IDENTIFICATION AND EVALUATION OF GENES INVOLVED IN SEED DEVELOPMENT AND SEEDLESSNESS PHENOTYPE IN *Vitis vinifera L.*

Thesis presented to the Pontificia Universidad Católica de Chile in partial compliance with the requirements for the degree of Doctor of Biological Sciences with mention in Molecular Genetics and Microbiology.

By

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IDENTIFICACIÓN Y EVALUACIÓN DE GENES INVOLUCRADOS EN EL DESARROLLO DE LA SEMILLA Y EL FENOTIPO DE APIRENIA EN *Vitis vinifera L.*

Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al grado de Doctor en Ciencias Biológicas con mención en Genética Molecular y Microbiología.

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To my parents and brothers, for their unconditional love and support.
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ABBREVIATIONS

A	:	Auxin
AG	:	*AGAMOUS*
AGL13	:	*AGAMOUS LIKE 13*
AGL6	:	*AGAMOUS LIKE 6*
AIL5	:	*AINTEGUMENTA LIKE 5*
ANT	:	*AINTEGUMENTA*
ATS	:	*ABERRANT TESTA SHAPE*
Bp	:	Base pairs
C	:	Cytokinins
CTAB	:	Cetyltrimethylammonium bromide
cDNA	:	Complementary Deoxyribonucleic acid.
CDS	:	Coding sequence
Col-0	:	Columbia ecotype.
CRC	:	*CRABS CLAW*
CUC	:	*CUP- SHAPED COTYLEDON 1*
Cv	:	cultivar
dCAPS	:	Derived cleaved amplified polymorphic sequences
DNA	:	Deoxyribonucleic acid
FS	:	Flame seedless
i.i.	:	inner integument
INO	:	*INNER NO OUTER*
LB	:	Luria-Bertani
Ler-0	:	Landsberg erecta ecotype
LG	:	Linkage group

LUG	:	*LEUNIG*
MMC	:	Megaspore mother cell
mRNA	:	messenger Ribonucleic acid
MS	:	Murashige and Skoog media.
o.i.	:	outer integument.
PCR	:	Polymerase chain reaction
qRT-PCR	:	quantitative Real time PCR
QTL	:	Quantitative trait locus
RG	:	Red globe
RNA	:	Ribonucleic acid
SHP1	:	*SHATTERPROOF1*
SHP2	:	*SHATTERPROOF2*
SIN	:	*SHORT INTEGUMENTS*
SPT	:	SPATULA
STK	:	*SEED-STICK*
SUP	:	*INNER NO OUTER*
TS	:	Thompson seedless
Ts	:	Thai seedless

RESUMEN

Las semillas, desarrolladas a partir de los óvulos florales, son estructuras con una importante función biológica, ya que permiten que las plantas puedan generar descendencia. La formación de los óvulos y las semillas, son por lo tanto, procesos complejos en el que participan múltiples genes.

La apirenia es un fenotipo caracterizado por la presencia de frutas sin semillas, con muy pocas semillas o con trazas seminales. Esta característica es altamente deseada por los consumidores de fruta.

Vitis vinifera L. es uno de los cultivos más importantes económicamente a nivel mundial. Naturalmente, las bayas de vid apirénicas se generan por dos mecanismos, Partenocarpia y Estenoespermocarpia, en los cuales se producen uvas sin semillas o con rudimentos seminales, respectivamente. Ambos mecanismos son el resultado de algún defecto ocurrido en etapas tempranas del desarrollo de la flor o la baya de vid.

El desarrollo del óvulo/semilla y el fenotipo de apirenia en *V. vinifera,* es un proceso que ha sido escasamente estudiado. Se cree que al igual que en otras especies como *Arabidopsis thaliana,* este es un proceso complejo en el cual participarían múltiples genes. A la fecha sólo han sido sugerido algunos genes candidatos , de los cuales sólo 3 han sido caracterizados.

Previamente, se realizó un análisis transcripcional masivo en nuestro laboratorio, utilizando tejido de flores y bayas jóvenes de vid, el cual permitió identificar genes candidatos que presentaron perfiles de expresión diferencial entre plantas con bayas semilladas y apirénicas.

En este contexto, la investigación realizada en esta tesis, consistió en caracterizar cuantitativamente el perfil de expresión de los genes candidatos que codifican para factores de transcripción putativos como *VvAGL6_1*, *VvAGL6_2*, *VvAIL5*, *VvINO*, o el correpresor transcripcional *VvLUG*, en el desarrollo temprano de la baya de vid y posteriormente, en determinar si alguno de estos genes afecta la formación de la semilla en la planta modelo *Arabidopsis thaliana*.

El estudio realizado permitió la identificación de dos genes, *VvAIL5* y *VvINO*, los cuales presentan una acumulación diferencial de transcritos entre individuos con fenotipo semillado/apirénico. En flores pre-polinizadas ambos genes exhibieron una disminución en la cantidad de transcritos en vides apirénicas, con respecto a vides semilladas. Por otra parte, en el estado de frutos en cuaja, sólo el gen *VvINO* exhibió disminución de los niveles de transcrito en bayas apirénicas en comparación a bayas semilladas. Estos resultados sugieren que existe una correlación entre una menor expresión de *VvAIL5* y *VvINO* y el desarrollo de bayas apirénicas, y a su vez, que ambos genes podrían contribuir a la formación de la semilla, participando en diferentes etapas del desarrollo de la flor y la baya.

Posteriormente, se seleccionó y evaluó la funcionalidad del gen *VvINO*, mediante expresión ectópica en la planta modelo *Arabidopsis thaliana*. A través del análisis *in silico* de la secuencia genómica de *VvINO*, se identificó múltiples elementos regulatorios en la región promotora que sugieren una interacción de este gen con otros factores de transcripción, así como regulaciones

a nivel hormonal. Finalmente, se obtuvieron plantas de Arabidopsis sobreexpresoras de *VvINO*, las cuales presentaron silicuas más grandes y con un mayor número de semillas en comparación a las *wild type.* Estos resultados, en conjunto con trabajos previamente publicados, permiten sugerir que *VvINO* afecta la formación del primordio del óvulo y por lo tanto, influye en el número total de las semillas en frutos maduros de *Arabidopsis thaliana*.

Los resultados generados en el marco de esta investigación constituyen una primera aproximación al estudio de genes de *Vitis vinifera*, candidatos a participar en el desarrollo reproductivo de la semilla, tema que hasta el momento ha sido escasamente estudiado. Así, este trabajo deja abiertas nuevas interrogantes para profundizar en el estudio de estos genes a futuro, lo que sin duda contribuirá a lograr una comprensión más acabada sobre los mecanismos moleculares y genéticos involucrados en la formación de la semilla en vid.

ABSTRACT

The seeds, developed from floral ovules, are structures with an essential biological role since they allow plants to generate offspring. The formation of ovules and seeds is, therefore, a complex process in which multiple genes participate.

Seedlessness is a phenotype characterized by the presence of fruits without seeds, with very few seeds or with seminal traces. This feature is highly desired by fruit consumers.

Vitis vinifera L. is one of the most economically important crops worldwide. In nature, seedless grapevine berries can be obtained by two mechanisms, Parthenocarpy and Stenospermocarpy, which generate berries completely seedless or with seminal rudiments, respectively. Both mechanisms are the consequence of a defect occurred in early reproductive stages across flower or berry development.

The ovule/seed development and seedlessness phenotype in grapevine is a process that has been poorly studied. It is believed that in other species such as *Arabidopsis thaliana*, this is a complex process in which multiple genes could participate, which some candidate genes have been suggested and only 3 of them have been characterized.

Previously, a massive transcriptional analysis was carried out in our laboratory, using tissue from young flowers and berries of grapevine, which allowed to identify candidate genes that presented differential expression profiles between plants with seed and seedless berries. In this context, the research carried out in this thesis, consisted first of quantitatively characterizing the

expression profile of the candidate genes that code for putative transcription factors such as *VvAGL6_1*, *VvAGL6_2*, *VvAIL5*, *VvINO*, or the transcriptional corepressor *VvLUG*, in the early development of the grapevine. Subsequently, the research focused on determining if any of these genes affect the formation of the seed in the model plant *Arabidopsis thaliana*.

The study allowed the identification of two genes, *VvAIL5* and *VvINO*, which present a differential accumulation of transcripts between individuals with seed/seedlessness phenotype. In pre-pollinated flowers, both genes exhibited a decrease in the accumulation of transcripts in seedless grapevine, concerning seed-bearing plants. On the other hand, in fruit set, only *VvINO* presented decreased transcript levels in seedless berries compared to seed fruits. These results suggest that there is a correlation between a lower expression of *VvAIL5* and *VvINO*, and the development of seedless berries, and in turn, that both genes could contribute to the formation of the seed, participating in different stages of flower and berry development.

Subsequently, *VvINO* was selected and functionality evaluated by ectopic expression in the model plant *Arabidopsis thaliana*. Through *in silico* analysis of *VvINO*, multiple putative regulatory elements were identified in the promoter region that suggest an interaction of this gene with other transcription factors, as well as hormonal regulations. Finally, Arabidopsis *VvINO* over-expressers lines were obtained, which presented larger siliques with a significantly higher number of seeds in comparison to *wild type*. These results, together with previously published studies, suggest that *VvINO* affects the formation of the primordium of the ovule and, therefore, influences the total number of seeds in mature Arabidopsis fruits.

The results generated in this investigation constitute a first approach about the study of genes of *Vitis vinifera*, candidates to participate in the reproductive development of the seed, a subject

that until now has been scarcely studied. Thus, this work opens new questions to deepen the study of these genes in the future, which will contribute to a better understanding of the molecular and genetic mechanisms involved in seed development in grapevine.

CHAPTER 1

GENERAL INTRODUCTION

1. Seed development in angiosperms

Seeds are fundamental part of the plant life cycle, as they contain the genetic information necessary for the next generation of plants and allow to produce offspring maintaining the species (Nonogaki, 2014). Also, seeds play key roles in agricultural production and crop improvement, since they constitute a simple and effective means of crop propagation, maintaining and transmitting genetic engineered enhancements. In addition, seeds are an important component of the global diet in the form of cereal grains (Bewley, 1997). **Despite the critical role that seeds play, seedless fruits have naturally emerged** (Varoquaux et al., 2000).

In flowering plants or 'Angiosperms'- the largest group of the Plantae Kingdom- the seeds are developed inside the ovary, the reproductive tissue of the flower. Therefore, flower development is a crucial process for a proper seed development. Morphologically, flowers have two kind of reproductive organs, gynoecium constitute the female organ while stamens are the male organs. The gynoecium is defined as the collective of all carpels that determine the fruit formation (Robinson-Beers et al., 1992b). It includes the stigma were pollen germination initiates, the style in which the pollen tube elongation occurs, and the ovary, the ovules containing structure (Figure 1. A and B). **Many efforts have been made to understand flower development since most of the reproductive plant cycle takes place here. The process has been mostly studied in *Arabidopsis thaliana*,** a core eudicot with a gynoecium including two laterals carpels fused (Figure 1. A) (Pfannebecker et al., 2016).

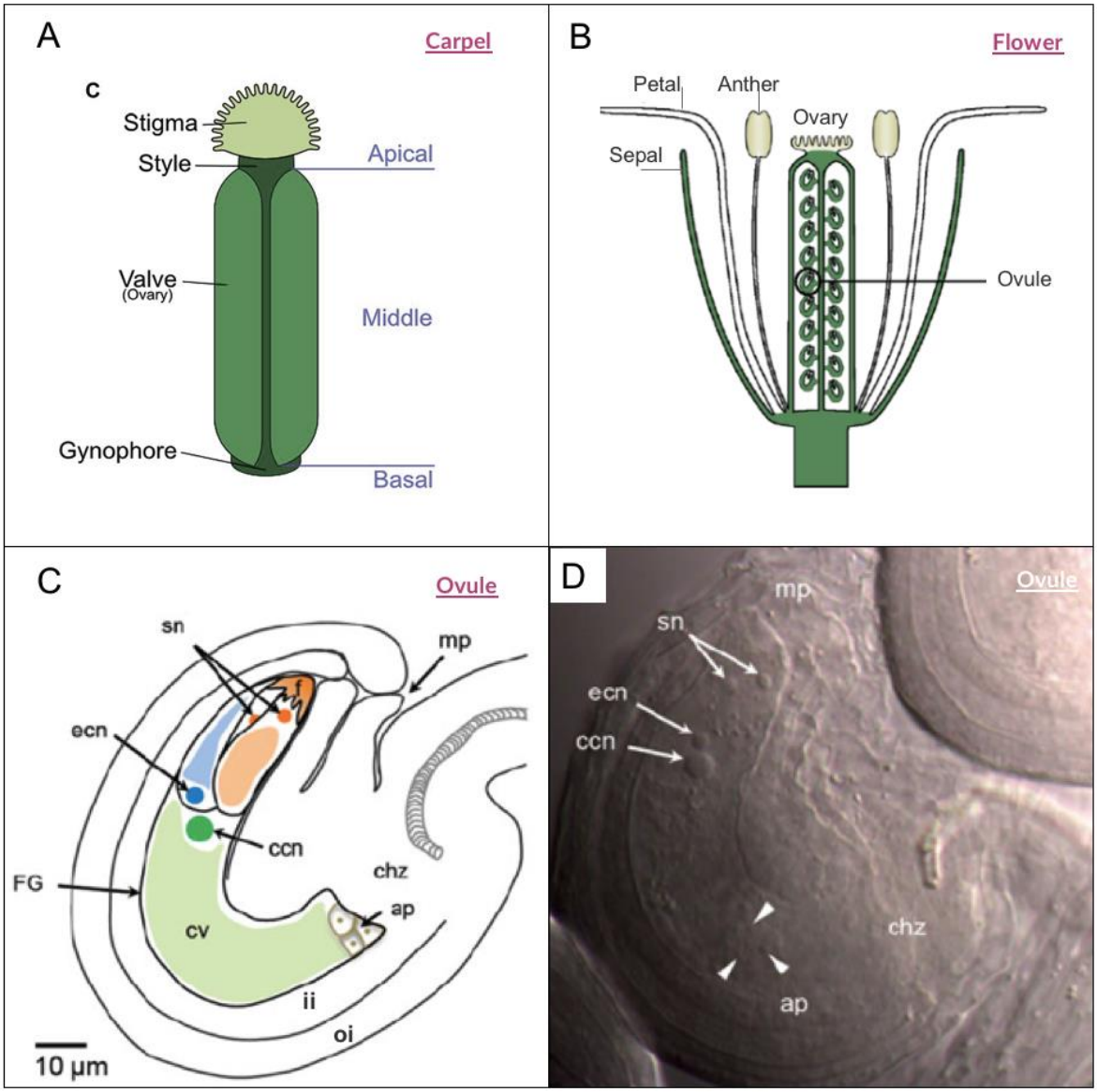

FIGURE 1. Arabidopsis female reproductive organs.
A) Schematic representation of the Arabidopsis gynoecium, showing stigma, style, ovary and ovules. **B)** Longitudinal diagram of Arabidopsis flowers. **C)** and **D)** Mature Ovule of Arabidopsis structures: inner integument (ii), outer integument (oi), mycropilar (mp), egg cell nucleus (ecn), synergid cells nuclei (sc), antypodal cells (ap) and central cell nucleus (ccn). The figures A and B ere adapted from Hawkins et al, 2014 and Schmid, 2012. C and D were adapted from Sprunck et al, 2011.

During floral development, organs are initiated in flower primordia at particular positions within concentric rings called ‘whorls.’ After organ initiation, those primordia become different floral organs, depending on the expression of distinct combinations of floral identity genes. This model was named ABC(DE), and most of the genes belong to the MADS-box transcription factors family, that function as quaternary complexes regulating different target genes (Immink et al., 2010). According to this model, all the genes control the formation of floral organs in a highly regulated manner at a spatial and temporal level. In other words, **their gene expression occurs in specific cell types and during precise moments to grant normal flower development.** The ABC(DE) model also states that **class D genes are ovule-specific** and, class C, D, and E specify ovule identity in whorl five at early stages of ovule development (Dreni and Zhang, 2016; Krizek, 2015).

Inside the floral carpels, ovules are formed. They are vital organs that enclose the female gametophyte and also develop into seeds after fertilization (Shi and Yang, 2011). In angiosperms, double fertilization occurs after **ovary becomes a fruit** (Chevalier et al., 2011). Then, the seeds play the role of propagating the species and the fruit has the important function of protecting seeds, helping in their dispersal. A developing seed contains three genetically distinct structures (Figueiredo and Köhler, 2018): the **embryo**, that has the potential to germinate and become a new organism (Chevalier et al., 2011), the **endosperm,** a nourishing tissue, and the **seed coat**, a maternal sporophytic tissue originated from the ovule integument(s) are cell layers that surround the seed, helping to protect it and facilitating the dispersal (Coen et al., 2017).

In general terms, the development of the fruit and the seed are synchronized processes, however, in some cases, the growth of the fruit is decoupled from the fertilization and development of the seed as indicated by the existence of seedless mutants and crops (Pandolfini, 2009)**.**

2. Main characteristics of the ovule and its development

2.1. Ovule characteristics and developmental stages during ovule formation

Ovule is the plant reproductive organ that develops into seeds after fertilization. In consequence, **defects in ovule's development generally result in female sterility, affecting seed formation and offspring propagation**. Hence the importance of deeply study ovule's formation.

Thus, ovule is the site where processes essential for sexual plant reproduction occur: megasporogenesis, megagametogenesis, fertilization and embryogenesis (Losa et al., 2010). This organ is composed by different sporophytic tissues: **the funiculus:** is the proximal region, which transports nutrients from the placental tissue to the ovule (Losa et al., 2010; Modrusan et al., 1994), **the chalaza:** which initiates two integuments, which are cellular layers enveloping the nucellus and **the nucellus,** within which the embryo sac or female gametophyte is developed, after megasporogenesis and megagametogenesis occur (Losa et al., 2010; Robinson-Beers et al., 1992b) (Figure 1. C and D).

Ovule´s development has been a complex process to study, due to both the small size and the difficulty of accessing inside the carpel. However, Schneitz and collaborators were able to propose a classification of four different stages of ovule development throw sectioning and microscopy techniques (Schneitz, K. Hülskamp, M. Pruitt, 1995). They identified four ovule development main stages, according to cellular characteristics. Also, Christensen and coworkers (1997), proposed a classification of female gametophyte developmental stages (Figure 2).

In general terms, ovule's development includes four stages. The first is the **Early phase (I)**, when the **ovule primordia arises** from the regions near to the carpel margins and at the end of this phase, they have recognizable pattern elements which will subsequently result in the nucellus, chalaza (flanked by initiating integuments) and the funiculus that will develop the vascular strand (Simon et al., 2017b). The second phase is called **Megasporogenesis (**II). Here, the megaspore mother cell (mmc) located in the nucellus, suffers two meiosis, a tetrad with four megaspores is formed, three of them will degenerated leaving one functional megaspora, this is also the Female gametophyte stage 1 (FG1) (Figure 2. C), Also, two integuments are initiated at this stage, arising from the chalaza (Simon et al., 2017b). The next phase is named **Megagametogenesis (III),** where resulting megaspore suffers three mitotic divisions, designated as stages FG2 to FG5 (Christensen et al., 1997) (Figure 2. D and E**). As a result of this phase the mature embryo sac is formed**, containing the egg cell, which gives rise to the embryo; the central cell, which give rise to endosperm and two accessory cells named synergid cells (Martin et al., 2014; Sundaresan and Alandete-Saez, 2010). Also the integuments development proceed during this stage (Schneitz, K. Hülskamp, M. Pruitt, 1995). **Therefore, at the end of the stage, the unfertilized mature ovule consists in a haploid embryo sac**

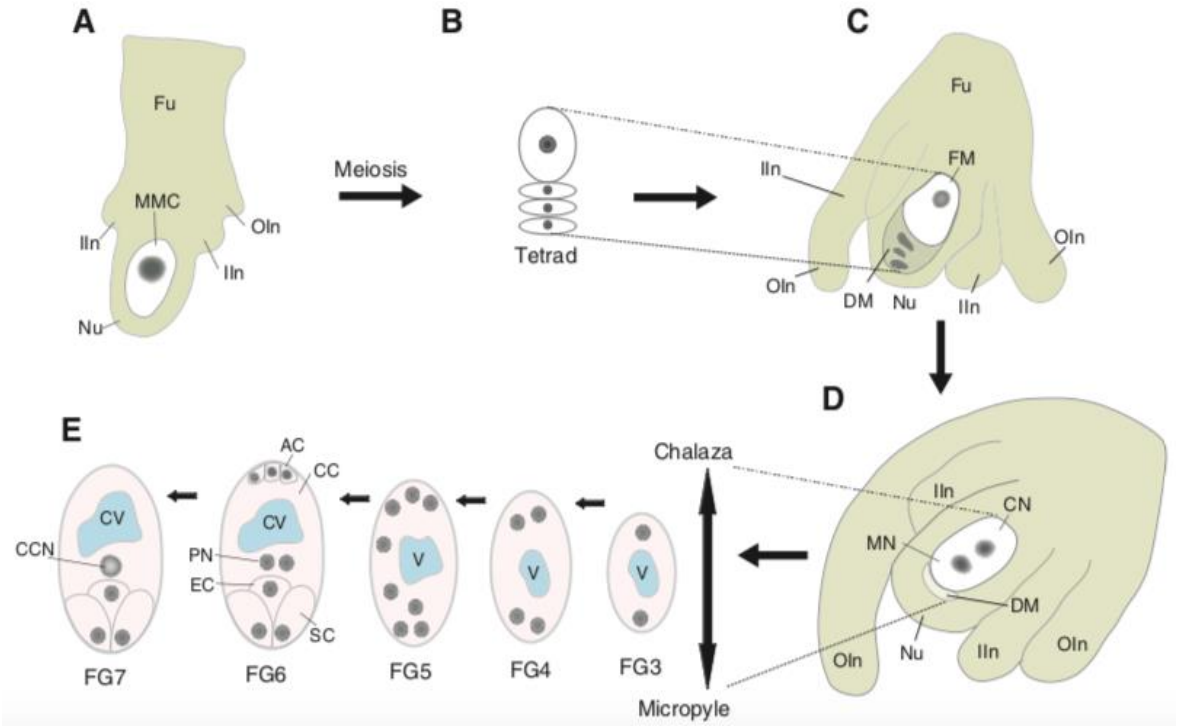

FIGURE 2. Schematic representation of female gametophyte development in Arabidopsis. **A)** The megaspore mother cell (MMC) is surrounded by epidermal cells of the nucellus (Nu) prior to undergoing meiosis to generate four megaspores. At this stage, the formation of the outer (OIn) and inner (IIn) integuments has just initiated. **B)** MMC suffers two meiosis that generates four spores (tetrad). Three of these undergo programmed cell death. The proximal (chalazal) megaspore becomes the functional megaspore (FM). **C)** FG1 stage. The FM undergoes the first mitotic division. **D)** FG2 stage. The female gametophyte comprises two nuclei. The nucellus (Nu) is enclosed by the OIn, but not the IIn integuments. **E)** Stages FG3 to FG7. The female gametophyte comprises two nuclei, separated by a large vacuole (V), that undergo second and third mitotic divisions to generate the eight-nucleate mature embryo sac at the FG5 stage. Subsequent cellularization (FG6 stage) results in the formation of seven cells: two synergid cells (SC); one egg cell (EC); one central cell (CC) carrying two polar nuclei (PN); and three antipodal cells (AC). By FG7, the two polar nuclei have fused to form the central cell nucleus (CCN), and the antipodal cells degenerate. CN, chalazal nucleus; CV, central vacuole; DM, degenerating megaspores; Fu, funiculus; IIn, inner integuments; MMC, megaspore mother cell; MN, micropylar nucleus; Nu, nucellus; OIn, outer integuments. Figure from Sundaresen et al, 2010.

surrounded by the integuments (Figure 1) (Grossniklaus and Schneitz, 1998).

Finally, there is a **Post-fertilization development phase (IV).** During fertilization, the pollen tube that contains two sperm cells reaches the mature ovule and leads to form a seed (Chevalier et al., 2011), where the fertilized cell egg develop into a diploid embryo and the central cell became in a triploid endosperm. Also, the integuments develops into the seed coat (Martin et al., 2014)

Angiosperms typically have "bitegmic ovules", with two integuments, which are important ovule's structures, that contributes to positioning and enable the formation of the female gametophyte. Also during pollination, they participate in the guidance of pollen tube towards female gametophyte in some species (Lora et al., 2018) and after fertilization, **the integuments become the seed coat**, which have different roles such us protection of the embryo, seed dissemination, and regulation of seed germination (Kelley and Gasser, 2009; Simon et al., 2017b).

2.2. Genes involved in ovule development

The study of ovule and female gametophyte is difficult mainly due to the small size of its structures. To understand the ovule's development, the main experimental approach used consists in large-scale genetic screens, through insertional mutagens. This allowed to identify single genes affecting ovule's structures, its fertilization capacity and seed formation (Sundaresan and Alandete-Saez, 2010). Thus, this approach has been useful to identify unique

genes whose mutations results into a phenotype, but not genes that act redundantly during this developmental process. Another alternative procedure is the comparison of expression profiles between wild type plants and previously identified mutants unable to form ovules. This has allowed to identify several genes that possibly participate in the formation of the embryo sac, many of them still remain without functional characterization (Sundaresan and Alandete-Saez, 2010). **In general, functional studies involve analysis of temporal and tissue- specific expression and loss-of-function mutants, complex techniques to perform in agronomic crops, leading to the use of models' plants like *Arabidopsis thaliana*.**

The mentioned approaches have allowed to identify **numerous genes participating in a coordinated way during the ovule formation, most of them are transcriptions factors**, summarized in Figure 3 (Baker et al., 1997; Cucinotta et al., 2014; Gaiser et al., 1995; Villanueva et al., 1999b). **Defects in most of these genes result in pleiotropic effects in flowers, defective ovules or both** (Baker et al., 1997; Gaiser et al., 1995; Gallagher and Gasser, 2008; Kelley et al., 2009). For example, *lug*- mutants exhibit a markedly decreased number of ovules (Cucinotta et al., 2014), *ant*- mutants have sterile ovules and a reduction in size and number of floral organs (Kelley and Gasser, 2009), in *ag*- loss function mutants, the carpels are replaced by new flowers (flowers inside flowers) (Franks et al., 2002) and in the triple mutant *stk shp1 shp2*, fewer ovules are initiated and ovule development is severely disrupted (Skinner et al., 2004).

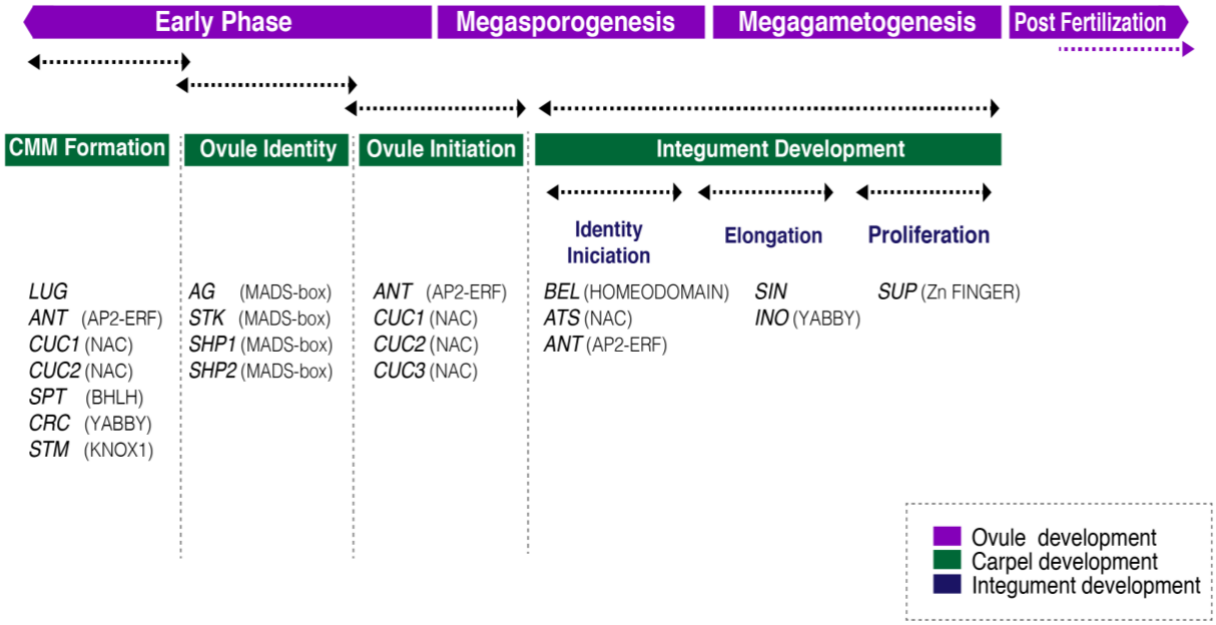

FIGURE 3. Principal stages of carpel and ovule development and genes identified. Some genes associated to different stages are schematized, in the case of transcription factors, their family is described in parentheses. The figure is of own elaboration, using the data of Gaiser and collaborators, 1995; Baker et al., 2007 and Cucinotta et al., 2014.

The formation of the integuments is a crucial stage during the development of the ovule since they contribute to the formation of the embryo sac. Several genes are participating at integument's establishment (Figure 3), and the **mutants in these genes have morphologically abnormal integuments that result in defective ovules. Even more important, most of them, such as *ant, bel, sin* and *ino* mutants, present female sterility** (Baker et al., 1997; Robinson-Beers et al., 1992b; Villanueva et al., 1999b), **an critical defect where ovules are not viable to be fertilized and therefore, incapable of becoming in seeds** (Robinson-Beers et al., 1992a). This phenomenon prevents plants from forming offspring, but it is desirable for agronomic crops, in which seedless fruits are highly desired (Varoquaux et al., 2000).

3. *Vitis vinifera* as a study model

Grapevine (*Vitis vinifera*) is one of the most economically important horticultural crops worldwide. The three main uses for grapes are dried fruit (raisins), winemaking, and fresh fruit also named 'table grapes'(Carmona et al., 2008). In Chile, towards 2015 there were more than 211.000 hectares of cultivated vineyards (OIV, 2016), is also our country the more substantial exporter of table grapes worldwide, with 14.4% of the total grapes exported abroad (Workman, 2018).

Regarding grapevine as a study plant, there are some advantages, such as that it is a plant whose genome has been sequenced (Jaillon et al., 2007). However, there are some technical difficulties that limit a better understanding of different physiological and molecular processes. For example, there is no availability of a mutant collection of *V. vinifera* that could support the study

or genes related to ovule and seed development hesitating to establish direct cause-effect relationships. Additionally, transgenesis in grapevine is a difficult process and therefore, the generation of overexpressing or silencing lines in genes of interest is a difficult task (Parada et al., 2017). Thus, usually, the genes of interest are identified and subsequently evaluated in model plants such as *Nicotiana benthamiana* or *Arabidopsis thaliana* (Hanania et al., 2007).

3.1. Morphological characteristics of grapevine reproductive organs

Morphologically, the ovary of grapevine flower has two locules, each containing two ovules generally, that will form up to four seeds (Figure 4. A). The anthesis or blooming, when the stamens are able to fertilize the female gametophyte, is a crucial stage because environmental and nutritional conditions at bloom time affect the success of fertilization and the resulting number of seeds per berry. After pollination and fertilization, each ovary develops to form a fleshy fruit called berry (Figure 4. C) (Boss et al., 2001; Poupin et al., 2011).

The ovule of the grapevine is 'anatropous,' that is, inverted with the micropyle towards the pedicel and with the funiculus (ovule stalk) joined to the outer integument forming a raphe (Figure 4.B). There are two integuments and a well- developed nucellus. The outer integument is joined on one side to the raphe for most of this length but is free at the micropylar end (Mullins et al., 1992). Also, *Vitis vinifera* has an endostomal micropylar in which the integuments do not

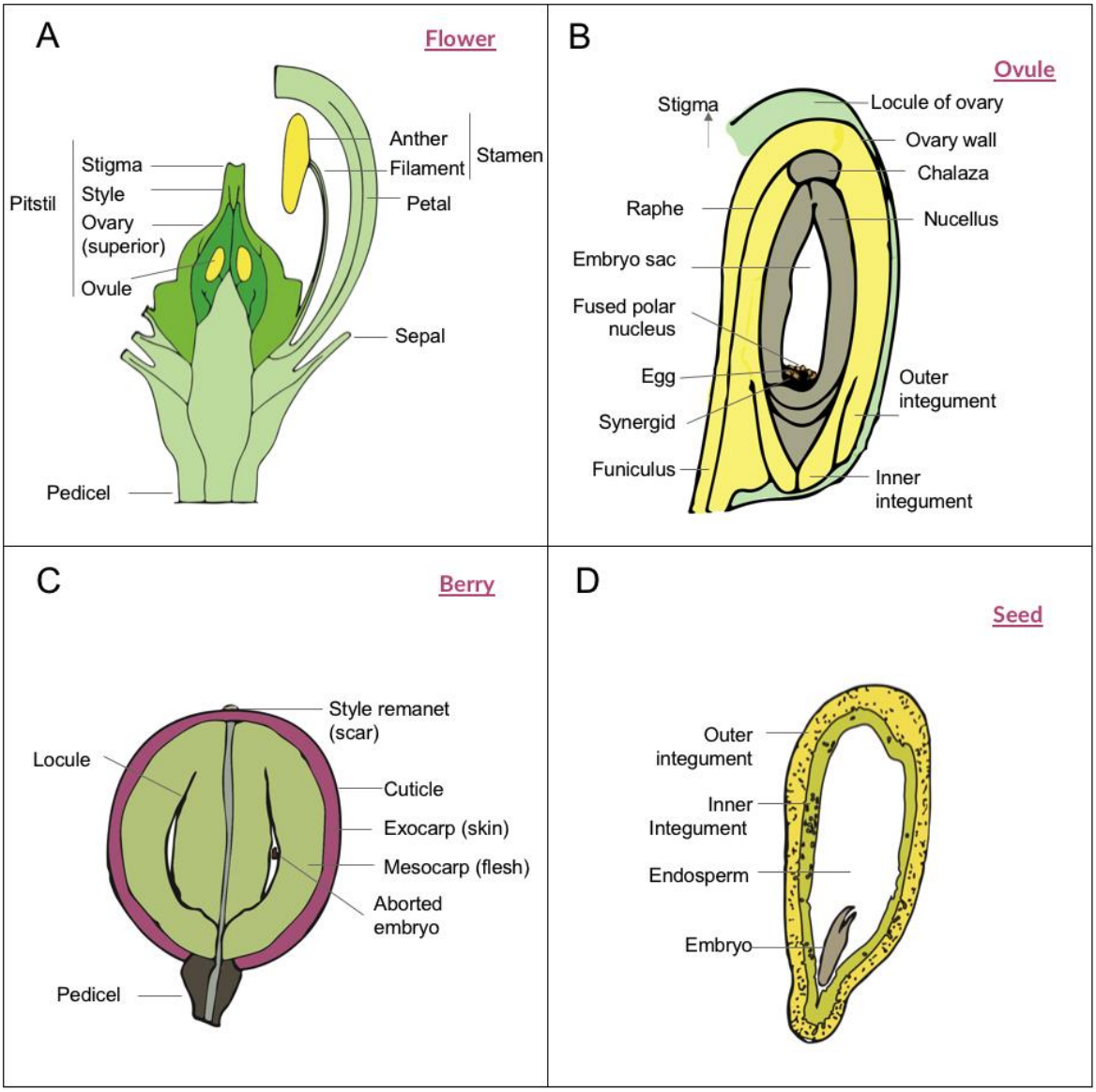

FIGURE 4. Flower, ovule and seed of grapevine.
A) Diagram of a longitudinal section of a mature grapevine flower. B) Longitudinal illustration of an ovule of the grape cultivar Concord at full bloom. C) Diagram of a longitudinal berry of a stenospermocarpic variety D) Longitudinal section of a grapevine seed. A, B and D were adapted from Pratt et al, 1971 and C was adapted from Dokoozlian et al, 2000.

entirely cover the ovule unlike Arabidopsis and therefore, it is presumed Arabidopsis and therefore, it is presumed do not participate in pollen tube attraction (Lora et al., 2011).

Flower formation is a critical process for the development of the resulting berries (Palumbo et al., 2019). As mentioned above, the morphology of the flower and particularly of its ovules are fundamental for the formation of fruits with seeds (Kennedy, 2002). **Particularly, abnormal ovules formed during flower development can conduce to seedlessness, through either the abnormal development of the nucellus or ovule integuments, or the degeneration of the egg in the embryo sac** (Ebadi et al., 1996).

3.2. Seed and berry development

Grape berry development consists of two successive sigmoidal growth periods separated by a lag phase (Coombe, 1992; Coombe and McCarthy, 2000) (Figure 5). Berry development starts after fertilization, with a process known as **fruit set** in which the ovary changes from a stationary state and experiences an abrupt increase in size that occurs due to cell division and enlargement, leading to rapid pericarp growth. During this period, seed development plays an important role, mainly because seeds produce auxins, gibberellins (GAs) and cytokinins, which have multiple functions in grape berry development (Keller, 2010). Both processes, seed and berry development are coordinated, and the changes that seeds undergo have an impact on fruit ontogeny (Serrano et al., 2017). In fact, the seeds also have three stages of development (Ristic and Iland, 2005) (Figure 5).

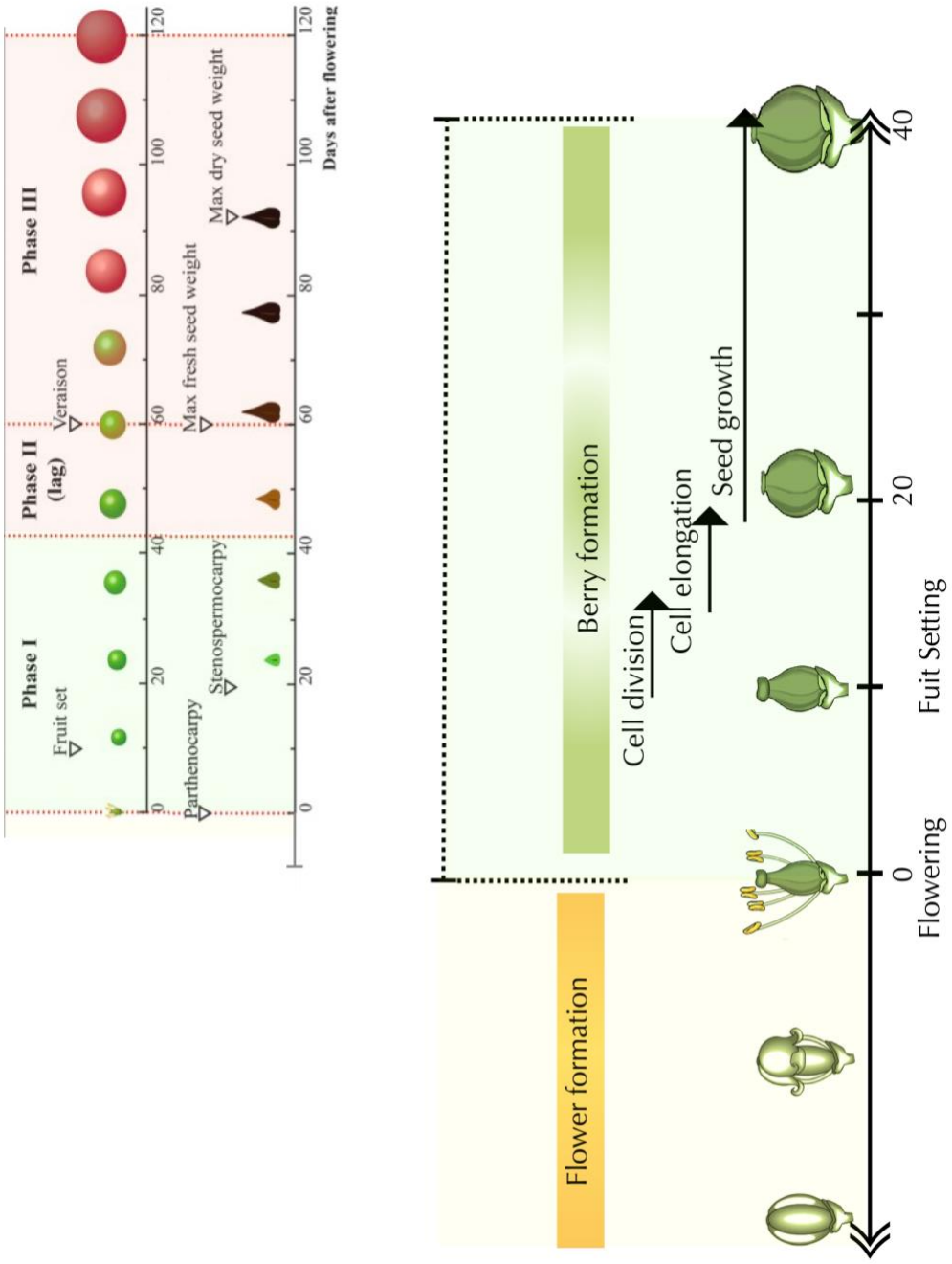

FIGURE 5. Flower, berry and seed development in *Vitis vinifera*.
The upper panel shows the three stages of berry and seed development of grapevine. The low panel shows the flower development before bloom and the early stages of berry development. Figure adapted from Serrano and collaborators, 2017.

3.2.1. Phase I

During this phase, the berry is formed, mainly by rapid cell division across the first weeks, and the total number of cells is established (Kennedy, 2002). At this period, seeds initiate their **growth phase**, mostly associated with cell division and differentiation (Ristic and Iland, 2005). Developing embryos produce phytohormones, which are released to the pericarp contributing to berry growth; therefore the pericarp growth correlates with the growth rate of developing seeds (Keller, 2010). Thus, this stage is characterized by a rapid increase in seed size, during which **embryogenesis and endosperm growth occur**. Towards the end of this stage, small embryos are formed (Conde et al., 2007; Coombe and McCarthy, 2000; Ristic and Iland, 2005; Staudt and Leidel, 1986).

3.2.2. Phase II

The second stage, also named ‘Lag Phase,’ is a short period where the berry growth ceases. The seeds undergo the **Transition Phase,** was there a small increase in dry weight together with a slight drop in water content occurs. The ‘notch’ or basal end of the seed expand, possibly to allow the accumulation of nutritional reserves. The end of this period coincides with the cessation of seed growth (Ristic and Iland, 2005).

3.2.3. Phase III

The berry faces a new period of growth, mainly due to volume increase. This stage is also called Ripening and starts with ‘veraison,’ a phenomenon in which the grape berry begins to gain color

and ends around 120 days after flowering (Figure 5) (Costantini et al., 2008a; Kennedy, 2002; Kuhn et al., 2014).

During this period, seeds go through a phase of **Drying and Maturation**. At the beginning of this stage, the endosperm accumulates reserves until the seeds turn dormant (Keller, 2010; Serrano et al., 2017). Then, after 70 - 100 days post- flowering, the growth of the embryo becomes rapid and reaches its peak. Thereby, the maximum berry weight coincides with the maximum seed dryness and the maximum embryo length (Ebadi et al., 1996; Ristic and Iland, 2005; Staudt and Leidel, 1986). Moreover, during berry ripening the seeds are being prepared to be released into the environment, the drying process of the seed takes place, which is associated with seed coat impermeability and seed dormancy (Ristic and Iland, 2005).

The three phases of the development have been studied in seed varieties; however, naturally, some grapes do not contain seeds or in which these are not fully developed.

3.3 Seedlessness phenotype

Fruit and seed development are processes that occur in a synchronized manner orchestrated by phytohormones and signals produced by developing embryos, after ovule fertilization (Pandolfini, 2009). **In some extraordinary cases, fruit growth occurs independently of the ovule's fertilization and seed development, as is observed in certain seedless mutant plants belonging to different species** (Varoquaux et al., 2000).

Seedlessness phenotype is characterized by fruits completely devoid of seeds, fruits containing a minimal number of seeds or presenting traces of aborted seeds (Pandolfini, 2009). A seedless

fruit can be obtained by **parthenocarpy,** when fruits are developed without fertilization or by **stenospermocarpy,** if the seeds are aborted after fertilization, leaving seed traces (Varoquaux et al., 2000).

For fruit consumption, seedlessness has become an important characteristic since in some seedless plants it has been reported better quality and flavor, increase in shelf life, less browning, among other features of interest (Grossniklaus and Schneitz, 1998; Pandolfini, 2009). However, **obtaining seedless fruits is difficult at the physiological level**, as the developing seeds play a role in the fruit formation during the first days, influencing cell division rate of the surrounding tissues (Gillaspy et al., 1993), and accordingly, the number of seeds affects the final size of the mature fruit (Varoquaux et al., 2000).

In *Vitis vinifera*, seedless grape varieties have arisen spontaneously in nature and have been preserved over the years through asexual propagation. Seedless berries develop naturally via parthenocarpy or stenospermocarpy, which generate berries without or with rudimentary seeds, respectively (Varoquaux et al., 2000). Interestingly, the frequency of grape's seedlessness phenotype is variable and does not relate to standard genetic ratios (Cain et al., 1983). **Despite the interest generated by this phenotype, there is limited knowledge about genetic mechanisms involved in grapevine seedlessness.**

3.3.1. Parthenocarpy

In parthenocarpic fruits, the stimulus of pollination is sufficient to trigger fruit set (Dokoozlian, 2000). Since the ovary is able to enlarge and form a berry without ovule fertilization**, there is**

no seed in the fruit (Varoquaux et al., 2000). Until now, few parthenocarpic grape cultivars have been, and they are mostly used as raisins. Therefore, there are few studies focused on this process. In one of these, two somatic variants -with and without seeds- were compared with transcriptomics. The investigators found about 2000 genes differentially expressed in pre-anthesis flowers, many of them associated with reproductive development were downregulated in seedless grapevine variant (Royo et al., 2016; Vargas et al., 2007). Also, 14 single-nucleotide polymorphisms (SNP) were identified between both genotypes, which could explain the parthenocarpy phenotype (Royo et al., 2016). **However, there are no functional studies of the candidate genes, and more studies are needed to characterize the contribution of these genes on the parthenocarpic phenotype**.

3.3.2. Stenospermocarpy

During stenospermocarpy, pollination and fertilization occur normally, but a few weeks later, **the embryo, the endosperm or both abort and the berries that have been generated possess only seed traces** (Varoquaux et al., 2000). It has been demonstrated that stenospermocarpy occurs in several seedless varieties, and is stable and unaffected by environmental factors (Zhang et al., 2013). However, **little is known about the molecular mechanisms that underlie stenospermocarpy in grapes**. The most accepted hypothesis proposes the existence of a dominant regulator gene called Seed Development Inhibitor (SDI), which could control three other recessive genes (Bouquet and Danglot, 1996). Different studies based on quantitative trait locus (QTL) analysis have reported a main QTL in linkage group 18 (LG18) (Cabezas et al., 2006; Costantini et al., 2008b; Doligez et al., 2002; Mejía et al., 2007), which could explain

between 50 and 70% of the seedlessness phenotype in grapes; LG18 could be considered as the SDI locus trait. In this context, *VvAGL11* (a MADS- BOX transcription factor, homolog to *STK*, a D-MADS box gene) was in silico mapped to the SDI locus, and it has been proposed as the main functional candidate gene for seedlessness in grapes (Mejía et al., 2011). In fact, it was demonstrated that the silencing of a *VvAGL11* homologous gene in tomato (*Solanum lycopersicum* L. cv Micro-Tom) generates fruits with few or rudimentary seeds (Ocarez and Mejía, 2016) and the ectopic expression of *VvAGL11* in *stk mutants* which have smaller siliques with slow number of seeds, restored the wild type phenotype, confirming a role in seed development (Malabarba et al., 2017). Based on genome sequencing data, it is known that in the stenospermocarpic variety cv. Thompson Seedless, the *VvAGL11* gene has an insertion in the 5'UTR, which could be the cause of the seedless phenotype (Di Genova et al., 2014). In addition, in cv. Sultanine Monococco, which is a seeded variety of Thompson, the *VvAGL11* transcript level is higher in comparison with the seedless variety (Ocarez and Mejía, 2016) as occurs when comparing seeded cv. Chardonnay with seedless cv. Thompson (Malabarba et al., 2017), supporting the hypothesis that this gene is one of the central regulators of seed formation in grapes. However, **this gene is incapable of explaining the seedlessness phenotype totally; thus there must be other genes involved that have not been identified** (Mejía et al., 2011).

The use of somatic variants in combination with current transcriptomic technologies has helped to discover new genes playing important roles in seed abortion. As an example, Hanania and collaborators (2007, 2009) compared inflorescences from somatic variants with and without seeds and identify the ch-Cpn21, a gene that encodes for a chloroplastic chaperonin, which is repressed in developing flowers of cv. Thompson Seedless in comparison with the seeded somatic variant. Likewise, the silencing of this gene in tobacco plants (*Nicotiana benthamiana*)

and tomato induces seed abortion (Hanania et al., 2007). Also, Costenaro-da-Silva et al. (2010) and Nwafor et al. (2014), founded several genes have been associated with early stages of grape berry development. These include transcription factors, ribosomal proteins, genes related to hormone signaling, among others. Many of these genes have putative pleiotropic effects, so it is difficult to estimate their specific molecular contribution to the stenospermocarpy phenotype. **Some of them could be involved in this process, but their functional characterization is needed to test this hypothesis** (Costenaro-da-Silva et al., 2010; Nwafor et al., 2014).

Until now, it has been challenging to understand the genetic and molecular mechanisms involved in ovule and seed development in *Vitis vinifera*. It is believed that, as in other species, many genes participate in this process acting in reproductive organs during ovule development at flowers or seed development at fruit formation.

4. Expression profile to identify candidate genes involved in the formation of the seed and seedlessness in *Vitis vinifera*

A previous study developed in our laboratory focused on analyzing the differential gene expression between two phenotypes of segregating plants, seeded and seedless, as a tool to identify novel genes associated with the seedlessness's phenotype. The plants were obtained from a cross between a seeded maternal line (cv. Moscatel rosada) and a seedless paternal line (cv. Ruby seedless). The study was performed using Affymetrix microarrays, comparing early developmental stages of berry: flower (bloom) and young berries (20 days post bloom). In this study, a total of 4077 differentially expressed genes were identified (Muñoz et al., 2012).

From the analysis of Affymetrix performed in our laboratory, a series of filters were applied to choose candidate genes. To do this, of the 4077 differentially expressed genes, those with a contrasting expression pattern were selected between plants that generate seedbed berries and rudiments, that is, genes that present an induction or repression between the stages of flower and fruit set in a type of plant, but that remain constant in the other class. This contrast profile is similar to the expression pattern of genes previously associated with seedlessness in grapevine, such as S27A and CPN21 (Hanania et al., 2007, 2009). Approximately 400 genes were found in this condition. Subsequently, these were filtered according to their expression levels, considering only those whose expression change (logarithm in base 2) is higher than ±1, obtaining a list of 148 genes. Within this list, ten genes encoding for putative transcription factors or transcriptional regulators, which were considered in this analysis, due to their importance in the modulation and regulation of gene expression. Finally, genes belonging to families of transcription factors that have been described as important in plant development processes were selected, and genes whose transcripts are accumulated in reproductive tissues linked to the development of the seed in model plants were preferably considered.

Considering this analysis and literature, we select new candidate genes whose expression changes could be responsible for the seedlessness phenotype. Candidate genes are putative transcription factors such as *VvAIL5, VvAGL6_1, VvAGL6_2, VvINO,* and transcriptional co-repressors like *VvLUG*.

4.1. *VvAGL6*

VvAGL6 encodes a putative MADS-box transcription factor. This gene family have a key role in flower and fruit development in different species (Boss et al., 2002; Roy Choudhury et al., 2012). Within this family, the *AGAMOUS like 6* clade includes to *AGL6* and *AGL13* as members (Hsu et al., 2014) and both genes are located in a genetic block suggesting a duplication event (Schauer et al., 2009).

Studies based on the molecular characterization and mutant analysis in species like petunia (*Petunia*), rice (*Oryza sativa*) and maize (*Zea mays*) suggest a function as E class genes during flower development. However, in order to test their specific functions in flower and ovule development, Arabidopsis null mutants must be generated and characterized (Dreni and Zhang, 2016). The rice *OsMADS6* is the most studied *AGL6* gene in seed plants and has expression inside the carpel in different tissues as ovule integuments (Ohmori et al., 2009). Strong *osmad6* mutant alleles show a partially lost in ovule's identity within the carpels, leading a female sterility, which suggest a role in ovule development (Li et al., 2011). Also, over-expression of *AGL6* homologues from orchid (*Oncidium Gower Ramsey*) and *Hyacinthus orientalis* L. in Arabidopsis, reveals a role in flowering timing and transition (Fan et al., 2007; Hsu et al., 2003).

Functional studies in Arabidopsis using over-expression or silencing lines of *AGL6* suggest a role in flowering timing and formation of lateral organs in Arabidopsis (Koo et al., 2010; Yoo et al., 2011). Silencing lines *35S:AGL13 RNAi* have half of the total ovules aborted at early stages, and pollen was also affected but viable (Hsu et al., 2014).

In Arabidopsis, *AGL6* is expressed in the endothelial layer of ovules, adjacent to the developing female gametophyte. Both *AGL6* and *AGL13* are co-expressed only in chalaza of the developing ovule (Schauer et al., 2009). *AGL13* is also expressed in the tapetum, the innermost anther wall layer in developing shoot apical meristem (Schauer et al., 2009), floral meristem, pollen and ovules (Smyth et al., 1990) (Hsu et al., 2014).

Little is known about *AGL6* homologous in *Vitis vinifera*. A first study identifies *VvMADS3* as a homologous with a high expression in grapevine's flowers, specifically in petals and carpels, and also in developing seeds, while it has no expression in vegetative tissues and berries. Also, this gene shared high similarity of sequence with both, *AGL6* and *AGL13,* suggesting a possible role in floral and seed development (Boss et al., 2002). A recent study identifies two genes named as *VviAGL6a (corresponds to VvMADs3)* and *VviAGL6b* as homologs of *AGL6* and *AGL13*. Transcripts of both genes are accumulated across flower development, suggesting a role during reproductive development (Palumbo et al., 2019). In this study, the genes will be named as *VvAGL6_1* and *VvAGL6_2*.

4.2. *VvAIL5*

Members of the AINTEGUMENTA-LIKE/PLETHORA (AIL/PLT) transcription factors have important roles in several plant development processes including flower development (Horstman et al., 2014; Krizek, 2015). *AINTEGUMENTA* (*ANT*), which belongs to the AP2/ ERF subfamily, is the most studied gene and is a key regulator of floral organs growth and a major ovule development regulator, since *ant* mutants have organs flowers with reduced size,

ovule defects and female sterility (Baker et al., 1997; Elliott et al., 1996) and its ectopic expression causes an increase in size of organs and seeds (Mizukami, 2000).

On the other hand, phenotypes of the ectopic expression of *AIL5* and *ANT* are similar, while *ant 5* mutants lines have no observable defects on flower development (Nole-Wilson et al., 2005; Prasad et al., 2011). Otherwise, it has been demonstrated that *AIL5* is involved in germination and seedling growth (Yamagishi et al., 2009; Yano et al., 2009), but it suggest a partially redundant role with other genes of this family (*AIL7* and *ANT)* during floral development (Krizek, 2015).

There is no much information about AIL/PLT family in *Vitis vinifera*. Licausi and co-workers carried out a genomic and transcriptomic study of the putative members of the AP2 / ERF family and quantified the expression of *VvAIL5*, identifying a highest expression in leaf, inflorescence and stem, but not in fruit tissues (Licausi et al., 2010). Also, the analysis made using data from The Grapevine Expression Atlas shows transcript's accumulation in reproductive tissues as carpel and developing seed, as well in vegetative tissue as root (Fasoli et al., 2012) (See Figure 6).

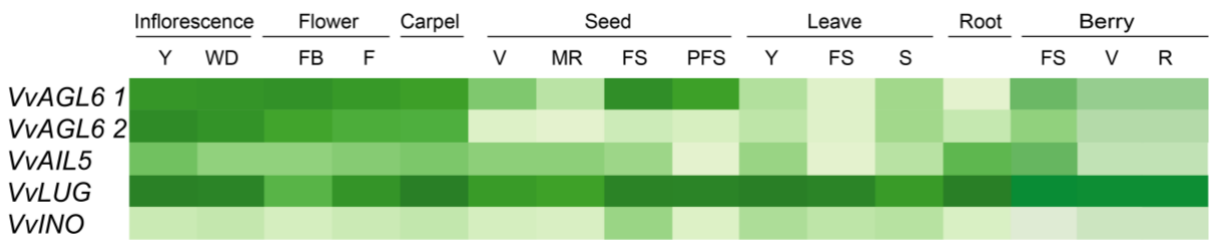

FIGURE 6. Profile expression of candidate genes in different tissues.
These analysis was made in this study using data from the Transcriptomic Atlas of Grapevine (Fasoli et al., 2012). Y: young WD: well developed, FB: flowering begins (10% cap off), F: flowering (50% caps off), V: veraison, MR: mid ripening, R: Ripening, FS: fruit set, PFS: post fruit set, S: Senescing.

4.3. *VvLUG*

LEUNIG (LUG) protein is similar in domain structure and biochemical function to the Groucho (Gro), Transducin-Like Enhancer of Split, and Tup1 family of corepressors in Drosophila, mammals, and yeast, respectively. These corepressors do not possess a DNA-binding domain and interact with DNA-bound transcription factors (Sitaraman et al., 2008).

LUG was firstly described it as a cadastral gene with important function in flower development, since *leunig* mutants' flowers have sepals transformed toward stamens and carpels, and petals staminoids or absent, between other pleiotropic effects. Also, a role in flower development, as an A and C class gene during ABC(DE) was proposed (Liu and Meyerowitz, 1995).

LUG is expressed in almost all plant tissues, with strong expression across floral development, including placenta and ovules (Conner and Liu, 2000; Pfannebecker et al., 2016).

Regarding this function, *LUG* was described as a negative regulator of *AGAMOUS* (*AG*, a C class gene) during floral development, acting in a complex with a Q- rich protein (Franks et al., 2002; Sridhar et al., 2004). Furthermore, it has been established that *LUG* have functions related to floral development, and embryo formation (Sitaraman et al., 2008). It has also assigned a role in carpel development (Sitaraman et al., 2008) and recently, a physical interaction between *LUG* and *INO* has been probed, suggesting its coordinated participation during ovule's integuments formation, possibly using a co-repressor as adapter (Simon et al., 2017a).

VvLUG corresponds to putative homologous of *LEUNIG* (*LUG*) in grapevines. So far, there have been no studies characterizing this gene in this model. Previous analysis doing during this work using data from Transcriptomic Atlas of Grapes showed that it is expressed in many tissues, vegetative and reproductive, as occurs in *LUG* from Arabidopsis (Fasoli et al., 2012) (See Figure 6).

4.4. VvINO

Villanueva and coworkers isolated the coding gene sequence (CDS) of *INNER NO OUTER* (*INO*) from *Arabidopsis thaliana* (Villanueva et al., 1999a). The CDS has 696 bp and encodes a 231 aminoacid protein that is a putative transcription factor belonging to the YABBY gene family, which is involved in the establishment of abaxial- adaxial polarity in lateral organs and asymmetric development (Bowman, 2000). Interestingly, *INO* is the only YABBY gene expressed in the ovule (Bowman and Smyth, 1999; Siegfried et al., 1999; Villanueva et al., 1999b).

The expression profile studied by *in situ* hybridization, shows that Arabidopsis *INO* mRNA is expressed on the abaxial zone of each ovule primordium, the area where the ovule's integuments initiation occurs, specifically on a group of approximately 15 epidermal cells during early phase of ovule's development, prior to visible integument initiation. During the next phases**,** when both integuments primordia are visible, *INO* mRNA is detected only in outer integument (abaxial side of ovule primordia). This transcript accumulation disappears during anthesis. Also,

INO mRNA is present in young embryos prior to globular stage, but not in other floral structures (Villanueva et al., 1999a).

The expression pattern of *INO* orthologs has been conserved in some other species like *Impatients* (McAbee et al., 2005), *Nymphacea* (Yamada et al., 2003), *Anonna* (Lora et al., 2011) and *Amborella* (Arnault et al., 2018), supporting the hypothesis that *INO* might have a conserved role in the o.i. among angiosperms (Kelley and Gasser, 2009; Lora et al., 2011; Yamada et al., 2003). Also, unitegmic ovules (plants with i.i. and o.i. fusioned) express *INO* gene (Lora et al., 2015; Skinner et al., 2016).

Two strong *INO* Arabidopsis mutants have been studied, *ino- 1* and *ino- 2*. The most studied, *ino-1*, has one nucleotide change (from G to A) in the fifth intron of a total of 6 introns, which creates a splicing acceptor site that results in a final transcript with 11 additional nucleotides. This generates a frameshift translation of the C- terminal region, near the YABBY domain, that cause an inactive protein (Villanueva et al., 1999a).

INO is a master regulator of o.i. formation required for both integument initiation and subsequent growth of this structure (Simon et al., 2017b; Villanueva et al., 1999a). Phenotypically, ***ino-1*** **completely lack of the o.i. and the gametophyte development is disrupted** (Villanueva et al., 1999b). In consequence, ino-1 have female sterility. Moreover, in ***ino*** **mutants there are no fruit development**, because o.i. is involved in pollen tube guidance in Arabidopsis, so in these mutants pollen tubes rarely target the ovules during pollination (Herrero, 2000; Lora et al., 2018; Skinner and Gasser, 2009).

In addition, ***Tai seedless*** **(Ts**), a spontaneous mutant of *Anonna squamosa* with a deletion of the

INO locus, generates unitegmic ovules, that no develops into seeds. Ts mutants have fruits completely developed, but without seeds(Lora et al., 2011). Unlike Arabidopsis, Anonna has an endostomal micropyle where the outer integument does not fully cover the inner integument and does not participate in the micropyle, and, presumably, in the pollen tube guidance. Hence, Ts mutant suffers the interruption of the reproductive program later than *ino* mutant, during the embryo and endosperm development and not during the embryo sac formation, which allows fruit development, despite disruption of seed development (Lora et al., 2011). *Vitis vinifera* has also an endostomal mycropile (Mullins et al., 1992) and therefore is likely that o.i. it does not have a role in the entry of pollen and subsequent fertilization.

The mechanisms that control *INO* expression are not completely known, however, there must be a precise spatio-temporal regulation. In fact, it has been proposed that several genes act by regulating their expression (Gaiser et al., 1995; Meister et al., 2002). This mechanism maintain the *INO* expression restricted to the gynobasal side of developing ovules, is required for initiation and asymmetric growth of outer integument (Meister et al., 2002).

Recently, Simon and coworkers, found a direct interaction between *INO* and co-repressors and co-activators. *INO* interacts with the co-repressors *LEUNIG (LUG)* and *SEUSS (SEU*) and with the co-activator protein *ADA2b*. Also, mutations in all this genes, generates a decrease in growth of the outer integument, suggesting a role in growth and extension of this integument (Simon et al., 2017b).

VvINO is the putative homologous of *INO* in grapevine and until now, it has not been studied. The analysis made using data from Fasoli and coworkers (shown in Figure 6) exhibit that *VvINO*

has a very low expression profile in the vegetative and reproductive tissues analyzed, presumably because it is expressed in specific cells inside the ovule, as in Arabidopsis.

In summary, regarding grapevine, seedlessness is characterized by the absence of normal seeds in grape berries, an attractive phenotype, which has been poorly studied. The transcriptional analyses previously carried out in our laboratory allowed the selection of candidate genes with differential expression between the seeded and seedless phenotypes (Muñoz et al., 2012)**. Therefore, we asked whether any of these selected genes participate in the formation of the seed in grapevine.**

CHAPTER 2

PROBLEM STATEMENT:

HYPOTHESIS AND OBJECTIVES

According to previously mentioned information in the general introduction, the biological question that directs this investigation is: Do any of the grapevine selected genes participate in the formation of the seed?

To address the biological question, the following hypothesis was proposed for this thesis work:

1. Hypothesis

The formation of the seed in the model plant *Arabidopsis thaliana* is affected by the expression of some of the genes of *Vitis vinifera* that are differentially expressed in seed and seedlessness grapevine: *VvAGL6_1, VvAGL6_2*, *VvAIL5*, *VvLUG*, and *VvINO*.

2. Objectives

The following main objective was proposed to answer the hypothesis:

Determine the expression profile of the genes; *VvAGL6_1*, *VvAGL6_2*, *VvAIL5*, *VvLUG* and *VvINO*; in seed and seedlessness grapevine and to evaluate if the expression of any of these affects the formation of the seed in *Arabidopsis thaliana.*

The general objective was divided into the following partial objectives:

1. Correlate the seeded or seedlessness phenotype and the expression of the selected genes in the early stages of the development of the grape berries.

2. To functionally evaluate the participation of one selected gene in the formation of the seeds in the model plant *Arabidopsis thaliana*.

CHAPTER 3

MATERIALS

1. Biological Material

1.1. Plant material

1.1.1. *Vitis vinifera*

The grapevine plants used in this work were obtained from an experimental field, located in Miraflores, Curacaví, at the Metropolitan valley of Chile (33°24'01.0"S 71°03'17.6"W).

Samples of the following varieties were collected: Moscatel rosada, Red globe, Thompson seedless and Flame seedless. Also, in this study plants obtained from a segregating population from crosses between Moscatel rosada x Flame seedless were used.

1.1.2. *Arabidopsis thaliana*

Seeds of Columbia (Col-0) and Landsberg erecta (Ler-0) ecotypes were obtained from the seed's collection of our laboratory. *ino-1* (CS3881) mutant was obtained from the Arabidopsis Biological Resource Center (ABRC) catalog (The Ohio State University).

1.2. Bacterial strains

1.2.1. *Escherichia coli* One Shot® TOP10 F–, *mcrA,* Δ*(mrr-hsdRMS-mcrBC),* Φ*80lacZ*Δ*M15,* Δ*lacX74, recA1, araD139,* Δ*(ara leu)7697, galU, galK, rpsL*, (*str*^{R})*, endA1, nupG*. Competent cells by calcium chloride, used for transformation with plasmids.

1.2.2. *Rhizobium radiobacter* **GV3101::pMP90** *pMP90 (pTiC58ΔT-DNA), rif*^{R}*, gm*^{R}. Electrocompetent cells, used for transformation with plasmids.

1.3. Plasmids

pGEM®-T Easy by Promega was used to clone DNA fragments from Polymerase chain reactions (PCR).

pENTR™/SD/D-TOPO®, PK7FWG2 was used to generate constructions using Gateway® technology, as entry clone and donor receptor respectively (Table 1).

TABLE 1. Vectors used in this work.

Vector	Selection marker in bacteria	Selection marker in plants	Application
pGEMT-easy	Amp	-	Cloning
pENTR™/SD/D-TOPO®	Kan	-	Cloning
pK7FWG2	Spec	Kan	Over-expression in plants (35S promoter)

Amp: ampicillin, Kan: kanamycin, Spec: spectinomycin.

2. Culture media

2.1. Culture media for plants

Arabidopsis *in vitro* media culture (MS 0,5 X) includes 2,2 g/L of MS salts supplemented with vitamins (Duchefa Biochemie B.V.), 15 g/L of sucrose, 0,1 g of myo-inositol; 0,7% w/v of agar-agar; pH 5,8 and antibiotics needed to make the selection for transgenic plants.

2.2. Culture media for bacteria

The bacteria culture media used correspond to Luria-Bertani (LB) Broth medium from MO BIO Laboratories (Carlsbad, California, United States.) supplemented with antibiotics for selection according to the vector, as shown in Table 1. LB solid media (25 g/L of LB Broth) was further supplemented with Merck agar (15 g/L) (Darmstadt, Germany).

The antibiotics used were gentamicin and rifampicin from PhytoTechnology Laboratories® (Shawnee Mission, Kansas, United States); ampicillin from Winkler (Santiago, Chile); kanamycin from United States Biological (Massachusetts, United States) and spectinomycin (Spec) from Duchefa Biochemie B.V. (Haarlem, Holland).

3. General reagents

3.1. Enzymes

The enzymes Taq DNA polymerase, T4 DNA ligase, SuperScript® II reverse transcriptase, Gateway® LR Clonase® II enzyme mix and Proteinase K were obtained from Invitrogen ™ (Carlsbad, California, United States). The enzyme deoxyribonuclease (DNase) TURBO DNA-free ™ DNase was obtained from Ambion®). Restriction endonuclease DdeI from New England BioLabs® (Ipswich, Massachusetts, United States) was also used.

3.2. Commercial Kits

The following commercial kits were used: pGEM®-T Easy Vector System from Promega (Madison, Wisconsin, United States), FavorPrep™ Plasmid DNA Extraction Mini Kit from Favorgen (Ping-Tung, Taiwan), SensiMix™ SYBR® Hi-ROX Bioline Kit from Bioline (London, United Kingdom).

3.3. General Reagents

The agarose was obtained from Bioline (London, United Kingdom).

From Biotium (Hayward, California, United States) the GelRed® nucleic acid staining solution was obtained.

From Calbiochem® (San Diego, California, United States), cetyltrimethylammonium bromide (CTAB) and β-mercaptoethanol were obtained.

From Invitrogen™ (Carlsbad, California, United States) the following reagents were obtained: (1) for PCR: deoxyribonucleotides dATP, dCTP, dGTP and dTTP (dNTPs), buffer 10X PCR buffer, magnesium chloride ($MgCl_2$) 50 millimolar (mM); (2) for complementary DNA(cDNA) synthesis: 5X First-Strand Buffer, dithiothreitol (DTT); (3) for RNA synthesis: TRIzol® reagent; and (4) for electrophoresis and gel staining: molecular mass marker for DNA 1 Kb Plus DNA Ladder and SYBR® Safe nucleic acid staining solution.

From Merck (Darmstadt, Germany) the following reagents were obtained: boric acid (H_3BO_3), hydrochloric acid (HCl), nuclease free water (used for molecular biology techniques), isoamyl alcohol (AIA), sodium carbonate (Na_2CO_3), chloroform ($CHCl_3$), calcium chloride dihydrate ($CaCl_2 \cdot 2H2O$), lithium chloride (LiCl), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H2O$), potassium chloride (KCl), sodium chloride (NaCl), dihydrogen phosphate ammonium ($NH_4H_2PO_4$), dimethylsulfoxide (DMSO), ethanol, disodium phosphate (Na_2HPO_4), monosodium phosphate monohydrate ($NaH_2PO_4 \cdot H2O$), glycerol, D (+) - glucose, sodium hydroxide (NaOH), isopropanol, methanol, copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 4H_2O$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), Tween® 20 and N,N,N',N'-TETRAMETILETILENDIAMINA (TEMED) and Ammonium persulfate ($(NH4)_2S_2O_8$).

The following reagents were obtained from Sigma-Aldrich (Saint Louis, United States): diethylpyrocarbonate (DEPC), spermidine, polyvinylpyrrolidone (PVP40),

polyvinylpolypyrrolidone (PVPP), sulfate manganese monohydrate ($MnSO_4 \cdot H2O$), Triton X-100.

From Winkler (Santiago, Chile) the following reagents were obtained: sodium acetate (NaOAc), 6X loading solution for nucleic acids (glycerol 30% w/v, bromophenol blue 0.25% w/v and xylen-cyanol 0,25% w/v), TAE 50X buffer (2 M Tris-acetate and 50 mM EDTA, pH 8), disodium salt of ethylenediaminetetraacetic acid dihydrate (EDTA), Tris (hydroxymethyl) aminomethane) and sodium hypochlorite 5%.

Reagent Silwett L-77 was obtained from commercial brands.

3.4. Oligonucleotides

The specific primers used in the PCR and qRT-PCR were obtained from Integrated DNA Technologies (Coralville, Iowa, United States) and are shown in detail in Table 2.

The mixture of random sequence hexanucleotide primers (Random Primers) was obtained from Invitrogen ™ (Carlsbad, California, United States).

4. Equipment

The 2720 Thermal Cycler from Applied Biosystems® (Carlsbad, California, United States) was used for the PCR and cDNA synthesis.

Homogenizer Precellys 24 (Bertin technologies) was used to grind plant tissue.

The UV transilluminator used to visualize agarose gels was obtained from Vilber Lourmat (Marne-la-Vallée Cedex 1, France) and the record of these gels was made with a Canon Power Shot G6 digital camera (Tokyo, Japan) with UV filter.

For the transformation of *Rhizobium radiobacter*, the MicroPulser™ Electroporator from Bio-Rad Laboratories (Hercules, California, United States) was used.

For the quantification of nucleic acids and optical density (D.O.) of cell cultures, the NanoDrop 2000c spectrophotometer from Thermo Scientific™ (Waltham, Massachusetts, United States) was used.

Arabidopsis seeds were visualized using a Dissecting Microscope Nikon SMZ800 and photographed with a Nikon digital DS-Fi1c Camera. For silique dissections, ophthalmological scalpel and tweezers from commercial brands were used.

5. Web sites, databases and softwares

For designing of specific primers for PCR and RT-PCR, the Primer3Plus tool (http://primer3plus.com/cgi-bin/dev/first3plus.cgi) and the primer-BLAST (NCBI) was used (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

For sequences analysis, the bioinformatic software CLC Workbench (Qiagen, Hilden, Germany) and Vector NTI® program from Invitrogen TM (Carlsbad, California, United States) were used. To search for regulatory elements in promoter sequences, we used the PLACE database (www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999). The search of motifs in predicted proteins, as well as Gene Ontology (GO) analysis were made on Phytozome, the Plant Comparative Genomics portal of Department of Energy's (University of California), which is provided by KOG, KEGG, ENZYME, Pathway and the InterPro family of protein analysis tools (https://phytozome.jgi.doe.gov/pz/portal.html). The search of the promoter predict sequence was made with Grape Genome Browser, from Genoscope (Centro National de Séquençage, France), INRA (L' Institut National de la Research agronomique, France) and the Institute of Applied Genomics (IGA), Italy (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/).

For statistical analysis, Prism 6.0 Software (GraphPad, Inc., La Jolla, CA, USA) was used.

CHAPTER 4

METHODS

1. Collection of Plant Material

1.1. Sampling of *Vitis vinifera*

During the season 2013, *Vitis vinifera* samples were collected. Phenological inflorescence stages used for theses analyses were **EL-18**, **EL-25** and **EL-27** (Figure 7), selected according to Coombe and co-workers (Coombe, 1995). Samples from independent branches were frozen in liquid nitrogen and stored at –80°C until required for further analysis.

As a model of study to characterize the expression profile of candidate genes, *Vitis vinifera* cv. ´Thompson seedless´ and cv. ´Flame seedless´ were used as seedless table grapes while cv. 'Red globe' was used as a seeded table grape. These varieties are widely used in the consumption of fresh grapes (OIV, 2016). For RT- q PCR, four independent clusters at each stage were used as a biological replica.

Also, samples from segregating plants descendants of Moscatel rosada x Flame seedless crosses, were collected during season 2013 and 2014. The phenotype of these plants was recorded in season 2013, and ripe berries were harvested and analyzed. For this, one cluster per plant was used, and 30 berries were randomly selected. The weight of each berry was determined and then a longitudinal cut was performed to extract and weight the seeds. The next season (2014), 2 biological replicas (bunch) per independent plant of each stage (EL-17, EL-25 and EL-27) were collected for expression assays.

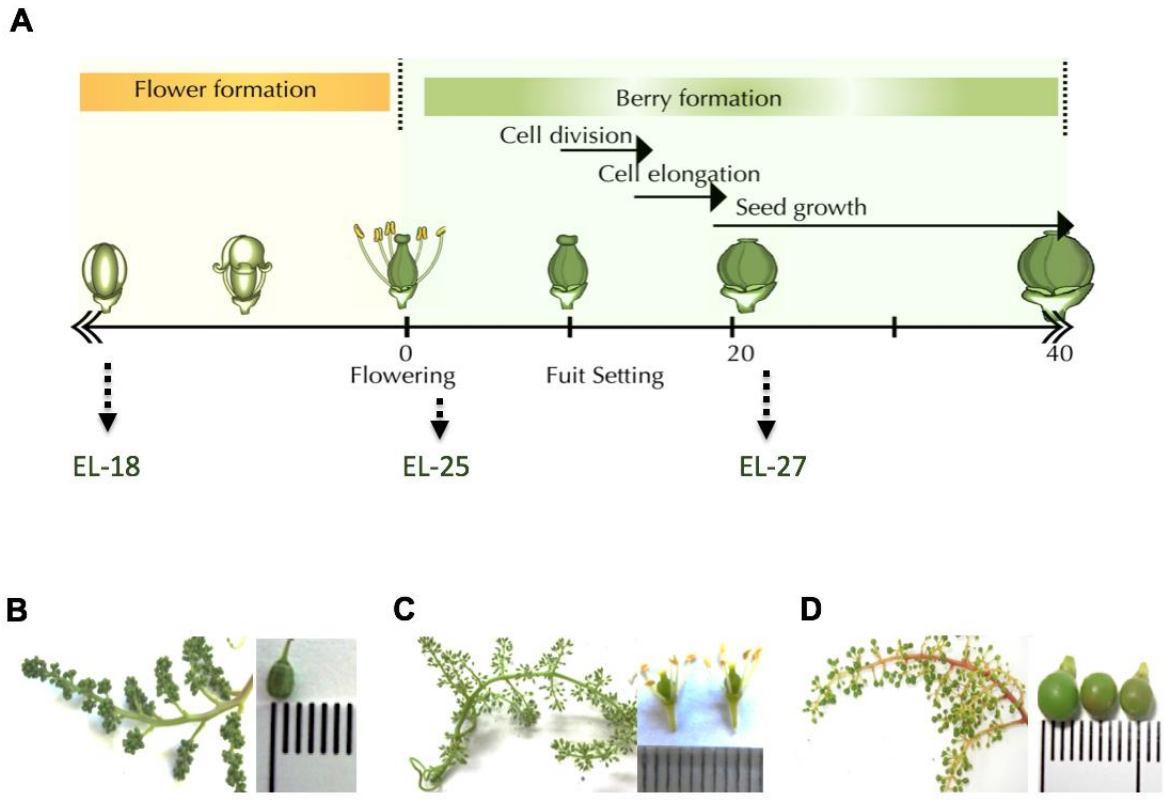

FIGURE 7. Phenological stages of *Vitis vinifera* used for this study
A) Time- line of first days of flower and berry development**. B)** Developmental berry stages analyzed in this study, according to literature (Coombe, 1995). **EL-18:** leaves separated; individuals flowers are separated, flower caps still in place, but cap color fading from green. **EL-25:** 80% caps off. More than 20 leaves separated. Mostly flowers are pollinated in this stage. **EL-27:** Setting; young berries enlarging (>2 mm diam.), bunch at right angles to stem. Young berries growing. Bunch at right angles to stem.

1.2. Sampling of *Arabidopsis thaliana*

In order to obtain Arabidopsis plants, seeds were sterilized in 1.5 mL polypropylene tubes with a 40% v/v sodium hypochlorite solution and 1% v/v of Tween-20® with shaking during 7 min and 4 washes with sterile distilled water for 3 min each. Subsequently, seeds were plated onto 0.5X MS medium incubated at 4 °C for 3 days in complete darkness, and then they were maintained in a growth chamber under controlled conditions, at 22 ± 2 °C in long day photoperiod (16/8h). For selection of transgenic plants, MS 0.5X medium was supplemented with 50 mg/L of Kanamycin.

After 10 days, plants were transferred to soil (a mix of peat moss and vermiculite, 2: 1) and maintained in a growth camber at 22 °C in long- day photoperiod.

2. Nucleic acid extraction, cDNA synthesis and gene expression analysis

2.1. DNA extraction

Genomic DNA was extracted from *Vitis vinifera* and *Arabidopsis thaliana* leaf tissues, according to a previously described method (Agurto et al., 2017). DNA concentration and integrity were determinate using Nanodrop spectrophotometer. A PCR amplification was performed using primers for a housekeeping gene (*VvActin* or *VvUBI-99 and 18S* for grapevine and Arabidopsis, respectively (Table 2) to confirm the viability of the DNA obtained.

2.2. RNA isolation, cDNA synthesis and q RT- PCR

Vitis vinifera total RNA was isolated from **EL-18, EL-25** and **EL-27** samples, according to a procedure previously described (Reid et al., 2006). *Arabidopsis thaliana* total RNA was isolated using TRIzol™ Reagent (Invitrogen) according to manufacturer's conditions.

For cDNA synthesis, 2 μg of total RNA were treated with TURBO DNA-free TM DNase (Ambion®) and reverse transcribed using random primers and SuperScript II RT (Invitrogen^{TM} Co., Carlsbad, California, U.S.A.), following the manufacturer's instructions. RT- qPCR analyses were performed using SensiMix^{TM} SYBR Hi-ROX Kit (Bioline, London, U.K.) and the Mx3000P qPCR system (Stratagene, Agilent Technologies Inc., Santa Clara, California, U.S.A.) according to the manufacturer's instructions.

Primers used in RT-qPCR are listed in Table 2 and correspond to VvSRP60, qVvEF1-*α*, qVvAIG1, qVvINO, q-VvLUG3, q-VvAGL6_1, qpcr3 AGL62, q-VvAIL5, Clat and AtActin 2.

Expression levels of all the evaluated genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), relative to *VvSRP60* housekeeping gene in the case of grapevine samples, and *Clatrin*, for Arabidopsis samples (Table 2) *VvSRP60* was selected from an analysis in which various housekeeping candidates (*VvEF1-α, VvAIG1*, *VvSRP60 and VvUBI99*) were compared using the software Normfinder (Andersen et al., 2004).

TABLE 2. Oligonucleotides used for PCR and RT-qPCR.

Vitis vinifera

Gene or region	ID Grape Browser 12X	Primers	Sequence (5'-3')	Ta (ºC)
VvACtin	GSVIVT01026580001	VvActin-F	ACCAGAATCCAGCACAATC	55
		VvActin-R	ATGGCCGATACTGAAGATAT	
VvSRP60	GSVIVT01035120001	VvSRP60-F	ATCTACCTCAAGCTCCTAGTC	55
		VvSRP60-R	CAATCTTGTCCTCCTTTCCT	
VvEF1-α	GSVIVT01025146001	qVvEF1-a-F*	GAACTGGGTGCTTGATAGGC	54
		qVvEF1-a-R*	AACCAAAATATCCGGAGTAAAAGA	
VvAIG1	GSVIVT01025947001	qVvAIG1-F*	GAAGATTATTTGGGCCGTGAG	54
		qVvAIG1-R*	CTTCTTGGCTTCATCCTTGGT	
VvINO	GSVIVT01013778001	VvINO_cdna_F	CACCATCTCCCCTTATTACATCAGAAAACT	57
		VvINO_cdna_R	TTCCGGGGTATCCCATGTTTC	
		Prom VvINO-F	TTAtGTTCGATACAATTCCTTTGCAGT	55
		Prom VvINO- R	GTTCTTCAGGAGAGAAAAGCAGC	
		qVvINO-F	GAGCTCAAAAAGCCTCAGAC	53
		qVvINO-R	GACATGGTGGACAGGAACTA	
VvLUG3	GSVIVT01035791001	q-VvLUG3-F	GGTAAAAACTGCCTCTGCAA	53
		q-VvLUG3-R	ACGGACAGAATGATCAACCA	
VvAGL6_1	GSVIVT01027577001	q-VvAGL6_1-F	GTGTAATGTGTGTATTGTGTTGAC	55
		q-VvAGL6_1-R	GTCCCCATGAAAGAGAAGCTA	
VvAGL6_2	GSVIVT01018450001	qpcr3 AGL62 F	ACTATGTTCCAGCAGAAGGGC	59
		qpcr3 AGL62 R	CACCCTTGGATGAAGTTGCTC	
VvAIL5	GSVIVT01029219001	q-VvAIL5-F	ATAGAGAGCAGGGTTTTCCG	58
		q-VvAIL5-R	CGAGAACTGATCGAGACCTG	
VvUBI-99	GSVIVT01038617001	*VvUBI-99-F*	TCTGAGGCTTCGTGGTGGTA	*55*
		VvUBI-99 -R	AGGCGTGCATAACATTTGCG	

Arabidopsis thaliana

Gene or region	TAIR locus ID	Primers	Sequence (5'-3')	Ta(ºC)
INO	AT1G23420	At INO-F	AAGCTCCCCAACATGACGAC	55
		At INO-R	TGCCCACATCTCACAGTCAC	
		ino 1dCAPS-F 5' **	TAACATTTTATGATTTCAATTTTTTGACTT	55
		ino 3'trunc R **	ATTTTGAAGCGAAAGTGATT	
18S	1005246134	18S-F	TAACGAGGATCCATTGGAGGGC	58
		18S-R	GCCCCCAACTTTCGTTCTTGA	
Clatrin	AT4G24550	Clat-F	AATACGCGCTGAGTTCCCTT	55
		Clat-R	AGCACCGGGTTCTAACTCAA	
Actin	AT3G18780	AT Actin2-R	AACAGCAGAGCGGGAAATTG	58
		AT Actin2-F	ACCAATCGTGATGACTTGCC	

Vectors Gene or region	ID	Primers	Sequence (5'-3')	Ta(ºC)
PK7FWG2	-	35 Prom- F	GACGTAAGGGATGACGCACAATC	57
		35 Term- R	CCTTATCTGGGAACTACTCACACA	
PK2GW7	-	Kan-F	AAAAGCGGCCATTTTCCACC	58
		Kan-R	GATGGATTGCACGCAGGTTC	
pENTRTM/SD/D- TOPO	-	M13 F	GTAAAACGACGGCCAG	50
		puc/M13 R	CAGGAAACAGCTATGAC	

Ta: Alignment Temperature

* Primers obtained from literature (González-agüero et al., 2013)

** Primers obtained from Charles Gasser, UC Davis

3. DNA recombinant techniques

3.1. DNA fragments amplification by Polymerase chain reaction (PCR)

For PCR, DNA, cDNA or bacteria grown in liquid medium were used as a template. The reactions were composed of 2.5 µL of 10X PCR buffer (200 mM Tris-HCl, pH 8,4 and 500 mM KCl), 0,5 µL of DMSO, 0,8 µL of 50 mM $MgCl_2$, 0,7 µL of dNTPs (10 mM each), 0,7 µL of each primer at a concentration of 10 µM, 10-500 ng of DNA or cDNA or 1 µL of bacterial culture, 0.1 µL of Taq DNA polymerase (5 U / µL) and sterile water until completing a final volume of 25 µL. The amplification was carried out in a thermal cycler using the following program: initial denaturation (95 ºC, 5 min), denaturation (95 ºC, 30 sec), hybridization (Ta of primers, 30 s), polymerization (72 ºC, 1 min/1000 bp of DNA), and final polymerization (72 ºC, 7 min). Once the PCR was finished, the amplified product was analyzed by electrophoresis in agarose gels. Primers used for PCR were listed on Table 2 and correspond to VvActin F and R, VvINO_cdna_F and R, At INO- F and R, ino1 dCAPS-F 5', ino3'trunc R, 18S F and R, Clatrin F and R and AtActin2 F and R.

3.2. DNA electrophoresis in agarose and acrylamide geles

3.2.1 Agarose gels

To visualize DNA fragments over 100 bp 1,5% w/v agarose gels were used. To prepare geles, 1,5 g of agarose was added to 100 mL of 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8). Once the agarose was gelled, the DNA was mixed with 6X loading solution (2 μL of loading solution for 8 μL of DNA) containing GelRed® staining according to manufacturer's instructions. The samples were loaded into the gel wells inside the electrophoresis chamber. Electrophoresis was performed in 0.5X TAE buffer at 100 V for 30 to 45 min. Finally, the DNA fragments were observed under the UV transilluminator and photographed using a digital camera with UV filter.

3.2.2. Polyacrylamide gels

To visualize DNA fragments less to 100 bp, 10% polyacrylamide gels were used. This gel was prepared using 2 mL of 30% (w/w) acrylamide/bis-acrylamide, 4 mL of 1X TBE (0,8 g/L of Tris, 5,5 g/L of Boric acid and 4 mL/L of 0,5 M Na_2EDTA pH 8.0), 5 μL of TEMED and 12 μL of ammonium persulfate 20%. Samples (8 μL) were mixed with 1 uL of loading buffer, in order to load into the gel. Electrophoresis was carried out at room temperature at a constant voltage of 100 V for 1 h. Finally, gels were stained with GelRed® and visualized at UV transilluminator.

3.3. Extraction and sequencing of plasmidial DNA

Plasmidial DNA was extracted from bacterial colonies grown for 12-18 hours at 37 °C in 5 mL of liquid LB medium (LB Broth 25 g/L) supplemented with the corresponding selection antibiotic. For DNA extraction, the FavorPrep ™ Plasmid DNA Extraction Mini Kit was used according to the manufacturer's instructions. DNA samples obtained by this method were stored at -20 °C until their later use. To sequence plasmids, the standard Macrogen sequencing service (Seoul, Korea) was used, with primers listed previously (Table 1).

3.4. Binary vector constructions

VvINO coding region sequence (1347 bp), was amplified by PCR from *Vitis vinifera* DNA using the primers VvINO-cdNA F and VvINOcDNA R (Table 2).

Then, the isolated sequence was cloned into pENTR™ Directional / SD/D-TOPO® (Invitrogen) as an entry vector, using chemically competent *E. coli*, and Plasmid DNA from the PCR positive colonies was purified and sequenced to corroborate that the cloned fragment presented the correct sequence. The obtained input plasmidial DNA from positive clones were used for a LR recombination reaction with pK7FWG2, the destination vector (Karimi et al., 2002) using Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen) and chemically competent *E. coli,* according to manufacturer's instruction. Positive colonies were used for plasmid extraction using FavorPrep™ Plasmid DNA Extraction Mini Kit according to manufacturer's instructions.

Clones with the expression vector P35S::*VvINO* were generated and used for transformation of *Rhizobium radiobacter*.

3.5. Transformation of competent cells of *Rhizobium radiobacter*

Competent cells of *R. radiobacter* GV3101 (100 µL aliquot) were thawed on ice and about 1 µg (5 µL) of the plasmid was added. The mixture was transferred to a 0.1 cm electroporation cuvette and a voltage of 2.2 kV was applied into an electroporator. Then, 1 mL of liquid LB was added and cells were transferred to a 1.5 mL polypropylene tube and incubated for 3 hours at 28 °C with shaking. Later, cells were pelleted by centrifugation and then plated on LB-agar plates supplemented with the antibiotics rifampicin (50 µg /mL), gentamicin (25 µg / mL) and spectinomycin or kanamycin (50 µg / mL), depending on the plasmid used (Table 1). The plates were incubated at 28 °C in the dark for 48 hours, or until the colonies were visible. To verify the presence of the plasmid in the colonies, these were grown in liquid medium supplemented with antibiotics and analyzed by PCR, using 1 µL of the liquid culture.

4. Transformation of Arabidopsis Plants

4.1. Transformation of Arabidopsis using Floral Dip

Arabidopsis Col-0 plants were grown in greenhouse conditions (22 ± 2 °C, long day photoperiod (16/8h)) and the first inflorescence shoots were removed as soon as they emerged.

Once the new inflorescence shoots reached a size of 7-10 cm, and the presence of many floral buds not yet opened was observed, the transformation of the plants was performed using Floral Dip method (Clough and Bent, 1998).

Two days prior to plant transformation, a colony of *R. radiobacter* carrying a binary vector (P35S::*VvINO*) was inoculate into a 5 mL- liquid LB media with antibiotics (rifampicin 50 μg/mL, gentamicin 25 μg/mL and kanamycin 50 μg/mL) and incubated at 28 ºC with vigorous agitation. Then 1 mL of the preculture were inoculated into 200 mL of LB medium and antibiotics for an additional 24 hours at 28 ºC or until reach an $O.D._{600}$ of 0,8 approximately.

Later, *R. radiobacter* was pelleted by centrifuging at 6000 rpm for 20 min at room temperature, using 50 mL propylene tubes. The cell pellet was resuspended in 400 mL of infiltration medium (glucose 50 g/L and Silwet L-77 500 μL/L) and the *R. radiobacter* suspension was transferred to vessel for dipping plants. The plants's pots were inverted, and the inflorescence shoots were dip into the suspension during 20 sec approximately. After about 3 weeks, seeds were collected.

4.2. Selection of transgenic plants

The seeds of the transformed plants were collected and grown in MS 0,5X plates supplemented with antibiotic (kanamycin 50 mg/mL). After 12 days, surviving plants were transferred to soil. One week later, one young leave per plant was used for DNA extraction and subsequent PCR. 18S primers were used to check the integrity of DNA and VvINO_cDNA F and R primers were used to amplify the transgene (Table 2). Finally, RT-qPCR were made to evaluate transgene expression levels as described in Section 2.2 of Methods.

5. Genotyping of *ino– 1* mutants using 'Derived Cleaved Amplified Polymorphic Sequences'

ino-1 mutants were generated as a point mutation with EMS method, and can be identified through Derived Cleaved Amplified Polymorphic Sequences (dCAPs), a technique that introduces a restriction site in DNA amplified by PCR, by using primers that contain one change in its nucleotide sequence (Neff et al., 1998).

Young leaves from *ino- 1* plants were collected and used for DNA isolation as described previously (section 2.1 of Methods). A 191 bp product was amplified with primers ino-1 dCAPS- F and ino 3'trunc- R. The PCR was carried according following conditions: 95 ºC for 30 secs, 55 ºC for 20 secs, 72 ºC for 30 secs, repeating 35 times.

Later, the PCR product was digested with DdeI enzyme according to manufacturer conditions and run in 10% polyacrylamide gel. A different band profile is generated depending on the genotype of the plants, wild type: two fragments of 149 bp and 42 bp; *ino-1-/-:* three fragments of 121 bp, 42 bp and 28 bp; and *ino-1+/-:* four fragments of 149 bp, 121 bp, 42 bp and 28 bp. This protocol was kindly provided by Dr. Charles Gasser, College of Biological Science, UC Davis.

6. Phenotypic analysis of Arabidopsis plants

The phenotypic analysis of the mutants was carried out by extracting 40 random mature siliques from each plant, mainly during growth stage 8 (Boyes et al., 2001). First, the size of the siliques was observed, and subsequently, a longitudinal section was made, in order to separate the two valves of the seed, and thus be able to visualize the septum where seeds are joined. Finally, the number of aberrant ovules (not fertilized), the number of aborted ovules and the number of normal seeds per silique (Figure 8) were determined under dissection microscope.

7. Statistical analysis

The data from RT- qPCR of grapevine tissues, were subjected to One- way ANOVA and Tukey when more of two groups were analyzed or T-Test and Holm -Sidak when two groups were compared using Prisma 6.0 Software (GraphPad, Inc., La Jolla, CA, USA). For RT-qPCR analysis of *oeVvINO*, the Dunnett test were used to compare each mean with the control mean using Prism 6.0 Software.

For comparisons of two plant groups, it was first verified if the data fit a Gaussian distribution. When the data followed a Gaussian distribution, unpaired t- test and parametric test was used. Otherwise, nonparametric test was used.

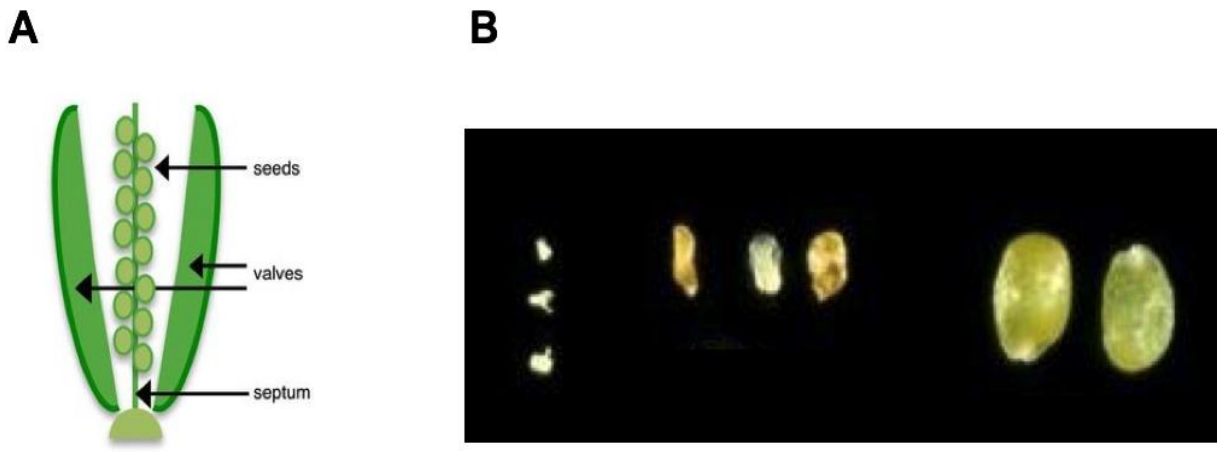

FIGURE 8. Phenotypic Evaluation of Arabidopsis ovules and seeds.
A) A representation of a silique showing two valves and septum with joined seeds. Adapted from (Ogutzen et al., 2018). **B)** A photography of aberrant no fertilized ovules (left), aborted or mutant seeds (medium) and normal seeds (right). Adapted from (Meinke et al., 2008)

In the case of comparisons between the number of unfertilized, aborted and normal seeds, two groups of plants were compared (wild type vs *ino-1* and wild type vs *oeVvINO)* using unpaired t- test, and a Welch correction which is used when SDs are not similar. For comparison between silique's size, nonparametric Mean Whitney test was used. In all cases, $p \leq 0.05$ indicated significant differences. The analyses were run on GraphPad Prism 6.0 Software (GraphPad, Inc., La Jolla, CA, USA)

8. *In silico* analysis

Vitis vinifera 12X genome sequences were obtained from Phytozome, the Plant Comparative Genomics portal of Department of Energy's, University of California. This platform delivers information about protein motifs, Gene Ontology, sequences and putative homologous in other species. Sequences of Arabidopsis genes were obtained from The Arabidopsis Information Resource (TAIR). The putative promoter region was identified using Grape Genome Browser database and was defined as 2 bk immediately upstream of the annotated 5' end of the gene. The analysis of *cis*- acting regulatory DNA elements in promotors were made using PLACE database (Higo et al., 1999).

CHAPTER 5

RESULTS

1. Correlate the seeded and seedless phenotype and the expression of the selected genes in the early stages of the development of the grape berries

The candidate genes analyzed in this study were selected from the analysis of the data obtained from a previous study performed in our laboratory (Muñoz et al., 2012). The main characteristics of these genes, such as their ID and identity with their Arabidopsis's related protein are summarized in Table 3, while their predicted functions were determined through Gene Ontology (GO) and are summarized in Table 4. The predicted protein products from *VvAGL6*, *VvERF19*, *VvAIL5* and *VvINO* contain motifs characteristic of transcription factors (Table 3), as well as possible functions of DNA interaction or development processes (Table 4). On the other hand, VvLUG predicted amino-acid sequence have protein binding sites according to its role as co-repressor of GroTLe family (Introduction. Section 4.3).

Early stages of berry development are key to form the mature fruit, as the phenomena during these days will allow the formation of a final berry with or without seeds. In order to understand if there is a correlation between the expression profile of the candidate genes and seeded or seedless berries phenotype in *Vitis vinifera*, early developmental stages were analyzed: EL-18 (pre-bloom inflorescence, flowers well separated and with caps), EL-25 (after- bloom flowers, 80% caps off and pollinated) and EL-27 (setting young berries) (See Figure 7).

TABLE 3. Characteristics of candidate genes.

Candidate Gene	ID (Genoscope 12 X)	Predict Protein Domains	Arabidopsis related	% Identity Protein
VvAGL6_1	GSVIVG01027577001	• AGAMOUS-LIKE MADS-BOX PROTEIN AGL13-RELATED (PTHR11945:SF146) • SRF-type transcription factor (DNA-binding and dimerization domain) (PF00319) K-box region (PF01486)	AT2G45650 (AGL6)	69,6
VvAGL6_2	GSVIVG01018450001	• MADS BOX PROTEIN(PTHR11945: SF165:) • SRF-type transcription factor (DNA-binding and dimerization domain) (PF00319) K-box region (PF01486)	AT2G45650 (AGL6)	50,8
VvAIL5	GSVIVG01029219001	• AP2-LIKE ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR AIL5 (PTHR32467:SF23) AP2 domain (PF00847)	AT5G57390 (AIL5)	56, 2
VvLUG	GSVIVG01035791001	• WD40 REPEAT PROTEIN(PTHR22847) WD domain, G-beta repeat (PF00400)	AT4G32551 (LUG)	72,4
VvINO	GSVIVG01013778001	• FAMILY NOT NAMED (PTHR31675) • AXIAL REGULATOR YABBY 4 (PTHR31675:SF8) HMG (high mobility group) box (PF00505)	AT1G23420 (INO)	68, 5

The ID of candidate genes was named according to 12X Genoscope annotation. Codes for Arabidopsis related genes were obtained from The Arabidopsis Information Resource. Predicted protein domains were analyzed using Phanter (PTHR) and PFAM (PH) databases. The information in parentheses corresponds to the codes of those databases.

TABLE 4. Predicted functions of candidate genes.

Candidate Gene	ID (Genoscope 12 X)	titulo	GO
VvAGL6_1	GSVIVG01027577001	AT2G45650	GO:0003677: Any molecular function by which a gene product interacts selectively and non-covalently with DNA (deoxyribonucleic acid). GO:0003700: Interacting selectively and non-covalently with a specific DNA sequence in order to modulate transcription. GO:0005634: A membrane-bounded organelle of eukaryotic cells in which chromosomes are housed and replicated. GO:0006355: Any process that modulates the frequency, rate or extent of cellular DNA-templated transcription GO:0046983: The formation of a protein dimer, a macromolecular structure consists of two noncovalently associated identical or nonidentical subunits. K09264: MADS-box transcription factor, plant
VvAGL6_2	GSVIVG01018450001	AT2G45650	GO:0003677: Any molecular function by which a gene product interacts selectively and non-covalently with DNA
VvAIL5	GSVIVG01029219001	AT5G57390	GO:0003700: Interacting selectively and non-covalently with a specific DNA sequence in order to modulate transcription. The transcription factor may or may not also interact selectively with a protein or macromolecular complex. GO:0006355: Any process that modulates the frequency, rate or extent of cellular DNA-templated transcription. GO:0007275:The biological process whose specific outcome is the progression of a multicellular organism over time from an initial condition (e.g. a zygote or a young adult) to a later condition (e.g. a multicellular animal or an aged adult).
VvLUG	GSVIVG01035791001	AT4G32551	GO:0005515: Interacting selectively and non-covalently with any protein or protein complex (a complex of two or more proteins that may include other nonprotein molecules).
VvINO	GSVIVG01013778001	AT1G23420	GO:0007275: The biological process whose specific outcome is the progression of a multicellular organism over time from an initial condition (e.g. a zygote or a young adult) to a later condition (e.g. a multicellular animal or an aged adult).

Gene Ontology was determinated by Phytozome platform which uses Quick GO and KEGG databases (European Bioinformatics Institute, EMBL- EBI). The information in parentheses corresponds to the codes of that database.

1.1. Determine the expression profile of the candidate genes during the early berry development, in different varieties of table grapes

In order to approach to understand the function of the candidate genes, their expression pattern throughout early development was analyzed in *Vitis vinifera*. Thus, it was analyzed whether the candidate genes are expressed in the early developmental stages of flower and fruit, with the aim of determining the phenological states where the transcripts accumulate preferentially. This approach was made using table grape varieties that are recognized as 'seeded' or 'seedless', where *Vitis vinifera* cv. Red globe (RG) was used as a model of seeded table grape and both, cv. Flame seedless (FS) and cv. Thompson seedless (TS) are models of seedlessness. The Characteristics of these varieties are described in Figure 9, according to the description from the Australian Table Grape Association.

1.1.1. Analysis of *VvAGL6_1* and *VvAGL6*_2 transcript accumulation during early grape berry development.

In this work, we will analyze two putative *AGL6* homologous, which will be named as *VvAGL6_1* and *VvAGL6_2*. The protein sequences encode both genes share an identity of 61%, while VvAGL6_1 and VvAGL6_2 have 69.6% and 50.8% of identity with AGL6 protein from Arabidopsis, respectively. Also, an *in silico* analysis of the protein reveals that both have characteristics associated with the MADS-box family, such as the MADS-box motif, the dimerization domain (Srf) and the K motif related to the formation of multimers (Immink et al., 2010) (See Table 3). Besides, the CDS sequence of *VvAGL6_1* was

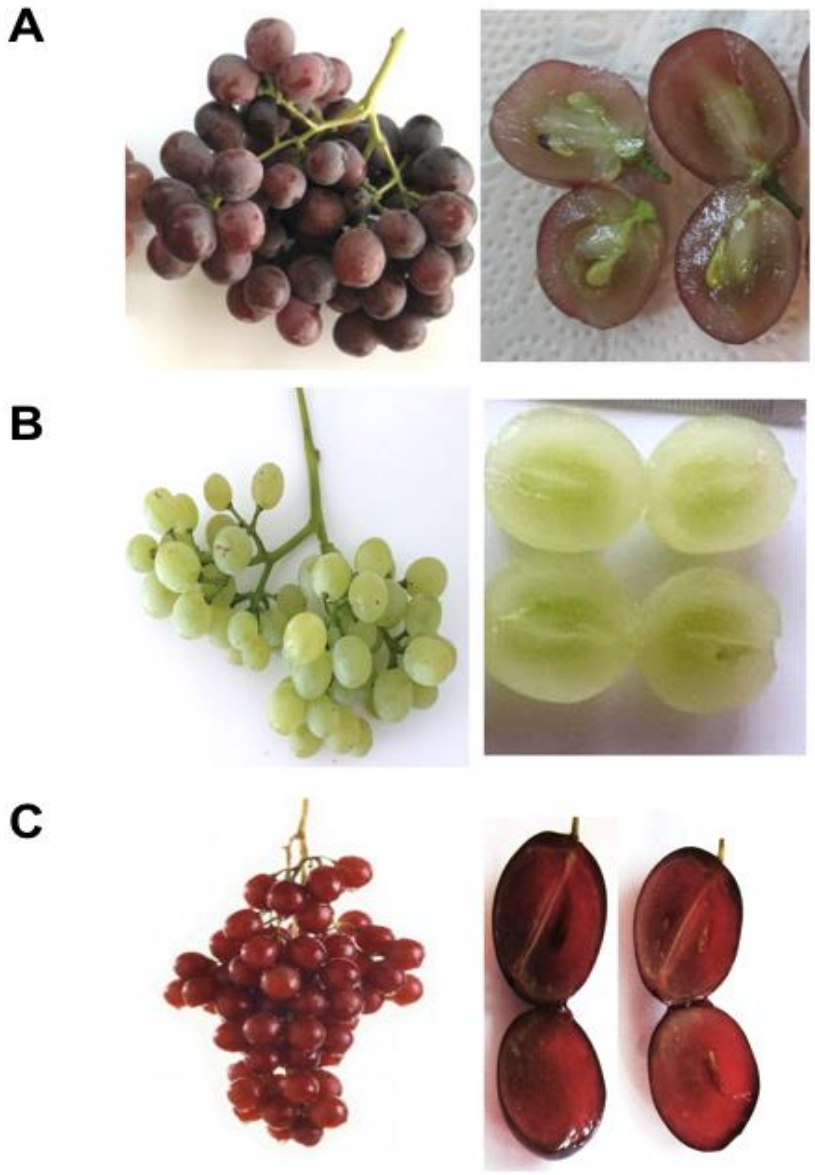

FIGURE 9. Characteristics of table grape varieties using in this study.
A) Cluster and berries of cv. Red globe: The grapes bear big, pinkish-red, very large (round) plum sized berries containing large seeds. **B)** Cluster and berries of cv. Thompson seedless: oval- shape berries, pale to amber green, seedless variety of grape widely cultivated worldwide for table use and for drying as raisins (rudimental seeds are present). **C)** Cluster and berries of cv. Flame seedless: It displays typical medium-sized, round and deep red, seedless berries (rudimental seeds are present).

isolated and cloned, showing a 100% identity with the predicted published sequence (Figure 31. Annexes).

From the expression analysis, it was possible to determine that transcripts of *VvAGL6_1* are accumulated mainly in closed and pre-pollinated young flowers (EL-18). Then, transcript abundance diminishes during development, showing significantly low expression in fruit set berries (EL-27). This profile is similar in all varieties studied (Figure 10. A, B, and C).

The expression profile of *VvAGL6_2* is also significantly low in berries at fruit set (EL-27) in FS and RG varieties, compared to inflorescences in EL-18 and EL-25 stages (Figure 10. E, and F). However, in TS, the expression profile is slightly different, with a peak during EL-25, but also with significant low levels in berries at fruit set (EL-27) compared to pollinated flowers (EL-25) (Figure 10. D).

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Comparing both genes, *VvAGL6_1* is mainly expressed in young inflorescences (EL-18), while *VvAGL6_2* is expressed at the same levels in young (EL-18) and blooming inflorescences (EL-25), except for TS which have higher levels during EL-25.

The expression profile during the analyzed stages leads us to suggest that *VvAGL6_1 and VvAGL6_2*, may be activated during inflorescence development, but after fertilization (EL-25) they are repressed, at least until the stage EL-27 (Figure 10). In Arabidopsis, *AGL6* has been related to flowering and ovule formation. Therefore, it is not strange to find a high expression in the early stages evaluated, where these processes take place.

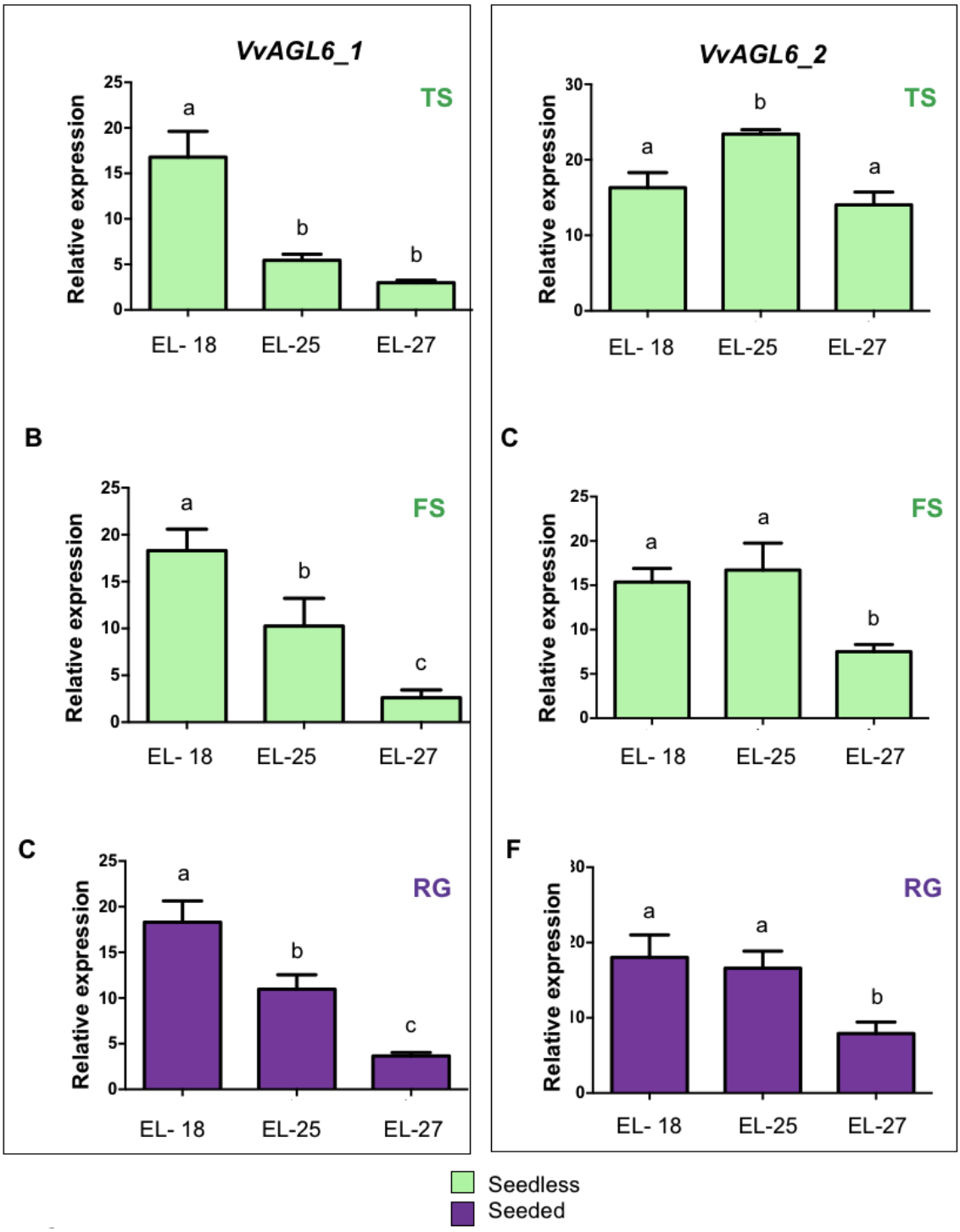

FIGURE 10. Relative quantification of *VvAGL6_1* and *VvAGL6_2* transcripts during early stages of flower and berry development.

Expression of *VvAGL6_1* in Thompson seedless **(A)**, Flame seedless **(B)** and Red globe **(C)**. Expression of *VvAGL6_2* in Thompson seedless **(D)**, Flame seedless **(E)** and Red globe **(F)**. Bars correspond to the mean ± standard error of 4 biological replicates. Transcript accumulation was normalized using *Vv60SRP* as reference gene. Different letters indicate significant differences (P<0,05, One- way ANOVA and Tukey Test).

1.1.2. Analysis of *VvAIL5* transcript accumulation during early grape berry development.

Regarding *VvAIL5*, an expression pattern was observed throughout development that is independent of the cultivar studied. In the seedless varieties (TS and FS) and the seeded variety (RG) the expression profile was similar, being low in pre-pollinated young flowers (EL-18) but increasing in mature post-pollinated flowers (EL-25) (Figure 11). Besides, in both TS and RG, the expression remained high in EL-27 stage, when berries and seeds are being formed. According to these results, we suggest that *VvAIL5* participates in floral and young fruit development in grapevines, which is also related to the function predicted by Gene Ontology (GO:0007275) (See Table 4).

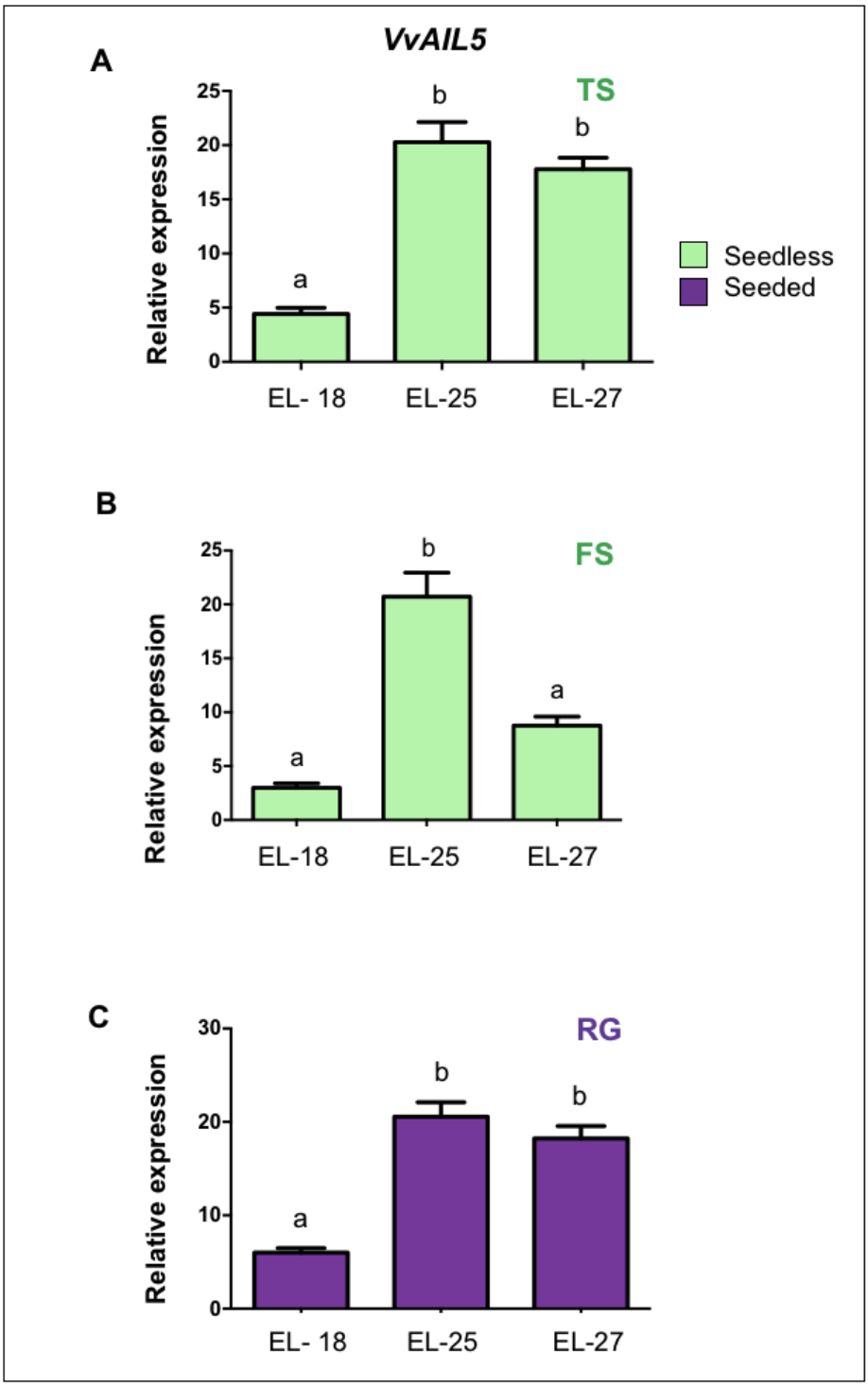

FIGURE 11. Relative quantification of *VvAIL5* transcripts during early stages of flower and berry development.

The expression of *VvAIL5* evaluated in Thompson seedless **(A)**, Flame seedless **(B)** and Red globe **(C).** Bars in this figure correspond to the mean ± standard error of 4 biological replicates. Transcript accumulation was normalized using *Vv60SRP* as reference gene. Different letters indicate significant differences (P<0,05, One- way ANOVA and Tukey Test).

1.1.3 Analysis of *VvLUG* transcript accumulation during early grape berry development.

Expression analysis of *VvLUG* throughout development, revealed that it remains constant in pre- and post-pollinated flowers (EL-17 and EL-25) in all varieties analyzed (Figure 12). This profile is consistent with previous studies in Arabidopsis, where a strong expression of *LUG* is described through floral development (Conner and Liu, 2000). Therefore, we might suggest that *VvLUG* is a gene involved in floral development of the grapevine.

Also, in both seedless varieties studied, FS and TS, the transcript accumulation remains similar until EL-27 (Figure 12. A and B). Only RG presents a significant decrease in transcript accumulation during EL-27, compared to the two previous stages (Figure 12. C). More analysis would be needed to verify if this decrease is associated with the formation of seeds in developing berries.

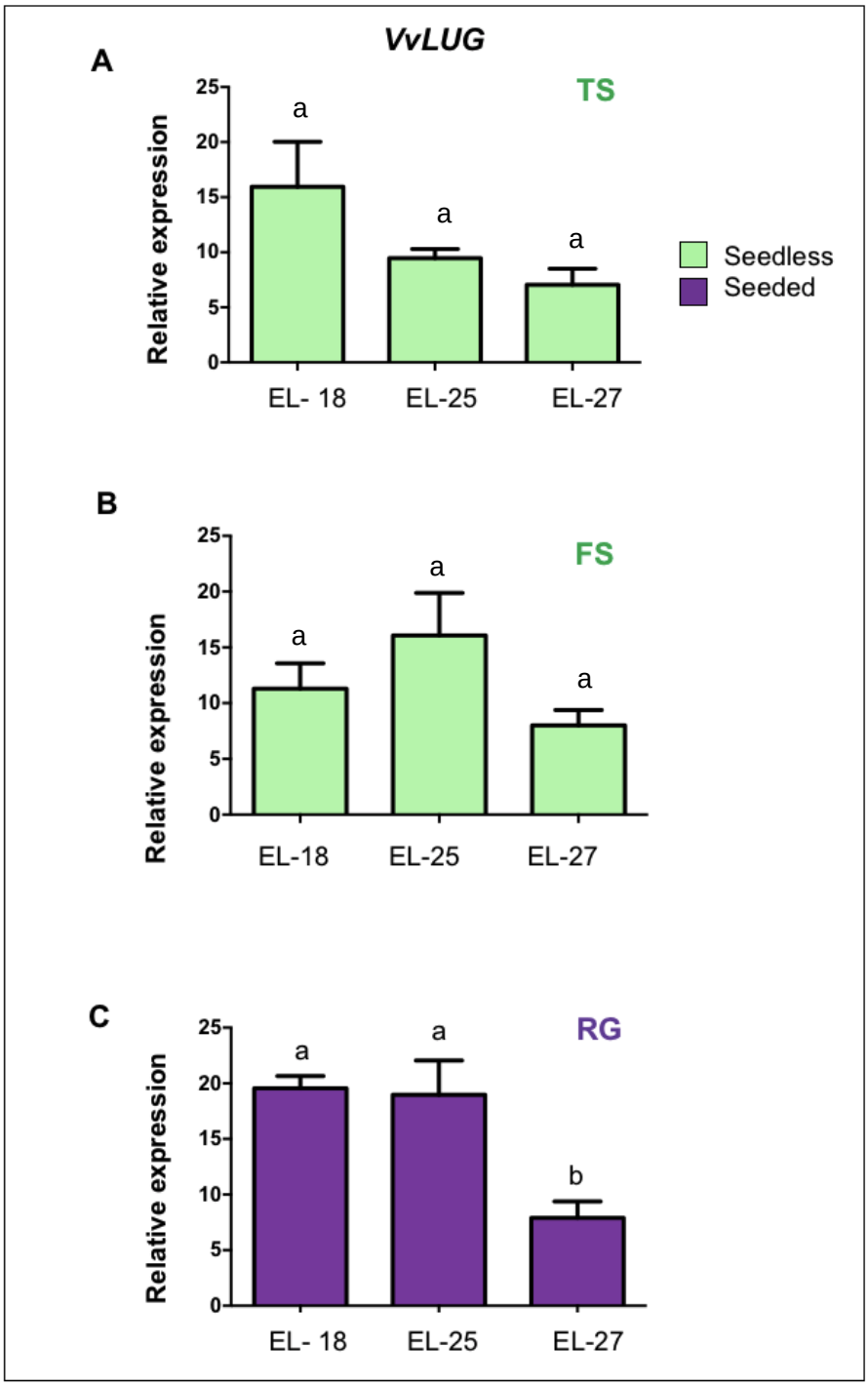

FIGURE 12. Relative quantification of *VvLUG* transcripts during early stages of flower and berry development.

The expression of *VvLUG* evaluated in Thompson seedless **(A)**, Flame seedless **(B)** and Red globe **(C)**. Bars in this figure correspond to the mean ± standard error of 4 biological replicates. Transcript accumulation was normalized using *Vv60SRP* as reference gene. Different letters indicate significant differences (P<0,05, One- way ANOVA and Tukey Test).

1.1.4. Analysis of *VvINO* transcript accumulation during early grape berry development

In the analysis of *VvINO* transcript accumulation, it was observed an expression pattern characterized by a decrease in transcript level that through the time, reaching the highest level in young flowers (EL-18) and the lowest level in berries at fruit set (EL-27). This profile was observed in the different varieties analyzed, regardless of their seedless/seeded phenotype (Figure 13).

As an overall result of activity 1.1, we can point out that transcripts of all the analyzed genes are accumulated in the reproductive tissues analyzed, corresponding to early stages of flower and berry development of the grapevine.

Besides, we can group the studied genes according to their profile of transcripts accumulation in 3 types:

1. Genes whose transcripts diminish throughout development, with preferential transcripts accumulation in young flowers EL-18 (pre-pollinated): In this group, we can find the MADS-box genes, *VvAGL6_1*, and *VvAGL6_2*, and the Yabby gene, VvINO.

2. Transcripts that increase throughout the development, with a more significant accumulation in fruit-set stage (EL-27): this is the case of *VvAIL5*, a gene of *AP2/ERF* family.

3. Transcripts that remain constant throughout the early development of the berry: this is the case of the corepressor *VvLUG*.

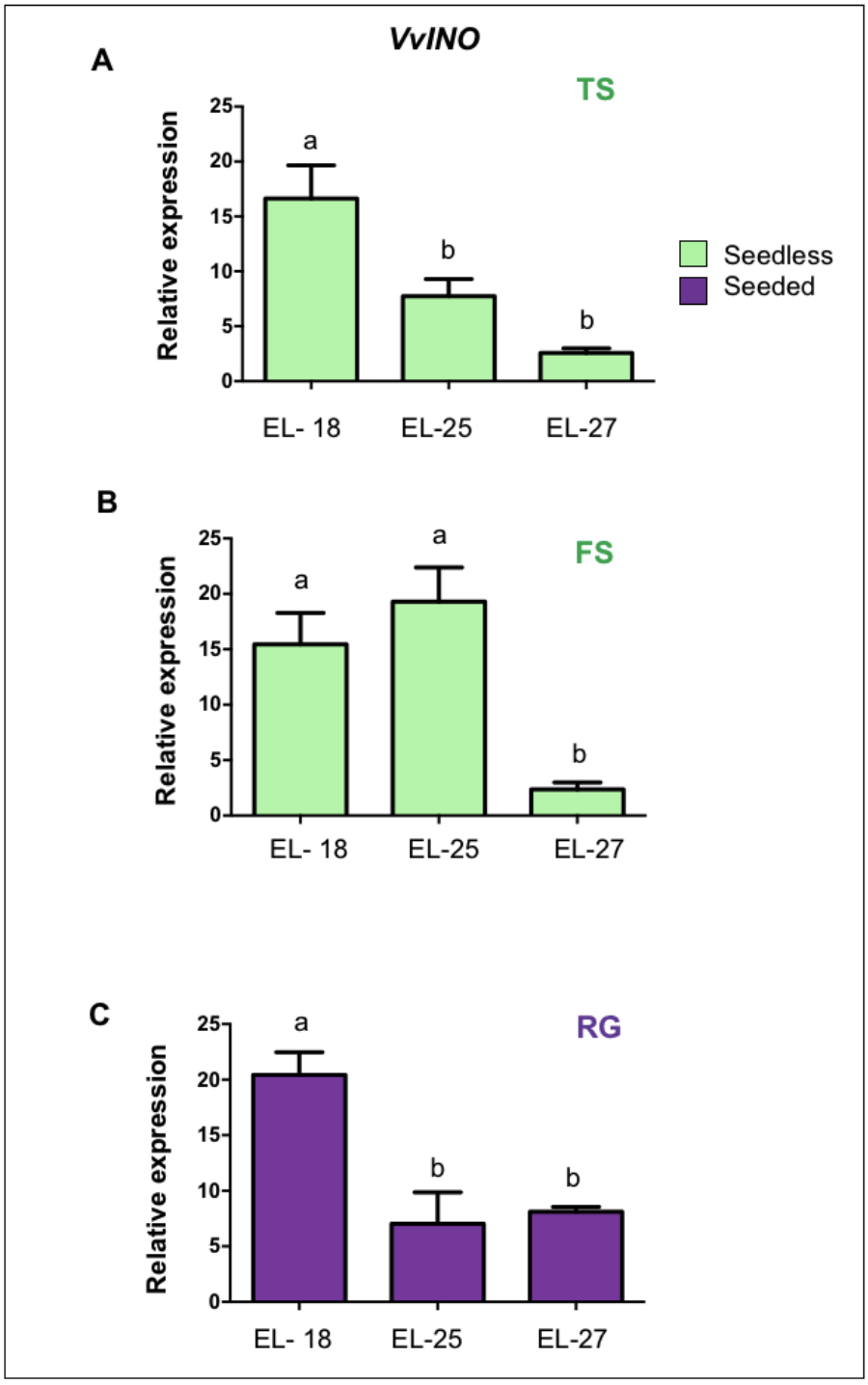

FIGURE 13.Relative quantification of *VvINO* transcripts during early stages of flower and berry development.

The expression of *VvINO* evaluated in Thompson seedless **(A)**, Flame seedless **(B)** and Red globe **(C)**. Bars in this figure correspond to the mean ± standard error of 4 biological replicates. Transcript accumulation was normalized using *Vv60SRP* as reference gene. Different letters indicate significant differences (P<0,05, One- way ANOVA and Tukey Test).

1.2. Compare the accumulation of transcripts in grapevine plants with seedless and seeded phenotype, during early developmental stages.

In order to determine whether exist a correlation between seeded or seedless phenotype and gene expression levels of the candidate genes, **we proceeded to make comparisons of transcript accumulation for each developmental stage individually** using the same varieties mentioned in the previous activity (Figure 7 and 9). Besides, gene expression comparisons by phenotype were also made in segregants lines from the crossing of Moscatel rosada x Flame seedless, as a manner to increase the number of plants analyzed by phenotype. In these segregants, its genetic background could be similar, even though its differences in seed content.

1.2.1 Evaluation of segregating plants phenotype

Individual segregating plants from a cross between seedless and seeded grapevine plants were used to investigate a correlation between gene expression of candidate genes and seed phenotype. During a previous season, the phenotype of eight individuals was confirmed, before the transcript accumulation analysis of candidate genes.

Significant differences between seeded and seedless segregating lines were observed in parameters such as berry weight and fresh seed weight per berry, and seed number per berry (Figure 14), as expected. Also, most of the seedless berries contained seminal rudiments, finding even two lines of plants, A2, and A4 without rudimental seeds, evidencing a complete absence of seeds (Figure 14). In this manner, the presence of four seeded plants and four seedless grapevine plants was verified, which were used for subsequent analyses.

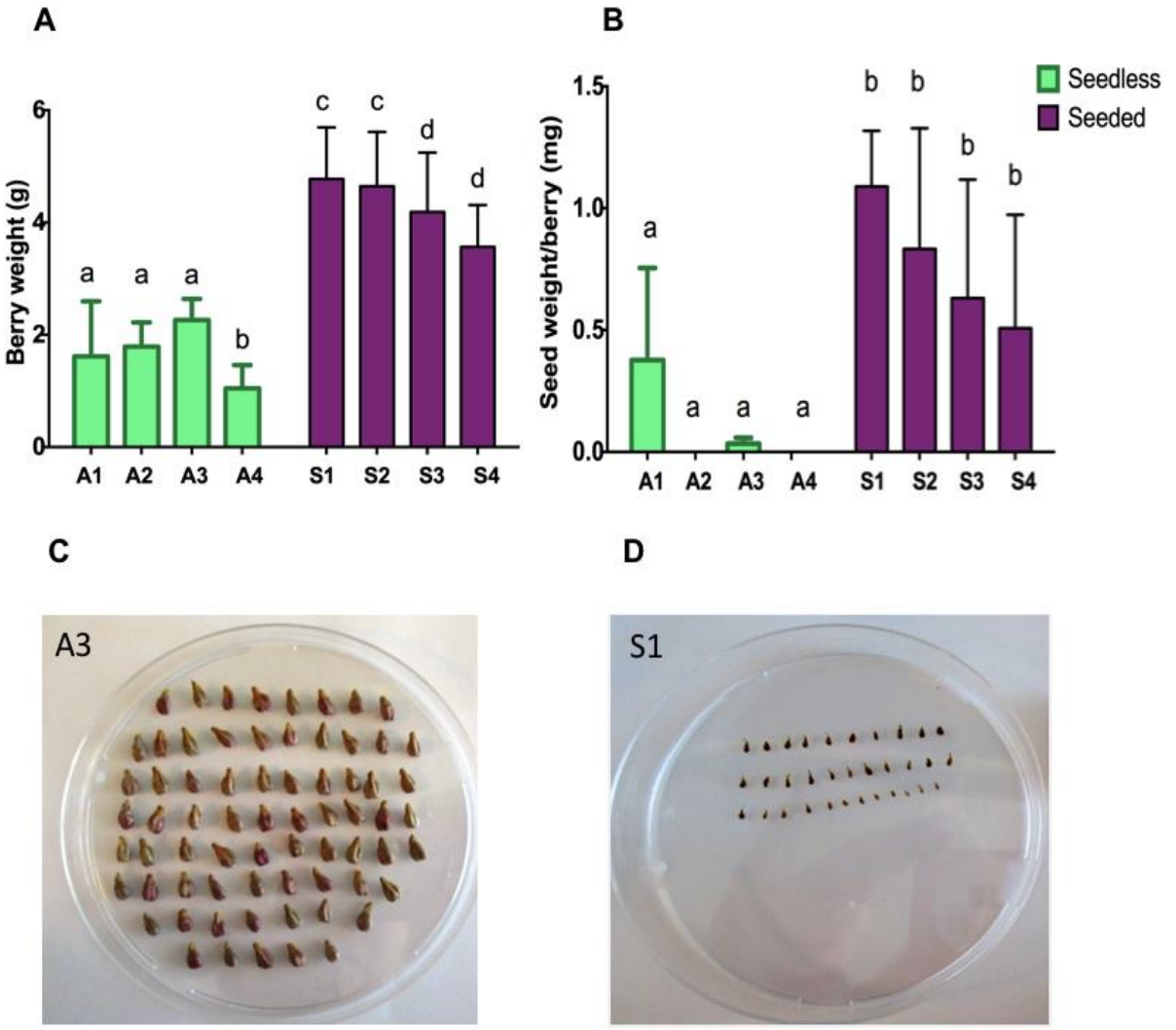

FIGURE 14. Phenotype of segregating lines from Moscatel rosada x Flame seedless.
Seeded plants are shown in purple and seedless plants in light green, each bar represents an independent line. **A)** Berry weight and **B)** Seed (or rudiment) weight per berry. The average weight was plotted with the standard deviation (SD). Statistical analysis was perform using one -way ANOVA and Tukey test, $p<0,05$ (n=30). **C)** Seeds from the seeded plant A3. **D)** Rudimental or seed traces from the S1 plant.

1.2.2. Comparison of *VvAGL6_1* and *VvAGL6_2* transcript accumulation in grapevine plants with seeded/seedless phenotype

In this activity, the transcript accumulation of the candidate genes was analyzed in each developmental stage, focusing on the comparison between seeded and seedless grapevine plants.

VvAGL6_1 transcript levels are diverse among cultivars and MO x FS segregants, being lower in FS in all developmental stages. In the previous activity, we observed that all varieties exhibit a similar transcript accumulation profile, which was decreasing with the development time (Figure 10). Analyzing every developmental stage, most of the studied plants, seeded, and seedless, have similar levels of *VvAGL6_1* transcripts (Figure 15. A, B, and C). According to these data, no correlation is observed between the phenotype studied and transcript accumulation of *VvAGL6_1* in the early stages of flower and berry development.

When evaluating each developmental stage independently, in general, no differences were observed in the accumulation of *VvAGL6_2* transcripts in seeded or seedless cultivars and segregants in any of the developmental phases (Figure 16. A, B, and, C). Thus, it could be thought that the expression of this gene would not have a clear correlation with the seed/seedless phenotype. Only one exception was observed during EL-27 stage, where a higher transcript level was observed in Thompson seedless (T1 and T2) and the seedless line A3 in comparison with other analyzed plants. However, more studies would be needed to find out if this result is also observed in other seedless grapevine plants.

According to these results, **both *VvAGL6* genes are expressed in all stages analyzed. However, no correlation was observed between the transcript accumulation of the genes and the seed/seedless phenotype observed in grapevine berries.**

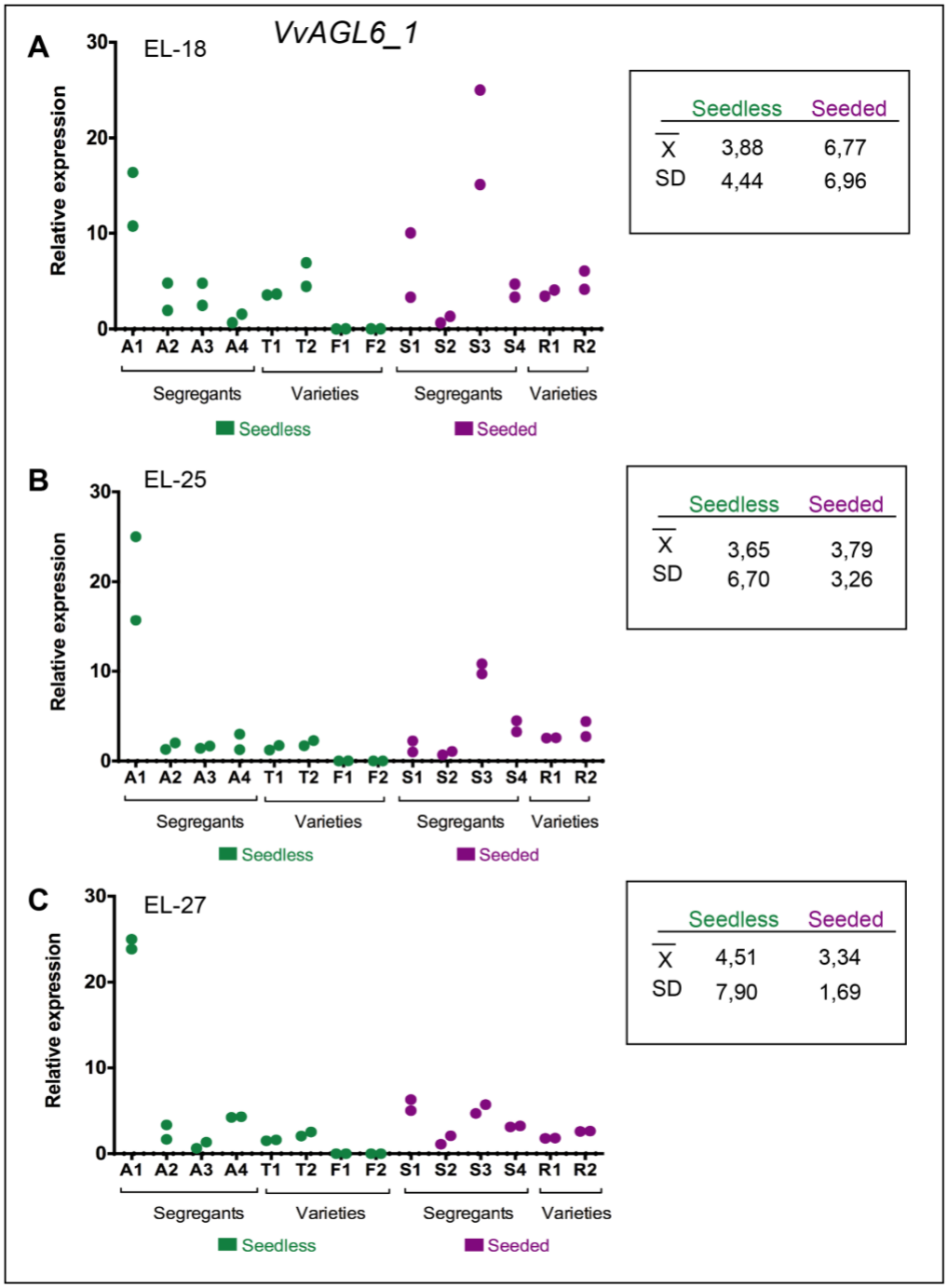

FIGURE 15. Comparison of the *VvAGL6_1* transcripts accumulation in grapevine plants with seeded and seedless phenotype.

Relative quantification of *VvAGL6_1* transcripts during EL-18 stage **(A)**, EL-25 **(B),** and EL-27 **(C)**. Seedless plants (green circles): A1 to A4 are MO x FS segregants, while Flame seedless (F1 and F2), and Thompson Seedless (T and T2) are seedless varieties. Seeded plants (purple circles): S1 to S4 are MO x FS segregants, and Red Globe (R1 and R2) is a seeded variety. Transcript accumulation was normalized using *Vv60SRP* as the reference gene. On the right, the mean and the standard deviation of the plotted values are presented. Circles represent an independent cluster of the plant.

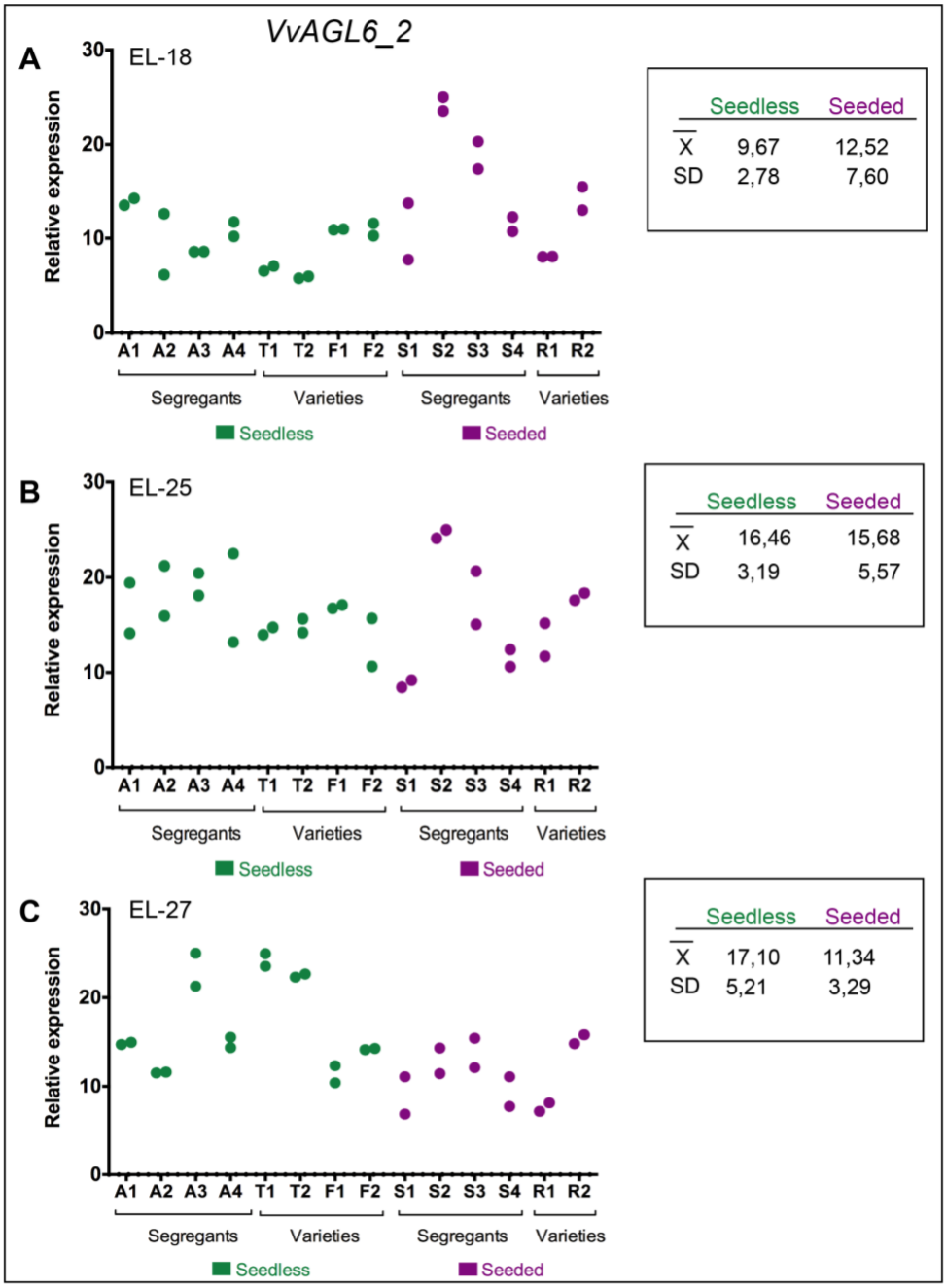

FIGURE 16. Comparison of the *VvAGL6_2* transcripts accumulation in grapevine plants with seeded and seedless phenotype.
Relative quantification of *VvAGL6_2* transcripts during EL-18 stage **(A)**, EL-25 **(B),** and EL-27 **(C)**. Seedless plants (green circles): A1 to A4 are MO x FS segregants, while Flame seedless (F1 and F2), and Thompson Seedless (T1 and T2) are seedless varieties. Seeded plants (purple circles): S1 to S4 are MO x FS segregants, and Red Globe (R1 and R2) is a seeded variety. Transcript accumulation was normalized using *Vv60SRP* as the reference gene. On the right, the mean and the standard deviation of the plotted values are presented. Circles represent an independent cluster of the plant.

1.2.3. Comparison of *VvAIL5* transcript accumulation in grapevine plants with seeded/seedless phenotype

Regarding the accumulation of *VvAIL5* transcripts in each stage, the following results are appreciated:

El-18 clearly shows a higher accumulation of transcripts in the seeded plants, both in MO x FS segregates and in the Red globe variety (R1 and R2) (Figure 17. A). When analyzing the mean of the data (Figure 17. A, right), it is also observed that these differences are significant.

In the stages EL-25 and EL-27, no consistent differences were observed between the groups of seeded and seedless plants, since variations of expression are found even within the plants of the same phenotype (Figure 17. B and, C).

Therefore, and considering the consistency of the results obtained in varieties and segregants, we propose a correlation between the decreased of *VvAIL5* transcripts in young flowers (EL-18) and the phenotype of seedlessness grapevine berries (EL-18) (Figure 17. A)

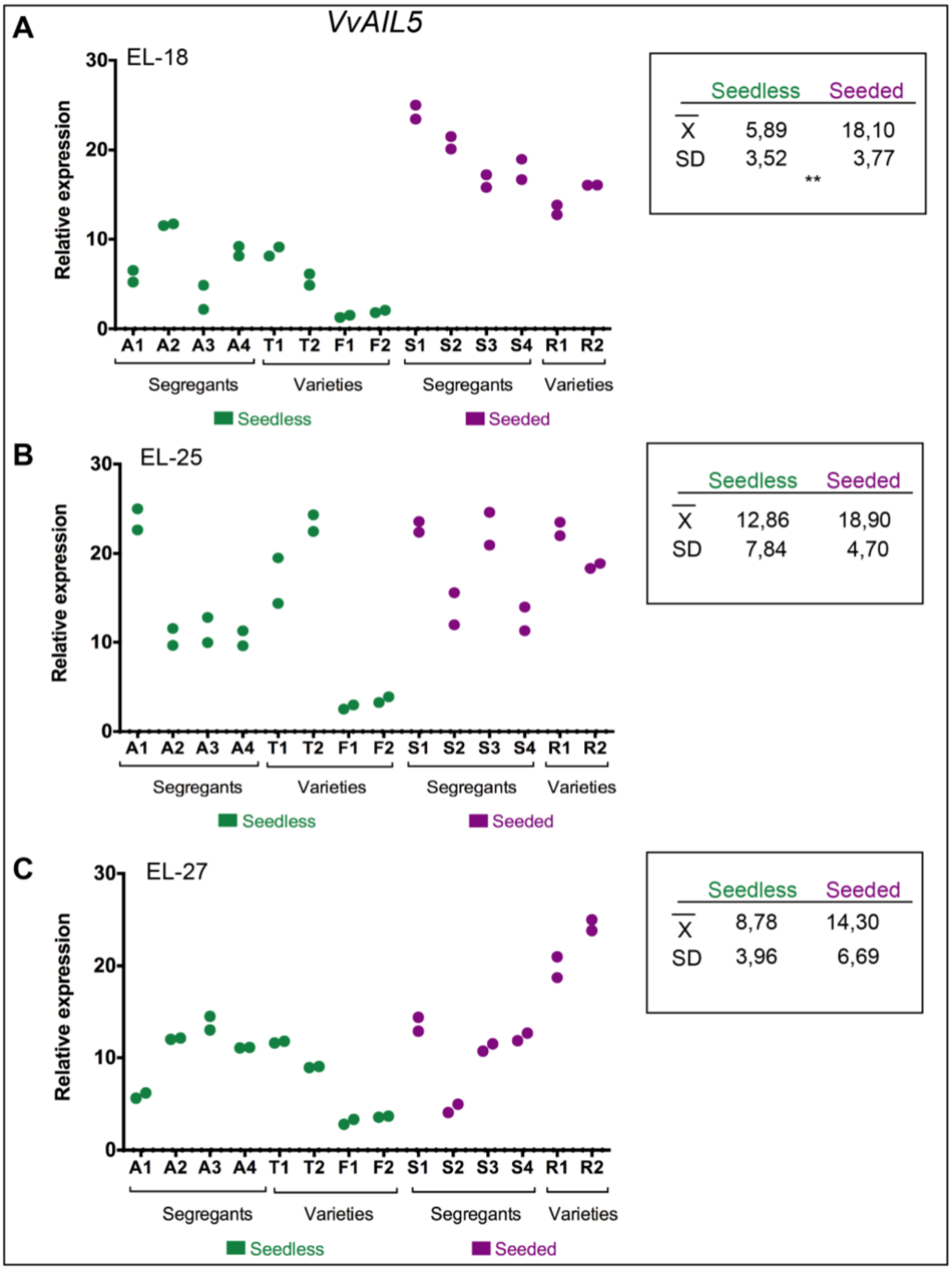

FIGURE 17. Comparison of the *VvAIL5* transcripts accumulation in grapevine plants with seeded and seedless phenotype

Relative quantification of *VvAAIL5* transcripts during EL-18 stage **(A)**, EL-25 **(B),** and EL-27 **(C)**. Seedless plants (green circles): A1 to A4 are MO x FS segregants, while Flame seedless (F1 and F2), and Thompson Seedless (T1 and T2) are seedless varieties. Seeded plants (purple circles): S1 to S4 are MO x FS segregants, and Red Globe (R1 and R2) is a seeded variety. Transcript accumulation was normalized using *Vv60SRP* as the reference gene. On the right, the mean and the standard deviation of the plotted values are presented. Significant differences between mean are showed as asterisks. (P value = 0.000052, Test T and Holm -Sidak). Circles represent an independent cluster of the plant.

1.2.4. Comparison of *VvLUG* transcript accumulation in grapevine plants with seeded/seedless phenotype

The transcripts of *VvLUG* present different levels of accumulation in each plant analyzed, with low expression levels in all the varieties at the three stages of development studied: Flame seedless (F1 and F2), Thompson seedless (T1 and T2) and Red globe (R1 and R2) (Figure 18. A, B, and C). The segregating lines present differences in the accumulation of transcripts within the same phenotypic group (seedless or seeded). Thus, it is not possible to associate the analyzed expression differences with a seed related phenotype. In particular, during EL-27 stage, the seedless segregants lines (A1, A2, A3, and A4) have higher expression levels than their seedless counterparts; however, this is not observed in the seedless varieties, Thompson (T1 and T2) and Flame seedless (F1 and F2) (Figure 18.C). More analyses are needed to establish if this expression differences results in changes in processes related to the formation of ovules and seeds.

Therefore, we cannot observe a clear correlation between *VvLUG* transcripts accumulation and the seeded/seedless phenotype in grapevines.

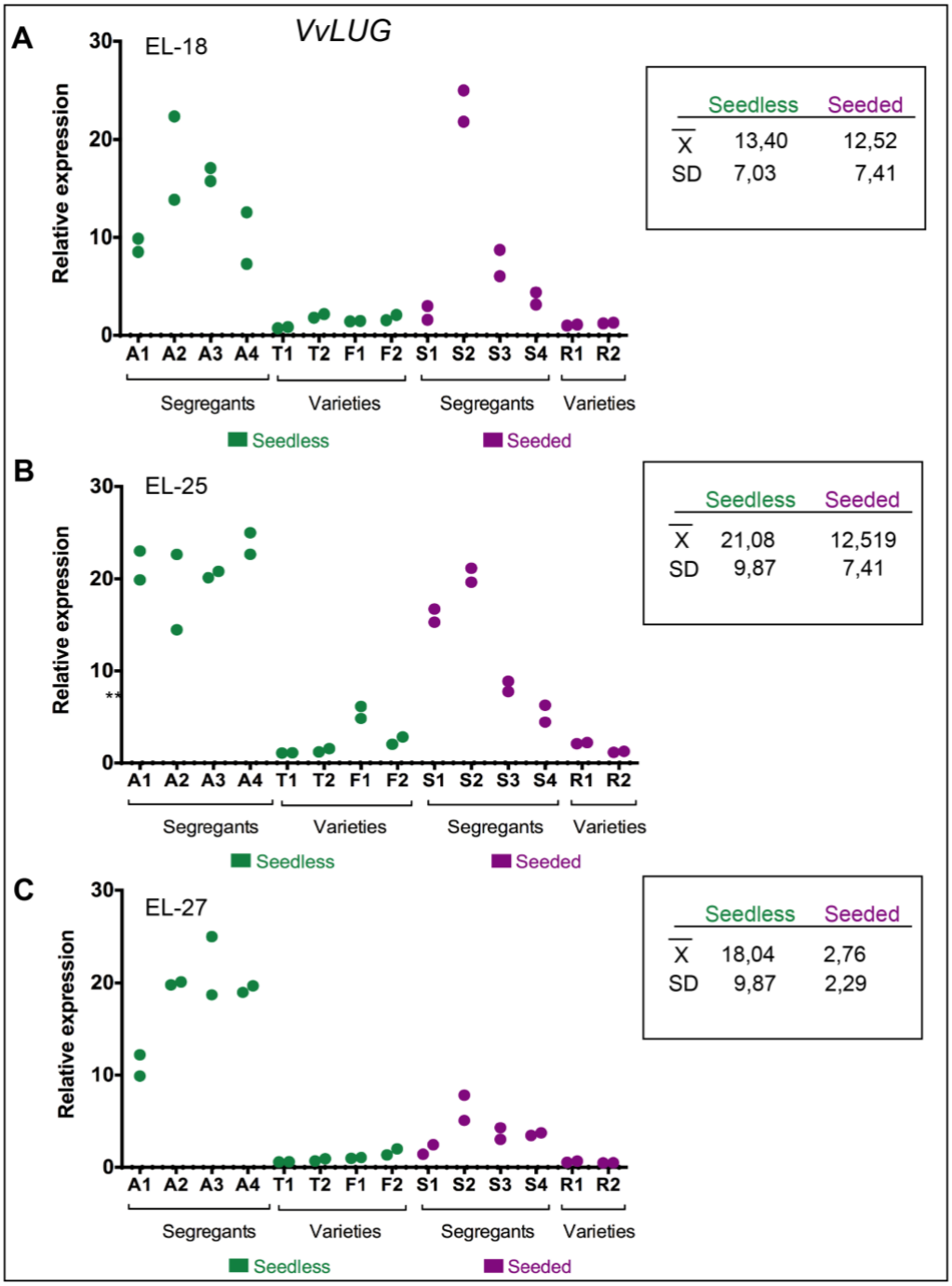

FIGURE 18. Comparison of the *VvLUG* transcripts accumulation in grapevine plants with seeded and seedless phenotype.

Relative quantification of *VvLUG* transcripts during EL-18 stage **(A)**, EL-25 **(B),** and EL-27 **(C)**. Seedless plants (green circles): A1 to A4 are MO x FS segregants, while Flame seedless (F1 and F2), and Thompson Seedless (T1 and T2) are seedless varieties. Seeded plants (purple circles): S1 to S4 are MO x FS segregants, and Red Globe (R1 and R2) is a seeded variety. Transcript accumulation was normalized using *Vv60SRP* as the reference gene. On the right, the mean and the standard deviation of the plotted values are presented. Circles represent an independent cluster of the plant.

1.2.5. Comparison of *VvINO* transcript accumulation in grapevine plants with seeded/seedless phenotype

The accumulation of *VvINO* transcripts was also analyzed in the three early stages of berry development.

In EL-18 and EL-27 it is evident that the seeded phenotypic group has a significantly higher accumulation of transcripts than seedlessness grapevine plants. These differences are significant when comparing the average of expression of both groups (Figure 19. A and C).

At the EL-25 stage, the accumulation of transcripts exhibits similar levels in each plant independently analyzed, regarding its phenotype. Except for seeded plants S1 and S4, which present higher levels, in the rest of the studied plants, there were not found apparent differences attributable to the phenotype (Figure 19. B).

Interestingly, in both, younger flowers (EL-18) and at fruit set (EL-27) stages, seeded grapevine plants showed significantly higher levels of *VvINO* compared to their seedless counterparts (Figure 19). It suggests a correlation between increased levels of *VvINO* transcripts in these early developmental stages and the formation of the seed. Regarding flowers, these results could also be related to the ovule's development, while in fruit set, these results could be correlated with the presence of developing embryos during the EL-27 stage in seeded grapevine plants. These results are not surprising considering that Arabidopsis *INO* mRNA accumulates in young embryos.

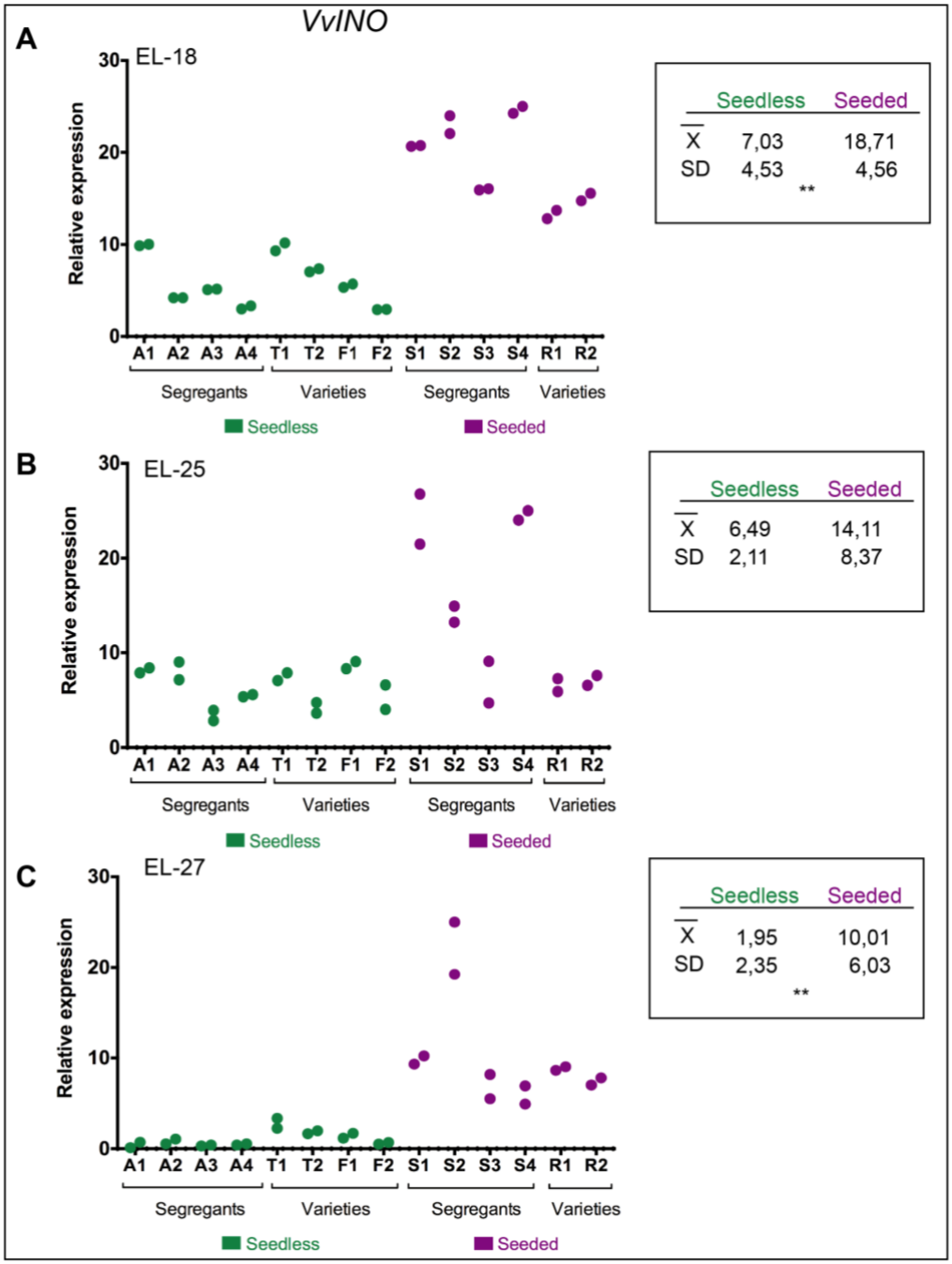

FIGURE 19. Comparison of the *VvINO* transcripts accumulation in grapevine plants with seeded and seedless phenotype.

Relative quantification of *VvINO* transcripts during EL-18 stage **(A)**, EL-25 **(B),** and EL-27 **(C)**. Seedless plants (green circles): A1 to A4 are MO x FS segregants, while Flame seedless (F1 and F2), and Thompson Seedless (T1 and T2) are seedless varieties. Seeded plants (purple circles): S1 to S4 are MO x FS segregants, and Red Globe (R1 and R2) is a seeded variety. Transcript accumulation was normalized using *Vv60SRP* as the reference gene. On the right, the mean and the standard deviation of the plotted values are presented. Significant differences between mean are showed as asterisks. (P value = 0.000618 (A) and 0.00049 (B), Test T and Holm -Sidak). Circles represent an independent cluster of the plant.

As a summary of activity 1.2, the expression analyses allowed the identification of two genes, *VvINO* and *VvAIL5*, with significant differential transcripts accumulation between seeded and seedless phenotypes. *VvINO* was selected for further analysis considering the following criteria:

1. Differential transcript accumulation in seed / seedless phenotype in *Vitis vinifera*:

- *VvAIL5* presents a significantly higher accumulation of transcripts in seeded young flowers (EL-18) compared to seedless flowers (Figure 17.A).

- *VvINO* accumulates significantly more, both, in young flowers (EL-18) and fruit set stage (EL-27) of seeded grapevine plants compared to their seedless counterparts (Figure 19. A and C).

2. Ovule specific expression in *Arabidopsis thaliana*:

According to the literature, both transcripts are accumulated in developing ovules in the model plant *A. thaliana* (Nole-Wilson et al., 2005; Villanueva et al., 1999a).

3. Mutants availability and their phenotype:

Previous analyses show that silencing of *AIL5* in *A. thaliana* does not cause a phenotype in reproductive tissue, which has been associated with possible gene redundancy between genes of the *AIL / PLT* family in *A. thaliana* (Elliott et al., 1996; Nole-Wilson et al., 2005).

On the other hand, the evidence provided by the authors Gaiser et al. (1995) and Lora et al. (2011), conclude that *INO* mutants in *A. thaliana* and its homologous in *A. squamosa* exhibit aberrant ovules, which lead to female sterility and incapacity to produce seeds. Therefore, it is

interesting to evaluate if *VvINO* gene also affects the formation of the seed in the Arabidopsis model.

2. To functionally evaluate the participation of one selected gene in the formation of the seeds in the model plant *Arabidopsis thaliana*.

2.1. Isolation and in silico characterization of *VvINO*

2.1.1. Isolation and cloning of genomic sequence of *VvINO*

Based on the *VvINO* sequence annotated in Genoscope (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), specific primers (VvINO_cdna-F and VvINO cDNA -R, see Table 2) were designed to amplify its genomic sequence from *Vitis vinifera* by PCR. The amplification of a fragment with the expected size was obtained, which was cloned into a directional cloning vector for entry to the Gateway® System (Figure 20.A). Seven positive clones were obtained and sequenced. A multiple alignment of these sequences performed in the AlignX tool of the Vector NTI® software allowed to obtain a consensus sequence of 1347 bp 100% identical to the annotated sequence in the grapevine genome (Figure 32. Annexes).

One clone was selected for subsequent experiments and was recombined into PK7FWG2 vector (Table 2), to generate the final construction as described in Methods, Section 3.4. The final vector P35S::*VvINO* was used to transform *Rhizobium radiobacter*, as shown in Figure 20. B.

2.1.2. *In silico* characterization of *VvINO*

The genomic sequence for *VvINO* has 1147 bp, including six exons and five introns. The CDS and amino-acid predicted sequences have 531 bp and 176 amino-acids, respectively. Also, a BlastP alignment of VvINO protein was carried out to compare its similarity with the most studied homologous proteins from *Arabidopsis thaliana* and *Anonna squamosa*. VvINO (GSVIVG01013778001) has a 68,51 % of sequence identity with INO (AT1G23420) from Arabidopsis (Table 3) and a 64,77% of identity with INO homologous from *Annona squamosa* (GU8228032). An *in silico* analysis of VvINO, using different databases, confirmed that VvINO belongs to YABBY Family of proteins (IPR006780, PTHR31675:SF8) and High mobility group box domain superfamily (IPR009071, PF00505) (Table 3). Besides, a role in biological processes related to progression between different development stages was predicted by Gene Ontology (Table 4), which is expected according to the known function of homologous genes in other species (Introduction. Section 4.4).

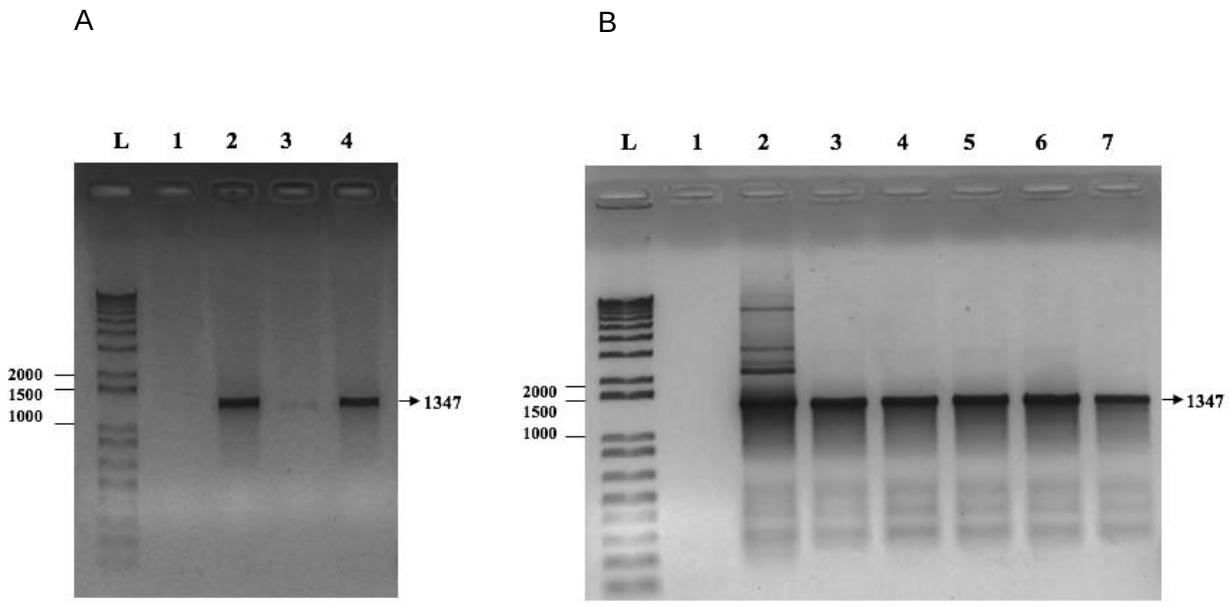

FIGURE 20. *VvINO* amplification and final P35S::VvINO construction.

A) PCR amplification from *Vitis vinifera* DNA. L: 1 kb plus DNA ladder**,** 1: negative control, 2: Cavernet sauvignon DNA, 3: Superior DNA, 4: Chardonnay DNA. **B)** PCR amplification in final expression vector in *R. radiobacter*. L: 1 kb plus DNA ladder, 1: negative control 2: positive control 3- 7: clones carrying final vector.

To gain broader insight into the possible role of *VvINO* in early berry and seed development, an analysis of plant cis- acting regulatory DNA elements in *VvINO* promoter was developed using PLACE database (Higo et al., 1999). In general terms, this study showed multiple putative binding sites for transcription factors, elements of light regulation, sites related to embryonic development and also, elements involved in hormonal regulation (Figure 21 and Table 5).

Regarding binding sites to transcription factors, it was found elements for different families: E2F, MYB, WRKY, Hight mobility group box (homeodomain), AP2, bZIP and MADs BOX (Table 5. Elements listed in green). Interestingly, there is a site that can be recognized by AGAMOUS (AG), the product of an important floral gene of C class with a role in in ovule identity (Introduction, Section 2.3).

In addition, the promoter has responsive elements to the phytohormones gibberellins, auxins, abscisic acid, ethylene and cytokinin, important hormones for several developmental processes (Figure 21 and Table 5, Elements listed in orange).

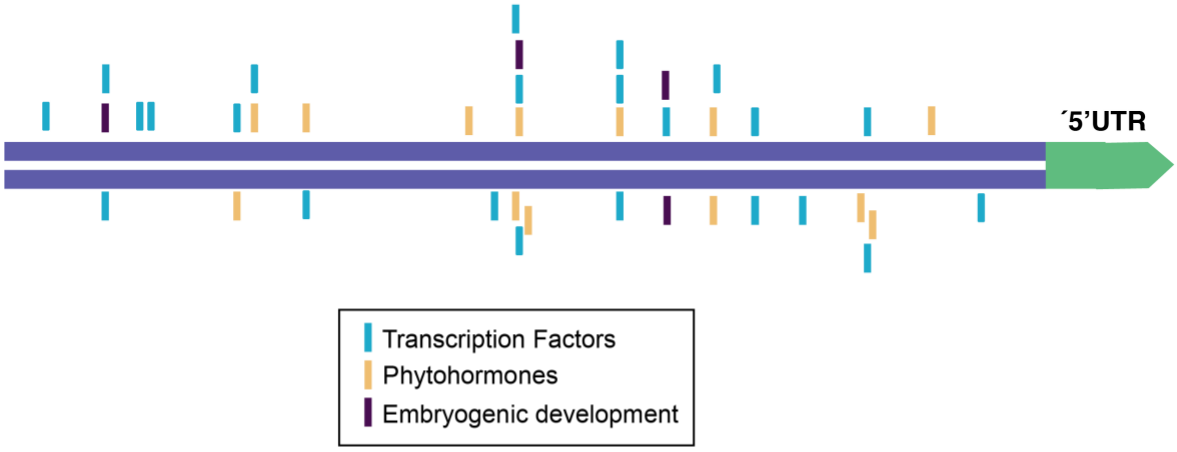

FIGURE 21. Diagram of plant cis-acting regulatory DNA elements in *VvINO* Promoter. In silico analysis of the *INO* promoter, using the PLACE platform (Higo et al, 1999). The promoter was defined as 2000 kb upstream of the start of transcription. The main cis acting elements related to transcription factors, hormonal regulation and embryogenesis are graphed.

2.2. Functional evaluation of *VvINO* through ectopic expression in *Arabidopsis thaliana*

2.2.1. Genotyping of *ino-1* mutants

Genotyping of *ino-1* seed stock was performed through dCaps technique (Methods. Section 5). Throughout this study, about 200 plants from the ABRC seed's stock were genotyped. Out of the total of analyzed plants, 53% were identified as heterozygous *ino-1* - /+ and 47% as wild type plants. It was no possible to find a homozygous *ino -1* -/-, which is consistent with the evidence that loss of *INO* function is associated with female sterility. In Figure 22, an example of the genotyping of ten plants is shown. Lines 1, 2, 3, 4, 5 and 9, identified as heterozygous mutants, were selected to perform the subsequent phenotypic analyses.

2.2.2. Phenotypic evaluation of *ino-1* mutants

Observations with the naked eye of mutants *ino-1* (background *Ler)* and wild type plants (*Ler-0*), do not allow to identify noticeable differences, possibly because the main differences are observed at a reproductive and non-at vegetative level (Figure 23). In order to deeply characterize the phenotype of mutants *ino-1*, we conducted observations under dissecting microscope to identify the characteristics of the final fruit or 'silique', determining its size, counting the number of aberrant or unfertilized ovules, that is, those abnormal ovules that did not become in seeds, the number of aborted ovules and the number of seeds with normal development.

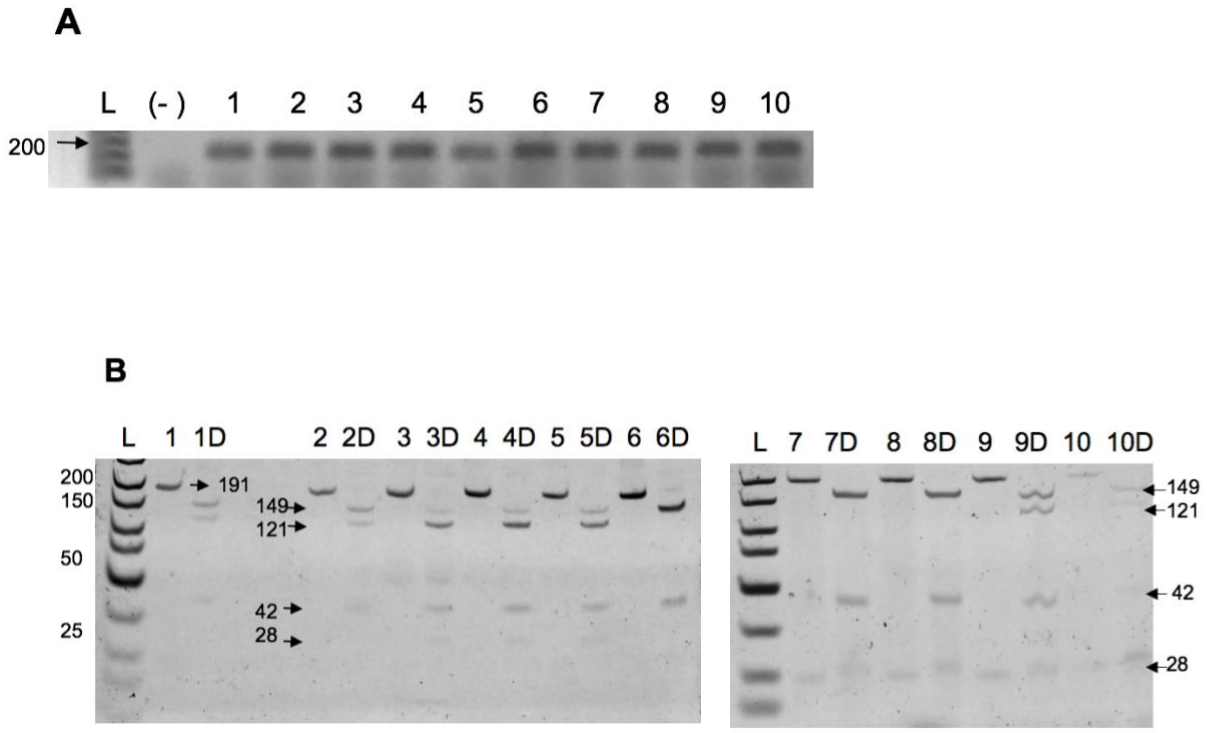

FIGURE 22. Genotypification of *ino-1* mutants.

A) Amplification of a fragment of *INO* through conventional PCR. Agarose 1,5%. L: 1 kb plus DNA ladder, (-): negative control without DNA, 1-10: DNA samples from plants grown from *ino- 1* seed's stock, showing the 191 bp fragment expected. **B)** Restriction analyses. Samples before and after DdeI using 10% polyacrylamide geles. Samples before (1-10) and after digestion (1D-10D) are shown. Plants 1, 2, 3, 4, 5, 9 and 10 were *ino-1* -/+.

FIGURE 23. Vegetative growth of *ino-1* -/+ and wild type plants.
ino-1+/- mutant (left) and a wilt type plant (right). Plants of 7 weeks, grown at long-day photoperiod.

In general terms and according to literature, the silique's size correlates with the number of seeds contained inside (Meinke et al., 2008). In this study, it was observed that the size of wild type siliques was larger compared to the size determined for *ino-1* +/- mutants (Figure 24). In addition, when counting the number of seeds with normal appearance per silique, **it was determined that wild type siliques have significantly higher amount of seeds than *ino-1* mutants** (about 44% more seeds in the wild type plant compared to the mutant), which is consistent with the smaller size of its fruits (Figure 25). Interestingly, two lines (1 and 4) present a complete absence of normal seeds (Figure 25 A). Therefore, the mutation of one allele in heterozygous plants is capable of decreasing the number of seeds.

A typical silique under optimal conditions contain 40-60 normal seeds and can include a few unfertilized ovules (Meinke et al., 2008), which is also concordant with the data obtained in this study for wild type plants (*Ler-0* ecotype) (Figure 25. A, B, and G, H).

Also, **as an important result, we noted a significantly higher number of unfertilized ovules per silique in *ino-1 mutants* related to wild type plants** (Figure 25. G, and H). This result correlates with the function of *INO*, which is necessary to form a viable ovule, containing normal embryo sacs. The partial loss of function of this gene in heterozygous lines would affect the formation of viable ovules, and therefore, these will not be able to be fertilized and form seeds after pollination. Thus, this leads to a significantly lower amount of normal seeds in *ino-1* plants compared to the wild type control (Figure 25. A).

Regarding the number of aborted ovules, no significant differences between wild type and mutant plants were appreciated (Figure 25. D and E).

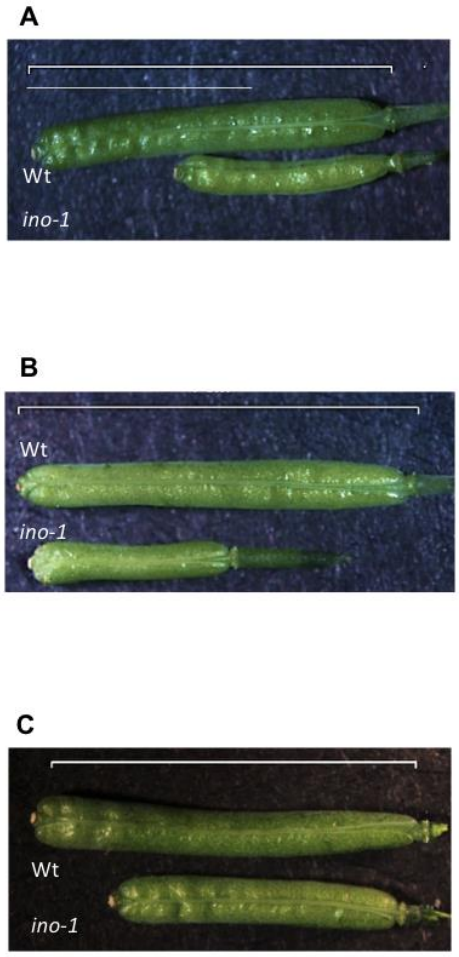

FIGURE 24. Silique size differences in *ino-1*+/- mutants and wilt type plants.
A) In the upper part, there is a silique from wild type plant. At the bottom side, there is a silique from mutant 1, measuring 5 mm. **B)** In the upper part, there is a silique from wild type plant. At the bottom side, there is a silique from mutant 3, measuring 6 mm. **C)** In the upper part, there is a silique from wild type plant. At the bottom side, there is a silique from mutant 5, measuring 8 mm. The white bar represents 1 centimeter.

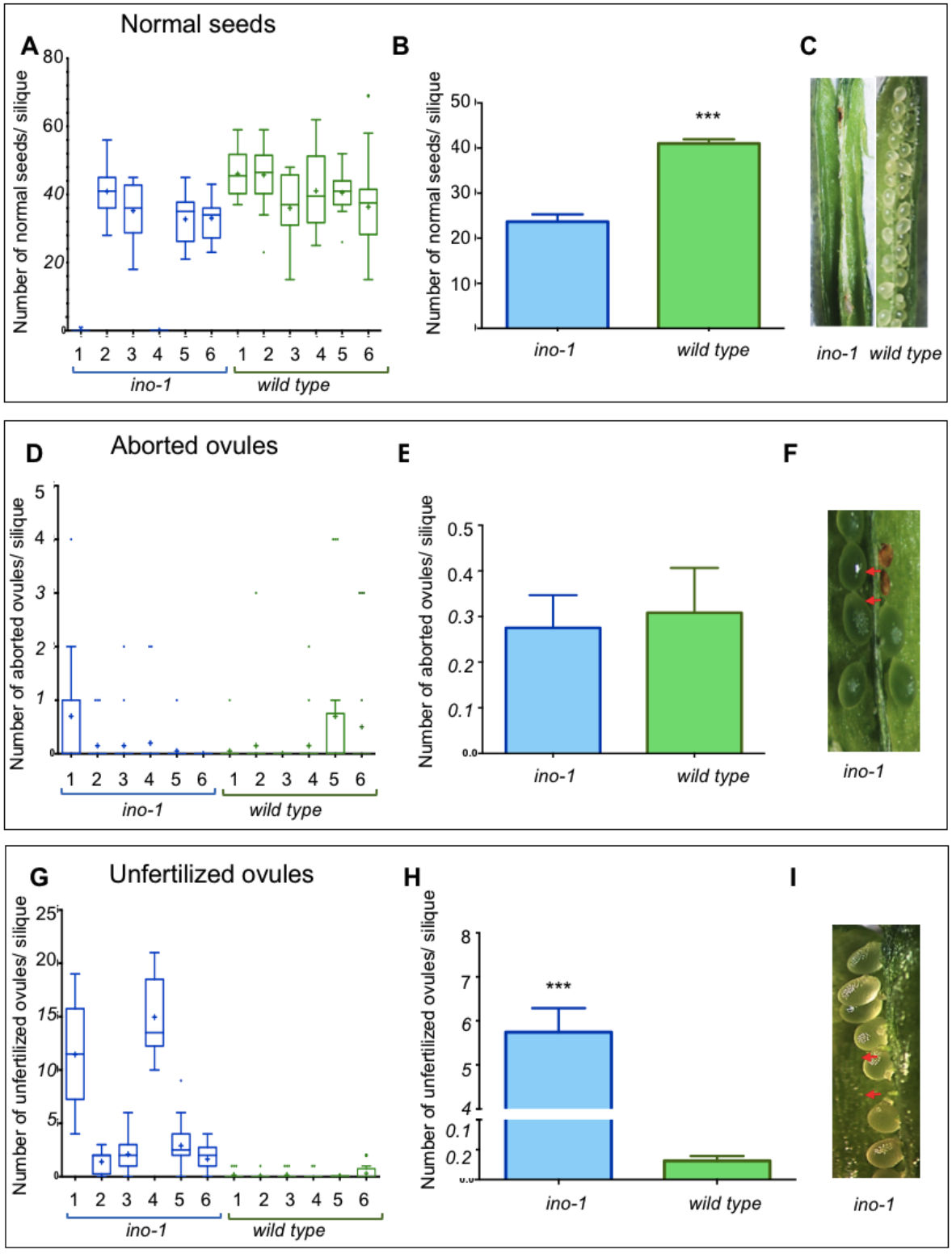
Normal seeds
A
B
C
Number of normal seeds/ silique
80
60
40
20
0
1 2 3 4 5 6 1 2 3 4 5 6
ino-1
wild type
50
40
30
20
10
***
ino-1
wild type
ino-1 wild type
Aborted ovules
D
E
F
Number of aborted ovules/ silique
5
4
3
2
1
0
1 2 3 4 5 6 1 2 3 4 5 6
ino-1
wild type
0.5
0.4
0.3
0.2
0.1
0.0
ino-1
wild type
ino-1
Unfertilized ovules
G
H
I
Number of unfertilized ovules/ silique
25
20
15
10
5
0
1 2 3 4 5 6 1 2 3 4 5 6
ino-1
wild type
8
7
6
5
4
0.1
0.2
***
ino-1
wild type
ino-1

FIGURE 25. Phenotypic evaluation of *ino-1* mutants.

A) Tukey Box- Plot showing the distribution of normal seeds by silique in plants analyzed independently. **B)** Average of normal seeds by silique in both types of plants, *ino-1* and wild type. **C)** On the left, image of mutant 1 showing complete absence of seeds and on the right, image of a wild type silique showing seeds with normal appearance **D)** Tukey Box plot showing the distribution of aborted ovules by silique in plants analyzed independently. **E)** Average of ovules aborted by silique in both types of plants, *ino-1* and wild type. **F)** Image of mutant 1 showing aborted ovules (red arrow). **G)** Tukey Box- plot showing the distribution of unfertilized ovules found by silique in plants analyzed independently. **H)** Average of unfertilized ovules found by silique in both types of plants, ino-1 and wild type. **I)** Image of mutant 3 showing unfertilized or aberrant ovules (red arrow). In **A, D and G** graphics, + symbol represents the media of the data, and the horizontal line inside the boxes represents the median of the data conjunct (N=40). **B, E, H** Data points represents means ± SEM considering N=240. Asterisks shows statistically significant difference between two genotypes as it was determinate by T test and Welch correction (P≤0,05). *ino-1* have Ler-background and wild type belongs to Ler-0 ecotype.

In summary, the data presented in Figure 25, suggest that partial loss of *INO* function in heterozygous mutants generates a diminished quantity of total seeds. As the number of unfertilized ovules is increased in *ino-1* related to wild type, this could be associated with defects in the development of some ovules, associated to the infeasibility of to be fertilized and not to the abortion of the ovule (or young embryo) after fertilization. However, those ovules that develop normally are viable to be fertilized and form regular seeds.

2.2.3. Ectopic expression of *VvINO* in Arabidopsis

In order to study the function of the *VvINO* gene, an over-expression strategy was carried out in Arabidopsis ecotype col-0. After obtaining the p35S::*VvINO* construction, *Arabidopsis thaliana* inflorescences were transformed using the floral dip method (Methods, section 4). The seeds obtained were selected with the corresponding antibiotic, and later, DNA were extracted from plants to check the presence of *VvINO* transgene. A total of 18 transgenic lines were obtained according to PCR assay and named as *oeVvINO* for subsequent analysis (Figure 26).

The accumulation of *VvINO* transcripts in the transgenic lines obtained was also determined by RT- qPCR. Most of the analyzed plants have higher levels of *VvINO* transcript than the wild type, with significant differences in eight different lines (3, 4, 5, 10, 11, 13, 15 and 18) (Figure 26. C).

According to the results obtained in this activity (Figure 26), it was possible to generate Arabidopsis plants that incorporated the *VvINO* transgene in their genome and also, it was expressed in significantly high levels in several of the analyzed plants.

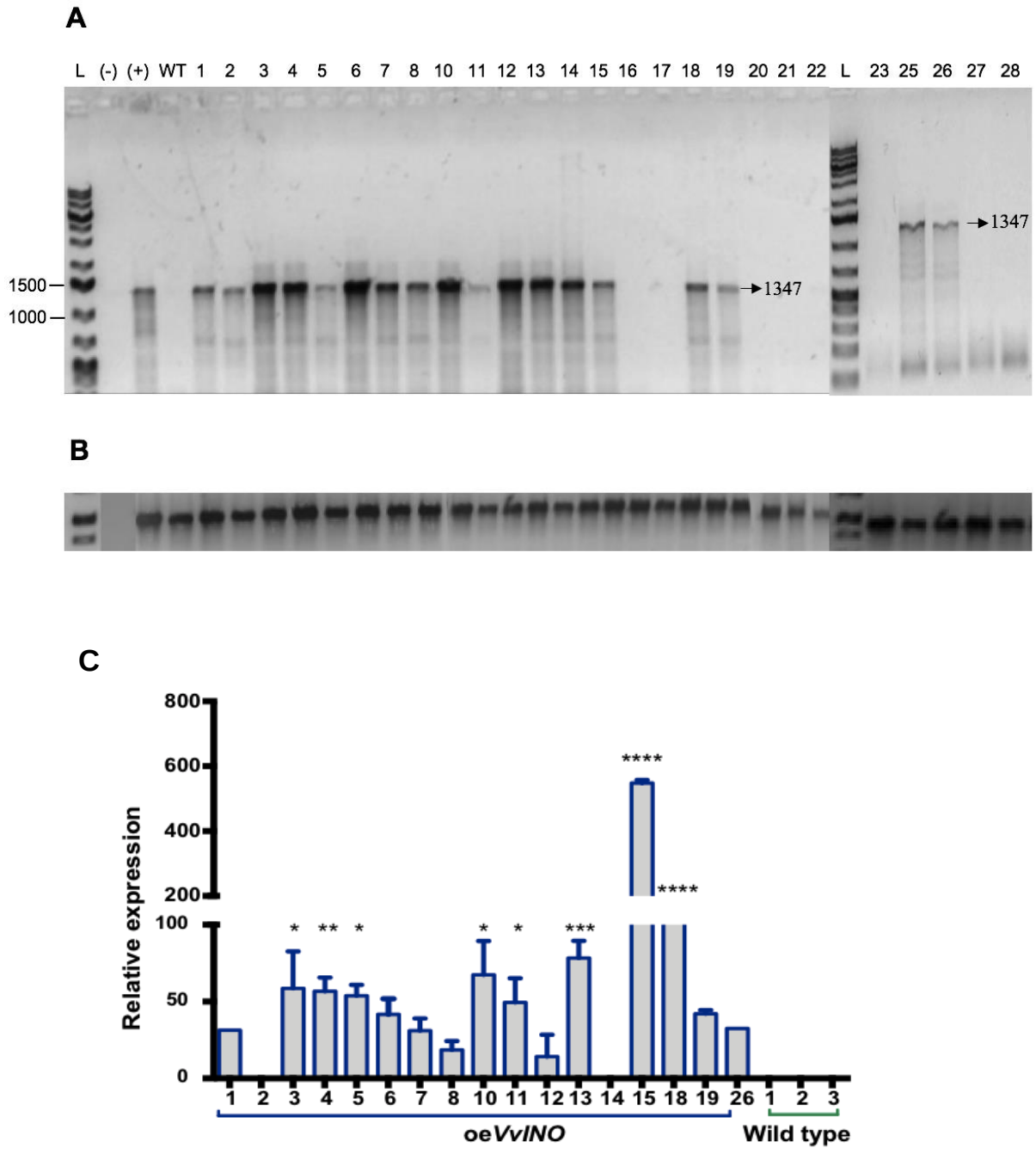

FIGURE 26. Selection and expression analysis of *P35S::VvINO* Arabidopsis lines.

A) Amplification of *VvINO* transgen in Arabidopsis plants. **B)** Amplification of *At18S* housekeeping gene as a measure of DNA integrity was verified in analyzed plants. L: 1 kb DNA ladder plus, (-): negative control without DNA, (+): positive control from *Vitis vinifera* DNA, WT: Arabidopsis wilt type plants (Col-0), 1-28: different DNA plants analyzed. **C)** *VvINO* expression in Arabidopsis selected plants related to Clatrin. RT-qPCR analysis was developed and media of three technical replicas per plant ± SEM was plotted. The asterisks show the plant lines with significant differences respect wild type. One- way ANOVA and Dunnett's multiple comparison test. (P≤0,05)

2.2.4. Phenotypic Evaluation of *oeVvINO*

Phenotypical analysis of silique´s size, number of unfertilized ovules, aborted ovules, and seeds with regular aspect were developed in *oeVvINO* lines selected by *PCR* (Figure 27 and 28). As fruit size typically correlates with seed number (Meinke et al., 2008), we first analyzed the silique size, and interestingly, transgenic *oeVvINO* has larger siliques in comparison with wild type plants (Figure 27).

Additionally, **the number of total seeds with normal appearance was significantly higher in plants over-expressers of *VvINO,* about 36% more seeds in comparison with *wild type.*** (Figure 28. E, and F). Other parameters analyzed, such as the number of unfertilized and aborted ovules per silique, did not have significant differences between wild type and transgenic plants (Figure 28. A, B, C, and D).

The data presented in this study allow us to establish a correlation between the presence of *VvINO* gene and the number of seeds in *Arabidopsis* fruit, by mechanisms that remain unclear. This is the first study where functional analysis of *VvINO* gene are carried out, and evidence about its incidence in the number of final seeds in mature fruits in a model plant is presented.

This work constitutes a first approach to understand the function of *VvINO*. However, more research is needed to understand the mechanisms of action and its interactions with other genes. *In silico* analyses of the promoter gave clues on a possible interaction of *VvINO* with other transcription factors, as well as suggest the probability that this gene has regulation by light and hormones.

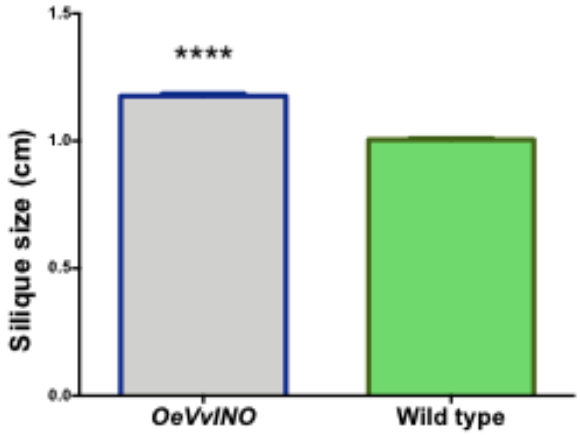

FIGURE 27. Silique size in *P35S::VvINO* and wild type Arabidopsis plants.
The mean ± standard error of size from 320 siliques were plotted. The statistical analysis was made using unpaired t-test and Man Whitney (P< 0,05). The asterisks show significant differences respect wild type plant.

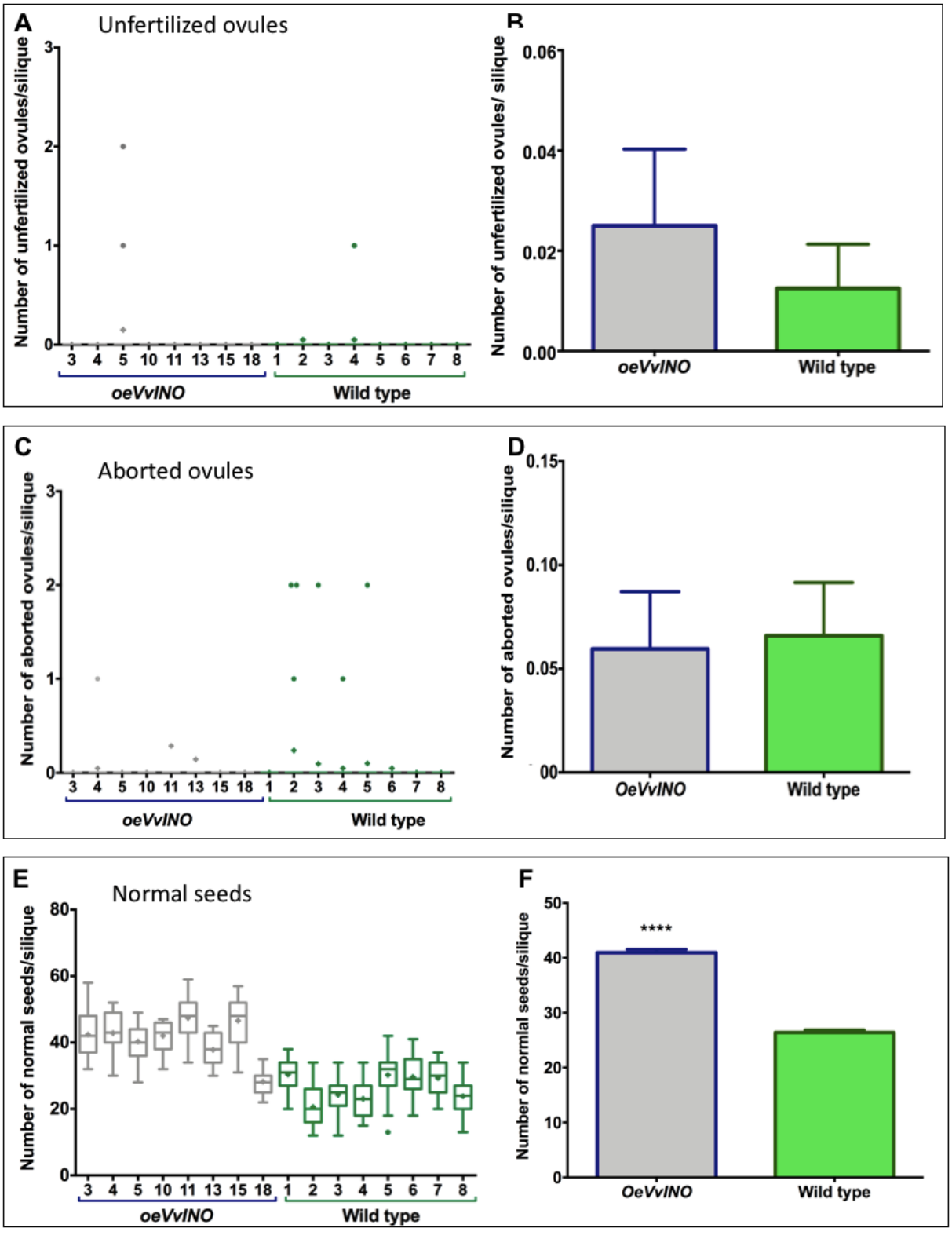
A
Unfertilized ovules
Number of unfertilized ovules/silique
3 4 5 10 11 13 15 18 1 2 3 4 5 6 7 8
oeVvINO
Wild type
B
Number of unfertilized ovules/ silique
0.06
0.04
0.02
0.00
oeVvINO
Wild type
C
Aborted ovules
Number of aborted ovules/silique
3 4 5 10 11 13 15 18 1 2 3 4 5 6 7 8
oeVvINO
Wild type
D
Number of aborted ovules/silique
0.15
0.10
0.05
00
OeVvINO
Wild type
E
Normal seeds
Number of normal seeds/silique
80
60
40
20
0
3 4 5 10 11 13 15 18 1 2 3 4 5 6 7 8
oeVvINO
Wild type
F
Number of normlal seeds/silique
****
50
40
30
20
10
0
OeVvINO
Wild type

FIGURE 28. Phenotypic evaluation of *P35S::VvINO* Arabidopsis lines
A) Tukey Box- Plot showing the distribution of unfertilized ovules by silique in plants analyzed independently. **B)** Average of ovules unfertilized by silique in both types of plants, *oeVvINO* and wild type. **C)** Tukey Box plot showing the distribution of aborted ovules by silique in plants analyzed independently. **D)** Average of aborted ovules by silique in both types of plants, *oeVvINO 1* and wild type. **E)** Tukey Box- plot showing the distribution of normal seeds found by silique in plants analyzed independently. **F)** Average of seeds with normal aspect found by silique in both types of plants, ino-1 and wild type. In **A, C and E** graphics, + symbol represents the media of the data, and the horizontal line inside the boxes represents the median of the data conjunct (N=40). **B, D, F** Data points represents means ± SEM considering N=320. Asterisks shows statistically significant difference between two genotypes as it was determinate by T test and Welch correction (P≤0,05). *ino-1* have Ler-background and wild type belongs to Ler-0 ecotype.

CHAPTER 6

DISCUSSION

DISCUSSION

Impact of seedless grapes and the limitations of its study

Seeds originate from fertilized ovules and constitute critical structures that allow the propagation of plant species. On the other hand, seedless fruits and mainly, seedless grapes are a desirable product for consumers. Nevertheless, studying seedlessness in grapevine has been very difficult, mostly because the life cycle of *Vitis vinifera* is extensive. Only the reproductive developmental cycle takes at least two years in adult plants (Carmona et al., 2008), which makes grapevine plants a complicated model for performing functional studies at a genetic level. Indeed, to date only three genes related to seed development have been characterized in grapevine. Moreover, in all these studies was necessary to conduct the characterization in other plant models as tobacco and tomato (Hanania et al., 2007; Ocarez and Mejía, 2016). Therefore, in this study, we used Arabidopsis to functional studies.

Another challenge is the variability between grapevine plants. The ideal study model for made molecular comparisons are the ‘somatic variants, that is, individuals vegetatively propagated that possess a similar genotype (Wenzel and Foroughi- Wehr, 1994). Somatic variants are produced when a grapevine plant suffers a spontaneous mutation that generates a new characteristic, for example, Thompson seedless and Thompson seeded studied by Hanania, 2007 and 2009. Unfortunately, it is impossible to have somatic variants intentionally, in this study

several well-known grapevine varieties were used, as well as MO x FS segregants sharing the same parents and also with similar genetic backgrounds, but whose genotype is not identical.

Candidate genes are expressed in the early stages of the grapevine reproductive cycle

Many studies have been made to improve our understanding of berry development, most of them from veraison to post-harvest time. However, early stages of flower development and berry formation have been poorly investigated, despite their importance.

This work aimed to identify genes of *Vitis vinifera* that could affect the formation of ovules and seeds in the model plant *Arabidopsis thaliana* as a first approach to answer the biological question of whether these genes would participate in the formation of the seed in grapevine.

As a first approach, the expression pattern of the candidate genes in early developmental stages was analyzed in a semi- quantitatively, with respect to a reference gene. In general terms, **the transcripts of all candidate genes evaluated in this study are accumulated in reproductive tissue of *Vitis vinifera*** and according with literature, presumably at low expression levels, because many genes involved in the ovule/seed and flower development have a particular spatio-temporal expression pattern (few cells with a particular timing). Therefore, the small expression differences between each stage found in this study can account for critical morphological changes in reproductive organs. However, to deeply know the specific tissue expression profile, it is recommended to carry out *in situ* hybridization assays with probes for each candidate gene. During this work, different attempts to do these analyses were made under the guidance of Dr.

Beth Krizek at the University of South Carolina, but technical difficulties did not allow obtaining results (data not shown). In fact, very few works have been published *in situ* hybridization studies in *Vitis vinifera* and woody plants (Fernandez et al., 2007; Hanania et al., 2009), because there is a strong background signal that impairs a clear signal identification (Colas et al., 2010; Fernandez et al., 2007). Another approach that could be carried out is the immunohistolocalization of candidate proteins, which is also a sophisticated technique because requires specific antibodies. Therefore, investigations in this field are still challenging.

Consequently, our assays of transcripts accumulation during the early development of flower and berry showed similar results with previously published data analyzed in this study (Figure 5) (Licausi et al., 2010). The results obtained in this work indicate that most genes analyzed have a characteristic expression pattern throughout early flower and berry development, which is similar independently of the plant type studied. **Interestingly, three putative transcription factors *VvAGL6_1, VvAGL6_2,* and *VvINO* are expressed mainly in young flowers and less in fruit set berries** (Figures 9 and 13).

In the case of *Arabidopsis*, in the work of Klepikova and collaborators (2016), who made a developmental transcriptome using RNAseq data, it was found that *AGL6* was expressed throughout floral development. Therefore, is not surprising to find a greater accumulation of *VvAGL6*_1 and *VvAGL6_2* transcripts in flowers. Also, in the siliques the expression is observed only in seeds at specific stages. ***VvINO* expression pattern obtained in this work was as expected, due to the higher transcripts accumulation in young flowers is concordant with previously described *INO* role in ovule's integument formation, a process that occurs during flower development prior to anthesis** (Villanueva et al., 1999b).

On the other hand, ***VvAIL5* has an opposite profile, because its transcripts are higher accumulated in fruit set berries than in younger flowers** (Figure 11). **This is interesting, because in Arabidopsis it has been suggested that *AIL5* has a role during floral and embryogenic development, but no further studies have been carried out to know its role in fruit formation.** Besides, the developmental transcriptome analysis of Arabidopsis reveals a predominant transcript accumulation of *AIL5* during seed development (Klepikova et al., 2016) and possibly the greater expression of *VvAIL5* in young fruits could be explained by the developing seed after fertilization.

For *VvLUG*, the expression profile during early development provide inconclusive information and we propose to carry out more assays including other varieties (Figure 12 and 18). Transcripts of *VvLUG* are equitably in all evaluated stages in seedless varieties, while it diminishes its expression in fruit set in the seeded variety Red globe (Figure 12). To further confirm if this decrease is associated with seed formation, more analyses are needed, including an evaluation of a greater number of seeded grapevine varieties, as well as studies of gene functionality. *LUG*, its homologous gene in *Arabidopsis* is highly expressed both in vegetative and reproductive tissue, although with greater expression in the carpels of all stages of development, which is consistent with the expression profile observed for *VvLUG* in grapevine (Klepikova et al., 2016).

All candidate genes are expressed in early stages of flower or fruit development, but long- term functional assays would be necessary for confirm if there are involved in flower or fruit development and seed formation in grapevine.

Two candidate genes have consistent differential expression when comparing grapevine seeded and seedless phenotype

To evaluate the contribution of the candidate genes in the final phenotype of grape berries (seeded or seedless berries), each early development stage was analyzed independently, comparing individuals with both phenotypical characteristics.

For this, we used varieties whose seed phenotype (absence or presence) is known and has been stable over the years. Also, segregants were analyzed, which are individuals descendants from the same crossing (same ancestors), thus should have a more similar genetic background than different varieties, which come from distinct ancestors.
The berries of these segregants were analyzed one season before the expression assays, and we observed that seedlessness is a strongly variable phenotype, ranging from the complete absence of seeds to the formation of small rudiments (Figure 14). It is interesting to mention that this variability is observed in plants that share the same parental, which accounts for the **complex genetic and molecular regulation that must exist to lead to the formation of the ovule and seed**. In addition to the absence of regular seeds, berries from seedless individuals are smaller as expected, which is explained because developing embryos participate in the synthesis of the growth hormone auxin (A), the cell division hormone cytokinin (C) and the cell expansion hormone gibberellin (GA), promoting the fruit growth for first days (Keller, 2010).

The comparative analyses gave the following results:

Limited information was obtained for *VvAGL6_1*, *VvAGL6_2*, and *VvLUG*, when comparing transcript accumulation between seedless and seeded grapevine plants (Figure

15, 16, and 18), because no clear difference of expression was observed in segregating plants and varieties with different phenotype. Besides there were differences even between lines of the same phenotype, which accounts the natural variability. As future work, it would be interesting to evaluate if there are differences in transcript accumulation in other varieties or segregants plants and ideally analyze somatic variants.

Interestingly, two genes presented consistent expression differences between seeded/seedless phenotypes, in both varieties and segregates plants: ***VvAIL5* and *VvINO*** (Figure 29). The first gene, *VvAIL5* showed significant differences in pre-pollinated flowers (EL-18), with a higher expression in seeded plants. *VvINO* transcripts levels are significantly higher in in pre-pollinated flowers (EL-18) and in developing seeded fruits (fruit- set, EL-27), in comparison with the seedless ones.

Considering these results, we suggest that both genes, *VvAIL5* and *VvINO* would be key genes during ovule's development in young inflorescences. Likewise, we suggest that the regulation of *VvINO* gene would be important in developing seeds after ovule fertilization, in young grape berries as evidence their higher transcript accumulation in EL-27. Therefore, these genes could contribute to seedlessness phenotype with different timing.

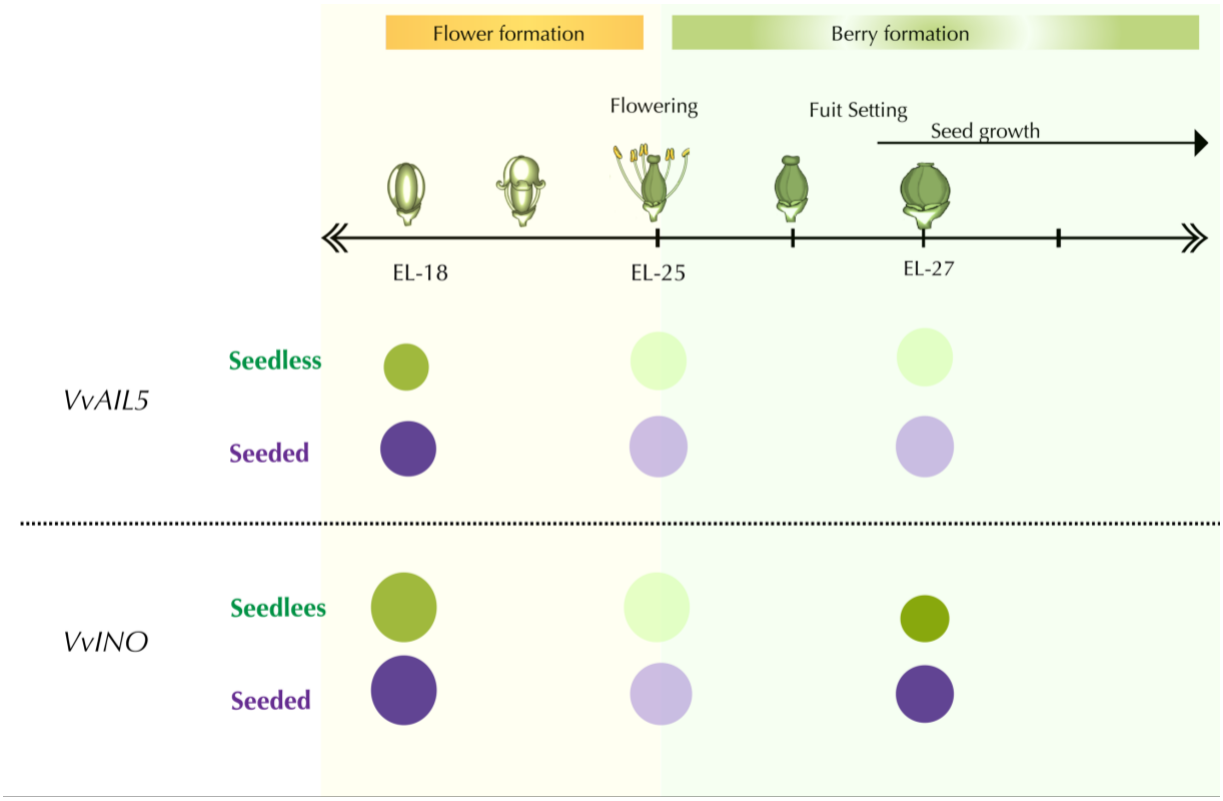

FIGURE 29. Representation of *VvAIL5* and *VvINO* expression profiles.
Green and purples circles represent the expression of each gene in seedless and seeded plants, respectively. The differences in circles' size describe the transcript accumulation differences between each stage. The circles with darker color represent the stage where significant differences of expression between both phenotypes were observed.

So far, in **Arabidopsis it is known that *AIL5* is expressed in flowers, particularly in ovule primordium and integuments and although there is no certainty about their specific role in these tissues**, it is believed that could participate in embryo sac formation such as *ANT,* another member of AP2 family with partially overlapped expression (Nole-Wilson et al., 2005). The expression profile found in this work, suggests new evidence about a possible participation of *VvAIL5* during flower formation. Since seedless flowers have lower levels of *VvAIL5*, we deduce a potential contribution of this gene during female gametophyte formation, as occurs with *ANT*. Thus, ***VvAIL5* could participate during ovule development in grapevine inflorescences** and therefore, a decrease in transcript levels of *VvAIL5* in young flowers could be linked to some defect in ovule formation and thus alter seeds development.

On the other hand, there are apparently some differences between *VvINO* and its counterpart in Arabidopsis, since in the latter there is no *INO* transcripts during anthesis, as we observed in grapevines, suggesting a new role for this gene in grapevine. This suggested function after anthesis might be related to the morphological differences between both species. Indeed, despite the conserved function between *INO* genes from different species, there are some differences in ovule that could result in different functions. For example, *ino* strong mutants (*Arabidopsis*) are incapable of form fruits while *Tai-seedless* mutant (*Anonna squamosa*) shows completely developed fruits but without seeds (Lora et al., 2011). These differences are presumably attributed to ovule characteristic, since *A. squamosa* and *V. vinifera* has an endostomal micropyle, where the outer integument does not completely cover the inner integument and therefore does not participate in the pollen guidance during fertilization unlike Arabidopsis.

Surprisingly, gene expression differences were found in fruit-set berries, when seed is already in formation, which is consistent with the expression reported for *INO* in Arabidopsis developing embryos (Villanueva et al., 1999a). In this case, young berries that form seeds (seeded plants) present higher transcript levels than young fruits that will not contain seeds (seedless plants). Thus, we suggest that the initial embryo formation is already absent or aborted in seedless berries at EL-27 as a result of *VvINO* lower expression in grapevine seedless plants at this stage. Therefore, ***VvINO* could contribute to the seed development during berry formation**.

In addition, a recent study in Arabidopsis proposes an indirect interaction between INO and LUG during flower development (Simon et al., 2017b), but more studies are needed to know if there is a combined participation of their grape homologous in ovule and seed development. The data provided in this thesis shows that both putative homologous genes are expressed during all the analyzed stages in grapevines. However, more studies are needed to establish a combined participation during seed development in *Vitis vinifera*.

In silico characterization of *VvINO* suggests a complex regulation

VvINO encodes for a putative transcription factor, that is, a protein with DNA binding capacity that controls the transcription of other specific genes. The analysis of VvINO protein sequence confirms the presence of a DNA binding region (HMG) as well as the YABBY motif that is found in all the transcription factors of this family (Table 3). In addition, the analysis of *VvINO* promoter region indicates that it has responsive elements to phytohormones such as ethylene

(ERE), gibberellins (GARE), abscisic acid (ABRE), as well as binding sites to Auxin Response Genes (ARF). This suggests a possible regulation of this gene, mediated by phytohormones. This is interesting, since there is evidence about the role of phytohormones in ovule/seed development:

Regarding auxins (A), there is evidence that participate in pattern formation in the female gametophyte, due to an hormone gradient that act to specify cell fates during gametophyte formation (Sundaresan and Alandete-Saez, 2010). Also, it is known that A is important for ovule primordia initiation, because an A maxima is required to primordium formation (through PIN transporter proteins) (Cucinotta et al., 2014). Moreover, cytokinins (CK) increase *PIN1* expression in ovules suggesting the existence of a cross- talk between CK and A during ovule primordia formation. Mutants with defects in CK production or perception have defective ovules and/or a reduction in the number of this organs (Cucinotta et al., 2014). According these evidences and the *in silico* analyses of *VvINO* promoter, we hypothesized a regulation of *VvINO* action during ovule development mediated by phytohormones. For example, during ovule initiation A could promote the expression of *VvINO,* but more studies will be needed to test this hypothesis.

Additionally, *in silico* analysis of *VvINO* promoter identified several transcription factors binding sites for MYB, bZIP, AP2 and MADS- box families. These transcription factors function in further regulation of several genes under particular development or stress conditions (Ambawat et al., 2013). Therefore, *VvINO* expression could be regulated by other transcription factors associated with developmental processes and in turn, *VvINO* could regulate the expression of other genes. This opens a new line of research to discover genes and proteins that

interacts with *VvINO* and its product. **Particularly, we found DNA motifs that are described as binding sites of AGAMOUS (AG*)* or AGAMOUS-like (AGL2), which is interesting because there are several evidences that *AG* is an important regulator of ovule and floral developmen**t** (Cucinotta et al., 2014). In grapevine, *VvMADS1* has homology with *AG* (Boss et al., 2001) and could be a possible partner for *VvINO*. Secondly, it has been proposed that *LUG*, the gene that encodes a protein recently described as INO partner, could negatively regulates *AG* expression (Nole-Wilson et al., 2005). However, LUG does not have DNA binding sites, so it has been mentioned that it must act in complexes with other proteins (Simon et al., 2017b).

The presence of putative binding sites in *VvINO* promoter suggest an interaction with AG- like proteins, hence new studies are required to determine if there is a combined participation between *VvINO*, *VvLUG* and *VvAG-like* genes to regulate ovule/seed formation. The possibility of a combined action between these genes and proteins is an interesting task to study in the future.

Ectopic expression of *VvINO* suggests a role in ovule primordia formation

During this work, at least 200 plants descendants from *ino-1+/-* were genotypified, and homozygous plants were never obtained because this mutant presents female sterility. When analyzing the offspring of heterozygous lines, about 50% of new heterozygous plants were obtained, which is the ratio reported for recessive mutations on lethal genes with defects in one gametophyte that does not follow the Mendelian segregation of 70% (Meinke et al., 2008). In

this case, it is known that *ino-1* mutants have defects in female gametophyte, therefore this rate was expected.

The heterozygous lines were studied, and we observed that they had smaller siliques with a reduced amount of seeds than wild type plants. Moreover, some heterozygous plants did not present any seed, but instead contained unfertilized ovules (Figure 25). This reflects that even the loss of function of one allele is capable of affecting the normal gametophyte formation of a fruit, leaving some or all aberrant ovules uncapable to be fertilized inside the mature fruit. It also accounts for the variability of the seedlessness phenotype, as happened in the case of segregating grapevine plants described above.

On the other hand, the phenotype of *INO* over- expression in Arabidopsis is not already reported. Thus, the characterization of *VvINO* ectopic expression is an important contribution to understand the function of *INO*-related genes. **Arabidopsis plants with ectopic *VvINO* expression presented larger siliques with more seeds than wild type plants** (Figure 27 and 28). No differences were observed in the number of unfertilized or aborted ovules, therefore the greater number of seeds is not due to a reversal of these parameters that occur spontaneously at low rates in wild type plants (Meinke et al., 2008). According to these results, we proposed that the higher seed rate of oe*VvINO* siliques could be related to a role of *VvINO* in ovule identification, were primordial ovule number is established and therefore, the potential seed number of the fruit. There is evidence about other genes (*ANT* and *HLL*) participating in integuments development - like *INO*- that also are involved in promoting the growth of ovule primordia by mechanisms that are not entirely clear (Huang et al., 2013).

Ovule formation is a highly regulated process

The formation of the ovules is a vital process for plants and consequently it is logical that it is highly regulated at the hormonal and molecular levels. For that reason, is not strange that several genes are part of this process.

At the hormonal level, A would play a key role in gynoecium and ovule development, since it has been demonstrated that NPA application (inhibitor of A transport) results in a drastic reduction in the number of ovules (Nemhauser et al., 2000). Also, auxin-related mutants have an ovule reduction or absence and thus, are female sterile (Cucinotta et al., 2014). A connection between *ANT* and A has been suggested, since *ant* mutants are more sensitive to alterations in auxin polar transport than wild type plants (Cucinotta et al., 2014; Nole-wilson et al., 2010). It has also been proposed that ANT protein participate in a positive regulation of *INO* expression, but without physic interaction (Meister et al., 2004). At a molecular level, it has been proposed that *ANT* could has a role as a master regulator of the ovule primordium formation, even though there are other genes involved in this function (Cucinotta et al., 2014).

Considering these antecedents, we suggest that *VvINO*, together with phytohormones and other genes and their proteins, can play a role in early ovule development, participating in the formation of the ovule primordia. On a molecular level, it could exist a positive regulation by some of the transcription factors that could interact with *VvINO* promoter as suggested the *in silico* analysis, for example an AP2 transcription factor ANT-related, such as *VvAIL5* protein product. At hormonal level, the action of *VvINO* is possibly affected by hormones like A, which participates during the establishment of the ovule primordia.

In addition, we suggest that *VvINO* participates at the initial stage of seed development, as suggested by the correlation between the higher expression in developing seeded berries in comparison with seedless ones (Figure 29).

As a projection of this thesis and as a way to confirm the function of *VvINO in* grapevine, we propose to conduct a long-term analysis by silencing or editing *VvINO* in grapevine. This experiment is technically difficult in the context of a thesis because it will take at least 6 years until have an adult grapevine mutant plant. However, this is a key experiment to determine the putative role of *VvINO* in seed formation and would allow the generation of new varieties of seedless grapes.

Finally, this work constitutes a first approach to identify genes of *Vitis vinifera* related to a reproductive development. In this study, we present interesting evidence about the effect of *VvINO* in Arabidopsis fruits, which affects the seed yield in siliques. More studies are needed to know if this gene is of the main contributors to the formation of the ovule and seed in grapevine.

CHAPTER 7

CONCLUSIONS

CONCLUSIONS

Ovule and seed formation are interesting processes to study because of their importance in the propagation of the species, as well as the value of the seedlessness phenotype in fruit consumption. In grapes, there is limited information about the molecular mechanisms regulating these processes.

In this work, we have identified genes of *Vitis vinifera* that until now have been scarcely or none studied, evaluating mainly its expression profile in early stages of berry development, a crucial phase for seed formation. The genes *VvAGL6_1*, *VvAGL6_2*, *VvAIL5*, *VvINO*, and *VvLUG* showed expression in reproductive tissues in these early stages of flower and berry development of the grapevine.

Besides, the expression of transcripts was compared in plants with different phenotypes, seeded and seedless. From this analysis, two genes, *VvINO* and *VvAIL5*, which exhibit differential transcripts accumulation between seeded/ seedless phenotype, both in varieties as well as in segregants grapevine plants. Both genes have a lower accumulation of transcripts in seedless grapevine plants in comparison with seeded plants in specific developmental stages. Therefore, we suggest that *VvINO* and *VvAIL5*, could participate in different stages of ovule and seed development; contributing to the seedlessness through different mechanisms.

VvINO was selected for functionality studies. First, we analyzed the phenotype of *ino-1* mutants from *Arabidopsis thaliana*, which have smaller fruits, with a higher quantity of unfertilized ovules and a lesser amount of normal seeds, in comparison to wild type plants.

Also, we isolated *VvINO* gene from *Vitis vinifera*, a putative transcription factor of the YABBY family. The ectopic expression of this gene in Arabidopsis was studied, and over-expressers lines (*oeVvINO*) presented larger fruits with a higher number of seeds in comparison with wild type plants. These results, along previously published work, allowed as to suggest a function of *VvINO* during ovule primordia formation and therefore, affecting seed number in mature fruits of Arabidopsis.

CHAPTER 8

ANEXXES

ANNEXES

TABLE 5. Plant cis-acting regulatory DNA elements in *VvINO* Promoter

Element	Repetitions	Position	Description	Reference
DOFCOREZM	27	19 (-) AAAG 94 (-) AAAG 204 (+) AAAG 685 (+) AAAG 822 (+) AAAG 876 (-) AAAG 908 (-) AAAG 927 (-) AAAG 1007 (+) AAAG 1047 (-) AAAG 1081 (+) AAAG 1295 (+) AAAG 1398 (+) AAAG 1405 (+) AAAG 1443 (+) AAAG 1487 (-) AAAG 1495 (+) AAAG 1518 (-) AAAG 1537 (-) AAAG 1573 (-) AAAG 1590 (-) AAAG 1664 (-) AAAG 1681 (-) AAAG 1741 (+) AAAG 1869 (+) AAAG 1883 (-) AAAG 2014 (-) AAAG	Core site required for binding of Dof proteins in maize (Z.m.). Dof proteins are DNA binding proteins, with presumably only one zinc finger and are unique to plants.	(Yanagisawa and Schmidt, 1999)
IBOX	1	48 (-) GATAAG	Conserved sequence upstream of light-regulated genes. Binding site of LeMYB1, that is a member of a novel class of myb-like proteins	(Scolnik and Cashmorea, 1988)
IBOXCORE	4	49 (-) GATAA 220 (+) GATAA 368 (-) GATAA 744 (-) GATAA	Conserved sequence upstream of light-regulated genes	(Lohuis and Miller, 1998)
E2FCONSENSUS	1	58 (-) WTTSSCSS	"E2F consensus sequence" of all different E2F-DP-binding motifs that were experimentally verified in plants. E2F transcription factors induce the transcription of genes required for cell cycle progression and DNA replication	(Vandepoele et al., 2005)
ARR1AT	17	278 (+) NGATT 455 (-) NGATT 467 (-) NGATT 501 (+) NGATT 520 (+) NGATT 600 (-) NGATT 1022 (+) NGATT 1044 (-) NGATT 1166 (+) NGATT 1206 (-) NGATT 1241 (-) NGATT 1248 (-) NGATT 1502 (-) NGATT 1803 (+) NGATT 1931 (-) NGATT	"ARR1-binding element". ARR1 is a response regulator that function as transcriptional activator	(Sakai et al., 2000)
SEF1MOTIF	1	250 (-) ATATTTAWW	"SEF1 (soybean embryo factor 1) binding motif"	(Allen et al., 1989; March-Díaz et al., 2007)

			In Arabidopsis SEF is a protein required for flowering repression.	
SEF4MOTIFGM7S	8	76 (+) RTTTTTR 263 (+) RTTTTTR 280 (+) RTTTTTR 317 (-) RTTTTTR 402 (-) RTTTTTR 475 (-) RTTTTTR 563 (-) RTTTTTR 1202 (-) RTTTTTR	"SEF4 binding site" (soybean embryo factor 4)	(Allen et al., 1989)
GT1CONSENSUS	22	134 (-) GRWAAW 135 (-) GRWAAW 220 (+) GRWAAW 330 (+) GRWAAW 338 (-) GRWAAW 367 (-) GRWAAW 503 (-) GRWAAW 510 (-) GRWAAW 630 (+) GRWAAW 666 (+) GRWAAW 743 (-) GRWAAW 861 (+) GRWAAW 922 (-) GRWAAW 928 (-) GRWAAW 942 (-) GRWAAW 1092 (+) GRWAAW 1093 (+) GRWAAW 1111 (+) GRWAAW 1120 (-) GRWAAW 1254 (-) GRWAAW 1746 (+) GRWAAW 1784 (+) GRWAAW	Consensus GT-1 binding site in many light-regulated genes	(Zhou, 1999)
CCA1ATLHCB1	3	278 (-) AAMAATCT 520 (-) AAMAATCT 1041(+) AAMAATCT	CCA1 binding site. CCA1 protein is a myb-related transcription factor involved in circadian clock and phytochrome regulation.	(Wang et al., 1997)
WBBOXPCWRKY1	3	310 (-) TTTGACY 472 (-) TTTGACY 1725 (+) TTTGACY	"WB box"; WRKY proteins bind specifically to the DNA sequence motif (T)(T)TGAC(C/T), which is known as the W box. WRKY proteins are transcription factors.	(Eulgem et al., 2000)
WRKY71OS	6	311 (-) TGAC 473 (-) TGAC 1026 (+) TGAC 1057 (+) TGAC 1469 (-) TGAC 1727 (+) TGAC	"A core of TGAC-containing W-box" of, e.g., Amy32b promoter; Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway	(Zhang et al., 2004)
MYB1AT	1	571 (-) WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in Arabidopsis. MYB recognition sites in the rd22 promoter region function as cis-acting elements in the drought- and ABA-induced gene expression of rd22	(Abe et al., 2003)
MYBCORE	1	604 (+) CNGTTR	Binding site for all animal MYB and at least two plant MYB proteins, ATMYB1 and ATMYB2, both isolated from Arabidopsis	(Lüscher and Eisenman, 1990)
CPBCSPOR	2	780 (-) TATTAG 1561 (-) TATTAG	The sequence critical for Cytokinin-enhanced Protein	(Fusada et al., 2005)

			Binding in vitro, found in the promoter of the cucumber (CS)*POR ge*ne (NADPH-protochlorophyllide reductase)	
ABRELATERD1	3	806 (+) ACGTG 1320 (-) ACGTG 1321 (+) ACGTG	ABRE-like sequence. (ABRE: ABA-response elements)	(Simpson et al., 2003)
MYBST1	2	834 (-) GGATA 1160 (-) GGATA	Core motif of MybSt1 (a potato MYB homolog) binding site, can function as a transcriptional activator	(Baranowskij et al., 1994)
ERELEE4	1	948 (+) AWTTCAAA	"ERE (ethylene responsive element)" of tomato (L.e.) E4 and carnation GST1 genes. GST1 is related to senescence. ERE motifs mediate ethylene-induced activation of the U3 promoter region	(Luo et al., 2005)
GAREAT GAREAT	1	1001 (-) TAACAAR	GARE (GA-responsive element); Occurrence of GARE in GA-inducible, GA-responsible, and GA-nonresponsive genes found in Arabidopsis seed germination was 20, 18, and 12%, respectively.	(Ogawa et al., 2003)
BIHD1OS	2	1026 (-) TGTCA	Binding site of *OsBIHD1*, a rice *BELL* homeodomain transcription factor, in disease resistance responses	(Luo et al., 2005)
RAV1AAT	4	1635 (+) CAACA 1780 (-) CAACA 1920 (+) CAACA	Binding consensus sequence of Arabidopsis transcription factor, RAV1. RAV1 protein contain AP2-like and B3-like domains; which recognize CAACA and CACCTG motifs, respectively.	(Kagaya et al., 1999)
ARFAT	3	1192 (-) TGTCTC 1876 (+) TGTCTC 1892 (-) TGTCTC	ARF (auxin response factor) binding site found in the promoters of primary/early auxin response genes of *Arabidopsis thaliana*.	(Ulmasov et al., 1999)
AGL2ATCONSENSUS	1	1254 (-) NNWNCCAWWWWTRGWWAN	Binding consensus sequence of Arabidopsis (A.t.) AGL2 (AGAMOUS-like 2); AGL2 contains MADS domain; AGL2 binds DNA as a dimer	(Huang et al., 1993)
AGATCONSENSUS	1	1255 (+) TTWCCWWWWNNGGWW	Binding consensus sequence for the product of the Arabidopsis floral homeotic gene *AGAMOUS* (*AG*)	(Huang et al., 1993)
CARGATCONSENSUS	2	1258 (-) CCWWWWWWGG 1258 (+) CCWWWWWWGG	"CArG consensus" sequence found in the promoter of Arabidopsis SOC1 which is the MADS-box flowering-time gene	(Hepworth et al., 2002)
GARE2OSREP1	1	1421 (+) TAACGTA	"Gibberellin-responsive element (GARE)" found in the promoter region of a cystein proteinase (REP-1) gene in rice	(Sutoh and Ã, 2003)
ABRERATCAL	1	1320 (+) MACGYGB	ABRE-related sequence" or "Repeated sequence motifs" identified in the upstream regions of 162 Ca^{+2} responsive upregulated	(Fluhr and Fromm, 2006)

			genes (ABRE: ABA-response elements)	
DPBFCOREDCDC3	1	1637 (+)ACACNNG	A novel class of bZIP transcription factors, DPBF-1 and 2 (Dc3 promoter-binding factor-1 and 2) binding core sequence found in carrot.	(Kim et al., 1997)
CIACADIANLELHC	1	1799 (-) CAANNNNATC	Region necessary for circadian expression of tomato (L.e.) Lhc gene	(Piechulla et al., 1998)
CACGTGMOTIF	2	1320 (-) CACGTG 1320 (+) CACGTG	"G-box" Binding site of Arabidopsis GBF4; CrGBF1 and CrGBF2 in *C. roseus* can act as transcriptional repressors of the Str promoter via direct interaction with G-box; essential for expression of β-phaseolin gene during embryogenesis in different plants	(Chandrasekharan et al., 2003)

This table describe the cis-acting elements, the repetitions or number of times the element is present in the promotor region, its position in the promotor sequence and its description according literature. Blue: light-regulated genes, Purple: Embryo development related gene, Green: Transcription factor, Orange: hormone signaling.

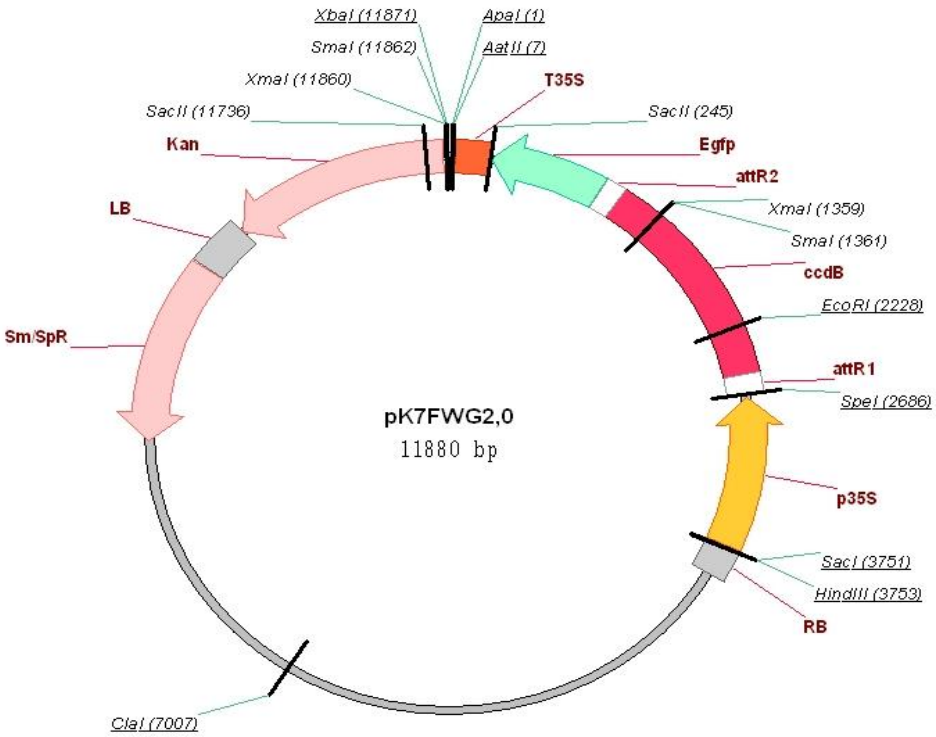

FIGURE 30. PK7FWG2 vector used for ectopic expression in Arabidopsis plants.

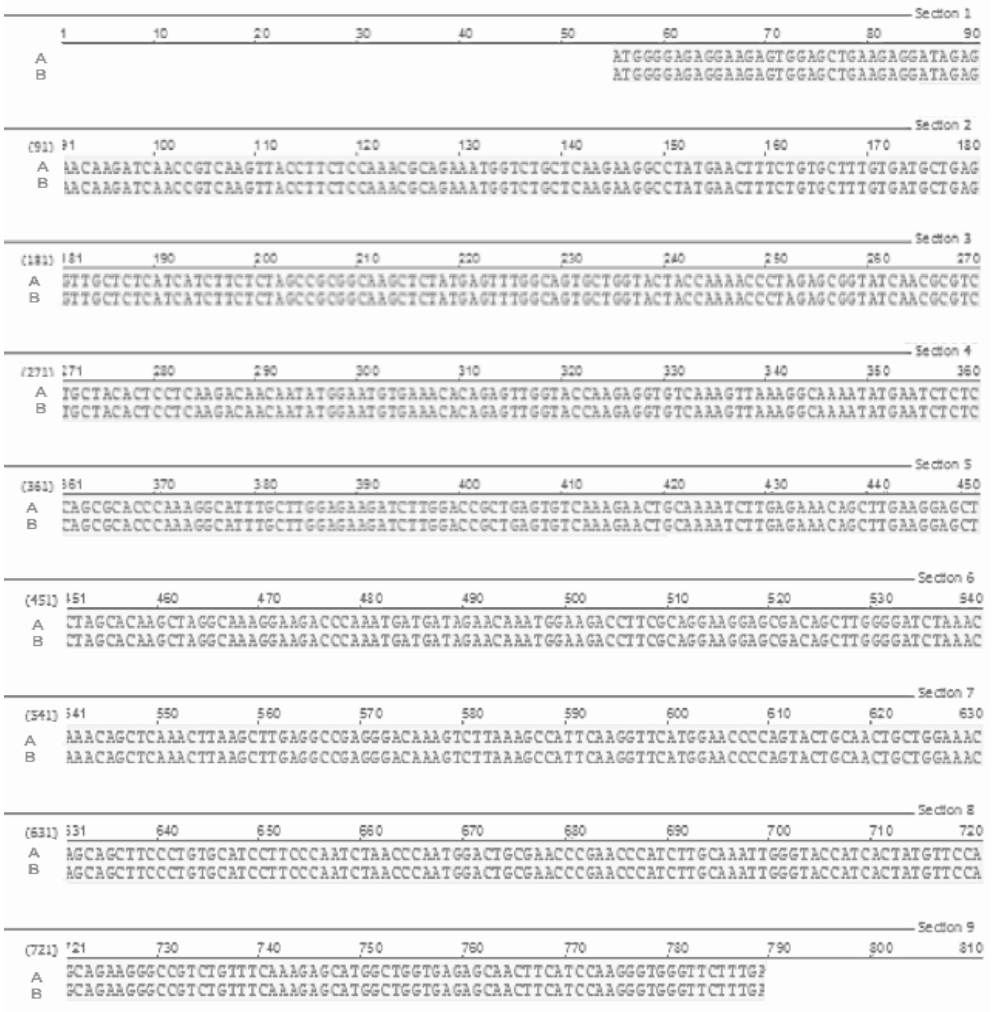

FIGURE 31. Aligning between the isolated *VvAGL6_1* and the predicted sequence.
A: Consensus CDS sequence obtained by cloning. B: predicted sequence of *VvAGL6_1INO* according to Genoscope database.

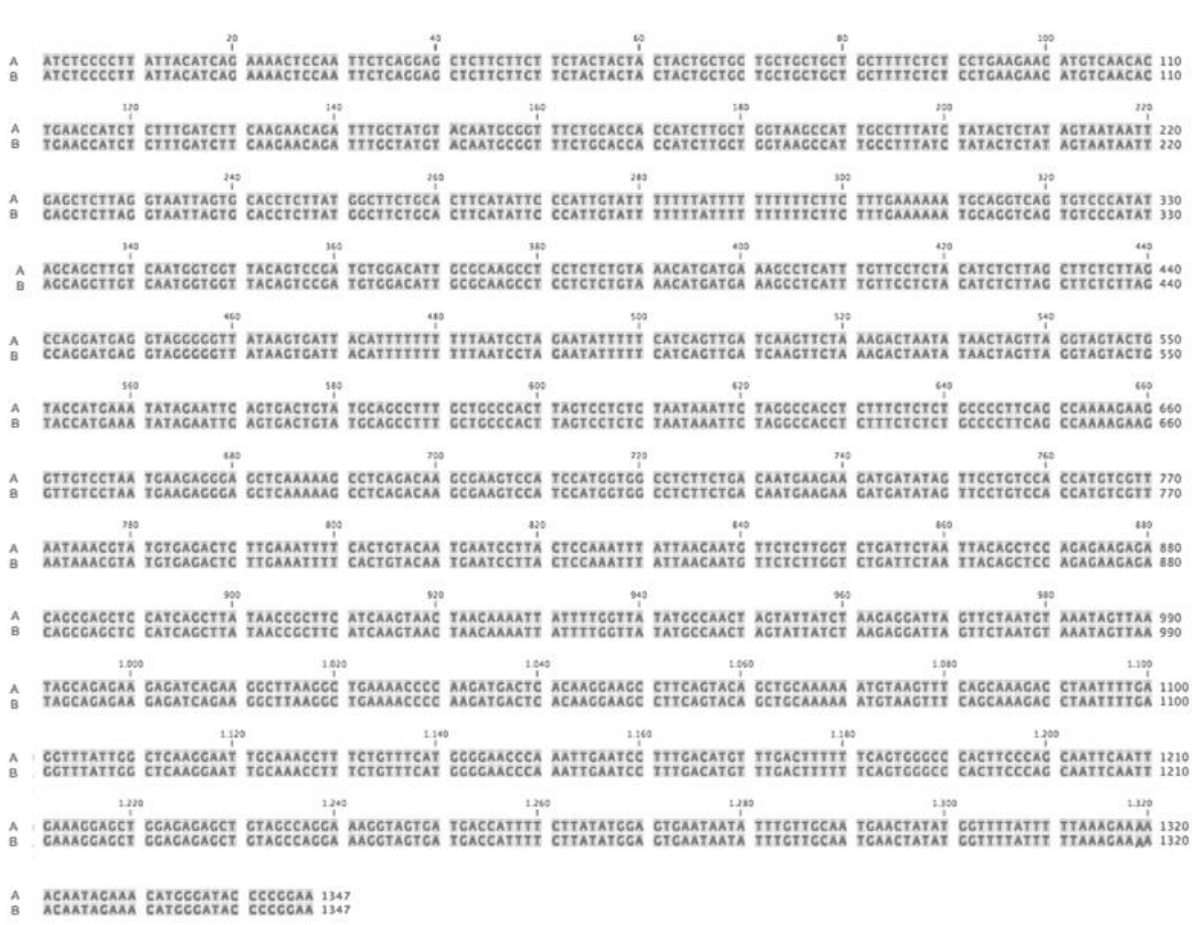

FIGURE 32. Aligning between the isolated *VvINO* and the predicted sequence.
A: Consensus genomic sequence obtained by cloning. B: predicted sequence of *VvINO* according to Genoscope database.

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