained importance for the production of biofuels and bioremediation. This work aimed to analyze the expression of AGP12 and AGP17 associated with the development of the female gametophyte in early stages of development through RT-PCR and qRT-PCR, in contrast to marker genes of the first cell divisions that act during megagametogenesis. It was found that both genes presented similar expression patterns, with a decrease in expression at the beginning of meiosis and another during the maturation of the ovule and emission of stigmas.

Keywords:

arabinogalactan-proteins, differentiation, megagametophyte, reproductive development.

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Introduction

The development of the female gametophyte is mediated by the expression of different genes corresponding to transcription factors and other protein families, including cell surface receptors. There are also other positional factors involving the response to chemical signals and hormone levels and auxin transport, which have demonstrated an ability to regulate critical functions in ovule emergence and morphogenesis.

Ovule diversification has been studied using regulatory gene orthologs in divergent angiosperm groups (Gasser and Skinner, 2019). The castor oil plant (*Ricinus communis* L.) is a crop that has gained attention due to its use in the production of biofuels, the adaptability it has had in Mexico, and the diversification of byproducts with commercial uses, in addition to being proposed as an alternative for soil bioremediation.

It belongs to the Euphorbiaceae family and is monoecious, so the study of reproductive development was easy. It can be grown as an annual plant or kept as a perennial, being productive in both cases (LEAL *et al*., 2022). The largest producer of *Ricinus* seed worldwide is India with 1 198 000 t; this country represents about 84.18% of the total production, the second largest producing country is Mozambique with only 85 436 t and it represents only 6%. Mexico ranks 25 th worldwide with a production of 282 t in 2018 (FAOSTAT, 2020).

Due to its economic importance, global projects have been promoted and the sequenced genome of *R. communis* (txid:3988) is already available, 31 237 genes have been identified in an approximate size of 350 Mb (Saadaoui *et al*., 2017). For these reasons, it emerges as an interesting study model with molecular tools now available. New technologies have enabled the study of plants using advanced genetic approaches, available genome information, transcriptome analyses, sophisticated cell isolation, and microscopic imaging have improved the understanding of the molecular mechanisms underlying gametophyte formation (Saadaoui *et al*., 2017; Erbasol *et al*., 2019).

The study of the development of gametophytes and seeds is considered an opportunity to improve yields, accelerate the domestication of genotypes adapted to the different regions of the country, and even modify the composition of oils, as the most valuable product and for which mass production is promoted.

The development of gametophytes takes place in the floral meristem once its development mediated by the wuschel (WUS) gene has finished (Thomson and Wellmer, 2019); the wus gene is regulated by the sporocyteless/nozzle (SPL/NZZ) transcription factor, which activates the expression of windhose (WIH1, 2) in the nucellus area of the ovule primordium; in this area, the WIH1 and WIH2 genes (encoding small peptides) and the tornado2 (TRN2) gene are responsible for the development of the megaspore mother cell (MMC).

Some genes have been identified as responsible for the development of the megaspore mother cell (MMC). In rice (*Oryza sativa* L.), the loss of functions of the multiple sporophyte1 (MSP1) gene presents a development of multiple cells similar to MMC, although they have the ability to undergo meiosis to generate embryo sacs, they are abnormal and partially sterile. These features support the existence of a lateral inhibition mechanism in the ovule primordium, allowing the MMC to repress the formation of additional MMCs in the surrounding tissue (Erbasol *et al*., 2019).

The mneme gene (MEM) has also been shown to restrict the development of nucellar cells adjacent to MMC, MEM is predominantly expressed in the MMC and encodes an RNA helicase of the dead-box family; dead-box RNA helicases have been shown to act on RNA-directed DNA methylation (Schmidt *et al*., 2011).

Members of the argonaute family (AGO4, 6 and 9) are associated with 24-nucleotide small interfering RNAs (siRNAs) to silence transposable elements (TEs). Loss-of-function mutants in all members of this group have defects similar to those observed in mem mutants (Hernández-Lagana *et al*., 2016).

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Similar to mutants affecting the AGO4 group, the loss of function of other components of the RdDM (RNA-directed DNA methylation) pathway, such as the suppressor of gene silencing3 (SGS3) and the RNA-dependent RNA polymerase6 (RDR6), results in multiple MMC-like cells and unreduced embryo sacs. In addition, MMC-like cells in the ago9, sgs3, and rdr6 mutants show a different chromatin state than surrounding nucellar cells (Olmedo-Monfil *et al*., 2010).

Once the MMC undergoes meiosis, four haploid megaspore cells that are marked by the accumulation of callose in their cell walls are generated (Rodkiewicz, 1970). Three of them degenerate through programmed cell death while one survives and gives rise to the functional megaspore (FM). The chromatin state of FM suggests that its selection involves specific and common chromatin features compared to MMC differentiation in *Arabidopsis thaliana* (She *et al*., 2013).

In addition to controlling entry into meiosis, kinase inhibitor-related proteins (KRPs) appear to be important for FM selection. In loss-of-function mutants of the highly redundant KRP family, the multiple MMC phenotype is confirmed and more than one megaspore often survives (Yang and Sundaresan, 2000). Surviving megaspores can form multiple embryo sacs, which, in rare cases, result in twin seedlings after fertilization.

In particular, in the wild type, KRP4 was specifically detected in degenerate megaspores but not in FM (Rodkiewicz, 1970; Yang and Sundaresan, 2000; She *et al*., 2013). Several *A. thaliana* mutants that affect FM selection have been identified. In the antikevorkian mutant, whose corresponding locus has yet to be determined, degradation of the four megaspore cells is prevented and all meiotic products appear capable of generating an embryo sac (Yang and Sundaresan, 2000).

A critical role for cytoskeletal elements in the organization of cytoplasmic arrangements and positioning of nuclei during megagametogenesis has been defined; in fact, nuclear division and cellularization are severely affected by cytoskeleton perturbations: mutations in *A. thaliana* genes encoding #-tubulin isoforms (TUBG1, TUBG2) or a central subunit of the #-tubulin-containing complex (GCP2) exhibit female gametophytic defects in mitosis, nuclear positioning, and cellularization. In addition, kinesin-encoding genes, such as atnack1/hinkel and stud/tetraspore/atnack2, have been shown to be necessary for proper cellularization and nuclear positioning during the development of female gametophytes (Erbasol *et al*., 2019).

Arabinogalactan-proteins (AGPs) are widely distributed in the plant kingdom, they are extracellular glycoproteins that constitute an amorphous part of the cell wall as structural proteins. They have up to 90% of their weight of sugar with heterogeneity in the protein skeleton and carbohydrate chains in addition to the presence of glycosyl-phosphatidyl-inositol (GPI) at the carboxy-terminal end, which allows anchoring to the cell membrane. The glycosidic motif consists mainly of a galactan skeleton with β-(1,3) bonds to which oligosaccharide or polysaccharide chains rich in galactose and arabinose are attached.

The protein core is composed of hydroxyproline, proline, serine, alanine, and threonine (Showalter and Basu, 2016; Ma *et al*., 2017; Su and Higashiyama, 2018; Leszczuk *et al*., 2018). In *Arabidopsis*, there are several studies of the expression of AGP genes in individual cells of different compartments of the female gametophyte, which allow us to know in detail the differential expression in each part of the ovule, for example, AGP1 clearly expresses itself in the funiculus and integuments near the micropyle, AGP12 in the chalazal region, and AGP19 in the wall of the ovary. It is mentioned that their interaction influenced the function as signal molecules by mediating the development and nutrition of the ovules.

Overexpression of arabinogalactan protein18 (AGP18), which encodes a glycosylated protein bound to the plasma membrane that is located in the integumentary cells of the meiotic ovule, results in the survival of multiple megaspore cells that can acquire FM identity (Demesa and Vielle 2013). Finally, in triple loss-of-function mutants of arabidopsis histone kinase (AHK) receptor genes, FM is often not specified, based on morphology and lack of marker expression, resulting in null or abnormal embryo sac development.

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The expression of all AHKs is detected in the chalazal sporophytic tissue and acts on the cytokinin signaling pathway (Cheng *et al*., 2013). The importance of AGP18 as a marker for the location of primordia for the formation of ovules and other organs is also mentioned (Acosta and Vielle, 2004; Pereira *et al*., 2014; Leszczuk *et al*., 2018). Recently, it has been reported that, in *Torenia fournieri*, AGP proteins that are expressed in the synergids of the female gametophyte exert an attractive role for the pollen tube by activating cysteine-rich peptides, called lure1 and lure3 (Zhong and Qu, 2019); this shows that, in addition to being important for development and differentiation, they also fulfill particular functions in the fertilization process.

The heterogeneous nature of AGPs is reflected in the multiple functions they fulfill. They are anchored in the cell membrane and can act as adhesion molecules that connect elements of the cell wall to the plasma membrane and tissue and subcellular localization have already been reported. In addition, it is recognized for its important role in the reproductive development of plants, both in male and female organs (Leszczuk *et al*., 2019).

It has been reported that the expression of AGPs occurs mainly in the growth pathway of the pollen tube during the period of greatest receptivity of the ovule, suggesting that they could act as a lubricant and also as a nutrient to create a more favorable environment for the growth of the pollen tube in the extracellular matrix along the pistil, which ensures that fertilization is achieved (Lopes *et al*., 2016). After fertilization, the accumulation of AGPs in the basal zone of the embryo in the globular stage has also been observed as an indicator of the transition to the triangular stage, which highlights the importance of these molecules in cell interaction during development, which includes cell communication, signal transduction, and material transport (Lopes *et al*., 2016).

Given that AGPs presented different characteristic expression patterns and their importance in differentiation, the objective of the research was to determine the possible role of the AGP12, AGP17, and AGP19 genes during the development of the female gametophyte of *R. communis*.

Materials and methods

The experiment was carried out in the agrifood molecular biology laboratory of the National Technological Institute of Mexico/ Roque Technological Institute, located at km 8 of the Celaya-Juventino Rosas highway, Celaya, Guanajuato. The collected material was inflorescences of *Ricinus communis* L., established in the experimental field of the Technological Institute of Roque; the collection was carried out between 40 and 70 days after planting during the period from 2018 to 2020 with management according to Raya-Pérez *et al*. (2016). Female flower buds were selected and classified into five stages of development: 2 mm, 3 mm, 4 mm, 5 mm and 6 mm in length.

Considering the size of 6 mm as the stage in which the flower releases the stigmas to be pollinated, at this stage the embryo sac must be well developed for proper fertilization. The flower buds were dissected by using a Leica^{MR} Zoom stereo microscope and a scalpel, the sepals and the capsule with prematurely developed spines were removed, and the embryo sacs were left exposed.

The ovules were fixed in FAA fixative solution (formaldehyde, acetic acid and ethanol, 1:1:18) for five minutes, the operation was repeated three times, and it was rinsed with 70% alcohol between each operation; the samples were dehydrated in ethanol serial solutions and clarified with ethanol-lactic acid (1:3, 1:2, 1:1) for 24, 2 and 2 h respectively. Observations were made in an Olympus 1X71® inverted microscope with different objectives ranging from 10X to 100X under Normanski interferential contrast optics, photodocumented with an Olympus DP-70 camera attached to the same microscope.

Total RNA extraction was performed in five different sizes (2 mm, 3 mm, 4 mm, 5 mm and 6 mm) in five replications with the TRIzol reagent (Invitrogen™) according to the manufacturer's recommendations. The extracts were resuspended in a final volume of 20 µl and the concentration was determined with a NanoDrop™ 1000c equipment, thus obtaining values for yield in ng μL^{-1} and the A260/280 absorbance ratio close to 2 and the integrity was verified through electrophoresis in 0.8% agarose gel. For expression analysis via RT-PCR and qRT-PCR, specific oligonucleotides were designed by homology with the corresponding Arabidopsis thaliana genes in the reference genome of Ricinus communis L. (taxid:3988) and the BLAST tool (ncbi).

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The synthesis of oligonucleotides (T4Oligo™) was ordered as follows: RcActina fw(5'-GCCTGATGGTCAGGTCATCAC-3'), Rev (5'-TAGAGCCACCGATCCAGACAC-3'); RcNZZ Fw (5'-TTGGCTATGCTATTGGGTTCTT-3'), Rev (5'-TGCTACACTTTTGGGGCTCTT-3'); RcSYN1 Fw (5'-GTCAGTACAAGGGAGC AGATT-3'), Rev (5'-CAGGGAAAGATAAACCAAGTAGTC-3'); AGP12 Fw (5'-ACAACTCAT CATTCGCACCA-3'), Rev (5'-CATCGGAAGTAGGACTTGG-3'); AtAGP17 Fw (5'-CCATT TCTCCAGCTGCTCCA-3'), Rev (5'-CGGGCGGAGCAGGCGGGCTT-3'); AtAGP19 Fw (5'-CCA CCAGCTCCAAAAGTTGC-3'), Rev (5'-GGGCATGGTGATGCCTTTTG-3').

Endpoint RT-PCR reactions were carried out with the MLV-reverse transcriptase (Invitrogen™) enzyme with the protocol provided by the manufacturer in the SimpliAmp thermal cycler (Life technologies™) and for quantitative RT-PCR, a comparative CT experiment (##CT) was designed through the StepOne™ V2.1 software by means of a kit (Mix SYBR green®) for each stage of development (five stages) to measure the expression of the genes studied (AGP12 and AGP19) and the reference gene (actin), with two replications.

In the experiment, the ROXTM fluorescent dye was used as a passive sample (included in the SYBR green® Mix). The cycling conditions were programmed into the StepOne™ real-time PCR equipment (Life Technologies™): initial denaturation 95c, 10:00; denaturation: 95c, 00:15; annealing: 58C, 01:00; 40 cycles. The following was considered for the melting temperature curve (Tm): denaturation 95c, 00:15; annealing 58c, 01:00; denaturation 95c, 00:15. The results of the analyses were examined with the StepOne™ v2.1 software.

Results and discussion

Determination of developmental stages

Microscopic analysis showed that, at the 2 mm stage, internal morphological development is not yet evident. The establishment of the micropylar-chalazal axis and the space of the embryo sac can be seen; regarding the external structures, the capsule's spines have not yet been extended and the microscopic analysis does not reveal clearly differentiated internal structures; however, the axes of development are positionally defined from earlier stages (Figure 1A).

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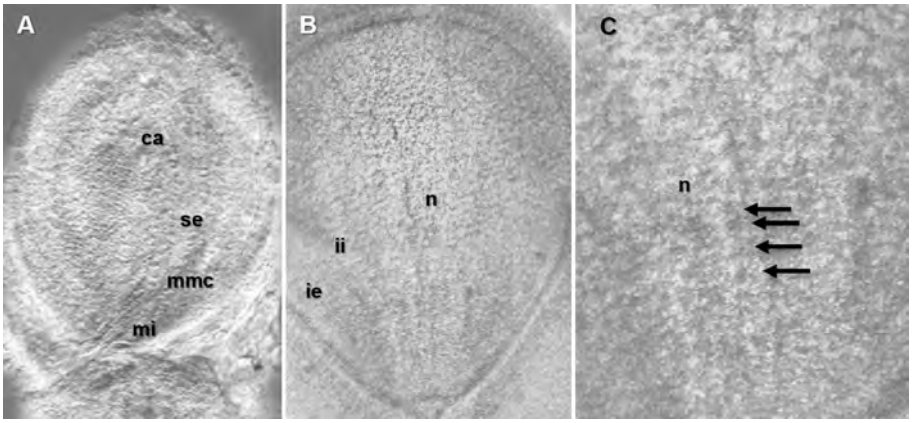
Figure 1. Differen a on in the early stages of development of the female gametophyte of R. communis. A) studied stage (2 mm) of the flower bud, it showed an early structure of the development of the female gametophyte in which the distribu on of the embryo sac (se), the chalaza zone (ca), the micropyle zone (mi), and the megaspore mother cell (mmc) were observed; B) 3 mm, the structure of the ovule and the internal (ii) and external (ie) integuments were appreciated and C) detail in the 3 mm stage. The arrows indicate the cells of the tetrad, subsequent to meiosis in the area of the nucellus (n).



In the second stage of development selected (3 mm), the cell differentiation that occurs inside the ovule is observed; in the area near the chalaza, a single differentiated cell is distinguished, corresponding to the megaspore mother cell MMC, this cell becomes evident by localized expansion and thickening of the cell wall (Figure 1B). In this same 3 mm stage, the first meiotic division of the MMC for the formation of a dyad is observed, which directs the progression in the development of the female gametophyte during sporogenesis (Figure 1C).

Expression analysis of AGP12, AGP17 and AGP19

Expression analysis of AGP12, 17, and 19 genes by RT-PCR indicated that all three genes are continuously expressed during flower bud development from the initial 2 mm stage to the last analyzed development stage corresponding to 6 mm, which differs from the synapsis1 (SYN) and sporocyteless/nozzle (SPL/NZZ) markers, which showed a clear temporality of expression, similar to that reported in *Arabidopsis* by Wils and Kaufmann (2016); the expression pattern of AGPs confirms the conservation of function and expression in different families of dicots. Nevertheless, interaction with other types of AGP induces changes in expression levels, which were verified by qRT-PCR.

The SYN gene is used as a developmental marker corresponding to the beginning of meiosis in the megaspore mother cell while SPL/NZZ are related to the differentiation of the archeospore cell, an event prior to the definition of the megaspore mother cell; it was observed that the expression of this gene is evident until after the 4 mm stage, when the meiotic divisions have already been completed (Figure 2).

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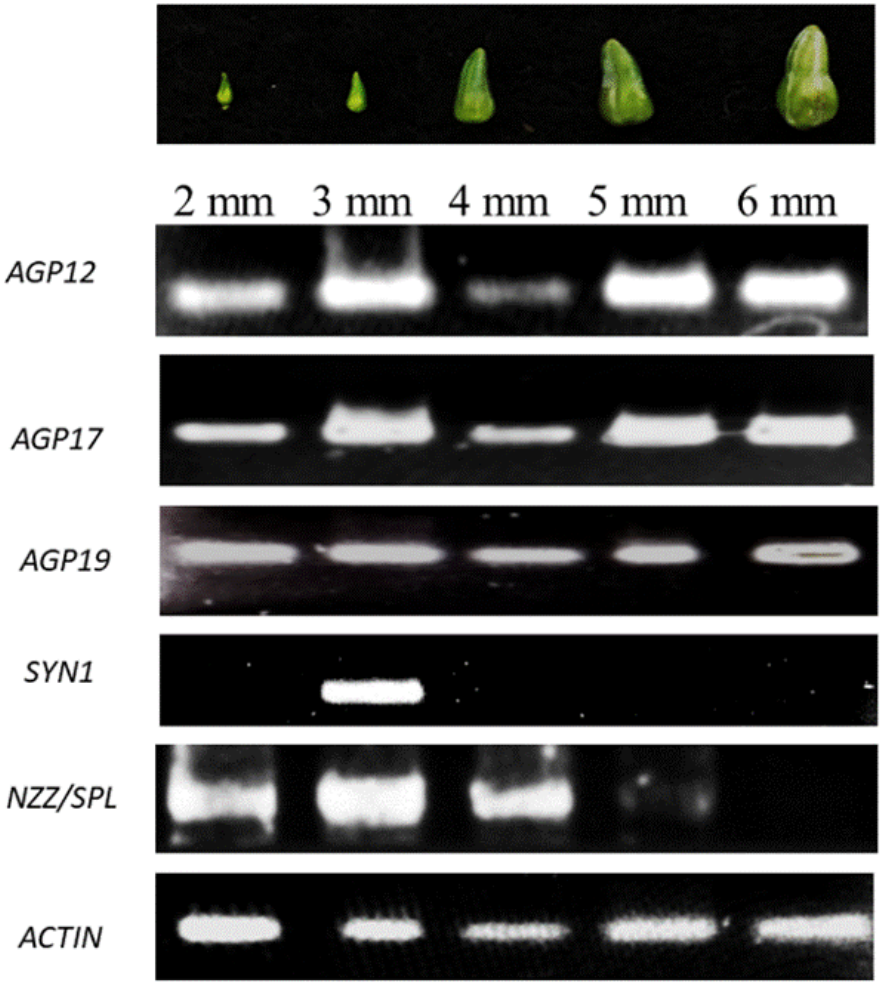
Figure 2. Gene expression during the development of the flower bud and female gametophyte. AGP 12, 17 and 19 genes show expression in the five stages prior to the emergence of s gmas, whereas SYN1 marks the first division of MMC and entry into meiosis; SPL/NZZ stops expression from the 5 mm stage. The ac n gene was used as an expression control in the RT-PCR assay. The upper part shows the appearance of the flower bud at each stage analyzed.



The maturation process of the female gametophyte in *R. communis* was perfectly comparable with that modeled in *Arabidopsis*, so each of the developmental stages can be distinguished and it is assumed that the gene expression reported is applicable in this study model; this was confirmed when performing the expression analyses.

In *Arabidopsis*, SPL/NZZ are involved in the processes of anther and ovule development, as well as in the identity of the stamen tissues (Wils and Kaufmann, 2016), which explains, to some extent, the fact that expression prevails beyond the specification of the archeospore cell. Although differences in intensity were observed in the amplified bands, they are not considered as evidence of differences in actual expression levels, so quantitative expression analyses were necessary.

The expression of AGP17 promotes membrane remodeling and has been reported to be expressed during the process of plant development and reproduction in species such as *Quercus suber* and *Capsicum annuum*; given the role they play in development, variations in expression levels were expected (Verdugo-Perales *et al*., 2018; Pérez-Pérez *et al*., 2019).

The specific activity of AGP19 reported is associated with the development of vascular bundles, particularly when differentiating xylem elements. AGP19 transcripts are most abundant in roots and flowers, moderately abundant in stems, seedlings, and siliques, and are virtually absent in leaves (Yang et al., 2011).

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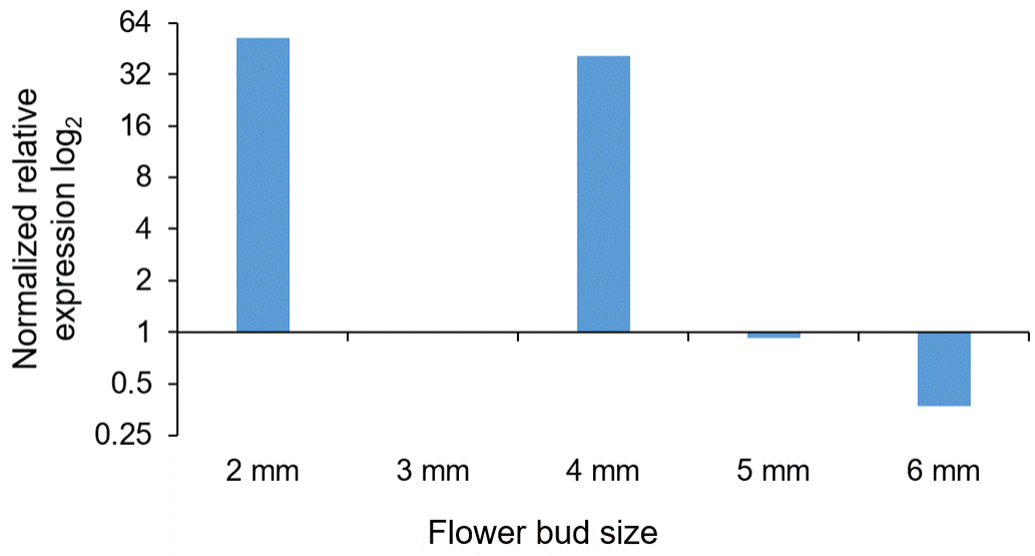
For the AGP12 gene, the expression pattern has not been published, but there are global expression data obtained by microarray-based strategies; according to data from The Arabidopsis Information Resource (TAIR), AGP12 is expressed in carpels, cauline leaf, leaf collecting structure, cotyledon, flower, pedicel, guard cells, hypocotyl, floral meristem, leaf apex, base of leaf blade, petal, petiole, embryo, pollen, root, seed, sepal, apical meristem, stamens, and stems; the greatest expression is detected in the filament of the stamen (TAIR, 2020), not to mention the temporality of the expression.

Recent studies have shown the differential expression of AGPs during the ripening of fruits, such as tomatoes; it is even suggested that AGPs may function as expression markers for the fruit ripening process (Kutyrieva-Nowak *et al*., 2023).

Relative quantification of the expression of the AGP12 and AGP17 genes

The analysis of the expression by qRT-PCR of the AGP12 gene, normalized with the reference gene, showed an intermittent pattern; in the stage prior to the onset of female gametogenesis (2 mm), it presents an expression 52 times higher than the 3 mm stage, where cell differentiation and meiosis begins; again, in the 4 mm stage, there was a 41-fold increase in expression compared to the same stage; in the 5 mm stage, the expression decreases practically as in the 3 mm stage; finally, in the 6 mm stage, the relative expression decreased to half of the reference stage (3 mm) (Figure 3).

Figure 3. Relative quantification of AGP12 expression. Flower buds of *Ricinus communis* L., during development prior to fertilization.



Physiologically, the stages where expression increases occur correspond to stages where differentiation events take place, whereas the stages with lower expression correspond to cell division events, meiosis in the 3 mm stage and mitotic divisions in the 4 mm stage, with a process similar to that observed in the development of the *A. thaliana* megagametophyte (Acosta and Vielle, 2004).

The results of the quantification of the AGP17 gene expression normalized with the actin gene show that, in the 2 mm stage, the expression is 29.8 times higher than in the 3 mm reference stage; in the stage after the beginning of the development of the female gametophyte (4 mm), the gene of interest increased its expression 31.2 times more than the reference stage; in the 5 mm stage, there

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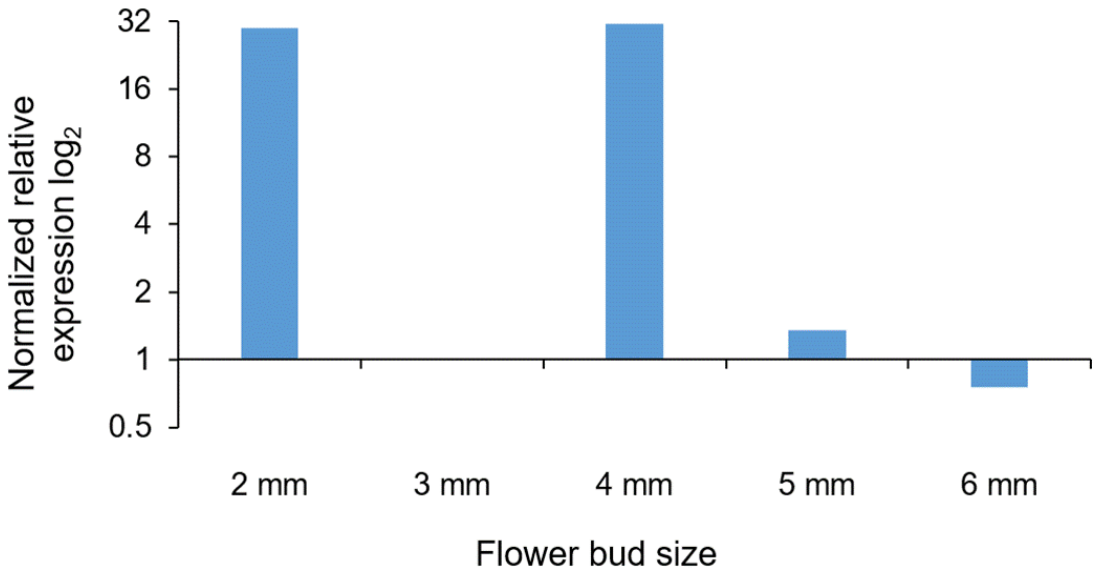
was an increase in expression of 1.3 times compared to the reference stage; like in the previous stage, at 6 mm of development, with an expression 0.8 times higher than the reference, there was no numerical variation between the 2 and 4 mm stages.

This finding allows us to relate AGP17 with the development of the female gametophyte since previous reports have only related homologs of this gene with intercellular signaling processes in defense processes and in the establishment of symbiotic relationships; however, it is mentioned, due to similarity in the glycosylation pattern, in the process of attracting the pollen tube for the double fertilization of the female gametophyte (Mizukami *et al*., 2016) (Figure 4).

Figure 4. Relative quantification of the AGP17 gene expression. During the development of the flower bud of *Ricinus communis* L., it exhibits an expression pattern similar to AGP12, with increased expression in the stages of cell differentiation.



The comparison of the relative expressions in the studied stages showed similar behavior for the AGP12 and AGP17 genes, but with variation in the level of induction; this suggests that both genes can act together in the stages shown; from the 5 mm stage onwards, they present a repressed expression (Figure 5).

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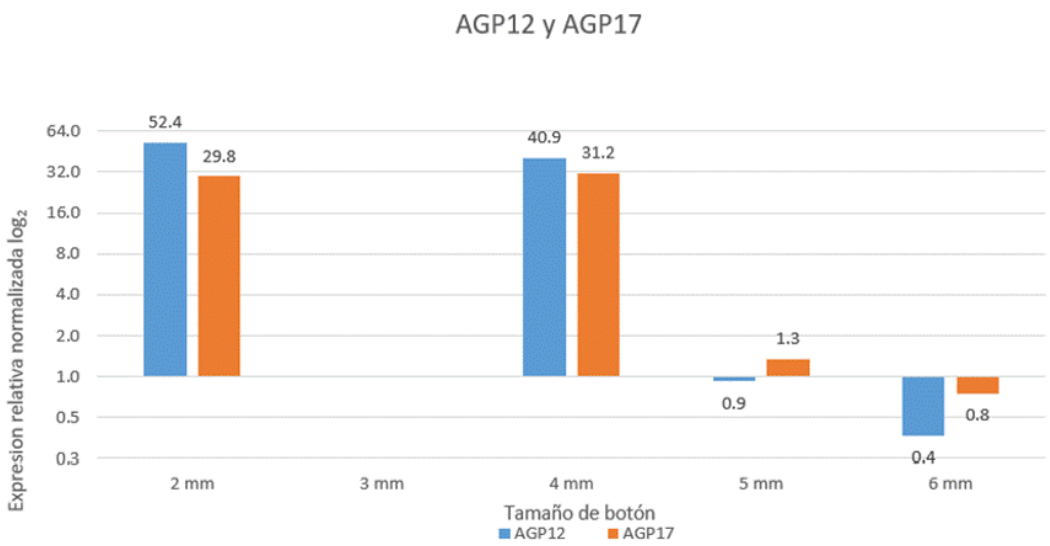
Figure 5. Comparison of the relative expression of the AGP12 and AGP17 genes. During gametophyte development.



The AGP12 and AGP17 genes are part of a family of genes of approximately 50 members, with functions related to the signaling, differentiation, and structure of plant tissues, so it is not difficult to understand that there may be redundant functions and at the same time, the importance of the processes in which they are involved is evident, so evolutionarily, these genes have been conserved and reiterated to ensure that the functions are covered.

Structurally, AGP17 is more similar to AGP18 and AGP19, but the expression pattern reported in this research was similar to AGP12. Through analyses with anti-AGP antibodies, AGPs have been in the dyad, tetrad, and functional megaspore, so the role in AGP signaling for the development of this cell is inferred (Pinto *et al*., 2019).

There are still many questions about the precise function of AGPs in the development of gametophytes, but the specific characterization of expression patterns is a key piece in the understanding of developmental processes. In addition, an increase in levels of AGPs due to the action of abiotic stress has been reported, such as the OsAGP1, OsAGP15, and OsELA3 genes reported in rice (*Oryza sativa*) when subjecting the plants to desiccation-rehydration cycles, whose expression also responded to the presence of ABA; this can be naturally related to the dehydration process that orthodox seeds undergo when they reach maturity.

Without ruling out that the high proportion of sugars could act in the protection of tissues and proteins during natural desiccation and the accumulation of ABA, which prevents the seed from germinating before completing its maturation process, a decrease in the levels of AGPs was also reported in tobacco plants (*Nicotiana tabacum*) subjected to salinity stress in relation to the inhibition of cell expansion characteristic of this condition and to lack of oxygen.

On the other hand, experiments carried out with tomato (*Solanum lycopersicum*) under conditions of hypoxia and anoxia are mentioned, which causes a decrease in the expression of specific AGP genes, (SlAGP1, SlAGP2, and SlAGP4); in addition, the expression of AGP genes decreased in response to other types of stress, such as that produced by heat and heavy metals (Mareri *et al*., 2019).

Conclusions

In *R. communis*, the SPL/NZZ and SYN1 genes, homologous to those corresponding to *Arabidopsis*, maintain patterns similar to the model plant and function as markers of the early stages of development of the female gametophyte. The AGP12 and AGP17 genes showed expression in

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the female gametophyte throughout its development, with a decrease in expression in the stage corresponding to the differentiation and initiation of the division of the megaspore mother cell to later increase the expression and decrease again to proceed with the maturation of the female gametophyte.

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