Running title: **CYP78A9 is involved in reproductive development**

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**Research Category:**

**Genes, Development, and Evolution**
Cytochrome P450 CYP78A9 is involved in Arabidopsis reproductive development

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Financial support

MSS was supported by the Mexican National Council of Science and Technology (CONACyT) fellowship (229496) and RACM by a CONACyT postdoctoral fellowship. This work was financed by the CONACyT grants 82826 and 177739, CONCyTEG grant 08-03-K662-116, support from Langebio intramural funds, and the European Union FP7 project EVOCODE (247587).

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Abstract

Synchronized communication between gametophytic and sporophytic tissue is crucial for successful reproduction and hormones seem to have a prominent role in it. Here, we studied the role of the Arabidopsis cytochrome P450 CYP78A9 enzyme during reproductive development. First, controlled pollination experiments indicate that CYP78A9 responds to fertilization. Second, while CYP78A9 overexpression can uncouple fruit development from fertilization, the cyp78a8 cyp78a9 loss-of-function mutant has reduced seed-set due to outer integument development arrest leading to female sterility. Moreover, CYP78A9 has a specific expression pattern in inner integuments in early steps of ovule development, as well as in the funiculus, embryo, and integuments of developing seeds. CYP78A9 overexpression did not change the response to the known hormones involved in flower development and fruit-set, and it did not seem to have much effect on the major known hormonal pathways. Furthermore, according to previous predictions, perturbations in the flavonol biosynthesis pathway were detected in cyp78a9, cyp78a8 cyp78a9, and es1-D mutants. However, it appeared that they do not cause the observed phenotypes. In summary, the results add new insights into the role of CYP78A9 in plant reproduction and present the first characterization of metabolite differences between mutants in this gene family.
Introduction

Angiosperms have evolved the processes of double fertilization and fruit development as pivotal steps of their survival and dispersal strategies. Pollination and fertilization are essential for fruit initiation, considering that the angiosperm flower initiates terminal senescence and abscission programs if pollination has not taken place (Vivian-Smith et al., 2001; Fuentes and Vivian-Smith, 2009). For centuries, humans endeavored to prevent this association in order to develop seedless fruits. Most research has concentrated on the role of endogenous phytohormones as triggers for fruit initiation after fertilization and different strategies as exogenous application or artificial overproduction of plant hormones (Fuentes and Vivian-Smith, 2009), mutation or misexpression of specific genes has been tested. The principal lines of evidence suggest that increased auxin and gibberellin content in ovules and ovary leads to parthenocarpic fruits in Arabidopsis (Varoquaux et al., 2000; Goetz et al., 2007; Alabadi et al., 2009; Dorcey et al., 2009; Fuentes and Vivian-Smith, 2009; Pandolfini et al., 2009; Carbonell-Bejerano et al., 2010). But even with this increasing insight, the available knowledge of the mechanisms that underlie parthenocarpy is still limited.

The Arabidopsis transposon activation tagged mutant empty siliques (es1-D) overexpresses the Cytochrome P450 CYP78A9 gene and is characterized by the development of parthenocarpic fruits (Marsch-Martinez et al., 2002; de Folter et al., 2004). When crossed to wild type plants, it results in the formation of viable seed. However, its fertility is reduced compared to wild type plants. Moreover, siliques that result from the pollination of mutant es1-D plants with wild type pollen grow larger than unpollinated mutant siliques and wider than wild type siliques (Marsch-Martinez et al., 2002). A T-DNA activation tagging screen also identified a mutant overexpressing CYP78A9 with a similar phenotype (Ito and Meyerowitz, 2000).

P450 Cytochromes represent a family of heme-containing enzymes belonging to the monooxygenase group, which are found in all kingdoms and show extraordinary diversity in their chemical reactions (Schuler et al., 2006; Mizutani and Ohta, 2010). In plants, they are involved in the metabolism of most phytohormones including auxins, gibberellins (GA), cytokinins (CK), brassinosteroids (BR), abscisic acid (ABA), and
jasmonic acid (JA) as well as many secondary metabolites (defensive compounds and fatty acids) (Werck-Reichhart et al., 2002; Bak et al., 2011).

Arabidopsis has six CYP78A genes (Bak et al., 2011). The overexpression of KLUH/CYP78A5, EOD3/CYP78A6, and CYP78A9 genes all produced a general growth phenotype. This phenotype included large siliques and short stamens, a delay in bud opening and organ abscission, reduced fertility, a more severe phenotype in basipetal flowers, and longevity. This shared phenotype between the different genes indicates that they might act on a related metabolic network (Zondlo and Irish, 1999; Ito and Meyerowitz, 2000; Marsch-Martinez et al., 2002; Fang et al., 2012). An insertional knockout line for KLUH/CYP78A5 showed a higher rate of leaf initiation (Wang et al., 2008). The smaller size of leaves, sepals, and petals in homozygous cyp78a5/klu plants was found to be due to a decreased cell number and not caused by a decreased cell size (Anastasiou et al., 2007). The use of cyp78a5/klu plants, in which KLUH/CYP78A5 expression can be induced, showed that CYP78A5 prevents a premature arrest of growth by maintaining cell proliferation. Comparison of regions of organ proliferation and CYP78A5 petal expression domains indicated that CYP78A5 acts non-cell-autonomously (Anastasiou et al., 2007). The same mode of action occurs in seeds. KLUH/CYP78A5 is expressed in the inner integument of developing ovules and stimulates cell proliferation, thereby determining seed size (Adamski et al., 2009). A loss-of-function mutant for the closely related gene CYP78A7 has also been isolated, though it does not show an evident phenotype (Wang et al., 2008). However, embryos of the double knockout mutant cyp78a5/klu cyp78a7 did not develop correctly. Their size was small and their shoot apical meristem continued to grow, resulting in supernumerary cotyledons. When some seedlings survived, they gave rise to small plants with compacted rosette leaves and an increased leaf initiation rate. It seems therefore that KLUH/CYP78A5 and CYP78A7 play redundant roles in regulating the relative growth of the shoot apical meristem and the rest of the plant (Wang et al., 2008). Adamski and coworkers (2009) reported that the homozygous insertional mutants of CYP78A9 showed a reduction in seed size. However, they did not detect any genetic interaction between CYP78A9 and KLUH/CYP78A5 (Adamski et al., 2009). On the contrary, Fang and coworkers (2012) proposed that CYP78A9 and EOD3/CYP78A6
have an overlapping function in the control of seed size, as noted by the synergistic enhancement of the *cyp78a6* seed size phenotype produced in *cyp78a9* background (Fang et al., 2012).

Although the catalytic function of the Arabidopsis CYP78A enzymes remains unknown, the expression pattern and *in vivo* effect of the related genes bring up their possible engagement in the biosynthesis of some unknown type of plant growth regulator (Zondlo and Irish, 1999; Ito and Meyerowitz, 2000; Anastasiou et al., 2007; Kai et al., 2009).

Hormones seem to have a prominent role in synchronizing fertilization and fruit growth. Dorcey and coworkers (2009) have proposed that a fertilization-dependent auxin response in ovules triggers fruit development through the stimulation of gibberellins metabolism in Arabidopsis (Dorcey et al., 2009). However, this does not explain the phenotype of *es1-D* Arabidopsis mutant in which fruit-set can be uncoupled from fertilization.

In this work, we provide a detailed description of the *CYP78A9* function during reproductive development. The genetic relation of *CYP78A9* with its closest paralogs, *CYP78A6* and *CYP78A8*, shows a redundant function controlling floral organ growth and integument development, which in turn affect fertility. All the defects observed in the *cyp78a8 cyp78a9* double homozygous mutant are related to the sporophyte before fertilization, which supports the idea that this family of genes acts maternally during reproductive development. Moreover, the expression pattern of *CYP78A9* studied in detail added a new insight into the possible function of this gene during reproductive development. *CYP78A9* expression was observed in integuments during ovule development and highlights the possible communication role of the *CYP78A9*-produced signal between sporophytic and gametophytic tissue. During seed development *CYP78A9* expression was also present in embryo and seed coat. In addition, the pattern of *pCYP78A9::GUS* visible in the funiculus 12 hours after hand-pollination, together with the fact that *CYP78A9* overexpression uncoupled fruit development from fertilization, supports the possible role of a *CYP78A9*-produced signal in synchronizing
fertilization and fruit development. Furthermore, metabolic profiling of mutants compared to wild type showed perturbation in flavonoid content. However, the differences in kaempferol and quercetin content did not explain the observed phenotypes of the mutants. In summary, the results add new insights to the role of CYP78A9 in plant reproduction and present the first metabolic characterization of mutants in this family of genes.

Results

Characterization of the Arabidopsis activation tagging line es1-D

In the activation tagged es1-D mutant (Figure S1; Marsch-Martinez et al., 2002; de Folter et al., 2004), androecia and gynoecia developed in an uncoordinated manner; anthers dehisce later than in wild type Arabidopsis plants, resulting in lack of pollination (Figure 1). Interestingly, fruits developed even without pollination, which never happens in wild type plants (Vivian-Smith et al., 2001). Despite the apparently normal carpel morphology, the majority of the mature fruits were empty (Figure 1A). These parthenocarpic fruits were wider and shorter than the wild type (Figure 1G), and when developing flowers were emasculated the produced empty fruits reached a longer and wider size (Figure 1B). At the end of the es1-D life cycle, pollination occurs but seed yield and fruit length are still reduced (Figure 1G and H). Reciprocal crosses were made to analyze whether the male and/or the female reproductive side was affected. The reduced fertility could not be rescued with wild type pollen (Figure 1H), suggesting that es1-D has defects during ovule development. Ovule number was not changed in es1-D, but had larger ovules and displayed outer integuments with more and larger cells than wild type (45 to 50 compared to 29 to 35 cells in wild type Ws-3) (Figure 2A). The ovule perimeter was significantly ($P < 0.001$ of Student’s t test) larger in es1-D, compared to wild type Ws-3 (Figure 2B). However, es1-D pollen also failed to produce normal fruit-set when crossed to wild type, only 17 to 20 seeds per fruit were observed (Figure 1H), compared to normally 40 to 50 seeds found in wild type fruits. Although the fruits of this cross (♀ Ws-3 x ♂ es1-D) had more seeds, they did not reach the length of a wild type
fruit (Figure 1G). These results indicate that both male and female reproductive development is affected in the es1-D mutant.

Furthermore, we observed that es1-D sepals, petals, pistils, and seeds were larger in comparison to wild type (Figure 1D,E,I). However, es1-D stamen filaments failed to elongate (Figure 1E). Moreover, basipetal floral buds were slow to open, and stamen filament elongation more severely affected. The more acropetal flowers were less severely affected, although the sepals and petals of those flowers did not abscise normally. In addition to the flower phenotypes, the es1-D plants showed larger dark green leaves and stout stems with a zig-zag pattern (Figure 1C), a phenotype that has also been observed when KLUH/CYP78A5 and EOD3/CYP78A6, CYP78A subfamily members, are overexpressed (Zondlo and Irish, 1999; Ito and Meyerowitz, 2000; Marsch-Martinez et al., 2002; Fang et al., 2012).

The es1-D mutant flowered later (21 days) than the wild type plants (16 days), but no difference in the number of leaves produced at the moment of flowering was observed (Figure S2). Furthermore, the period of flower production and total number of flowers produced in the main inflorescence of mature es1-D and wild type plants was compared. At week 5 after flower initiation, wild type plants stopped producing flowers, while at week 8 the main inflorescence of a still growing es1-D plant was producing flowers (Figure 3B). Data presented in Figure 3 shows that es1-D clearly produces flowers over a longer period and that the growth of its main inflorescence is extended by several weeks (Figure 3A) and therefore produced more flowers, but not because of a faster growth. This phenotype was also described by Hensel et al. (1994) for mutants that have reduced fertility, whereby mutations that reduced the number of seeds per silique by more than 50% were associated with an increased proliferative capacity of the main inflorescence stem.

To confirm that the observed phenotypes in es1-D were due to CYP78A9 overexpression, the entire genomic region was cloned and expressed constitutively under the 35S promoter (35S::gCYP78A9). We obtained 21 independent lines and 17 of them displayed the es1-D phenotype (Figure S3).
The phenotypes observed in the activation tagging mutant es1-D as well as in the transgenic lines constitutively overexpressing CYP78A9, suggest that CYP78A9 has a function controlling floral organ size and, moreover, that it is involved in producing a signal that can overcome the pistil senescence program that initiates when in wild type plants fertilization does not occur (Vivian-Smith et al., 2001; Fuentes and Vivian-Smith, 2009).

**Characterization of Arabidopsis cyp78a9, cyp78a6, and cyp78a8 single and double mutants**

To better understand the function of CYP78A9, T-DNA insertion loss-of-function mutants for CYP78A9 and for its closest paralogs, CYP78A6 and CYP78A8, were analyzed (Figure S1B-E). Homozygous plants were identified by PCR genotyping and RT-PCR experiments confirmed that they were true knock-out mutants (Figure S4).

Regarding fruit development, no evident loss-of-function phenotype was observed in cyp78a9, cyp78a6, and cyp78a8 single mutants compared to wild type Col-0. All single mutants showed no difference in fruit-length and seed number per fruit compared to each other and to wild type (p< 0.001) (Figure 4A,B and Figure S5A,B). To test whether CYP78A6 or CYP78A8 act redundantly with CYP78A9 regulating fruit-length and seed-set we crossed cyp78a9 to cyp78a6 and to cyp78a8. A reduction in both fruit-length and seed-set was already evident in F1 plants cyp78a8 (+/-) cyp78a9 (+/-) compared to wild type and single mutants (p≤0.001). These results were also confirmed in F2 double heterozygous plants (Figure 4A,B).

This reduction in seed-set was related with defects in ovule development. From a total of 194 cyp78a8 (+/-) cyp78a9 (+/-) ovules analyzed, 109 (56%) presented different levels of integument development arrest. The most abundant phenotype, 105 of 194 ovules (54%), were those that still conserved at some extent the asymmetry of a wild type ovule, but presented short integuments that failed to accommodate the developing embryo sac resulting in physical restriction of the gametophyte leading to female sterility (Figure 5B). The affected ovules (56%) were smaller, the reduction of the cyp78a8 (+/-)
cyp78a9 (+/-) ovule perimeter was significantly different (at $P < 0.001$ of Student's t test) compared to wild type Col-0 (Figure 5H). Furthermore, in severe cases (around 1% of ovules), the outer integument interrupted its growth and the ovule developed a somehow orthotropic morphology (Figure 5C). In other cases (around 1% of ovules) tracheid-like cells appeared at the position of the embryo sac (Figure 5D).

Interestingly, the cyp78a8 (-/-) cyp78a9 (-/-) double homozygous mutant showed alterations in silique length and seed-set, but to a lesser extent than in the cyp78a8 (+/-) cyp78a9 (+/-) double mutant (Figure 4A,B). In the double homozygous mutant, ovules were also affected in outer integument development. From 213 analyzed ovules, 74 (35%) were significantly ($P < 0.001$ of Student's t test) reduced in size (Figure 5H) due to less cells in the outer integument (15 to 20 compared to 29 to 33 cells in wild type Col-0), but still presented a normal embryo sac (Figure 5E). Seventy seven (36%) developed short integuments that failed to accommodate the developing embryo sac (Figure 5F), and 3 (1.4%) presented tracheid-like cells at the position of the embryo sac (Figure 5G). The frequency of the phenotype severity correlates well with the observed seed-set.

In addition, the cyp78a8 (+/-) cyp78a9 (+/-) and cyp78a8 (+/-) cyp78a9 (-/-) double mutants presented a reduction in floral organ size compared to wild type (Figure 4D-M), which is the opposite effect seen when CYP78A9 is overexpressed (Figure 1). This phenomenon is also reported for mutants with outer integument defects, as for AINTEGUMENTA (ANT) in which strong mutants presented random reduction of organ size in the outer whorls (Klucher et al., 1996). Furthermore, a change in color of the seed testa was observed in cyp78a8 (-/-) cyp78a9 (+/-) and cyp78a8 (-/-) cyp78a9 (-/-) (pale brown; Figure 4C), which is normally seen in mutants related to the phenylpropanoid pathway (Buer et al., 2010).

The analysis of the cross between cyp78a6 and cyp78a9 showed no reduction in silique length although the number of seeds produced per silique was slightly diminished in F1 generation plants (Figure S5A,B). However, Fang and coworkers (2012) showed that cyp78a6 cyp78a9 had no reduced seed-set but reduced seed-size due to smaller cells
in the integuments of the developing seeds and they proposed that cyp78a9 synergistically enhanced the seed-size phenotype of cyp78a6. In our work, the analysis of the cyp78a8 (-/-) cyp78a9 (-/-) and cyp78a8 (+/-) cyp78a9 (+/-) double mutants showed a clear genetic interaction between these genes controlling seed-set. Moreover, the defects observed in cyp78a8 (-/-) cyp78a9 (-/-) and cyp78a8 (+/-) cyp78a9 (+/-) double mutants are linked to the sporophyte before fertilization, which supports the idea that this family of genes acts maternally during reproductive development.

**CYP78A9 expression during reproductive development**

In order to learn more about the CYP78A9 gene, we first made a transcriptional fusion with the GUS reporter under the control of the putative 3 kb CYP78A9 promoter region (CYP78A9::GFP:GUS) (Figure 6A-G). Transgenic plants were analyzed during reproductive development and GUS staining was observed first at the floral developmental stage 11 according to (Smyth et al., 1990). At stage 11 the GUS staining was localized in anthers and around stage 12 staining was also observed at the gynoecium (Figure 6A) and the inner integuments of the ovules (Figure 6B). At stage 13, when flowers open and fertilization occurs, the staining was localized at the placenta and the funiculi (Figure 6C). During seed development, strong staining was observed in the embryo, from the globular stage till the curly leaf stage of the embryo (Figure 6C-G, arrows), with a peak at the torpedo stage (Figure 6F, arrow). Furthermore, signal was also evident at the maternal chalazal region, the endosperm, and the developing testa (Figure 6D-F, arrowheads).

A more detailed expression analysis by in situ hybridization confirmed the observed GUS patterns and gave additional information on CYP78A9 expression (Figure 6H-O). Floral buds at stage 10 showed that CYP78A9 was expressed in the tapetum cells of the anthers (Figure 6I). Moreover, at this stage, CYP78A9 mRNA was localized in ovules, placenta, and also in the valves and stigma. After fertilization, signal in the developing seeds was stronger compared to mature ovules and was localized to the chalazal and micropilar regions (Figure 6J-K). During seed development, mRNA localization was observed in the embryo in the same manner as seen in transgenic
plants with CYP78A9::GFP::GUS, however, the highest in situ hybridization signal was localized at the epidermis of developing embryos (Figure 6L-O). Furthermore, signal was detected in endosperm, in the testa of the seed, and in valves of developing fruits. Notably, mRNA expression analysis in es1-D mutant floral buds presented the same expression pattern compared to wild type, though with around twofold increase in signal intensity, confirming that the es1-D phenotype is due to the altered amount of mRNA and not to ectopic expression of CYP78A9 (Figure S6).

Furthermore, we analyzed the protein localization for CYP78A9. For this, a transient expression assay was performed by infecting tobacco leaves with Agrobacterium tumefaciens containing a 35S::gCYP78A9:GFP translational GFP fusion construct. GFP fluorescence signal was detected by confocal microscopy at both sides of the celluar wall of consecutive cells, suggesting that CYP78A9 could be a plasma membrane-associated protein (Figure 7).

**CYP78A9 responds to the fertilization event**

Because of the fertility related phenotypes observed, we investigated what happened with the expression of CYP78A9 during fertilization. In situ and CYP78A9::GFP::GUS analyses (Figure 6) demonstrated signal in mature ovules and in the septum before fertilization. After fertilization, signal was observed in developing seed and septum, but also in the funiculus and placental tissue. The funiculus is the connection between the mother plant, via the placenta, and the developing offspring and is important for nutrition. Stadler and coworkers (2005) demonstrated that the release of photoassimilates and macromolecules to the developing seed is mediated by the outer integument that represents a symplastic extension of the funicular phloem (Stadler et al., 2005).

Developing flowers of transgenic CYP78A9::GFP::GUS plants were emasculated and observed 12 hours after hand-pollination. At this time point, fertilization has normally taken place (Faure et al., 2002). As a control, CYP78A9::GFP::GUS plants were emasculated and stained after 12 hours. In these control plants, GUS signal in the ovary was observed only in the septum (Figure 8A,B). Interestingly, in emasculated and
afterwards hand-pollinated pistils, GUS signal was observed also in the funiculus and in the placental tissue (Figure 8C,D). These results indicate that the CYP78A9 promoter responds to the fertilization event.

The CYP78A9-produced signal seems to be different from the known hormones that trigger fruit growth after fertilization

To determine whether CYP78A9 was involved in known pathways regulating fruit growth, the response pattern of two of the major hormones involved in this process, auxins and gibberellins, were analyzed in the es1-D mutant.

A cross between es1-D and the auxin-response marker DR5::GUS (Ulmasov et al., 1997) showed a similar expression pattern to wild type DR5::GUS plants throughout flower development (Figure S7). The only noted difference was observed in stage 14 flowers. In wild type DR5::GUS plants, where normal fertilization occurs at floral stage 13, GUS signal was seen in fertilized ovules (developing seeds) and in funiculi, while there was no signal observed in this tissue in es1-D DR5::GUS plants due to the absence of pollination (Figure S7). However, it is possible that the auxin increase required to produce parthenocarpic fruit development occurs earlier in the es1-D mutant.

We analyzed the expression of the GA biosynthesis marker line GA20ox1::GUS (Desgagné-Penix et al., 2005) in the es1-D background. The same expression pattern was observed during flower and fruit development in the wild type background as well as in es1-D background (Figure S7). This data suggests that GAs cannot be attributed as the cause for the uncoupled growth of es1-D fruit from fertilization.

On the other hand, we evaluated the transcriptional effect of the application of different hormones, as well as hormone inhibitors in seedlings, on the expression of CYP78A9. For this, publicly available microarray data present in the eFP browser (Winter et al., 2007) was used. We observed that CYP78A9 responded transcriptionally to the application of many hormones (Figure S8) as well as hormone inhibitors (Figure S9).
CYP78A9 expression changes observed did not allow us to identify if CYP78A9 might be involved in one of these hormone pathways, however, a hypothesis about CYP78A9 expression being regulated by inputs from multiple hormone signals could be made.

**Microarray expression analysis in the es1-D mutant**

In order to increase our knowledge about the transcriptional responses to CYP78A9 activity, a microarray experiment comparing es1-D and wild type closed floral buds from young primary inflorescences was performed. CYP78A9 responsive genes were defined as genes that were significantly differentially expressed in es1-D (z-score > 2SD) compared to wild type, which resulted in 409 upregulated and 825 downregulated genes (Table S1).

Some hormone and secondary metabolite enzymes were identified. Among the upregulated genes we can mention CYP724A1, encoding a brassinosteroid C-22 hydroxylase (Zhang et al., 2012); FMO GS-OX1, a glucosinolate oxigenase (Hansen et al., 2007); and MIOX1, a MYO-INOSITOL OXIGENASE 1 involved as an entry point of ascorbate biosynthesis (Endres and Tenhaken, 2009), converting myoinositol into D-GlcUA, which is an important precursor for cell walls and regulates the flux of inositol into the phosphoinositol pathway (Alimohammadi et al., 2012).

The downregulated genes included YUC1 (Cheng et al., 2006, 2007), AT3G51730 (SAUR-like response factor), IAA3/SHY2 (Tian et al., 2002), and IAA27/PAP2 (Liscum and Reed, 2002) involved in auxin metabolism and signaling; Jasmonate-regulated gene 21 (JRG21) and a lipooxygenase (LOX1) involved in jasmonic acid biosynthesis (Melan et al., 1993); BRI1 associated to brassinosteroid signaling (Noguchi et al., 1999); and AT2G39540 that encodes a gibberellin regulated protein (Roxrud et al., 2007).

Interestingly, CYP78A8 was downregulated in response to the overexpression of CYP78A9 as well as chalcone isomerase (CHI), responsible of converting chalcones into flavonones (Buer et al., 2010), and CYP71B16 reported as co-expressed with the phenylpropanoid pathway (Ehlting et al., 2006). A relationship between the phenylpropanoid pathway and parthenocarpy was suggested also by the gene expression profiles of highly and weakly parthenocarpic pear cultivars. The highly
parthenocarpic group showed changes in genes located in the junctions of the metabolic routes leading to the production of flavonoids or monolignols and sinapolymalate (Nishitani et al., 2012).

Several genes related to ovule and stamen development were changed. Concerning to ovule integument development, we found *HUELLENLOS (HLL)* upregulated (Skinner et al., 2001) and *AINTEGUMENTA (ANT)* downregulated (Elliott et al., 1996).

In relation to stamen development and to the early defects observed in *es1-D*, short filaments and delay in anther dehiscence, *MYB21* and *MYB65*, were downregulated (Millar and Gubler, 2005; Song et al., 2011). With regard to pollen development and pollen tube growth, *TCP16* (Takeda et al., 2006), *AT1G10770* (pectin metylesterase/invertase inhibitor) (Zhang et al., 2010), phospholipase (*PLA2-GAMMA*) (Kim et al., 2011), and the arabinogalactan proteins (*AGP7, AGP11, AGP22, and AGP23*) were found as downregulated (Pereira et al., 2006; Seifert and Roberts, 2007; Coimbra et al., 2009; Ellis M., 2010).

Furthermore, a comparison was made between the data obtained in *es1-D* and the described sets of hormone-regulated genes (Nemhauser et al., 2006), but no clear overlap was found (Table S2).

Previous information on transcriptional responses to *CYP78A9* was obtained by de Folter et al. (2004) in fruits, using a macro-array containing probes to analyze expression of over 1100 transcription factors. By means of comparison between the micro-(this study) and the macro-array data (de Folter et al., 2004), 28 downregulated and 16 upregulated genes were down- and upregulated in both datasets, respectively (Table S3). Among these, we found a series of transcription factors that could be related to the altered phenotypes seen in the *es1-D* mutant. For instance, *ANT* and *BEL1-like (SAWTOOTH 1 (BLH2), SAWTOOTH 2 (BLH4), and BEL1-LIKE HOMEODOMAIN 5 (BLH5))* transcription factors related to integument development (Elliott et al., 1996). Furthermore, *MYB3*, downregulated in both experiments, is a transcriptional repressor of proanthocyanidin biosynthesis (Feller et al., 2011). Interestingly, two enzymes involved in flavonol production were identified in the common gene list, a family 1 glicosyl transferase (upregulated) and chalcone-flavonone isomerase (*TT5*; downregulated).
In summary, the microarray experiment showed an altered transcriptional pattern of genes associated to male and female reproductive development. Related to hormones, some genes were found to be downregulated, though, no clear trends could be observed. A set of sixteen P450 enzymes changed its level of expression in the es1-D mutant. Some of them were not annotated as being part of a specific biosynthetic pathway and the ones that were annotated participate each one in a different pathway (CYP79F1: glucosinolate biosynthesis; CYP710A2 brassinosteroid biosynthesis; CYP86A7: fatty acid metabolic process). Interestingly, CYP78A8, one of CYP78A9 closest paralogs, was downregulated in response to CYP78A9 overexpression. Furthermore, enzymes involved in phenylpropanoid pathway as chalcone isomerase (TT5) and other P450 enzymes as CYP71B16 and CYP716A1 reported as co-expressed with this pathway responded to the overexpression of CYP78A9.

**CYP78A9 function prediction using public database analyses**

Another strategy undertaken for the identification of the role of CYP78A9 was the analysis of genes co-expressed with CYP78A9 based on floral tissue public microarray data. 88 genes (Table S4) were found to be co-expressed with CYP78A9 using ARACNE (Algorithm for the Reconstruction of Accurate Cellular Networks; Margolin et al., 2006a; Margolin et al., 2006b). These genes were then annotated and grouped as being part of 5 functional categories using DAVID (Gene Functional Classification Tool; Huang et al., 2008, 2009). Group 1 was enriched for genes linked to cyanoamino acid metabolism, phenylpropanoid, starch and sucrose biosynthesis, and lipid metabolism processes. Group 2 included genes involved in fatty acid metabolism, alkaloid and flavonoid biosynthetic processes. Protein kinase signaling pathway and ABC transporter G (in animals related to eye pigment transport; Schmitz et al., 2001) formed part of Group 3. Flavonoid and metylglglyxal catabolic process, protein kinase related to fatty acid signaling pathway, triacylglycerol and ketone body metabolism and phenylalanine, tyrosine and tryptophan biosynthesis genes formed Group 4. Finally, genes belonging to Group 5 were implicated with calcium dependent lipid binding signaling involved in cell death.
Predictions made by Aracyc, a metabolic pathway reference database (Zhang et al., 2005; Rhee et al., 2006), positioned the CYP78A9 protein in the phenylpropanoid pathway, more specifically in the flavonol biosynthesis pathway, at the conversion of dihydrokaempferol to dihydroquercetin step and/or in luteolin biosynthesis and in eriodictyol formation from naringenin, steps known to be catalyzed by the enzyme flavonoid 3'-monooxygenase (F3'H) (Figure 9). In the case of CYP78A8, the Cytochrome P450 Expression Database (CYPedia; Ehlting et al., 2006) showed that the top scoring co-expressed pathway was the mono-/sesqui-/di-terpene biosynthesis, and KEGG (Kanehisa and Goto, 2000; Kanehisa et al., 2012) predicted its function in Stilbenoid, diarylheptanoid, and gingerol biosynthesis. For CYP78A6 there are no predictions available.

All these data, together with bioinformatics predictions reported in CYPedia (Ehlting et al., 2006), suggest that CYP78A9 could be an enzyme in the core phenylpropanoid pathway (Figure 9). Although, we cannot discard its function in other predicted pathways like cell wall carbohydrates metabolism, lipid, fatty acid, and isoprenoid biosynthesis.

**Kaempferol and quercetin content in leaves and inflorescences of cyp78a9, cyp78a8, cyp78a8 cyp78a9, and es1-D mutants**

The response pattern of DR5::GUS and GA20ox::GUS as well as the transcriptional responses to the overexpression of CYP78A9 indicates that the CYP78A9-produced metabolite seems to be different from the known hormones that trigger fruit growth after fertilization. The group of genes found to be co-expressed with CYP78A9 in public flower microarray data showed that this gene could be involved in many processes including secondary metabolism and signaling pathways. Moreover, Aracyc predicted the function of CYP78A9 to the same point in phenylpropanoid pathway as F3'H. All these data together with the changes observed in the testa color of the double mutant that are similar to that observed in the tt7 mutant (mutated in F3'H), motivated us to explore whether CYP78A9 could act in the flavonol biosynthesis pathway.
Metabolic profiling was performed using LC-MS and multiple fragmentation. Acetone crude extracts from leaves and inflorescences were screened by LC-MS (ACQUITY UPLC-LCT Premier™ XE equipment, Waters). The analyses of the spectra obtained both from the mutant and its respective wild type ecotype was performed using MarkerLynx™ (Waters) and an integrated package comparing and discriminating data sets using multivariable statistics such as Principal Component Analysis (PCA). Selected metabolites were further fragmented using a SYNAPT HDMS system (Waters) and fragmentation spectra of highlighted metabolites were compared to authentic kaempferol and quercetin standards.

In order to find out if metabolic fluxes were guided towards a specific direction in the phenylpropanoid pathway, we analyzed hydrolyzed acetone extracts, which reveals the total pool of kaempferol and quercetin in the samples. *cyp78a9* and *cyp78a8* (-/-) *cyp78a9*(-/-) leaves have a 40 and 50% kaempferol content reduction, respectively, compared to wild type Col-0 (p<0.05), in contrast to the 120% increase of kaempferol content that presented *es1-D* leaves with respect to wild type Ws-3 (p<0.05) (Figure 10A). Even though *cyp78a8* leaves showed a tendency of kaempferol content reduction compared to wild type, this difference was not statistically significant (Figure 10A). It has been reported that Arabidopsis leaves have a reduced flux through the flavonoid biosynthetic pathway, and accumulate a higher ratio of kaempferol to quercetin derivatives than flowers and seedlings (Sheahan and Cheong, 1998). In line with this, in our study, hardly any quercetin intensities in leaves were detected and also no difference by PCA analysis was found.

Inflorescences presented less difference than leaves with respect to kaempferol content. However, *cyp78a9* inflorescences showed a significant reduction in quercetin content with respect to wild type (Figure 10B).

In summary, although we observed alterations in the kaempferol and quercetin content in *cyp78a9*, *cyp78a8*(-/-) *cyp78a9*(-/-), and *es1-D* mutants, which supports the predictions of CYP78A9 having a certain function in this pathway, the phenotypes of the mutants in terms of seed-set are not in accordance with the ones observed for the
known tt7 and tt4 mutants that do not show these levels of seed-set impairment (Ylstra et al., 1996).

Genetic interaction between CYP78A9 and chalcone synthase (tt4)?

Given the prediction of ARAcyc that positioned CYP78A9 in the flavonol biosynthesis pathway, at the conversion of dihydrokaempferol to dihydroquercetin step, and the perturbations in kaempferol levels observed in both es1-D (CYP78A9 overexpression), cyp78a9, and cyp78a8 (-/-) cyp78a9 (-/-) mutant, we wanted to further clarify the relationship between CYP78A9 and the flavonoid branch of the phenylpropanoid pathway. To test this, we examined the cross between es1-D (CYP78A9 overexpression) and tt4-1 (null mutant that has a lesion in chalcone synthase (CHS) resulting in no flavonoid production and known for its yellowish seed color) (Peer et al., 2001). The phenotype of the tt4-1 es1-D double mutant plants, found in the F2 generation, presented the characteristic yellow testa seed color of the tt4-1 mutant, however, also the reduction in fertility, the larger seed, the zig-zag stem, and the floral organ size phenotypes of the es1-D mutant (Figure 11). This means that there is no genetic interaction between CYP78A9 and tt4-1, because the double mutant showed a purely additive phenotype. Metabolic analysis of this double mutant showed no kaempferol neither quercetin accumulation (Figure 10), suggesting that the alterations in kaempferol observed in es1-D cannot be responsible for the CYP78A9 overexpression phenotypes. Moreover, previous studies showed that the Arabidopsis tt4-1 mutant, which lacks all downstream compounds of the flavonoid pathway, has no impairment in seed-set (Burbulis et al., 1996; Ylstra et al., 1996).

Preliminary metabolic screen

A preliminary metabolic screen was performed in es1-D mutant using LC-MS and multiple fragmentations (Table 1). Acetone crude extracts from leaves and inflorescences were screened by LC-MS (ACQUITY UPLC-LCT Premier™ XE
equipment, Waters). Following the same procedure as described before, after PCA analysis selected metabolites were further fragmented using a SYNAPT HDMS system (Waters) and fragmentation spectra of highlighted metabolites were compared to authentic standards (kaempferol and quercetin) and public databases, and recently published data (von Roepenack-Lahaye et al., 2004; Beekwilder et al., 2008; Kachlicki et al., 2008; Bollinger et al., 2009; McNab et al., 2009; Gouveia and Castilho, 2010). A distinct pattern of metabolite markers was observed by PCA for the mutants when compared to wild type plants (Table 1), indicating that the genetic alterations gave rise to changes in metabolite composition and concentration. Among the variations in metabolite concentration, the data highlighted a difference in flavonol content between es1-D and its wild type ecotype. Kaempferol glycosylated derivatives were the most abundant markers during the MarkerLynx PCA analysis (Table1). However, the presence of quercetin derivatives was also notable even if found at lower proportion in the es1-D overexpressing mutant compared with wild type. A unique accumulation profile was observed in different tissues of es1-D and wild type (Table1). Inflorescence tissue showed more diversity in the nature of the flavonols aglycones than the leaves did. Finally, compounds, not assigned as part of the flavonol pathway, were found in varying concentration in the es1-D mutant and wild type. Within the data collected in the ESI negative mode of inflorescence tissue, the presence of lysophosphatidic acids (LPAs) was confirmed by the typical fragmentation of LPA showing peaks at m/z 153 and 79. As an example, the concentration of LPA (m/z 734.29) was higher in es1-D mutant than in wild type (Table 1). Also, the presence of glucohirsutin m/z 492.10, a glucosinolate, was confirmed by its fragmentation pattern m/z 428 and 311 and reported as being more concentrated in es1-D inflorescences (Table 1).

In summary, this work provides the first characterization of metabolite differences between mutants in this gene family, giving interesting indicators for future investigation of the reaction(s) they catalyze.

Discussion
**Parthenocarpic fruit development in CYP78A9 overexpression line, and the hormonal context**

Overexpression of the cytochrome P450 CYP78A9 gene allows fruit growth to be uncoupled from fertilization. In the es1-D activation tagging mutant, where the CYP78A9 gene is overexpressed, androecia and gynoecia develop in an uncoordinated manner. Anthers present a dehiscence delay and the filaments never reach the stigma so pollination does not occur. In wild type plants during the maturation and receptive periods, specific molecular pathways restrict the growth of the pistil and accessory tissues and stop them from developing into fruits (Vivian-Smith et al., 2001; Goetz et al., 2006). Apparently though, CYP78A9 overexpression can overcome this restriction allowing the pistil to grow before the androecium is mature. Dorcey and collaborators (2009) further showed that a fertilization-dependent auxin signal induces gibberellin (GA) biosynthesis (via GA20ox1, GA20ox2, and GA3ox1) that in turn triggers fruit growth. However, es1-D DR5::GUS plants did not show the characteristic expression pattern in ovules and funiculus as the wild type, but fruits were developed. Moreover, es1-D GA20ox1::GUS plants showed the same expression pattern as wild type plants suggesting that GAs cannot be attributed as the cause for the uncoupled growth of es1-D fruit from fertilization. However, both auxin and GA probably act in conjunction with the CYP78A9-produced signal in the fruit growth program.

**CYP78A6, CYP78A8, and CYP78A9 are involved in reproductive development**

In the more acropetal flowers of es1-D plants, at the end of its life cycle, pollination and fertilization occur, although fertility is still reduced. Cross-pollination experiments showed that both pollen and ovules of es1-D were affected. The most relevant altered feature in es1-D ovules was integument size, with more and larger cells than wild type. This fact also has been recently reported for eod31-D, the activation tagging mutant for CYP78A6 (the closest paralog of CYP78A9) in which the ovules have more and larger integument cells (Fang et al., 2012), and has also been reported for plants with directed expression of KLUH/CYP78A5 to the outer integuments (Adamski et al., 2009).
contrast, when *cyp78a6 cyp78a9* and *cyp78a8 cyp78a9* double mutants were analyzed, a reduction in fertility was detected and was more severe in the latter. The reduction of fertility in *cyp78a8 cyp78a9* double mutant was correlated with the growth arrest of outer integuments that fail to accommodate the embryo sac.

Arabidopsis mutants with impaired integument initiation and outgrowth such as *ant*, *ino* (*INNER NO OUTER*; a YABBY transcription factor), and *bel1* are associated with aborted embryo sac development and reduced fertility (Ray et al., 1994; Elliott et al., 1996; Baker et al., 1997; Villanueva et al., 1999). Other mutants with altered integuments have been proven to present abnormalities during seed development as is the case of the *abs stk* double mutant, that completely lacks endothelium (Mizzotti et al., 2011) or the *ttg2* mutation that affects primarily cell elongation in the integuments by altered proanthocyanidin synthesis (Garcia et al., 2005). The gametophytic mutants characterized until now all have normal development of sporophytic tissues. Accordingly, a hierarchical communication between the two generations has been suggested, assigning a higher order to the sporophytic maternal tissues (Bencivenga et al., 2011). The *FRUIT WITHOUT FERTILIZATION* (*fwf/arf8*) Arabidopsis parthenocarpic mutant showed extended outer integuments (Vivian-Smith et al., 2001) as observed in *es1-D*. Interestingly, two integument defective mutants have been reported to affect parthenocarpic fruit development of the Arabidopsis *fwf/arf8* mutant. The *ABERRANT TESTA SHAPE* (*ats-1/kan4-1*) and the *bel1* mutant enhanced parthenocarpic fruit development. The need of emasculation is negated when the double mutant of 3-*KETOACYL-COA SYNTHASE 6* (*pop1*) and -*fwf-1* is combined with the *ats-1* mutant, which only develops one integument (Vivian-Smith et al., 2001). On account of this evidence, the third floral whorl prevents fruit initiation possibly by a shared pathway with the integuments mediating this communication. Nevertheless, which class of messengers are involved e.g., metabolites, small peptides, or hormones has not been deciphered yet (Vivian-Smith et al., 2001; Bencivenga et al., 2011; Mizzotti et al., 2011).

Previous work using *cyp78a* mutants and *CYP78A*-overexpressing plants proposed that *CYP78A* family members are involved in the generation of novel signaling compounds that are mainly related to the control of organ size and shape as well as plant...
architecture (Ito and Meyerowitz, 2000; Miyoshi et al., 2004; Anastasiou et al., 2007; Adamski et al., 2009). A KLUH/CYP78A5-dependent signal has been demonstrated to act in a non-cell autonomous manner to shape and size floral organs and to maternally limit the size of the outer integuments to control seed size (Adamski et al., 2009). While klu/cyp78a5 had reduced petal and integument cell number, transgenic plants that overexpressed KLUH/CYP78A5 had an increased number of petal and integument cells, indicating that this gene regulates organ size through cell proliferation (Zondlo and Irish, 1999; Anastasiou et al., 2007; Adamski et al., 2009). The maternal control of seed size has also been demonstrated for CYP78A6 and CYP78A9-dependent signals (Adamski et al., 2009; Fang et al., 2012). There is also a report of the biological role of members of this family in Physcomytrella patens, where the two CYP78A members act redundantly during protonemal growth and gametophore development. In this study the cyp78a27 cyp78a28 double mutant as well as both overexpression lines resulted in impaired gametophore development and affected the endogenous levels of several plant hormones (Lohmann et al., 2010; Katsumata et al., 2011). So it seems that the CYP78A family of P450-monoxigenases has a conserved role across the plant kingdom. In this work we confirmed the role of CYP78A9 controlling floral organ size and its role during integument development. The analysis of the cyp78a8 cyp78a9 double mutant showed a clear genetic interaction between these genes controlling seed-set via outer integument development. The defects observed in cyp78a8 cyp78a9 double mutants are linked to the sporophyte before fertilization, which supports the idea that this family of genes act maternally during reproductive development. Although, the defects detected in cyp78a8 cyp78a9 mutant were linked to the sporophyte before fertilization, the expression pattern detected during seed and embryo development suggest that CYP78A9 could have a possible communication role between the embryo and the seed coat while they develop. Furthermore, the fact that CYP78A9::GFP:GUS responds to the fertilization event, indicates the possible communication role of CYP78A9 between the placenta, funiculus, and ovule during the fertilization process.

CYP78A8 and CYP78A9 in metabolic processes
Many studies suggest that members of the CYP78A family produce a novel kind of signal. Anastasiou and colleagues (2007) demonstrated that there is no clear overlap between the KLUH/CYP78A5-regulated genes and the genes controlling the known plant hormones, and the klu/cyp78a5 phenotype could not be rescued by the application of the known hormones. Microarray data obtained in the present work also showed no clear changes in hormonal pathways due to the overexpression of CYP78A9. Imaishi and colleagues (2000) reported that the recombinant CYP78A1 protein in yeast is able to catalyze the 12-hydroxilation of lauric acid. KLUH/CYP78A5, CYP78A7, and CYP78A10 produced in insect cells are also able to catalyze the ω-hydroxylation of short chain fatty acids with lauric acid as the preferred substrate (Kai et al., 2009). However, no rescue of klu/cyp78a5 phenotype was obtained with the application of 12-hydroxyl-lauric acid, pointing out that this might not be the only substrate in planta. Su and coworkers (2010) showed that transient expression in petals in Phalaenopsis of CYP78A2 cloned from the same species increased anthocyanin content in that organ. CYP78A2 boosts the pathway without the biosynthesis of any new anthocyanin and the effect is not limited to Phalaenopsis, but also occurs in rose and carnation (Su and Hsu, 2010). However, they could not prove if CYP78A2 acts directly in this pathway or indirectly via the production of another hormone or secondary metabolite (Su and Hsu, 2010).

In an effort to confirm the prediction that CYP78A9 has an overlapping function with F3’H - in the conversion of dihydrokaempferol to dihydroquercetin- we performed a metabolic exploration of the flavonoid pathway. Here, one would expect that a partial block of the pathway results in an increase of dihydrokaempferol or kaempferol. Still, quercetin can be present, as the double mutant is in a F3’H-intact background. Interestingly, hydrolyzed extracts showed that cyp78a9 and cyp78a8 (-/-) cyp78a9(-/-) leaves have a 40 and 50% kaempferol content reduction, respectively, compared to wild type Col-0 (p<0.05), in contrast to the 120% increase of kaempferol content that presented es1-D leaves with respect to wild type Ws-3 (p<0.05). However, cyp78a9 inflorescences showed a significant reduction in quercetin content with respect to wild type. Flavonoids are plant secondary metabolites that comprise both pigments such as chalcones and anthocyanins as well as colorless molecules such as flavonones,
flavones, and flavonols (Buer et al., 2010). Presence of flavonoids has been reported in pollen and pistils of many plant species. In petunia (Petunia hybrida) and maize (Zea mays), chalcone synthase mutants are blocked in the first step of flavonoid biosynthesis and are defective in pollen tube growth. Kaempferol was identified as the pollen germination-inducing factor when applied to mutant stigma (Mo et al., 1992). Biochemical experiments and analyses of auxin fluxes in flavonoid-deficient mutants suggest that flavonols negatively regulate auxin transport processes, which is increased in tt4 mutants (Jacobs and Rubery, 1988; Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004). Recently, experiments in Arabidopsis have shown the ability of unglycosylated kaempferol and quercetin to compete with the auxin transport inhibitor NPA for a high-affinity binding site found in a protein complex containing PGP1, PGP2, and MDR1/PGP19, proteins that belong to the ATP-binding-cassette (ABC) transporters (Noh et al., 2001; Murphy et al., 2002; Geisler et al., 2005). Flavonols are believed to directly modulate auxin transport, a process that is reduced in the kaempferol-overaccumulating mutant tt7 defective in F3’H (Peer et al., 2004; Santelia et al., 2008).

Alterations in seed size and development were observed in mutants defective in proanthocyanidin synthesis or accumulation, as is the case for ttg2 which is affected in integument cell elongation (Garcia et al., 2005) and for the abs stk double mutant that has severely reduced its fertility and completely lacks endothelium (Mizzotti et al., 2011). A link between parthenocarpy and the flavonoid pathway has been established in tomato by Schijlen and coworkers (2007). Downregulation of the flavonoid pathway using RNA interference (RNAi)-mediated suppression of chalcone synthase (CHS) rendered plants with impaired pollen tube growth and pollination dependent parthenocarpic fruit development (Schijlen et al., 2007). Parthenocarpy can also be achieved by overexpression of the grape stilbene synthase (STS) gene (Ingrosso et al., 2011). This enzyme competes for the same substrates as CHS, and its overexpression produced a decrease in flavonoid content that leads to male sterile pollen in tobacco and a decreased seed-set in tomato fruits. This connection could not be made in Arabidopsis as the tt4 (CHS) mutant did not present male sterility neither parthenocarpic development, though, a slight reduction in seed-set was observed (Burbulis et al., 1996; Ylstra et al., 1996). Recently, Mahajan and colleagues (2011) have proposed a new
strategy to generate fruits with reduced seed-set in tobacco. By mean of post-transcriptional gene silencing of *FLS* (*Flavonol synthase*) they obtained plants reduced in quercetin and anthocyanidins content, but increased in catechin, epi-catechin, and epi-gallocatechin. Interestingly, *FLS* silenced lines were significantly reduced in seed number (Mahajan et al., 2011). In summary, flavonoids are important metabolites that have diverse biological functions. Within the past few years, increasing interest in these metabolites has been reported because their possible role during reproductive development and the implied potential of biotechnologically control seed-set by manipulating this pathway (Taylor and Grotewold, 2005; Falcone Ferreyra et al., 2012).

Although, the metabolic data showed differential accumulation of flavonoids between wild type and mutants, some contradictions did not support these alterations to be in line with the CYP78A9 catalytic bioinformatics prediction or CYP78A9 as being the direct cause of the phenotypes observed in the mutants. First, kaempferol levels of *cyp78a9* and *cyp78a8 cyp78a9* mutants were not in accordance with the data reported for the *tt7* mutant, deficient in F3’H activity, which overaccumulates kaempferol and does not produce quercetin (Shirley et al., 1995; Peer et al., 2001). In this sense, *cyp78a8 cyp78a9* kaempferol profile resembles more the *tt5* mutant that shows a drastically reduced flux through the biosynthetic pathway in relation to wild type (Sheahan and Cheong, 1998), however, has not the same change in testa color, *cyp78a8 cyp78a9* has pale brown testa and *tt5* has yellowish testa. The profiles reported for *tt3* and *tt6* mutants did not resemble the situation of our mutant, as *tt3* has been reported to accumulate excess of quercetin and kaempferol (Peer et al., 2001), and *tt6* has reduced kaempferol content and has no quercetin (Shirley et al., 1995). Moreover, the *tt7* (kaempferol overaccumulator) and *tt4* (devoided of kaempferol) mutants did not show the levels of seed-set impairment that showed the *cyp78a8 cyp78a9* double mutant (Ylstra et al., 1996). Second, the *tt4-1 es1-D* double mutant showed a purely additive phenotype and did neither accumulate kaempferol nor quercetin, suggesting that the alterations in flavonoids present in the overexpression mutant are not responsible for the observed phenotypes. Third, variations in other metabolites like glucohirsutin (a glucosinolate), lysophosphatidic acids (LPAs), unknown flavonoid aglycones, and unidentified compounds were found in the analyses. And
fourth, Kai and coworkers (2009) showed that CYP78A5/KLUH, CYP78A7, and CYP78A10 catalyze the ω-hydroxylation of short chain fatty acids, and proposed that this family of enzymes modifies a fatty-acid related molecule, which could participate in another biosynthetic pathway.

Although CYP78A9 was predicted to possibly be chloroplast localized (Schuler et al., 2006), our work suggest its localization to the plasma membrane. Based on this information, together with experimental evidence that CYP78A9 forms a protein-protein interaction with CALMODULIN 7 (CAM7; At3g43810) and with the protein kinase superfamily protein (At1g48210), localized also in the plasma membrane (Popescu et al., 2007), and predictions made by ARACNE that this protein could be implicated with calcium dependent lipid binding signaling involved in cell death, makes this an interesting case to explore.

Furthermore, future studies should elucidate whether a specific metabolite causes all the observed morphological phenotypes or different metabolites cause particular phenotypes.

**Conclusions**

Our findings suggest that *CYP78A9* has a function during reproductive development. The genetic evidence supports the idea that *CYP78A9* and its closest paralogs participate in a pathway that control floral organ size and ovule integuments development as denoted by the phenotypes of *es1-D* overexpression and *cyp78a8 cyp78a9* double mutants. The *CYP78A9* specific expression pattern suggests that the produced signal coordinates growth between sporophytic and gametophytic tissue, and between the structures that protect the ovules and the seed while they develop. Studies with the *CYP78A9* promoter line suggest an interesting function of the gene in communication between the placenta, funiculus, and ovule during the fertilization process. Metabolic analyses of the mutants showed the existence of alterations in flavonoid content with respect to wild type. However, these alterations seem not to cause the observed
phenotypes, as the tt4-1 es1-D double mutant presents purely additive phenotypes without having kaempferol and quercetin contents. Despite this, the work presented here contributes to the first characterization of metabolite differences between mutants in this gene family, giving interesting indicators for future investigation of the reaction(s) they catalyze.

Materials and Methods

Plant growth and plant material

Plants were germinated in soil (3 peat moss:1perlite:1vermiculite) in a growth chamber at 22°C under long day conditions (8 hrs dark/16 hrs light) and transferred to standard greenhouse conditions (22 to 27°C, natural light). The flowering time assay was performed in growth chamber at 22°C under long day conditions. The empty siliques (es1-D) mutant was identified by activation tagging in Arabidopsis thaliana Wassilewskija (Ws-3) background (Marsch-Martinez et al., 2002). The SALK line (cyp78a9, SALK_0665880), SAIL lines (cyp78a6, CS833552; cyp78a8, CS823514), the marker line DR5::GUS (Ulmasov et al., 1997), and GA20ox1::GUS (CS57942) (Desgagné-Penix et al., 2005) are all in the Columbia (Col-0) background.

Constructs and transformation

For the promoter analysis, 3020 bp directly upstream of the CYP78A9 (At3g61880) translational start was amplified and cloned into the pENTR-D vector (Invitrogen) using the specific primers (forward 5’-GGTGGGATACCGGTCAAGTG-3’ and reverse 5’-GGATGCAGAGGAACAAGAGAG-3’). The resulting construct was sequenced verified and recombined with the binary vector pBGWFS7.0 (Karimi et al., 2002), resulting in the vector CYP78A9::GFP::GUS. Wild type Arabidopsis plants (ecotype Ws-3) were transformed using the floral dip method (Clough and Bent, 1998) and transformants were identified through BASTA selection.
To confirm that the phenotype of es1-D was due to the overexpression of CYP78A9 gene the entire genomic region was cloned using the forward primer 5'-ATGGCCACCAAGCTCGACAC-3' and the reverse primer 5'-TCATACACTAAAACGTGCCTGG-3', into the pENTR-D vector (Invitrogen), verified by sequencing and recombined with the Gateway plasmid pK7WG2D (Karimi et al., 2002), resulting in the vector 35S::gCYP78A9. Wild type Arabidopsis plants (ecotype Ws-3) were transformed using the floral dip method (Clough and Bent, 1998) and transformants were identified through kanamycin selection.

For protein localization, the pENTR-D + gCYP78A9 (entire genomic region) plasmid was recombined with the Gateway binary vector pB7FWG2 (Karimi et al., 2002), resulting in the vector 35S::gCYP78A9:GFP. Agrobacterium containing this construct was grown till OD>1 in 5 mL LB medium with appropriate antibiotics. The culture was centrifuged at 4000 rpm for 10 min at room temperature and the pellet resuspended in 3 ml solution of 10 mM MgCl₂, 10 mM MES pH= 5.6, and acetosyringone to a final concentration of 200 µM added. This suspension was left for 3 hours at room temperature with weak shaking. Tobacco leaves were infiltrated with a syringe of 10 mL on the abaxial face. Fluorescence signal was observed with confocal microscopy (TCS SPE Leica) after 3 days of incubation in greenhouse conditions. A 488 nm argon laser line was used for excitation and emission was detected between 505 and 550 nm.

**Histology**

For GUS analysis, Arabidopsis tissues were incubated overnight at 37°C with an X-Gluc solution (Gold Biotechnology, St. Louis, MO; (Jefferson et al., 1987). For clearings, tissue was treated overnight with Hoyer’s solution (Anderson, 1954) plus 20% lactic acid. Light pictures from GUS stained tissues and from plant phenotypes were obtained using a Leica EZ4 D stereomicroscope (Leica, Germany). Pictures of ovules were obtained using a Leica CTR6000 equipped with Differential Interference Contrast (Nomarski) optics.
RNA extraction and RT-PCR

RNA was isolated using LiCl (Verwoerd et al., 1989). Around 1 µg RNA was treated with DNAse I (Invitrogen) and 1/10 of the treated RNA was used for cDNA synthesis with M-MLV Reverse Transcriptase or Superscript II Rnase H-Reverse Transcriptase (both from Invitrogen), following the supplier’s instructions. The obtained cDNA was used for gene expression analyses. PCR experiments were performed using cDNA from wild type and from mutant tissues. A PCR using ACTIN (forward 5’-GTGTTGGACTCTGGAGATGGTGTG-3’ and reverse 5’-GCCAAAGCAGTGATCTCTTTGCTC-3’) primers for all the samples was used as a control. The reactions were performed as follows: 95ºC 3min, (95ºC 30 sec, 55ºC 40 sec, 72ºC 2 min) 30 cycles, and 72ºC 5 min.

In situ hybridization

Arabidopsis tissue was collected and for siliques, transverse cuts were made in order to remove the apical and basal tips (to allow better infiltration), fixed, and embedded in paraplast. In summary, the samples were placed in 10 mL vials and fixation was performed with FAA solution (50% Ethanol, 5% Glacial Acetic Acid, 3.7% Formaldehyde), vacuum was applied twice for 15 min, followed by replacement of fixative and then the samples were left overnight at 4ºC. Before imbibing the tissue in paraplast the samples were dehydrated in a series of ethanol (50%, 60%, 70%, 80%, 90%, and 95%), 30 min between each step, followed by incubation overnight in 95% ethanol at 4ºC. Afterwards, samples were incubated twice in 100% ethanol, 30 min between steps, and then left overnight at 4ºC. Next, the tissue samples were taken through a histoclear series of 1 hour each of 25% histoclear: 75% ethanol, 50% histoclear: 50% ethanol, 75% histoclear:25% ethanol, and finally 100% histoclear. Infiltration was made with 100% fresh histoclear and 10 to 15 chips of paraplast and incubated overnight at room temperature. The next day, the solution was replaced with 100% paraplast and repeated 5 times during a time span of 6 hours at 60ºC, followed by
making the molds. After this, sections (10 μm) were made on a rotary microtome (Leica RM 2025) and mounted on slides.

A CYP78A9 PCR fragment corresponding to nucleotides 1755 to 1960 (using forward primer 5’-CACTATAGGGCAACACGTATCAAGATGTTAGTTTA-3’ and reverse primer 5’-CCAGGCAGTGTAGTTA-3’) was cloned into PGEM-T easy vector (Promega). The sense and antisense probes were synthesized by an in vitro transcription reaction using T7 and SP6 polymerase (Metabion), respectively. Prior to hybridization, the slides were dewaxed in histoclear twice for 10 min, 100% ethanol 2 min and ethanol series (95% to 30%; 1 min each), transferred to saline solution (NaCl 8.5 g/L) for 15 min, and then 5 minutes in PBS 1x. To improve probe penetration into the tissue, slides were incubated with proteinase K (1 μg/mL) at 37°C for 30 min. The proteinase K digestion was stopped by keeping the slides for 2 min in 2 mg/mL glycine-PBS 1x. For postfixation, the slides were transferred to fresh fixation solution and kept for 10 min at room temperature. Next, the slides were washed twice with 1x PBS for 5 min and transferred to 0.1 M triethanolamine plus 1 mL acetic anhydride for 10 min. Followed by 5 min wash in 1x PBS and a reverse ethanol series (from 30%, 50%, 85%, 95% to 100%) 2 min each, a step in 0.5% NaCl for 2 min and 2 min 1x PBS. Hybridization was done in a humidified box with a digoxigenin-labeled probe at 52°C overnight. Immunological detection was performed with 1:1250 antibody final concentration (Anti-Digoxigenin-AP, Roche) in BSA (10 g/L) solution for 2 hours and detected with an overnight incubation in NBT/BCIP containing solution as described previously (Coen et al., 1990).

Expression Analysis (microarray)

RNA was extracted (Verwoerd et al., 1989) from pooled inflorescences of 20 plants containing close buds only, comparing the es1-D with wild type Arabidopsis plants (Ws-3). Labeled cDNA (Alexa555 and Alexa647) was used for hybridization (in duplicate) to the 70-mer oligo Arabidopsis thaliana V.3.0.3 microarrays, which was performed at the Unidad de Microarreglos de DNA, Instituto de Fisiologia, UNAM, Mexico (http://microarrays.ifc.unam.mx/).
Microarray data analysis was performed with free software genArise, developed in the Computing Unit of Cellular Physiology Institute of UNAM (http://www.ifc.unam.mx/genarise/). GenArise carries out a number of transformations: background correction, lowess normalization, intensity filter, analysis of replicates, and selection of differentially expressed genes. The software identifies differentially expressed genes by calculating an intensity-dependent z-score using a sliding window algorithm to calculate the mean and standard deviation within a window surrounding each data point, and then defines a z-score where z measures the number of standard deviations a data point has from the mean. \( z_i = \frac{R_i - \text{mean}(R)}{\text{sd}(R)} \), where \( z_i \) is the z-score for each element, \( R_i \) is the log-ratio for each element, and \( \text{sd}(R) \) is the standard deviation of the log-ratio. Ratio calculations of significant changes in gene expression derived from globally normalized data are performed by simply computing the ratio of the average of all of the measurements from one condition or sample to another. With this criterion, the elements with a z-score > 2 standard deviation are considered significantly differentially expressed genes (Cheadle et al., 2003).

**PCR based genotyping**

Identification of the *cyp78a9* mutant allele was performed by PCR analysis using the primer LBB1 5’-GCGTGGACCGCTTGCAACT-3’ on the T-DNA left border and the primer 5’-TCATAAACTGCGCTGG-3’. The *CYP78A9* wild-type allele was identified using the primer 5’-ATGGCCACCAAGCTAGAC-3’ in combination with the primer 5’-TCATAAAACTGCGCTGG-3’. The *cyp78a6* mutant allele was identified using primers LB3 5’-ATTTTGCCGATTTCCGAAC-3’ and 5’-CCGTAAAGATCGGCTTAC-3’, and the *CYP78A6* wild type allele using the primer combination 5’-AACCCTGTTGGAAGCTTTCACCAACCAC-3’ and 5’-CCGTAAAGATCGGCTTAC-3’. For the identification of the *cyp78a8* mutant allele LB3 5’-ATTTTGCCGATTTCCGAAC-3’ in combination with 5’-CTGAGATGTAACGCAAGC-3’ primer was used, and for the *CYP78A8* wild type
allele the primers 5’-ATAGCCCACATGTGACCAC-3’ and 5’-CTGAGATGAGTAACGCAAGC-3’ were used.

Metabolite analysis by LC-MS and MSMS using ACQUITY UPLC-LCT Premier™ XE and SYNAPT HDMS system, respectively

Sample preparation. Frozen plant material (fully expanded leaves after flowering, and whole flowers) was ground in liquid nitrogen. For each 100 mg of fresh tissue, 300 µL of cold acetone was added, and the mixture was vortexed, sonicated for 5 min, and then centrifuged at 16,100 g to separate the crude extract from the tissue. The supernatant was used for analysis (crude extracts) and for the hydrolysis of aglycones from the glycosylated flavonoid compounds. The hydrolysis was performed following the protocol of Burbulis et al. (1996) by heating equal volumes of crude extract and 2 N HCl at 70°C for 40 min. The flavonoids were separated from the aqueous volume with an equal volume of ethyl acetate by vortexing and centrifuging at 16,100 g for 10 min. The upper organic layer was removed and placed in new Eppendorf tubes, and lyophilized. The lyophilized samples were dissolved in 1000 µL of 100% MeOH and filtered through a 0.22 µm filter before the injection into the chromatographic column. For each sample three biological replicas were included. Every sample was injected in triplicate.

Chromatography. Chromatographic separation was performed on a ACQUITY BEH C-18 column (2.1 x 50 mm i.d., 1.7 µm, Waters, Mexico) using an ACQUITY UPLC system (Waters Corps., Mexico). The column was maintained at 50°C and eluted with a 10 min linear gradient for hydrolyzed samples (method 1) or it was maintained at 35°C and eluted with a 30 min gradient applied to the crude extracts (method 2). Method 1: the mobile phase, at a flow rate of 0.5 mL/min, consisted of H₂O : MeOH (75 : 25; + 0.125% formic acid), and was maintained for 10 min. The volume of sample injected onto the column was 5 µL. Method 2: the mobile phase, at a flow rate of 0.2 mL/min, consisted of a starting mixture of solvents A : B (MeOH : H₂O; 1 : 9; A: 100% MeOH; and B : H₂O +
0.1% formic acid). A decrease of solvent B up to 20% over 15 min was then performed. Solvent B was returned to its initial composition over 1 min and the initial condition was maintained for 15 min in order to equilibrate the column. The volume of sample injected onto the column was 5 μL.

**Mass spectrometry.** The eluent was introduced into the Q-Tof mass spectrometer (LCT Premier™ XE, Waters Corps. Mexico) by electrospray ionization, with capillary and cone voltages set in the positive ion mode to 3100 V and 70 V, and for the negative mode to 3300 V and 40 V. The desolvation gas was set to 850 L/h at a temperature of 350°C for the positive mode, and 650 L/h and 200°C for the negative mode. The cone gas was set to 10 L/h, and the source temperature was set to 80°C for the positive mode, and 10 L/h and 100°C for the negative mode. Continuum data were acquired from m/z 50 - 1000 using an accumulation time of 0.2 s per spectrum. All spectra were mass corrected in real-time by reference to leucine enkephalin (2 μg/mL), infused at 5 μL/min through an independent reference electrospray. The resolution of the system was of 11000 for the positive mode and 10500 for the negative mode.

Data processing was performed by MarkerLynx, an application manager of the MassLynx 4.1 software (Waters, Inc.) with the following parameters: retention time (Rt) range 0.1 - 9.80 min for the hydrolyzed samples and from 0.64 - 30 min for the crude extract; mass tolerance 0.30 Da; peak width and baseline noise automatically calculated by the program; mass window at 0.05 Da; Rt window at 0.2 min. Automatic smoothing was applied and isotopic peaks were removed from the data. The data were analyzed by principal component analysis (PCA) within the Marker Lynx application manager on the mean centre of the peak area intensities with pare to scaling. Fragmentation analysis was performed on MarkerLynx selected metabolites using a SYNAPT HDMS system (Waters Corps. Mexico). The mass spectra were obtained applying a cone voltage ranging from 15 to 80 V, leading to the fragmentation of the base peak of interest. The fragmentation spectra of highlighted metabolites were compared to authentic standards (for kaempferol and quercetin), to public databases, and recently published data for the other metabolites found (von Roepenack-Lahaye et al., 2004; Beekwilder et al., 2008; Kachlicki et al., 2008; Bollinger et al., 2009; McNab et al., 2009;
Gouveia and Castilho, 2010). The collected peak lists with m/z, and peak area intensities were further processed with Excel software, and the statistically significant differences in individual markers between the wild type and the mutants were demonstrated by pairwise t-test (two-tailed, two-sample unequal variance).

**Accession numbers**

Sequence data for this article can be found in The Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: CYP78A9 (At3g61880), CYP78A6 (At2g46660), and CYP78A8 (At1g01190). Microarray data submission number E-MEXP-3770 (ArrayExpress Database).

**Acknowledgements**

We would like to thank the SALK institute, ABRC, and the NASC stock centre for seeds. We thank Jorge Ramirez for the microarray service at the Unidad de Microareglos, Instituto de Fisiologia, UNAM, Mexico. Furthermore, we thank Gerardo Acosta García for discussions, Paulo Cázares-Flores for the 35S::gCYP78A9 construct, Daniela Ramos-Cruz for the flowering time assay, and Edmundo Lozoya-Gloria and Yolanda Rodriguez-Aza for HPLC standards.

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Figure legends:

Figure 1. Flower and fruit phenotypes of es1-D and wild type plants. A, Open mature Ws-3 and es1-D fruits. B, es1-D fruit development: 1, es1-D pistil at stage 13; 2, es1-D non-emasculated developed fruit; 3, es1-D developed fruit from a pistil emasculated at stage 13; 4, wild type developed fruit. C, Detail of the apical part of the
main inflorescence stem from wild type and es1-D plants. Note the zig-zag pattern in the es1-D mutant. D, Stage 14 wild type and es1-D flowers. The uncoordinated development of androecium and gynoecium in the es1-D mutant is visible. E, Sepals, petals, stamens, and pistils of the flowers showed in D. F, close-up view of a wild type and es1-D inflorescence showing a strong delay in bud opening in es1-D compared to wild type. G, Silique length comparison between es1-D parthenocarpic, self-pollinated, and wild type or es1-D cross-pollinated fruits. H, Seed-set comparison between es1-D parthenocarpic, self-pollinated, and wild type or es1-D cross-pollinated fruits. I, Representative es1-D and wild type seeds. Scale bar represents 0.2 cm in A,B,F, 2 cm in C, 0.2 mm in D and E, 1 mm in I. Values represent mean+SEM (n=10 for autopolinated Ws-3, autopollinated es1-D, and parthenocarpic es1-D; n=6 for reciprocal crosses).

**Figure 2.** es1-D mutant has larger ovules and develops outer integuments with more and larger cells. A, Mature ovules from Ws-3 and es1-D plants, showing bigger es1-D ovules with more cells in the outer integument. B, Ovule perimeter comparison between Ws-3 and es1-D, showing that ovule perimeter was significantly larger in es1-D than in Ws-3 plants ("P<0.001). CC, central cell; EC egg cell; SC, synergid cell; II, inner integument; OI, outer integument. Scale bar represents 10 µm in A. Values represent mean+SEM (n=40).

**Figure 3.** Increased longevity of es1-D plants. A, Inflorescence length of wild type (bars) and es1-D (cross) plants. es1-D mutant lives longer and produces longer inflorescence stems than wild type. B, Number of flowers per main inflorescence stem in wild type and es1-D plants. es1-D produces more flowers. Values represent the mean+SEM (n=10).
Figure 4. Flower and fruit phenotypes of cyp78a9, cyp78a8, and double mutants. A, Mature silique length for wild type, cyp78a9, cyp78a8 single and double mutants. Silique length is reduced in the cyp78a8 (-/-) cyp78a9 (-/-) and cyp78a8 (+/-) cyp78a9 (+/-) double mutants. B, Seed yield, the number of seeds per silique is severely reduced in cyp78a8 (-/-) cyp78a9 (-/-) and cyp78a8 (+/-) cyp78a9 (+/-) double mutants. C, Mature seeds of wild type, cyp78a9, cyp78a8 single and double mutants. Note the pale testa color of the double mutant. D, Flower phenotypes of the cyp78a8 (+/-) cyp78a9 (+/-) double heterozygous mutant compared to Col-0 wild type. E, Flower phenotypes of the cyp78a8 (-/-) cyp78a9 (-/-) double homozygous mutant compared to Col-0 wild type. F-M, Sepals, petals, stamens, and pistils of the flowers presented in D and E. Note the reduction of organ size in the double homozygous and double heterozygous mutants. Scale bar represents 0.2 mm in D-M and 1 mm in C. Values represent mean+SEM (n=20). *Significantly different from Col-0 at P< 0.001.

Figure 5. Ovule phenotypes of cyp78a9, cyp78a8, and double mutants. A, Mature ovules from wild type (Col-0) B-D, Mature ovules from cyp78a8 (+/-) cyp78a9 (+/-) double heterozygous mutant. From 194 ovules analyzed, 105 (54%) have shorter outer integuments that failed to accommodate the embryo sac but still conserve ovule characteristic asymmetry (B), around 1% have severe arrest of outer integument growth (C), and in some cases (1%) tracheid-like cells appear at the position of the embryo sac (D). E-G, Mature ovules from cyp78a8 (-/-) cyp78a9 (-/-) double homozygous mutant. From 213 ovules analyzed, 74 (35%) have shorter outer integuments than wild type but still a normal embryo sac (E), 77 (36%) presented growth arrest of outer integument and embryo sac abortion (F), and 3 (1.4%) presented tracheid like cells in the position of the embryo sac (G). H, Comparison of ovule perimeter between Col-0 and double homozygous and heterozygous mutants CC, central cell; EC, egg cell; SC, synergid cell; II, inner integument; OI, outer integumen; trc, tracheid-like cell. Scale bar represents 10 µm in A-G. *Significantly different from Col-0 at P< 0.001. Values represent mean+SEM (n=40).
Figure 6. Expression patterns of *CYP78A9* in flower, fruit, and seed tissues using promoter::GUS analysis (A-G) and in-situ hybridization (H-O). A, *pCYP78A9::GUS* flowers showing GUS signal in anthers at stage 11 and 12 (arrowheads). At stage 12 the signal is also localized in pistils. B, Ovules of a stage 10 bud showing expression in the inner integuments (arrowheads). C, Ovule 24 hours after pollination showing signal in the funiculus and placenta (arrowheads). D, Developing seed showing *CYP78A9* expression in integuments, funiculus, endosperm, and the embryo at globular stage (arrowheads). E, Seed with heart stage embryo. The GUS signal is localized in the embryo (arrow) and in the integuments (arrowhead). F, Seed with torpedo stage embryo. At this stage the signal is stronger in the embryo (arrow) and endosperm (arrowhead) but still localized in the integuments (arrowhead). G, Seed with curly stage embryo. The signal is localized in the cotyledons and the radical (arrowheads). H, *CYP78A9* sense probe hybridized to longitudinal sections of wild type gynoecium from a closed bud. No signal is observed. I, *CYP78A9* antisense probe hybridized to longitudinal sections of wild type closed bud. *CYP78A9* is expressed in ovules, stigma, marginal tissue, and tapetum (arrowheads). J, *CYP78A9* antisense probe hybridized to a wild type pollinated flower showing expression in developing seeds and valves (arrowheads). K, *CYP78A9* antisense probe hybridized to an early developing seed showing expression in chalaza and micropila regions (arrowheads). L-O, *CYP78A9* antisense probe hybridized to wild fruits showing seeds with embryo at heart, torpedo, and curly stage. *CYP78A9* is expressed in the embryo and strongly in the epidermis of wild type embryo (arrowheads) with the strongest signal observed in torpedo stage embryos (arrowhead) (O). Expression can also be seen in the integuments of the developing seeds and in the valves of the fruit (arrowheads). Scale bar represents 1 mm in A-G, and 10 µm in H-O.

Figure 7. Cellular *CYP78A9* protein localization. A,B, Background fluorescence signal of non-infected tobacco leaves, (A) bright field and (B) dark field view. C,D, Fluorescence GFP signal of gCYP78A9:GFP in infected tobacco leaves with 35S::gCYP78A9:GFP, (C) bright field and (D) dark field view. The fluorescence signal
detected suggests that the CYP78A9 protein is associated with the plasma membrane. Scale bar represents 25 µm.

**Figure 8.** *CYP78A9 promoter (pCYP78A9::GUS) responds to the fertilization event.*

**A,** Pistil 12 hours after emasculation showing expression in the stigma, septum (arrowhead), but not in funiculus. **B,** Close-up view of ovules from the emasculated pistil (in C). Signal is only present in the septum but not in ovules. **C,** Pistil 12 hours after hand-pollination showing expression in the stigma, placenta, and funiculus (arrowheads). **D,** Close-up view of ovules of the pollinated pistil (in A) showing clear GUS signal localization in the funiculus and the placenta (arrowheads), but not in ovules. Scale bar represents 1 mm.

**Figure 9. The flavonoid branch of the phenylpropanoid pathway.** Known location of Arabidopsis *transparent testa* (*tt*) mutations are indicated in parenthesis on the phenylpropanoid pathway. CYP78A9 and CYP78A8 are located in the pathway based on the ARACyc and KEGG bioinformatic predictions, respectively.

**Figure 10.** Kaempferol and quercetin perturbations were found in *cyp78a9, cyp78a8 cyp78a9,* and *es1-D* mutants. **A,** Fragment ions intensity of kaempferol from hydrolyzed samples from leaves of Ws-3, Col-0, *cyp78a8, cyp78a9, cyp78a8* (-/-) *cyp78a9* (-/-), *es1-D,* and *tt4-1 es1-D.* **B,** Fragment ions intensity of kaempferol and quercetin from hydrolyzed samples from inflorescences of Ws-3, Col-0, *cyp78a8, cyp78a9, cyp78a8* (-/-) *cyp78a9* (-/-), *es1-D,* and *tt4-1 es1-D.* *Significantly different from wild type at P< 0.05. Values represent mean+SD in percentage from the wild type (n=9).
Figure 11. Phenotype of the *tt4-1 es1-D* double mutant. **A**, *tt4-1* and **B**, *tt4-1 es1-D* flowers. Note the *tt4-1 es1-D* bigger organ size. **C**, *tt4-1 es1-D* plant showing the characteristic zig-zag pattern as seen in the *es1-D* stem. **D**, *tt4-1* and *tt4-1 es1-D* fruits. Note the characteristic phenotype from *es1-D* in which fruits are wider and shorter than *tt4-1* fruits. **E**, Seeds from Col-0, single and double mutants, showing the additive phenotype present in *tt4-1 es1-D* double mutant. Scale bars represent 0.2 mm in A and B, 2 cm in C, 0.2 cm in D, and 1 mm in E.
Table 1. Relative amounts of metabolites identified in crude extracts of Arabidopsis leaves and inflorescences by UPLC-qTOF-premier XE (Waters). The values are averages of the relative peak response area in three replicates with standard error values. The up (↑) or down (↓) regulation ratio is shown in bold when there is a statistically significant difference (P <0.05). NS means not significant. The fragmentation spectra of metabolites were compared to authentic standards (for kaempferol and quercetin), to public databases, and recently published data for the other metabolites found (von Roepenack-Lahaye et al., 2004; Beekwilder et al., 2008; Kachlicki et al., 2008; Bollinger et al., 2009; McNab et al., 2009; Gouveia and Castilho, 2010).

| Tissues          | m/z esi | MS2                  | I.D.                              | Ws-3 (mean±SD) | est1-D (mean±SD) | Ratio     | P-value   |
|------------------|---------|----------------------|-----------------------------------|----------------|-------------------|-----------|-----------|
| **leaves**       |         |                      |                                    |                |                   |           |           |
| 624.16           | 539, 503, 341, 313 | Unknown flavanoid aglycone | 122.1 ± 17.5                       | 258.9 ± 12.4   | 2.12               | 1277E-05  |
| 819.16           | 749, 477, 455, 277 | Unknown               | 106.6 ± 42.7                       | 172.9 ± 9.4    | 1.62               | 0.0494    |
| 820.46           | 477, 461, 313   | Iso-rhamnoetin 3-O-glucoside-7-O-rhamnoside [M-H]- | 241.3 ± 11.1                       | 161.1 ± 57.8   | NS                 |           |
| 431.15           | 341, 285, 153 | Kaempferol rhamnoside [M-H]- | 66.8 ± 4.9                        | 86.9 ± 6.0     | 1.3                | 0.0023    |
| 785.21           | 447, 341      | unknown               | 84.8 ± 6.6                         | 44.8 ± 1.6     | 0.53               | 0.0008    |
|                  | 624.16      | 477, 461, 313   | Iso-rhamnoetin 3-O-glucoside-7-O-rhamnoside | 407.6 ± 17.4 | 382.2 ± 20.5 | 0.94 | 0.0439 |
|                  | 786.21      | 609,447, 421,341 | Unknown                           | 228.5 ± 12.4 | 222.9 ± 13.3 | NS        |           |
|                  | 492.10      | 428, 234,97,80     | Glucohirsutin                     | 24.7 ± 7.3    | 165.6 ± 78.1 | 6.71 | 0.0067 |
|                  | 593.11      | 447, 285           | Kaempferol 3-O-glucoside-7-O-rhamnoside | 139.3 ± 7.7 | 115.5 ± 31.3 | NS        |           |
|                  | 669.41      | 381, 255, 153, 79 | LPA                               | 114.4 ± 6.3  | 105.0 ± 11.9 | NS        |           |
|                  | 639.16      | 607, 461, 422, 341, 297 | Unknown                           | 109.1 ± 5.3  | 99.3 ± 9.4    | NS        |           |
|                  | 734.29      | 422, 377, 277, 153, 79 | LPA                               | 91.5 ± 8.5    | 103.8 ± 9.8   | 1.13   | 0.0433 |
|                  | 663.17      | 477, 341, 311, 153, 79 | LPA                               | 101.9 ± 6.7  | 86.6 ± 20.4   | NS        |           |
|                  | 786.22      | 609, 447, 341, 153, 79 | LPA                               | 82.7 ± 4.3    | 83.7 ± 4.9    | NS        |           |
| **Inflorescence**|         |                      |                                    |                |                   |           |           |
| 624.16           | 539, 503, 341, 313 | Unknown flavanoid aglycone | 235.1 ± 10.9                       | 247.9 ± 12.0   | NS                 |           |
| 327.16           | [Quercetin +Na + 2]2+ | Unknown                  | 67.9 ± 28.9                       | 75.4 ± 15.2   | NS                 |           |
| 593.28           | 533, 460   | Unknown               | 140.7 ± 3.6                        | 128.2 ± 7.7  | 0.87               | 0.0537    |
| 329.15           | Unknown    | Unknown               | 140.2 ± 7.1                        | 92.9 ± 15.2   | 0.66               | 0.0041    |
|                  | 579.25      | 433, 381, 287   | Kaempferol 3-O-rhamnopyanoside-7-O-rhamnopyanoside | 235.1 ± 10.9 | 247.9 ± 12.0 | NS        |           |
|                  | 741.23      | 595, 433, 381, 287 | Kaempferol O-rhamnoside O-hexosyl-rhamnoside hexoside (K-Rha-Gly-3-Rha-7) | 179.9 ± 12.0 | 182.1 ± 11.9 | NS        |           |
|                  | 599.21      | 433, 365, 287   | Kaempferol-3-O-glycosyl-7-O-rhamnoside | 90.1 ± 14.9 | 135.3 ± 47.0 | NS        |           |
|                  | 625.18      | 463, 381, 365, 316 | Rhamnosyl hexosyl methyl quercetin | 109.8 ± 6.9  | 98.9 ± 9.9    | NS        |           |
|                  | 580.15      | 433, 287       | neoeriocitrin                     | 79.6 ± 5.8    | 96.5 ± 17.5   | NS        |           |
|                  | 742.23      | 570, 433, 287   | Kaempferol rhamnoside-3-O-glycoside-7-O-rhamnoside | 66.6 ± 4.3  | 67.1 ± 5.3    | NS        |           |
|                  | 611.16      | 449, 381, 355, 303, 221 | Quercetin conjugate               | 67.9 ± 4.6    | 53.9 ± 5.7    | NS        |           |
|                  | 323.19      | 223, 192, 138, 130, 84 | Unknown                           | 63.8 ± 12.2  | 64.4 ± 12.6   | NS        |           |
|                  | 609.18      | 355, 303, 281, 221 | Quercetin conjugate               | 58.2 ± 2.9    | 53.9 ± 5.7    | NS        |           |
Supplementary data:

Table S1. Microarray upregulated and downregulated genes 2SD.
Table S2. Comparison of CYP78A9-regulated and phythormone responsive genes.
Table S3. Gene list derived for the comparison between macro and microarray data.
Table S4. CYP78A9 co-expressed genes in flower tissue.
Table S5. Gene functional classification derived from the genes co-expressed with CYP78A9 in flower tissue.

Figure S1. Position of AIE affecting CYP78A9 gene. Neighbor joining tree of Arabidopsis CYP78A clade.
Figure S2. es1-D flowering time.
Figure S3. 35S::gCYP78A9 phenotype.
Figure S4. T-DNA insertional lines genotyping and RT-PCR data.
Figure S5. Fruit size and seed yield from wild type and cyp78a9, cyp78a6 single and double mutants.
Figure S6. Comparison of in situ hybridization between Ws-3 and es1-D buds.
Figure S7. Expression pattern of auxin and gibberellin in es1-D compared to wild type (Ws-3) during flower development.

Figure S8. CYP78A9 transcriptional effect upon application of different hormones to seedlings. Evaluation made using publicly available microarray data present in the eFP browser (Winter et al., 2007).

Figure S9. CYP78A9 transcriptional effect upon application of different hormone inhibitors to seedlings. Evaluation made using publicly available microarray data present in the eFP browser (Winter et al., 2007).
