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Toward understanding the role of *CYP78A9* during *Arabidopsis* reproduction

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After fertilization in *Arabidopsis*, auxin response in ovules triggers fruit development through the stimulation of gibberellin metabolism. In a recent work, we showed that this model could not explain why *CYP78A9* overexpression can uncouple these processes. The specific expression pattern of *CYP78A9* suggests its involvement during reproductive development. Moreover, controlled pollination showed that *CYP78A9* responds to fertilization. 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. Furthermore, according to previous predictions, perturbations in the flavonol biosynthesis pathway were detected in *cyp78a9, cyp78a8 cyp78a9* and *es1-D* mutants. However, they do not cause the observed phenotypes. Our 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.

Keywords: *CYP78A9, empty siliques,* parthenocarpy, integument development, *Arabidopsis* reproduction, P450 monoxigenase

Abbreviations: *es1-D*, *empty siliques*; *CYP78A9*, activation tagged mutant; GA, gibberellins; *GA20ox1*, *gibberellin 20-oxidase 1; GA20ox2*, *gibberellin 20 oxidase 2*; *GA3ox1*, *gibberellin 3-oxidase 1*; GFP, green fluorescent protein; *ga1-3*, mutant in *GA1 GA requiring 1*; *tt4-1*, mutant in *transparent testa 4*

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Successful sexual plant reproduction depends on fruit-set, an essential process that can be defined as the activation of a developmental program to convert the pistil or gynoecium into a developing fruit with seeds. This transition comprises two different and coordinated processes: the fertilization of the ovule and the growth of structures that will protect the developing seeds. In most species, the coordination between these two events relies on the signal that promotes fruit growth, which exclusively originates from the developing seeds. It is currently accepted that, in *Arabidopsis*, the coordinated action of growth signals triggers fruit-set and growth, so in the absence of pollination and fertilization, the ovary will cease cell division and abscise.[1-5]

In a recent article we showed the involvement of *CYP78A9* controlling floral organ size and its role during reproductive development.[6] 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 the *cyp78a8 cyp78a9* double mutant are linked to the sporophyte before fertilization, which supports the idea that this family of genes act maternally during reproductive development.[7,8] Although the defects detected in *cyp78a8 cyp78a9* 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 *pCYP78A9::GUS* (transcriptional fusion under the control of *CYP78A9* promoter) responds to the fertilization event, indicates the possible communication role of *CYP78A9* between the placenta, the funiculus and the ovule during the fertilization process. This work also revealed that in the *es1-D* activation tagging mutant, where the *CYP78A9*

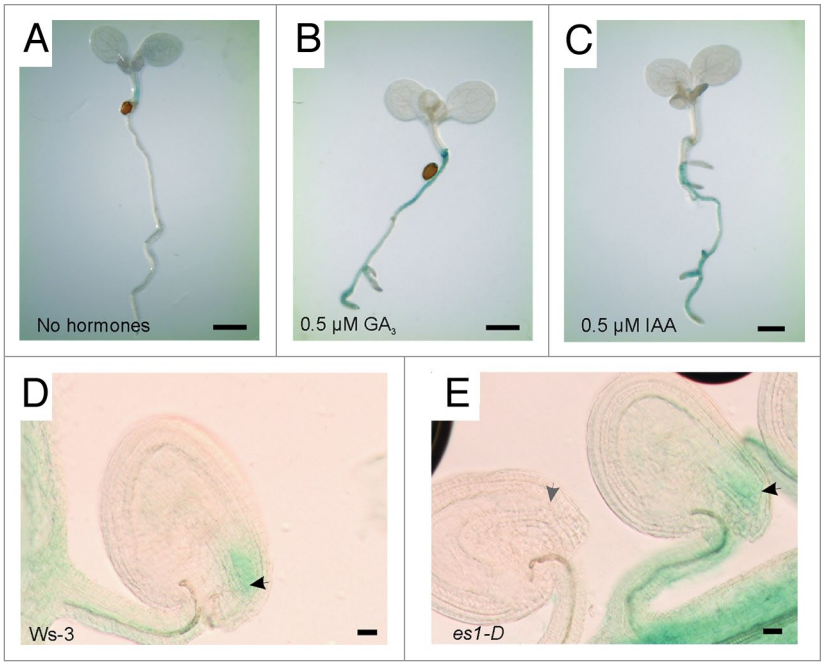

Figure 1. Hormone treatments and pollination experiments. (**A–C**) The 3 kb *CYP78A9 promoter* (*pCYP78A9::GUS*) responds to gibberellin and auxin treatments. (**A**) Control *pCYP78A9::GUS* 12-d-old seedling showing signal in the hypocotyl. (**B and C**) *pCYP78A9::GUS* 12-d-old seedling 20 h after 0.5 µM GA_3 addition (**B**) and 20 h after 0.5 µM IAA addition (**C**), showing that the GUS signal is now present in roots. (**D and E**) Outer integument extension in *es1-D* seems to change the position of the micropyle preventing pollen tube attraction. (**D**) Wild type ovule crossed with *LAT52::GUS* pollen, showing the signal of a pollen tube that entered the embryo sac (black arrowhead). (**E**) *es1-D* ovule showing no attraction of pollen tubes; no LAT52 signal is observed (left, gray arrowhead). The difference between the two type of ovules seems to be the length of the integuments, which is longer in the ovule on the left (*es1-D*) than on the right (wild type). Scale bar represents 0.5 cm in (**A–C**) and 10 µm in (**D and E**).

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, preventing them from developing into a fruit.[9,10] However, *CYP78A9* overexpression can overcome this restriction allowing the pistil to grow before the androecium is mature. Dorcey and collaborators (2009) showed that a fertilization-dependent auxin signal induces gibberellin (GA) biosynthesis (via *GA20ox1*, *GA20ox2* and *GA3ox1)* that in turn triggers fruit growth.[5] However, in our work using the *DR5::GUS* reporter line, we observed that *es1-D* plants did not show the characteristic auxin expression pattern in ovules and funiculi as the wild type, but fruits still developed.[6] Moreover, the expression pattern of the *GA20ox1::GUS* marker line was not changed in *es1-D* compared with wild type plants. Furthermore, *es1-D* could not recover the wild type phenotype of *ga1–3* plants that are unable to synthesize GA. In addition, microarray data of plants overexpressing *CYP78A9* compared with wild type showed no clear changes in hormonal pathways, suggesting that these hormones might not be the cause for the uncoupled growth of *es1-D* fruit from fertilization. Bioinformatic studies showed that *CYP78A9* responded transcriptionally to the application of different hormones as well as hormone inhibitors. Interestingly, here we observed that *CYP78A9* responds to the application of auxin and GA, based on an altered pattern of GUS signal in *pCYP78A9::GUS* seedlings (**Fig. 1A–C**). Twelve day old seedlings grown in MS medium without hormones showed GUS signal in the hypocotyl (**Fig. 1A**). However, after 20 h of auxin or GA application, the GUS signal was not observed in the hypocotyls anymore but was found in roots (**Fig. 1B and C**). Therefore, the *CYP78A9* promoter is responsive to these hormones in vegetative tissues. We could speculate, that during reproductive development, both auxin and GA might be related to the *CYP78A9*-produced signal in the fruit growth program.

Fertility is affected in the gain-of-function mutant and 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.[6] Here we further investigated the communication between ovules and pollen to obtain more insights about the reduced fertility. For this, we used the *LAT52::GUS,* a pollen specific marker line.[11] We followed GUS staining after hand-pollinating pistils of *es1-D* emasculated flowers with *LAT52::GUS* pollen. In wild type pistils *LAT52::GUS* pollen tubes normally reach and penetrate the ovules. Their content is discharged inside the ovule and therefore, GUS staining is clearly observed (**Fig. 1D**). In affected *es1-D* ovules, i.e., ovules with large outer integuments, GUS staining was not observed (left ovule in **Fig. 1E**), meaning that pollen tubes were not able to reach and penetrate them. This interesting observation could be explained by at least three different scenarios. First, it may be that these ovules are not able to chemically attract the pollen tubes, which normally occurs.[12,13] Second, the structural defects of these ovules, such as the altered position of the micropyle, may constitute a physical barrier for proper pollen tube penetration. A third, attractive scenario would be that *es1-D* ovules produce a signal that repels the pollen tubes, which in *Arabidopsis*, occurs when ovules are already fertilized.[12,14,15]

Various studies suggest that members of the CYP78A family produce a novel kind of signal.[7,16-18] Predictions made by Aracyc, a metabolic pathway reference database,[19,20] positioned the CYP78A9 enzyme in the phenylpropanoid pathway. The enzyme was positioned in the flavonol biosynthesis pathway, as having a putative overlapping function with F3'H in the conversion of dihydrokaempferol to dihydroquercetin. Although the metabolic

data showed indeed differential accumulation of flavonoids between wild type and mutants, other metabolites were also differentially accumulated.[6] Moreover, the *es1-D* characteristic parthenocarpic phenotype was unaffected in the *transparent testa 4* (*tt4-1*) mutant background, where the flavonoid pathway is blocked and no kaempferol or quercetin is produced.[21] Therefore, the flavonoid alterations observed according to the CYP78A9 function prediction are not the direct cause of the phenotypes observed in the gain-of-function mutant.

Conclusions

P450 cytochrome *CYP78A9* is involved in controlling floral organ size and is important during reproductive development. Our own data and data on other CYP78A family members, suggest that they are involved in the synthesis of a novel signal other than the known hormones controlling fruit-set. Metabolic profiling revealed that *CYP78A9* is able to alter the flavonoid pathway, though it appears that these alterations do not cause the observed phenotypes. It has been shown that the hormones auxin and GA are essential for normal fruit-set. Interestingly, they are able to regulate *CYP78A9*, because hormone treatments caused an altered expression pattern in *pCYP78A9::GUS* seedlings (this work). Moreover, the communication between defective *es1-D* ovules and pollen tubes, and therefore fertilization, is impaired. Future research on the novel role of these genes during reproductive development and their possible coordinated action with known growth and communication signals will contribute to the further understanding of the complexity of the processes that occur before and finally lead to fruit-set.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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