Comparative analysis of the expression of sex candidate genes in flower of dioecious and hermaphrodite grapevine (Vitis vinifera L. ssp.)

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Abstract

Vitis vinifera L. can be divided into two subspecies, *V. vinifera* subsp. vinifera, the cultivated grapevine, and its wild ancestor, *V. vinifera* subsp. sylvestris. Three flower types have been described: hermaphrodite and female in some varieties of vinifera, and male or female flowers in sylvestris. We have conducted an expression analysis of the functional genes candidate to sex determination in the newly defined sex locus described by Picq et al (2014) using four flower types. The candidate gene Ethylene overproducer-1 (ETO1) localized in the sex locus region and which inhibits the enzyme activity of the enzyme ACS (1-aminocyclopropane-1-carboxylic acid synthase) was showed highly significantly different expression pattern according to the sex flower. Other genes studied in the sex locus do not reveal significant different expression patterns. For genes located outside of the sex locus, only the SAUR (Small auxin up RNAs) protein and the ACS gene showed different expression among sex flowers. Therefore, as ETO1 is only expressed in female and hermaphrodite flowers, it could be a good candidate for the recessive female fertility mutation and ACS copy could be implied in the reaction cascade leading to the inhibition of stamens in female flowers. However, the ETO1 only negatively interacts with type 2 ACS and our ACS phylogeny analysis confirmed that the VviACS copy is not type 2. Therefore, it is unlikely that there is such molecular interaction in grapevine. Another hypothesis could be that the molecular mechanisms that regulated the activity of VviACS2 are induced by the VvETO1 protein regulating the activity of both families of ACS type I and type 2. The last gene showing differential expression according to sex is the SAUR protein. This gene consists in early auxin response genes family playing key role in hormonal and environmental signals. Our results pointed out that one gene (ETO1) inside of the flower sex locus region and two genes (ACS, SAUR) located outside of the sex locus region, could be considered as putative candidate genes for the control of sexual traits in grapevine.

1 Introduction

Among flowering plants, dioecy, i.e male and female flowers on separate individuals, occurs in only 5-6 % of the species, and has evolved from hermaphroditism independently in many phyla (Renner 2014). The emergence of dioecy is thought to follow the perfect linkage between two mutations with complementary dominance: a recessive mutation resulting in male sterility and a dominant female-suppressing factor (Charlesworth and Charlesworth 1978a). This two-factor sex determination model has been confirmed in some plant species as in garden asparagus (*Asparagus officinalis*) or in date palm (*phoenix* ssp.) (Harkess et al. 2017; Torres et al. 2018). In *Actinidia* ssp. (kiwifruit), a cytokinin response regulator, named *Shy Girl* (*SyGI*) acts as the suppressor of female development, while a fasciclin-like gene named *Friendly boy* (*FrBy*) enables the maintenance of the male functions (Akagi et al. 2018; Akagi et al. 2019). However, the two-factor model is not the only path to dioecy, a single factor is a possible alternative (Charlesworth and Charlesworth 1978b). In diploid persimmon (*Diospyros lotus*), the *OGI* gene encodes a small RNA that regulates in dosage-dependent fashion the *MeGI*, a homeodomain transcription factor regulating anther fertility (Akagi et al. 2014). Thus, in the past few years, the discovery of genetic mechanisms for sex determination in a handful of plant species has strengthen the two main theoretical models advanced to explain the emergence of dioecy (one or two-factor model). The study of additional species in a wider taxonomic sample will no doubt be valuable to perfect our understanding of the dioecy evolution in Angiosperm.

The wild grapevine *Vitis vinifera* subsp. *sylvestris* is the wild ancestor of the domesticated grapevine *Vitis vinifera* subsp. *vinifera* (Levadoux 1956), cultivated for wine and table production. During grapevine domestication, the sexual system has incurred a radical evolution, with the change from dieocy, to hermaphroditism i.e. flowers with both functional sexes (This et al. 2006).). For grape cultivation, the switch to hermaphroditism ensures greater yield given that all individuals contribute to fruiting and to pollination (This et al., 2006). The male flowers in wild grapevines possess erected stamen producing fertile pollen, and a pistil reduced to a very small but viable ovary on which the style and the stigma do not develop (Valleau, 1916; Levadoux 1956; Gallardo et al. 2009). The carpel becomes sterile as a result of the embryo sac abortion in a fully developed ovule (Caporali et al. 2003). Interestingly, certain male flowers present a more developed pistil (android), and in favorable conditions can produce fruit (Levadoux, 1946; Picq, pers. Com.). In female flowers, the pistil is well developed, and the stamens are curved and produce ovale sterile pollen (Valleau, 1916; Levadoux 1956; Gallardo et al. 2009). Pollen infertility is cause by an abnormal microspore cell wall architecture (Caporali et al. 2003). Thus, the abortion of reproductive organs resulting in unisexual flower occurs in the very last stages of flower

development. Based on the inheritance of sex in different progenies, sex determination in *Vitis* was supposed to be controlled by an unique sex locus with three alleles, M (male), H (hermaphrodite) and F (female), in the following dominance relationship: M>H>F (Valleau 1916; Oberle 1938; Antcliff 1980; Carbonneau 1983). Genetic map and population genomics analyses confirmed the presence of a single sex-determining region of about 150 kb located on the chromosome 2 between position ~4.90 and 5.05 Mb (Fechter et al., 2012; Picq et al., 2014; Zhou et al. 2017; Zhou et al. 2019). These same studies also support the existence of three alleles with the allelic combination for each sex: MF or MH for male, HF or HH for hermaphrodite, and FF for female. The sex locus of the grapevine displays haplotype diversity, linkage disequilibrium and differentiation (Picq et al. 2014; Zhou et al. 2017; Zhou et al. 2019) that typically correspond to a small XY non-recombining region (Ming et al. 2011). Such a region is expected under the "two-factor model" of sex determination in dioecious species (Charlesworth and Charlesworth 1978a). Thus, assuming a two-factor model in *Vitis*, the F allele contains a recessive, "loss-of-function" type, male sterility mutation, while the M allele harbors a fully-functioning male fertility allele coming together with a dominant sterility female mutation (Charlesworth 2013). The allele H may derive from the allele M through the loss of the dominant female sterility mutation. This is coherent with genetic diversity analyses revealing a closer proximity between the H and M allele (Picq et al. 2014). In the sex locus, several genes have been already suggested as good functional candidates for flower sex determination in grapevine: the *flavin-containing monooxygenase* (FMO), the *adenine phosphoribosil transferase* (VviAPRT3), and the *Ethylene Overproducer-like 1* (ETO1) (Fechter et al., 2012; Picq et al. 2014). Indeed, the expression pattern of VviAPRT3 assessed by RT-qPCR revealed a higher expression in the carpel primordia of male plants suggesting a possible role in the abortion of the pistil (Coito et al. 2017). For the FMO gene, transcriptomic analyses showed differential expression among sex (Ramos et al. 2014; Zhou et al. 2017; Zhou et al. 2019). However, the gene expression are female or male biased according to the reference genome used, the F haplotype in the PN40024 12X (Jaillon et al. 2007) or the H haplotype in the Char04 reference (Zhou et al. 2019) respectively.

In order to get a more detailed understanding of flower sex locus in grapevine we have analyzed several candidate genes which were predicted in the genomic region described by Picq et al., (2014) and they were used in order to know which one could be involved in the formation of flower sex. The present study used qPCR analysis for a comparation of differentially expressed genes during different developmental stages in the male, female and hermaphrodite flowers of grapevine.

2 MATERIALS AND METHODS.

2.1 *Tissue collection.*

The plant material consisted of 2 female and 4 male wild grapevines (*V. v. sylvestris*), and 1 hermaphrodite cultivated grapevine (*V. v. vinifera*; Pinot noir cutivar). These 7 accessions are maintained at the germplasm bank of El Encín (IMIDRA, Madrid, Spain). The 6 wild grapevines were originally collected in two different sites in Spain (north vs. south; 1 female accession CA1.6 and 2 males accessions CA2.2 and H7.7 (android) from the South of Spain and 1 female accession LE1.6 and 2 males accession S 2.9 and NA2.5 (android) from the North of Spain, and introduced 16 years ago in the germplasm center. Regarding the male individuals, we have included male type and androids (pistil uncompletedly aborted).

Floral buds were collected in 2010 at 6 inflorescence developmental stages (Figure 1): rosette of leaf tips visible (stage 5), shoots10 cm long with 5 unfolded leaves and inflorescence visible (stage 12), shoot with 8 unfolded leaves and single flowers in compacted groups (stage 15), 14 unfolded leaves and flower caps still in place but their colour fading from green (stage 18),17-20 leaves separated and 50% caps off (stages 23), and cap fall complete (stage 26). We have choose those developmental stages because the morphological differentiation between male and female flowers of the dioecious grapevine can only be identified at a late stage of flower development, since at early stages a hermaphrodite development pattern is observed (Caporali et al., 2003). The growth stages description and numerical codification follow the scale developed by Combe et al. (1995). In all this work we will make the assumption that some stages are more important such as 15 and 18 for biological reason of flower development (Caporali et al., 2003). Until early 15 stage there are no morphologic cues that allow distinguish male from female plants or even from hermaphrodite ones (Figure 1). At a later developmental stage (stage 18), male and female flowers show the first morphological indication before blooming. Floral bud samples were immediately frozen in liquid nitrogen and maintained at -80ºC until their analysis.

2.2 *Total RNA isolation, purificacion and cDNA synthesis.*

RNA was extracted from grounded frozen floral buds following the protocol developed by Zeng and Yang (2002). Then, the total RNA was purified and concentrate using a MicroElute® RNA Clean up Kit (Omega bio-tek, Norcross, USA). To eliminate genomic DNA from total RNA preparation, DNA digestion was done with theDNase I digestion set (Sigma-Aldrich, St. Louis, USA). RNA concentration and purity were assessed using a NanoDrop ND-1000 spectrophotometer

(NanoDrop Technologies, Wilmingon, DE, USA). RNA integrity was checked by electrophoresis in 1% agarose gels. Eventually, cDNAs were synthesized from 0,5-1µg of total RNA using the Superscript^{TM} III first-strand Super-Mix for qRT-PCR (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions.

2.3 *PCR primer design.*

We have used the grapevine reference genome (12X.v1) to annotation and design primers. Primers for the genes *flavin-containing monooxygenase* (FMO;VIT_02s0154g00160), *Ethylene overproducer-1* (ETO-1; VIT200s0233g00090), *WRKY transcriptional factor* (VIT_02s0154g00210), *SAUR protein* (VIT_02s0154g00010), *NAC domain protein* (VIT_02s0154g00020), AGAMOUS (VIT_02s0025g04980); YABBY (VIT_02S0154G00070), *1-aminocyclopropane-1-carboxylic acid synthase* (ACS) gene (VIT_02s0234g00090), ACS-X (VIT_02s0025g04650) and ACS-S (VIT_02s0025g04980; identified by Marguerit et al. 2009) were designed using PRIMER3 software (Misener et al., 2003; Table 1). VviAPRT3 and VviFSEX primers used were described by Coito et al. (2017). The housekeeping gene EF1-α was employed as control gene, using published primers (Reid et al., 2006).

2.4 *qPCR conditions and analysis*.

The expression pattern of selected genes was validated by quantitative PCR method using three independent biological replicates for each flower gender accession and developmental stage, mean of each gender and stage, except hermaphrodite, were calculated. PCR reactions were performed in 96-well plates with an ABI PRISM® 7300 Real Time PCR system (Applied Biosystems, Foster City, USA) using SYBR® Green to detect dsDNA synthesis. Reactions were done in 20µl volumes containing 0.8 µl of each primer 5µM, 10µl of 2× PerfeCTaTM SYBR Green SuperMix with ROX (Quantabio, Beverly, USA) and 2µl of cDNA (corresponding to ~6 ng). Reactions conditions were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Dissociation curve was obtained to verify the specificity of each amplification reaction. Each PCR reaction was completed in duplicate. Data were analyzed using the SDS v1.4 software (Applied Biosystems, Foster City, USA). Expression levels were determined as the number of amplification cycles needed to reach a fixed threshold in the exponential phase of the PCR reaction (Ct). All amplification plots were analyzed with an Rn threshold of 0.2 to obtain Ct values. The PCR efficiency was determined for each gene with LinReg software (Ramakers et al., 2003), which uses absolute fluorescence data captured during the exponential phase of amplification of each reaction. Relative expression was obtained as Ct GeneEfficiency/ Ct EF1-αEF1-α Efficiency (Pfaffl, 2001) and this

value was corrected with the value obtained for the Pinot noir control in both experiments. In this study, we have used a T-student analysis with the cutoff for statistical significance p value is < 0.05.

2.5 *Sequence analysis*

The PCR amplified fragments for the candidate gene ACS were sequenced in both directions to ensure sequence authenticity. We have sequenced the ACS gene in 8 wild grapevine accessions (GenBank accession MN539724-MN539735) that correspond 2 female accessions (CA1.6 ;CA2.4) and 2 males accessions (CA2.2; H7.7) from the South of Spain and 1 female accession (LE1.6) and 3 males accessions (S 2.9; NA2.5; SS3.5). Sequence analysis was carried out using BLAST searches (http.//www.ncbi.nlm.nih.gov/BLAST/). Nucleotide and amino-acid sequences were aligned using ClustalW (Thompson et al., 1994). The orthology analysis according MCMC (Arvestad et al., 2003) were developed using Bayesian software program MrBayes 3.2.7 (Ronquist et al. 2012).

1 RESULTS.

3.1 *Expression analysis of candidate genes*

In the present study, we propose to characterize expression of genes located in the sex-determining region of about 150 kb for male, female and hermaphrodite flower using qPCR technology in order to identify candidate for sex determinism in grapevine. Based on previous work in grapevine and knowledge in other plant species, we selected the adenine phosphoribosil transferase (VviAPRT3), flavin-containing monooxygenase (FMO, VIT_02s0154g00160), the Ethylene Overproducer-like 1 (ETO1) (VIT_200s0233g00090), VviFSEX (VIT_02s0154g00200) and the WRKY transcription factor (VIT_02s0154g00210) (Figure 2). We also considered genes outside of the sex locus in the chromosome 2 as they are potentially implied downstream in sex determination pathway: SAUR protein (VIT_02s0154g00010), NAC domain protein (VIT_02s0154g00020), AGAMOUS protein (VIT_02S0025G04650), and the YABBY protein (VIT_02s0154g00070) already identified by Battilana et al. (2013) (Fig 2). Eventually, we added the 1-aminocyclopropane-1-carboxylic acid synthase (ACS-S; VIT_02s0234g04980; Marguerit et al.2009) known to be involved in flower sex determination in melon (*Cucumis melo*, Boualem et al., 2009). We also considered two other ACS copy also located in chromosome 2: the ACS (VIT_02s0234g00090) and the ACS-X (VIT_02S0025G00360) (Figure 2)

In order to test if the differential expressions for the candidate genes in the sex locus region and outside of the region, the expression patterns of the candidates genes were analyzed at the six developmental stages of the female, male and hermaphrodite floral buds by qPCR (Figure 3). The results from these qPCR experiments showed that in the sex locus, the gene *Ethylene overproducer-1* (ETO1) showed significant different expression pattern according to the sex flower (Figure 3B). This gene is highly expressed in stage 18 in female e flowers and in stage 15 in hermaphrodite flowers. The SAUR protein showed different expression among sex flowers. This gene was more strongly expressed in the stage 18 for female flowers. The other genes studied in the sex locus i.e. the *adenine phosphoribosil transferase* (VviAPRT3), *flavin-containing monooxygenase* (FMO), VviFSEX and the *WRKY transcription factors* (Figure 3C, D, E, H) does not reveal significant different expression patterns. For genes located outside of the sex locus and possibly implied downstream in sex determination pathway, only the *1-aminocyclopropane-1-carboxylic acid synthase* (ACS) gene (VIT_02s0234g00090) showed different expression among sex flowers. This gene is more strongly expressed in female flowers in the stage 15 and 18 (Figure 3A). Regarding the ACS copies we have found no differential RNA expression for the ACS-S and ACS-X copies outside of the sex locus region. These elements converge to exclude these ACS-S and ACS-X copies as functional candidate genes for sex determinism in grapevine.

The genes related with flower development as YABBY and AGAMOUS genes appeared to have a higher expression in the stage 15 in the hermaphrodite flowers (Figure 3I and J) at the late stages of flower development. These results pointed out that sexual determination can occur during great part of flower development.

In our analysis, we have included the phenotype called "android" (male with pistil uncompletedly aborted) we have observed different expression level between male and android flowers of the putative candidate genes located in the flower sex locus as APRT3 and FSEX in early developmental stages and outside of the flower sex locus as YABBY and AGAMOUS in the late developmental stages. (Figure 3 C, D, I and J).

3.2 *Isolation and characterization of the VvACS gene copies*

Taking in consideration that the ACS gene was associated with sexual dimorphism in different species as melon (Yoshida et al., 2005) we have analysed the sequence of the different ACS copies. We have designed PCR primers to amplify a 2420-bp genomic fragment. Based on the amino acid sequence of C-terminal region the ACS proteins can be divided into three types (type 1, 2 and 3) (Yoshida et al., 2005). After sequencing, phylogenetic and molecular analyses were conducted using MEGA version Mrbayes program, by comparing the three ACS-like copies outside of the sex locus region with ACS-like sequences from different plant species (Figure 4). The phylogenetic tree, representing 88 ACS sequences, belonged ACS, ACS-like, and Alanine Aminotransferases from different plant species showed that ACS-X and ACS copy are similar to ACS peptides involved in the synthesis of ACC (1-aminocyclopropane-1-carboxylate). The results indicated that the ACS copy showed the highest homology value with CsACS2 (Figure 4) and they are inside type 1 ACS group. Thus, we have concluded that ACS copy is likely the cucumber orthologue of CsACS2, and for that reason we have renamed the ACS copy as VviACS2. The ACS-X had the lowest homology to known ACS are related with AtACS7 and belong to type 3 ACS. Finally, the ACS-S was closely matched to AtACS10 and AtACS12, which are presumed as putative amino acid transferases without ACS activity (Boualen et al., 2008).

Sequence analysis of VviACS2 showed that it has a 73% homology to the ACS copy from melon (ACS2). In addition, the predicted protein of VviACS2 (506 amino acid) and the ACS protein in cucumber, CsACS2 (445 amino acid), share 98% of identity and only differ in eight residues, all of which are located in non-conserved positions among seed plants.

4 DISCUSSION.

The objective in this work was to identify the candidate genes in sex specification in *Vitis vinifera* subs sylvestris by comparation with *Vitis vinifera* subs sativa using expression patterns analysis. Three genes possibly playing a role in sex specification have been detected: VviETO1; VviACS2 and VviSAUR.

Sex determinism in *Vitis* is supposed to be controlled by a single sex-determining region with three alleles, M (male), H (hermaphrodite) and F (female) and located on the chromosome 2 between position ~4.90 and 5.05 Mb (Fechter et al., 2012; Picq et al., 2014; Zhou et al. 2017; Zhou et al. 2019). Based on previous work, we analyzed expression pattern of genes that might contribute to sex determination of grapevine flower (Fechter et al., 2012; Picq et al., 2014;Coito et al. 2017; Zhou et al. 2017; Zhou et al. 2019). Among the 5 genes studied in the sex locus, only the *Ethylene overproducer-1* (ETO1) revealed highly different expression between sexes: this gene is over-expressed in female and hermaphrodite flower in the last stages of development, stage 18 and stage 15 respectively. As mentioned in the introduction, if we assume a two-factor model for sex determination in grapevine, the F allele contains a recessive male sterility mutation, while the M allele harbors a fully-functioning male fertility allele coming together with a dominant sterility female mutation (Charlesworth 2013). The allele H may derive from the allele M through the loss of the dominant female sterility mutation. Thus, as ETO1 is only expressed in female and hermaphrodite flowers, it could be a good candidate for the recessive female fertility mutation shared by the F and H alleles. Indeed, the *eto1* is a recessive mutation in Arabidopsis that results in a 10-fold ethylene overproduction (Guzman and Ecker, 1990), and in Cucumis ethylene favors development of female organs (see Henry et al. 2018). However, in previous studies, sequence diversity and gene expression analysis did not find differences between female/hermaphrodite and male individuals (Picq et al. 2014, Zhou et al. 2019). Future works will have to be conducted to understand this discrepancy between studies.

The gene VviACS2 involved in ethylene hormonal production (Kende, 1993), shown higher expression in female flowers and hermaphrodite, but particularly in female, that in male and android at the stage 15 and 18. This observation suggests that this gene could be involved in pollen sterility. Moreover, the gene encoding the enzyme 1-aminocyclopropane-1-carboxylic acid synthase (ACS), involved in melon male organ sterility and located close to the grapevine sex locus, could be considered as a putative candidate gene for the control of flower sex in grapevine. Indeed, most of the molecular studies in cucumber have targeted the role of ethylene in sex determination, specifically the role of the key regulatory enzyme of ethylene biosynthesis, ACS (Knopf and Trebitsh, 2006; Li et al., 2009; Martin et al., 2009; Mibus and Tatlioglu, 2004; Shiber et al., 2016; Trebitsh et al., 1997). It has been described that the genes involved in sex

249 determination are related to ethylene biosynthesis and perception (Ando and Sakai, 2002; Saito et al., 2007; Yamasaki et
250 al., 2000) such as CsACS1 and CsACS2 (Li et al., 2009; Martin et al., 2009; Saito et al., 2007; Shiber et al., 2016;
251 Trebitsh et al., 1997). Our sequence analyses showed that VviACS2 gene is ortholog to CsACS2 which correspond to the
252 type 1 isoform from melon in which it is differential expressed in grape flower development. Although this gene is
253 outside of the flower sex locus, it could be possible that some proteins from the sex locus region affect the ACS gene
254 expression. In fact, one gene in the sex locus region, annotated as ETO1, has been described as a protein that specifically
255 inhibits the enzyme activity of ACS (Yoshida et al., 2005). The results suggest that the high expression of ETO1 in the
256 stage 15 of hermaphrodite flower could induce the repression of the ACS in the stage 18 allowing the hermaphrodite
257 flower development. In the other hand, the significant high expression in the female flower in the stage 18 allowed the
258 development of female flower and putative involved pollen sterility. However, Yoshida et al (2005) showed the
259 interaction between ETO1 and ACS protein family is restricted to type 2 ACS isozymes which possess specific C-
260 terminal amino acid sequences. Yoshida et al (2005) also showed that the suppression of a type 2 ACC synthase, in
261 transgenic tomato was produced by the constitutive expression of ETO1. These results suggest that members of the ETO1
262 protein family are negative regulation of type 2 ACC synthases in the plant kingdom. A negative interaction between
263 ACS and ETO1 in grapevine would be not easy to explain if we suppose that ETO1 is possibly the recessive female
264 fertility mutation and ACS is involved in the inhibition of stamens in female flowers. Actually, the ETO1 only negatively
265 interacts with type 2 ACS (Yoshida et al. 2005), and our ACS phylogeny analysis combined with a previous work by Xu
266 and Wang (2012) confirmed that the ACS copy studied here is not type 2. Therefore, it is unlikely that there is such an
267 interaction in grapevine. Another hypothesis could be that the molecular mechanisms that regulate the activity of
268 VviACS2 are induced by the VvETO1 protein regulating the activity of both families of ACS type I and ACS type 2 (Li
269 et al., 2011), this coordinate regulation is necessary for flower development. In this way, our results suggest that the gene
270 VviACS2 could be involved in sex development but the molecular mechanisms is unclear. The VviACS2 expression data
271 agree with those reported by Saito et al (2007) for CsACS2 on melon, of which our candidate gene VviACS2 is
272 orthologous. Saito et al (2007) proposed that the mechanisms of action of CsACS2 was that the expression of CsACS2
273 was mainly accumulates just under the pistil primordia of flower buds at the stage 6 in cucumbers which correspond with
274 sexual determination stage (Bai et al., 2004). Saito et al (2007) found that the persistent accumulation of CsACS2 mRNA
275 was correlated with the expression of the active enzyme inhibits the development of male organs and is not required for
276 carpel development. These findings suggest the relationship between the permanent arrest of stamen development and the
277 expression of CsACS2. In addition, Li et al. (2012) suggested a positive feedback mechanism for CsACS2 gene leading

to a stable level of transcription, and this level might produce ethylene constantly, and then continually prevent the stamen development. These results suggest that the ethylene-responsive elements (EREs) in the cucumber CsACS2 promoter had a conserved function. However, the model for melon may be not completely adapted for grapevine, since the ACC synthase will not allow enough ethylene accumulation to eliminate the development of stamen primordia, because in grape female flowers the suppression of maleness appears to be the consequence of pollen sterility (Caporali et al., 2003). In this way, further studies should be done in the future.

The last gene showing differential expression according to sex is the SAUR protein (*SMALL AUXIN UP RNAs*). SAURS consists in a large early auxin response genes family playing key role in hormonal and environmental signals that regulate plant development and growth (Ren and Gray, 2015). In the current state of knowledge, it is difficult to explain the role of SAUR proteins in the determinism of sex in the grapevine. However, it has been described in grapevine that a synthetic kinin, SD 8339, at 1000 parts per million in alcohol solution, applied to flower clusters of a male grapevine about 3 weeks before anthesis, completely converted the flower sex from male to hermaphrodite (Negi and Olmo, 1966). Therefore, sex reversion by hormonal application may indicate that this gene can be involved in hormonal signaling and could be important in the development of male and female flowers. Recently, It has been described by (Ni et al., 2018) that cytokinin regulated the biosynthesis, transportation and signaling of other phytohormones in the regulation of sex determination in *S. sebiferum* (oil plant) then they suggest some cross talk between different hormones including ethylene that could be involved in flower development. Taking into consideration the cross talk between hormones, we cannot exclude that flower type and sex specification may be controlled through hormone regulation.

Two of the most obvious candidate genes are the flowering-related genes which map close to important regions controlling flower sex in grapevine, *AGAMOUS and YABBY*, which along with other genes in *A. thaliana* are involved in the specification of stamens, carpels and ovules (Mizukami and Ma 1992; Ray et al. 1994; Boss et al. 2001). We have showed that these genes involved in different carpel structures development showed significant expression difference in the stage 15 during grapevine hermaphrodite flower development. The high expression level of these genes in this stage could be linked to their role in determining the carpel. Finally, we have to consider that could be possible that the genome assembly and annotation could be fragmented or incorrect at this 150 kb sex-linked region as has been described previously (Ramos et al., 2014).

Conclusions

The results pointed out that one gene (ETO1) inside of the flower sex locus region and two genes (ACS, SAUR) located outside of the sex locus region, could be considered as putative candidate genes for the control of sexual traits in grapevine. All these genes are related with hormone biosynthesis or signaling. However, it is difficult to distinguish normal floral development pathways from the abnormal carpel formation through this approach, since these pathways seem dependent on an expression balance of hormone related genes (Coito et al., 2019). The mechanism of sex determination is of great interest to researchers. However, the direct regulators and the molecular details in grape remain poorly understood. Nevertheless, other genes in other regions could be involved in flower sex determins in *Vitis*.

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7. Author Contributions Statement

RA conceived the idea and design of the study and wrote the manuscript; DC, AB and AV developed the expression analysis, performed the phylogenic analysis studies and participated in the drafting of the manuscript. SP, RB and PT were involved in discussion and interpretation of the results and oversaw the final draft and revisions.

8. Conflict of Interest Statement

The authors declare that they have no conflict of interests

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Figures

Figure 1. Sampled points from the shoot and inflorescence development for the gene expression study. Adapted from Coombe et al (1995).

Figure 2. Genes located in the sex locus defined by Fetcher et al. (2012) and Picq et al. (2014).

Figure 3. Expression levels of the genes A) *VvACS*; B) *VvETO* ; C) *VvFSEX* **;**D) *VvAPTR*3 ; E) *VvWRKY* ; F) *VvSAUR* ; G) *VvNAC4* ; H) *VvFMO* ; I) *VvYABBY* and J) *VvAGAMOUS* throughout flower development in four genders evaluated by qPCR using three independent biological replicates for each flower gender.

Figure 4. Phylogenetic analyses of 88 ACS copies from different species: Arabidopsis, tomato, rice, *Amborella trichopoda*, conifers, the lycophyte *Selaginella moelendorfii*, the mosss *Physcomitrella patens*, humans, the cnidarian *Nematostella vectensis*, and the green alga *Ostreococcus lucimarinus, Chlamidomonas and Volvox carteri.* The arrows showed the *Vitis vinifera* L ACS copies. Clade values are indicated at nodes.

Table 1. Primers used on qPCR. The primers for APTR3 and FSEX were described in Coito et al (2017).

Primers	Sequence
YAB Fw	5´ACG CCT TCT TCT CTC CTT CC 3´
YAB Rv	5´AAG TCA TTT GCG GTG GTC TG 3´
AGAM Fw	5´CGC TAC CAA AGT AAA GCC AAG 3´
AGAM Rv	5´CAA ACA TTC GCC TAA TAG TCT TCG 3´
FMO Fw	5´CGG TGT TCT CTC CGA TCG GAT TA 3´
FMO Rv	5´AGC CAT TGT ACT CGA ACA GAT GGG 3´
SAUR Fw	5´GCG AAA TCA AAG TCC GAG AG 3´
SAUR Rv	5´GGA AAA CAG AGC CCC TTA GC 3´
NAC Fw	5´ATT GAG CCA TGG GAT CTT CA 3´
NAC Rv	5´CAG AAT CCG GCT TTT GTA GC 3´
WRKY Fw	5´CTT TCA GAC TGG CCA TCC AT 3´
WRKY Rv	5´TGA TCC AAG ATG CAA CAA GC 3´
ETO-Fw	5´CAG GCC CTT AAC AAC CTT GGC 3´
ETO-Rv	5´AAT GAA CCC TAG CAA GGC CC 3´
ACS-X Fw	5´GAT CCT GGT GAT GCA TTC CT 3´
ACS-X Rv	5´TGT TGT CCT CTT GGG CTT TC 3´
ACS Fw	5´CCG GCA ATG AAA TAC TCA CA 3´
ACS Rv	5´TAT CCA CCC CAG TTC TCC AC 3´

Figure 1

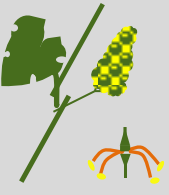
Stage 5: Rosette of leaves tips visible.

Stage 12: Shoots about 10cm long. Inflorescence clear, 5 leaves separated

Stage 15: 8 leaves separated;Shoot elongating rapidly; single flowers in compact groups

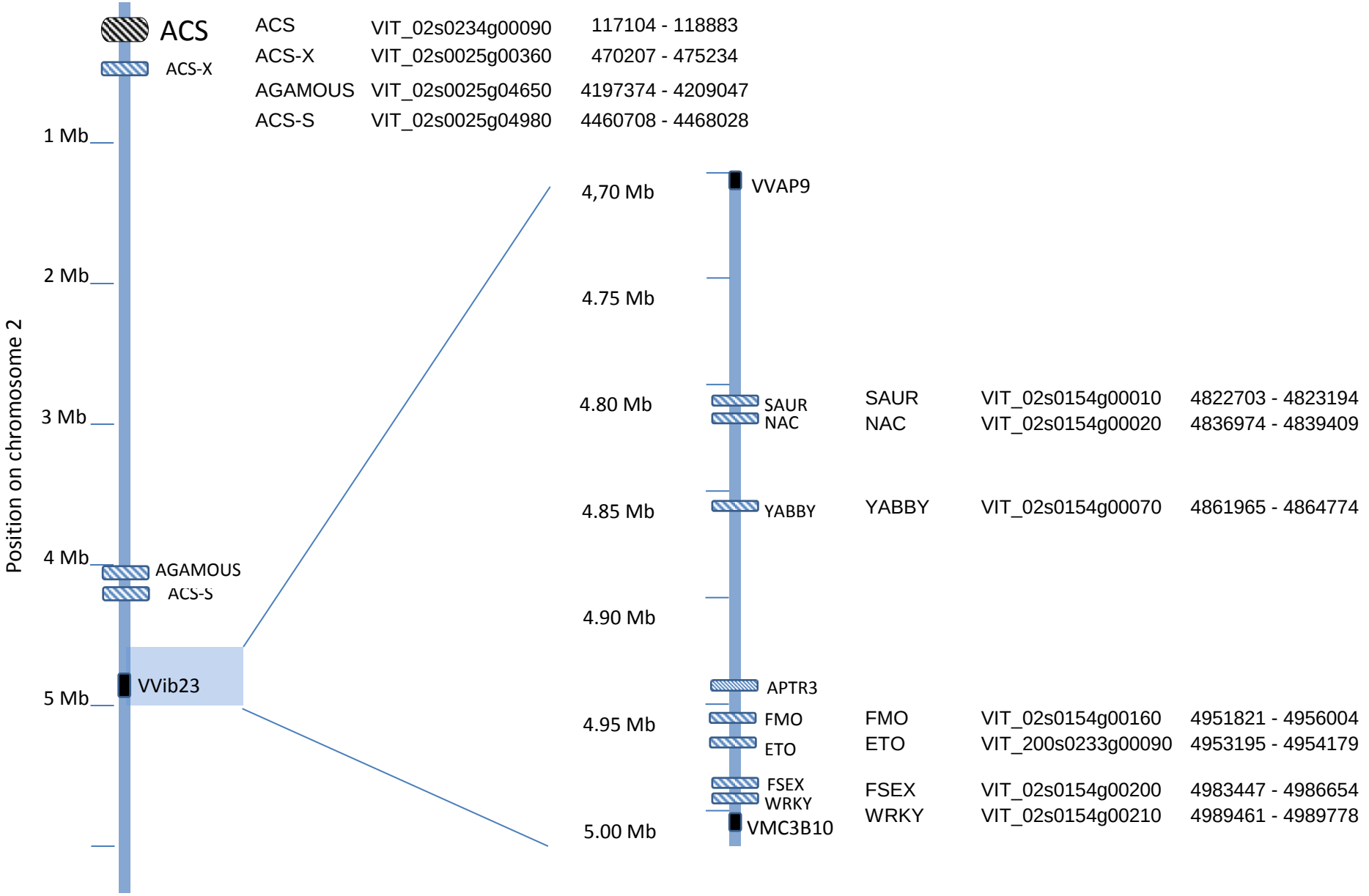
Stage 18: 14 leaves separated; flower caps still in place, but cap colour fading from green.

Stage 23: 17-20 leaves separated; 50% caps off (=full bloom)



Stage 26: Cap fall complete

Figure 2


ACS
ACS-X
AGAMOUS
ACS-S
VVib23
1 Mb
2 Mb
3 Mb
4 Mb
5 Mb
Position on chromosome 2
ACS VIT_02s0234g00090 117104 - 118883
ACS-X VIT_02s0025g00360 470207 - 475234
AGAMOUS VIT_02s0025g04650 4197374 - 4209047
ACS-S VIT_02s0025g04980 4460708 - 4468028
4,70 Mb
4.75 Mb
4.80 Mb
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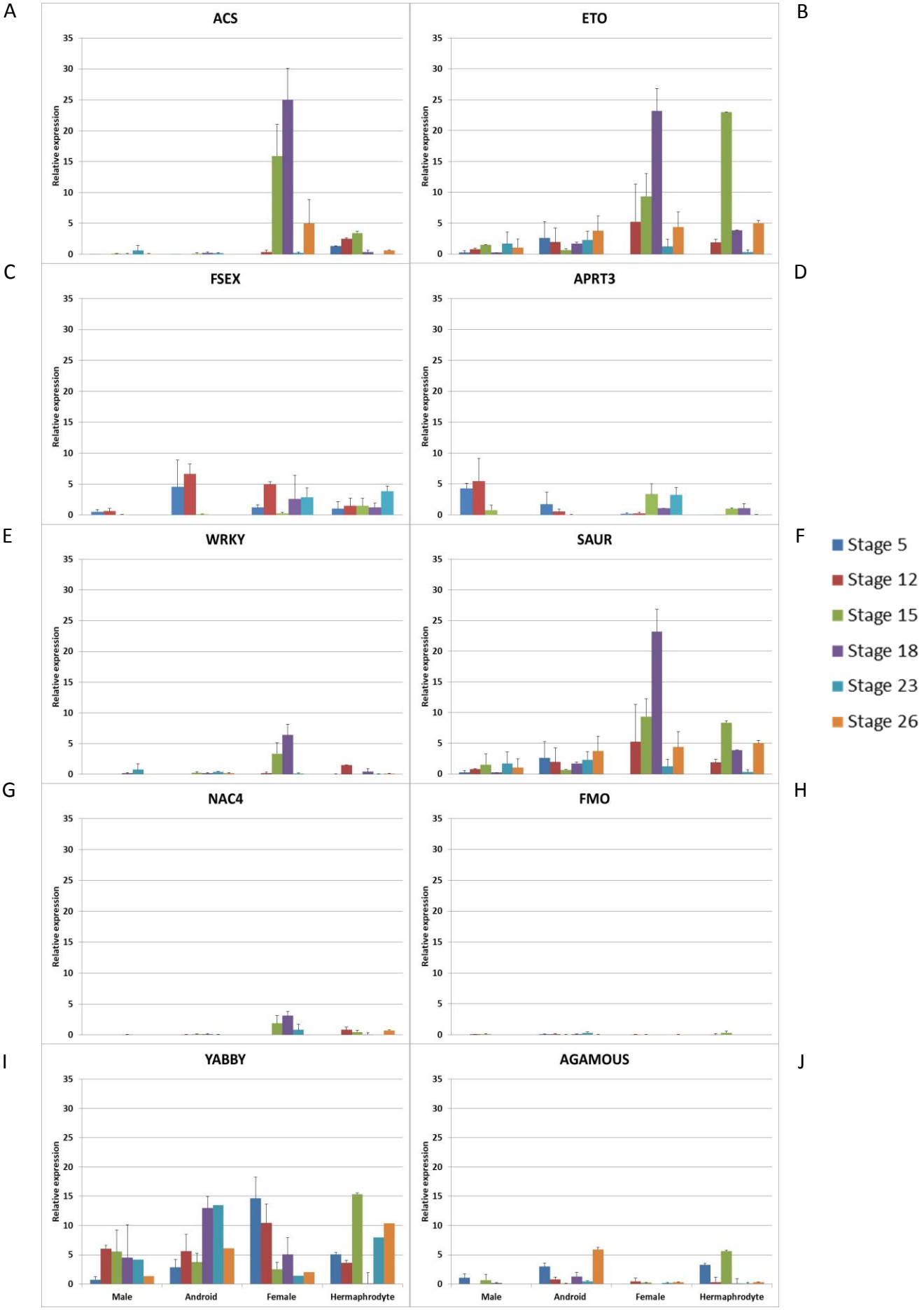
VVAP9
SAUR
NAC
YABBY
APTR3
FMO
ETO
FSEX
WRKY
VMC3B10
SAUR VIT_02s0154g00010 4822703 - 4823194
NAC VIT_02s0154g00020 4836974 - 4839409
YABBY VIT_02s0154g00070 4861965 - 4864774
FMO VIT_02s0154g00160 4951821 - 4956004
ETO VIT_200s0233g00090 4953195 - 4954179
FSEX VIT_02s0154g00200 4983447 - 4986654
WRKY VIT_02s0154g00210 4989461 - 4989778

Figure 3


A
ACS
B
ETO
C
FSEX
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APRT3
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WRKY
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NAC4
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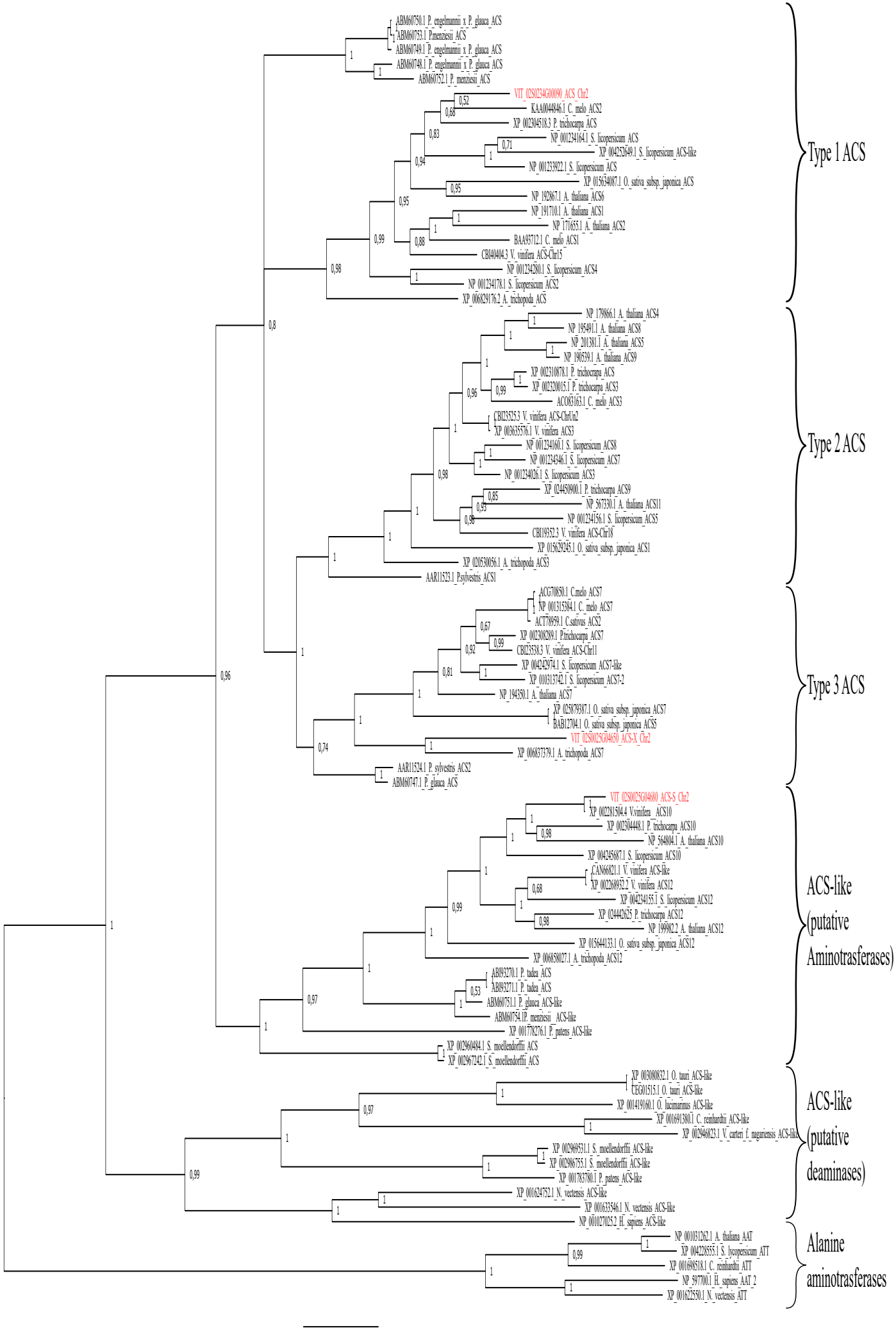
YABBY
J
AGAMOUS
Relative expression
0
5
10
15
20
25
30
35
Male
Android
Female
Hermaphrodyte
Stage 5
Stage 12
Stage 15
Stage 18
Stage 23
Stage 26

Figure 4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ABM60753.1 P.menziesii ACS
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ABM60752.1 P. menziesii ACS
VIT_02s0234G00090 ACS Chr2
KAA0044846.1 C. melo ACS2
XP_002304518.3 P. trichocarpa ACS
NP_001234164.1 S. licopersicum ACS
XP_004252649.1 S. licopersicum ACS-like
NP_001233922.1 S. licopersicum ACS
XP_015634087.1 O. sativa subsp. japonica ACS
NP_192867.1 A. thaliana ACS6
NP_191710.1 A. thaliana ACS1
NP_171655.1 A. thaliana ACS2
BAA93712.1 C. melo ACS1
CBI40404.3 V. vinifera ACS-Chr15
NP_001234280.1 S. licopersicum ACS4
NP_001234178.1 S. licopersicum ACS2
XP_006829176.2 A. trichopoda ACS
Type 1 ACS
NP_179866.1 A. thaliana ACS4
NP_195491.1 A. thaliana ACS8
NP_201381.1 A. thaliana ACS5
NP_190539.1 A. thaliana ACS9
XP_002310878.1 P. trichocarpa ACS
XP_002320015.1 P. trichocarpa ACS3
ACO83163.1 C. melo ACS3
CBI23525.3 V. vinifera ACS-ChrUn2
XP_003635576.1 V. vinifera ACS3
NP_001234160.1 S. licopersicum ACS8
NP_001234346.1 S. licopersicum ACS7
NP_001234026.1 S. licopersicum ACS3
XP_024450900.1 P. trichocarpa ACS9
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NP_001234156.1 S. licopersicum ACS5
CBI19352.3 V. vinifera ACS-Chr18
XP_015629245.1 O. sativa subsp. japonica ACS1
XP_020530056.1 A. trichopoda ACS3
AAR11523.1 P.sylvestris ACS1
Type 2 ACS
ACG70850.1 C.melo ACS7
NP_001315304.1 C. melo ACS7
ACT78959.1 C.sativus ACS2
XP_002308289.1 P.trichocarpa ACS7
CBI23538.3 V. vinifera ACS-Chr11
XP_004242974.1 S. licopersicum ACS7-like
XP_010313742.1 S. licopersicum ACS7-2
NP_194350.1 A. thaliana ACS7
XP_023879387.1 O. sativa subsp. japonica ACS7
BAB12704.1 O. sativa subsp. japonica ACS5
VIT_02s0025G04650 ACS-X Chr2
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AAR11524.1 P. sylvestris ACS2
ABM60747.1 P. glauca ACS
Type 3 ACS
VIT_02s0025G04680 ACS-5 Chr2
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XP_002304448.1 P. trichocarpa ACS10
NP_564804.1 A. thaliana ACS10
XP_004245687.1 S. licopersicum ACS10
CAN66821.1 V. vinifera ACS-like
XP_002268932.2 V. vinifera ACS12
XP_004234155.1 S. licopersicum ACS12
XP_024442625 P. trichocarpa ACS12
NP_198982.2 A. thaliana ACS12
XP_015644133.1 O. sativa subsp. japonica ACS12
XP_006850827.1 A. trichopoda ACS12
ABI93270.1 P. tadea ACS
ABI93271.1 P. tadea ACS
ABM60751.1 P. glauca ACS-like
ABM60754.1P. menziesii ACS-like
XP_001778276.1 P. patens ACS-like
XP_002960494.1 S. moellendorffii ACS
XP_002967242.1 S. moellendorffii ACS
ACS-like (putative Aminotrasferases)
XP_003080832.1 O. tauri ACS-like
CEG01515.1 O. tauri ACS-like
XP_001419160.1 O. lucimarinus ACS-like
XP_001691380.1 C. reinhardtii ACS-like
XP_002946823.1 V. carteri f. nagariensis ACS-like
XP_002969531.1 S. moellendorffii ACS-like
XP_002986755.1 S. moellendorffii ACS-like
XP_001783780.1 P. patens ACS-like
XP_001624752.1 N. vectensis ACS-like
XP_001633546.1 N. vectensis ACS-like
NP_001027025.2 H. sapiens ACS-like
ACS-like (putative deaminases)
NP_001012621.2 A. thaliana AAT
XP_004228555.1 S. lycopersicum ATT
XP_001698518.1 C. reinhardtii ATT
NP_597700.1 H. sapiens AAT 2
XP_001622550.1 N. vectensis ATT
Alanine aminotrasferases
0.2

***Declaration of Interest Statement D Carrasco**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Firmado por CARRASCO GATA, DAVID (FIRMA) el día 08/04/2020 con un certificado emitido por AC DNIE 006