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MOLECULAR BASIS OF FRUIT DEVELOPMENT

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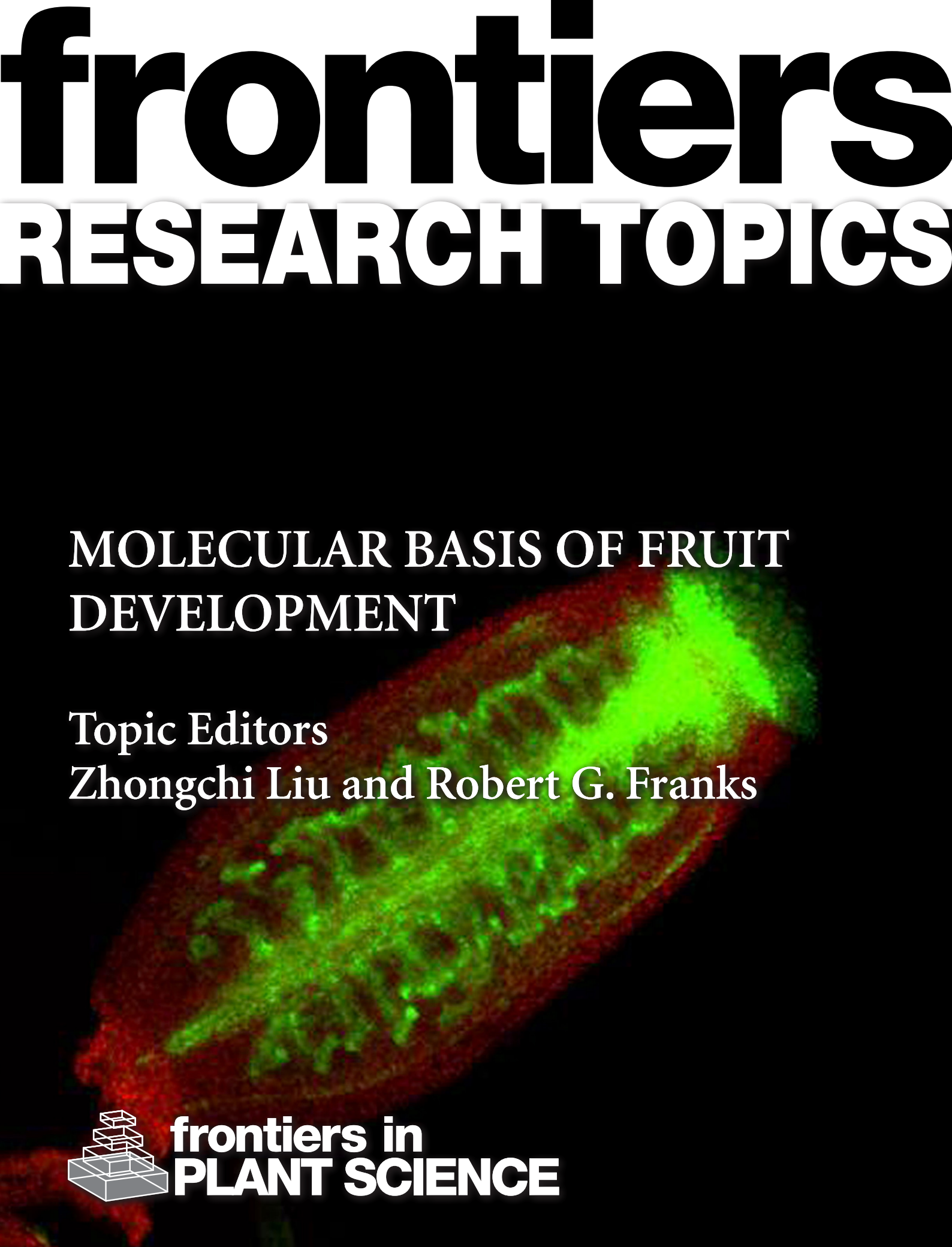
Zhongchi Liu and Robert G. Franks



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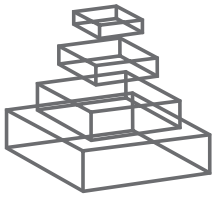

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MOLECULAR BASIS OF FRUIT DEVELOPMENT

Topic Editors:
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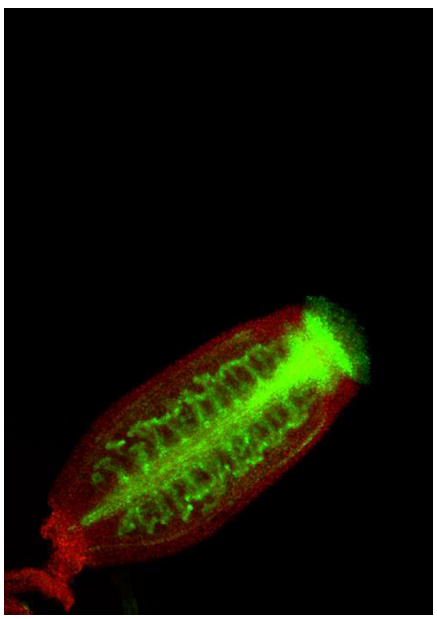

Expression of pTAA1::GFP:TAA1 in a developing gynoecium (stage 11) of an Arabidopsis thaliana line that overexpress the NGATHA3 transcription factor. Marginal tissues (stigma, transmitting tract, funiculi and ovules) are highlighted. TAA1 (tryptophan aminotransferase) encodes an enzyme that catalyzes the conversion of Trp to IPA, which is then converted to auxin by YUCCA.

Photo credit: Cristina Ferrandiz. The image is related to the article by Martinez-Fernandez et al., (2014) in this research topic.

The fruit is an important plant structure. Not only does it provide a suitable environment for seeds to develop and serve as a vehicle for seed disposal, but it is also an indispensable part of the human diet. Despite its agronomic and nutritional value and centuries of intensive genetic selection, little is known about the molecular mechanism of its development or the evolution of its diverse forms. The last few years have witnessed a surge of investigations on the early stages of fruit development propelled by the advancement of high throughput sequencing technology, genome sequencing of fruit bearing species, and detailed molecular insights based on studies of model organisms. This research topic is focused on early stage fruit development that ranges from pre-fertilization patterning of the female ovary through post-fertilization fruit initiation and growth. Provided by the renowned experts in the field, these papers are intended to highlight recent progress and shed light on different aspects of fruit development from structure, function, to molecular genetics, and evolution.

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EDITORIAL
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Molecular basis of fruit development

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Keywords: fruit evolution, endocarp, auxin, cytokinin, morphogenesis, ovule, gynoecium, carpel margin meristem

The fruit is a vital plant structure that supports seed development and dispersal, and is an indispensable part of the human diet. The 11 articles within this special research topic focus on the molecular mechanisms of early fruit development and span a diversity of species and experimental approaches. Since the gynoecium, the female floral structure, is the precursor of all or part of the fruit, several articles are focused on mechanisms of gynoecium development. The articles can be organized into several groups based on common themes highlighted below.

PATTERNING OF THE GYNOECIUM

The gynoecium consists of one to several carpels, usually fused together and topped with style and stigma. The botanical fruit is derived from the carpel wall (pericarp) and genes that regulate gynoecium development ultimately affect fruit size, shape, and dispersal mode. Hence, one could not discuss fruit development without understanding the mechanism controlling the gynoecium development.

The gynoecium is a three dimensional structure with three positional axes: basal-apical; medial-lateral; and abaxial-adaxial. Auxin synthesis, transport, and signaling have been implicated in the regulation of all three axes. Previously, the auxin gradient model (Nemhauser et al., 2000) proposed that auxin was synthesized at the apical tip of the gynoecium and then transported basally, forming a gradient from high auxin concentrations at the apex to low concentrations at the base. The differential cellular responses to the auxin gradient resulted in the apical-basal patterning of the gynoecium.

In this research topic, Zuniga-Mayo et al. (2014) added a new dimension to this model by showing that exogenous cytokinin application (benzyl amino purine, BAP) to the *Arabidopsis* inflorescence caused a phenotype similar to that caused by the auxin transport inhibitor NPA (1-*N*-naphtylphtalamic acid). Hence, cytokinin may reduce auxin transport and thus also be involved in gynoecium apical-basal patterning.

Hawkins and Liu (2014) proposed an alternative model in lieu of the auxin gradient model. They pointed out that the gynoecium apical-basal axis determination likely occurred very early, long before auxin biosynthesis occurs at the apical tip. This new model suggests that, much like leaf patterning, it is the role of auxin in the abaxial-adaxial polarity establishment that determines proper apical to basal patterning of the gynoecium.

At later stages of the gynoecium development, auxin biosynthesis at the apex may be critical to formation of the style and stigma. In *NGATHA* (*NGA*) gain- or loss-of-function mutants of *Arabidopsis*, when apical development is disrupted, Martinez-Fernandez et al. (2014) identified 2449 genes whose expression levels were altered. Their analysis of these genes suggests that the NGA proteins regulate gynoecial development via the control of auxin homeostasis.

CARPEL MARGIN MERISTEMS (CMMs) AND SHOOT APICAL MERISTEM (SAM) ARE REGULATED WITH SIMILAR MECHANISMS

Carpel margin meristems (CMMs) are the meristematic medial portions of the gynoecium that give rise to the ovules. Arnaud and Pautot (2014) reviewed the roles of TALE HD (three amino acid loop extension homeodomain) transcription factors in regulating CMMs of *Arabidopsis*, highlighting similar molecular mechanisms underlying CMM and SAM (shoot apical meristem) with respect to gibberellic acid and cytokinin signaling. Another similarity between the CMM and the SAM was highlighted by Kamiuchi et al. (2014) as they defined the role of *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2* genes in initiating and positioning the CMM and in the regulation of *SHOOTMERISTEMLESS* (*STM*) expression in the gynoecium. Wynn et al. (2014) reveal a role for the transcription factor *PERIANTHIA* (*PAN*) during CMM development as well as during floral meristem determinancy. Their work suggested that proper termination of the floral meristem may be required for the complete development of the CMM and ovules.

Cucinotta et al. (2014) reviewed the early ovule development with a focus on the formation of the CMM and the initiation of ovule primordia in *Arabidopsis*. They presented a model of ovule initiation that relates the functions of *CUC* and *ANT* genes as well as the action of auxin, cytokinin, and brassinosteroid hormones (Galbiati et al., 2013; Cucinotta et al., 2014). Their model posits a role for *ANT* in the growth of the organ primordia, and *CUC* genes in the specification of the boundary zones between ovules. The interactions between the primordial and boundary regions, as well as cytokinin regulation are required for the proper expression and localization of the *PIN-FORMED1* (*PIN1*) auxin transporter and thus for

proper auxin fluxes. Brassinosteroid signaling is proposed to support ovule initiation through the stimulation of *ANT* activity in ovule primordia (Huang et al., 2013; Cucinotta et al., 2014).

FRUIT SHAPE, SIZE, AND RIPENING

Two articles each provided a unique perspective on fruit development and ripening. Van Der Knaap et al. (2014) discussed six key genes and their mechanisms that regulate tomato fruit shape and weight. Some of the genes also act during floral meristem and floral organ development, highlighting the close connection between floral organ initiation, specification and later fruit shape and sizes.

The review by Pesaresi et al. (2014) focused on the retrograde (plastids to nucleus) and anterograde (nucleus to plastids) communication pathways during fruit ripening, areas that were not yet fully explored. In the anterograde pathway, nuclear-encoded regulators alter plastid function and specify plastid types (Leon et al., 1998; Raynaud et al., 2007). The retrograde pathways allow for the transfer of information from the plastid to the nucleus regarding the functional or physiological status of the plastids (Chi et al., 2013).

EVOLUTIONARY PERSPECTIVES OF FRUIT DEVELOPMENT

Upon fertilization, the carpel in many species transitions from an ovule-containing vessel to the seed containing fruit. Dardick and Callahan (2014) reviewed molecular mechanisms that regulate endocarp differentiation in each of three species from the families Brassicaceae, Rosaceae, and Solanaceae. The endocarp is the innermost cell layer of the carpel wall. They discussed in detail current understanding of the "stone" endocarp in peach and suggested that the regulatory genes and pathways controlling the lignified valve margin layer of Brassica's dry fruit are similar to those controlling lignified "stone" endocarp in peach.

Pabon-Mora et al. (2014) took a phylogenetic approach to analyze the transcription factor genes that regulate carpel valve margins of dry fruit in Arabidopsis. Through comprehensive searches for homologs across core-eudicots, basal eudicots, monocots, and basal angiosperms and phylogenetic tree construction, the authors suggested conservation of certain fruit development pathways and established the foundation for future functional tests.

SUMMARY

The diverse perspectives presented in this research topic provide an in depth understanding of ongoing researches in this exciting and evolving field. One common theme emerging from several articles is that distinct structures do not always result from entirely distinct regulatory networks, (e.g., similar genes regulate SAM, FM, and CMM development, similar mechanisms may underlie patterning of carpels and leaves, and conserved networks are required for the stone endocarp in peach and the dry fruit valve margin in Brassica). Because of the broad biological questions addressed as well as the potential applications to agricultural problems, this field will likely attract further interest and funding, and yield important discoveries in the future.

ACKNOWLEDGMENTS

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Cytokinin treatments affect the apical-basal patterning of the *Arabidopsis* gynoecium and resemble the effects of polar auxin transport inhibition

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The apical-basal axis of the *Arabidopsis* gynoecium is established early during development and is divided into four elements from the bottom to the top: the gynophore, the ovary, the style, and the stigma. Currently, it is proposed that the hormone auxin plays a critical role in the correct apical-basal patterning through a concentration gradient from the apical to the basal part of the gynoecium, as chemical inhibition of polar auxin transport through 1-*N*-naphtylphtalamic acid (NPA) application, severely affects the apical-basal patterning of the gynoecium. In this work, we show that the apical-basal patterning of gynoecia is also sensitive to exogenous cytokinin (benzyl amino purine, BAP) application in a similar way as to NPA. BAP and NPA treatments were performed in different mutant backgrounds where either cytokinin perception or auxin transport and perception were affected. We observed that cytokinin and auxin signaling mutants are hypersensitive to NPA treatment, and auxin transport and signaling mutants are hypersensitive to BAP treatment. BAP effects in apical-basal gynoecium patterning are very similar to the effects of NPA, therefore, it is possible that BAP affects auxin transport in the gynoecium. Indeed, not only the cytokinin-response *TCS::GFP* marker, but also the auxin efflux carrier PIN1 (*PIN1::PIN1:GFP*) were both affected in BAP-induced valveless gynoecia, suggesting that the BAP treatment producing the morphological changes has an impact on both in the response pattern to cytokinin and on auxin transport. In summary, we show that cytokinin affects proper apical-basal gynoecium patterning in *Arabidopsis* in a similar way to the inhibition of polar auxin transport, and that auxin and cytokinin mutants and markers suggest a relation between both hormones in this process.

Keywords: apical-basal patterning, gynoecium, *Arabidopsis*, plant developmental biology, auxin, cytokinin

INTRODUCTION

The gynoecium is the female reproductive organ of the flower. Different axes can be distinguished during the development of the *Arabidopsis thaliana* gynoecium and one of them is the apical-basal axis. This axis can be divided into four domains: the stigma at the apical part, consisting of a single layer of elongated cells called papillae, followed by a solid cylinder below, called the style, then there is the ovary which is the most complex part of the gynoecium and contains the ovules, and finally in the basal part the gynophore, which is a short stalk-like structure connecting the gynoecium with the rest of the plant (Balanza et al., 2006; Roeder and Yanofsky, 2006; Alvarez-Buylla et al., 2010).

Plants produce different hormones, which are involved in many developmental processes throughout their life cycle (Durbak et al., 2012; Lee et al., 2013). One of the most widely studied hormones is auxin (Tromas and Perrot-Rechenmann, 2010; Sauer et al., 2013). It has been reported that alterations in polar auxin transport, as occurs in the *pin1* mutant (Okada et al., 1991), or treatment with the polar auxin transport inhibitor 1-*N*-naphtylphtalamic acid (NPA; Nemhauser et al., 2000), or alterations in auxin signaling, occurring in the *ettin* mutant (Sessions and Zambryski, 1995), or deficiency in auxin biosynthesis, shown in the *yuc1 yuc4* (Cheng et al., 2006) and the *wei8 tar2* (Stepanova et al., 2008) mutants, have strong impact on gynoecium development, affecting the establishment of their apical-basal patterning. It has been proposed that auxins act through a gradient in the establishment of apical-basal patterning of the gynoecium, where the highest concentration of auxin is in the apical end and decreases towards the basal part of the gynoecium (Nemhauser et al., 2000), though modified views have evolved related to the presence of an auxin gradient (Ostergaard, 2009; Larsson et al., 2013). Alterations in the apical-basal patterning of the gynoecium are distinguished by an increase in the style and gynophore domain sizes at the expense of the ovary, which in severe cases even completely disappears.

Another well-studied plant hormone is cytokinin, which is involved in different developmental processes such as shoot meristem formation and maintenance, organ formation, and seed germination, among others (Mok and Mok, 2001; Hwang et al., 2012; El-Showk et al., 2013). Recently, it has been reported that cytokinins are involved in the regulation of floral organ size,

ovule number, and ovule development in the gynoecium (Bartrina et al., 2011; Bencivenga et al., 2012). Furthermore, cytokinins are involved in medial tissue proliferation at early stages of the developing gynoecium and at more mature stages in valve margin differentiation (Marsch-Martinez et al., 2012a,b; Reyes-Olalde et al., 2013).

In recent years special attention has been paid to the study of interactions between different hormones. Hormonal crosstalk provides an extra level of regulation in biological processes conferring robustness and stability, as well as flexibility (Moubayidin et al., 2009; Wolters and Jurgens, 2009; Depuydt and Hardtke, 2011; Vanstraelen and Benkova, 2012). The cytokinin–auxin crosstalk is important for the establishment and maintenance of the root apical meristem (RAM) and the shoot apical meristem (SAM). These two hormones act antagonistically in the RAM, cytokinin by promoting cell differentiation and auxin by promoting cell division (Dello Ioio et al., 2007; Ruzicka et al., 2009). Conversely, in the SAM, auxin increases cytokinin response through the repression of cytokinin signaling repressors (Zhao et al., 2010). Several studies have demonstrated that the cytokinin–auxin crosstalk can occur at different levels, cytokinin can affect auxin synthesis, transport or signaling, and *vice versa*, auxin can affect cytokinin synthesis, degradation, or signaling (Hwang et al., 2012; El-Showk et al., 2013).

Despite the large number of studies on the role of cytokinins in plant development, their functions in gynoecium development are just beginning to be explored (Marsch-Martinez et al., 2012a; Reyes-Olalde et al., 2013), while it's possible interactions with other hormones in this organ have not been studied yet. In this study we analyzed the possible role of cytokinin in apical-basal patterning of the gynoecium and its possible interaction with auxin through exogenous application of the cytokinin benzyl amino purine (BAP) and the auxin transport inhibitor NPA to different mutants and cytokinin and auxin signaling markers. The results suggest that cytokinins are also involved in apical-basal patterning of the gynoecium, which is more evident when the auxin transport or signaling is affected.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS

All wild type and mutant plants used in this study are *Arabidopsis thaliana* ecotype Columbia. Plants were germinated in soil under long-day conditions (16–8 h, light–dark) in a growth chamber at 22°C. One week after germination, the plants were transferred to the greenhouse with a temperature range from 22 to 28°C, long-day conditions (13–11 h, light–dark approximately) and natural light.

HORMONE TREATMENTS

One week after bolting, wild type, mutant and marker line inflorescences were dipped five consecutive days in BAP, NPA, or mock solutions. The BAP and NPA solutions contained 100 μM benzylaminopurine (BAP; Duchefa Biochemie, http://www.duchefa.com) or 100 μM NPA (Sigma–Aldrich, St. Louis, MO, USA) respectively, and 0.01% Silwet L-77 (Lehle Seeds, Round Rock, TX, USA). The mock solution contained only 0.01% Silwet L-77. All treated plants with their respective controls were grown simultaneously under the same conditions. For each mutant background five plants were treated, of which 10–15 main and secondary inflorescences were analyzed. The gynoecia were analyzed after anthesis. The treated plants were frequently monitored; the apical-basal patterning phenotypes began to be observed after 2 weeks.

The standard deviation was calculated considering the phenotype frequency percentages between each inflorescence analyzed. To determine whether there was a significant difference in the different phenotypes between wild type plants and the different treated mutants a Student's *t*-test was performed comparing the phenotype frequency percentages of each mutant background versus wild type plants. The treatments for each mutant were performed twice with similar results. The results presented here are from one experiment.

MICROSCOPY

For light pictures and phenotype analysis the plant material was dissected and observed using a Leica EZ4 D stereomicroscope (Leica, Wetzlar, Germany). Scanning electron microscopy images were captured using a Zeiss EVO40 environmental scanning electron microscope (Carl Zeiss, Oberkochen, Germany) with a 20 kV beam, and the signal was collected using the BSD detector, for which plant tissue was collected and directly observed in the microscope. For fluorescent microscopy, the images were captured using a LSM 510 META confocal scanning laser inverted microscope (Carl Zeiss, Oberkochen, Germany). Propidium iodide (PI) was excited using a 514-nm line and GFP was excited using a 488-nm line of an Argon laser. PI emission was filtered with a 575-nm long-pass (LP) filter and GFP emission was filtered with a 500–550-nm bandpass (BP) filter.

RESULTS

EXOGENOUS APPLICATION OF CYTOKININ AFFECTS THE APICAL-BASAL PATTERNING OF THE *Arabidopsis* GYNOECIUM

Recently, we reported that cytokinins are important for the proliferation at the medial tissues in the gynoecium and for proper valve margin differentiation in *Arabidopsis* fruits (Marsch-Martinez et al., 2012a). It has been shown that auxin plays an important role in establishing the correct apical-basal patterning of the gynoecium (Nemhauser et al., 2000). Furthermore, it is known that cytokinin and auxin cross-talk at different levels in several developmental processes (El-Showk et al., 2013). With this in mind, we decided to analyze the effect of exogenous cytokinin applications on the apical-basal patterning of the *Arabidopsis* gynoecium. Inflorescences of wild type plants were treated once a day for a period of 5 days with 100 μM BAP solution. In parallel, we carried out a treatment with 100 μM NPA under the same conditions; this compound blocks the polar auxin transport, causing apical-basal patterning defects in the gynoecium (Nemhauser et al., 2000). This treatment was performed in order to compare the effect of exogenous cytokinin application versus polar auxin transport blocking.

We previously reported that prolonged BAP application (3–4 weeks) produced gynoecia with conspicuous tissue proliferation (Marsch-Martinez et al., 2012a). However, when the wild type inflorescences were treated with BAP during a shorter time (5 days)

a gradient of phenotypes were observed. The first open flowers (flowers 1–5) after the treatment contained gynoecia with no obvious phenotype. The next floral buds to open (flowers 6–18) contained gynoecia that showed the proliferation that was reported previously. However, floral buds that opened later (flowers 19–31) contained gynoecia that showed apical-basal defects which are the focus of this study. In some cases we observed gynoecia with both phenotypes, the proliferation and the apical-basal defects; these gynoecia were developed in the transition zone of these two phenotypes. Finally normal gynoecia were developed.

Two weeks after each treatment, the gynoecia of treated floral buds were analyzed. In both cases for wild type plants twelve to fifteen gynoecia per inflorescence showed apical-basal defects with different severities. The observed phenotypes were classified according to previously reported by Sohlberg et al. (2006). The classification consists of three categories based on valve development: (1) If the length of the valves was more than 50% the length of the gynoecium, but less than the length of valves of mock-treated gynoecium, were named "reduced valves"; (2) This category includes gynoecia with one valve and gynoecia with two small valves that occupied less than half of its length; and (3) If the gynoecium did not develop any valves the phenotype was named "valveless" (**Figures 1** and **2**).

The BAP-treated wild type gynoecia presenting apical-basal defects were analyzed, and the majority of them (88%) showed reduced valves, 10% developed very reduced valves and almost 2% were classified as valveless (**Figures 2** and **3A**). In the case of NPA-treated wild type gynoecia, 59% of them showed reduced valves, 25% developed very reduced valves, and 16% showed the valveless phenotype (**Figure 3B**). The data obtained for the NPA treatment (**Figures 1** and **3**) are similar to those previously reported (Sohlberg et al., 2006). Comparing the frequencies of the phenotypes in both treatments, the defects observed due to BAP are less severe than the defects due to NPA, however, the occurrence of these phenotypes are constant between BAP treatments and significantly higher than the frequency in which they appear in untreated plants. These results indicate that, like NPA, exogenously applied cytokinin affects proper establishment of the apical-basal patterning in the *Arabidopsis* gynoecium.

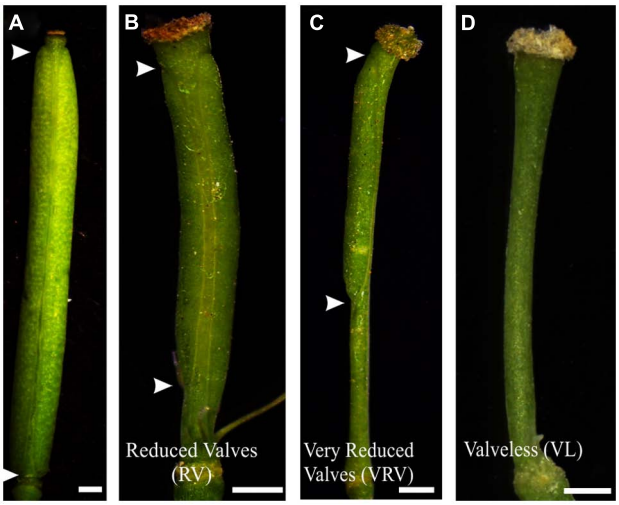

FIGURE 2 | Apical-basal phenotypes caused by exogenous BAP application. (A) Mock-treated wild type gynoecium. **(B)** A gynoecium with the "Reduced Valves" (RV) phenotype. **(C)** Gynoecium with a "Very Reduced Valves" (VRV) phenotype. **(D)** Gynoecium with the "Valveless" (VL) phenotype. The arrowheads indicate the beginning and the end of valves. Scale bars: **(A)** 1 mm; **(B,C)** 400 μm; **(D)** 200 μm.

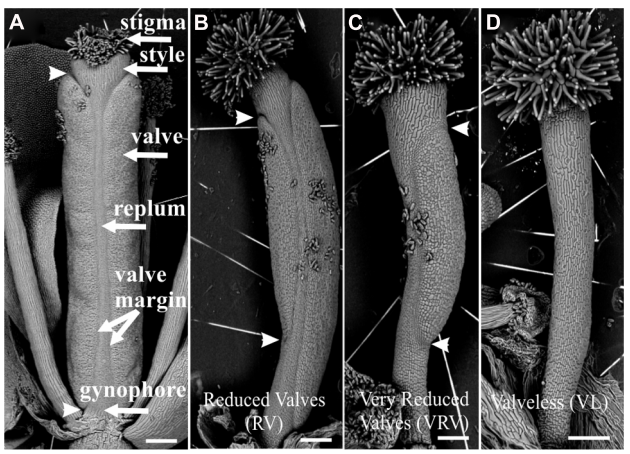

FIGURE 1 | Scanning electron micrographs of classification of apical-basal phenotypes in the *Arabidopsis* gynoecium. **(A)** Mock-treated wild type gynoecium. **(B)** Gynoecium presenting a "Reduced Valves" (RV) phenotype. **(C)** Gynoecium with the "Very Reduced Valves" (VRV) phenotype. **(D)** Gynoecium with the "Valveless" (VL) phenotype. These gynoecia were treated with NPA. The arrowheads indicate the beginning and the end of valves. Scale bars: **(A–D)** 200 μm.

BAP AND NPA APPLICATIONS HAVE SIMILAR EFFECTS IN AUXIN TRANSPORT AND SIGNALING MUTANTS

It has been reported that the apical-basal gynoecium patterning of auxin biosynthesis or signaling mutants gynoecia is hypersensitive to NPA treatment (Staldal et al., 2008). In order to know whether the the BAP effect on the apical-basal patterning was related with any auxin related processes, we performed BAP treatments in different auxin transport and signaling mutants.

In *Arabidopsis*, polar auxin transport requires the activity of polarly localized PIN-FORMED (PIN) auxin efflux transporters (Benkova et al., 2003; Friml, 2003). The *pin1* mutant produces hardly any flowers (Okada et al., 1991), so it was discarded for this study. On the other hand, the *pin3 pin7* double mutant gynoecia show alterations in apical-basal patterning, but its reproductive development is also severely affected (Benkova et al., 2003). However, the *pin3* and *pin7* single mutants do not exhibit visible apical-basal defects. Therefore, these two mutants represent an opportunity to explore the effect of BAP application in a background where polar auxin transport is affected but development is not severely altered. When the *pin7* mutant was treated with BAP, 39% of gynoecia developed reduced valves, 37% developed very reduced valves, and 24% showed the valveless phenotype

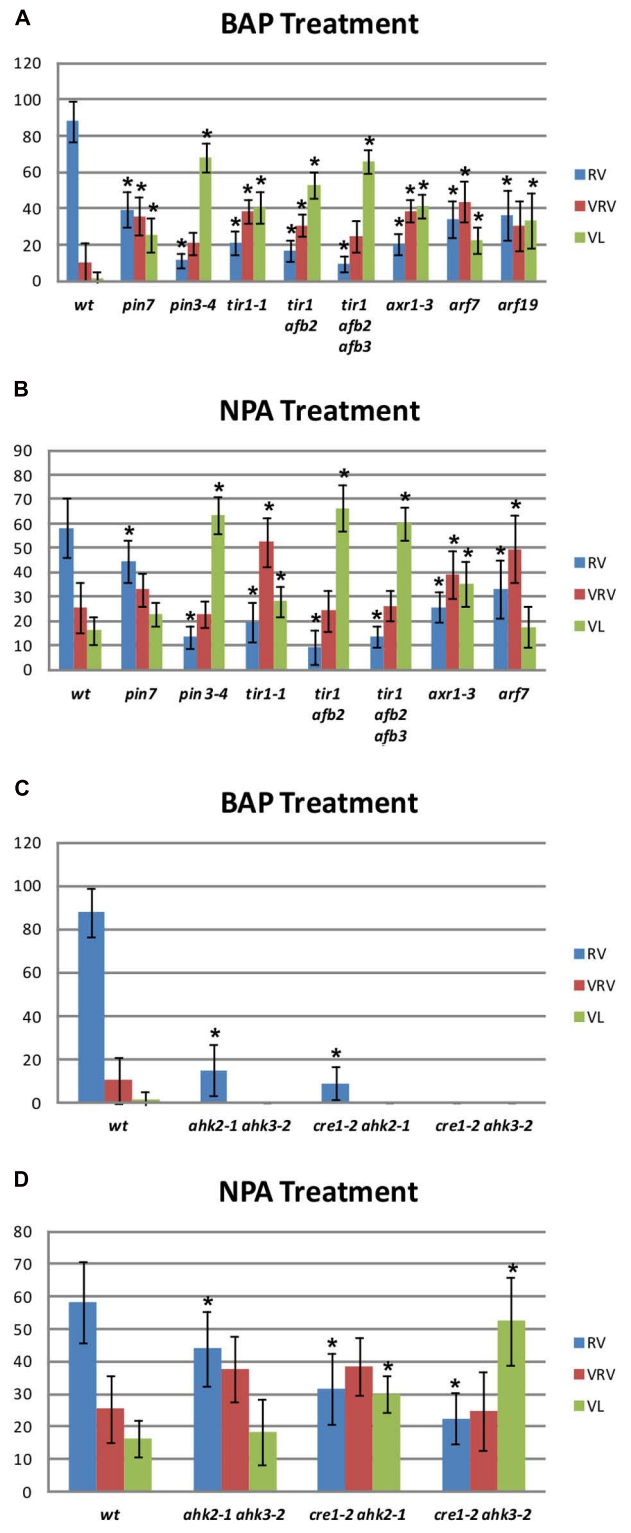

FIGURE 3 | Apical-basal gynoecium patterning phenotype frequency of NPA and BAP treatments in wild type and mutant backgrounds. (A,C) Distribution of the different categories of apical-basal phenotypes in BAP-treated gynoecia. **(A)** Auxin signaling mutants. Col, $n = 204$; *pin7*, $n = 111$; *pin3*, $n = 204$; *tir1*, $n = 145$; *tir1 afb2*, $n = 299$; *tir1 afb2 afb3*, $n = 383$; *axr1*, $n = 372$; *arf7*, $n = 122$. **(C)** Cytokinin signaling mutants. *ahk2 ahk3*, $n = 224$; *cre1 ahk2*, $n = 288$; *cre1 ahk3*, $n = 495$. **(B,D)** Distribution of the different categories of apical-basal phenotypes in NPA-treated gynoecia. **(B)** Auxin signaling mutants Col, $n = 231$; *pin7*, $n = 225$; *pin3*, $n = 258$; *tir1*, $n = 314$; *tir1 afb2*, $n = 557$; *tir1 afb2 afb3*, $n = 889$; *axr1*, $n = 406$; *arf7*, $n = 317$; *arf19*, $n = 434$. **(C)** Cytokinin signaling mutants *ahk2 ahk3*, $n = 163$; *cre1 ahk2*, $n = 148$; *cre1 ahk3*, $n = 177$. RV, Reduced Valves; VRV, Very Reduced Valves; VL, Valveless. Error bars represent standard deviation. The "*n*" indicates the total number of analyzed gynoecia for each background. Values on the *y*-axis are percentages. The asterisk (*) indicates significant difference.

(**Figure 3A**). In the *pin3* mutant 11% of gynoecia showed reduced valves, 21% developed very reduced valves, and 68% showed the valveless phenotype (**Figure 3A**). These same mutants were also treated with NPA (**Figure 3B**). In the *pin7* mutant 22% of gynoecia did not develop valves, whereas this alteration was observed in 64% of *pin3* mutant gynoecia. These results indicate that the apical-basal patterning of *pin3* and *pin7* gynoecia is hypersensitive to both treatments and the valveless phenotype frequencies are similar for both treatments in the same mutant. In addition, the *pin3* mutant appears to be more sensitive than the *pin7* mutant to both treatments, suggesting that PIN3 plays a more relevant role in the establishment of apical-basal gynoecium patterning than PIN7.Furthermore, auxin signaling mutants were treated with BAP or NPA. First, different auxin receptor mutants were treated: the single mutant *transport inhibitor response 1* (*tir1*; Ruegger et al., 1998), the double mutant *tir1 auxin signaling F-box protein 2* (*afb2*), and the triple mutant *tir1 afb2 afb3* (Dharmasiri et al., 2005). The untreated *tir1* and *tir1 afb2* gynoecia did not exhibit obvious apical-basal defects, while *tir1 afb2 afb3* gynoecia occasionally showed apical-basal defects under our growth conditions. However, all three genotypes were hypersensitive to BAP treatment, and the frequency of the more severe phenotype (valveless) increased when auxin perception decreased, such that in *tir1*, *tir1 afb2*, and *tir1 afb2 afb3* plants 40, 53, and 64% of gynoecia, respectively, showed the valveless phenotype (**Figure 3A**). When the mutants were treated with NPA, in *tir1, tir1 afb2*, and *tir1 afb2 afb3* plants 28, 66, and 61% of gynoecia, respectively, showed the valveless phenotype (**Figure 3B**), indicating that these mutants are also hypersensitive to the NPA treatment.

In addition, mutants affected in auxin signaling, downstream perception, were treated with BAP and NPA. These mutants were *auxin resistant 1* (*axr1*), where a protein related to the ubiquitin-activating enzyme E1 is affected, and *auxin response factor 7* (*arf7*) and *arf19* mutants, where transcription factors that mediate auxin response are affected (Leyser et al., 1993; Harper et al., 2000; Okushima et al., 2005). Untreated *axr1* gynoecia occasionally showed apical-basal defects under our growth conditions, but this was not observed for *arf7* and *arf19*. Regarding the BAP treatment, the *axr1* mutant developed 41%, the *arf7* mutant 24%, and the *arf19* mutant 35% of gynoecia without valves (**Figure 3A**). These results indicate that these three mutants are hypersensitive to the BAP treatment. In the case of the NPA treatment, the *axr1* mutant developed 34% and the *arf7* mutant 18% of valveless gynoecia (**Figure 3B**), indicating that *axr1* is hypersensitive to

NPA treatment. For the *arf19* mutant no data were obtained due to technical reasons.

In summary, the results indicate that the gynoecia of auxin transport and signaling mutants are hypersensitive to BAP application, resulting in apical-basal patterning defects. This phenomenon was already reported for NPA application (Staldal et al., 2008), therefore in this study NPA was used as reference, and produced similar results as seen for the BAP application.

THE ABSENCE OF CYTOKININ RECEPTORS ALTERS THE RESPONSE TO BAP AND NPA APPLICATIONS

The above results suggest that disruption of auxin transport or signaling has an impact on the effect caused by BAP treatments on the apical-basal patterning of the gynoecium, as had been reported and was also observed here for NPA treatments. The next step was to explore the possibility that disturbances in processes related to cytokinin perception might also have an impact on the effect of these treatments. For this purpose, the *cytokinin response 1* (*cre1*) *Arabidopsis histidine kinase 2* (*ahk2*), *cre1 ahk3*, and *ahk2 ahk3* cytokinin receptor double mutants (Higuchi et al., 2004; Nishimura et al., 2004) were treated. Untreated double mutant gynoecia never presented apical-basal defects under our growth conditions. After BAP treatment, two of the three cytokinin receptor double mutants showed slight apical-basal defects, but none of them developed gynoecia with severe apical-basal phenotypes. In *ahk2 ahk3* and *cre1 ahk2* mutants 15 and 9% of gynoecia developed reduced valves, respectively (**Figure 3C**). The *cre1 ahk3* mutant gynoecia did not show visible apical-basal phenotypes (**Figure 3C**). These results suggest that the cytokinin receptors CRE1, AHK2, and AHK3 are required for the full effect of exogenous BAP application on the establishment of apical-basal patterning of gynoecia observed in wild type plants. An opposite response was observed when the cytokinin receptor mutants were treated with NPA. In the *ahk2 ahk3*, *cre1 ahk2*, and *cre1 ahk3* mutants 19, 30, and 53% of the gynoecia, respectively, showed the severe valveless phenotype (**Figure 3D**), in comparison to only 16% in wild type plants. These results suggest that adequate cytokinin perception is necessary to attenuate the impact of the reduction in polar auxin transport on the establishment of apical-basal patterning of the gynoecium.

BAP AND NPA APPLICATIONS AFFECT THE EXPRESSION PATTERN OF CYTOKININ (*TCS::GFP*) AND AUXIN-RESPONSE MARKERS (*DR5::GFP*) AND THE AUXIN TRANSPORTER PIN1 (*PIN1::PIN1:GFP*) IN THE GYNOECIUM

It has been described that the cytokinin (*TCS::GFP*) and auxin-response (*DR5::GFP*) markers have well defined and mutually exclusive expression patterns in some regions of the gynoecium during development (Marsch-Martinez et al., 2012a). Besides, the auxin efflux carrier PIN1 is important for gynoecium development, because the *pin1* mutant produces almost no flowers and when flowers are produced their gynoecium show severe apical-basal patterning defects (Okada et al., 1991). We analyzed whether BAP or NPA application were able to cause changes in the expression pattern of PIN1 and the hormonal-response markers, and whether these changes could be related to the apical-basal gynoecium defects due to these treatments. For this purpose, each marker line was treated once a day for a period of 5 days, as done for the treatments described above, with the BAP or NPA solution for *TCS::GFP* and *DR5::GFP* and with BAP for *PIN1::PIN1:GFP*. The expression patterns of these marker lines were analyzed using confocal laser scanning microscopy when gynoecia with apical-basal defects were observed.

In wild type gynoecia between floral stages 8–10 (Smyth et al., 1990) the TCS::GFP signal was observed at the center, where the medial tissues are developing from the carpel marginal meristem (CMM), as we have observed before (Marsch-Martinez et al., 2012a; **Figures 4A,D**). After BAP or NPA treatment, the TCS::GFP signal was increased in the central zone of valveless gynoecia. However, these gynoecia had reduced development of the internal medial tissues (**Figures 4B,C,E**). For the DR5::GFP auxin-response marker in untreated gynoecia between stages 9–12 the signal was observed at the apical end of gynoecia and in the vasculature, as we have observed before (Marsch-Martinez et al., 2012a; **Figure 4H**). After BAP or NPA treatment, the DR5::GFP signal did not show obvious changes in these experiments (**Figures 4I,J**). However, in the wild type gynoecium at stage 10, the auxin efflux carrier PIN1 is expressed in the tissue that will give rise to the replum (**Figure 4F**), and after BAP treatment the *PIN1::PIN1:GFP* signal was observed in the whole valveless gynoecium (**Figure 4G**).

In summary, BAP and NPA application had comparable effects in the hormone reporter lines, this is, an increase in TCS::GFP activity in the central region of the gynoecium, but no detectable change in the DR5::GFP signal. Moreover, BAP application caused an increase in expression level and alteration of the localization of PIN1 in the gynoecium. These results correlate well with the observation that BAP and NPA treatments cause similar apical-basal patterning defects.

DISCUSSION

IMPACT OF CYTOKININ AND NPA APPLICATION ON APICAL-BASAL GYNOECIUM PATTERNING IN AUXIN TRANSPORT AND SIGNALING MUTANTS

Cytokinin is involved in different developmental processes throughout the *Arabidopsis* life cycle (Hwang et al., 2012; El-Showk et al., 2013), including proper gynoecium and fruit development (Marsch-Martinez et al., 2012a,b; Reyes-Olalde et al., 2013). Here, we evaluated the effect of exogenous cytokinin application on the establishment of apical-basal patterning of the *Arabidopsis* gynoecium.

BAP-treated gynoecia present the same apical-basal defects observed as when treated with NPA, but the frequencies in which altered phenotypes are observed are lower. Because the role of NPA is to block polar auxin transport and the phenotypes caused by both BAP and NPA treatments are similar, the results suggest that exogenously applied cytokinin might affect polar auxin transport and thereby cause the observed patterning phenotypes.

It has been reported that auxin biosynthesis or signaling mutant gynoecia are hypersensitive to NPA treatment in regard to apical-basal patterning (Staldal et al., 2008). In this study, we observed that the auxin transport mutants *pin3* and *pin7* were hypersensitive to both BAP and NPA treatments, and the sensitivity level was similar between treatments but different between mutants. In this case, the *pin3* mutant was more sensitive to either treatment

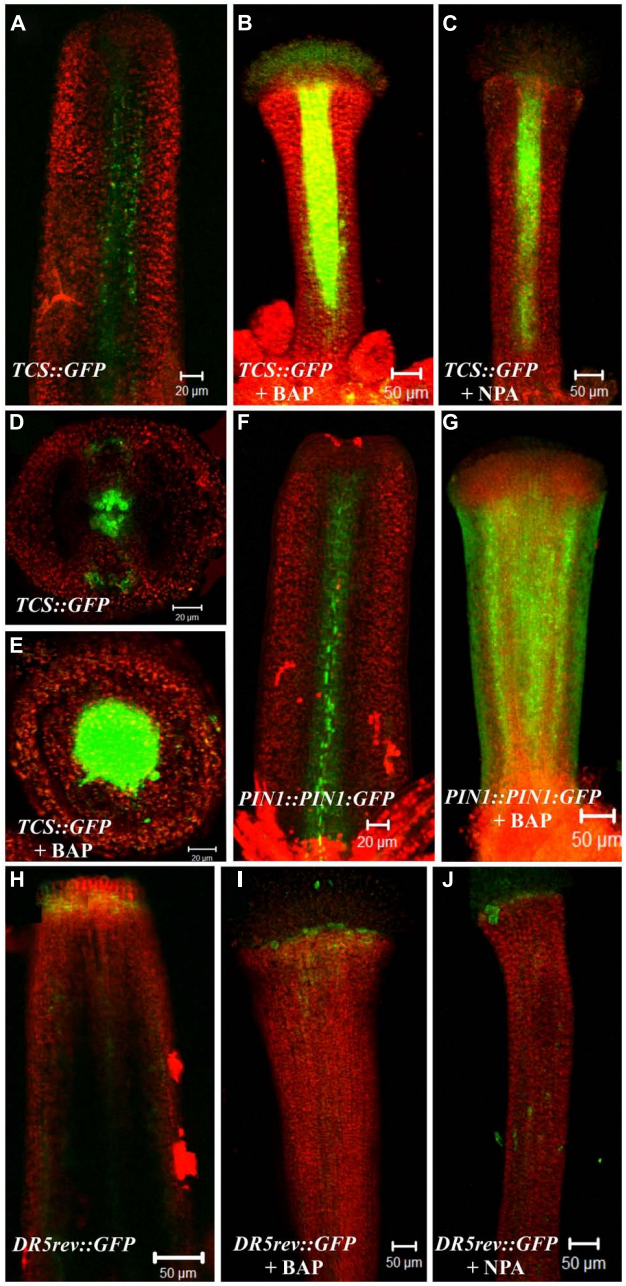

FIGURE 4 | Effect of cytokinin (BAP) and NPA application on the PIN1 (*PIN1::PIN1:GFP*), cytokinin (*TCS::GFP*) and the auxin-response markers (*DR5::GFP*). (A–E) The fluorescence signal of the cytokinin response marker *TCS::GFP* observed in the wild type gynoecium at floral stage 10 in a longitudinal view **(A)** and transverse view **(D)**. Valveless gynoecium at floral stage 11 caused by BAP treatment in a longitudinal view **(B)** and transverse view **(E)**. Valveless gynoecium at floral stage 11 caused by NPA treatment in a longitudinal view **(C)**. **(F,G)** The fluorescence signal detection of the PIN1 marker *PIN1::PIN1:GFP* observed in the wild type gynoecium at floral stage 10 **(F)**. Valveless gynoecium at floral stage 10 caused by BAP treatment **(G)**. **(H–J)** The fluorescence signal detection of the auxin response marker *DR5::GFP* observed in wild type gynoecium at stage 12 **(H)**. Valveless gynoecium at floral stage 12 caused by BAP treatment **(I)**. Valveless gynoecium at floral stage 12 caused by NPA treatment **(J)**. Scale bars: **(A,D–F)** 20 µm; **(B,C,G–J)** 50 µm.

compared to the *pin7* mutant, indicating that in the absence of the PIN3 function the imbalance caused by both BAP and NPA application has a greater impact on the establishment of apical-basal gynoecium patterning. This suggests that PIN3 and PIN7 contribute to different extent to proper gynoecium apical-basal patterning.

Furthermore, the different auxin signaling mutants analyzed in this study were also sensitive to both treatments. In the case of the auxin receptor mutants, only the mock-treated *tir1 afb2 afb3* gynoecia occasionally showed some apical-basal gynoecium patterning defects. However, the three different mutants were hypersensitive to BAP and NPA, suggesting that the proper establishment of the apical-basal gynoecium pattern is a robust process that even when auxin perception is severely affected can be carried out without major defects. However, when perturbations such as those caused by cytokinin application or by auxin transport inhibition occur, it becomes evident that a change in the level of auxin perception affects proper gynoecium development.

Auxin Response Factors (ARFs) are transcription factors that regulate transcription in an auxin-dependent manner. It is known that the *ARF7* and *ARF19* genes are involved in cell growth of leaves and in lateral root formation (Wilmoth et al., 2005; Okushima et al., 2007), and *ARF7* acts redundantly with *MONOPTEROS* (*MP/ARF5*) in the axial patterning of the embryo (Hardtke et al., 2004). We observed that the *arf7* and *arf19* mutants are hypersensitive to BAP application regarding apical-basal gynoecium patterning, suggesting a role of these genes in this process.

IMPACT OF CYTOKININ AND NPA APPLICATION ON APICAL-BASAL GYNOECIUM PATTERNING IN CYTOKININ SIGNALING MUTANTS

When the cytokinin receptor mutants were treated with BAP, less severe or no alterations were observed in apical-basal gynoecium patterning, suggesting that the exogenous cytokinin needs to be perceived by the plant to trigger these changes. Interestingly, the altered apical-basal patterning phenotypes caused by NPA treatments were increased in the cytokinin receptor mutants.

A comparison of the effects of both treatments in the different cytokinin receptor mutant backgrounds, suggested a negative correlation between the ability to respond to cytokinin and the severity of the phenotype caused by auxin transport inhibition. In the mutants where cytokinin perception was more affected, i.e., less alteration in patterning caused by BAP (least phenotypic effect observed in *cre1 ahk3*), the effect of NPA was increased, i.e., more visible alterations in patterning.

This may indicate that cytokinin (perception) buffers the effect of decreased auxin polar transport in apical-basal patterning.

IMPACT OF CYTOKININ AND NPA APPLICATION ON CYTOKININ (*TCS::GFP*) AND AUXIN-RESPONSE MARKERS (*DR5::GFP*) AND THE AUXIN TRANSPORTER PIN1 (*PIN1::PIN1:GFP*) IN THE GYNOECIUM

The cytokinin (*TCS::GFP*) and auxin-response (*DR5::GFP*) and PIN1 (*PIN1::PIN1:GFP*), markers were analyzed in gynoecia presenting apical-basal defects. The TCS::GFP signal was detected in the medial tissues during normal gynoecium development at

early stages. We followed the TCS::GFP signal in the BAP and NPA induced valveless gynoecia. In these gynoecia the medial tissue showed reduced development. However, the TCS::GFP signal was not only maintained, but interestingly, it was increased.

NPA treatments have been shown to inhibit the formation of lateral organs in shoot apical meristems (Reinhardt et al., 2000). The valves of gynoecia are considered lateral organs (Benkova et al., 2003), and NPA has a comparable effect, producing valveless gynoecia. In the shoot apical meristem context, NPA does not affect the meristematic activity as shown by the maintenance of the activity of various meristem markers (Reinhardt et al., 2000). At the gynoecium, the activity of the *TCS::GFP* marker suggests that a similar situation occurs in this tissue, i.e., that the valves are not formed, but the meristematic activity at the medial tissues continues. Interestingly, the cytokinin signaling was not only maintained after the NPA treatment, but seemed to increase, as revealed by the increased fluorescence observed at the medial tissues.

After BAP and NPA application, no evident changes were detected in the DR5::GFP signal in the abaxial (external) side of the valveless gynoecia, compared to the wild type. The model proposed by Sessions in 1997 suggests that the apical-basal patterning of the gynoecia is determined through the specification of two boundaries that are specified very early, during floral stage 6 when the gynoecial primordium is a radially symmetric dome of cells (Sessions, 1997; Larsson et al., 2013). Based on this, one possible explanation is that changes in auxin signaling (*DR5::GFP)* may occur in early stages (stage 5–7) during BAP or NPA-treated gynoecium development causing the apical-basal defects and such changes cannot be detected at later stages of gynoecium development. In order to test this hypothesis it would be necessary to analyze auxin signaling during earlier valveless gynoecia development, which is technically challenging, or by using a more sensitive auxin signaling marker like the DII-VENUS sensor (Brunoud et al., 2012).

On the other hand, cytokinin negatively affects PIN expression and localization in the root meristem (Laplaze et al., 2007; Dello Ioio et al., 2008; Ruzicka et al., 2009). In contrast, here we observed that the auxin efflux carrier PIN1 expression was increased and localized in whole valveless gynoecia due to cytokinin application. This suggests that cytokinin has an opposite effect on PIN1 expression in the gynoecium versus the root meristem, as similarly observed in the root vasculature (Bishopp et al., 2011).

The cytokinin–auxin interaction can occur at different levels, i.e., cytokinin can affect auxin synthesis, transport or signaling, and auxin can affect cytokinin synthesis, degradation or signaling (Hwang et al., 2012; El-Showk et al., 2013). With the generated data so far we cannot rule out any of these possibilities related to apical-basal gynoecium patterning. However, because the NPA role is to block polar auxin transport and the phenotypes caused by both treatments were very similar, the observations obtained from our experiments suggest that the exogenous BAP application may be able to affect polar auxin transport and therefore cause apical-basal gynoecium patterning defects. Supporting this hypothesis is the observation that cytokinin can affect PIN expression and localization in gynoecia. Further support comes from the fact that the different auxin transport or signaling mutants tested in this work showed a similar sensitivity level for both treatments and the TCS::GFP and DR5::GFP expression pattern, respectively, were also similar for both treatments. Another possibility is that exogenous BAP application affects auxin on more than one action level and that the induced apical-basal gynoecium patterning defects are due to the sum of these changes. Future work should give more insights into the molecular mechanisms.

AUTHOR CONTRIBUTIONS

Victor M. Zúñiga-Mayo and J. Irepan Reyes-Olalde performed experiments; all authors analyzed data; Victor M. Zúñiga-Mayo, Nayelli Marsch-Martinez, and Stefan de Folter drafted the manuscript. All authors provided intellectual content and contributed to manuscript revisions. All authors provided final approval of the manuscript. All authors agree to be accountable for all aspects of the work, including ensuring the accuracy and integrity of the work.

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A model for an early role of auxin in *Arabidopsis* gynoecium morphogenesis

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The female reproductive organ of angiosperms, the gynoecium, often consists of the fusion of multiple ovule-bearing carpels. It serves the important function of producing and protecting ovules as well as mediating pollination. The gynoecium has likely contributed to the tremendous success of angiosperms over their 160 million year history. In addition, being a highly complex plant organ, the gynoecium is well suited to serving as a model system for use in the investigation of plant morphogenesis and development. The longstanding model of gynoecium morphogenesis in *Arabidopsis* holds that apically localized auxin biosynthesis in the gynoecium results in an apical to basal gradient of auxin that serves to specify along its length the development of style, ovary, and gynophore in a concentration-dependent manner. This model is based primarily on the observed effects of the auxin transport blocker *N*-1-naphthylphthalamic acid (NPA) as well as analyses of mutants of *Auxin Response Factor (ARF) 3/ETTIN (ETT).* Both NPA treatment and *ett* mutation disrupt gynoecium morphological patterns along the apical–basal axis. More than a decade after the model's initial proposal, however, the auxin gradient on which the model critically depends remains elusive. Furthermore, multiple observations are inconsistent with such an auxin-gradient model. Chiefly, the timing of gynoecium emergence and patterning occurs at a very early stage when the organ has little-to-no apical–basal dimension. Based on these observations and current models of early leaf patterning, we propose an alternate model for gynoecial patterning. Under this model, the action of auxin is necessary for the early establishment of adaxial–abaxial patterning of the carpel primordium. In this case, the observed gynoecial phenotypes caused by NPA and *ett* are due to the disruption of this early adaxial–abaxial patterning of the carpel primordia. Here we present the case for this model based on recent literature and current models of leaf development.

Keywords: gynoecium, auxin, ETTIN, abaxial, adaxial

THE STRUCTURE OF *Arabidopsis* GYNOECIUM

Angiosperms, plants that produce flowers, are far and away the most diverse division of plants today, with even the most conservative estimates placing the number of known extant species at more than 223,000 (Scotland and Wortley, 2003). In addition to being an incredibly successful group in nature, flowering plants account for the vast majority of plants used and cultivated by humans, both for agricultural and for horticultural purposes. For this reason, there is great promise in the prospect of engineering angiosperm development to increase productivity, fecundity, and survivability. To do that in any systematic way, it is necessary to understand the genetic machinery that drives angiosperm development and that allows these plants to shape themselves into the vast diversity of forms seen in nature.

Evolutionarily, the flower consists of a complex of organs that are derived from leaves growing from a single stem (Coen and Meyerowitz, 1991; Honma and Goto, 2001; Pelaz et al., 2001; Scutt et al., 2006). A complete flower consists of the stem itself, divided into the pedicel and receptacle, and four different types of leaf-derived floral organs arranged in four concentric whorls around the stem. These are, from outermost to innermost: The sepals, which protect the flower; the petals, which serve as a display to attract pollinators; the stamens, which produce pollen; and the carpels, which contain the ovules that later develop into the seeds when they are fertilized. Carpels are of particular interest and significance as they constitute the angiosperms' defining feature. In many species, the carpels are fused into a single structure called the gynoecium. This structure is of critical economic importance, as it is the source of fruits and of seeds, including nuts, beans, and cereal grains. The interactions of genes and hormones that shape the structure, however, are not completely understood. *Arabidopsis thaliana*, a flowering weed and a model plant, has thus been under intensive investigation to address the underlying molecular mechanisms.

Like the other floral organs, the carpels are widely thought to represent modified leaves or sporophylls (Balanzá et al., 2006; Scutt et al., 2006; Vialette-Guiraud and Scutt, 2009; Reyes-Olalde et al., 2013). The ancestral carpel is most likely ascidiate, meaning it represents an invagination of a leaf to form a hollow structure sealed by a secretion (Endress and Igersheim, 2000; Endress and Doyle, 2009; Doyle, 2012). There are a number of possibilities as to how exactly this occurred, including curled leaf borne

on axillary branch or curled leaflets borne along the rachis of a compound leaf (Doyle, 2012). Examples of ascidiate carpels can be found in the basal extant angiosperms such as *Amborella* and water lilies. Most "higher" angiosperms, however, including most monocots and eudicots (*Arabidopsis* among them), instead possess plicate carpels (Endress and Doyle, 2009; Doyle, 2012). Rather than being an invagination of the leaf, the plicate carpel is curled or folded along its length into a tube-like or book-like shape, enclosing the ovules within (**Figure 1A**). This type of structure appears to have evolved by elongation of the apical end of the primitive ascidiate carpel. In angiosperms, irrespective of carpel type, the ovule-bearing surface is strictly adaxial (Doyle, 2012).

In *Arabidopsis*, two carpels are fused congenitally to form the gynoecium (Sattler, 1973; **Figure 1B**), and each carpel is homologous to an ancestral spore-bearing leaf (sporophyll; compare **Figure 1A** with **Figure 1B**). The adaxial tissues near the margins of the fused carpels are meristematic and are thus called the carpel margin meristem (CMM; **Figure 1B**). The CMM is responsible for generating the placenta, ovules, septum, transmitting tract, style, and stigma; these tissues are critical for the reproductive competence of the gynoecium (Wynn et al., 2011; Reyes-Olalde et al., 2013). From the base to the apex of the gynoecium are three morphologically distinct regions (**Figure 1C**). The basal-most region is the gynophore, a short stalk that connects the rest of the gynoecium to the flower. The apical-most region of the gynoecium consists of the style and stigma. In the middle of the gynoecium is the ovary; a cross section of the ovary (**Figure 1B**) shows two valves (also called ovary valves or carpel valves) separated externally by the replum and internally by a septum, dividing the interior into two locules. Each locule protects two rows of ovules initiated along the carpel edges from the CMM.

The homology between carpels and leaf-like lateral organs extends to the resemblance of carpel valves to leaf blades (lamina) and the CMM to the leaf margins. In certain angiosperm species such as *Kalanchoe daigremontiana*, also known as "mother of thousands," leaf margins produce plantlets and express the meristem marker gene *SHOOT MERISTEMLESS* (*STM*) in a small group of leaf margin cells that were initiating plantlets (Garcês et al., 2007), much like the *STM*-expressing placenta along the *Arabidopsis* carpel margins (Long et al., 1996). The possibility of conserved molecular mechanisms that specify the basic organ plan of the leaf and carpel draws support from several prior observations: Firstly, *N*-1-naphthylphthalamic acid (NPA) treatment causes the formation of both needle-like leaves without a lamina and of stalk-like gynoecia without valves (Okada et al., 1991). Further, NPA treated young leaves showed increased density of veins along their margins and multiple parallel midveins, much like NPA-treated gynoecia where the veins linking the gynoecium to the receptacle are increased in number (Nemhauser et al., 2000). Secondly, when one manipulates the expression of A, B, C, and E-class floral homeotic genes, floral organs can be turned into leaves or vice versa (reviewed in Goto et al., 2001). Thirdly, single sepals can be readily turned into single, free carpels, such as in *Arabidopsis ap2-2* mutants (Bowman et al., 1989).

AUXIN REGULATES GYNOECIUM DEVELOPMENT

Of critical importance to the development of the plant is auxin, a family of hormones of which the most common is indole-3-acetic acid (IAA). This tryptophan-derived chemical is needed for many different processes in the plant, including lateral organ initiation and morphogenesis, phototropism, lateral root initiation, xylem formation, and apical dominance (Arteca, 1996; Benková

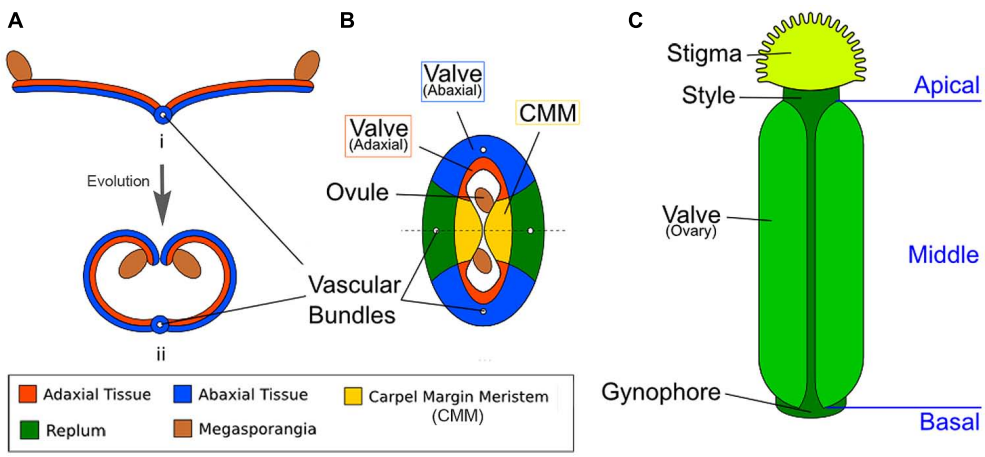

FIGURE 1 | Diagrams illustrating the homology between modern carpels and ancestral leaves. (A) Hypothetical evolution of a single plicate carpel based on Scagel (1965). (i) A cross section of an ancestral plant's spore-bearing leaf (sporophyll), showing megasporangia at the leaf edge. (ii) Over evolutionary time, inward curling of a megasporangia-bearing leaf and subsequent fusion at the leaf margin led to a one-chamber ovary with two rows of megasporangia on the interior (adaxial side). The actual evolutionary path is more complicated and not fully settled. **(B)** The cross section view of the *Arabidopsis gynoecium*, consisting of two fused carpels enclosing two locules. Note the vascular bundles. Although there are four rows of ovules, only two ovules are visible in the cross-section since the rows alternate within each locule. **(C)** A diagram of the *Arabidopsis gynoecium*, showing that it consists of three regions along the basal-to-apical axis. The basal section consists of a short stalk, the gynophore, the middle section is the ovary, and the apex consists of style and stigma.

et al., 2003; Friml, 2003). Auxin was the first plant hormone to be identified and has classically been characterized as a hormone synthesized in growing apices and transported down toward the roots.

AUXIN BIOSYNTHESIS

The IAA biosynthetic pathway begins with tryptophan or a tryptophan precursor (Bartel, 1997; Ljung, 2013). Recent reports suggest that auxin biosynthesis in plants involves only a two-step pathway, in which *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1)* and its four homologs *TAR1-4* convert tryptophan to indole-3-propionic acid (IPA). Members of the *YUCCA (YUC)* family of flavin monooxygenases then catalyze the conversion of IPA to auxin (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Zhao, 2012).

Analyses of the expression and mutant phenotypes of auxin biosynthesis genes indicate that localized synthesis of auxin is critical to proper gynoecium morphogenesis. Among the 10 *YUC*-family genes, *YUC1* and *YUC4* appear to play important roles in gynoecium development (Cheng et al., 2006) as double *yuc1 yuc4* mutants show a stalk-like gynoecium (**Figures 2A,C**), completely missing the ovary valves. *In situ* hybridization and promoter-*GUS* (β-glucuronidase) fusions have revealed that both *YUC1* and *YUC4* are expressed in inflorescence apices and young floral primordia. Most interestingly, *YUC1* and *YUC4* are expressed at the base of young floral organs including carpel primordia (Cheng et al., 2006). This specific expression pattern at the base of emerging floral organs is likely critical to proper floral organ initiation and apical–basal patterning (see later sections). In older flowers, *YUC4* expression is concentrated at the apical tip of carpels, stamens, and sepals (Cheng et al., 2006) and may be involved in later proper differentiation of floral organs.

Likewise, double mutants of TAA1/TAR family genes exhibit stalk-like gynoecia similar to those of *yuc1 yuc4* double mutants (Stepanova et al., 2008). The TAA1-GFP protein is localized in a few cells located at the apex (L1 layer) of young floral primordia as early as floral stage 2. This localized expression continues to floral stage 4, when a few epidermal cells at the central dome of the carpel primordium express TAA1. Since floral stage 4 is when carpel primordia emerge, this localized TAA1 expression may be involved in the apical–basal patterning of the gynoecium. Later, at floral stages 5–9, TAA1-GFP is prominently expressed in the medial ridge region of the gynoecium; this later stage expression maybe relevant to the development of marginal tissues including ovules, styles, and stigma. Based on localized and specific expression patterns of TAA1/TAR, Stepanova et al. (2008) suggested that auxin is synthesized in different regions at different developmental times and that localized auxin biosynthesis may represent a mechanism redundant to auxin transport in ensuring that robust local auxin maxima are able to form.

AUXIN SIGNALING

Auxin signaling consists of a system of the TIR/AFB family of receptors, the IAA family of repressors, and the *ARF* family of transcription factors. ARFs contain a DNA binding domain but most require homodimerization to bind DNA (Ulmasov et al., 1999). IAA-family repressor proteins bind to ARFs and competitively inhibit their ability to homodimerize. The TIR/AFB family of auxin receptors, when bound by auxin, induces the ubiquitination and degradation of the IAA repressors, thus freeing the ARFs to bind DNA. This may result in transcriptional activation or repression of target genes, depending on the co-factors bound to the ARF (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Mockaitis and Estelle, 2008; Calderón Villalobos et al., 2012). AUXIN BINDING PROTEIN1 (ABP1) represents a second type of auxin receptor, which acts as part of a system of rapid and local auxin responses on the plasma membrane (Dahlke et al., 2010; Xu et al., 2010; Effendi and Scherer, 2011; Shi and Yang, 2011; Craddock et al., 2012). The plasma membrane localized TMK1 receptor-like kinase was recently found to physically associate with ABP1 at the cell surface to regulate ROP GTPase signaling in response to auxin (Xu et al., 2014). In addition, ABP1 also acts to negatively regulate

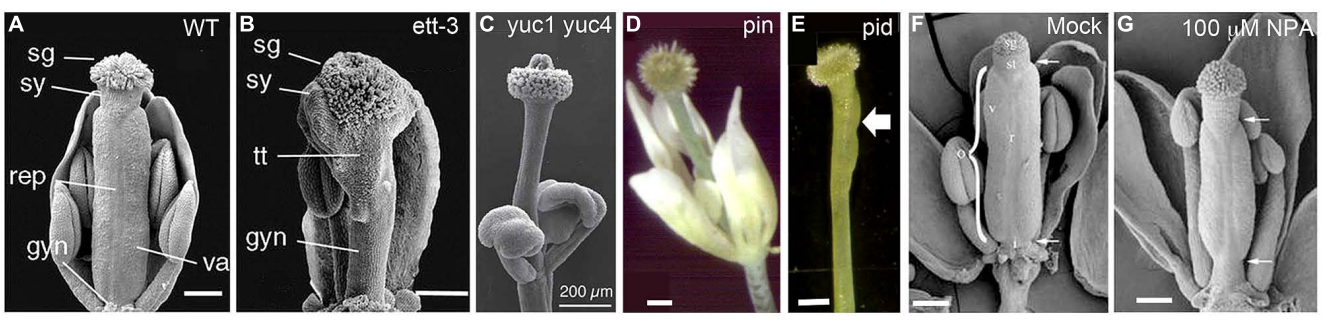

FIGURE 2 | Gynoecium phenotypes of mutants defective in auxin biosynthesis, transport, or signaling. (A) Wild-type gynoecium at stage 12 with the parts labeled as stigma (sg), style (sy), replum (rep), valves (va), and gynophore (gyn). **(B)** ett-3 gynoecium at stage 12, showing an elongated gynophore, a diminished valve pushed toward the apex, and expanded stigma, style, and transmitting track (tt) tissue. **(C)** Gynoecium of a yuc1-1 yuc4-1 double mutant, showing the complete absence of ovary valve and an enlarged apical stigma. **(D)** A weak pin mutant showing a gynoecium without any ovary valve tissue. **(E)** A pid gynoecium with one small ovary valve (arrow). **(F,G)** NPA-treated wild type *Arabidopsis* gynoecium. The apical and basal boundaries of the ovary are marked by a pair of arrows. The various tissues are indicated with letters: ovary (o), replum (r), valve (v), style (st), and stigma (sg). Images are reproduced from Heisler et al. (2001; **A,B**), Cheng et al. (2006; **C**); Roeder and Yanofsky (2006; **D,E**), and Nemhauser et al. (2000; **F,G**) with permissions from Copyright Clearance Center or Creative Commons Attribution-Non-Commercial 4.0 International License. Scale bars: 200 μm **(A–C)**; 250 μm **(D,E)**; 165 μm **(F)** and 140 μm **(G)**.

the SCF (TIR/AFB)-mediated auxin signaling pathway (Tromas et al., 2013).

ETTIN (*ETT*), also known as *ARF3*, is a member of the ARF family. Its closest in-paralog is *ARF4*, from which it appears to have split early in angiosperm evolution (Finet et al., 2010). *ETT* and *ARF4* are also expressed in the abaxial domain of leaves and floral organs, where they are believed to function as abaxialization factors in lateral organ development (Sessions et al., 1997; Pekker et al., 2005; Hunter et al., 2006). In the gynoecium, *ett* mutants show diminished or absent carpel valve tissue and an expansion of stigma, stylar, and basal gynophore (**Figure 2B**; Sessions and Zambryski, 1995; Sessions, 1997; Sessions et al., 1997; Heisler et al., 2001). The severe gynoecium phenotype of *ett* provided one of the earliest clues pointing to auxin as a critical regulator of gynoecium morphogenesis.

AUXIN TRANSPORT

Auxin travels through the plant via a cell-to-cell, "bucket brigade" style of transport. According to the chemiosmotic model, first proposed by Rubery and Sheldrake (1974), the acidic environment of the extracellular space (the apoplast) protonates the auxin, allowing IAA to diffuse across the plasma membrane into adjacent cells. Once inside a cell, it is exposed to a more alkaline pH and becomes deprotonated. The resulting anionic IAA^- is unable to cross the lipid bilayer without the help of efflux carriers. There are two different families of efflux transport proteins. The *PIN-FORMED* (*PIN)* family of efflux carriers is localized to a particular pole of the cell, exporting IAA selectively in the direction corresponding to PIN's localization (Wiśniewska et al., 2006; Löfke et al., 2013). The ATP Binding Cassette B (ABCB) transporters represent the second type of auxin efflux transporters. ABCB and PIN can independently as well as coordinately transport auxin (Titapiwatanakun and Murphy, 2009; Peer et al., 2011). Distinct modes of directional auxin transport operate in different developmental contexts. "Up-the-gradient" PIN1-based transport generates auxin maxima at lateral organ initiation sites, while "with-the-flux" PIN1 polarization operates in leaf midvein patterning (Bayer et al., 2009).

A third class of auxin transport proteins is the AUX1/LAX family of auxin uptake symporters. Though IAA is believed to be capable of entering a cell from the apoplast by passing through the membrane on its own (Rubery and Sheldrake, 1974), these auxin uptake symporters are still necessary for a number of developmental processes due to their ability to create sinks for auxin to flow into (reviewed in Titapiwatanakun and Murphy, 2009; Peer et al., 2011). In addition, AUX1 was proposed to play a role in restricting auxin to the epidermis of vegetative meristems by counter-acting the loss of auxin caused by diffusion into the meristem inner layers (Reinhardt et al., 2003).

Strong null mutants of *PIN1* produce no lateral organs or axillary shoots, resulting in the bare, pin-like shoot that gives the mutants their name (Okada et al., 1991; Gälweiler et al., 1998; Palme and Gälweiler, 1999; Benková et al., 2003). In weak *pin* mutants, lateral organs can develop but the gynoecium is often valveless and topped with stigmatic tissues, which is reminiscent of the abnormal gynoecium of auxin biosynthesis mutants described above (compare **Figures 2C,D**). *PINOID (PID)*, an AGC3-type protein kinase, acts to phosphorylate PIN to regulate PIN's polar localization in the cell (Friml et al., 2004; Huang et al., 2010). Interestingly a similar gynoecial phenotype was observed in *pid* mutants (**Figure 2E**; Bennett et al., 1995; Benjamins et al., 2001). The action of *PIN* proteins in transporting auxin may be blocked via the application of NPA. Application of NPA to wild type *Arabidopsis* mimics *pin* mutant phenotypes (Okada et al., 1991; Nemhauser et al., 2000) with pin-like shoots as well as abnormal gynoecia without any valve or with reduced valves (**Figures 2F,G**). Taken together, while severe disruption of polar auxin transport abolishes all lateral organ initiation and hence results in the formation of pin-like shoots, milder disruption of polar auxin transport allows lateral organ initiation but blocks proper lateral organ morphogenesis, resulting in stalk-like gynoecia (**Figures 2D,E**). The weaker *pin* and *pid* mutant phenotypes provide strong evidence that polar auxin transport is critical for gynoecium morphogenesis.

THE NEMHAUSER MODEL OF GYNOECIAL PATTERNING

Multiple lines of evidence strongly indicate that the action of auxin is critical for proper development and apical to basal patterning of the gynoecium. Mutants of biosynthesis (*yuc* or *taa/tar*) and transport (*pin* and *pid*) genes show the strongest gynoecium phenotype, a phenotype that is nearly identical between them: their valveless gynoecium is basically a thin and round stalk topped with stigmatic tissues (**Figures 2C–E**). Application of the polar auxin transport inhibitor NPA shows a similar but weaker phenotype with reduced ovary valves (**Figures 2F,G**). While mutations in the auxin signaling gene *ett/arf3* cause a similar effect to those of auxin biosynthesis (*yuc/taa/tar*) or transport (*pin/pid*) in reducing ovary valve, *ett/arf3* mutants appeared to exhibit more expanded stigma and stylar tissues (**Figure 2B**).

Based on the phenotype of *ett/arf3* and the effect of NPA treatment on wild type and *ett/arf3* gynoecia, Nemhauser et al. (2000) proposed a model wherein auxin biosynthesized locally at the apex of the gynoecium is transported basipetally, resulting in a gradient of auxin concentration with a maximum at the apex, mid-range level in the middle, and a minimum at the base (**Figure 3A**). The high auxin level at the apex specifies stigma/style, while the mid-range level promotes valve formation. At the base when auxin level is low, gynophore develops. *ETT* is partly responsible for interpreting this gradient, and promotes the formation of valve tissue in the middle region of gynoecium where there is a mid-range level of auxin. Under this model, when the gynoecium is exposed to NPA, the auxin produced at the apex is not transported down as readily, resulting in a steeper and up-shifted gradient (**Figures 3A–C**). This results in the observed phenotype of a smaller amount of valve tissue being formed near the apex of the gynoecium and a "bushier" stigma, which could be explained under this model by pooling and accumulating a higher level of apically synthesized auxin at the gynoecium apex. Because of the shift of auxin gradient toward the apex, the basal region, the gynophore, is expanded (**Figures 3A–C**). Mutants of *ETT*, under this model, show a similar phenotype because the job of *ETT* is to interpret the mid-range auxin gradient in the middle segment of the gynoecium to promote valve formation. In the absence of *ETT*, therefore, the auxin gradient is invisible to the plant, and valve fails to form (**Figure 3C**).

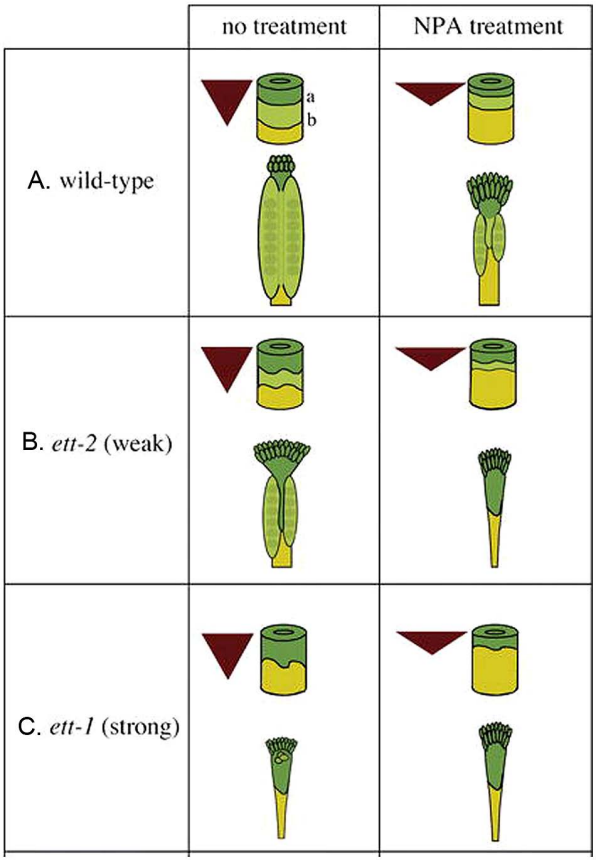

FIGURE 3 | The auxin gradient model. Auxin is produced at the apex and transported toward the base, creating a morphogenic gradient that provides positional information, which is interpreted in part by *ETT* to specify ovary valve. The triangle represents the auxin gradient within the gynoecium. The cylinder represents the gynoecium with border marked "a" between the style (dark green) and ovary (light green) and border marked "b" between the ovary and gynophore (yellow). **(A)** Wild-type gynoecia with and without NPA treatment. **(B)** Weak *ett-2* mutants with a mild phenotype (left); the phenotype is significantly enhanced when *ett-2* mutants were treated with NPA (right). **(C)** Strong *ett-1* mutants with a strong phenotype with or without NPA treatment. The figure is reproduced from Nemhauser et al. (2000) with permission from Copyright Clearance Center.

This model was reasonably consistent with the data available at the time. Since then, however, additional information has emerged. The auxin biosynthesis gene *YUC4* is expressed (among other places) in a small region at the tip of multiple lateral organs, including cotyledons, and stamens. However, it does so largely when the organs are close to maturity (Cheng et al., 2006). In the gynoecium, the apical *YUC4* expression is not visible until after the gynoecial apical-to-basal patterning is largely determined (after stage 7–8; Cheng et al., 2006) and thus is not likely to be responsible for the initial pattern formation of the gynoecium. At earlier stages of floral meristem development (stages 3–7; staging based on Smyth et al., 1990), *YUC4* as well as *YUC1* are expressed at the bases of young floral organ primordia, including the base of young gynoecia. In light of the timing and the dramatic gynoecium phenotype of *yuc1 yuc4* double mutants (**Figure 2C**), the early expression pattern around young floral primordia maybe more relevant to gynoecial apical-to-basal patterning than the later-stage *YUC4* expression at the apex. Further, if auxin is made at the apex and responsible for stigma formation, we would expect to see a reduced or diminished stigmatic tissue in *yuc1 yuc4* double mutants. However, *yuc1 yuc4* double mutants as well as *taa/tar* double mutants produce heads of stigmatic tissue even larger than wild type and their phenotypes are little different from those of plants that fail to transport auxin and therefore supposedly pool the auxin at the apex due to a lack of downward transport (compare **Figure 2C** with **Figures 2D,E**; Cheng et al., 2006; Stepanova et al., 2008).

Various attempts have been made to visualize the proposed auxin gradient using the *DR5* reporter. DR5 consists of tandem direct repeats of an 11-bp auxin-responsive element and, when used to drive a reporter gene, serves to report local auxin response (Ulmasov et al., 1997). Larsson et al. (2013) examined auxin distribution during early stage gynoecium development (about stage 7) using the *DR5rev::GFP* reporter. Two weak foci were detected at the apical tips of stage 7 flowers. At later stages (about stage 8), *DR5rev::GFP* expression was expanded into four foci (both medial and lateral domains) and in the pro-vasculature. Throughout the development, no gradient was observed. Other experimental work has also shown localization of auxin only to the apex of gynoecia in flowers at stage 6 or older, without showing a gradient along the apical-to-basal axis at any stage (Benková et al., 2003; Girin et al., 2011; Grieneisen et al., 2013). These data do not support the auxin gradient model.

Finally, the auxin gradient model proposed that the auxin is transported in a basipetal direction. Yet studies of the polar localization of auxin efflux carrier PIN1 show accumulation in the apical side of the replum cells (Sorefan et al., 2009; Grieneisen et al., 2013), indicating upward transport.

Fourteen years after the proposal of the auxin gradient model, accumulating new data suggest that this model, while highly attractive at the time it was proposed, should be revised or re-evaluated. Alternative models that better interpret and incorporate these new observations should be proposed.

OTHER ALTERNATIVE MODELS

Prior to the Nemhauser's auxin gradient model, Sessions (1997) proposed a "boundary" model, in which *ETT* was proposed to regulate the two boundary lines that trisect the gynoecium into three regions, with one boundary (the apical line) dividing the ovary from the stylar tissues and the second boundary (the basal line) dividing the gynophore from the ovary above it. Sessions (1997) further proposed that the two boundaries are set as early as stage 6 of flower development, when the effects of *ett* begin to be observed. Based on this model, the effect of *ett* was interpreted as simultaneously lowering the apical boundary line and raising the basal boundary line. These two lines are also proposed in the Nemhauser model (**Figure 3**), which was built upon Sessions' "boundary" model. Since the molecular identify of *ETT* as an ARF was not published at the time when the "boundary" model was proposed, the connection to auxin was not proposed. Although Sessions (1997) mentioned an adaxial/abaxial boundary located at the distal tip of the carpel

primordia, *ETT* was not proposed to regulate the adaxial/abaxial boundary.

Recently, Larsson et al. (2013), unable to detect an auxin gradient along the apical-to-basal axis of early stage gynoecium using the *DR5rev:GFP* reporter described above, pointed out that their data did not strongly support the Nemhauser gradient model. In addition, Larsson et al. (2013) noted the fact that auxin biosynthesis genes are expressed in regions not limited to the gynoecium apex as another inconsistency with the Nemhauser gradient model. They then proposed several alternative ideas/models. One was the proposal of an abaxial domain KANADI (KAN)–ETT complex that regulates PIN activity and localization during positional axis determination in gynoecia. This idea directly links AD/AB polarity with auxin in the determination of the apical-to-basal axis of gynoecia and is similar to what is being proposed below. Another idea put forth by Larsson et al. (2013) was the differential sensitivity or response of the lateral vs. medial tissues of gynoecium to auxin polar transport inhibitors.

LESSONS FROM LEAF MORPHOGENESIS

Auxin has long been known to play a role in leaf initiation. Auxin is observed to pool in small areas (maxima) on the shoot apical meristem, and the appearance of such an auxin maximum presages the formation of each lateral organ primordium (Reinhardt et al., 2000, 2003; Benková et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Smith et al., 2006). An auxin maximum in the L1 layer of the meristem is the earliest mark of a new lateral organ primordium. The formation of such auxin maxima correlates with localization of the membrane-associated auxin efflux carrier PIN1, in each epidermal cell, to the side of the cell that faces toward the neighbor with a higher auxin concentration. This "up-the-gradient" transport helps to amplify the localized concentration of auxin. Heisler et al. (2005) showed *pPIN1::PIN-GFP* localization in the L1 layer toward incipient primordia starting at incipient primordium stage 3 (I3; from youngest to oldest, the stages are I3, I2, I1, budding-primordium1 (P1, P2, etc.). The signal intensity of the polarized PIN-GFP toward the auxin maxima increased steadily until primordial stage P1. The PIN1-GFP in the adaxial domain of lateral organ primordia then showed a brief reversal of transport, switching from being directed toward the primordium to being directed away from the primordium. These two waves of auxin transport suggest that auxin may act twice in lateral organ development, first in organ primordium initiation and then possibly in organ growth. If so, the timing and specific context of auxin flow may affect different processes of organ development.

The function of auxin maxima and polar auxin transport in lateral organ initiation and growth was demonstrated by examining *pin* mutants, where auxin maxima as well as lateral organ formation were absent. Further, application of auxin to the peripheral zone of the meristem induces lateral organ formation (Reinhardt et al., 2000, 2003; Smith et al., 2006). However, Smith et al. (2006) showed that short-term NPA treatment failed to abolish the auxin maxima, suggesting the presence of additional mechanisms that help redistribute auxin within the epidermis of the shoot apical meristem. On reaching their convergence point, the auxin flows switch the direction and go basipetally toward the roots (**Figures 4A–D**; Berleth et al., 2007). The internal auxin flows are responsible for the leaf midvein formation and utilize the "with-the-flux" transport mode (Bayer et al., 2009).

Soon after a leaf primordium is initiated, one of the first signs of patterning appears in the specification of the adaxial (upper; AD) and abaxial (lower; AB) halves of the leaf. This early patterning is believed to happen in response to a signal generated at the apex or shoot apical meristem (Sussex, 1951; reviewed in Husbands et al., 2009). If the path from shoot apex to primordium is blocked, such as by a cut made directly above the incipient primordium, the adaxial–abaxial patterning of the leaf will be disrupted. The identity of this signal is still unknown but auxin remains a possibility (Husbands et al., 2009).

The AD and AB domains not only exhibit characteristic cell morphology but also express cohorts of domain-specific genes (reviewed in Kidner and Timmermans, 2007; Liu et al., 2012). These gene cohorts, generally mutually repressive, will remain associated with the AD and AB sides of the leaf as they develop. Therefore, the earliest differentiation of the AD and AB domains in lateral organ primordia can be detected by examining AD-

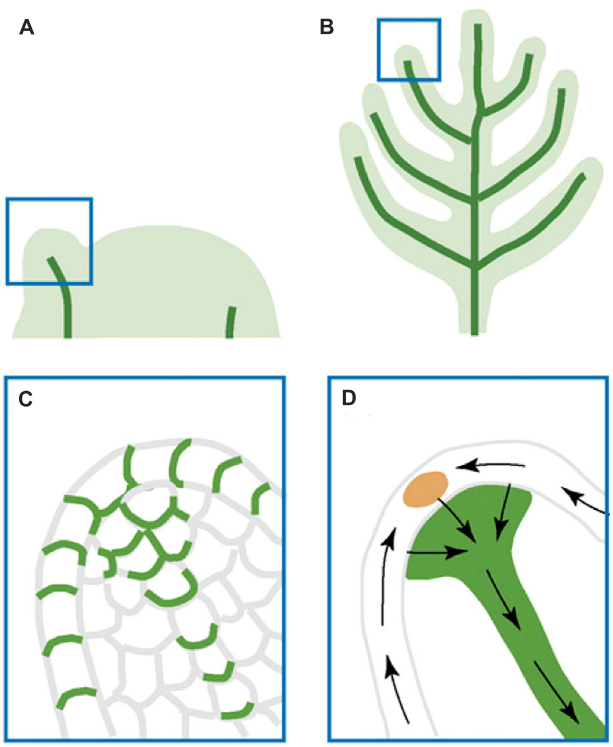

FIGURE 4 | Illustration of auxin transport during leaf and lateral organ initiation. (A) Leaf primordial initiation. **(B)** Lateral organ initiation. **(C)** A zoom-in diagram of the leaf primordium tip showing PIN:GFP (green) polar localization that indicates auxin transport routes. **(D)** Inferred auxin transport routes (black arrows) based on PIN:GFP localization. The epidermal convergence of two counter-oriented auxin flows results in a change of auxin transport direction toward the internal base of the primordium. This internal flow is responsible for the formation of the midvein. The figure is reproduced from Berleth et al. (2007) with permission from Copyright Clearance Center.

and AB-specific marker genes. As early as stage I1, the adaxial marker *REVOLUTA (REV; pREV::REV-VENUS)* was found to be visibly expressed in the adaxial domain of incipient primordia while the abaxial marker gene *FILAMENTOUS FLOWER (FIL; pFIL::DsRED-N7*) was expressed in the abaxial domain (Heisler et al., 2005). Further, *pPIN1::PIN1-GFP* expression was found to mark the boundary between AD and AB domains marked, respectively, by *pREV::REV-VENUS* and *FIL::dsRED-N7* (Heisler et al., 2005). Based on these results, Heisler et al. (2005) proposed that the auxin transport route plays a role in positioning the boundary between adaxial and abaxial cells. Barton (2010) also noted that the AD/AB boundary in a primordium coincides with the point in the primordium on which the epidermal auxin flows from opposite directions converge. If causal, this would indicate that a specific role of auxin transport is to establish the AD/AB boundary in incipient organ primordia.

Proper specification of the AD/AB domains is critical for proper leaf development because it generates the AD/AB boundary and the juxtaposition of AD and AB domain is essential for leaf blade formation (Waites and Hudson, 1995). Many of these AD/AB polarity genes are required for the leaf to grow a blade (lamina), and disruption of one or more of them often creates needle-like structures, with the lamina absent or severely reduced. Examples of this include single mutants of the adaxialization factor *PHANTASTICA* in *A. majus* (Waites and Hudson, 1995), double or triple mutants of the abaxialization factor family *KAN* (Eshed et al., 2004; Pekker et al., 2005), mutants of the HD-ZIPIII adaxially localized proteins (McConnell and Barton, 1998; Emery et al., 2003), and mutants of YABBY genes (Stahle et al., 2009; Sarojam et al., 2010).

ETT/ARF3 and its paralog *ARF4*, both auxin signaling components, have been suggested as the essential intermediaries for the gradual establishment of abaxial identity in lateral organs initiated by *KAN*. *KAN* encodes a GARP transcription factor and plays a key role in the abaxial identity specification of leaves, carpels, embryos, and vasculature (Eshed et al., 2001, 2004; Kerstetter et al., 2001; Ilegems et al., 2010). Since *KAN* does not regulate *ETT*/*ARF4* transcription, and over-expression of *ETT* or *ARF4* cannot rescue *kan1 kan2* double mutants, they are thought to act cooperatively (Pekker et al., 2005). Interestingly, ETT has been found to physically interact with a KAN family protein, ATS/KAN4 (Kelley et al., 2012). This ETT–KAN complex likely acts in different developmental contexts, embryogenesis, integument development, and leaf lamina growth, by promoting abaxial fate and repressing adaxial fate (Kelley et al., 2012).

Recently it was shown that KAN1 and the adaxial HD-ZIPIII factor, REV, oppositely regulate genes in auxin biosynthesis, transport, and signaling (Merelo et al., 2013; Huang et al., 2014). KAN was shown to regulate PIN1 expression and localization during embryo as well as vascular development (Izhaki and Bowman, 2007; Ilegems et al., 2010). Additionally, the AS1–AS2 nuclear protein complex involved in leaf AD/AB polarity specification was recently shown to directly and negatively regulate *ETT* (Iwasaki et al., 2013). These experiments indicate that proper AD/AB polarity establishment and maintenance in leaves critically depend on proper regulation of auxin synthesis, transport, and signaling. Thus, dynamic auxin regulation and AD/AB polarity specification and maintenance appear to regulate each other in a feedback loop in different tissue and developmental contexts. Any disruption in auxin synthesis, transport, and signaling will affect AD/AB polarity and vice versa.

A NEW MODEL: THE EARLY ACTION MODEL OF AUXIN ON GYNOECIUM PATTERNING

The evolutionary derivation of floral organs from leaf-like lateral organs suggests that the basic molecular tenets of the regulation of lateral organ polarity may be conserved. Indeed, carpels, like leaves, express members of the same gene families that control leaf AB/AD polarity. *ETT* and *ARF4* are clearly involved in carpel development and show abaxial domain-specific expression around the outer side of the tube of the developing gynoecium, the side that is equivalent to the underside of the leaf (Pekker et al., 2005). Similarly, the expression of class III HD-ZIP adaxialization factor *PHABULOSA* (*PHB*) and the abaxialization factor *YABBY1* (*YAB1*) are detected in the carpels in an equivalent configuration to that of members of their respective families found in the leaf (Franks et al., 2006; Nole-Wilson et al., 2010).

If an individual carpel primordium develops in an analogous manner to that of a leaf primordium, the AD/AB boundary of the carpels should be set very early in their development, at the incipient carpel primordium stage (approximately at floral stage 3–4). Further, auxin should have a major role to play at this stage in specifying the initial AD/AB boundary. The expression of the *YUC1* and *YUC4* genes suggests that auxin production is likely localized to the base of individual floral organ primordia at the very beginning of the primordial initiation (Cheng et al., 2006); this local auxin production and subsequent transport may contribute, at least partly, to the establishment of the AD/AB boundary in developing carpel primordia. As suggested by Stepanova et al. (2008), localized auxin biosynthesis and transport may represent a mechanism redundant to the transport of auxin from elsewhere to ensure robust local auxin maxima at the organ primordia. The site of auxin maximum at the incipient carpel primordium may set the sharp AD/AB boundary, as has been proposed for leaves and lateral organs (Heisler et al., 2005; Barton, 2010).

Based on the ideas put forward by Larsson et al. (2013) linking AD/AB polarity to auxin in the determination of the apical-to-basal axis of gynoecia, we further propose that proper AD/AB polarity establishment and boundary juxtaposition in carpels is necessary for the upward growth of the carpel valve, analogous to the requirement of AD/AB boundary juxtaposition in leaf lamina formation. The valveless gynoecia in auxin pathway mutants are therefore much like the bladeless leaves of polarity mutants. Since the two carpels are congenitally fused, their primordia rise as a circular ring (**Figure 5A**; Sessions, 1997). We propose that the AD/AB boundary likely resides at the apical ridge of the ring. The close juxtaposition of AD and AB domains on either side of this boundary causes the ring ridge to grow vertically as a long hollow tube with adaxial tissues facing inward (**Figure 5C**). However, at the base of the gynoecium primordium, the AD/AB boundary is diffuse, resulting in the base of the primordium developing into a single radially symmetric and non-hollow gynophore. If the AD/AB boundary is

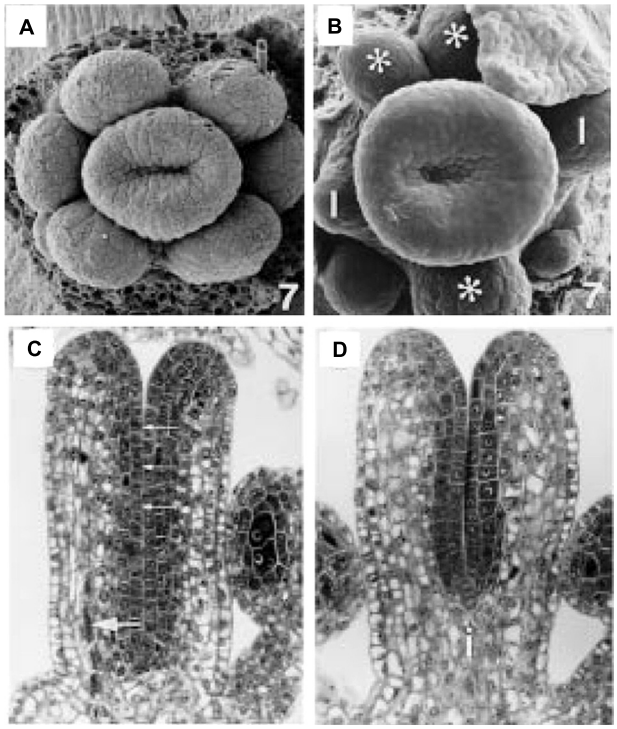

FIGURE 5 | Early stage wild type and *ett-1* gynoecium development. **(A)** Stage 7 wild type floral meristem showing upward growth of the gynoecial tube. **(B)** Stage 7 *ett-1* floral meristem showing a shallower gynoeciual tube. Aberrant stamen is marked with *. Scale bar is 22 μM **(A)** and 30 μm **(B)**, respectively. **(C)** Section of the medial plane of a stage 8 wild type gynoecium showing inner surface (small arrows) and medial vascular bundle (large arrow). **(D)** Section in the medial plane of a stage 8 *ett-1* gynoecium showing a shorter tube. The basal gynophore (i) is more prominent. Images reproduced from Sessions (1997) with permission from *American Journal of Botany*.

disrupted, for example in *ett* mutants, the upward growth of the ring ridge fails to occur, or only occurs to limited extent resulting in a shallower tube (**Figures 5B,D**). The elongation of the gynophore may be regulated by a separate mechanism related to the proximal–distal growth similar to the elongation of needle-like leaves in polarity mutants. **Figure 6** depicts the early action model in wild type and different auxin pathway mutants. In wild type (**Figure 6A**), each incipient carpel primordium is divided into AD and AB domains at the site of convergence of the two opposing auxin flows (indicated by the yellow arrows). The sharp AD and AB boundary marked by a black line is located near the apical surface of the incipient primordium and responsible for the upward growth of the hollow tube. Mutants of the auxin signaling component and abaxialization factor *ETT/ARF3* have compromised abaxial identity (Pekker et al., 2005), which may lead to partially adaxialized carpels and hence enlarged adaxial tissues like stigma and style. In weak *ett* mutants (**Figure 6B**), a compromised abaxial domain means a reduced AD/AB boundary at the time of carpel primordium emergence (approximately floral stages 3–4). This is indicated by a short black line (AD/AB boundary) at the apical surface of the incipient primordium (compare **Figure 6Bi** with **Figure 6Ai**) and a shorter gynoecium tube (**Figure 6Bii**). In support of an early role of AD/AB polarity in specifying gynoecium patterning, double mutants of the *KAN* gene family with compromised abaxial identity also exhibit similar gynoecium phenotypes to *ett* mutants (Eshed et al., 2001; Pekker et al., 2005).

Mutants defective in auxin polar transport (in *pin* or *pid* mutants, or by NPA treatment) exhibit weakened or absent auxin flows into the incipient carpel primordium (**Figures 6Ci,iii**), which will lead to a lack of a clear AD/AB boundary in the incipient carpel primordium indicated by a lack of the black line. As a result no valve or a reduced valve will form. Mutants of auxin biosynthesis (in *yuc1 yuc4* or *taa/tar* mutants) likely have insufficient auxin to be transported toward the incipient primordium, resulting in the absence of AD/AB domains and hence a lack of gynoecium tube (**Figures 6Di,iii**).

In all auxin-pathway mutants (*yuc, taa/tar, pin, pid,* and *ett*), the severity of the defects caused by different alleles negatively correlates the extent to which an AD/AB boundary remains in the primordium. The stronger the defects, the smaller the AD/AB boundary is at the apex, and the smaller the valve. The resulting non-polarized zone at the base of the primordium may lead to a longer gynophore at the base. Gynophore elongation may be regulated by a separate growth mechanism that is related to the proximal–distal growth and independent of the AD/AB polarity.

This early action model cannot explain why the *yuc1 yuc4* or *pin,* or *pid* mutants are still capable of developing almost normal amount of stigmatic tissues at the apex, other than by proposing that the stigma development may occur later, after the apical to basal patterning of gynoecium is established. *STYLISH1/2* and *NGA3* transcription factors are known to activate the late-stage *YUC* gene expression required for stigma development (Sohlberg et al., 2006; Trigueros et al., 2009; Eklund et al., 2010). The fact that *yuc4 yuc1* double mutants still develop stigmatic tissues hints at additional redundancy in sources of auxin for the apex of the gynoecium. This redundancy could be caused by other *YUC* genes such as *YUC2,* which is expressed broadly in floral primordia (Cheng et al., 2006), or by upward transport of auxin via PIN1 localized to the replum cells (Grieneisen et al., 2013). As the replum represents the medial edge of the carpels, this pattern of upward transport is strikingly reminiscent of the Berleth et al. (2007) model of auxin's movement in aerial organs discussed earlier, which has auxin from the stem being transported up the leaf along its medial edges.

This early action model could be evaluated experimentally by looking at the expression of genes in the AD/AB cohorts at very early stages of gynoecial development. Under this model, we would expect that *pin1*, *pid*, or *yuc1 yuc4* double mutants fail to show a clear AD/AB boundary in carpel primordia and that *ett* mutants express expanded adaxial-specific molecular markers and shrinking abaxial-specific markers due to adaxialization of carpels. In contrast, the Nemhauser apical gradient model does not imply such a result.

CONCLUSION

Fourteen years ago, Nemhauser et al. (2000) proposed the auxin gradient model to explain the apical-to-basal morphogenesis of the *Arabidopsis gynoecium.* While it is a highly attractive model,

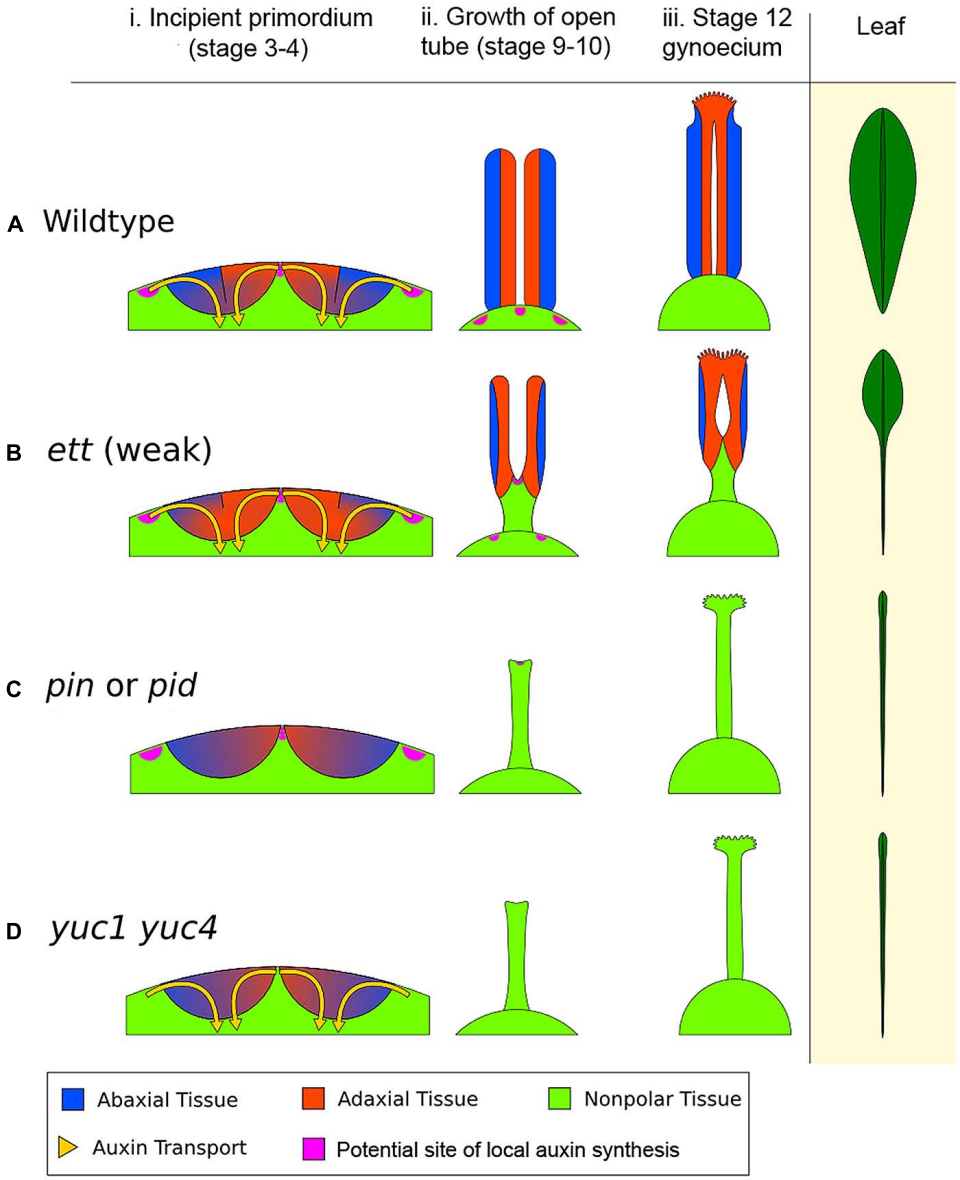

FIGURE 6 | The early action model of gynoecium patterning. (A) Wild type (WT) gynoecium development. The diagram in (i) depicts a young floral meristem giving rise to the two incipient carpel primordia, viewed as an enlarged longitudinal section of the floral meristem apex. In WT, opposing auxin flows (indicated by the yellow arrows) converge on the epidermal center of each carpel primordium. The convergence site likely marks the AD/AB boundary, shown as a black line between blue (AB) and orange (AD) domains. The sharp AD/AB boundary ensures upward growth of carpel tube, forming a long tube with AD domain facing interior (ii). Later the cylindrical tube differentiates into stigma/style at the apex and barely visible gynophore at the base (iii). The phenotypic analogy to a normal *Arabidopsis* leaf with lamina along its entire length is shown on the right. **(B)** In a weak *ett* mutant (*ett-2*), abaxial identity is compromised (but not eliminated entirely), resulting in partial adaxialization of the carpel primordia indicated by expansion of orange color (AD) area (i). As a result, there is diminishing AD/AB boundary, indicated by a shorter boundary line (i). Consequently, only a small area of the carpel primordium near the primordial apex has a clear AD/AB boundary. This shorter (or fuzzier) AD/AB boundary results in limited upward growth and hence a shorter (shallower) tube (ii), and subsequently a reduced ovary valve (iii). This phenotypically resembles leaf polarity mutants (such as double mutants of *KAN*) with a diminished lamina pushed to the leaf tip. **(C)** In auxin polar transport mutants such as in *pin* or *pid* mutants, the two counter-oriented auxin flows are compromised, resulting in failure to form a sharp AD/AB boundary as well as a lack of clear AD or AB identity, which is indicated by mixed blue-orange color in the primordia (i). Since the AD/AB boundary is required for valve formation, a lack of the AD/AB boundary resulted in only radialized gynophore (ii and iii), which exhibits no AD/AB polarity. **(D)** In auxin biosynthesis mutants such as in the *yuc1 yuc4* double mutants, a lack of local auxin biosynthesis, and hence a reduced auxin flow, results in little or no AD and AB identity being formed and no AD/AB boundary being established, as indicated by the mixed blue-orange color (i). Without the AD and AB polarity boundary, there is little to no carpel valve growth (ii, iii), analogous to a leaf without lamina (Waites and Hudson, 1995), shown on the right diagram. The pink patches highlight putative local auxin synthesis sites based on Cheng et al. (2006). The medial region expression of *TAA1* in gynoecium at floral stages 5–9 (Stepanova et al., 2008) is not shown.

the auxin gradient, on which the Nemhauser model heavily relies, remains elusive and multiple observations made since are inconsistent with aspects of the model. Here, we have proposed an alternative model, the early action model, based on three observations. One is the timing of the apical-to-basal patterning, which occurs much earlier than the observed auxin biosynthesis at the gynoecium apex. Another is the already-established evolutionary homology between carpel and leaf-like lateral organs. The third is the set of emerging models of auxin's role in leaf and lateral organ development, including the link between auxin transport, synthesis, and signaling and lateral organs' AD/AB boundary establishment. Our model emphasizes auxin's early effects on AD/AB boundary establishment as an explanation for the defects of gynoecium in apical–basal patterning induced by auxin-disrupting mutations and chemicals. Furthermore, the early action model unifies the development of carpels with current models of the development of other lateral organs.

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The effect of NGATHA altered activity on auxin signaling pathways within the Arabidopsis gynoecium

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The four *NGATHA* genes (*NGA)* form a small subfamily within the large family of B3-domain transcription factors of *Arabidopsis thaliana*. *NGA* genes act redundantly to direct the development of the apical tissues of the gynoecium, the style, and the stigma. Previous studies indicate that *NGA* genes could exert this function at least partially by directing the synthesis of auxin at the distal end of the developing gynoecium through the upregulation of two different *YUCCA* genes, which encode flavin monooxygenases involved in auxin biosynthesis. We have compared three developing pistil transcriptome data sets from wildtype, *nga* quadruple mutants, and a *35S::NGA3 line*. The differentially expressed genes showed a significant enrichment for auxin-related genes, supporting the idea of *NGA* genes as major regulators of auxin accumulation and distribution within the developing gynoecium. We have introduced reporter lines for several of these differentially expressed genes involved in synthesis, transport and response to auxin in *NGA* gain- and loss-of-function backgrounds. We present here a detailed map of the response of these reporters to *NGA* misregulation that could help to clarify the role of *NGA* in auxin-mediated gynoecium morphogenesis. Our data point to a very reduced auxin synthesis in the developing apical gynoecium of *nga* mutants, likely responsible for the lack of *DR5rev::GFP* reporter activity observed in these mutants. In addition, NGA altered activity affects the expression of protein kinases that regulate the cellular localization of auxin efflux regulators, and thus likely impact auxin transport. Finally, protein accumulation in pistils of several ARFs was differentially affected by *nga* mutations or *NGA* overexpression, suggesting that these accumulation patterns depend not only on auxin distribution but could be also regulated by transcriptional networks involving NGA factors.

Keywords: gynoecium development, NGATHA, auxin synthesis, auxin transport, AUXIN RESPONSE FACTORS

INTRODUCTION

The carpel is the female reproductive organ of the angiosperm flower and its most distinctive feature. Carpels typically occur at the center of the flower forming the gynoecium, most commonly fused into a single pistil (a syncarpic gynoecium) or less frequently as individual organs that collectively form an apocarpic gynoecium composed of several pistils. The gynoecium confers major advantages to flowering plants: provides protection for the ovules; enables pollen capture and pollen tube guidance and supports self- and inter-specific incompatibility; finally, after fertilization of the ovules, the gynoecium develops into a fruit, which protects the developing seeds and facilitates seed dispersal (Ferrandiz et al., 2010). To accomplish these roles, gynoecium development involves the differentiation of specialized functional modules: stigma forms at the apex of pistils to capture and germinate pollen grains; immediately below, the style is rich in transmitting tissues that conduct pollen tubes to the ovary, which is a basal structure that contains the ovules. In addition to these specialized tissues, other structures also develop in some pistils, such as those that will form the dehiscence zones in shattering fruits, or the septa that divide the ovary in locules (Sundberg and Ferrándiz, 2009; Ferrandiz et al., 2010).

To achieve differentiation and coordinated growth of the functional modules found in pistils, a suite of regulatory networks has to be in place. Most of our current knowledge on the major players in these networks comes from work carried out in *Arabidopsis thaliana*. A number of transcription factors have been identified with a role in the differentiation of the specialized tissues found in gynoecia or in the specification of polarity axes, and, while the picture is far from complete, we are now beginning to understand how their regulatory hierarchies and functional interactions work (reviewed in Balanzá et al., 2006; Ferrandiz et al., 2010). In addition to transcriptional regulation, the phytohormone auxin has been regarded as one of the major morphogens instructing gynoecium patterning and post-fertilization developmental events (Alabadi et al., 2009; Larsson et al., 2013). Local auxin maxima and minima have been shown to be instrumental for valve margin development and dehiscence (Sorefan et al., 2009). Most importantly, it is also known that auxin controls polarity in the apical-basal axis of the developing gynoecium. More

than one decade ago, (Nemhauser et al., 2000) proposed a model for auxin-dependent distribution of tissues based on the phenotypes of *ettin (ett)* mutants, affected in the *AUXIN RESPONSE FACTOR 3* gene; the phenotypes of mutants defective in auxin transport such as *pinoid (pid)* or *pin-formed1 (pin1)*; and on the effects of inhibiting polar auxin transport (PAT) in gynoecium morphology. According to this model, auxin would be produced in the apical end of the pistil and transported basipetally, creating a gradient along the apical-basal axis that would be translated into the differentiation of the different functional modules: high apical auxin levels would direct the differentiation of style and stigma, intermediate levels would specify the ovary, and low basal levels, the gynophore. The Nemhauser model has been very useful to frame the role of different players in Arabidopsis carpel development, but conclusive proof of the proposed auxin gradient has never been obtained. Actually, detailed descriptions of auxin accumulation throughout gynoecium development using a *DR5rev::GFP* reporter have shown that auxin maxima are formed in the apical domain, first as isolated foci and later as a continuous apical ring, while the proposed gradient cannot be observed (Girin et al., 2011; Marsch-Martinez et al., 2012a; Larsson et al., 2013). In addition, several recent studies indicate that the dynamics of auxin accumulation, homeostasis and response within the developing gynoecium are highly complex and we are still far from fully comprehending how positional information is translated into developmental outputs in gynoecium differentiation (Sohlberg et al., 2006; Ståldal et al., 2008; Ståldal and Sundberg, 2009; Marsch-Martinez et al., 2012b). In any case, although detailed understanding of these mechanisms is still lacking, the pivotal role of auxin in apical-basal gynoecium patterning is widely acknowledged.

Among the transcriptional regulators directing carpel patterning, two small families of unrelated factors have been shown to be essential for apical tissue differentiation. The four *NGATHA* genes belong to the RAV clade of the large B3-domain transcription factor family and are redundantly required for the specification of style and stigma. *nga* quadruple mutants form apparently undisturbed ovaries but completely lack style and stigma, and the gynoecium ends apically as an open structure with several protrusions of valve-like tissue (Alvarez et al., 2009; Trigueros et al., 2009). Almost identical phenotypes are found in multiple mutants of the SHI/STY family of RING finger-like zinc finger motif transcription factors (Kuusk et al., 2006). *NGA* and *SHI/STY* genes also share similar expression patterns, which include the apical domain of developing gynoecia from stage 6 to stage 11–12, when style and stigma specification and differentiation take place (**Figures 1C–F**) (Kuusk et al., 2002; Alvarez et al., 2009; Trigueros et al., 2009). Interestingly, both NGA and SHI/STY factors have important connections to auxin. STY1 has been shown to directly regulate *YUCCA4 (YUC4)*, a gene encoding a flavin monooxygenase-like enzyme involved in auxin synthesis (Eklund et al., 2010). Likewise, *YUC4* and *YUC2* are not expressed in the gynoecium apex of lines where *NGA* genes were downregulated, a regulatory interaction that appears to be conserved also in other dicot species (Trigueros et al., 2009; Fourquin and Ferrandiz, 2014). Moreover, *NGA3* overexpression carpel phenotypes resemble the effects of PAT inhibition and of weak *ett* mutant alleles, and also affect other auxin-related processes in the plant, such as apical dominance, leaf morphology, or secondary root development, suggesting that *NGA* genes may interact with auxin signaling at multiple levels (Alvarez et al., 2009; Trigueros et al., 2009). In this study, we aim to characterize in detail the response of several components of the auxin signaling network to altered levels of NGA activity in the gynoecium, hoping to clarify the mechanisms of NGA action in auxin-mediated carpel morphogenesis.

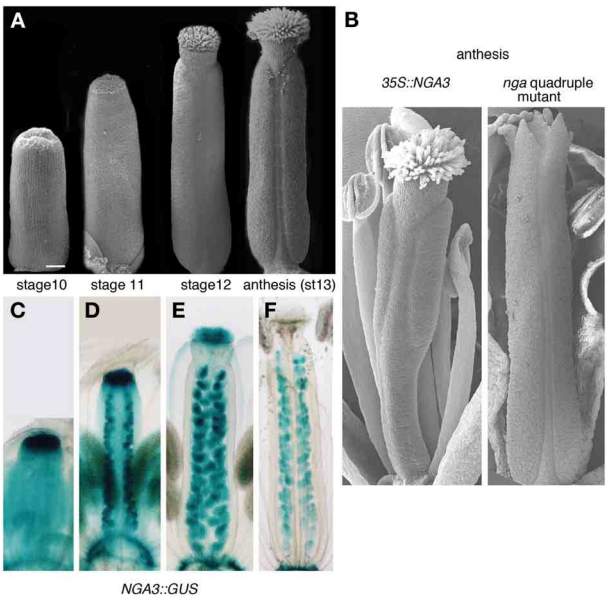

FIGURE 1 | Gynoecium development in Arabidopsis and NGA-related mutants. (A) Scanning electron micrographs of wildtype gynoecia at different stages of development. **(B)** Phenotypes at anthesis of *35S::NGA3* (left) and *nga* quadruple mutant pistils (right). **(C–F)** Expression of *NGA3* in gynoecia at the same stages shown in **(A)**, revealed by the histochemical activity of a GUS reporter gene directed by *NGA3* regulatory sequences (Trigueros et al., 2009).

MATERIALS AND METHODS

PLANT MATERIAL

nga1-4 (line WiscDsLox429G06), *nga2-2* (line SM.20993) *nga3-3* (AMAZE En-1 line 6AAi79), and *nga4-3* (AMAZE En-1 line 6AAB133i) alleles were used to generate *nga* quadruple mutants. Genotyping was performed as previously described (Trigueros et al., 2009). All reporter lines used in this study have been previously described: *YUC8::GUS* (Rawat et al., 2009), *TAA1::GFP:TAA1* (Stepanova et al., 2008), *AMI1::GUS* (Hoffmann et al., 2010), *DR5rev::GFP* (Benková et al., 2003), *PID::GUS*, *PID::PID:GFP* (Lee and Cho, 2006), *WAG2::GUS* (Santner and Watson, 2006), *PIN3::PIN3:GFP* (Lee and Cho, 2006), *ARF8::GUS*, *ARF11::ARF11:GFP*, *ARF18::ARF18:GFP* (Rademacher et al., 2011).

RNAseq ANALYSIS

Arabidopsis carpels between stages 8–13 from wildtype, *nga* mutant and *35S::NGA3* plants were collected manually from 15

developing inflorescences and stored transiently in cold extraction buffer of the Qiagen RNA extraction kit. RNA extraction was made with the Qiagen RNA extraction kit. RNA was analyzed for yield and quality on a Bioanalyzer 2100 (Agilent 2100). Libraries for sequencing were prepared from 2–4 μg total RNA using Illumina TruSeq RNA kits and sequenced with Illumina HiSeq2000. Quality control on the raw sequence data was done using FastQC (Babraham Bioinformatics). Reads were aligned to whole genome sequences from the TAIR10 A. thaliana database (www.arabidopsis.org) and analyzed using the CLC Genomics workbench (www.clcbio.com). RPKM (reads per kilobase per million) was considered as expression values. Two biological replicates for wildtype and three for *nga* and *35S::NGA3* were used for sequencing. After normalization, Bagerley's test and a FDR correction were used for statistical analysis of samples. Genes with a corrected FDR p-value < 0.05 and with a fold change >1.4 or <-1.4 were selected for gene ontology analysis with the agriGO toolkit (Du et al., 2010).

REPORTER ACTIVITY DETECTION

GUS histochemical detection was performed as previously described (Trigueros et al., 2009).

For GFP detection, fluorescent images were captured using an LSM 780 confocal scanning laser inverted microscope (Zeiss). GFP was excited using a 488 nm line of an argon ion laser. GFP emission spectra were collected between 500–550 nm and plastid autofluorescence was collected between 601 and 790 nm.

AUXIN MICRO-APPLICATION

For the micro-application experiment, 80 mg of indolacetic acid (IAA) (Sigma, St. Louis, MO, USA) were dissolved in 2 mL of ethanol. IAA, or ethanol for mock treatment, was added to 10 gr of lanolin containing 2.5% liquid paraffin. The lanolin paste was applied to the apical end of stage 8–10 gynoecia using plastic pipette tips under a dissecting microscope, resulting in apical parts completely covered by lanolin paste. The gynoecia were observed after 2 weeks and photographed under a dissecting scope.

RESULTS

To identify genes involved in gynoecium development that are expressed under the control of NGA factors, we compared the expression profiles in stage 8–13 dissected pistils from wildtype, quadruple *nga* mutants and plants overexpressing *NGA3* (stages defined after Smyth et al., 1990; **Figures 1A,B**). We selected transcripts with a fold change of >1.4 or <-1.4 and a corrected FDR p-value < 0.05. With those thresholds, we identified 1889 genes differentially expressed between wildtype and the quadruple *nga* mutant, 554 between wildtype and *35S::NGA3* and 637 between the quadruple *nga* mutant and *35S::NGA3*. Combining the results of the three comparisons, a list of 2449 genes were identified as putative targets of NGA regulation. With this final list we conducted a gene ontology analysis with the agriGO toolkit, finding that auxin-related genes were overrepresented in the dataset of differentially expressed transcripts (Suppl. Figure 1). Among them, genes related to auxin synthesis, transport, and response were identified, confirming previous reports of a functional relationship of NGA and auxin signaling (Alvarez et al., 2009; Trigueros et al., 2009), and suggesting that this interaction could occur at multiple levels.

To characterize in more detail how altered NGA activity influenced the spatial and temporal patterns of expression of their putative auxin-related targets, we introduced in *nga* mutants and in the *35S::NGA3* line reporter lines for several of these differentially expressed and other related genes, as well as markers for auxin accumulation.

NGA MUTATIONS AFFECT THE EXPRESSION OF GENES INVOLVED IN AUXIN SYNTHESIS

It has been reported that *nga* mutations severely modify the expression patterns of *YUC2* and *YUC4* genes in the apical gynoecium (Trigueros et al., 2009). *YUC* enzymes catalyze the rate-limiting step in Trp-dependent auxin biosynthesis (**Figure 2A**) (Zhao et al., 2001; Mano and Nemoto, 2012). Only *YUC2* and *YUC4* have been shown to be strongly expressed in the apical developing gynoecium, suggesting that they could be essential contributors to auxin synthesis in this domain (Cheng et al., 2006). However, *yuc2 yuc4* double mutants show no evident phenotypes in floral development, indicating that other *YUC* genes may also be important to direct auxin synthesis in the pistil (Cheng et al., 2006). In the RNAseq dataset, the expression of one additional *YUC* gene, *YUC8*, was found to be strongly reduced in the quadruple *nga* mutant. In *YUC8::GUS* lines, expression could be observed in the ovules and in two small foci in the basal part of the style in stage 11–12 wildtype pistils (**Figure 2B**). This expression was completely absent in *nga* quadruple mutants (**Figure 2C**), while appeared unchanged or slightly increased in *35S::NGA3* pistils (**Figure 2D**). At later stages (13–15), expression in ovules was maintained in wildtype and *35S::NGA3* fruits (**Figures 2E–G**), although GUS activity was clearly stronger in the overexpression lines. These results confirm that *YUC8* is also upregulated by NGA factors at least in the apical gynoecium and likely in the ovules.

In addition to *YUC8*, RNAseq data revealed that expression of *TAA1* was also affected by *NGA* loss of function. *TAA1* encodes an enzyme that catalyzes the conversion of Trp to IPA, the proposed substrate for YUC enzymes (Mano et al., 2010; Stepanova et al., 2011). In wildtype developing gynoecia, a *TAA1::GFP:TAA1* reporter showed strong expression in the apical domain and in two longitudinal bands at medial positions in the developing gynoecial cylinder at stage 9–10 (**Figure 2H**). Later in development (st. 11), expression became restricted to a cell layer at the style/stigma junction and to the medial vascular bundles and the vascular veins of the funiculi (**Figure 2K**). In post-anthesis young fruits, expression was detected in the developing dehiscence zone at both sides of the replum (**Figure 2N**). *TAA1::GFP:TAA1* expression was reduced but not absent from the apical and medial domains of *nga* quadruple mutants at stage 8–9 (**Figure 2I**). In stage 11 *nga* gynoecia, since style and stigma do not form properly, the single cell layer of GFP expression below stigmatic cells could not be detected, but GFP accumulated at the tips of the valve protrusions and was mostly unchanged in other domains (**Figures 2L,O**). In *35S::NGA3* pistils, *TAA1::GFP:TAA1* expression was found in the same spatial

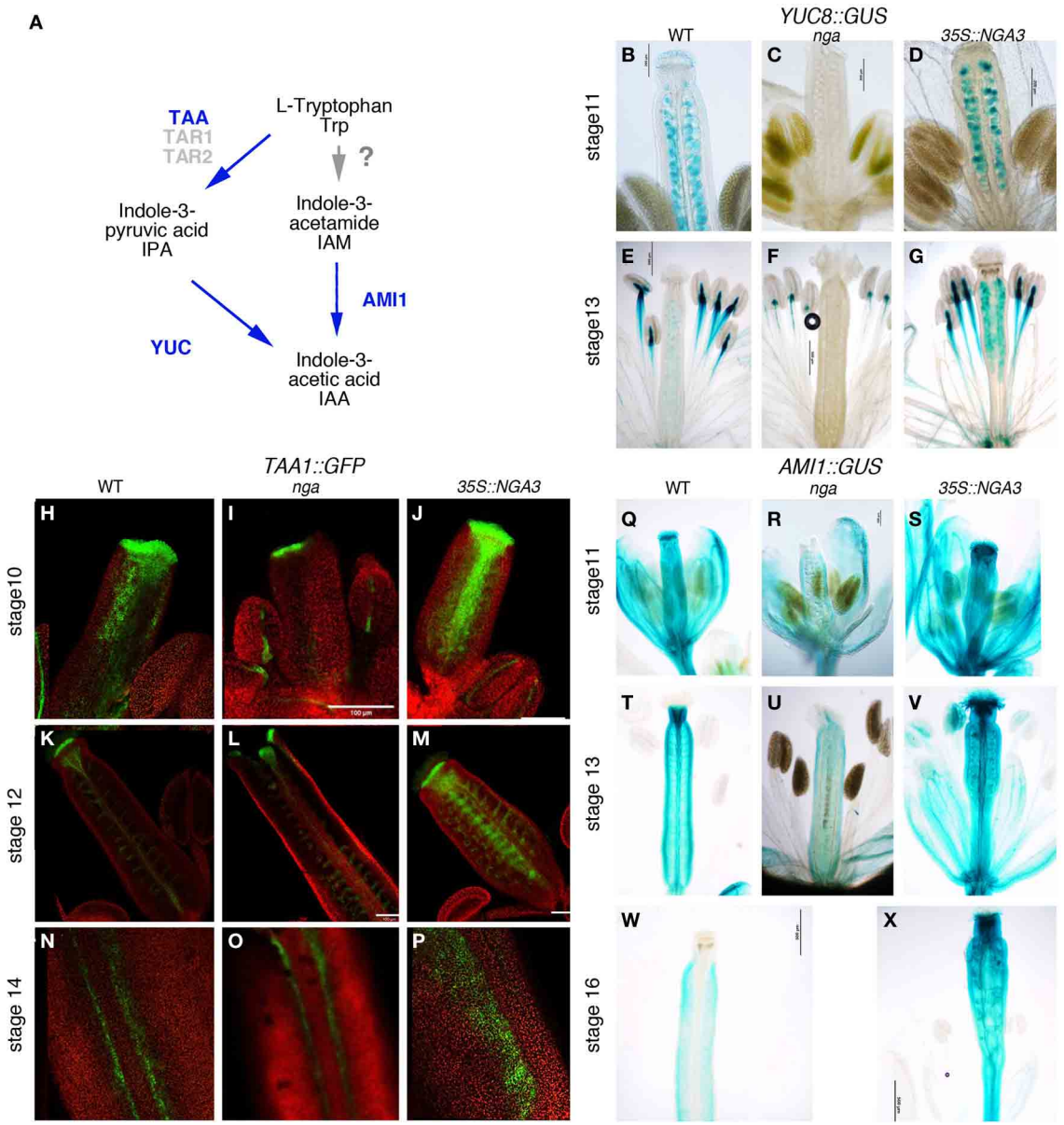

FIGURE 2 | Effect of NGA altered activity on the expression of genes involved in local auxin synthesis. (A) Simplified scheme of the presumptive pathways for IAA biosynthesis studied in this work, adapted from Mano and Nemoto (2012). Genetic functions analyzed in this work are noted in blue. Question mark on the TRP>IAA pathway denotes that the conversion of TRP to IAM has not been demonstrated in plants **(B–G)** Histochemical detection of GUS activity driven by the *YUC8* promoter in wildtype **(B,E)**, *nga* quadruple mutants **(C,F)** and *35S::NGA3* gynoecia **(D,G)** at stage 11 **(B–D)** and at anthesis **(E–G)**. **(H–P)** *TAA1::GFP:TAA1* expression in wildtype **(H,K,N)**, *nga* quadruple mutants **(I,L,O)**, and *35S::NGA3* gynoecia **(J,M,P)** at stage 10 **(H–J)** and stage 12 **(K–M)**. **(N–P)** show close up views of the valve margins in the ovary region of anthesis pistils. **(Q–X)** Histochemical detection of GUS activity driven by the *AMI1* promoter in wildtype **(Q,T,W)**, *nga* quadruple mutants **(R,U)** and *35S::NGA3* gynoecia **(S,V,X)** at stage 11 **(Q–R)**, at anthesis **(T–V)**, and post-fertilization, at around stage 16 **(W–X)**.

pattern as in wildtype, although GFP signal appeared to be stronger in all domains and maintained for longer (**Figures 2J,M**). Thus, stage 11 *35S::NGA3* gynoecia still showed GFP signal in the medial region (**Figure 2M**), and in post-anthesis young *35S::NGA3* fruits, strong expression could be detected in the funiculi and expanding to the valves (**Figure 2P**).

AMIDASE1 (AMI1) encodes an enzyme that catalyzes the conversion of IAM to IAA and it has been proposed to contribute to auxin synthesis through an alternative pathway to the TAA1/YUC route (**Figure 2A**) (Mano et al., 2010). An *AMI1::GUS* reporter line showed *AMI1* promoter to be active in most floral organs. In the gynoecium, GUS activity could be detected at medium levels throughout the gynoecial tube, while in the style region it accumulated strongly from stages 10–11 (**Figure 2Q**). In anthesis flowers, GUS signal was very high in the style and it could also be detected in the vascular bundles (**Figure 2T**). Apical expression

disappeared in developing fruits, while in the ovary, low levels of expression could still be detected (**Figure 2W**). In *nga* quadruple mutants, the strong expression in apical gynoecium typical of wildtype pistils was absent in preanthesis or anthesis pistils (**Figures 2R,U**). Conversely, *35S::NGA3* lines showed stronger GUS signal that was maintained in developing *35S::NGA3* fruits (**Figures 2S,V,X**).

AUXIN ACCUMULATION IS REDUCED IN THE APICAL DOMAIN OF THE NGA MUTANT GYNOECIA

Altogether, our results pointed to a greatly reduced or absent auxin synthesis in the apical domain of *nga* mutants and possibly a sustained increased auxin synthesis in the overexpression lines. If this was true, we could expect a reduced auxin accumulation in the apical domain of *nga* developing gynoecia and higher auxin levels in pistils and fruits. To test this hypothesis, we compared the activity of a *DR5rev::GFP* reporter in wildtype (**Figures 3A–C**), *nga* quadruple (**Figures 3D–F**) and *35S::NGA3* backgrounds (**Figures 3G–I**). *DR5rev::GFP* activity during Arabidopsis gynoecium development has been described (Benková et al., 2003; Girin et al., 2011; Marsch-Martinez et al., 2012a; Larsson et al., 2013). GFP expression is first detected as two lateral apical foci (stage 7), which at stage 8 also comprise two additional medial apical foci (**Figure 3A**), and at stage 9 extends as a continuous apical ring (**Figure 3B**). In *nga* gynoecia, the apical foci in stage 7–8 could be barely detected (**Figure 3D**), and the formation of the apical ring was never observed (**Figure 3E**). Surprisingly, in *35S::NGA* pistils, *DR5rev::GFP* activity was very similar to wildtype, indicating that in spite of the apparently increased auxin synthesis that could be expected from the stronger expression of *TAA1*, *YUC8*, or *AMI1*, the response of the reporter was not enhanced (**Figures 3G–I**). Interestingly, when *DR5rev::GFP* activity in ovule primordia of wildtype and *nga* mutants was compared, a reduction of GFP levels in *nga* ovules was observed, but not the absence of the distal auxin maxima (**Figures 3J–M**). This result suggested that, in spite of the absence of *YUC8* expression in *nga* ovules, the persisting expression of *YUC4* previously reported in this domain (Trigueros et al., 2009) was sufficient to direct auxin synthesis and allow ovule development.

Low auxin levels have been related to the *nga* phenotypes in style and stigma. Thus, reduced apical tissues were observed in a transgenic line where the *NGA3* promoter drove the expression of *iaaL*, a bacterial gene that encodes an enzyme that inactivates free auxin (Jensen et al., 1998; Trigueros et al., 2009). To test whether exogenous auxin treatments could restore style and stigma development, we performed micro-applications of IAA dissolved in droplets of lanolin to the tip of young developing *nga* gynoecia (stages 9–10). A limited partial rescue of the *nga* phenotypes was observed, with restored apical closure of the gynoecium, but no development of style or stigma typical cells, suggesting that the lack of auxin accumulation in this domain was probably not the only factor causing the *nga* phenotypes (**Figures 3N,O**).

In summary, auxin synthesis was likely very reduced in the apical domain of *nga* mutants, both through the TAA/YUC pathway and the presumptive AMI1 pathway, thus leading to low auxin levels in the distal region of *nga* developing pistils as deduced from the greatly reduced activity of the of *DR5rev::GFP* reporter. However, *NGA3* overexpression did not have a high impact in distal auxin accumulation, suggesting that NGA factors could also be interfering with other components of the auxin transport/response pathways.

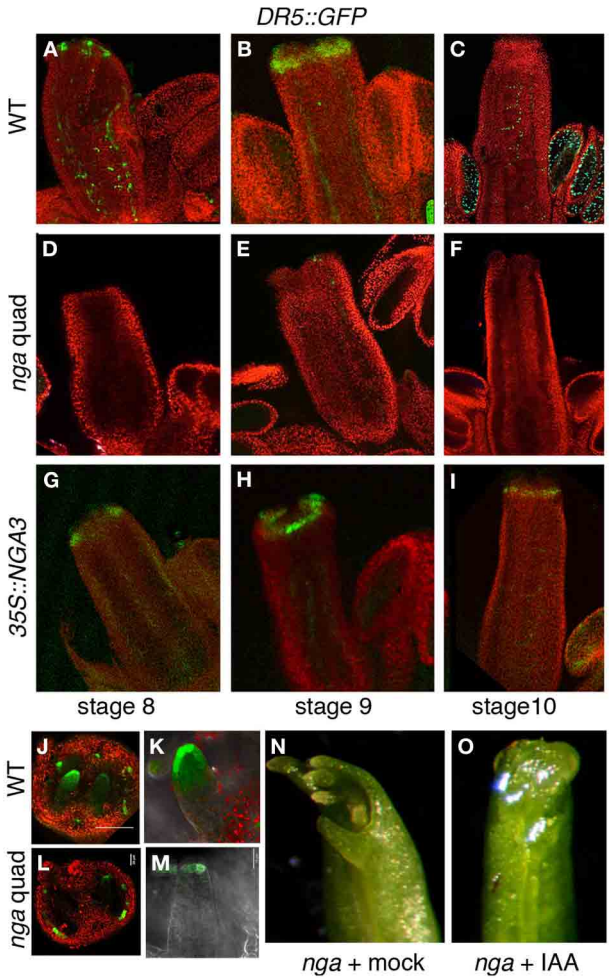

FIGURE 3 | Effect of NGA altered activity on *DR5rev::GFP* expression in gynoecium development. (A–C) *DR5rev::GFP* in wildtype pistil development. **(A)** *DR5rev:GFP* is detected as discrete foci in stage 8 gynoecium **(B)** GFP is detected as a continuous apical ring in stage 9 gynoecium and in longitudinal strands at sites of main vascular development. **(C)** At stage 10, apical GFP expression is barely detected and signal is only clear in the funiculi of ovules. **(D–F)** *DR5rev::GFP* expression in *nga* quadruple mutants. GFP is virtually undetectable at stage 8 **(D)**, stage 9 **(E)**, or stage 10 **(F)** gynoecia. **(G–I)** *DR5rev::GFP* expression in *35S::NGA3* gynoecia. GFP expression is detected in similar patterns as wildtype in stage 8 **(G)** and stage 9 **(H)** gynoecia, while it appears to be slightly more persistent in the apical cells of stage 10 pistils **(I)**. **(J,K)** *DR5rev::GFP* expression in ovules of stage 10 wildtype **(J,K)** and nga quadruple mutants **(L,M)**. Note the reduced but detectable GFP activity at the tip of *nga* developing ovules. **(N,O)** Effect of micro-application of auxin to the apical end of *nga* mutant pistils. **(N)** Mock treated pistils, showing completely unfused apical ends with no signs of style or stigma development. **(O)** IAA-treated pistils show apical closure and very limited style development, but no stigmatic cells are able to develop.

NGA MUTATIONS AFFECT THE EXPRESSION OF GENES INVOLVED IN AUXIN TRANSPORT

Members of the PIN protein family of auxin efflux regulators have been shown to mediate various developmental processes, including carpel patterning. Polar, subcellular localization of PIN proteins determines the direction of auxin flux (reviewed in Friml, 2003) and this localization is partially regulated by their phosphorylation status, which depends on the antagonistic action of the PP2AA phosphatases, and kinases such as PINOID, WAG1, WAG2, and PID2 (Santner and Watson, 2006; Michniewicz et al., 2007). *PID* and *WAG2* expression has been reported in apical tissues of Arabidopsis developing gynoecia (Girin et al., 2011). Moreover, *pid* mutants show severe carpel patterning phenotypes similar to those found in *pin1* or *pin3 pin7* mutants (Okada et al., 1991; Bennett et al., 1995; Benková et al., 2003). RNAseq analyses revealed altered expression levels of both *PID* and *WAG2* in either *nga* or *35S::NGA3* pistils, and therefore, we introduced *PID* and *WAG2* reporters into these backgrounds. *PID::GUS* activity was weakly detected in the style of stage 11 wildtype carpels (**Figure 4A**). In *nga* mutants, expression could still be detected in the apical protrusions typical of *nga* gynoecia (**Figure 4B**), while in *35S::NGA3* pistils, expression was absent in the style but present in the stigma (**Figure 4C**). Moreover, a *PID::PID:GFP* reporter line showed a substantially reduced GFP signal in *35S::NGA3* lines when compared to wildtype, suggesting that NGA3 could be preventing PID accumulation (**Figures 4D–G**). *WAG2::GUS* showed early expression in the distal end of the stage 9 gynoecial tube in wildtype, *nga*, or *35S::NGA3* (**Figures 4H–J**). This apical expression was maintained until stage 11 in wildtype or *35S::NGA3* pistils (**Figures 4K,M**), while clearly reduced in *nga* mutants (**Figure 4L**). These results suggested that NGA factors could regulate *PID* and *WAG2* in opposite directions, repressing *PID* while activating *WAG2*, similarly to what it has been described for the bHLH transcription factors INDEHISCENT and SPATULA (Girin et al., 2011).

The differences in *PID* and *WAG* expression caused by altered NGA activity suggested that PIN protein localization could also be affected. PIN1 protein localization has been described in developing fruits, but no detailed patterns of expression have been described for any of the PIN transporters throughout gynoecium development (Sorefan et al., 2009). We compared *PIN1::PIN1:GFP* in wildtype, *nga*, and *35S::NGA3* backgrounds, but no clear differences could be observed (Suppl. Figure 2). Likewise, the *PIN7::PIN7:GFP* reporter line available to us showed very low levels of GFP activity and we could not obtain conclusive results. Finally, we determined *PIN3::PIN3:GFP* expression in wildtype developing gynoecia. PIN3:GFP protein was localized in a narrow apical ring and two longitudinal stripes at epidermal medial positions in stage nine pistils (**Figures 5A,B**). Apical expression was maintained at stage 12, restricted to the stigma and the underlying layers in the style, and also in the replum domain, although at lower levels (**Figures 5C,D**). It was difficult to determine the subcellular orientation of PIN3 both in the apical ring and in the replum domain at early stages, and therefore the direction of the auxin flux was not easily deduced. At stage 12, however, PIN3:GFP protein was mostly localized in the basal side of cells in the style and the replum, suggesting that auxin flux would be directed toward the basal part of the ovary (**Figure 5D**). We failed to introduce the *PIN3::PIN3:GFP* reporter in the *nga* quadruple mutants background, but PIN3:GFP accumulation was studied in the *35S::NGA3* background. *35S::NGA3* pistils showed similar accumulation patterns of PIN3 at stage 9 (**Figures 5E,F**). However, from stage 11, PIN3 accumulation appeared to be increased both in the apical domain and the replum region, where it comprised a higher number of cell rows, suggesting that basipetal auxin flux could be facilitated in the *35S::NGA3* background (**Figures 5G,H**).

NGA MUTATIONS AFFECT IN DIFFERENT WAYS THE EXPRESSION OF AUXIN RESPONSE FACTORS (ARFs) THROUGHOUT GYNOECIUM DEVELOPMENT

Finally, we took advantage of the recently created collection of ARF reporters described by Rademacher et al. (2011) to examine the effect of NGA altered activity on the protein expression patterns of several ARFs expressed in the apical domain of developing gynoecia, namely ARF1, ARF8, ARF11, and ARF18. We also included in our analyses ARF3/ETT, since it was also expressed in the developing pistil, but we did not observe significant changes in reporter activity in the *nga* or *35S::NGA3* backgrounds (Suppl Figure 3).

ARF8::ARF8:GUS reporter activity in wildtype gynoecium development has been already described (Goetz et al., 2006). ARF8 protein appears strongly associated to transmitting tissues, specially stigma and transmitting tract, and, at lower levels, in the ovary walls and the ovules (**Figures 6A,C**). Loss of *NGA* function mainly affected the accumulation of ARF8:GUS in the apical end of the gynoecium, which appeared reduced at anthesis although maintaining foci of expression at the apical end of valve protrusions, while did not alter significantly ARF8:GUS levels in the ovary or the ovules (**Figures 6B,D**). Crosses between *ARF8::ARF8:GUS* and *35S::NGA3* line failed and therefore we were not able to analyze the activity of the reporter in this background for this work.

ARF1::ARF1:GFP reporter showed activity in medial and epidermal tissues of stage 10 wildtype gynoecia, with higher levels of GFP signal at the apical end of the gynoecial tube (**Figure 6E**). In anthesis wildtype pistils, signal was mainly associated with stigmatic cells and valve margins, with low but consistent accumulation of ARF1:GFP detected in the epidermal cells of style and valves (**Figures 6H,K**). In *nga* mutants, ARF1:GFP patterns were very similar to wildtype in preanthesis and anthesis stages, although likely due to the lack of stigmatic cells, no strong signal was detected in apical cells of anthesis *nga* gynoecia (**Figures 6F,I,L**). As for *35S::NGA3* lines, ARF1:GFP expression was found at similar domains, although it appeared to be increased in level (**Figures 6G,J**). This stronger expression was more conspicuous at anthesis, where valves showed clearly enhanced fluorescent signal (**Figure 6M**).

ARF11::ARF11:GFP reporter activity was detected already at stage 9–10 in the presumptive developing style and the valve margins (**Figure 6N**), accumulating below the stigmatic cells until stage 12 (**Figure 6Q**), and becoming barely detected at anthesis and later stages. In *nga* quadruple mutants, ARF11:GFP protein could be only detected at very reduced levels in a small apical

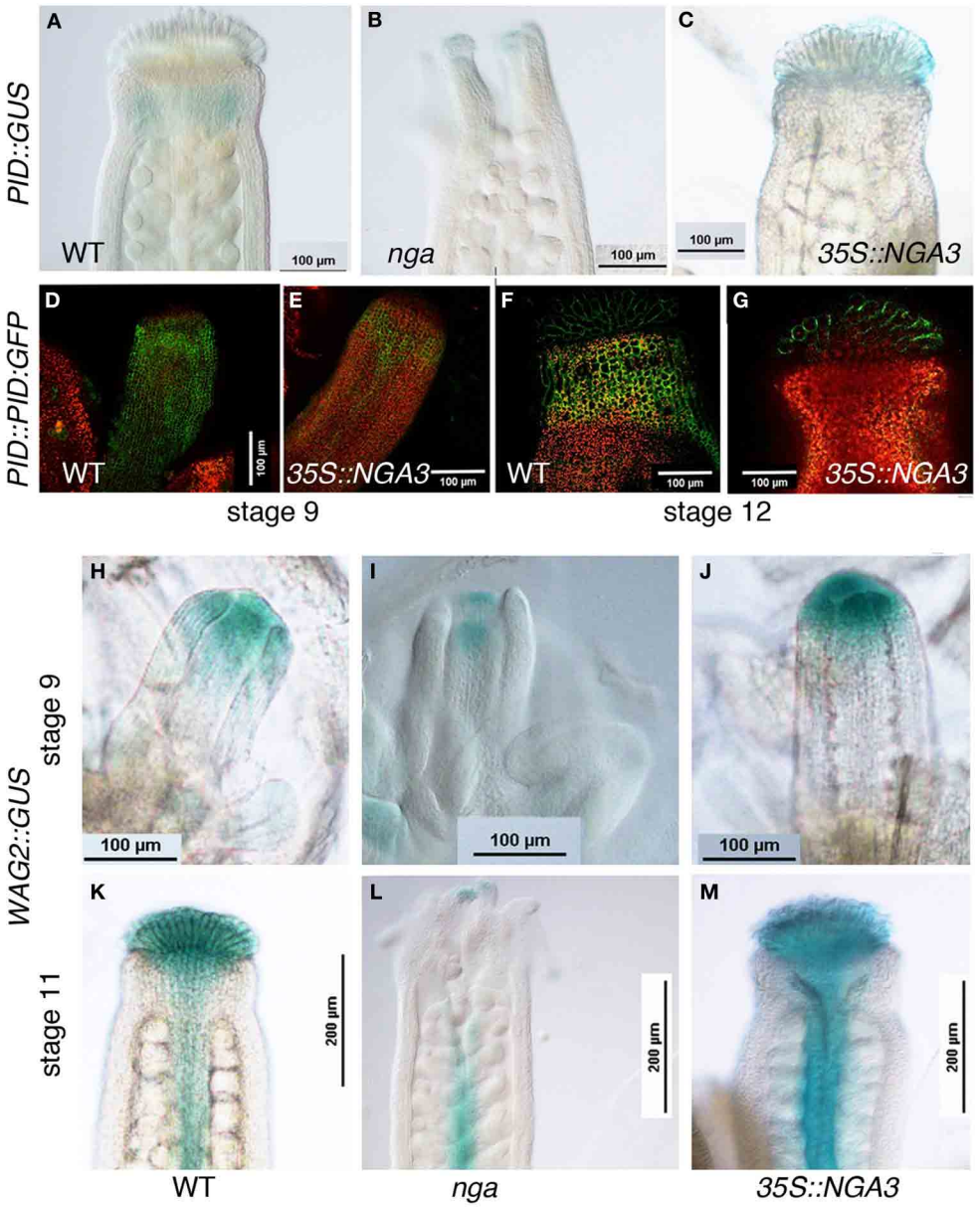

FIGURE 4 | Effect of NGA altered activity on the expression of protein kinases involved in the regulation of PIN subcellular polarization. (A–C) Histochemical detection of GUS activity driven by the *PID* promoter in wildtype **(A)**, *nga* quadruple mutants **(B)** and *35S::NGA3* apical region of stage 12 gynoecia **(C)**. **(D–G)** *PID::PID:GFP* expression in stage 9 wildtype **(D)** or *35S::NGA3* gynoecia and in the style/stigma region of stage 12 wildtype **(F)** or *35S::NGA3* **(G)** pistils. **(H–M)** Histochemical detection of GUS activity driven by the *WAG2* promoter in wildtype **(H,K)**, *nga* quadruple mutants **(I,L)** and *35S::NGA3* **(J,M)** gynoecia at stage 9 **(H–J)** and stage 11 **(K–M)**.

domain of stage 10 pistils (**Figures 6O,R**), while in *35S::NGA3*, ARF11:GFP protein accumulated similarly to wildtype in the apical tissues although in an expanded domain (**Figure 6P**). Interestingly, and unlike from wildtype, ARF11:GFP accumulated in the valve margins of *35S::NGA3* gynoecia from early stages of development, where it could still be strongly detected prior and at anthesis (**Figures 6P,S**).

ARF18::ARF18:GFP reporter drove a strong GFP signal in the apical domain of stage 10 wildtype pistils (style and stigmatic cells) and at the valve margins (**Figure 6T**). At later stages (stage 13 and postanthesis), ARF18:GFP could still be detected in the stigmatic cells and the differentiating dehiscence zones, restricted to a few cell rows (**Figure 6W**). In *nga* quadruple mutants, ARF18:GFP accumulation was similar to that observed in wildtype, in spite of the absence of style and stigma, and signal was detected in the valve protrusions that developed apically. In stage 11 *nga* gynoecia, a strong GFP signal could be observed in the apical domain and weakly at the valve margins (**Figure 6U**). Apical expression could still be weakly detected in anthesis *nga* pistils, to become restricted to the dehiscence

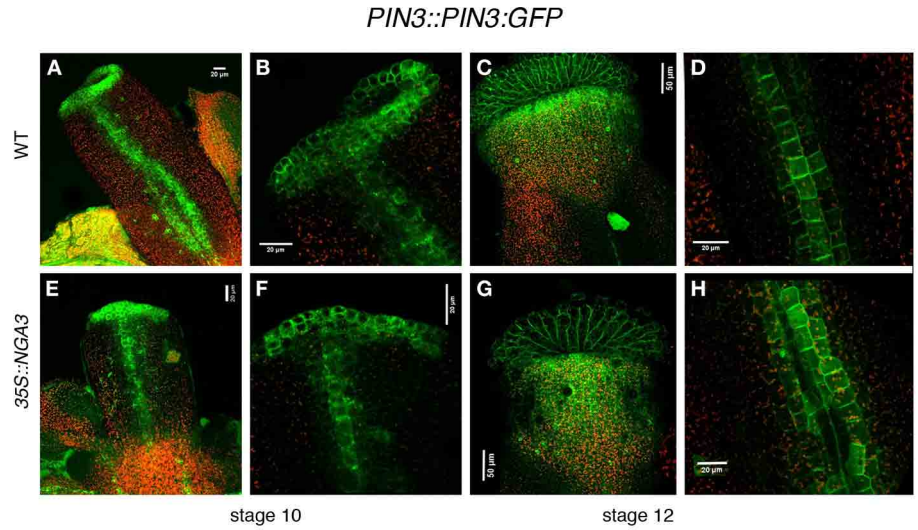

FIGURE 5 | Effect of NGA altered activity on PIN3 protein localization. *PIN3::PIN3:GFP* expression was observed in wildtype **(A–D)** and *35S::NGA3* **(E–H)** developing gynoecia at stage 10 **(A,B,E,F)** and stage 12 **(C,D,G,H)**. Close view of the apical ring in wildtype **(B)** or *35S::NGA3* **(F)** stage 10 pistils did not show a clear PIN3 subcellular polarization, although in the longitudinal stripes of cells running along the ovary, PIN3 appears to be predominantly at the basal side of cells. **(C)** Stage 12 wildtype gynoecia showed strong GFP signal in stigmatic cells and in a domain in the style just below the stigma. **(D)** In wildtype stage 12 ovaries three rows of cells showed PIN3:GFP expression, where PIN3:GPF protein appeared to be localized at the basal side of cells. **(G)** In *35S::NGA3* stage 12 pistils, PIN3:GFP is detected in stigma and a broader domain of the style **(H)** In *35S::NGA3* stage 12 ovaries, PIN3:GFP expands to 4–5 cell longitudinal rows, also apparently localized to the basal side of cells.

zones in post-anthesis stages (**Figure 6X**). *35S::NGA3* pistils also showed similar patterns of ARF18:GFP accumulation in apical domains and in the valve margins (**Figure 6V**). In anthesis and post-anthesis stages, however, the accumulation of ARF18:GFP was found in a broader area at the valve margins, correlating with the lateral expansion of the dehiscence zones in *35S::NGA3* fruits (**Figure 6Y**) (Trigueros et al., 2009).

DISCUSSION

The study carried out in this work shows how alterations in NGA function have significant effects in auxin signaling throughout gynoecium development and that these interactions likely occur at multiple levels.

First, we have shown that the apical auxin maxima that forms in stage 8–9 wildtype gynoecia cannot be detected in *nga* quadruple mutants. While it is not conclusively proven that this maxima is directly responsible for style and stigma differentiation, it is clearly temporally correlated with the development of these tissues. Moreover, the inability of *nga* mutants to form this maxima and to differentiate apical tissues that we show in this work, together with the *nga*-like phenotypes of lines where the *NGA3* promoter directed the expression of *iaaL*, an enzyme that inactivates the pool of active auxin (Jensen et al., 1998), supports this direct causative link. On the other hand, the partial rescue of *nga* apical defects by local auxin application might suggest that the absence of auxin maxima is not the only cause of *nga* phenotypic defects. However, the method that we used for local auxin treatment is coarse and may not reproduce properly the spatial distribution or the timing of auxin accumulation dynamics, therefore providing a partial picture of the expected effects and limiting the validity of these conclusions. Interestingly, the generation of auxin maxima as revealed by *DR5rev::GFP* in developing gynoecia that constitutively express *NGA3* is not significantly different from that of wildtype. *35S::NGA3* pistils do not show a dramatic overproliferation of style and stigma, thus indirectly reinforcing the idea of the putative instructive role of auxin accumulation in these tissues to direct the development of apical tissues (Trigueros et al., 2009).

The likely failure to accumulate auxin in the apical domain of *nga* mutants can also explain the insensitivity of *nga* mutants to PAT inhibition (Alvarez et al., 2009): since no auxin is present, it can be expected that no basipetal transport takes place and therefore, no phenotypic defects result from this inhibition. It has been shown that *shi/sty* mutants are hypersensitive to NPA treatment (Ståldal et al., 2008). This situation is opposite to that found in *nga* mutants, in spite of the almost identical phenotypes found in gynoecium development and the apparent convergent regulation of YUC-mediated auxin synthesis by both NGA and SHI/STY factors (Kuusk et al., 2006; Sohlberg et al., 2006; Trigueros et al., 2009; Eklund et al., 2010). This could reflect a different role of NGA and SHI/STY factors in the establishment of auxin maxima or in the regulation of downstream effectors in response to those. To understand these mechanistical differences, it would be useful to describe auxin accumulation throughout gynoecium development in *shi/sty* multiple mutants.

Local auxin synthesis appears to be strongly reduced in apical tissues of *nga* mutants. It already has been shown that *NGA* downregulation leads to the loss of *YUC2* and *YUC4* activation in the apical domain of developing gynoecia (Trigueros et al., 2009). We show here that *YUC8* expression is completely absent in *nga* mutant pistils, while slightly increased in *35S::NGA3* lines. It could be envisioned that the lack of apical auxin maxima in

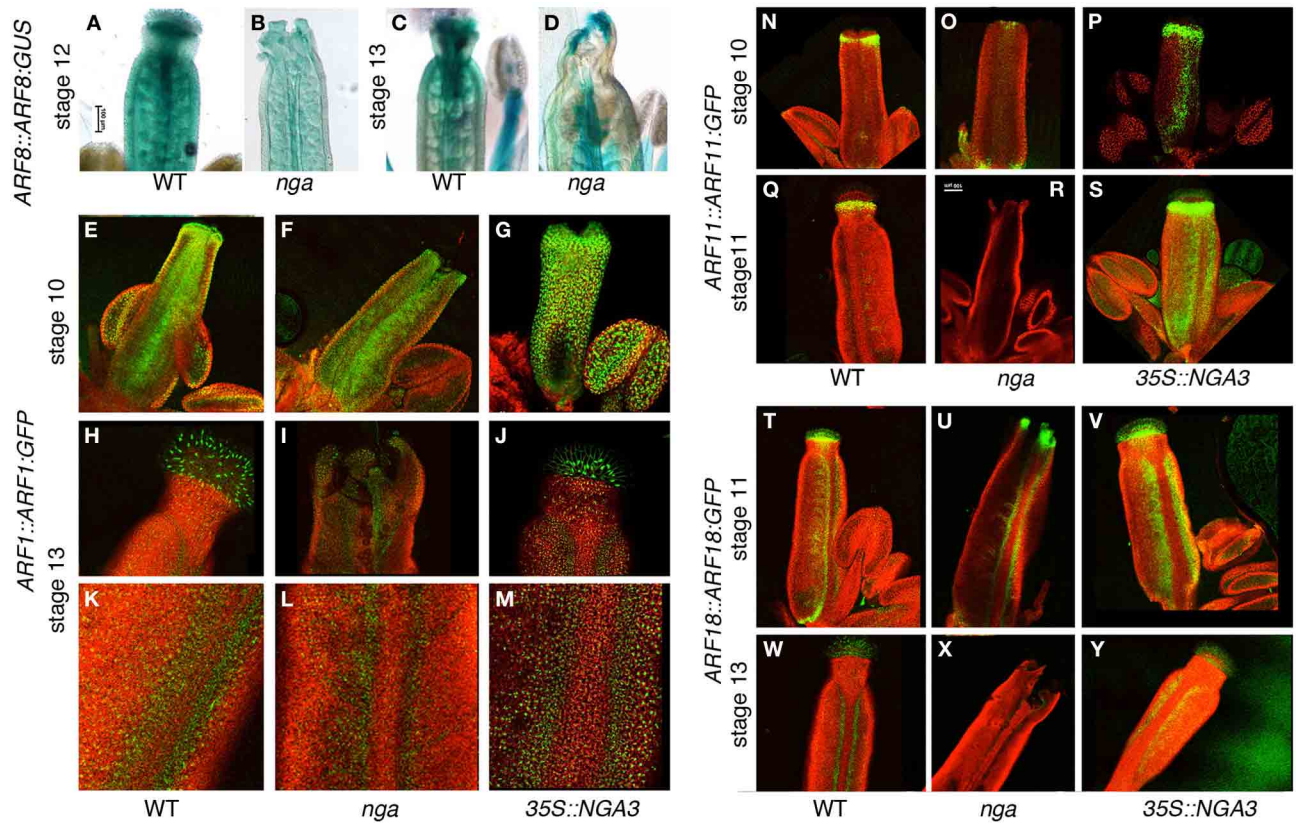

FIGURE 6 | Effect of NGA altered activity on the expression of ARFs throughout gynoecium development. (A–D) Histochemical detection of *ARF8::ARF8:GUS* activity in wildtype **(A,C)**, and *nga* quadruple mutants **(B,D)** gynoecia at stage 12 **(A,B)** and at anthesis **(C,D)**. While in stage 12 pistils ARF8 apical accumulation is clearly reduced **(B)**, at anthesis shows some expression at valve protrusions formed in the nga quadruple mutant **(D)**. **(E–M)** *ARF1::ARF1:GFP* expression in wildtype **(E,H,K)**, *nga* quadruple mutants **(F,I,L)** and *35S::NGA3* gynoecia **(G,J,M)** at stage 10 **(E–G)** and at anthesis **(H–M)**. **(H–J)** show close up views of the apical domain, and **(K–M)** show close up views of valve margins in the ovary region of anthesis pistils. **(N–S)** *ARF11::ARF11:GFP* expression in wildtype **(N,Q)**, *nga* quadruple mutants **(O,R)** and *35S::NGA3* gynoecia **(P,S)** at stage 10 **(N–P)** and at stage 11 **(Q–S)**. Note the strongly reduced GFP signal in the apical region of *nga* mutants **(O,R)** and the strong signal associated with valve margins of *35S::NGA3* developing gynoecia **(P,S)**. **(T–Y)** *ARF18::ARF18:GFP* expression in wildtype **(T,W)**, *nga* quadruple mutants **(U,X)** and *35S::NGA3* gynoecia **(V,Y)** at stage 11 **(T–V)** and at anthesis **(W–Y)**. Note expanded GFP signal associated with valve margins of *35S::NGA3* pistils **(V,Y)**.

nga pistils could be due to the absence of YUC-mediated auxin synthesis in this domain. The phenotypes of *yuc2 yuc4 yuc8* triple mutants have not been described, so it is not possible to directly compare both scenarios. Even in the triple mutants, since *YUC2* and *YUC4* are normally expressed in *nga* mutants outside the apical gynoecium, loss of *YUC2* and *YUC4* function could have additional effects that might obscure the specific role of *YUC2/4/8* in style and stigma differentiation, so in addition to generating and characterizing the *yuc2 yuc4 yuc8* triple mutants, it might be necessary to inactivate specifically all three enzymes in the apical developing gynoecium. In addition to the effect of *nga* mutations on *YUC* gene expression, *TAA1* and *AMI1* also appear to be under NGA direct or indirect regulation. TAA1 has been recently placed in the same biosynthetic route as the YUC enzymes (Stepanova et al., 2011) and thus it would be possible that the moderate effects of NGA altered function in *TAA1* expression would not lead to dramatic differences in auxin synthesis rates through this TAA-YUC pathway. Unlike the *YUC* genes for which detailed expression patterns in carpels have been reported, *TAA1* is expressed in carpel margins of stage 12 and postanthesis wildtype pistils. Interestingly, this valve margin expression is reduced in *nga* mutants while increased in *NGA3* overexpressors. While the role of this putative local auxin synthesis at the valve margin is currently unknown, as well as the precise role of *NGA* in valve margin development, the possible altered auxin synthesis in valve margins in response to NGA differential activity could partly explain the changes in the expression levels of *ARF1*, *ARF11* or *ARF18* in the different *NGA* backgrounds revealed in this study. Finally, a third putative contributor to local auxin synthesis is the AMI1 enzyme, which catalyzes the transformation of IAM to IAA. It is still unclear whether AMI1 activity significantly contributes to auxin synthesis in inflorescence development (Mano et al., 2010; Zhao, 2010), but the strong *AMI1* expression in developing gynoecia and, specially, in apical and transmitting tissues, suggests that it may have a role in auxin production in this domain. We show here that *nga* mutations significantly reduce *AMI1* accumulation in the apical pistil, while *NGA3* overexpression leads to increased

and persistent levels of *AMI1* expression, thus indicating that NGA could also positively regulate AMI1 activity in these tissues and hence putative auxin synthesis through this pathway. It has been described that *ami1* mutants (aka *attoc64-I*) do not show phenotypic defects (Aronsson et al., 2007), which could be due to redundancy with other members of the family, and therefore is its premature to speculate at this point about the relevance of the NGA-AMI1 functional relationship. However, the convergent effect of *NGA* mutations on the regulation of *TAA1*, *YUC,* and *AMI1* strongly suggests that NGA factors may function as strong positive regulators of auxin synthesis in the apical gynoecium.

The strongly reduced or absent local auxin synthesis in the apical developing *nga* mutant gynoecia probably contributes to the reduced auxin accumulation observed in these tissues, although it is unlikely to be the only cause. It is generally accepted that auxin maxima are mainly produced by PAT (Grieneisen et al., 2013), and there are examples of these maxima directing auxin synthesis that could reinforce auxin accumulation patterns (Grieneisen et al., 2007). In this work, it has been shown how enzymes involved in auxin synthesis are still expressed in the apical gynoecium after the *DR5rev::GFP* reporter signal has faded or is very reduced, suggesting that additional mechanisms have also an impact in auxin distribution downstream auxin synthesis. Clearly more work would be needed to resolve the interplay between transport, synthesis and probably other components of the pathway. Such further work should include a detailed characterization of auxin flux as directed by auxin transporters such as several PIN-family members or other transporters. Unfortunately, our analyses on the effect of *NGA* loss or gain of function on PIN proteins have not produced clear conclusions. Still, our results indicate that auxin transport is likely altered in *nga* mutants or the *35S::NGA3* line, since the expression of *PID* and *WAG2*, major regulators of PIN polarization, as well as the expression domain of PIN3 are affected by *NGA* altered function. In this sense, the expanded domain of expression of PIN3 observed in *35S::NGA3* pistils could facilitate auxin depletion from the apical domain through increased basipetal auxin transport, thus providing a hint on the mechanisms that could explain the wildtype-like response of *DR5rev::GFP* observed in *35S::NGA3* developing gynoecia.

Finally, protein accumulation patterns for several ARFs expressed through gynoecium development have been described in wildtype, *nga* mutants and *35S::NGA3* lines. We have found that NGA factors appear to differentially regulate the accumulation of the different ARFs under study. Thus, the apical domain of accumulation found for ARF11 is completely lost in *nga* mutants, while apical expression of *ARF8* and *ARF1* (and only slightly that of *ARF18*) are reduced, but not absent, from *nga* mutant apical gynoecia. Interestingly, *NGA3* constitutive expression appears to induce the expanded expression at valve margins of *ARF1*, *ARF11*, and *ARF18*, similarly to what was observed for *TAA1* expression, suggesting that they might be responsive to local auxin synthesis putatively mediated by TAA1. It has been described that *35S::NGA3* fruits have enlarged dehiscence zones and thus, this expanded expression domains could be also due to an indirect effect of *NGA3* overexpression on fruit morphology. Because no function has been assigned yet to *ARF1*, *ARF11*, or *ARF18* in gynoecium development, it remains to be studied whether ARF regulation may mediate *NGA* functions in this process.

In summary, our work shows that NGA factors impact on auxin signaling pathways at multiple levels throughout pistil development. First, and more importantly, NGA factors appear to be essential, but not sufficient for auxin synthesis in the apical developing gynoecium, since several members of the *YUC* family, as well as *TAA1* and *AMI1* were not expressed in this domain in *nga* quadruple mutants, but only showed moderately increased expression in *35S::NGA3* lines. Accordingly, *DR5rev::GFP* showed no activity in *nga* mutants but no significant differences in *35S::NGA3* pistils when compared to wildtype. It is thus tempting to speculate that NGA could only direct auxin synthesis in the presence of other factors, for which SHI/STY family members are strong candidates. In addition, NGA altered activity affected the expression of *PID* and *WAG2*, regulators of PIN subcellular localization, and thus likely had an impact on auxin transport in parallel to the effect on auxin synthesis. Finally, protein accumulation in pistils of several ARFs was differentially affected by *nga* mutations or *NGA* overexpression, suggesting that these accumulation patterns depend not only on auxin distribution but could be also regulated by transcriptional networks involving NGA factors. Again, *NGA3* constitutive expression did not result in wide activation of ARF expression in the gynoecium, reinforcing the idea of NGA requiring additional factors to exert their regulatory functions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Journal/10.3389/fpls.2014.00210/abstract

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Ring the BELL and tie the KNOX: roles for TALEs in gynoecium development

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Carpels are leaf-like structures that bear ovules, and thus play a crucial role in the plant life cycle. In angiosperms, carpels are the last organs produced by the floral meristem and they differentiate a specialized meristematic tissue from which ovules develop. Members of the three-amino-acid-loop-extension (TALE) class of homeoproteins constitute major regulators of meristematic activity. This family contains KNOTTED-like (KNOX) and BEL1-like (BLH or BELL) homeodomain proteins, which function as heterodimers. KNOX proteins can have different BELL partners, leading to multiple combinations with distinct activities, and thus regulate many aspects of plant morphogenesis, including gynoecium development. TALE proteins act primarily through direct regulation of hormonal pathways and key transcriptional regulators. This review focuses on the contribution of TALE proteins to gynoecium development and connects TALE transcription factors to carpel gene regulatory networks.

Keywords: carpel, TALE, transcription factors, development, *Arabidopsis*

INTRODUCTION

In *Arabidopsis*, the female reproductive organ, or gynoecium, consists of an apical stigma, a style, and a basal ovary (**Figure 1** and for reviews, Ferrándiz et al., 1999; Roeder and Yanofsky, 2006; Girin et al., 2009; Ferrándiz et al., 2010). The ovary is composed of two fused carpels (termed valves after fertilization) whose margins are joined by the replum. The inner (adaxial) side of the replum has a typical meristematic layered structure. This meristem gives rise to ovules and to two septum primordia, which grow and fuse to create the septum that divides the ovary into two locules. Two rows of ovules arise along the septum inside each locule. The septum differentiates a central transmitting tract tissue, which guides pollen tubes from the style to the ovule. Upon fertilization, ovules develop into seeds, and gynoecium structure changes dramatically: the fruit enlarges both longitudinally and laterally to accommodate seed growth and the valve margins undergo cell wall changes required for silique dehiscence and seed dispersal.

In multicellular organisms, development relies on stem cells, which are defined by their ability to renew themselves and to give rise to daughter cells that contribute to organ production. In plants, stem cells are maintained within structures called meristems, and new organs are produced at the meristem periphery (for review, Sablowski, 2011). The shoot apical meristem (SAM) produces leaves and axillary meristems. Following floral evocation, the SAM becomes an inflorescence meristem (IM), which produces flower meristems (FMs) that give rise to flowers containing gynoecia. Carpels are thought to be modified leaves with their margins representing a lateral organ boundary (Frohlich, 2003). As such, similar interactions occurring between SAM-boundary-leaf apply to fruit patterning.

Within meristems, cell proliferation and differentiation are tightly controlled by networks of transcription factors (TFs), which integrate developmental cues such as position, differentiation, and growth (Sablowski, 2011). The *KNOTTED1* (*KN1*) gene in maize was the first regulator of meristem activity identified in plants (Hake and Vollbrecht, 1989). In *Arabidopsis*, *SHOOT MERISTEMLESS* (*STM*), which is functionally related to *KN1*, and *WUSCHEL* (*WUS*) control meristem activity (for review, Aichinger et al., 2012). *WUS* is required to maintain the stem-cell population, as *wus* mutants lack stem cells at the center of the shoot apices while *STM* is required for SAM initiation and its maintenance in an undifferentiated state, as strong *stm* mutants fail to develop a meristem during embryogenesis and fail to produce lateral organs (Endrizzi et al., 1996; Long et al., 1996). *STM* is expressed in SAM, IM, FM, and in the inner side of the replum (Endrizzi et al., 1996; Long et al., 1996; Ragni et al., 2008). *STM* is down-regulated when cells become specified as primordium founder cells (Long et al., 1996).

STM belongs to the "Three-Amino-acid-Loop-Extension" (TALE) homeodomain superclass of TFs, which in *Arabidopsis* comprises 9 KNOTTED-like (KNAT or KNOX) and 13 BEL1-like (BLH or BELL) members (**Box 1**). The TALE factors function as KNOX-BELL heterodimers (for reviews, Hay and Tsiantis, 2010; Hamant and Pautot, 2010; Di Giacomo et al., 2013). STM maintains the pool of indeterminate meristematic cells through repression of gibberellin (GA) biosynthesis, activation of GA catabolism, and activation of cytokinin (CK) biosynthesis (Sakamoto et al., 2001; Chen et al., 2004; Jasinski et al., 2005; Bolduc and Hake, 2009). In addition, in the SAM, STM represses the *ASYMMETRIC LEAVES1* (*AS1*) gene, which encodes a MYB TF involved in leaf patterning. AS1 represses other TALE-family members such as *KNAT1/BREVIPEDICELLUS* (*BP*), *KNAT2*, and *KNAT6* in leaves (Byrne et al., 2000; Phelps-Durr et al., 2005). Subsequent organ initiation requires high auxin and GA levels

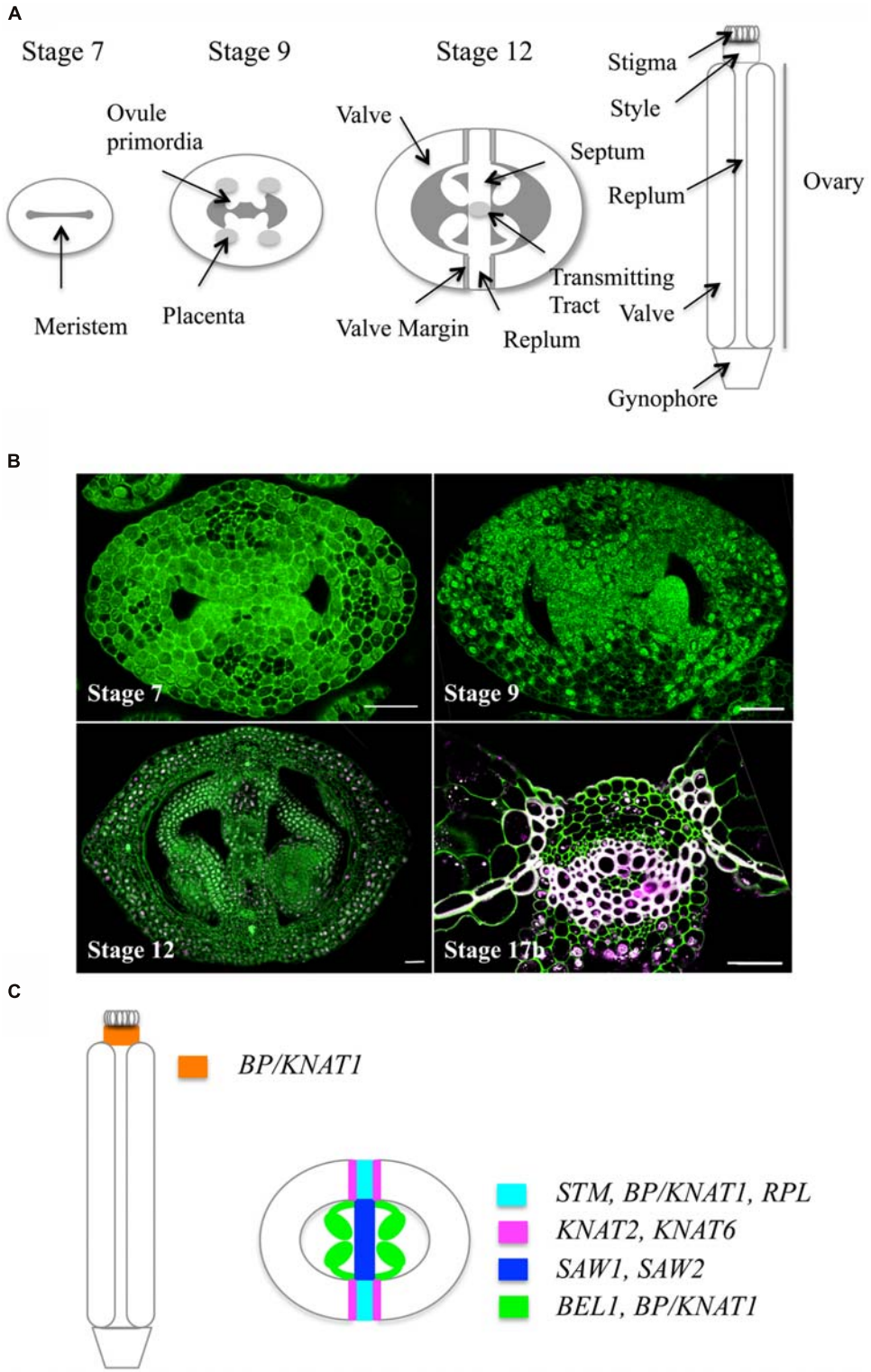

FIGURE 1 | *Arabidopsis* gynoecium development. (A) Schematic cross sections showing the different tissues of the gynoecium at three developmental stages according to Smyth et al. (1990). **(B)** Optical cross sections through the *Arabidopsis* gynoecium at four developmental stages stained with iodine green and carmine alum: upper left, stage 7, showing the layered structure of the meristem; upper right, stage 9, showing ovule primordia initiating from the placenta; lower left, stage 12, lower left, close-up of the medial tissue (stage 17b) showing the replum and lignin deposition at the valve margins and at the endocarp b layer. Scale bars represent 25 μm. **(C)** Schematic representation of expression patterns of *TALE* genes in the *Arabidopsis* gynoecium (stage 12).

BOX 1 | Meet the TALE gene family.

The TALE family is a superclass of homeodomain TFs which comprises eight KNOTTED-like proteins in *Arabidopsis thaliana* (KNAT or KNOX) plus a mini KNAT lacking the homeodomain (KNATM) and 13 BEL1-like (BLH or BELL) members (for a detailed review of the structure of this gene family, see Mukherjee et al. (2009) and for a phylogenetic tree of the *Arabidopsis* TALE family, see Hamant and Pautot, 2010). This family controls development in all eukaryotic lineages (Hay and Tsiantis, 2010). KNOX and BELL families occur in single copy in Green algae and have diversified in land plant (Lee et al., 2008). The KNOX family is divided into three classes based on sequence similarity and gene expression pattern (Hake et al., 2004; Magnani and Hake, 2008): Class I includes STM, BP/KNAT1, KNAT2 and KNAT6; Class II includes KNAT3, 4, 5, and 7. Class III contains KNATM, which can interact with other TALE members to modulate their activity (Kimura et al., 2008; Magnani and Hake, 2008). The BELL family comprises RPL/BLH9, PNF/BLH8, ATH1, SAW1/BLH2, SAW2/BLH8, BEL1 – whose functions have been characterized- and BLH1, BLH3, BLH5, BLH6, BLH7, BLH10, and BLH11 – whose functions are not yet known (Hamant and Pautot, 2010). The interaction of KNOX and BELL proteins is critical for their nuclear localization and their binding affinity to DNA, thereby imparting their activity (Smith et al., 2002; Rutjens et al., 2009; Kim et al., 2013). TALEs can also form complexes with other TFs, such as MADS-Box family members, to control ovule development (Brambilla et al., 2007) and with OVATE proteins, which negatively regulate KNOX-BELL heterodimers by relocalizing them from the nucleus to the cytoplasm (Hackbusch et al., 2005). STM protein traffics selectively through plasmodesmata, and this cell-to-cell movement, which involves chaperonins belonging to a group of cytosolic chaperones, is critical to maintain of the SAM (Xu et al., 2011).

and down regulation of *STM* and related TFs (Hay and Tsiantis, 2010).

Multiple combinations of TALE heterodimers with distinct activities are produced throughout the plant life cycle, controlling diverse developmental processes, such as SAM and boundary maintenance, leaf development and flowering. This review discusses the contribution of TALE TFs to gynoecium development in *Arabidopsis*, and links these proteins to the other key molecular players of carpel development.

CARPEL INITIATION: KNOX AND BELL INTERACTIONS WITH AGAMOUS

Carpels are the last organs to be produced by floral meristems. Weak *STM* alleles or weak *STM* RNAi lines show no carpel formation due to premature differentiation of meristematic cells (Endrizzi et al., 1996; Scofield et al., 2007). Consistent with this, CLAVATA (CLV) receptors control the proliferation and number of organs in developing gynoecia through STM activity. Mutations in CLV1, CLV2, and CORYNE (CRN) receptors lead to increased meristem size correlated with an enlarged *STM* expression pattern (Durbak and Tax, 2011). Unlike the SAM, which is indeterminate, the FM terminates after carpel initiation. This determinacy depends on a negative feedback loop involving the C-function homeotic MADS domain TF, AGAMOUS (AG) which acts in part via activation of the zinc finger protein KNUCKLES (KNU) to repress *WUS* expression (Lenhard et al., 2001; Lohmann et al., 2001; Sun et al., 2009). AG controls carpel identity in combination with another MADS BOX TF, SEPALLATA3 (SEP3) (Bowman et al., 1989; Honma and Goto, 2001; Pelaz et al., 2001). *AG* expression is first detectable in developing flowers at early stage 3, flower stages defined by Smyth et al. (1990), where it is initially localized in the center of the FM, and is later restricted to stamen and carpel primordia (Bowman et al., 1991; Drews et al., 1991). At late stage 5, the floral meristem forms a flattened oval where the gynoecium initiates (Smyth et al., 1990). This stage coincides presumably with the generation of auxin maxima similar to those observed at the initiation of other organs, although no expression of auxin-signaling reporters at stages 5–7 has been described (for review, Larsson et al., 2013). The BELL member, REPLUMLESS (RPL), also known as PENNYWISE (PNY), BELLRINGER (BLR), VAAMANA (VAN), or LARSON (LSN) and its close relative POUNDFOOLISH (PNF) together with STM, function in parallel with LEAFY (LFY) and WUS to promote carpel formation through positive regulation of *AG* (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bao et al., 2004; Bhatt et al., 2004; Yu et al., 2009). Interestingly, a previous report showed that RPL represses *AG* together with LEUNIG and SEUSS, two transcriptional co-regulators of AG (Bao et al., 2004). This study was based on analysis of two recessive *rpl* alleles (*blr-4* and *blr-5*) whose flowers exhibit homeotic conversion of sepals to carpels at high temperature during late-stage flower development. This suggests that RPL could have two antagonistic activities depending presumably on its partner. However, no ectopic *AG* expression has been reported so far in null *rpl* mutants. An alternative hypothesis is that the point mutations within the homeobox region in *blr-4* and *blr-5* mutants cause the production of abnormal protein with regulatory defects.

GYNOECIUM PATTERNING

Once initiated, the gynoecium developmental program promotes correct patterning of the future fruit. Several specific tissues are formed (see above and **Figure 1**), some of which require the activity of TALE TFs. At stage 6, the gynoecium forms as a ridge of raised cells around a central cleft and starts to acquire its mediolateral symmetry, comprising replum, valve margins and valves. In the transverse plane, the adaxial inner side of the replum has a typical meristematic layered structure (**Figure 1B**), and accordingly expresses the meristematic genes *STM, CLV1/2*, and *CRN* (Long et al., 1996; Durbak and Tax, 2011; Romera-Branchat et al., 2012). However, *WUS* is not expressed in the replum (Groß-Hardt et al., 2002). Recently, a role for *WUS-LIKE HOMEOBOX13 (WOX13)* in replum was reported (Romera-Branchat et al., 2012). Unlike *WUS*, which marks a few cells in the SAM, defining its organizing center, *WOX13* has a broad expression pattern in replum, suggesting that the medial region of the gynoecium does not show typical SAM organization.

Consistent with a role for *STM* in initiating and maintaining meristems, weak alleles of *STM* or weak *STM* RNAi lines produce fewer ovules than the wild type (Endrizzi et al., 1996; Scofield et al., 2007). Two other TALE genes, *RPL* and *BP*, are also expressed in the replum. The *rpl* mutant shows defects in replum differentiation and in septum fusion (Roeder et al., 2003). RPL promotes replum identity through restriction of expression of the MADS-BOX genes *SHATTERPROOF1/2* (*SHP1/2*) and the basic helix-loop-helix (bHLH) gene *INDEHISCENT* (*IND*), to the

valve margins (Roeder et al., 2003; Liljegren et al., 2004; Dinneny et al., 2005), but RPL is not required *per se* for replum specification, since double or triple mutant combinations including *rpl* alleles develop a normal replum. In addition, RPL represses several valves-associated genes in the replum: *JAGGED* (*JAG*), and genes conferring abaxial fate *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*), which promote *FRUITFULL* (*FUL*), *SHP1/2* and *IND* expression in the presumptive valve and valve margin tissues, respectively (Dinneny et al., 2005). BP, which interacts with RPL and activates its expression, contributes redundantly with RPL to replum development (Alonso-Cantabrana et al., 2007). Similarly to their role at the leaf/SAM interface, AS1 and the lateral organ boundary (LOB)-domain protein asymmetric leaves2 (AS2) restrict the expression of *BP* to the replum, exemplifying the co-option of this regulatory module in the SAM and carpel (Alonso-Cantabrana et al., 2007; González-Reig et al., 2012; Luo et al., 2012; Lodha et al., 2013). Together these studies led to proposition of a model in which antagonism between the lateral factors (JAG/FIL and AS1/2) and the medial factors (BP and RPL) determines the medio-lateral fruit pattern by regulating the formation and size of three domains: valve, valve margin and replum. Furthermore, APETALA2, a member of the AP2/Ethylene-responsive element binding protein (EREBP) TF family, limits growth of both replum and valve margins by repressing *BP* and *RPL* in the replum and *SHP1/2* and *IND* in valve margins (Ripoll et al., 2011). Although *BP*, together with *RPL*, contributes to replum development, single *bp* loss-of-function mutants have wild-type repla (Alonso-Cantabrana et al., 2007; Ripoll et al., 2011). BP is also expressed in the style where it is required for radial growth (Venglat et al., 2002).

From the maternal side, optimal seed production relies on adequate generation of ovules. Ovule primordia formation depends on auxin maxima (Bencivenga et al., 2012). Auxin levels are modulated by the combined activity of CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 TFs, which are redundantly required to regulate the polar auxin transporter *pin-formed1* (*PIN1*) expression (Galbiati et al., 2013). CK also regulates *PIN1* expression during early stages of ovule development (Bencivenga et al., 2012). The interplay between hormones and TFs forms an integrative framework enabling ovule primordia initiation. Once initiated, an ovule differentiates a central nucellus containing the embryo sac, two integuments that envelop the nucellus, and a funiculus that connects the ovule to the placenta (for reviews, Colombo et al., 2008; Shi and Yang, 2011). Correct ovule development requires the activity of the *BEL1* gene, the founding member of the BELL family. *BEL1* is expressed in ovule integument primordia, and controls ovule integument identity (Robinson-Beers et al., 1992; Reiser et al., 1995). The *bel1* mutant exhibits bell-shaped ovules – hence its name – caused by the abnormal development of integuments (Reiser et al., 1995). Occasionally, the *bel1* mutant shows homeotic conversion of ovules into carpeloid structures due to prolonged *AG* expression during ovule development (Modrusan et al., 1994; Ray et al., 1994; Brambilla et al., 2007). BEL1 is required for auxin and CK signaling pathways during ovule development; the level and localization of *PIN1* expression are controlled by CK in part via BEL1 activity (Bencivenga et al., 2012).

Inside the future fruit, tissues required for successful fertilization and fruit compartmentalization are formed concomitantly. Two placenta ridges develop in the medial plane to give rise to a specialized structure compartmentalizing the fruit, the septum, which divides the fruit into two halves. In its center, the transmitting tract differentiates in the apical-basal axis to guide pollen tube growth. To date, little is known about the role of *TALE* genes in septum development. SAWTOOTH1 (SAW1)/BLH2 and SAW2/BLH4, members of the BELL family, are expressed in the transmitting tract, and interact with STM and BP, but their exact role in medial tissue development remains to be determined (Kumar et al., 2007).

POST-FERTILIZATION EVENTS

Upon fertilization, the gynoecium will develop into a fruit that contains the seeds. Gynoecium enlargement to accommodate the developing seeds relies on the coordinated growth of the entire organ, which strongly depends on hormonal balances (for review, Reyes-olalde et al., 2013). For instance, GA-deficient mutants show reduced fruit size, indicating that fruit development involves extensive GA-activated cell elongation (Koornneef and van der Veen, 1980; Chiang et al., 1995). While the fruit enlarges, differentiation processes take place to ensure efficient release of the seeds (Reyes-olalde et al., 2013). At the cellular level, this includes the differentiation of the dehiscence zone at the valve margins. This process depends on the activity of IND, which is responsible of the formation of a local auxin minimum at the valve margins through the regulation of *PINOID* and *WAG2* kinases (Sorefan et al., 2009). The dehiscence zone consists of two cell layers: the lignified and the separation layers. The lignified layer, located at the boundary with the valve, is continuous with the lignified internal layer (endocarp b) and contributes to tension that builds up in the silique until dehiscence. The layer located on the replum side, which constitutes the separation layer, is composed of isodiametric cells that undergo middle lamella breakdown. This separation process involves the activity of specialized cell wall enzymes such as polygalacturonases (PGs) and pectin methylesterases (PMEs) that increase the ability of PGs to break down pectin (Ogawa et al., 2009 and for review, Wolf et al., 2009). A link between TALE proteins and cell wall modifications has been shown in studies of internode patterning in *rpl* and *bp* mutants (Mele et al., 2003; Smith and Hake, 2003; Peaucelle et al., 2011). BP prevents premature deposition of lignin during internode growth by direct repression of genes involved in lignin biosynthesis, and regulates other cell-wall-specific genes such as ones encoding PMEs or cellulose synthetase (Mele et al., 2003; Wang et al., 2006). RPL is involved in maintaining normal phyllotaxy via the regulation of PMEs, which are involved in the cell wall loosening necessary to allow growth (Peaucelle et al., 2011). Interestingly, *KNAT6* and *KNAT2*, which act antagonistically to *BP* and *RPL* in stems, are expressed in valve margins (Ragni et al., 2008). This is consistent with *KNAT6* expression in SAM and its role in maintaining boundaries between SAM and lateral organs (Belles-Boix et al., 2006). Inactivation of *KNAT6* rescues replum formation in *rpl* mutants, showing that the antagonistic interaction between *KNAT6* and *RPL* also controls fruit architecture. Consistent with their expression in valve margins, KNAT6, and KNAT2 positively regulate lignin deposition

(Khan et al., 2012a,b). These factors also act antagonistically to BP during floral organ abscission, a process that also requires cell wall remodeling. *BP* regulates the timing of floral abscission by controlling abscission zone cell size. Upon activation of a signaling pathway including inflorescence deficient in abscission (IDA) and two receptor-like kinases, HAESA and HAESA-LIKE2 (HAESL2), BP is inactivated, leading to an increase of *KNAT2* and *KNAT6* expression, which act as positive regulators of floral organ separation (Shi et al., 2011). The link between TALEs and cell wall remodeling enzymes was further confirmed with the identification of STM, KN1, and RPL targets, which include several genes involved in cell wall modifications (Spinelli et al., 2011; Bolduc et al., 2012; Etchells et al., 2012).

FUTURE DIRECTIONS

Gynoecium development is critical for Angiosperm reproductive success, and is therefore tightly controlled by interconnected networks of TFs. Here, we reviewed the role of TALE TFs in the control of carpel development, and present the state of knowledge of the molecular interactions within this gene regulatory network. To date, the studies concerning the contribution of TALE TFs to carpel development focused on a few members of this family. Despite the number of studies, several pieces of the puzzle that will be needed to decipher the entire carpel regulatory network are still missing. In particular, the role of the KNAT class II members in carpels has not been investigated. Although several TALE members are expressed in carpels, the detailed expression pattern remains to be characterized for most of them. A precise map of TALE expression and co-localization of KNOX and BELL in the gynoecium will provide clues about putative partners and redundancies. Despite evidence linking TALE TFs, CK and GA pathways, the exact role of this regulatory node and its precise contribution to carpel development are not yet well established. Recently, the direct targets of KN1 in maize inflorescences were identified, and these data confirm that TALE TFs function as major orchestrators of hormone synthesis or response (Bolduc et al., 2012). Importantly, a clear link between KN1 and the auxin pathway was demonstrated. Furthermore, key developmental regulators such as homeodomain TFs are highly represented among KN1 targets, suggesting that KN1 orchestrates upper levels of regulatory networks controlling development. New strategies based on next generation sequencing to identify targets of TFs have begun to shed light on the molecular interactions downstream of key TFs, providing crucial insight into the mechanisms controlling development and opening new perspectives regarding carpel development. The integration of these data into comprehensive models accounting for spatial and temporal information represents a challenge to fully understand how fruits develop. Developing mathematical models will be particularly useful for understanding how fruit morphology can vary and how their astonishing diversity of shape can be achieved among plant species.

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The *CUC1* and *CUC2* genes promote carpel margin meristem formation during *Arabidopsis* gynoecium development

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Carpel margin meristems (CMMs), a pair of meristematic tissues present along the margins of two fused carpel primordia of *Arabidopsis thaliana*, are essential for the formation of ovules and the septum, two major internal structures of the gynoecium. Although a number of regulatory factors involved in shoot meristem activity are known to be required for the formation of these gynoecial structures, their direct roles in CMM development have yet to be addressed. Here we show that the *CUP-SHAPED COTYLEDON* genes *CUC1* and *CUC2*, which are essential for shoot meristem initiation, are also required for formation and stable positioning of the CMMs. Early in CMM formation, *CUC1* and *CUC2* are also required for expression of the *SHOOT MERISTEMLESS* gene, a central regulator for stem cell maintenance in the shoot meristem. Moreover, plants carrying miR164-resistant forms of *CUC1* and *CUC2* resulted in extra CMM activity with altered positioning. Our results thus demonstrate that the two regulatory proteins controlling shoot meristem activity also play critical roles in elaboration of the female reproductive organ through the control of meristematic activity.

Keywords: *Arabidopsis thaliana*, carpel margin meristem, shoot meristem, leaf development, MicroRNA (miRNA), fruit development

INTRODUCTION

In the plant shoot, leaves and floral organs are produced from the shoot and floral meristems, respectively. These meristems maintain pluripotent stem cells at the center and differentiate appropriate types of lateral organs at their periphery depending on the developmental context. Although diverse in their shape and function, floral organs (sepals, petals, stamens, and carpels) are considered to be modified leaves and their specific characters are conferred by combinatorial actions of homeotic genes (Krizek and Fletcher, 2005). How unique shapes of individual organ types are generated is a central question in understanding plant shoot development.

The carpel is a component of the gynoecium, a highly complex organ system dedicated to reproduction. Either single or multiple carpel(s) fuse to form the gynoecium and enclose ovules inside. Ovules are formed by meristematic tissues located within or adjacent to carpel primordia (Yamaki et al., 2011) and in *Arabidopsis thaliana*, they are produced by a pair of meristematic tissues called carpel margin meristems (CMMs; also called medial ridges), which are present along the fused margins of the two carpel primordia (Nole-Wilson et al., 2010). In addition to producing ovules laterally, the CMM pair terminates and fuses with each other along their tip, forming the septum that acts as transmitting tissue for pollen tubes.

Several regulators of shoot meristem activity are involved in carpel margin development (reviewed in Reyes-Olalde et al., 2013). The *REPLUMLESS* gene (*RPL*; also known as *BLR* and *PNY*) encoding a BELL-type homeodomain protein is expressed in carpel margins and is required for replum development (Roeder et al., 2003). The RPL protein physically interacts with a class I KNOX protein BREVIPEDICELLUS (BP) and the activity of the two proteins counteracts with *JAGGED* (*JAG*), *FILAMENTOUS FLOWER* (*FIL*), *YABBY3* (*YAB3*), and *ASYMMETRIC LEAVES1/2* (*AS1* and *AS2*) genes, which promote the fate of adjacent valve and valve margin tissues (Dinneny et al., 2005; Alonso-Cantabrana et al., 2007; Gonzalez-Reig et al., 2012). Both *RPL* and *BP* genes are also expressed in the shoot meristem and affect internode length (Smith and Hake, 2003). The RPL protein interacts with another class I KNOX protein SHOOT MERISTEMLESS (STM) and they act together to maintain stem cells in the shoot meristem (Byrne et al., 2003). Although strong mutant alleles of *stm* do not produce flowers due to their strong shoot meristem defects, function of *STM* in carpel development has been accessed using weak *stm* alleles or inducible RNAi plants, which produce abnormal flowers. In these flowers, carpels often fail to fuse at their margins and develop few ovules, indicating that *STM* is required for proper formation of carpel margins (Endrizzi et al., 1996; Scofield et al., 2007).

The *CUP-SHAPED COTYLEDON* genes *CUC1* and *CUC2* encoding a pair of paralogous NAC transcription factors are required for shoot meristem initiation through promoting *STM* expression (Hibara et al., 2003). Because of their functional redundancy, seedlings of each single mutant show little morphological phenotype while their double mutants completely lack a shoot meristem and produce severely fused cotyledons. Viable shoots with flowers can be regenerated from double mutant calli and these flowers produce carpels with severe reduction of ovules, septum and replum (Ishida et al., 2000; **Figure 1**). The Auxin

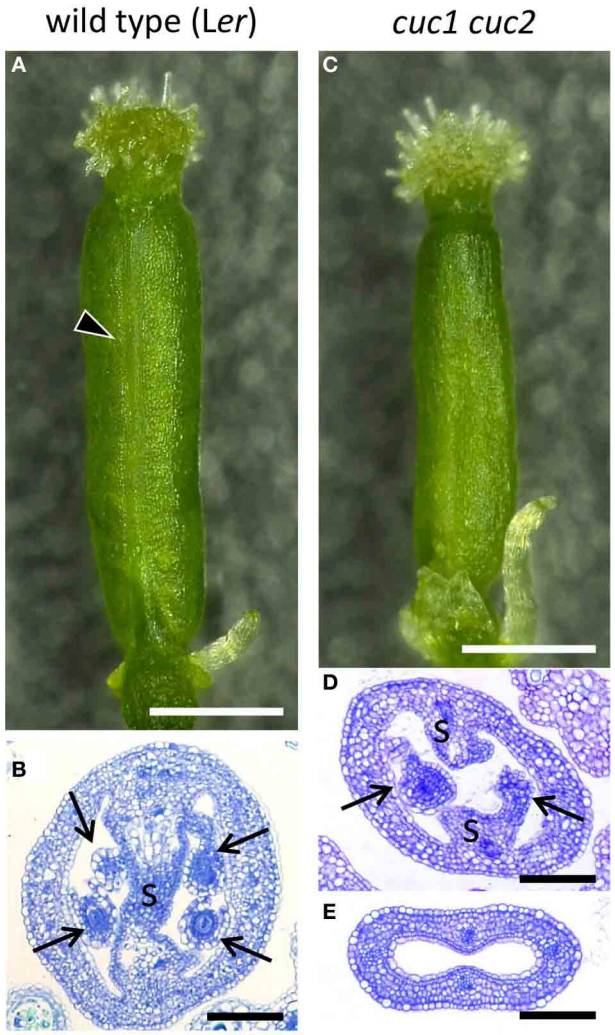

FIGURE 1 | Gynoecium phenotype of *cuc1 cuc2*. Mature gynoecia from regenerated plants of wild type L*er* **(A,B)** and *cuc1 cuc2* **(C–E)**. Whole view **(A,C)** and transverse sections **(B,D,E)**. Arrowhead in **(A)** indicates the replum. Arrow, ovule; s, septum. Scale bars are: **(A,C)**, 500 μm; **(B**,**D,E)**, 100 μm.

Response Factor MONOPTEROS (MP) is required for *CUC1* and *CUC2* expression possibly through its direct binding to the gene promoters and the *CUC* genes in turn affect expression and polarity of the auxin transport protein PIN1 in ovule primordia (Galbiati et al., 2013). In addition, both *CUC1* and *CUC2* are negatively regulated by the microRNA miR164, which is encoded by three loci in *Arabidopsis*. Disruption of miR164 encoding genes or that of its target sequences in *CUC1* and *CUC2* causes misregulation of their expression, resulting in various developmental defects including abnormal carpel development (Mallory et al., 2004; Baker et al., 2005; Nikovics et al., 2006; Sieber et al., 2007; Larue et al., 2009).

The above results point to the importance of *CUC1* and *CUC2* and in formation of carpel margin structures. However, the developmental basis of the roles for these factors has not been fully investigated. Notably, whether the factors directly affect CMM formation and if so, how they interact during the process remains unknown. Here we investigated the roles for *CUC1* and *CUC2* in gynoecium development by loss and gain of function approaches. The results demonstrate that the *CUC1* and *CUC2* genes are critical for normal CMM development.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

The *Arabidopsis thaliana* accessions Landsberg *erecta* (L*er*) and Columbia (Col) were used as the wild type strains. The *cuc1 cuc2* double mutant (*cuc1-1 cuc2-1*) is in the L*er* background (Aida et al., 1997). *CUC2g-m4* is in the Col background and was described previously (Nikovics et al., 2006). For construction of *CUC1g-m7*, the miR164 target sequence (AG CAC GTG TCC TGT TTC TCC A) of *CUC1* was replaced by a mutant sequence (AG CAC GTG AGT TGT TTT AGT A), which contains seven silent mutations (underlined). The mutated genomic fragment corresponding to the nucleotides 5108201..5112019 of chromosome 3 (TAIR 10) was cloned into pGreenII 0229 (Hellens et al., 2000) and transformed into Col. A transgenic line displaying extra petal number and reduced sepal growth, a typical phenotype described for miR164 resistant *5mCUC1* (Mallory et al., 2004), was selected and subjected to analysis. This line accumulated *CUC1* mRNA ∼9.5 fold of the wild-type level in inflorescence apices. Seeds were surface sterilized and sown on MS plates as previously described (Fukaki et al., 1996). After incubation for 2 days at 4°C in the dark, plants were grown in a growth chamber at 23°C under constant white light. Ten- to fourteen-day-old seedlings were transferred onto soil and grown at 23°C under constant white light. Induction of calli from root explants and subsequent shoot regeneration was performed as previously described (Aida et al., 1997). Flower stages were determined as previously described (Smyth et al., 1990). Stage 9 was further subdivided into early, mid and late substages, each corresponding to stages 5, 6, and 7 of anther development (Sanders et al., 1999).

HISTOLOGICAL ANALYSIS

Histological sections (3 μm) were prepared as previously described(Aida et al., 1997), except that formalin/acetic acid/alcohol (FAA) was used as a fixative. Scanning electron microscopy was carried out as described previously (Aida et al., 1997). *In situ* hybridization was performed as previously described (Ishida et al., 2000) with following modifications: 6 instead of 8 μm sections were prepared and hybridized at 45°C instead of 42°C. Probes for *STM* and *FIL* have been described previously (Long et al., 1996; Sawa et al., 1999). Templates for *CUC1* and *CUC2* probes were the full-length coding sequences. In the wild type and *cuc1 cuc2*, coloring reaction was performed for 36 h, with the initial 12 h at room temperature and the remaining at 4°C. In *CUC1g-m7* and *CUC2g-m4*, coloring reaction was carried out for 12 h at room temperature.

RESULTS

CUC1 AND *CUC2* ARE REQUIRED FOR THE INITIATION OF THE CMMs

To compare gynoecium development of the wild type (L*er*) and *cuc1 cuc2*, we used inflorescence shoots regenerated from calli of

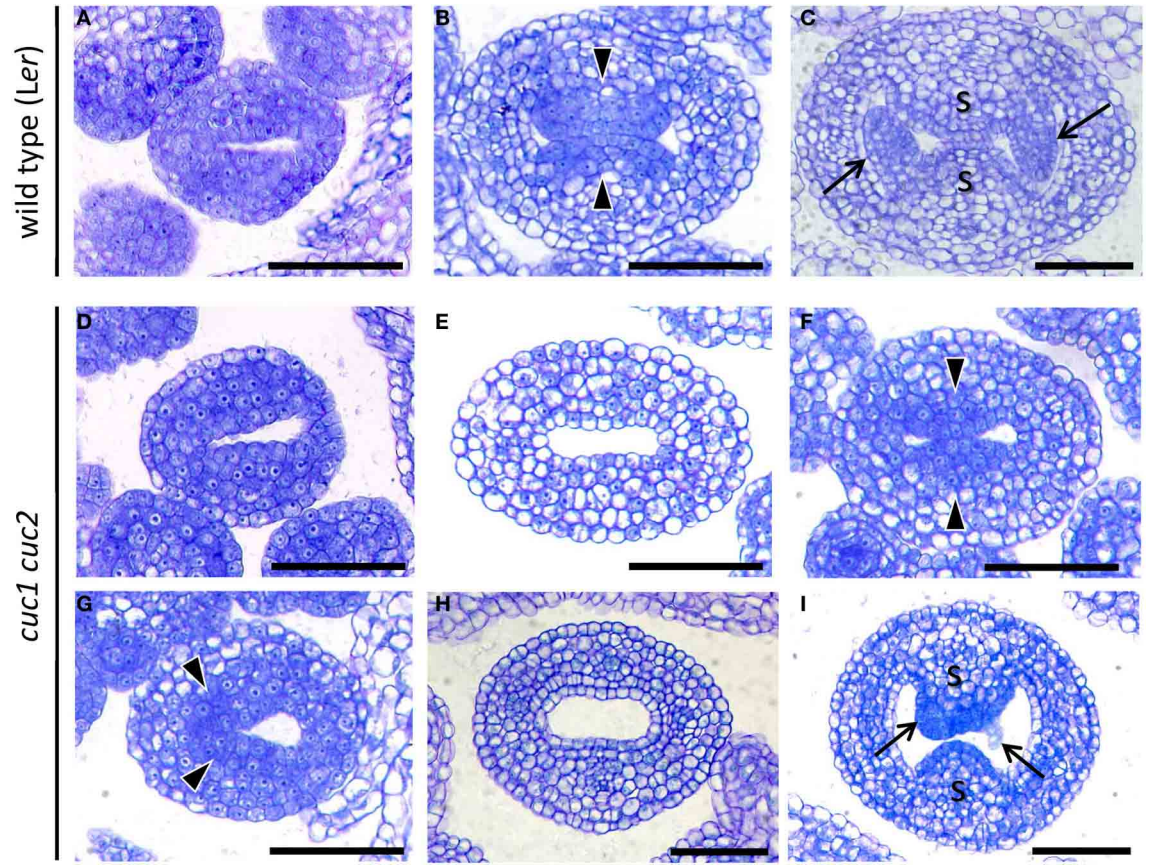

FIGURE 2 | *CUC1* and *CUC2* are required for formation and stable positioning of CMM. Transverse sections of developing gynoecium at stage 7 **(A,D)**, stage 8 **(G)**, early stage 9 **(B,E,F)**, mid stage 9 **(H),** and late stage 9 **(C,I)**. Sections are prepared from wild type **(A–C)** and *cuc1 cuc2* **(D–I)**. Arrow, ovule; arrowhead, CMM; s, septum. Scale bars are 50 μm.

Table 1 | Effect of *cuc1 cuc2* on CMM formation.

Genotype	Stage*	CMM formation			CMM contact**	
		Both sides	One side	None	Yes	No
wild type	8	9 (100%)	0	0	8 (89%)	1 (11%)
	Early 9	14 (100%)	0	0	14 (100%)	0
	Mid 9	7 (100%)	0	0	7 (100%)	0
cuc1 cuc2	8	3 (33%)	3 (33%)	3 (33%)	3 (100%)	0
	Early 9	7 (39%)	7 (39%)	4 (22%)	7 (100%)	0
	Mid 9	6 (55%)	4 (36%)	1 (9%)	3 (50%)	3 (50%)

*Flower stage (Smyth et al., 1990). Stages early 9 and mid 9 correspond to anther stage 5 and 6, respectively, (Sanders et al., 1999).

**Scored only when CMM ridges are formed on the both sides.

each genotype (Aida et al., 1997; Ishida et al., 2000). Wild-type gynoecia in regenerated plants showed essentially the same morphology as those from non-regenerated plants (**Figures 1A,B**) and followed normal developmental stages (**Figure 2**; Smyth et al., 1990). Mature gynoecia of *cuc1 cuc2* were somewhat smaller than those of wild type and tended to lose replum tissues most prominently in the apical region of the ovary (compare **Figures 1A and 1C**; Ishida et al., 2000). In histological sections, the septum and ovules were severely reduced (**Figure 1D**) or completely missing (**Figure 1E**).

Gynoecium primordia of *cuc1 cuc2* were indistinguishable from those of the wild type up to stage 7, at which both wild type and *cuc1 cuc2* formed a cylindrical primordium consisting mostly of densely cytoplasmic cells (**Figures 2A,D**). Deviation of the mutant phenotype began at stage 8 to early stage 9, when the wild type initiated two bulges of CMM from the adaxial wall. In the wild-type, cells in the CMMs remained cytoplasmically dense whereas the rest of the cells started vacuolation, which was a sign of cell differentiation (**Figure 2B**). On the other hand, *cuc1 cuc2* frequently failed to form either one or both of the CMMs and cells in the corresponding regions became vacuolated (**Figure 2E**; **Table 1**). In some mutant gynoecia, CMMs were formed on both sides, but their size was smaller than that of the wild type (**Figure 2F**). In addition, the positioning of CMM initiation was often asymmetric (**Figure 2G**). When the wild type initiated ovule primordia, cells at the contacting surfaces of the two CMMs underwent post-genital fusion to form the septum (**Figure 2C**). On the other hand, a significant fraction of *cuc1 cuc2* gynoecia still failed to initiate CMMs

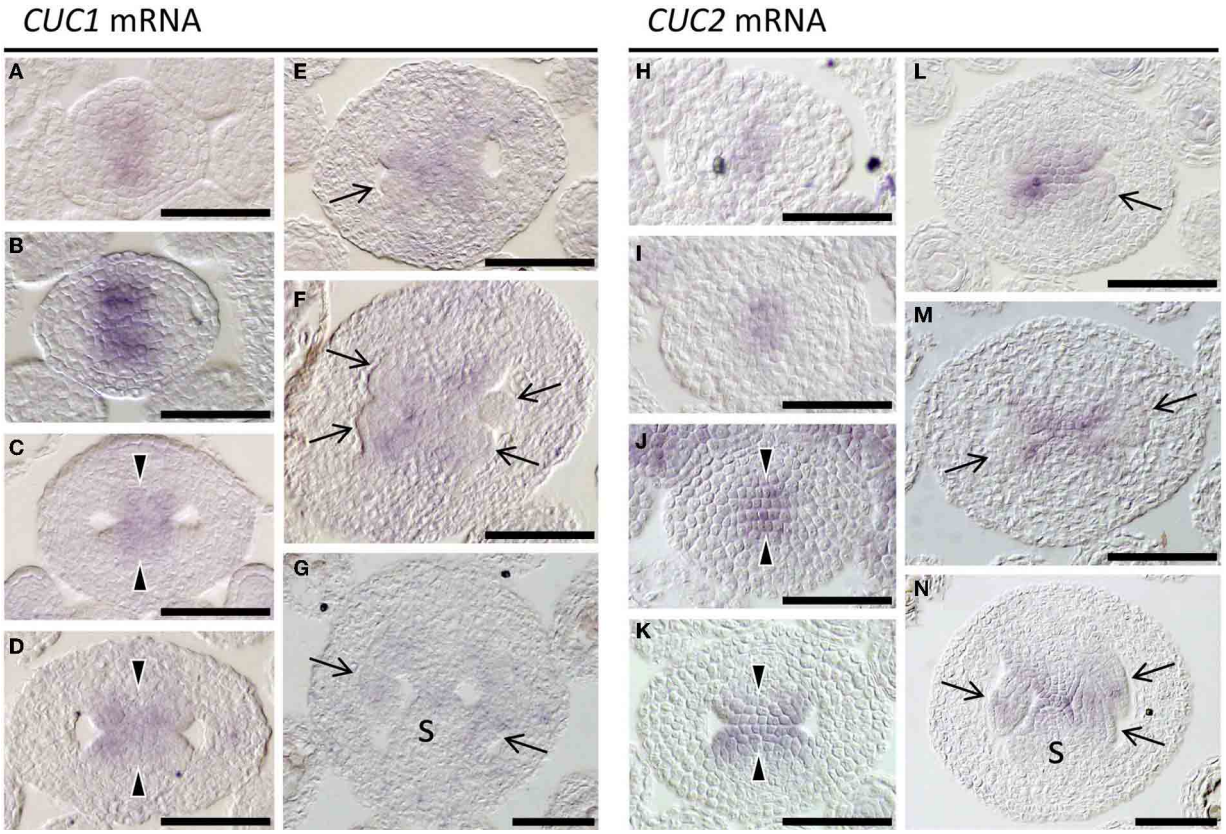

FIGURE 3 | Expression of *CUC1* and *CUC2*. *In situ* hybridization on transverse sections of developing wild type (L*er*) gynoecia regenerated from calli. Probed with *CUC1* **(A–G)** and *CUC2* **(H–N)**. Sections are prepared from gynoecia at stage 6 **(A,H)**, stage 7 **(B,I)**, stage 8 **(C,J)**, early stage 9 **(D,K)**, mid stage 9 **(E,L)**, late stage 9 **(F,M),** and stage 10 **(G,N)**. Arrow, ovule; arrowhead, CMM; s, septum. Scale bars are 50 μm.

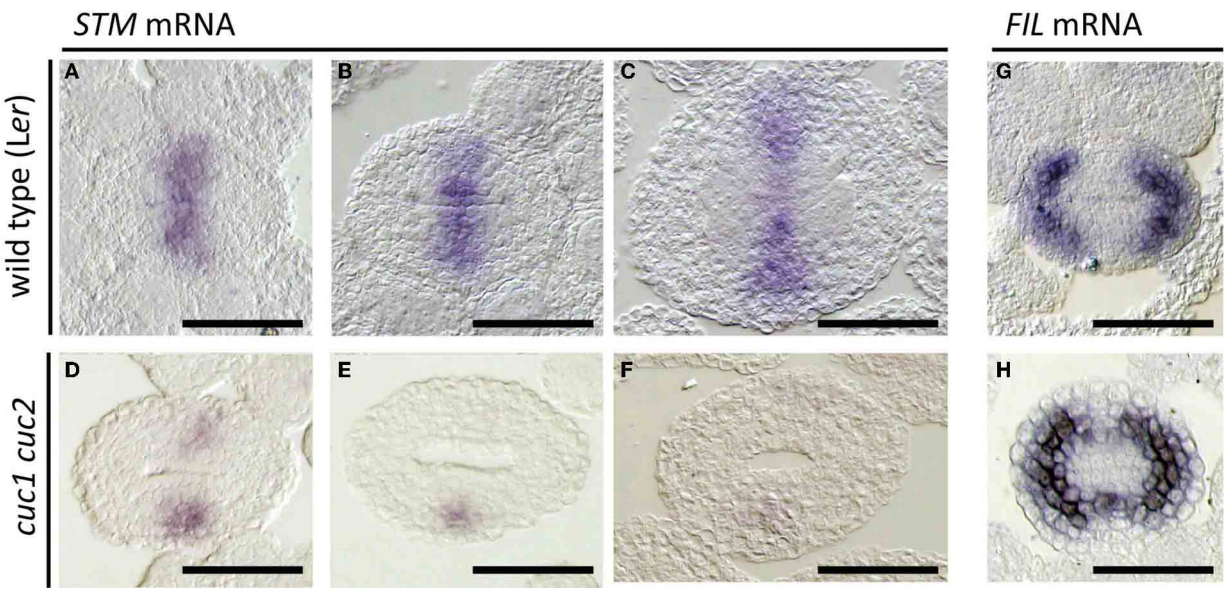

FIGURE 4 | Effect of *cuc1 cuc2* on *STM* and *FIL* expression. *In situ* hybridization on transverse sections of developing wild type L*er* **(A–C,G)** and *cuc1 cuc2* **(D–F,H)** gynoecia regenerated from calli. Probed with *STM* **(A–F)** and *FIL* **(G,H)**. Sections are prepared from gynoecia at stage 8 **(A,D,G,H)**, early stage 9 **(B,E)** and mid stage 9 **(C,F)**. Scale bars are 50 μm.

(**Figure 2H**; **Table 1**). Some mutant gynoecia developed small bumps at the corresponding positions, but their surfaces often failed to contact (**Figure 2I**; **Table 1**). These bumps were likely to retain organogenic activity as indicated by the presence of densely cytoplasmic cells, and might produce ovule-like primordia from their flanks. Taken together, these results show that *CUC1* and *CUC2* are required for CMM initiation. Occasional CMM formation at asymmetric positions indicates that the *CUC* gene activities are also required for stable positioning of the CMMs.

EXPRESSION OF *CUC1* AND *CUC2* PREDICTS THE SITES OF CMM INITIATION

Expression patterns of *CUC1* and *CUC2* during gynoecium development have been reported only partially (Ishida et al., 2000; Takada et al., 2001; Nahar et al., 2012; Galbiati et al., 2013). We therefore carried out detailed expression analysis. Upon initiation of the gynoecial primordium, their expression was detected at its apical center, where the cleft of the future gynoecium cavity will form (**Figures 3A,H**). When the primordium became cylindrical, *CUC1* and *CUC2* expression was detected in the adaxial region of the medial wall, from which the CMM develops (**Figures 3B,I**). The area of *CUC1* expression domain was broader than that of *CUC2*. When the CMMs began to form, expression of both genes was detected throughout the bulge (**Figures 3C,D,J,K**). Their expression was missing in the developing ovule primordia but present in the remaining part of the CMMs (**Figures 3E,L**). Later, transcripts of *CUC1* and *CUC2* were both detected at the base of ovule primordia, the fused region of the septum, and in ovules (**Figures 3F,G,M,N**). These results are consistent with the role for *CUC1* and *CUC2* in CMM formation. Expression patterns from stage 7 to stage 10 are summarized in Supplementary Figure 1 and Supplementary Table 1.

CUC1 AND *CUC2* ARE REQUIRED FOR STM EXPRESSION AND PREVENT DIFFERENTIATION OF CMM CELLS

The class I KNOX gene *STM* plays a critical role in maintaining shoot meristem activity and is also required for proper gynoecium development. As reported previously, *STM* expression was detected along the carpel margins of early gynoecia (**Figure 4A**; Long et al., 1996) and continued in the CMM while it was missing in ovule primordia (**Figures 4B,C**). In *cuc1 cuc2*, by contrast, *STM* expression was greatly reduced (**Figures 4D–F**). Notably, its expression tended to be absent on the adaxial side of the carpel margins while it remained on the abaxial side. These results show that *CUC1* and *CUC2* are required for *STM* expression in the CMM.

Expression of the *FIL* gene is detected in the future valve region while it is excluded from the carpel margins (Dinneny et al., 2005; **Figure 4G**). Together with its close homolog *YAB3*, it is required for valve development. In *cuc1 cuc2*, *FIL* expression extended toward the carpel margins and formed a continuous ring (**Figure 4H**). These results are consistent with the reduction of carpel margin structures in *cuc1 cuc2* and indicate that *CUC1* and *CUC2* prevent valve differentiation at the carpel margins.

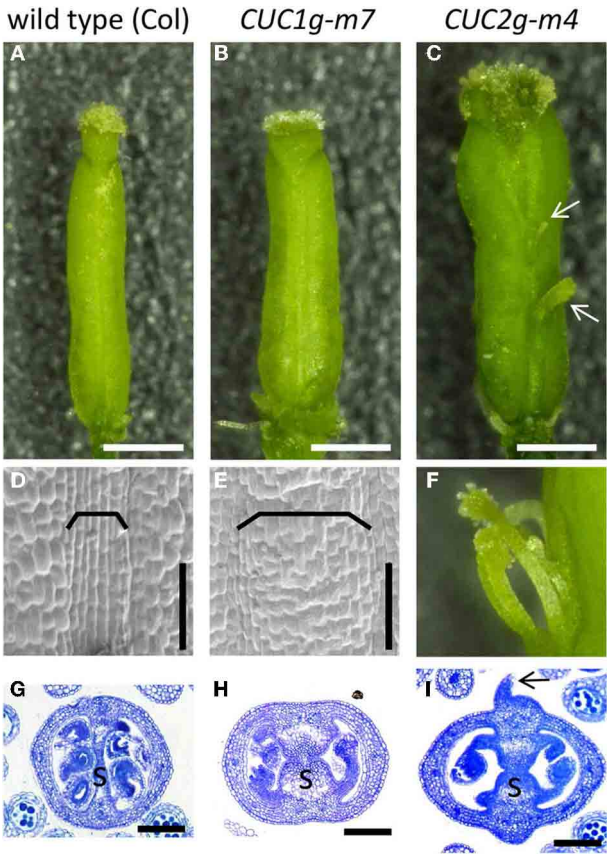

FIGURE 5 | Gynoecium phenotype of *CUC1g-m7* and *CUC2g-m4*. Mature gynoecia of wild type Col **(A,D,G)**, *CUC1g-m7* **(B,E,H),** and *CUC2g-m4* **(C,F,I)**. Whole view **(A–C)**, scanning electron micrograph of replum **(D,E)**, close up view of filamentous structures **(F)** and transverse sections of ovary **(G–I)**. Arrows in **(C,I)** indicate filamentous structures. Brackets indicate repla. s, septum. Scale bars are: **(A–C)**, 500 μm; **(D,E,G–I)**, 100 μm.

MicroRNA RESISTANT VERSIONS OF *CUC1* AND *CUC2* GENOMIC FRAGMENTS CAUSE EXPANSION OF THE CMMs

We next examined the role of microRNA-dependent regulation of *CUC1* and *CUC2* in CMM formation. To this end, we used transgenic plants carrying genomic fragments of *CUC1* or *CUC2* that carry silent mutations in the target sequences of miR164 (*CUC1g-m7* and *CUC2g-m4*, respectively). These plants exhibited expansion of carpel margin structures including the replum (**Figures 5A–F**) and the septum (**Figures 5G–I**). In addition, the abaxial surface of carpel margins in *CUC2g-m4* was swollen and produced filamentous structures (**Figures 5C,F,I**; Nikovics et al., 2006).

Expression of *CUC1* and *CUC2* was examined to access the effect of the silent mutations introduced into the transgenes. In *CUC1g-m7*, *CUC1* mRNA initially accumulated in a broad region around the carpel margins with four peaks of staining (**Figure 6A**), which later dissolved into four discrete spots (**Figure 6B**). In *CUC2g-m4*, *CUC2* mRNA was first detected broadly throughout the carpel margins (**Figure 6C**) and later it

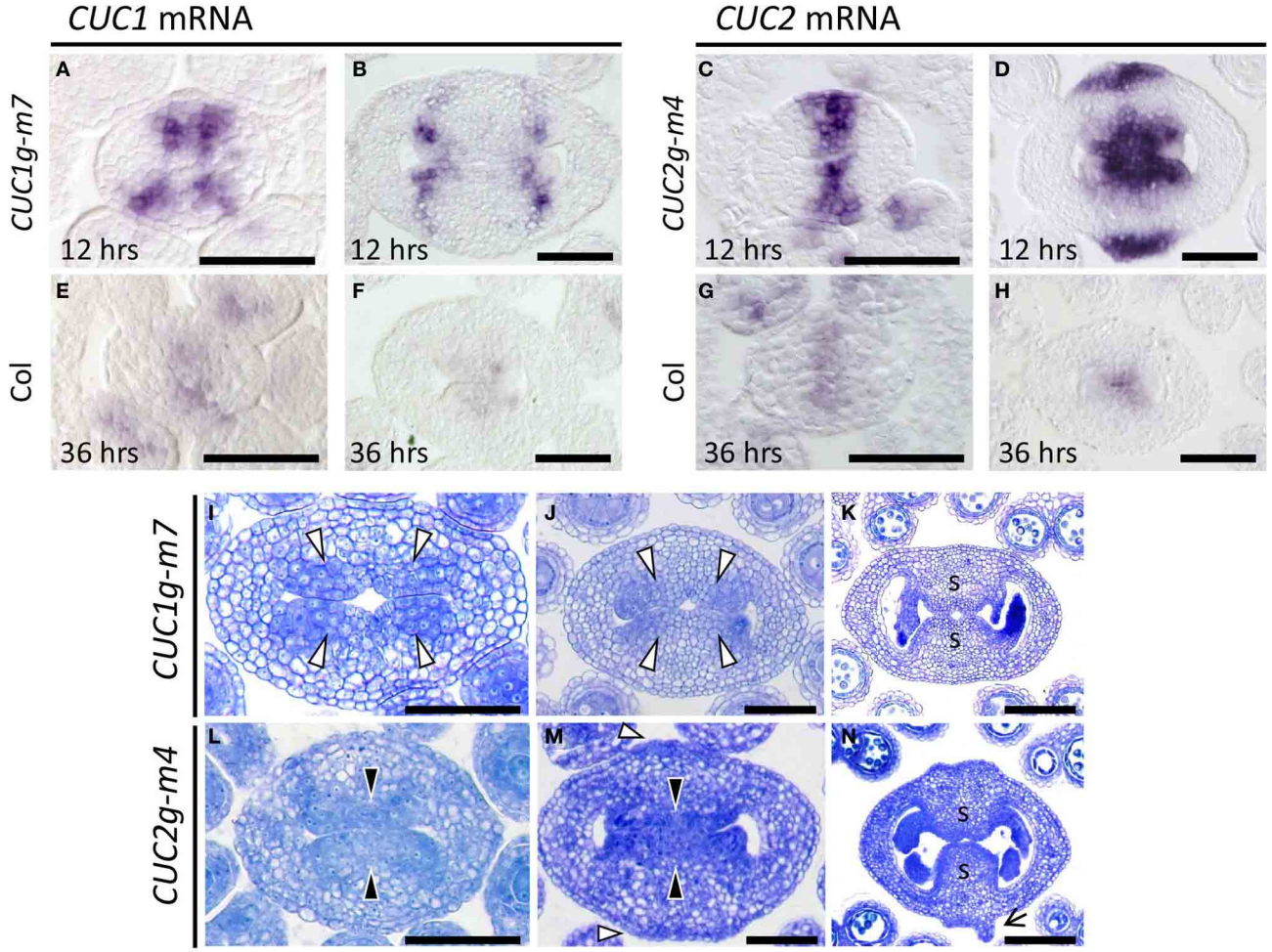

FIGURE 6 | CMM development in *CUC1g-m7* and *CUC2g-m4*. (A–H) *In situ* hybridization on transverse sections from developing gynoecia of *CUC1g-m7* **(A,B)**, *CUC2g-m4* **(C,D)** and wild type Col **(E–H)** probed with *CUC1* **(A,B,E,F)** and *CUC2* **(C,D,G,H)**. Staining reaction was carried out for 12 h in *CUC1g-m7* **(A,B)** and *CUC2g-m4* **(C,D)**, and for 36 h in Col **(E–H)**. Sections are prepared from gynoecia at stage 6 **(A,C,E,G)** and late stage 9 **(B,D,F,H)**. **(I–N)** Transverse sections from developing gynoecia of *CUC1g-m7* **(I–K)** and *CUC2g-m4* **(L–N)** at early stage 9 **(I,L)**, late stage 9 **(J,M)**, and stage 11 **(K,N)**. Black arrowheads indicate CMMs at normal positions and white arrowheads indicate those at abnormal positions. Arrow in **(N)** indicates a primordium of a filamentous structure. s, septum. Scale bars are 50 μm.

split into adaxial and abaxial ends (**Figure 6D**). In both transgenic plants, stronger signals were observed in shorter staining time than in wild type (12 vs. 36 h), indicating that the levels of *CUC1* and *CUC2* mRNA was significantly elevated (compare **Figures 6A–D** with **6E–H**).

We next examined early gynoecium development in these transgenic plants. In *CUC1g-m7*, CMMs were duplicated and initiated at four positions (**Figure 6I**, white arrowheads) that corresponded to the peaks of *CUC1* mRNA accumulation (**Figure 6A**). Each pair of the duplicated CMMs grew adaxially as a congenitally fused tissue and contacted each other at the center to undergo post-genital fusion, forming a thicker septum than that of the wild type (**Figures 6J,K**). The boundary of the fused CMMs was slightly depressed, forming a small central space after the fusion (**Figure 6K**). In *CUC2g-m4*, CMMs developed on the adaxial side and were broader compared to the wild type (**Figure 6L**). In addition, *CUC2g-m4* produced ectopic meristematic tissues as indicated by densely cytoplasmic cells on the abaxial side (**Figure 6M**, white arrowheads). These meristematic tissues further expanded and initiated primordia of filamentous structures on their flanks (**Figure 6N**, arrow). We interpret these meristematic tissues as ectopic CMMs, although they lack ability to form ovules. Together, the results show that disruption of miR164-mediated regulation of *CUC1* and *CUC2* strongly affected the size, positioning, and number of the CMMs.

In *CUC1g-m7*, expression of *STM* was laterally extended compared to that in wild type (compare **Figures 7A** with **4B**), and four strong staining peaks were found within the expression domain, showing a strong correlation with the pattern of *CUC1* expression in this background (compare **Figures 7C** with **6B**). Expression of *STM* was also broader in *CUC2g-m4* than in wild type and was detected throughout the carpel margins including outermost cells on the abaxial side, in which wild type did not

accumulate *STM* transcripts (compare **Figures 7B** to **4B**). This ectopic expression of *STM* continued in ectopic CMMs on the abaxial side (**Figure 7D**). On the other hand, *FIL* expression was not detected in the carpel margins of *CUC1g-m5* and *CUC2g-m7* as in the wild type (**Figures 7E,F**). These results indicate that elevated and ectopic levels of *CUC* gene expression in *CUC1g-m7* and *CUC2g-m4* cause increased meristematic activity of the CMMs.

DISCUSSION

Our results demonstrate that *CUC1* and *CUC2* play critical roles in formation and positioning of CMMs, which are central tissues for generating internal gynoecial organs. The loss of *CUC1* and *CUC2* activity caused severe reduction and altered positioning of CMMs, indicating that the previously reported defects in ovule and septum formation (Ishida et al., 2000; Galbiati et al., 2013) were due to the failure of forming these meristematic tissues. Moreover, *CUC1g-m7* and *CUC2g-m4* plants resulted in duplication and expansion of CMMs, and the positions of the duplicated CMMs in each transgenic plant are associated with the peaks of *CUC1* or *CUC2* transcripts, respectively. These results indicate that *CUC1* and *CUC2* promote CMM formation and, possively through the interaction with miR164, they are required for correct positioning of the CMMs.

Expression of *STM* was strictly dependent on *CUC1* and *CUC2* activities in the CMMs. This result is consistent with the previously reported function of *STM* in ovule formation (Scofield et al., 2007) and supports that *CUC1* and *CUC2* act upstream of *STM* in CMM formation. It has been suggested that *CUC1* and *CUC2* promote ovule development partly though activating a cytokinin pathway (Galbiati et al., 2013) and *STM* can promote cytokinin biosynthesis genes in seedling apices (Jasinski et al., 2005; Yanai et al., 2005). Our results are thus consistent with the idea that activation of *STM* expression by *CUC1* and *CUC2* promotes cytokinin production, which in turn contributes to ovule formation. Despite significant reduction of *STM* transcripts in *cuc1 cuc2* double mutant gynoecia, they do not show a split carpel phenotype, which has been reported for weak *stm* mutant alleles (Endrizzi et al., 1996) and inducible *STM* RNAi plants (Scofield et al., 2007). This aspect of carpel phenotype may reflect earlier function of *STM*, which is already expressed in the floral meristem before carpel initiation (Long et al., 1996). Alternatively, the remaining *STM* expression on the abaxial side of *cuc1 cuc2* carpel margins (**Figures 4D–F**) may be sufficient to prevent split of carpels.

In contrast to *STM*, the area of *FIL* expressing cells was greatly reduced in *cuc1 cuc2*, indicating that *CUC1* and *CUC2* negatively affect *FIL* expression. This result fits to the model in which factors responsible for carpel margin formation and those responsible for valve/valve margin formation counteract each other, as has been proposed based on interactions among *RPL*, *BP*, *FIL*, *JAG*, and *AS1/2* genes (Alonso-Cantabrana et al., 2007; Gonzalez-Reig et al., 2012). Because the STM protein has shown to physically interact with the carpel margin factor RPL (Byrne et al., 2003; Smith and Hake, 2003), it would be possible that the activation of *STM* expression by the *CUC* gene increases the amount of the STM/RPL complex in CMMs, thereby antagonizing the valve/valve margin factors including *FIL*.

Our results show that activation of the class I KNOX gene *STM* by *CUC1* and *CUC2*, a critical regulatory step during embryonic shoot meristem formation (Aida et al., 1999; Takada et al., 2001; Hibara et al., 2003), also occurs during CMM formation. The same regulatory relationship is also found in the formation of leaf margin structures (Kawamura et al., 2010) and is conserved among eudicots (Blein et al., 2008). Furthermore, the important roles for auxin and miR164 in regulating expression of *CUC* genes are also conserved among the processes of shoot meristem, leaf margin and carpel margin formation (Aida et al., 2002; Furutani et al., 2004; Nikovics et al., 2006; Larue et al., 2009; Koyama et al., 2010; Bilsborough et al., 2011; Galbiati et al., 2013). Further investigation on how these common regulatory factors are integrated into each developmental context and their possible relation with context-specific regulatory factors such as floral homeotic genes will be important to understand how unique shapes of different organs are formed.

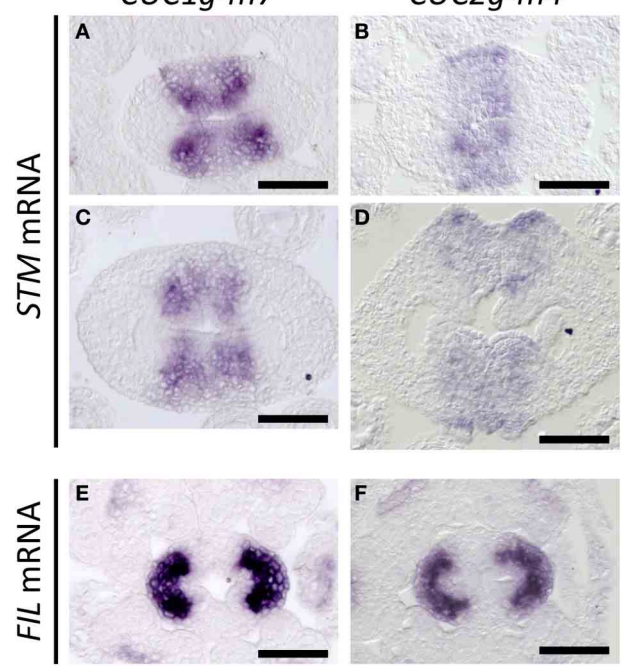

FIGURE 7 | *STM* and *FIL* expression in *CUC1g-m7* and *CUC2g-m4*. *In situ* hybridization on transverse sections from developing gynoecia of *CUC1g-m7* **(A,C,E)** and *CUC2g-m4* **(B,D,F)** probed with *STM* **(A–D)** and with *FIL* **(E,F)** at early stage 9 **(A,B)**, late stage 9 **(C,D)** and stage 8 **(E,F)**. Scale bars are 50 μm.

AUTHOR CONTRIBUTORS

Yuri Kamiuchi, Masao Tasaka, and Mitsuhiro Aida designed the research. Yuri Kamiuchi, Kayo Yamamoto, Masahiko Furutani, and Mitsuhiro Aida performed the research. Yuri Kamiuchi and Mitsuhiro Aida analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00165/abstract

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Novel functional roles for *PERIANTHIA* and *SEUSS* during floral organ identity specification, floral meristem termination, and gynoecial development

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The gynoecium is the female reproductive structure of angiosperm flowers. In *Arabidopsis thaliana* the gynoecium is composed of two carpels that are fused into a tube-like structure. As the gynoecial primordium arises from the floral meristem, a specialized meristematic structure, the carpel margin meristem (CMM), develops from portions of the medial gynoecial domain. The CMM is critical for reproductive competence because it gives rise to the ovules, the precursors of the seeds. Here we report a functional role for the transcription factor *PERIANTHIA* (*PAN*) in the development of the gynoecial medial domain and the formation of ovule primordia. This function of *PAN* is revealed in *pan aintegumenta (ant)* as well as *seuss (seu) pan* double mutants that form reduced numbers of ovules. Previously, *PAN* was identified as a regulator of perianth organ number and as a direct activator of *AGAMOUS (AG)* expression in floral whorl four. However, the *seu pan* double mutants display enhanced ectopic *AG* expression in developing sepals and the partial transformation of sepals to petals indicating a novel role for *PAN* in the repression of *AG* in floral whorl one. These results indicate that *PAN* functions as an activator or repressor of *AG* expression in a whorl-specific fashion. The *seu pan* double mutants also display enhanced floral indeterminacy, resulting in the formation of "fifth whorl" structures and disruption of *WUSCHEL* (*WUS)* expression patterns revealing a novel role for *SEU* in floral meristem termination.

Keywords: ovule, gynoecium, flowers, agamous, wuschel, organ identity, indeterminate growth

INTRODUCTION

In *Arabidopsis thaliana*, as with most angiosperms, reproductive competence depends on the proper development of the flower. Arabidopsis flowers develop from floral meristems, specialized structures that contain organized groups of undifferentiated cells that give rise to the four types of floral organs: sepals, petals, stamens, and carpels (Vaughn, 1955; Hill and Lord, 1988; Bowman et al., 1989). These four organ types develop in concentrically-organized circular fields, termed whorls. Each organ type is produced in a separate whorl of the flower: four sepals in the exterior-most whorl (whorl 1); four petals (whorl 2); six stamens (whorl 3); and finally two carpels in the inner-most whorl (whorl 4).

The proper development of the complete complement of 16 floral organs requires a balance within the floral meristem between the generation of floral organ primordia and the maintenance or renewal of undifferentiated stem cells (Sablowski, 2007). After the initiation of the two carpels in the innermost whorl, the floral meristem terminates. Thus, the ability of the floral meristem to produce cells that will become floral organs is temporally-limited and is genetically-predetermined. This type of developmental floral program is termed a determinate program and generates a fixed number of floral organs. A failure to terminate the floral meristem can result in indeterminacy, or the formation of additional (supernumerary) organs in the central-most positions of the flower.

AGAMOUS SPECIFIES STAMEN AND CARPEL IDENTITY AND BRINGS ABOUT TERMINATION OF THE FLORAL MERISTEM

AGAMOUS (AG) encodes a MADS-domain containing protein that plays at least two key roles during floral genesis: participation in regulatory complexes that specify the identity of stamens and carpels; and bringing about termination of the floral meristem and thus generating a determinant floral structure (Yanofsky et al., 1990; Bowman et al., 1991, 2012; Coen and Meyerowitz, 1991; Drews et al., 1991; Meyerowitz et al., 1991). Loss of *AG* activity results in indeterminate flowers within which additional whorls of floral organs are generated from perdurant meristematic cells.

AG is predominately expressed in the inner-most two whorls of the flower (whorls 3 and 4) in the cells that will give rise to the stamens and carpels (Yanofsky et al., 1990). At least three genes have been shown to have a role in the activation of *AG* transcription within the flower: *LEAFY* (*LFY*) (Weigel and Meyerowitz, 1993); *WUSCHEL* (*WUS*) (Lenhard et al., 2001) and *PERIANTHIA* (*PAN*) (Das et al., 2009; Maier et al., 2009). All three directly bind to cis-regulatory elements located in the *AG* second intron (Busch et al., 1999; Lohmann et al., 2001; Das et al., 2009; Maier

et al., 2009). *pan* single mutant plants exhibited an incompletely penetrant floral meristem indeterminacy phenotype (Das et al., 2009) and the penetrance and severity of *pan* phenotypes are modified by environmental growth conditions, particularly day length (Maier et al., 2009). Additionally *pan* mutant phenotypes are enhanced by hypomorphic *lfy* alleles, indicating a functional similarity between *LFY* and *PAN* during the activation of *AG* (Das et al., 2009).

REPRESSION OF *AG* IN WHORLS ONE AND TWO

A number of genes have been shown to play a role in the repression of *AG* within floral whorls one and two (for review see Liu and Mara, 2010). One of these genes, *SEUSS (SEU),* encodes a transcriptional adaptor protein, that physically interacts with several MADS domain proteins including APETALA1 (AP1), SEPALLATA3 (SEP3), AGAMOUS-LIKE24 (AGL24) and SHORT VEGETATIVE PHASE (SVP) (Sridhar et al., 2004; Gregis et al., 2006; Sridhar et al., 2006; Gregis et al., 2009). As a transcriptional adaptor, SEU is not thought to bind DNA directly but rather is recruited to cis-regulatory elements located within the *AG* second intron through interactions with these MADS domain containing DNA transcriptional regulators (Liu and Meyerowitz, 1995; Franks et al., 2002; Gregis et al., 2006; Sridhar et al., 2006). SEU functions as a bridging protein that recruits the transcriptional repressor LEUNIG (LUG) to the complex and brings about transcriptional repression of *AG* in whorls one and two (Sridhar et al., 2004, 2006). *seu* mutants display weak homeotic transformations of perianth organs caused by ectopic expression of *AG* in the perianth, as well as a variety of additional pleiotropic phenotypes (Franks et al., 2002). *SEU* is widely expressed within the developing plant and likely functions in many developmental events.

SEU AND *ANT* FUNCTION DURING THE DEVELOPMENT OF THE GYNOECIAL MEDIAL DOMAIN

In *Arabidopsis thaliana* the female reproductive floral structure is the gynoecium, a composite structure formed from the congenital fusion of two carpel organs into a tube-like structure (Bowman et al., 1999). A specialized meristematic tissue termed the carpel margin meristem (CMM) develops within the medial portions of the gynoecial tube and gives rise to ovules (Bowman et al., 1999; Liu et al., 2000; Azhakanandam et al., 2008). The ovules are the immature, prefertilized precursors of seeds. Many groups have contributed to the understanding of the molecular mechanisms that support the specification and development of the medial gynoecial domain and the subsequent initiation of ovules (reviewed in Reyes-Olalde et al., 2013), but our mechanistic understanding of this important developmental process is incomplete.

SEU, in addition to its function in the specification of floral organ identity through the repression of *AG,* functions to promote ovule formation in the CMM (Azhakanandam et al., 2008). *SEU* works in a partially redundant manner with *AINTEGUMENTA* (*ANT*), another transcription factor, to regulate the expression of downstream genes critical for the formation of ovules (Azhakanandam et al., 2008; Wynn et al., 2011). In contrast to *SEU* which does not have a DNA binding domain (Sridhar et al., 2006), *ANT* encodes an *AP2*-like transcription factor containing a sequence-specific DNA binding domain. *ANT* activity supports the establishment of proper organ size in lateral organs by controlling the period of developmental time during which cells of the organ primordia are competent to grow and divide (Elliott et al., 1996; Klucher et al., 1996; Mizukami and Fischer, 2000; Nole-Wilson and Krizek, 2000; Krizek and Eaddy, 2012). The loss of either *SEU* or *ANT* activity, individually, results in a reduction of ovule number, however, the combined loss of *SEU* and *ANT* activity results in the complete loss of ovule formation (Azhakanandam et al., 2008).

Although both *SEU* and *ANT* function in *AG* repression, it is unlikely that the alteration of CMM development in the *seu ant* double mutant is due to the de-repression of *AG* expression (Azhakanandam et al., 2008). Rather additional gene regulatory alterations in the *seu ant* double mutants are likely to engender the altered development of the medial domain. Published transcriptomics experiments have identified genes that are misregulated in the *seu ant* gynoecia relative to the single mutant parents (Wynn et al., 2011). Many of these genes are expressed within the developing medial gynoecial domain and thus are likely candidates for regulators of medial domain development. *PAN* encodes one such candidate. *PAN* is a member of the bZIP transcription factor super-family of proteins (Hurst, 1995; Chuang et al., 1999). *pan* mutants display alterations in the spacing, position, and number of perianth organs formed, but do not condition a severe gynoecial phenotype (Running and Meyerowitz, 1996; Meyerowitz, 1997; Roe et al., 1997; Chuang et al., 1999; Maier et al., 2009, 2011; Wynn et al., 2011). As *PAN* is expressed strongly in the developing gynoecial medial domain, placenta, and ovules is it possible that *PAN* plays a functional role during gynoecial development that is not observed in the *pan* single mutant due to functional redundancy.

In order to better assay the functional role of *PAN* during gynoecial development, we have generated *seu pan* and *pan ant* double mutant plants and examined floral development with a focus on gynoecial development and ovule formation. Our analyses of *seu pan* and *pan ant* double mutants indeed support the tenet that *PAN* plays a functional role during gynoecial and ovule development that can be revealed when either the activity of *SEU* or of *ANT* is compromised. We also report that *SEU* plays a previously unanticipated role in floral meristem termination. This is revealed by altered patterns of *WUS* expression and the strong enhancement of the *PAN* indeterminacy phenotype in the *seu pan* double mutants, particularly under short-day conditions. Additionally, our data suggests that *PAN* can act as a repressor of *AG* within sepals, in contrast to previous work indicating a role for *PAN* in the activation of *AG* in whorl 4. Our data suggest that both *PAN* and *SEU* have whorl-specific functions during the regulation of *AG* that are critical for generating the Arabidopsis flower. Furthermore, the role of *PAN* and *SEU* during both floral meristem termination and CMM development suggest a possible link between these developmental events.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Plants were grown under long-day conditions of 16 h of light or under short-day conditions of 8 h of light. Temperature in the growth chambers were kept between 22 and 26°C, however the temperature experienced by the plants is lower when the lights are off, thus short-day grown plants may be grown at a slightly lower average temperature than long-day grown plants. The *ant-1* and *seu-3* alleles were previously characterized (Klucher et al., 1996; Pfluger and Zambryski, 2004). The *pan* alleles used are SALK_031380, SAIL_247, and SALK_057190 with T-DNA insertions in the 5′UTR, 7th intron and the 3rd intron, respectively, (McElver et al., 2001; Sessions et al., 2002; Alonso et al., 2003). PCR was used to confirm genotypes (**Table 1**). The *pan 057190* (SALK_057190) allele was previously characterized as a RNA null allele via *in situ* hybridization to inflorescence and floral tissues (Maier et al., 2009, 2011). Plants for rosette leaf counts were grown under short-day conditions until after bolting. Rosette leaves were removed, with care to only count rosette leaves and not axillary, cauline, or cotyledon leaves.

IN SITU HYBRIDIZATION

The protocol for *in situ* hybridization was described previously (Wynn et al., 2011). A more detailed protocol is located at http://www4.ncsu.edu/∼rgfranks/research/protocols.html. The *AG* antisense probe was *in vitro* transcribed using the T7 promoter from the pCIT565 plasmid linearized with HindIII. The *AG* antisense probe generated is complementary to the *AG* cDNA sequence from +140 (relative to the ATG) through the 3′ end of cDNA. The antisense *WUS* probe is complementary to the entire *WUS* cDNA clone and is derived from BamHI cut pMHwus16 plasmid, a gift of Jenn Fletcher. The *AG* sense strand control probe was generated from a linearized pCIT565 plasmid (cut with XhoI) using the Sp6 promoter and contains the full length cDNA sequences. To generate the *PAN* antisense probe, the plasmid G50929 (ABRC) a full-length sequence-confirmed ORF/cDNA clone (Yamada et al., 2003) was cut with SalI to linearize and then the antisense probe was generated using the T7 polymerase. Due to the position of the SalI site, the *PAN* antisense probe generated is complementary to sequences from +746 through +1353 relative to the ATG start codon in the *PAN* cDNA.

TISSUE FIXING AND CLEARING

Tissue was fixed in 9 parts ethanol:1 part acetic acid for 2 h, then washed in 90% ethanol twice. Gynoecia were hand-dissected in ethanol and then moved into Hoyer's solution (70% ethanol, 5% gum arabic, 4% glycerol) for clearing and mounting on slides for visualization. Slides were examined with an Axioscop2 microscope (Zeiss) with Nomarski optics. Ovule counts were made from stages 11–14 gynoecia fixed on slides. Analysis of carpel bending and splitting was done under dissecting scope. Gynoecia were rated from 1 to 4 independently for bending as well as splitting. A severity score was given based on the following scoring system: 1, no defect; 2, mild defect; 3, moderate defect; 4, severe defect. All gynoecia were scored by the same individual, at the same time without knowledge of the genotype. All photos were captured with Q Capture software on a 5.0 RTV digital camera (Q Imaging, Surrey, BC, Canada). Data analysis was conducted in JMP Pro 10 (SAS Institute Incorporated, Cary, NC, USA) using multiple pair-wise comparison of the means with a Tukey-Kramer HSD test at an alpha of 0.05 or with a Student's *T*-test.

RESULTS

pan MUTANT ALLELES CONDITION ENHANCED REDUCTION OF OVULE NUMBER IN *seu* AND *ant* MUTANT BACKGROUNDS

In order to assay gynoecial development in *pan* mutant plants we characterized three available *pan* alleles (See Materials and Methods, **Table 1**) (McElver et al., 2001; Sessions et al., 2002; Alonso et al., 2003). Under our long-day growth conditions Col-0 plants averaged 46.9 ± 5.9 ovules per gynoecium. As has been previously reported, *ant* single mutants displayed significantly fewer (35 ± 8.7) ovules per gynoecium (**Figure 1A**) (Elliott et al., 1996; Klucher et al., 1996; Azhakanandam et al., 2008). Previously published characterizations of *pan* mutants did not report a reduction in ovule formation. We counted ovule primordia in the gynoecia of three different *pan* mutant alleles (**Figure 1A**). Although we detected slight reductions in ovule number in two out of three of the *pan* alleles we tested, these differences were not statistically different from Col-0. However, all three of these *pan* mutant alleles conditioned an enhancement of ovule loss in the *ant* mutant background (**Figure 1A**). Thus, in the *ant* mutant background *PAN* appears to provide an activity that supports ovule formation.

In a second set of experiments, we examined the function of *PAN* in the *seu* mutant background by assaying *seu pan* double

Table 1 | Genotyping primers.

Allele name	Genotyping Oligos FW (5′–3′)	Genotyping Oligo RV (5′–3′)	Genotyping Oligo internal	Size of Fragments (in base pairs)
ant-1	TTCCCTCAAACCAGAAACCA	GGGCTCATGGATAAGCTCAG	N/A	Wt: 131 bp Mutant: 109
seu-3	GAATTTGCTGCGGTTCCAACT	GAAAATGTTCCGCCTTCGAT	Restrict with *BslI*	Wt: 235 and 345 Mutant: 580
pan 031380 (in 5′ UTR)	CGGTAACACACATGACACATATG	ATGGTGAAAACCATTGACTGG	LbB1	Wt: 1228 Mutant: 550
pan SAIL_247 (in 7th intron)	TTGCCTCAATAAATCAGCCTG	GAATTCTTGGCAGACACTTCG	pCSA110LB2	Wt: 1138 Mutant: 500
pan 057190 (in 3rd intron)	ACATCAACACGGCCAAGTAAC	TCTCTCCTCACTCCCTCCTTC	LbB1	Wt: 1219 Mutant: 650

The oligonucleotides and size of the PCR fragments from genotyping for each of the alleles we utilized in this paper.

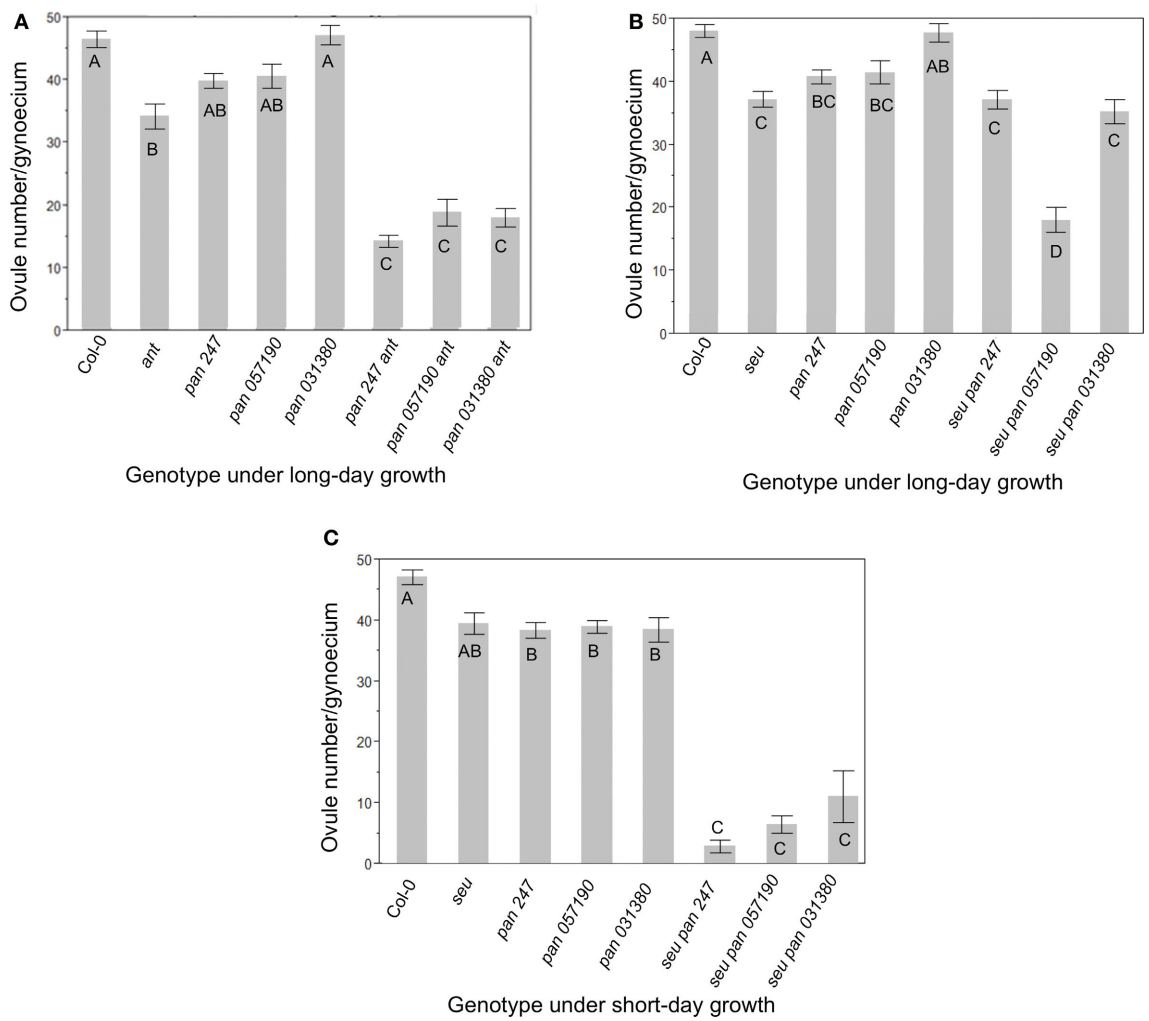

FIGURE 1 | Ovule number is decreased in *pan ant* and *seu pan* double mutants. (A) Under long-day growth conditions *pan ant* double mutants displayed an enhanced loss of ovules relative to single mutant parents for all three *pan* alleles examined. **(B)** Under long-day growth conditions only the *seu pan 057190* plants showed a statistical reduction in ovule number compared to single mutants parents. **(C)** Under short-day growth conditions, the *seu pan* double mutants displayed an enhanced loss of ovules relative to single mutant parents for all three *pan* alleles examined. Comparisons for statistical differences across genotypes were made via pair-wise mean testing and the Tukey HSD *post-hoc* test—different letters indicate statistically different categories. Each error bar is constructed using 1 standard error from the mean.

mutants and the single mutant parents (**Figure 1B**). We again detected a slight reduction in ovule number in the *pan* single mutants relative to wild type, however this time the reduction was statistically significant in both the *pan 057190* and the *pan SAIL_247* alleles. The *seu* single mutant also conditioned a significant loss of ovules relative to Col-0. Furthermore, ovule loss in the *seu pan* double mutant was significantly enhanced by one of the three *pan* alleles (*057190*) that we tested under our long-day growth conditions (**Figure 1B**).

Short-day growing conditions have been previously shown to enhance the severity of mutant phenotypes of *pan* mutants (Maier et al., 2009, 2011). Under short-day conditions all three *pan* alleles displayed a statistically-significant reduction in ovule number relative to Col-0 grown under similar conditions (**Figure 1C**). Furthermore, all three alleles of the *seu pan* double mutant gynoecia exhibited an enhanced loss of ovules relative to either the *pan* or the *seu* single mutants. Thus, under the short-day growing conditions *pan* single mutants displayed a modest but significant reduction in ovule number relative to wild type, while ovule loss was enhanced in *seu pan* double mutants relative to the single mutants.

pan MUTANT ALLELES CONDITION ENHANCED DISRUPTIONS OF GYNOECIAL MORPHOLOGY IN *seu* AND *ant* MUTANT BACKGROUNDS

The wild type Arabidopsis gynoecium is composed of two carpels that are fused along the carpel margins. The fused margins of the carpels are situated within the medial portion of the gynoecium. The growth of the medial gynoecial domain is reduced in the *seu ant* mutant resulting in gynoecial splitting and a loss of ovule formation (Azhakanandam et al., 2008). Thus, we

examined the *pan* single and *seu pan* and *pan ant* double mutant gynoecia for gynoecial splitting and other gross morphology disruptions. Alterations to the overall gynoecial morphology were evident under both long- and short-day conditions, but they were more pronounced in plants grown under short-day conditions (**Figure 2**).

Under long-day growth conditions, we evaluated gynoecia for both carpel splitting and carpel bending phenotypes employing a severity index from 1 to 4 (See Materials and Methods). We then used this severity index to generate a mean severity score for the comparison of genotypes of interest. Carpel bending and carpel splitting phenotypes were not observed in the Col-0 gynoecia that we assayed. Under the long-day growth conditions, the *ant* single and *pan* single mutants did display a mild degree of carpel splitting (**Figure 3A**). However, the *pan ant* double mutants displayed a statistically significant enhancement of carpel splitting compared to the single mutant gynoecia (**Figures 2C**, **3A**). This is manifested by a greater proportion of double mutant gynoecia for which splitting was characterized as moderate or severe. Although *seu* single mutants displayed a mild degree of carpel splitting (**Figure 2D**), the carpel splitting phenotype was not enhanced in the *seu pan* double mutant under long-day conditions (**Figure 2F**).

Using a similar severity index we evaluated carpel bending. The *seu pan 057190* showed statistically significant enhancement in carpel bending compared to the *pan* single mutant (*seu* single mutants rarely display a bending phenotype under long-day) (**Figure 3B**). The bending phenotype appears to be the result of the gynoecium consisting of only one carpel that fuses to itself (data not shown). These data suggest that *PAN* and *SEU* play a role in both promotion of medial domain development and in the proper formation of two carpels in whorl four.

Under short-day growing conditions the severity and penetrance of *seu pan* gynoecial defects were enhanced relative to the long-day conditions (**Figures 2G–O**). (We did not examine the development of the *pan ant* double mutants under the short-day growing conditions.) To analyze the *seu pan* phenotypes under short-day conditions we scored the gynoecia for the occurrence of four phenotypes; complete loss of ovules, carpel bending, severe loss of valves (based on external cell-type morphology), and indeterminate growth from internal gynoecial positions. We did not observe any of these phenotypes in the Col-0 plants and they were found infrequently (5%) in *seu* or *pan* single mutants (**Table 2**). The *seu pan* double mutants, however, frequently displayed severe ovule loss, loss of the external valve tissue morphology, carpel bending, and/or indeterminacy phenotypes (**Figures 2G–K**; **Table 2**). In the Col-0 and single mutant gynoecia the surface morphology of the abaxial replum (ar) cells is distinct from that of the valve (v) cells (**Figures 2L–M**). Thus, these cell fates can be distinguished by the external cell surface morphology. In a subset of the *seu pan* gynoecia from short-day grown plants, the cells of the valve and abaxial replum could still be distinguished (**Figure 2N**). However, between 33 and 63% (depending on the mutant *pan* allele) of the *seu pan* double mutants displayed a severe alteration in the morphology of the external valve cells (**Figure 2O**). In these gynoecia we could not identify cells with the surface morphology that is indicative of valve cell identity. Instead all of the external gynoecial cells appeared to resemble abaxial replum cells (ar-like in **Figure 2O**).

Other phenotypes observed in the *seu pan* double mutant under short-day growth conditions included five sepals, a reduction in petal size and number (typically two reduced petals per flower) and reduced production of pollen from anthers (data not shown). Additionally, the *seu pan* double mutant plants displayed an enhanced delay in the transition from vegetative to reproductive development as determined by counting the number of rosette leaves formed before bolting (**Figure S1**).

FLORAL MERISTEM INDETERMINACY IS ENHANCED IN THE *seu pan* DOUBLE MUTANTS UNDER SHORT-DAY GROWING CONDITIONS

In the wild type Arabidopsis flower, the floral meristem terminates after the formation of the gynoecium. *AG* is required to promote the termination of the floral meristem (floral determinacy) by repressing the expression of *WUS*, a stem cell maintenance gene within the floral meristem (Laux et al., 1996; Mayer et al., 1998; Parcy et al., 1998; Busch et al., 1999; Lohmann et al., 2001; Lenhard et al., 2002). We examined *seu pan* double mutants to determine the extent of floral indeterminacy under both long-day and short-day conditions. We characterized fifth whorl structures as an over-proliferation of cells at the base of the gynoecium (**Figures 2F–K**). These structures were typically enclosed within the gynoecial tube.

These fifth whorl structures were observed in 15% of *seu pan 057190* and *seu pan 247* double mutants in long-day tissues, but not seen in either single mutant. Under short-day conditions the presence of fifth whorl structures was significantly more frequent in *seu pan* plants compared to both the frequency in the single mutants as well as to the double mutants grown in long-day conditions (**Table 2**). The fifth whorl structures appeared larger and more elaborated under short-day conditions. This data suggests that both *PAN* and *SEU* function to promote floral meristem determinacy and that this phenotype is more penetrant under the short-day growing conditions.

WUS EXPRESSION AND INFLORESCENCE MERISTEM STRUCTURE ARE ALTERED IN *seu pan* DOUBLE MUTANT PLANTS

The fifth whorl structures formed in the *seu pan* double mutants gynoecia were also examined for *WUS* expression as a marker for indeterminacy and a persistent functioning meristem. We occasionally were able to detect ectopic *WUS* expression in the fifth whorl structures (**Figure 4B**) suggesting that ectopic WUS expression may contribute to the formation of fifth whorl structures. However, we found additional examples of fifth whorl structures that did not express *WUS* (data not shown). Thus, although we were able to document cases of perdurant *WUS* expression, our data suggests that this ectopic expression is likely relatively short in duration and that *WUS* expression is not continuously maintained in the fifth whorl structures.

We also detected *WUS* expression in the center of the shoot apical meristem (SAM) and in early stage floral primordia (**Figure 4**). In Col-0, *WUS* is tightly expressed in a small number of cells in both the SAM and floral meristem (Laux et al., 1996; Mayer et al., 1998) (**Figures 4D–H**). In the *seu pan* mutant, the domain of *WUS* expression within both the SAM and the floral

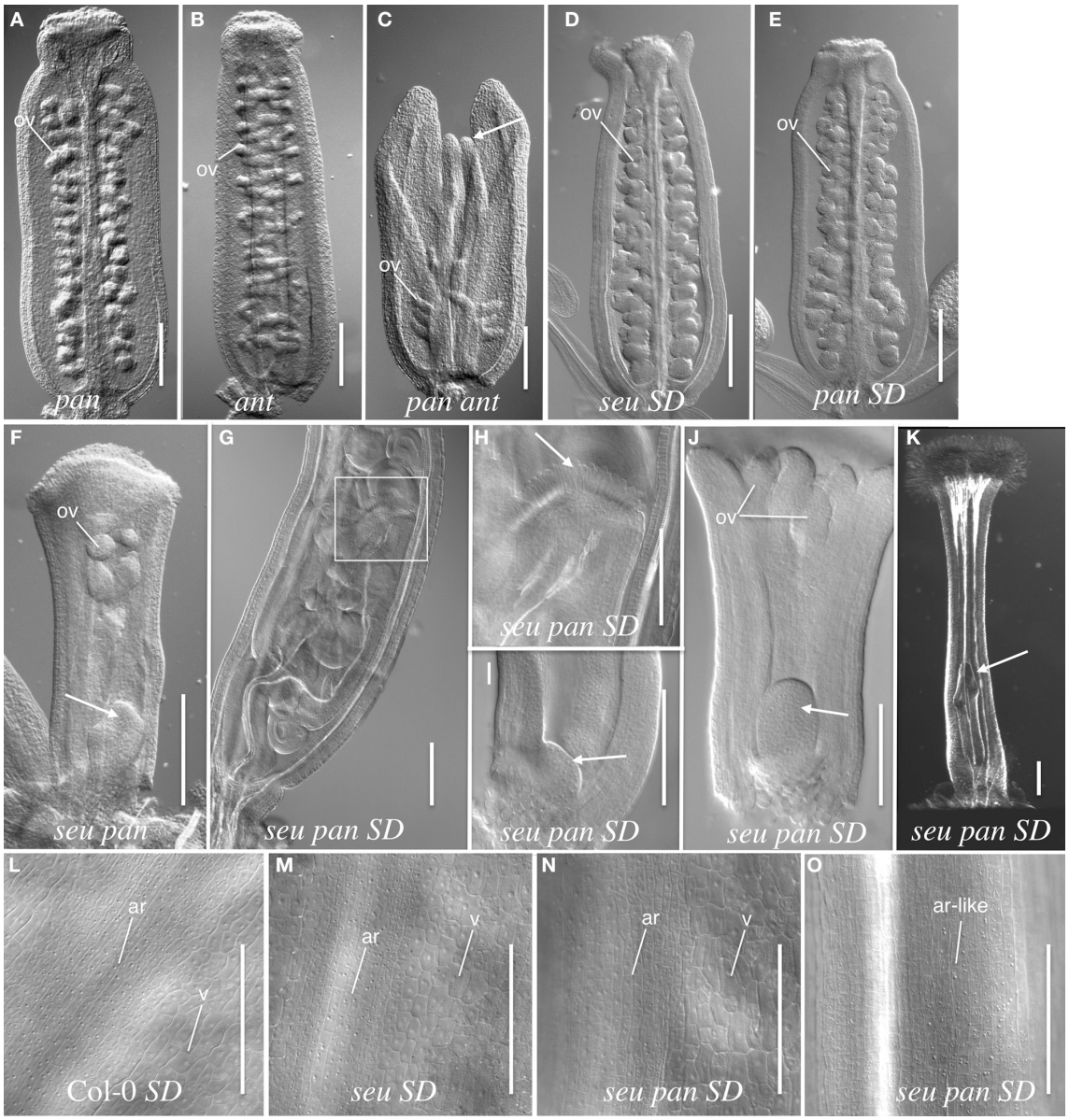

FIGURE 2 | Gynoecial and ovule phenotypes in *pan ant* and *seu pan* double mutants. (A) *pan 057190* single mutant gynoecium. **(B)** *ant* single mutant gynoecium. **(C)** *pan 057190 ant* double mutant gynoecium displays reduced ovule number, a reduction in the growth of the medial domain (arrow) and enhanced splitting of the gynoecium. Arrow in panel **(C)** indicates the apical extent of the medial domain of the carpel. **(D)** *seu* single mutant gynoecium grown under short-day (SD) conditions shows slight splitting of the gynoecial apex. **(E)** SD-grown *pan* single mutant displays near wild type phenotype. **(F)** *seu pan 057190* double mutant (long-day conditions) displays a "fifth whorl" structure inside the gynoecium (arrow). **(G)** SD-grown *seu pan 057190* double mutant displays a well-developed "fifth whorl" structure inside the gynoecium. **(H)** A higher magnification image of the boxed area shown in panel **(G)**. Arrow indicates the stigmatic tissue at the apex of second gynoecium developing within the primary gynoecium. **(I)** SD-grown *seu pan* 057190 double mutant (stage 8) arrow indicates early stage of fifth whorl structure. **(J)** SD-grown *seu pan* 057190 double mutant (stage 9) arrow indicates fifth whorl structure. **(K)** SD-grown *seu pan 057190* double mutant displays a complete loss of ovule primordia, the loss of normal external valve cell surface morphology, and the presence of a "fifth whorl" structure (arrow). **(L–O)** epidermal cell morphology of external (abaxial) surface of the gynoecium. Distinctive cell surface morphology is observed in valve (v) and abaxial replum (ar) regions in Col-0 wild type **(L)**, *seu* single mutant **(M)** and a subset of *seu pan* double mutants **(N)**. However, in severely disrupted *seu pan* double mutants **(O)** the cell surface morphology of valve cells is not observed and all cells display an abaxial replum-like (ar-like) morphology. Scale bars in all panels are 200 microns, except for panels **(H–J)** where scale bars are 100 microns. ov, ovule; SD, Short-day growth conditions; ar, abaxial replum; v, valve.

meristem appeared broader and more diffuse than it was in the wild type samples (**Figures 4I–M**). Although we did not quantify the size of the IFM, or observations of *in situ* sections showed instances where the size of the *seu pan* IFM was enlarged relative to the Col-0 or single mutant parents (compare 4D to 4I). In some of the *seu pan* inflorescence meristems the *WUS* expressing region was expanded and appeared to be punctuated as if several organizing centers had been formed within the potentially compound

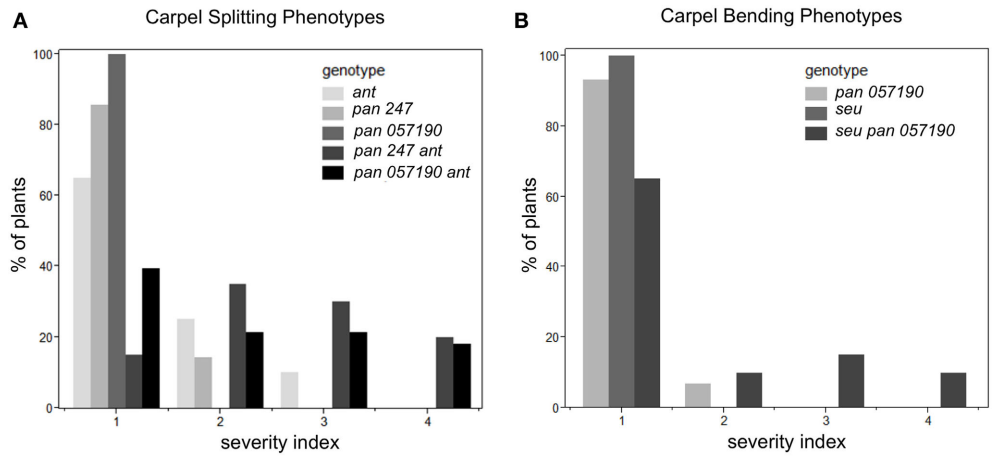

FIGURE 3 | Enhanced severity of carpel splitting in *pan ant* and carpel bending in *seu pan* double mutants under long-day growth conditions. **(A)** The *pan ant* double mutants showed a statistically more severe carpel splitting than the single mutant parents. **(B)** *seu pan* double mutant plants showed statistically more severe carpel bending than the single mutant parents. Severity index for the carpel splitting: 1, no splitting; 2, mild splitting (flared tips or slight split); 3, moderate splitting (highly flared); 4, severe or completely split. Severity index for carpel bending: 1, no bending; 2, slight bending; 3, moderate bending; 4, severe bending. Statistical analysis was conducted with a Student's *T*-test and *p*-values less than 0.05.

Table 2 | Gynoecial disruption in short-day grown plants.

Genotype	Complete loss of ovules (%)	Carpel bending (%)	Loss of external valve tissue morphology (%)	Fifth whorl structure within the carpels (%)	Number of gynoecia counted
Col-0	-	-	-	-	18
Seu	-	5	-	-	20
pan 057190	-	-	-	5	21
pan 247	-	-	-	5	20
seu pan 051790	35	33	33	71	31
seu pan 247	52	63	63	32	19

Categorization of the significant gynoecial disruptions. Note: phenotypes are not mutually exclusive and thus percentages can add up to more than 100%.

inflorescence meristem (**Figure 4C**; arrows). Thus, the disruption of *WUS* expression or accumulation in the IFM and early stage floral meristems as well as an ectopic persistence of expression within the floral meristem may contribute to the morphological disruptions observed in the *seu pan* mutant flowers.

AGAMOUS IS MIS-EXPRESSED IN WHORL ONE OF THE *seu pan* DOUBLE MUTANT FLOWERS

In light of the known role of *AG* in regulating floral determinacy, and *SEU* and *PAN* acting as regulators of *AG* expression, we examined *AG* expression patterns in *seu pan* plants via *in situ* hybridization. A dominant negatively-acting *PAN-RD* transgene, in which a transcriptional repression domain has been fused to the PAN coding sequences, has been shown to condition floral indeterminacy that was correlated with a reduction of *AG* expression within whorl four (Das et al., 2009). We also sought to determine if the levels of *AG* were reduced in whorl four in the *pan seu* double mutants. However, we could not detect a consistent reduction in the levels of *AG* expression in whorl 4 under long-day or short-day growth conditions.

Somewhat unexpectedly, we frequently observed instances of ectopic *AG* expression in *seu pan* double mutant whorl 1 organs (**Figures 5D,F,H**). Upon closer examination of the external cell morphology of floral organs from the *seu pan* flowers, we detected instances of chimeric organs in whorls one including partially petaloid and stamenoid organs (**Figure 6**). These partial homeotic organ transformations have been reported in the *seu* single mutant previously (Franks et al., 2002) but are rarely seen in the *seu-3* allele in the Col-0 background that we have used in this study (Pfluger and Zambryski, 2004). We found the 30% ($N = 43$) of the *seu pan* whorl one floral organs exhibited partial homeotic transformations based on the cell surface morphologies (**Table 3**). These homeotic transformations were not observed in the *seu* and *pan* single mutant parents under our growth conditions. Based on the organ type specific cell surface morphology, the *seu pan* whorl one organs appeared to be sepals that were partially converted to petals (**Figure 6**). The presence of cells with the classic petal cell morphology was most often observed on the adaxial and marginal portions of the sepals (**Figures 6A–C**). These data are consistent with *PAN* acting in a partially redundant

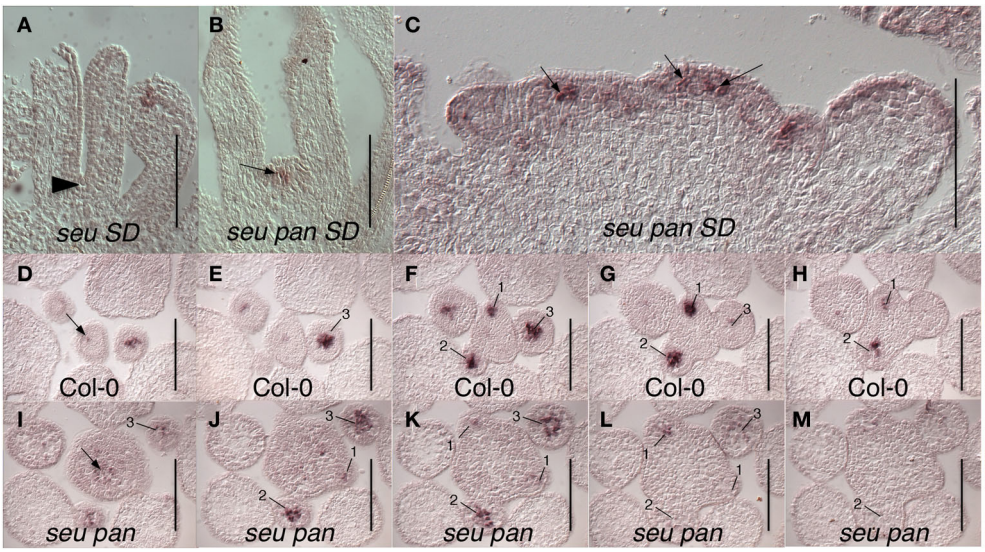

FIGURE 4 | *WUS* expression in *seu pan* double mutants. (A) No *WUS* expression is detected at the base of the stage 8 gynoecium (arrowhead) in this longitudinal section of a Col-0 flower. **(B)** *WUS* expression is detected in fifth whorl structure (arrow) at the base of the stage 8 *seu pan* gynoecium. **(C)** Longitudinal section of a fasciated inflorescence meristem (IFM) from *seu pan* double mutant where *WUS* expression appears in multiple foci (arrows) as well as diffusely throughout IFM. **(D–M)** Serial cross sections through Col-0 **(D–H)** and *seu pan* **(I–M)** inflorescences. Within a given genotype each cross section is 8 microns below the preceding cross section. **(D–H)** WUS expression is detected in the organizing center of the IFM (arrow) and in central zones of stage 1–3 floral meristems (numbered). **(I–M)** In the *seu pan* double mutants, the region of *WUS* expression appears more diffuse and somewhat expanded, both in the INF (arrow) and in the developing floral meristems (numbered). Note also that the IFM is larger in the *seu pan* double mutant. Scale bars are 100 microns in all panels.

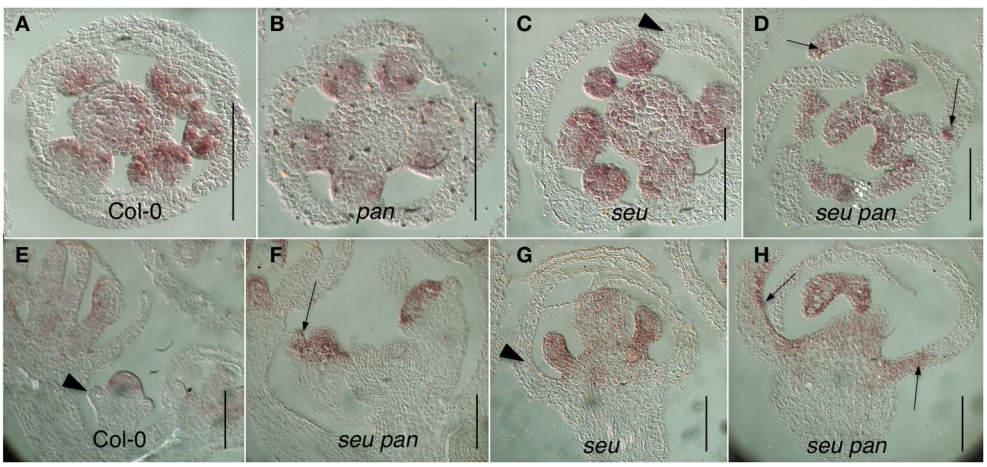

FIGURE 5 | Ectopic *AG* expression in the *seu pan* double mutants. (A–D) Floral cross sections **(E–H)** Floral longitudinal sections. Arrowheads indicate sepals within which *AG* expression is not detected. Arrows indicate sepals within which ectopic *AG* expression is detected. Ectopic expression of *AG* is most strongly detected in the adaxial and marginal portions of the developing first whorl organs in the *seu pan 057190* double mutants (panels **D,F,H**). Scale bars are 40 microns in length.

fashion with *SEU* during the repression of *AG* in the developing sepals. As previous accounts of *PAN* expression (Chuang et al., 1999) did not report *PAN* expression in whorl one organs, we examined expression of *PAN* via *in situ* hybridization to look carefully at the developing whorl one organs. We detected expression of *PAN* in portions of the developing whorl one primordia in wild type floral buds during stages 3–6. This was chiefly confined to the adaxial and marginal portions of the developing sepals (**Figures 6D–F**). Thus, there is a good correlation between the expression domain of the *PAN* transcript in wild type and the presence of homeotic cell type transformation in the *seu pan* mutant.

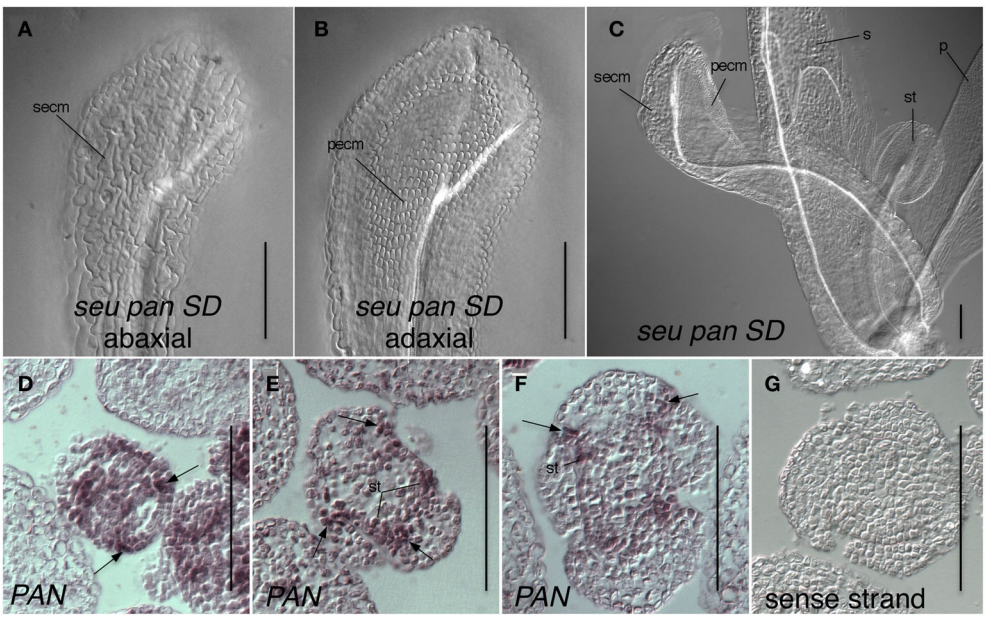

FIGURE 6 | Expression of *PAN* in adaxial portions of sepals is consistent with a role for *PAN* in the repression of *AG* and specification of organ identity. (A–C) Differential interference contrast optics microscopic image of chloral hydrate cleared tissue. **(A)** Abaxial surface of whorl one organ in *seu pan* short-day (SD)-grown flower displays cells with sepal epidermal cell morphology (secm). **(B)** Adaxial surface of same organ as in panel **(A)** displays petal epidermal cell morphology (pecm). **(C)** A *seu pan* flower displays a partial homeotic transformation of whorl one organ on adaxial surface as indicated by the presence of petal epidermal cell morphology (pecm). Morphologically normal petal (p), sepal (s), and stamen (st) are indicated. **(D)** Stage 3 floral cross section. *PAN* expression is detected in adaxial and marginal portions of developing sepals (arrows) as well as in stamen anlagen and floral meristem. **(E,F)** *PAN* expression is detected in adaxial and marginal portions of developing sepals (arrows) as well as in stamen primordia (st) in stage 5 **(E)** and stage 6 **(F)** floral cross sections. **(G)** sense strand control hybridization on stage 5 floral cross section. Scale bars are 100 microns in all panels.

Table 3 | Chimeric floral organs in *seu pan* double mutants under short-day conditions.

Genotype	% Chimeric sepals	% Chimeric petals
Col-0	0 ($N = 19$)	0 ($N = 8$)
seu-3	0 ($N = 32$)	0 ($N = 26$)
pan 057190	0 ($N = 23$)	0 ($N = 11$)
seu-3 pan 057190	30 ($N = 43$)	0 ($N = 11$)

DISCUSSION

ROLE OF *PAN* IN GYNOECIAL MEDIAL DOMAIN DEVELOPMENT

The enhancement of gynoecial defects observed in the *seu pan* and *pan ant* double mutants relative to the single mutant parents indicates (1) that *PAN* plays a role in the development of the medial domain of the gynoecium and that (2) this role is revealed when the activity of *SEU* or *ANT* is compromised. The dependence of the phenotype on the loss of function of *SEU* or *ANT* suggests that the function of *PAN* during gynoecial development is partially overlapping with that of*SEU* and *ANT*. The defects of the *seu pan* double mutant are similar to, but distinct from the *ant pan* double mutant, indicating a differential sharing of functions between these three genes.

The defects observed in the *pan ant* double mutants include an enhanced loss of ovules and enhanced splitting of the gynoecial tube relative to the single mutant parents (**Figures 1A**, **3A**). Both of these we interpret as the result of a reduced growth of the medial domain of the gynoecium. In the *pan ant* double mutants the medial domain does not grow to the same extent as the neighboring lateral domains (**Figure 2C**). This may result in the failure of the gynoecial tube to fuse completely, as well as contribute to a loss of ovule primordia.

The *seu pan* double mutant also displays an enhanced loss of ovules, although this is not as severe as that observed in the *pan ant* double mutant. Gynoecial splitting was not enhanced in the *seu pan* double mutants. The severity of carpel bending was enhanced in the *seu pan* double mutants relative to the parental genotypes. The enhanced curving of the gynoecial tube resulted from the loss of one of the two component carpels and the fusion of the remaining carpel upon itself (data not shown). This data suggests that *SEU* and *PAN* share a function in the regulation of carpel number. In the most severely affected *seu pan* double mutants, there is a loss of the characteristic morphology of the abaxial valve epidermal cells and these cells develop as cells that are morphologically similar to abaxial replum cells (**Figures 2L–O**) suggesting that patterning along the medial lateral extent of the gynoecium may be affected. The analysis of additional markers of the medial and lateral domains in the *seu pan* double mutant would help to determine if there is an alteration in medial/lateral patterning events in the *seu pan* double mutant.

PAN is expressed within the adaxial portions of the medial domain and in the developing ovule primordia (Chuang et al., 1999; Wynn et al., 2011) and thus may directly regulate genes within the medial domain that support medial domain development. Alternatively, as *PAN* is also expressed within the vegetative shoot apex, as well as the IFM and developing floral meristems (Chuang et al., 1999; Fulcher and Sablowski, 2009; Maier et al., 2009) (**Figure 6**), the effects of *PAN* on earlier stage floral meristems or perhaps the IFM may lead to later effects on the development of ovules from the medial domain.

Previous analyses of *pan ettin (ett)* and *pan tousled* (*tsl*) double mutants indicated that *PAN* also shares overlapping functional roles with *TSL* and *ETT* during medial domain development (Roe et al., 1997; Sessions et al., 1997). The carpels of the *pan tsl* double mutant gynoecia are completely unfused and serrated at their margins (Roe et al., 1997). The CMM-derived tissues are also significantly reduced in these gynoecia and very few ovules develop. Similarly Sessions et al. reported a synergistic loss of ovule and placental development in the *pan ett* double mutant (Sessions et al., 1997). Thus, a variety of non-additive genetic interactions affecting gynoecial development have been described for the *seu*, *lug*, *pan*, *ant*, *ett*, and *tsl* higher order mutants (Roe et al., 1997; Sessions et al., 1997; Liu et al., 2000; Franks et al., 2002; Pfluger and Zambryski, 2004; Azhakanandam et al., 2008).

ROLE OF *SEU* AND *PAN* IN FLORAL MERISTEM DETERMINACY

PAN has been previously shown to function in the termination of the floral meristem and to function as an activator of *AG* (Das et al., 2009; Maier et al., 2009). The direct binding of PAN to conserved regulatory elements within the *AG* second intron and the functional importance of these elements in generating the *AG* expression pattern strongly suggests that PAN directly functions as an activator of *AG* expression. Consistent with this Maier et al. reported a reduction in *AG* transcript in the *pan* single mutant, but only when this mutant was grown under short-day conditions (Maier et al., 2009). Das et al. did not detect a reduction in the *pan* single mutant, but did observe a reduction of *AG* expression within whorl 4 in plants that carried a dominant negative *PAN-RD* construct in which *PAN* is fused to a strong transcriptional repressor domain (Das et al., 2009). These results suggest that the function of redundant regulatory elements within the *AG* second intron (Sieburth and Meyerowitz, 1997; Bomblies et al., 1999; Deyholos and Sieburth, 2000) and of redundant bZIP family members (Das et al., 2009; Maier et al., 2009) may reduce the phenotypic consequences of the loss of *PAN* function on *AG* expression. Das et al. and Maier et al. both reported ectopic expression of *WUS* in fifth whorl structures in *pan* single mutants. However, they did not report alterations to *WUS* expression patterns at earlier stages of floral development. We also did not observe altered *WUS* expression in *pan* single mutants. However, we observed alterations of the *WUS* expression patterns that are evident in the *seu pan* double mutant, particularly when grown under short-day conditions. In these cases *WUS* expression was often more diffusely localized within the IFM and the developing floral meristems. Additionally we observed instances of perdurant *WUS* expression within the developing fifth whorl structures. Thus, a deregulation of *WUS* expression or localization is likely to contribute to the indeterminacy phenotypes observed in the *seu pan* double mutants.

As *PAN* functions as an activator of *AG* transcription, and *AG* as a repressor of *WUS* expression, this deregulation of *WUS* may be caused by a reduction in *AG* transcription or by post-transcriptional regulation of *AG* activity or both. We did not detect a consistent reduction in the levels of *AG* expression in either the *pan* single mutant or the *seu pan* double mutant under either long-day or short-day growth conditions. Yet the fifth whorl indeterminacy phenotypes were clearly enhanced in the *seu pan* double mutant under the short-day growing conditions. It is possible that the *in situ* hybridization assay is not sensitive enough to detect modest, yet biologically-significant reductions in *AG* transcript. Alternatively, as SEU can physically interact with several MADS domain-containing proteins that dimerize with AG (e.g., AP1, SEP3, SVP, and AGL24) (Gregis et al., 2006; Sridhar et al., 2006; Smaczniak et al., 2012), we propose that the SEU protein may regulate the ability of AG to function via physical interactions with these MADS domain proteins. Thus, the ability of the AG protein to repress *WUS* expression and thus bring about floral stem cell termination may be compromised in the *seu* mutant background. This would be consistent with the ectopic persistence of *WUS* expression observed in the *seu pan* fifth whorl structures. It also might contribute to the fasciation defects we observed and to the expansion of the *WUS* expression domain in the IFM and floral meristems.

WHORL SPECIFIC ACTION OF *PAN* AND *SEU* IN THE REPRESSION OF *AG* EXPRESSION AND SPECIFICATION OF ORGAN IDENTITY

SEU functions as a transcriptional adaptor required for the repression of *AG* transcription within whorls 1 and 2 (Franks et al., 2002; Gregis et al., 2006; Sridhar et al., 2006; Gonzalez et al., 2007). SEU forms a complex with several MADS domain-containing proteins and the transcriptional co-repressor LUG to bind to the second intron sequences of *AG*. This brings about repression via histone deacetlyation (Sridhar et al., 2006; Gonzalez et al., 2007). The loss of *PAN* activity in the *seu pan* double mutant enhances the de-repression of *AG* in whorl 1 structures and leads to the partial homeotic transformation of sepals (**Figures 5**, **6**). Thus, we suggest that *PAN* functions in the repression of *AG* in whorl 1, a function that is partially overlapping with *SEU*. As we have detected *PAN* transcript in the adaxial and marginal portions of the developing sepals, a direct role for *PAN* in the repression of *AG* is plausible.

We have been unable to demonstrate a physical interaction between SEU and PAN in a yeast two hybrid assay (data not shown). However, both *PAN,* as well as SEU-containing complexes have been shown to bind directly to the DNA regulatory elements found within the *AG* second intron (Sridhar et al., 2004, 2006; Das et al., 2009; Maier et al., 2009). Thus, we favor a model in which both SEU and PAN via direct interaction with the *AG* second intron bring about the repression of *AG* expression within developing whorl one organs. The action of additional redundant regulators of *AG* repression (*AP2*, and *SEUSS-LIKE* family members) (See for review Liu and Mara, 2010) likely buffers the extent of *AG* de-repression that is observed in the *seu pan* double mutant. Furthermore, the mis-specification of

petal cell identity in the whorl 1 structures suggests that B-class genes required for petal identity specification (e.g., *PI* and *AP3*) are also likely to be de-repressed in the *seu pan* double mutant, although we have not yet confirmed this with *in situ* hybridization experiments. Ectopic expression of B-class genes in whorl one organs could be caused by ectopic *AG* expression. Previously *lug* alleles were shown to condition the partial transformation of whorl one organs to petaloid and stamenoid chimeric organs and ectopic expressions of the B-class genes *PISTILLATA* (*PI*) and *APETALA3* (*AP3*) were detected in developing whorl one structures (Liu and Meyerowitz, 1995). Liu and Meyerowitz demonstrated that the petaloid characteristics of the whorl one organs in *lug* mutants were dependent on ectopic *AG* expression (i.e., the petaloid characteristics were not observed in the *lug ag* double mutants). They propose that the ectopic *AG* brings about an ectopic expression of the B-Class genes and this is consistent with the identification of *PI* and *AP3* as targets of *AG* regulation by Gomez-Mena et al. (2005).

Our data suggests that *SEU* and *PAN* function in a whorl specific fashion in the regulation of *AG* transcription or activity. *SEU* and *PAN* activities are required for efficient repression of *AG* in whorl 1 while their activities are required for efficient activation of *AG* function in whorl four. We propose that the differential action of these proteins is due to whorl specific co-factors or post-transcriptional modifications. The identity of these whorl specific modifiers remains to be elucidated.

RELATIONSHIP BETWEEN FLORAL MERISTEM TERMINATION AND CMM DEVELOPMENT

Zuniga-Mayo et al. previously suggested a relationship between the proper termination of the floral meristem and the subsequent development of the CMM (Zuniga-Mayo et al., 2012).

This was based on their analysis of *jaiba crabs claw* double mutants that display a loss of floral determinacy as well as defects in the development the CMM. Our investigation of the *seu pan* double mutant further supports this possibility. When ovules develop in the *seu pan* double mutants, they arise at apical positions within the gynoecium, and thus at a distance from the basally-located fifth whorl structures. Thus, if the floral meristem fails to properly terminate, the cells furthest from the perdurant meristem are more likely to form ovules than those closer to the meristem. This could suggest a gradient of an inhibitor from the floral meristem. However, it is equally likely that a temporal effect explains the difference. As the gynoecium grows from the apex, cells that divided temporally later in development will also be found in more apical positions. Thus, the decay over time of any inhibitory effect of the floral meristem might also result in the formation of ovules only at the apex of the *seu pan* double mutants.

AUTHOR CONTRIBUTIONS

April N. Wynn and Robert G. Franks: conceived and designed the experiments, analyzed the data, and wrote the paper; April N. Wynn, Andrew A. Seaman, Ashley L. Jones and Robert G. Franks: Performed the experiments.

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SUPPLEMENTARY MATERIAL

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Figure S1 | Short-day growth conditions reveal a floral transition delay in seu *pan 057190* double mutants Time to floral transition (in number of rosette leaves produced) indicates that both the *seu* single mutants and the *seu pan* double mutants have a delay in the transition to flowering. The *seu pan* plants are significantly more delayed than the *seu* single mutants. Comparisons for statistical differences across genotypes were made via pair-wise mean testing and the Tukey HSD *post-hoc* test—different letters indicate statistically different categories. Each error bar is constructed using 1 standard error from the mean. *pan* mutant alleles enhance the floral transition delay observed in *seu* single mutants. When examining *seu pan* plants grown in short-day conditions it was apparent that the *seu pan* double mutant plants were slower to transition to the reproductive growth phase. To quantify this delay, we counted the number of rosette leaves produced before the plants transition to a reproductive growth phase. The reproductive growth phase is characterized by the formation of cauline leaves and internode elongation. Under the short-day growing conditions the Col-0 plants produce on average 40.8 ± 4.7 rosette leaves before transition to a reproductive growth phase (**Figure S1**). *pan* mutants show no statistically significant difference when compared to Col-0 (43.2 ± 4.5 leaves). In contrast *seu* mutants exhibit a statistically significant delay in flowering relative to Col-0, producing 50.5 ± 4.0 leaves prior to bolting. Mutations in *SEU* also caused a delay in the floral transition in long-day growth conditions (Wynn and Franks, unpublished). When we examined the *seu pan* double mutants we observed that they were significantly delayed, relative to the single mutants, producing on average 60.4 ± 5.1 leaves before the transition. These results suggest that both *PAN* and *SEU* function to promote the transition to reproductive growth phase under short-day growing conditions.

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Ovule development, a new model for lateral organ formation

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In spermatophytes the ovules upon fertilization give rise to the seeds. It is essential to understand the mechanisms that control ovule number and development as they ultimately determine the final number of seeds and, thereby, the yield in crop plants. In *Arabidopsis thaliana*, ovules arise laterally from a meristematic tissue within the carpel referred to as placenta. For a correct determination of the number of ovules, a precise establishment of the positions where ovule primordia emerge is needed, and a tight definition of the boundaries between ovules is therefore also required. In the last decades, few factors have been identified to be involved in the determination of ovule number. Recently, plant hormones have also been revealed as fundamental players in the control of the initiation of ovule formation. In this review we summarize the current knowledge about both the molecular and hormonal mechanisms that control ovule formation in *Arabidopsis thaliana*.

Keywords: Arabidopsis, ovule primordia, ovule number, development, transcription factors, hormones

INTRODUCTION

Fruits are a major evolutionary acquisition of flowering plants (Angiosperms). They likely evolved to protect the developing seeds and to ensure seed dispersal (Knapp, 2002). Fruits derive mostly from the fertilized mature gynoecium although, especially in fleshy fruits, additional floral components have frequently been recruited. The gynoecium (or pistil), the female reproductive organ, is composed of a single carpel or a number of carpels that are often fused. Carpels are essential for sexual plant reproduction because they house the ovules and upon fertilization the carpel develops into the fruit that protects, nourishes and ultimately disperses the seeds.

In Arabidopsis, the fundamental processes leading to the formation of a complete developed set of ovules can be summarized in a few main steps (**Figure 1**). First of all, the lateral margins of the carpels, containing a meristematic tissue named the medial ridge or carpel margin meristem (CMM), give rise to the placenta, the septum and transmitting track. The CMM formation is known to be controlled by the interaction of genetic and hormonal networks (reviewed by Reyes-Olalde et al., 2013). Once the placenta is formed, some mechanisms, still poorly understood, are needed for the definition of boundary regions that will separate the ovule primordia. The ovule primordia are initiated by periclinal divisions from the subepidermal tissue of the placenta. During the early growth phase of primordia formation a series of predominantly anticlinal divisions take place. Later on, the relatively homogenous mass of cells of the primordium will be organized in three different regions along the proximal-distal axis: the funiculus, the chalaza and the nucellus (**Figure 1**). Within the nucellus, megasporogenesis and megagametogenesis take place, and finally the mature haploid embryo sac is formed. From the chalaza region the two integuments, progenitors of the seed coat, develop, while the funiculus connects the ovule to the mother plant.

In the last decades, several studies have identified genes involved in ovule identity determination and development in different species such as Arabidopsis, Petunia and rice (Bowman et al., 1991; Angenent et al., 1995; Colombo et al., 1995; Angenent and Colombo, 1996; Pinyopich et al., 2003; Dreni et al., 2007). However, the players that determine the number of ovules are largely unknown, due to the difficulties that the studies tempting to genetically dissect ovule initiation and development have encountered. On one hand, many genes that control ovule development are also involved in initiation and growth of other floral organs, masking their effects on ovules. On the other hand, it is difficult to establish if a mutation in a gene causes a reduction in ovule number in mutants that already display an altered gynoecium phenotype.

Nonetheless, understanding the factors that control ovule initiation is of great importance from an agricultural and economical point of view, as the ovule number will determine the number of seeds that develop in a fruit, and thus the crop yield.

With this review on ovule initiation we aim to summarize the current knowledge about the factors and the hormonal pathways that have been identified to be involved in the determination of ovule numbers in *Arabidopsis thaliana*, and the cross-talk between these hormonal and regulatory pathways.

CARPEL MARGIN MERISTEM FORMATION

GENETIC FACTORS CONTROLLING CARPEL MARGIN MERISTEM FORMATION

As already mentioned, the establishment and maintenance of the meristematic tissues of the CMM is inherently correlated to the generation of ovule primordia. CMM development is

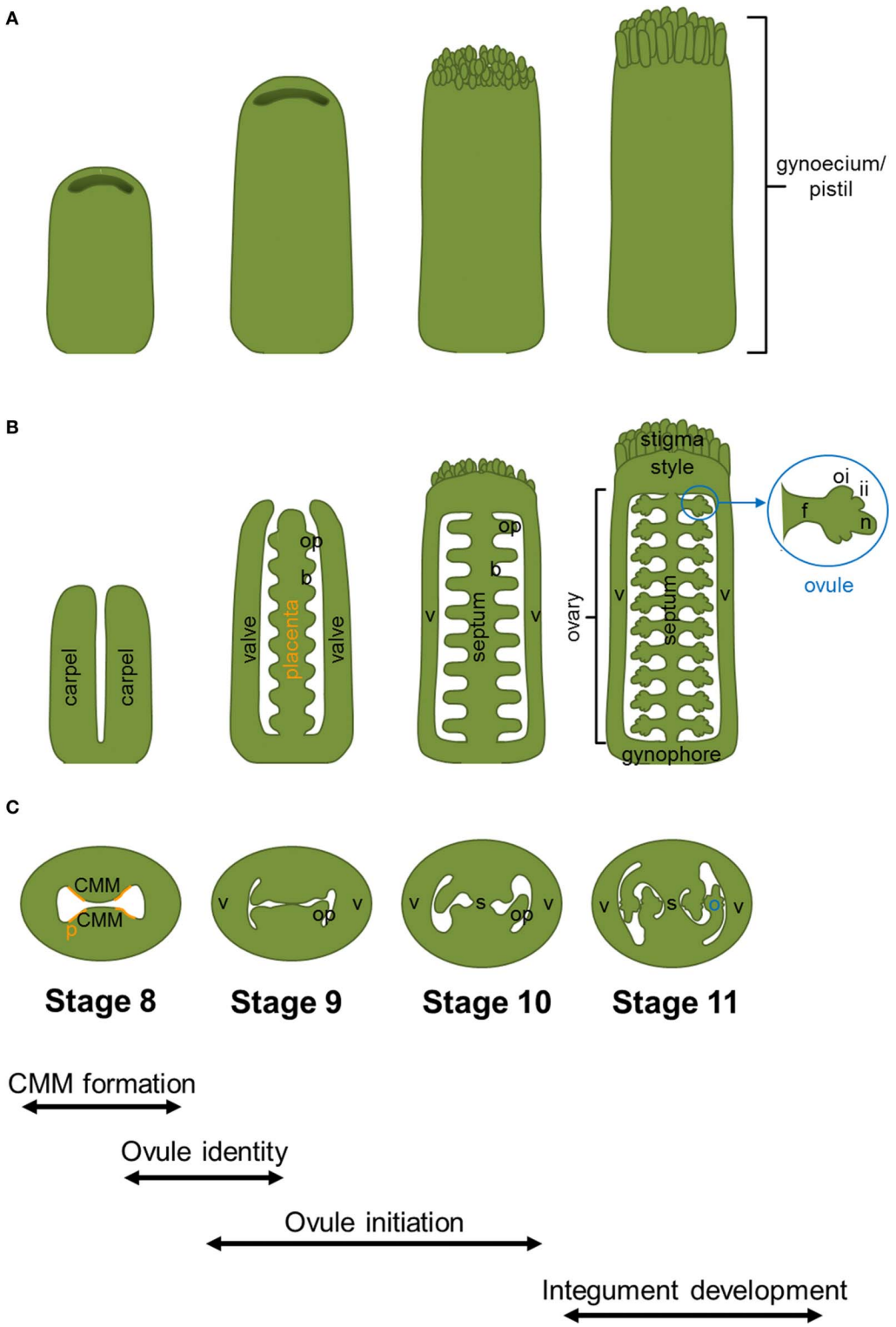

FIGURE 1 | ***Arabidopsis thaliana*** **gynoecium development.** Cartoons displaying wild-type gynoecia **(A)**, in longitudinal sections **(B)**, and transversal sections **(C)** from stage 8 to 11. The different stages and processes of gynoecium and ovule development are indicated at the bottom of the figure. Abbreviations: b, boundary; CMM, carpel margin meristem; f, funiculus; ii, inner integument; n, nucellus; o, ovule; oi, outer integument; op, ovule primordium; p, placenta; s, septum; v, valve. The region of the CMM where placenta is formed is indicated with orange lines.

known to be controlled at the transcriptional level and by hormones as reviewed by Reyes-Olalde et al. (2013). Several single and higher order mutant combinations with strongly reduced carpel marginal tissue development have been described in literature. One of the them is the *aintegumenta* (*ant*) mutant. ANT is a transcription factor that contains two AP2 domains that controls organ initiation and promotes cellular divisions during organ development (Klucher et al., 1996). Interestingly, the *ant-9* mutant has medial ridges that are frequently unfused to each other with a consequent reduction in functional CMM tissue. It has also been reported that the *ant* mutant displays enhanced morphological defects when combined with a mutation in *REVOLUTA* (*REV*), a member of the class III Homeodomain-Leucine Zipper (HD-ZIP III) family. In the *ant rev* double mutant a partial disruption of CMM and placenta development causes the reduced development of ovule primordia (Nole-Wilson et al., 2010).

An unfused carpels phenotype due to the compromised fusion between the two medial ridges was also observed in the mutants for *LEUNIG* (*LUG*), a floral organ identity gene that encodes a glutamine-rich protein with seven WD repeats, typical of transcriptional co-repressors (Liu et al., 2000). Despite this failure in ridge fusion, ovules are formed from the placenta although in a markedly decreased number in both *lug-1* (intermediate-strength allele) and *lug-3* (strong allele) mutants (**Table 1**). The simultaneous loss of *LUG* and *ANT* functions enhanced the defects in flower development in respect to the single *lug* and *ant* mutants. While the double mutant *lug-3 ant-9* did not form any ovules, septum or stigma, nearly 50% of the *lug-1 ant-9* pistils could develop normal medial ridges, that gave rise to partially formed septal tissues, although ovules, stigma and style were never present (Liu et al., 2000) (**Table 1**).

ANT also interacts synergistically with SEUSS (SEU), a transcriptional coregulator functionally similar to LEU, in the control of organ size of the flower. While the *seu-3* single mutant shows on average ovule numbers not significantly different from wild-type Col-0, the double mutant *seu-3 ant-1* results in a complete loss of ovule initiation, caused by severe defects in early gynoecia development. In the weaker allelic combination *seu-3 ant-3*, employing the *ant-3* hypomorphic allele, placenta formation is not compromised but defects such as ovule initiation and gametogenesis are present at later stages (**Table 1**) (Azhakanandam et al., 2008).

Other two players in CMM development are CUP-SHAPED COTYLEDON1 (CUC1) and CUC2, two transcription factors that belong to the NAC transcription factor family. The *cuc1* and *cuc2* single mutants display almost no phenotype, while the *cuc1 cuc2* double mutant completely lacks the shoot apical meristem (SAM) and the cotyledons are fused along their margin forming a cup-shaped structure. These seedlings die a few days after germination (Aida et al., 1997). Studying gynoecium development in the *cuc1 cuc2* double mutant was only possible using plants obtained by *in vitro* regeneration. They presented defects in the formation of the septum and in ovule development (Ishida et al., 2000). A gene that has been described to play a role with *CUC1* and *CUC2* in promoting the formation of carpel marginal structures and thus facilitating septum and ovule development is *SPATULA* (*SPT*), which encodes a basic helix–loop–helix (bHLH)

Table 1 | Ovule numbers phenotype of the mutants cited in these article.

Genotype	Ovule number per fruit	Ovule number per carpel	References
L*er*		26.4 ± 1.3	Liu et al., 2000
lug-1		15.4 ± 4.2	
lug-3		14.9 ± 3.1	
ant-9		14.8 ± 3.1	
lug-1 ant-9		0.0 ± 0	
lug-3 ant-9		0.0 ± 0	
Col-0		25 ± 2.0	Azhakanandam et al., 2008
Col-*gl*		21 ± 3.0	
ant-1		12 ± 1.3	
ant-3		20 ± 2.7	
seu-3		23 ± 1.8	
seu-3 ant-1		0.0 ± 0.0	
seu-3 ant-3		13 ± 3.4	
Col-0	55.66 ± 0.83		Nahar et al., 2012
spt-2	48.38 ± 0.61		
cuc1-1 spt-2	36.44 ± 0.59		
cuc2-1 spt-2	34.31 ± 0.49		
Col-0	∼ 30		Ishida et al., 2000*
cuc1	∼ 31		
cuc2	∼ 32		
cuc1cuc2	∼ 10		
Ler	51.8 ± 0.6		Galbiati et al., 2013
ant-4	17.8 ± 0.7		
*cuc2-1 ant-4***	20 ± 3		
cuc2-1 pSTK::CUC1_RNAi	41.7 ± 0.9		
*cuc2-1 ant-4 pSTK::CUC1_RNAi***	8 ± 1		
pin1-5	8.6 ± 2		
Ler	39.9 ± 1.1		Elliott et al., 1996
ant-9	15.0 ± 0.8		
hll-1	10% less than *wt*		Skinner et al., 2001
hll-3	10% less than *wt*		
L*er*	54 ± 4		Broadhvest et al., 2000
sin-2	33 ± 7		
Col-0	48		Bencivenga et al., 2012
cre1-12 ahk2-2 ahk3-3	5.5		
pin1-5	9.35		
Col-0	110		Bartrina et al., 2011***
ckx3-1 ckx5-1	65		
Col-0	52.95		Huang et al., 2012
bzr1-1D	68.06		
bin2	29.07		
det2	52		
WS	46.4		
bri1-5	32.2		
ap2-5	60.4		
bzr1-1D ap2-5	74.8		

(Continued)

Table 1 | Continued

Genotype	Ovule number per fruit	Ovule number per carpel	References
Cvi L*er*	55.5 ± 5.2 66.4 ± 3.9		Alonso-Blanco et al., 1999
ashh2-1, ashh2-2, ashh2-5	80% less than *wt*		Grini et al., 2009

*Mutants presenting defects in the gynoecia or ovule development also reported to be affected at the level of ovule number. *plants regenerated from calli; **Galbiati F. personal communication; ***the number refers to seeds.*

transcription factor. Mutations in *SPT* cause a split carpel phenotype in the apical part of the gynoecium. Moreover, *spt* plants have slightly fewer ovules than the wild type, from which only a small fraction develop into seeds (Nahar et al., 2012). When combined with *cuc1* and *cuc2* single mutants, the average number of ovules decreases. Thus, while the *spt* single mutant shows an average of 48 ovules per carpel, *spt cuc1* and *spt cuc2* present 36 and 32 respectively (**Table 1**), indicating that CUC1, CUC2, and SPT are together required for ovule development. Another mutant that displays an unfused gynoecium at the apex is *crabs claw* (*crc*) (Alvarez and Smyth, 1999). *CRC* encodes a transcription factor of the YABBY family and the characterization of different mutant alleles showed that, besides the failure of the fusion of the stylar region, *crc* mutants present a gradation of phenotypes with wider and shorter gynoecia that contain fewer ovules compared to the wild type (Alvarez and Smyth, 1999; Bowman and Smyth, 1999).

Thereby, the phenotype of ovule reduction that we frequently observe in the mutants defective in medial ridge fusion and thus in CMM formation could be due, at least in part, to their role in regulating cell proliferation in the medial ridges, from which septum and ovules originate.

CMM FORMATION AND THE AUXIN GRADIENT

Auxin is a key hormone for plant development, and it is also fundamental for gynoecium and thereby CMM and ovule development. In the last two decades several studies have demonstrated that local auxin biosynthesis and polar transport are responsible for the correct apical–basal patterning of the gynoecium. The auxin gradient hypothesis supports that high levels of auxin in the gynoecium apical regions control stigma and style formation; medium levels direct ovary formation whereas low levels of the hormone are responsible of gynophore development at the gynoecium base (Nemhauser et al., 2000). Indeed, all mutants in which the auxin synthetic pathway or transport are compromised have a similar severe gynoecium phenotype forming a pistil-like structure with reduction/absence of the valves, expansion of the gynophore and stylar regions and serious vasculature defects (reviewed in Balanzá et al., 2006; Larsson et al., 2013). This phenotype was characterized for the first time in the flowers of *pin-formed1-1* (*pin1-1*), a strong mutant allele of the auxin efflux carrier PIN1 (Okada et al., 1991) and in the *pinoid* mutant, a knock-out line for a serine/threonine kinase that regulates PINs polarity (Bennett et al., 1995). Other examples of mutants with similar pistil-like structure phenotypes are the *yucca1 yucca4* (*yuc1 yuc4*) and *weak ethylene insensitive8 tryptophan aminotransferase related2* (*wei8 tar2*) double mutants, in which local auxin production is impaired (Cheng et al., 2006; Stepanova et al., 2008). Predictably, in most of these auxin-related mutants the severe defects in gynoecium formation lead to a pistil with a reduction or complete absence of ovules and the consequent complete sterility.

Nemhauser et al. (2000) confirmed the importance of polar auxin transport (PAT) in gynoecium development through an experiment in which they used 1-napthylphthalamic acid (NPA), an inhibitor of the auxin transport. They showed that NPA application caused significant loss of ovules. The authors also highlighted that ovules seemed more sensitive to disruption in PAT, with respect to the other tissues of the gynoecium. Indeed, treated carpels were largely devoid of ovules but were still able to produce valves. In 2010 Nole-Wilson and collaborators proposed the connection between ANT and the hormone auxin on the base of the observation that the *ant* mutant is more sensitive than the wild type to alteration in PAT. Moreover, the expression of a subset of auxin-related genes was altered in the *ant* single and *ant rev* double mutant gynoecia, indicating that the morphological defects of the *ant rev* double mutants, at least in part, are due to an alteration in auxin homeostasis in these plants.

Auxin signaling is primarily regulated by the *AUXIN RESPONSE FACTOR* (*ARF*) gene family products, together with the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins. The phenotype of *ARF5/MONOPTEROS (MP)* strong mutant alleles results in an embryo lethal phenotype, while *mp* partial loss of function mutants have normal embryo development whereas that their reproductive development is compromised (Hardtke and Berleth, 1998). In the pistil of the *mpS319* weak allele the CMM does not develop, and placenta and ovules are completely missing (Cole et al., 2009; Galbiati et al., 2013). Interestingly, MP has been demonstrated to directly activate the *ANT, CUC1* and *CUC2* transcription factors encoding genes (Galbiati et al., 2013) Their role as major players in ovule primordia initiation and ovule number determination will be discussed in the following sections.

OVULE IDENTITY ESTABLISHMENT

The ovule cell fate is controlled by the ovule identity genes *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*), that belong to the MADS-box gene family of transcription factors. While in the single and double mutant combinations of these genes there is no detectable ovule phenotype, in the *stk shp1 shp2* triple mutant the ovule integuments are converted into leaf/carpel-like structures (Pinyopich et al., 2003; Brambilla et al., 2007). Moreover, ectopic expression of these MADS box genes results in ovule formation on sepals (Favaro et al., 2003; Pinyopich et al., 2003; Battaglia et al., 2006). *STK*, *SHP1*, and *SHP2* have overlapping expression patterns in the placenta and ovule primordia also with *AGAMOUS (AG)* (Rounsley et al., 1995; Savidge et al., 1995; Theißen et al., 1996; Pinyopich et al., 2003), one of the first identified MADS-box factors that determines stamen and carpel identity (Yanofsky et al., 1990). It has been shown that also

AG plays a role in ovule development by experiments in which the *apetala2* (*ap2*) single mutant was compared with the *ap2 ag* double mutant. Thus, in the *ap2* single mutant petals were mostly absent, while sepals were converted into carpel structures bearing ectopic ovules, some of which were transformed into carpelloid structures. Interestingly, the sepals (or first-whorl organs) of the *ap2 ag* double mutant still presented carpel identity, and the number of ovules converted into carpel structures was significantly higher, indicating that AG activity also contributes to ovule identity establishment (Bowman et al., 1991; Pinyopich et al., 2003). Interestingly, Skinner et al. (2004) suggested that when the functions of *stk, shp1*, and *shp2* were lost in a triple mutant, fewer ovules initiated and ovule development is severely disrupted.

OVULE PRIMORDIA INITIATION

THE ESTABLISHMENT OF THE BOUNDARIES

When new organ primordia are originated in the plant, two different regions, the boundaries and the zone of primordia outgrowth, need to be defined. The organ boundary is defined as the region between the meristem and the developing organ, or, as in the case of ovules, as the region between two adjacent ovule primordia. As Aida and Tasaka nicely reviewed in 2006, the "boundary cells" need to have peculiar characteristics respect to the surrounding cells, usually displaying reduced cell division and expansion. Another important aspect is the arrangement of the plasmodesmata that regulates the movement of transcription factors between cells. For example, the boundaries in the inflorescence meristem seem to restrict the passage of proteins into flower primordia (Wu, 2003).

The boundary-specific regulatory genes play a critical role in orchestrating several morphogenetic and patterning events and their spatial coordination. When this coordination is missing, fusion between organs is the most frequent observed phenotype (Aida et al., 1997). The *CUC* gene family was the first discovered to have a fundamental role in organ boundary establishment. In fact, in the *cuc1cuc2* double mutant embryo the cotyledons do not separate (Aida et al., 1997).

The transcripts of *CUC1* and *CUC2* were detected by *in situ* hybridization in the anlagen placenta and in ovules at stage 1-II and later on, starting from stage 2-I, restricted to the boundary between two ovules (Ishida et al., 2000; Galbiati et al., 2013). As we already mentioned, the study of the gynoecium phenotype of the *cuc1 cuc2* double mutant was only possible on plants regenerated *in vitro*. They showed defects in the formation of the septum and in ovule development; most of the gynoecia having less than 10 ovules (**Table 1**). However, the *cuc1 cuc2* double mutant plants never gave seeds (Ishida et al., 2000). A further demonstration that CUC1 and CUC2 are directly linked to the determination of ovule number in a direct way came from the work of Galbiati et al. (2013). In order to study the ovule phenotype in absence of both *CUC1* and *CUC2, CUC1* was silenced in a *cuc2-1* mutant background using a *CUC1* specific RNAi construct under the control of the ovule-specific *SEEDSTICK* promoter (*pSTK:CUC1_RNAi*) which is already active in the placenta before ovule primordia arise. The analysis of *cuc2-1 pSTK:CUC1_RNAi* plants revealed a reduction in ovule number of 20% (**Table 1**). Furthermore, *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants were generated in order to analyze the possible additive role of ANT to CUC function in the regulation of ovule primordia formation. The *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants displayed a further dramatic reduction in the number of developing ovules (a mean of seven ovule primordia per pistil), while the single mutant *ant-4* and the plants *cuc2-1 pSTK::CUC1_RNAi* showed 20 and 30 ovules per pistil, respectively (**Table 1**). Despite the reduction in ovule number in the different mutant backgrounds, the size of the pistils was not reduced. Therefore, the ovules were more distantly spaced compared to those in wild-type pistils (Galbiati et al., 2013). These studies of the characterization of the *ant* single and *cuc1 cuc2* double mutants, as well as *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants prove that ANT, CUC1 and CUC2 are key players in the control of the number of ovule primordia that develop from the placenta and that they act additively (Elliott et al., 1996; Ishida et al., 2000; Galbiati et al., 2013). All the information about these factors taken together indicates that they work in different ways: while ANT promotes ovule primordia growth, the CUCs play a role in the establishment of the ovule primordia boundaries (**Figure 2**).

CUC3, another putative NAC-domain transcription factor member of the *CUC* family, is expressed in an extensive range of boundaries in adult plants. Besides, the function of CUC3 is partially redundant with that of its homologous CUC1 and CUC2 in the establishment of the cotyledon boundary (Vroemen et al., 2003). Several studies revealed that *CUC* expression is controlled and restricted to the boundaries in several ways. For instance, in the SAM *CUC1* and *CUC2* but not *CUC3* are regulated by *miR164*, which restricts the expression of *CUC1* and *CUC2* mRNAs to the boundary domain (Laufs et al., 2004). In the carpel, in a similar way to the pattern already described

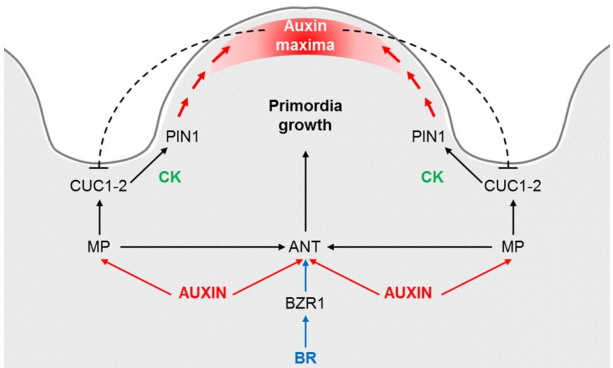

FIGURE 2 | Proposed model for the control of the ovule primordia initiation. Auxin triggers ANT and MP expression, which in its turn is required for *ANT, CUC1* and *CUC2* expression during the early stages of placenta development and ovule primordia formation. *ANT* expression is also regulated by brassinosteroids (BR), specifically being directly regulated by BZR1. ANT controls cell proliferation in the placenta and ovules, whereas CUC1 and CUC2 establish the boundaries and control *PIN1* expression, which is required for primordia formation. Cytokinin (CK) may act downstream of CUC proteins in promoting *PIN1* expression. Once the primordia have formed, auxin accumulates at the edge of the developing ovule. An inhibitory loop of auxins on CUC1 and CUC2, as it is postulated for the leaf serration, could be happening at the ovule boundaries. Adapted from Galbiati et al. (2013).

for *CUC1* and *CUC2, CUC3* expression marks the boundaries between ovule primordia. Therefore, it would be interesting to study also the contribution of *CUC3* in the regulation of defining ovule boundaries.

In 2008, Xu and Shen showed that three different transcription factors, ASSYMETRIC LEAVES 1 (AS1), AS2, and JAGGED (JAG), support normal sepal and petal growth by restricting the expression domain of the boundary-specifying genes *CUC1* and *CUC2*. AS1 and AS2 were already suggested to have roles in boundary control, given that they positive regulate, within the shoot apex, the members of the *LATERAL ORGAN BOUNDARIES* (*LOB*) gene family, a plant-specific family of transcription factors that are expressed in the boundaries (Byrne et al., 2002). *LOB*, the gene that names the family, is expressed at the base of all lateral organs. Interestingly, plants overexpressing *LOB* produced abnormal flowers with reduced floral organs and they were sterile even when fertilized with wild-type pollen (Shuai et al., 2002). Lee et al. in 2009 identified two new MYB transcription factors involved in lateral organ separation: *LATERAL ORGAN FUSION 1* (*LOF1*) and *LOF2*. The single mutant *lof1* exhibits a novel fusion between the axillary stem and the cauline leaf. Additional fusions resulted when *lof1* was combined with *lof2*, *cuc2* or *cuc3,* indicating the existence of overlapping roles for *LOF1, CUC2*, and *CUC3* to control organ separation during reproductive development.

Despite the identification of a number of boundary-specific transcription factors, boundary formation and maintenance is still a poorly understood process, and only CUC1 and CUC2 have been demonstrated to have a role in ovule boundary establishment. The factors that have been described to regulate or interact with the CUCs in a different developmental context could also have a role during ovule initiation, and some of them, like *AS1* and *AS2* are already known to be expressed in the gynoecium and ovules (Xu and Shen, 2008).

AINTEGUMENTA, A MASTER REGULATOR OF PRIMORDIA FORMATION

In Arabidopsis many genes have been described to play roles in the different phases of ovule development, although most of them do not determine directly the number of ovules (Schneitz et al., 1997; reviewed in Shi and Yang, 2011). However, the ANT transcription factor has been described to have a clear role in ovule primordia formation. *In situ* hybridization experiments showed that within the carpel it is expressed in the placenta and in the integuments of the developing ovules. In *ant* plants ovules do not develop integuments and megasporogenesis is blocked at the tetrad stage leading to complete female-sterility (Elliott et al., 1996). ANT is not only required for ovule development but it is also involved in ovule primordia formation. Indeed, in the *ant-9* mutant the number of ovules per carpel is reduced by more than half in respect to the wild-type (**Table 1**). Given that the *ant* gynoecia have the same length as those of wild type, the ovules that do arise in *ant* are more distantly spaced than in wild-type plants (Liu et al., 2000).

In addition to *ANT*, another essential gene for the regulation of ovule primordia outgrowth and for the control of integument formation is *HUELLENLOS* (*HLL*), a gene that encodes a mitochondrial ribosomal protein. Thus, plants presenting mutations in *HLL* display a phenotype similar to *ant* at the level of ovule integuments (Schneitz et al., 1997, 1998). Moreover, *hll-1* and *hll-3* mutant alleles display a reduction of about 10% in the number of ovules, although the authors also describe that *hll* plants display smaller gynoecia, which could contribute to the development of fewer ovules (**Table 1**). The phenotype of the double mutant *hll ant* was more severe at the level of primordia outgrowth however, nothing was described regarding ovule number (Schneitz et al., 1998; Skinner et al., 2001). A similar phenotype to *hll* was observed in the *short integuments 2* (*sin2*) mutants. Apart of an arrest in cell division in both ovule integuments, *sin2* plants presented shorter pistils bearing less ovules than the wild type (**Table 1**). Moreover, the authors describe an abnormal distribution of the ovules along the placenta, being the distance between ovules bigger than in wild-type plants (Broadhvest et al., 2000). Thus, in this particular case the shorter carpel might not be the only cause of reduced ovule numbers. The double mutant *sin2 ant-5* was not different from *ant-5* single mutant, indicating that *ANT* is epistatic to *SIN2* with respect to ovule development. On the contrary, *sin2 hll-1* double mutant had a stronger effect on ovule development than *sin2* or *hll-1* single mutants (Broadhvest et al., 2000). All these experiments taken together indicate that although ANT plays a master role, SIN2 and HLL also contribute to ovule primordia formation.

THE ROLE OF HORMONES IN OVULE PRIMORDIA FORMATION

AUXIN IS REQUIRED FOR OVULE PRIMORDIA FORMATION

As we previously underlined, the boundary region and the primordia formation zone are highly interconnected. It has been demonstrated that a fundamental role of the "boundary transcription factors" is to organize PAT, mediated by PIN proteins, in order to create a zone of auxin maximum where organ founder cells will be selected. Auxin maxima are fundamental for the formation of primordia, and auxin action has been well described for lateral roots (LR) and flower primordia (reviewed in Benková et al., 2003, 2009; Yamaguchi et al., 2013). The directionality of auxin flux depends principally on the polar localization of the PIN proteins. In Arabidopsis there are eight PIN proteins (PIN1-8), from which only *PIN1* and *PIN3* are expressed in the pistil and ovules (Benková et al., 2003; Ceccato et al., 2013). PIN1 protein is localized at the membrane of placenta cells and later on, in the developing ovules, it is restricted to the lateral-apical membranes of nucellus cells. PIN3 is also present in few cells at the tip of the developing nucellus shortly after ovule primordia emergence but, contrary to PIN1, it is not expressed in the placenta cells (Ceccato et al., 2013). PIN-dependent efflux mediates primordium development by supplying auxin to the tip creating an auxin maxima; indeed in plants expressing the *GFP* reporter gene downstream the auxin-responsive *DR5* promoter (*pDR5::GFP*), the GFP signal is detected at the tip of all ovule primordia (Benková et al., 2003). The weak *pin1-5* mutant allele is able to develop some flowers in which the pistils have slightly reduced valves but normal styles and stigmas (Sohlberg et al., 2006). The pistils of the *pin1-5* weak allele have an average of 9 ovules per carpel (**Table 1**) (Bencivenga et al., 2012). In addition, Galbiati et al. (2013) demonstrated that the reduced number

of ovules in *cuc2-1 pSTK:CUC1_RNAi* was caused by a down-regulation of *PIN1* and an incorrect PIN1 protein localization. CUC1 and CUC2 promote *PIN1* expression and localization to correctly form the auxin maximum where primordium will form (**Figure 2**). In the same way, a CUC2-dependent regulatory pathway controlling PIN1-mediated auxin efflux has been described to explain leaf serrations (Bilsborough et al., 2011). Moreover, in the newly formed primordia of the SAM the auxin maxima, in a negative feed-back loop, repress *CUC2* expression and restricts it to the boundaries (Vernoux et al., 2000; Heisler et al., 2005; and reviewed in Aida and Tasaka, 2006; Rast and Simon, 2008). A similar inhibitory loop could control *CUC* expression at the ovule boundaries (**Figure 2**). The phenotype of *cuc2-1 pSTK:CUC1_RNAi* was completely recovered by cytokinin (CK) application, since CK has been demonstrated to increase *PIN1* expression in the ovules (Bencivenga et al., 2012). These experiments evidence a convergence of two different plant hormones in the regulation of ovule primordia formation. In the next paragraph we will delve deeper into the role of CK in the formation and determination of ovule number.

CYTOKININ POSITIVELY REGULATES OVULE NUMBER

CK is an essential hormone for plant growth and development as it has a central role in the regulation of cell division and differentiation. In the last 10 years, several studies have clearly proven that CK has also a significant role during ovule development. As it will be explained in this paragraph, it has been demonstrated that in plants that are defective in the production or perception of this hormone, correct ovule formation is compromised and/or the number of ovule is drastically reduced. CK signaling, which has been recently summarized in a detailed review article (Hwang et al., 2012), is mediated by a two-component signaling pathway: histidine protein kinases (AHKs) work as CK receptors, while histidine phosphotransfer proteins (AHPs) transmit the signal from AHKs to nuclear response regulators (ARRs), which are able to regulate transcription. In *Arabidopsis* the CK signal is perceived by three histidine kinases: *ARABIDOPSIS HISTIDINE KINASE4* (*AHK4*, also known as *CYTOKININ RESPONSE1*, *CRE1*/*WOODEN LEG*, *WOL*), *AHK2* and *AHK3*. These three genes are all expressed in inflorescences, carpels and developing ovules (Higuchi et al., 2004; Nishimura et al., 2004). More precisely, *AHK2* and *AHK3* are expressed during all stages of ovule development, starting from early primordia stages to ovule maturity, whereas *CRE1* expression remains restricted to the chalazal region and later to the integuments of ovules during all the developmental stages (Bencivenga et al., 2012). The single and double mutants of *AHKs* do not present any phenotype at the level of the ovules (Higuchi et al., 2004). However, mutants lacking all three receptors exhibit no perception of CK and present a strong slowdown of shoot and root growth. The resulting miniature plants also show delayed flower induction and impaired fertility (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Thus, the triple mutant *cre1-12 ahk2-2 ahk3-3* do not produce seeds (Higuchi et al., 2004) because the gametophyte arrests at stage FG1-FG2 (Bencivenga et al., 2012). Moreover, a severe reduction in the ovule number, an average of 5 ovule per pistil, was noticed in these triple mutant plants (**Table 1**) (Bencivenga et al., 2012). A similar sterile phenotype was also observed for another allelic combination: the *ahk4-1 ahk2-1 ahk3-1* triple mutant (Nishimura et al., 2004). Differently, Riefler et al. (2006) obtained a weaker triple mutant *cre1-2 ahk2-5 ahk3-7* that self-fertilized and formed few seeds, suggesting that infertility of the histidine kinase triple mutants is a phenotype associated with specific mutant alleles.

Attention has also been given to the importance of CK catabolism. In *Arabidopsis* the irreversible degradation of CK is catalyzed by the oxidase/dehydrogenase (CKX). The *CKX* gene family of Arabidopsis consists of seven members (*CKX1* to *CKX7*), and by *promoter:GUS* fusion constructs it was shown that *CKX1*, *CKX5*, and *CKX6* (At3g63440, previously called *AtCKX7*) are expressed in flower tissues, being *CKX6* the only one reported to be expressed in the carpel and ovules, in particular in the funiculus (Werner et al., 2003). Werner and colleagues engineered transgenic *Arabidopsis* plants that individually overexpressed six different *CKXs* in order to enhance CK degradation. As expected, these plants manifested phenotypes linked to CK deficiency, like delayed vegetative growth and leaf expansion, diminished activity and size of the SAM but increased overall root system. The reproductive development of CK-deficient plants was also altered. In *35S::CKX1* and *35S::CKX3* plants, flowering was strongly delayed and furthermore the fertility of flowers was heavily reduced, partially due to the lack of pollen. *35S::CKX1* and *35S::CKX3* siliques were not filled completely and they formed approximately 8–20 viable seeds, whereas the wild-type siliques harbored up to 60 seeds. Although the number of ovules formed in these plants was not reported in this work, the expression patterns together with the phenotypes in the flowers and fruits indicate once more that CK play a role during reproduction. Moreover, the authors suggest a role for ANT in the observed reduced cell division in the leaves of *ckx* plants. Considering the documented role of ANT in ovule primordia initiation already introduced in this article, it will be very interesting to analyze also its role in the reproductive tissues of these plants.

With an opposite experimental approach, the simultaneous mutations of two *CKX* genes, it was demonstrated that plants with an increased level of CK had an enhanced activity of the reproductive meristem (Bartrina et al., 2011). Indeed, the *ckx3-1 ckx5-1* double mutant produced more flowers due to a larger inflorescence meristem with more cells than the wild type. Moreover, flowers were bigger and so were the gynoecia. Besides, double mutant gynoecia contained twice as many ovules as wild-type ones, indicating an increased activity of their placental tissue. The *ckx3-1* and *ckx5-1* single mutants already developed more ovules than the wild-type, and the flower size and the number of ovules was reflected into the length of the fruits (siliques of *ckx3 ckx5* were 20 mm long compared with the 17 mm of the wild type) and the seed number (110 seeds in the *ckx3 ckx5* mutant siliques compared with an average of 65 seeds in wild-type siliques, **Table 1**) The authors suggested that CKX3 and CKX5 may regulate the activity of meristematic cells in the placenta thus affecting organogenic capacity and ovule primordia formation.

A conclusive evidence about the relationship between the levels of CK and the initiation of ovule formation was obtained from

experiments in which inflorescences were treated with synthetic CK (6-Benzylaminopurine, BAP). The treatment resulted in the formation of new primordia, 20 ± 3 primordia in average in each pistil, positioned between the ovules already formed before the CK application (Bencivenga et al., 2012). An equivalent CK treatment was also able to increase the ovule number in *pSTK::CUC1_RNAi cuc2* plants already described in this review, by acting on the expression and localization of the auxin efflux carrier PIN1 (Galbiati et al., 2013). These results point out the importance of the cross-talk between CK and auxin during ovule primordia formation. However, the hormonal cross-talk is not limited to auxin and CK since very recently it has been demonstrated that also brassinosteroids (BR) play a crucial role in ovule and seed formation by regulating the expression of genes that control ovule development (Huang et al., 2012), as will be explained in the next paragraph.

THE ROLE OF BRASSINOSTEROIDS

BRs are hormones known to control general plant development. More specifically, they have been described as involved in the control of the initiation and formation of reproductive organs (Szekeres et al., 1996; Kim et al., 2005). Huang et al. (2012) found that the BR-deficient and -insensitive mutants have smaller and less seeds, while BR-enhanced mutants have more seeds. The analysis of the number of ovules and seeds and the morphological analysis of the siliques of *det-2* (a BR-deficient mutant involved in BR biosynthesis), *bri1-5* (the mutant for the BR receptor), heterozygous plants for *bin2-1* (a gain of function mutant deficient in BR signaling) and *bzr1-1D* (a BR signal-enhanced mutant) leaded to the conclusion that BR signaling positively regulates ovule number (**Table 1**) (Huang et al., 2012). Specifically, it was found that the transcription factor BRZ1 plays an important role in ovule and seed number determination, depending on its state of phosphorylation/dephosphorylation (more dephosphorylation implying more activity and more ovules and seeds).

By treating plants with BR it was shown that BR influences ovule development through regulating the transcription of genes such as *HLL* and *ANT*, which are redundant in the control of ovule primordia growth as already introduced in this review (Schneitz et al., 1998), and *AP2*, that affects floral organ (including ovule) pattern formation (Modrusan et al., 1994). *HLL* and *ANT* are clearly induced by BR, while *AP2* is slightly repressed by BR. These genes appeared to be targets of BRZ1, and its state of phosphorylation/dephosphorylation influences the expression of these genes. Further analysis indicated that *AP2* and *ANT* are direct targets of BRZ1, while *HLL* is regulated by an indirect way. The analysis of ovule number of *bzr1-1D* and *ap2-5* single mutants and *bzr1-1D ap2-5* double mutant (**Table 1**), together with other molecular proofs, indicate that BZR1 and AP2 play antagonistic effects in ovule number determination, being BZR1 (and HLL and ANT) promoters and AP2 inhibitor of ovule primordia formation (Huang et al., 2012).

A model for ovule primordia formation that integrates the molecular and hormonal networks has been proposed by Galbiati et al. (2013): MP is required for *ANT*, *CUC1* and *CUC2* expression during the early stages of placenta development and ovule primordia formation, being ANT expressed in the ovule primordia, whereas *CUC1* and *CUC2* in the ovule boundaries. CUC1 and CUC2 may be involved in the increase of CKs required for proper *PIN1* expression needed for primordia formation. Once the primordia have formed, auxin accumulates at the edge of the developing ovule. This model can be easily extended with the recently discovered role of the plant hormones BR, which positively regulate the number of ovule primordia, in part by the direct regulation of *ANT* by BZR1 (**Figure 2**).

OTHER MECHANISMS CONTROLLING OVULE NUMBER: THE EPIGENETIC REGULATION

Interestingly, in different Arabidopsis ecotypes (diploid accessions) a variation in ovule numbers can be observed. Alonso-Blanco et al. (1999) found that the Lansberg *erecta* ecotype presents 20% more ovules than the Cape Verde Islands (Cvi) one (**Table 1**). Recently a considerable genetic variation in ovule number was described in selfed F1 triploids of different *A. thaliana* genotypes (Duszynska et al., 2013). Triploids were obtained by crossing a tetraploid L*er*-0 line (used as a male or female parent) with different diploid accessions. Interestingly, it was observed an effect of the parental genome excess (2m:1p *vs.* 1m:2p) in the determination of the total ovule number in genetically identical F1 hybrid offsprings. These were the first parent-of-origin effects on ovule number in reciprocal triploids of plants. The authors postulate that such effects may represent epigenetic effects, because changes in DNA sequence cannot explain mitotically and/or meiotically heritable changes in gene function but they might be due to changes in DNA methylation, for example (Duszynska et al., 2013). Indeed, in Arabidopsis the ASH1 class of proteins, that can methylate lysine residues on histone tails, maintains an active transcriptional state during development. One of its members, ASH1 HOMOLOG 2 (ASHH2), has been described as a controller of reproductive development via H3K36 trimethylation. Plants homozygous for *ashh2* null alleles presented an 80% reduction in ovule numbers when compared to wild-type plants (**Table 1**) (Grini et al., 2009). These data altogether indicate that epigenetics may also play a role in the control of ovule number, and they open up a new interesting field of research.

CONCLUDING REMARKS AND FURTHER PERSPECTIVES

In the past years, several genes such as *AG*, *STK*, *SHP1*, and *SHP2* have been identified as ovule identity genes, and *ANT*, *HLL*, *SIN2*, *INNER NO OUTER* (*INO*), and *SUPERMAN* (*SUP*) as regulators of ovule outgrowth (Elliott et al., 1996; Schneitz et al., 1997, 1998; Broadhvest et al., 2000; Pinyopich et al., 2003). Nevertheless, most of their targets, which might be the genes that determine the correct development of the ovule, remain to be uncovered. Another quite unknown process is the regulation of the ovule primordia initiation. As explained in this review, only a few regulators, such as the transcription factors ANT, CUC1, CUC2, AP2 and the mitochondrial ribosomal protein HLL have been identified (Elliott et al., 1996; Schneitz et al., 1998; Galbiati et al., 2013). The majority of them are transcription factors, and the transcriptional cascades triggered by them, that will determine the regulation of the morphogenetic parameters such as cell division

and expansion, or expression patterns of identity genes of particular organs, are also largely unknown. Therefore, one of the next challenges would be the identification of downstream targets of these transcription factors by genetic or molecular biological approaches, including suppressor/enhancer mutant screenings or RNA-sequencing transcriptome analyses. It is worth to highlight that these regulators are not exclusively transcription factors, but also mitochondrial proteins or chromatin remodeling factors, indicating that a correct ovule initiation depends on a complex genetic and molecular network.

One of the difficulties of the genetic dissection of ovule initiation and development is that many mutations that affect ovule initiation have already pleiotropic effects on earlier stages of the development of the reproductive tissues, causing floral aberrations that may mask their effects on ovules. Thus, many genes that control ovule development are also involved in primordium initiation and growth of other floral organs (Elliott et al., 1996; Schneitz et al., 1998; Alvarez and Smyth, 1999). Moreover, it is difficult to establish if a mutation in a gene causes a reduction in ovule number if this mutant already has an altered gynoecium phenotype (Alvarez and Smyth, 1999; Western and Haughn, 1999; Broadhvest et al., 2000; Liu et al., 2000; Pinyopich et al., 2003; Nole-Wilson et al., 2010; Nahar et al., 2012). The reanalysis of these carpel mutants, measuring the space between ovules, or expressing the ovule number as the ovule number per millimeter of gynoecium, as some authors already presented (Huang et al., 2012), could contribute to resolve this uncertainty. The use of specialized vectors, for instance containing placenta-specific promoters to obtain milder vegetative and/or floral effects of these mutations would help to uncover the role of specific factors in ovule development. Besides, a reverse-genetic strategy using RNA interference or insertional mutants can be used to identify new regulators of ovule numbers determination.

Ovule boundary establishment is still a poorly understood process, and only CUC1 and CUC2 have been demonstrated to play a role (Galbiati et al., 2013). The contribution to the determination of ovule boundaries of the genes that have been described to regulate or interact with the *CUCs* in other organ boundaries would be worth to be analyzed, by means of the study of their patterns of expression and how these are accurately determined. The identification and characterization of single and multiple mutants, as has been done for the *CUC* genes (Aida et al., 1997; Galbiati et al., 2013) is also key to study their roles. Moreover, the analysis of their incidence at the cellular level will help to define the effects on cell behavior (i.e., division or expansion) that these factors could have. It has also been widely demonstrated that hormones play a role in the regulation of ovule primordia initiation, being auxin, CK and more recently also BR identified as the important hormonal players in this process. The crosstalk between these hormones, as Bencivenga et al. (2012) and Galbiati et al. (2013) present in their works, is starting to be revealed (**Figure 2**) and it will be very interesting to investigate in the future how auxin, CK and BR interact. Moreover, it will be important to explore if hormone and gene expression levels are responsible for the variation in ovule numbers described for the different ecotypes (Alonso-Blanco et al., 1999), and to identify QTLs linked to this trait.

Based on the experimental data exposed in this review, a similarity between ovule initiation and the initiation of other lateral organs in the plant can be proposed. The strongest pieces of evidence are the triggering role of auxins and the conservation in the genes that establish the boundaries and promote new organ growth. Although further studies will be needed in order to identify the common and specific players of the different lateral organ initiation processes, conserved modules can be already suggested. In the case of flower primordia initiation, similarly to what happens during ovule primordia formation, the coordinate action of MP and ANT is required. In particular, at the reproductive shoot apex, auxin-activated MP directly induces *ANT*, other two key regulators of floral growth, *LEAFY* (LFY) and *AINTEGUMENTA LIKE-6* (*AIL6*), and probably a forth unknown factor, which together lead the flower primordium initiation (Yamaguchi et al., 2013). Also the factors determining the new organ boundary seem conserved between ovule and flower primordia initiation: the coordinated spatial and temporal action of auxin, PIN transporters and CUC proteins is required (Heisler et al., 2005; Galbiati et al., 2013). If we instead compare the initiation of ovule primordia with the initiation of LR we also find many common players, despite the clear fact that ovule primordia arise from the naked placenta while LR have to pass through several cell layers to emerge. Thus, we find an auxin maxima that precedes organ formation (Benková et al., 2003). Also other hormones, such as BR and CK play a role in both ovule and LR initiation, although CK play opposite roles (it activates ovule primordia formation while inhibits LR initiation) (Werner et al., 2003; Higuchi et al., 2004; Bartrina et al., 2011; Huang et al., 2012; Bianco et al., 2013; Chang et al., 2013). Besides, the participation of IAA/AUX-ARF modules exists in both processes, and MP seems to be a regulator of the two of them (De Smet et al., 2010; Galbiati et al., 2013). Other members of the ARF family, as well as the NAC and the MADS-box transcription factors could be conserved in both processes, as some introductory works seem to indicate (Pinyopich et al., 2003; Moreno-Risueno et al., 2010; reviewed in Benková and Bielach, 2010). Finally, downstream the auxin signaling cascades, the activation of cell cycle genes will take place in order to promote organ growth, as it is starting to be revealed in the case of LR (Rast and Simon, 2008). Thereby, the analysis of the expression of cell cycle genes during ovule primordia formation would be very revealing. Apart of the hormonal and molecular pathways controlling LR formation, the influence of the environmental factors on this process is of extreme importance for the plant. How environment influences ovule primordia formation would be for sure a very challenging topic of research.

With this work we wanted to point out the little specific information available about the factors that control ovule primordia initiation, due to the difficulties to identify mutants presenting defects only in this particular step of ovule formation. Here we propose different experimental approaches to overcome the severity of some mutant phenotypes as well as to investigate these processes from a new point of view. The contribution and conservation of chromatin remodeling changes to the regulation of ovule number is starting to be elucidated and opens an extremely interesting field of research. Moreover, the most recent progresses

in the fields of ovule, flower and root development strongly suggest common hormonal and molecular signals in all these organ initiation processes; such as a crosstalk between auxin and CK and probably also BR and the factors that establish organ boundaries and those that promote new organ outgrowth.

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What lies beyond the eye: the molecular mechanisms regulating tomato fruit weight and shape

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Domestication of fruit and vegetables resulted in a huge diversity of shapes and sizes of the produce. Selections that took place over thousands of years of alleles that increased fruit weight and altered shape for specific culinary uses provide a wealth of resources to study the molecular bases of this diversity. Tomato (*Solanum lycopersicum*) evolved from a wild ancestor (*S. pimpinellifolium*) bearing small and round edible fruit. Molecular genetic studies led to the identification of two genes selected for fruit weight: *FW2.2* encoding a member of the Cell Number Regulator family; and *FW3.2* encoding a P450 enzyme and the ortholog of KLUH. Four genes were identified that were selected for fruit shape: *SUN* encoding a member of the IQD family of calmodulin-binding proteins leading to fruit elongation; *OVATE* encoding a member of the OVATE family proteins involved in transcriptional repression leading to fruit elongation; *LC* encoding most likely the ortholog of WUSCHEL controlling meristem size and locule number; *FAS* encoding a member in the YABBY family controlling locule number leading to flat or oxheart shape. For this article, we will provide an overview of the putative function of the known genes, when during floral and fruit development they are hypothesized to act and their potential importance in regulating morphological diversity in other fruit and vegetable crops.

Keywords: tomato, fruit morphology, gene regulation

INTRODUCTION

Angiosperm plants vary tremendously in morphological traits related to their reproduction. The floral appearance is driven by evolutionary aspects of the pollination syndrome whereas distinct dispersal modes drive the evolution of phenotypes associated with the fruit. In natural settings, the main functions of the fruit are to protect the developing seeds and to act as a dispersal agent. The onset of the change to an agricultural lifestyle, approximately 10,000 years ago, provided strong selection pressures on the fruit of incipient vegetable and fruit crops. The selections made by early farmers offer a great opportunity to identify the molecular basis of a range of phenotypic traits, especially those related to fruit morphology and flavor. For example, selections against bitter taste resulted in palatable eggplant and cucumber (Wang et al., 2008; Qi et al., 2013). Yet, the underlying principle for nearly all cultivated vegetable and fruit crops was the selection for larger and more nutritious fruits featuring a variety of shapes (Paran and Van Der Knaap, 2007; Pickersgill, 2007; Meyer and Purugganan, 2013) (**Figures 1A–C**). The larger fruit became more nutritious as a result of the increase in the edible and fleshy part of the fruit at the expense of the seed part for most domesticated fruits and vegetables.

The focus of the "hypothesis and theory" article is to summarize the current knowledge on the function of genes that change tomato fruit weight and shape resulting from domestication and diversification process. The focus on tomato is based on the extensive research that resulted in the cloning of six fruit shape and weight genes from this species in recent years. The predicted function of these genes will be discussed in the context of the phases of development where we hypothesize the impact of the mutant alleles is most critical. It is important to recognize that the mutations are not often resulting in complete nulls, i.e., a loss-of-function allele. Thus, the complete repertoire of functions of the tomato fruit shape and weight genes may not be apparent from the phenotype observed in the natural mutants. We will propose the pathways in which the shape and weight proteins function. We will also include the molecular basis of the underlying mutations that gave rise to the derived alleles and demonstrate that inversions, duplications, as well as single nucleotide polymorphisms (SNPs) in promoters and coding regions underlie the phenotypic diversity of the tomato fruit.

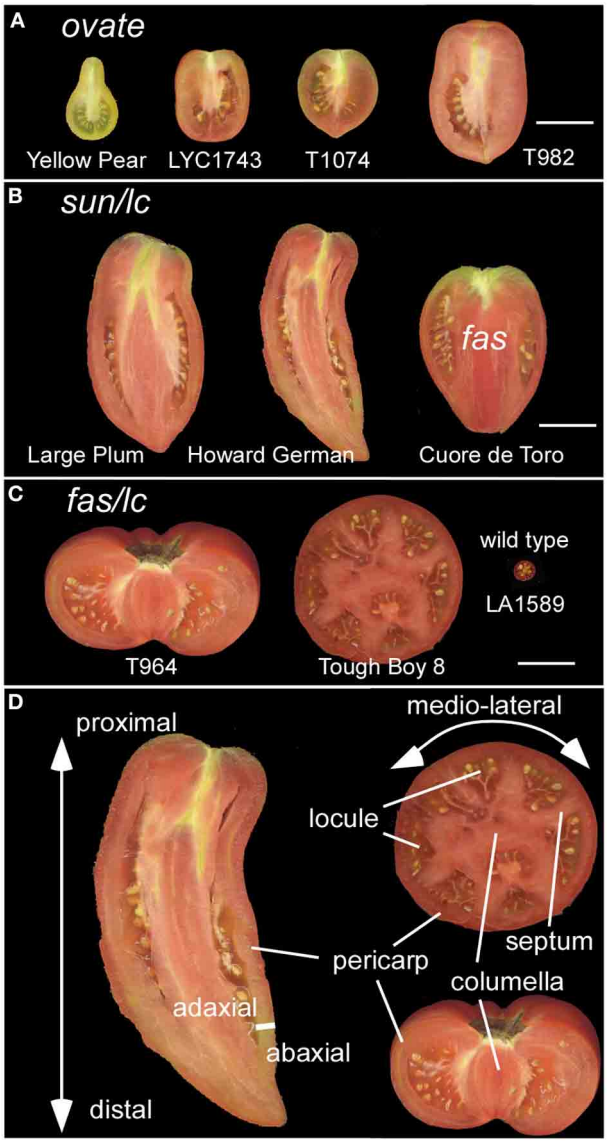

FIGURE 1 | Diversity in tomato fruit shapes. (A) Tomato varieties carrying the *ovate* mutation result in obovoid and ellipsoid fruit shapes. Size bar = 3 cm. **(B)** Tomato varieties carrying the *sun* and *lc* mutation result in a long fruit shape. The oxheart shaped tomato also carries *fas* in addition to *sun* and *lc*. Size bar = 3 cm. **(C)** Tomato varieties carrying *fas* and *lc* result in a flat fruit with many locules. The wild type represents the fruit from an ancestor of cultivated tomato, *S. pimpinellifolium* LA1589. Size bar = 3 cm. **(D)** The axes of growth: proximal (closest to the stem) to distal (farthest away from the stem); medio-lateral; and the adaxial (closest to the meristem) to abaxial (farthest from the meristem). The fruit tissues that give structure to the organ are highlighted as the pericarp, septum, columella and locule. Not indicated is the placenta which is the tissue extending from the columella and surrounds the seeds.

OVERVIEW OF TOMATO DEVELOPMENT

Even though the fruit is a terminal structure that forms relatively late in the plant's lifecycle, the formation of this organ and the parameters that determine its final dimensions are rooted much earlier in the plant's lifespan. Therefore, it is important to view tomato fruit development in the context of overall plant development starting after germination. Plant growth in tomato and other Solanaceous plants is characterized by a sympodial shoot architecture where after formation of 8–10 leaves, the shoot apical meristem (SAM) terminates into the inflorescence meristem (IM), and growth continues from lateral meristems called sympodial meristems (SYM). Meanwhile, the IM terminates into the floral meristem (FM) generating the flower (Schmitz and Theres, 1999). The tomato inflorescence also features a sympodial structure since a new IM emerges simultaneously from the flank of the first FM, terminating again in the second FM on the inflorescence and so on (**Figure 2A**). This growth pattern is referred to as cymose and results in a zigzag of flowers on a tomato inflorescence (Welty et al., 2007; Lippman et al., 2008; Castel et al., 2010). In most angiosperm species, FMs give rise to four whorls: the sepals, petals, stamens and carpels. Organ identity genes play critical roles to ensure that carpel primordia arise from specified founder cells within the FM (Causier et al., 2010) (**Figures 2A,B**). In addition to cell specification, the establishment of the boundaries between and within the primordia is required to ensure that the appropriate identities and division patterns are initiated and maintained throughout gynoecium growth (Dinneny and Yanofsky, 2005; Balanza et al., 2006; Girin et al., 2009). This step is critical to lay the foundation of growth of the organs along three axes: the proximal-distal, the medio-lateral and the abaxial-adaxial axis (**Figure 1D**). A mature tomato gynoecium coincides with flower opening which marks the anthesis and pollen release stage (Xiao et al., 2009). Following pollination and fertilization of the ovules, fruit development is initiated which is marked by a rapid increase in cell proliferation followed by cell enlargement (Gillaspy et al., 1993; Xiao et al., 2009) (**Figures 2G,H**). In most fruit tissues such as the pericarp, cell division ceases 5–10 days after anthesis and growth of the fruit continues by extensive cell enlargements that last for three to 5 weeks until the fruit ripening stage (Gillaspy et al., 1993; Xiao et al., 2009).

CRITICAL DEVELOPMENTAL STAGES BEFORE ANTHESIS AT WHICH THE FINAL SHAPE AND WEIGHT OF FRUIT IS REGULATED

The final dimensions of the fruit are regulated during multiple stages throughout the development of the plant. These stages occur before and after anthesis, and may be initiated as early as in the SAM. Thus, the first stage of regulation of the final fruit dimensions is likely to occur in the meristems as a result of their size (**Figure 2A**; **Table 1**). Since the gynoecium is a terminal structure, the size of the FM may impact the number of cells that are specified to form a carpel primordium as well as the number of primordia (Szymkowiak and Sussex, 1992; Clark et al., 1993; Taguchi-Shiobara et al., 2001; Suzaki et al., 2004). Cell identity and the positioning of organ primordia *per se* however are not controlled by the size of the FM. Therefore, the second stage of regulation is likely controlled by the organization within the meristem which relates to where and how often in the meristem the cells that are destined to become carpel primordia arise (**Figures 2A,B**). Similarly as for leaf primordia initiation, localized auxin maxima controlled by the auxin efflux protein PIN1

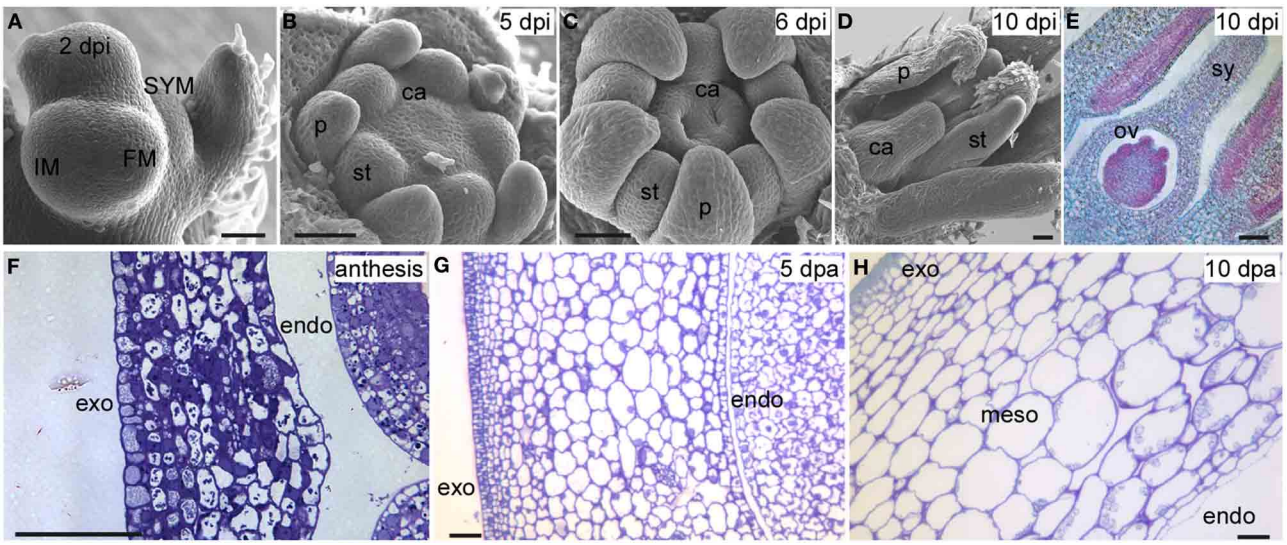

FIGURE 2 | Tomato floral and fruit development in *S. pimpinellifolium* LA1589. (A) The IM terminates into the FM, which develops into a floral bud 2 dpi. The SYM is emerging from the flank of the youngest leaf primordium. **(B)** Emergence of carpel primordia 5 dpi. **(C)** Growth of carpels and formation of two locules 6 dpi. **(D)** Growth of the style. **(E)** Formation of the ovules 10 dpi. **(F)** A transverse section of the pericarp at anthesis. **(G)** Transverse section of the pericarp 5 dpa. **(H)** Transverse section of the pericarp 10 dpa. SYM, sympodial meristem; IM, inflorescence meristem; FM, floral meristem; dpi, days post floral initiation; dpa, days post anthesis; p, petal; st, stamen; ca, carpel; sy, style; ov, ovule; exo, exocarp; endo, endocarp; meso, mesocarp. Size bar is 50 μm.

Table 1 | Developmental phases proposed to control fruit shape and weight.

Critical regulatory phases of fruit shape and weight	Developmental event[1]	Landmark[1]	Cellular events in the ovary or fruit	Days after meristem initiation	Stage-specific fruit shape and weight genes
Phase 1	Inflorescence and floral meristem formation	Floral landmark 1	Cell number, size of the stem cell niche	0	*LC/FAS/CNR*
Phase 2	Floral meristem organization	Floral landmark 1	Cell identity and boundary information	1	*FAS/CNR*
Phase 3	Gynoecium initiation	Floral landmark 5	Cell proliferation and enlargement	5–6	*OVATE/SUN*
Phase 4	Gynoecium growth	Floral landmark 6–9	Redifferentiation of tissue types	8–16	
Phase 5	Anthesis	Floral landmark 10 and fruit landmark 1	Flower opening	19	
Phase 6	Fertilization and 4–16 cell stage embryo	Fruit landmark 2–3	Cell proliferation	20–25	*SUN/KLUH*
Phase 7	Globular to coiled stage embryo	Fruit landmark 4–7	Cell enlargement	25–39	

[1] *From Xiao et al. (2009).*

and the expression of PLETHORA/AINTEGUMENTA transcription factors are thought to control floral organ positioning (Benkova et al., 2003; Krizek, 2011; Van Mourik et al., 2012; Hofhuis et al., 2013). The areas of low auxin coincide with the boundaries between primordia which are also tightly controlled processes (Nahar et al., 2012; Zadnikova and Simon, 2014). Misalignment during this stage would result in changes in final fruit morphology. The third stage is the phase that transmits positioning information to gynoecium growth (**Figures 2B,C**, **Table 1**). During this phase, the three axes of growth have been specified along which cell proliferation and enlargement occurs (Dinneny et al., 2005; Ostergaard, 2009). Cell proliferation, which is characteristic of this stage, consists of the rate and duration of the cell divisions within the developing ovary impacting final organ dimensions (**Figure 2C**). Also critical are the differential rates and duration of cell division within distinct tissues in the developing ovary, resulting in alternatively shaped fruit. For example, ovary and fruit length is determined by the degree of growth along the proximal-distal axis whereas width is determined by the degree of growth in the mediolateral axis (**Figure 1D**). The degree of the pericarp thickness and other internal tissues is determined along the abaxial-adaxial axis. Therefore, enhanced cell divisions preferentially along one axis of growth are proposed to lead to a different shape fruit as opposed to enhanced cell divisions along all three axes of growth. The fourth stage occurs concomitantly with the third stage which

is the continued specification of new tissue types through reactivation of the meristematic potential leading to the formation of many tissue types (Girin et al., 2009) (**Figures 2D,E**). Along the proximal-distal axis, the gynoecium develops two additional regions: the stigma and style. Along the medio-lateral axis, the ovary develops the placenta, ovules and transmitting track tissues. Along the abaxial-adaxial axis the ovary continues to maintain the polarities within the different tissues such as the pericarp, septum, placenta and ovules. The reinforcement to maintain the different zones is mediated by transcription factors in conjunction with boundary genes (Heisler et al., 2001; Nahar et al., 2012).

CRITICAL DEVELOPMENTAL STAGES AFTER ANTHESIS AT WHICH THE FINAL SHAPE AND WEIGHT OF FRUIT IS REGULATED

The anthesis/pollination/fertilization phase marks the end of ovary development and the beginning of fruit development. Lack of or poor fertilization leads to changes in fruit shape and reduced weight, marking the fifth phase. Aborted fruit is terminal and should not been considered to be part of phase 5. The first stage post-anthesis is the sixth phase proposed to correspond to the cell proliferation stage, a rapid increase in cell division throughout the developing fruit that follows immediately after fertilization (Gillaspy et al., 1993; Xiao et al., 2009) (**Figures 2F,G**). As in the ovary, this stage is comprised of differing cell division rates and duration in the tissues of the fruit that would greatly impact final fruit shape. The seventh and final stage is proposed to be cell enlargement which impacts overall fruit size the most (**Figure 2H**). Cell enlargement is regulated differentially in the various tissues within the fruit, and rates and duration determine the final fruit dimensions. For example, the columella and placenta tissues contain more large cells than the pericarp. Additionally within the pericarp, the exocarp cells (constituting the epidermis) are very small whereas the mesocarp cells are large (**Figure 2H**).

TOMATO FRUIT WEIGHT AND SHAPE ALLELES ACTING PRE-ANTHESIS

LOCULE NUMBER

LOCULE NUMBER (*LC*) controls the number of carpel primordia and a mutation results in a fruit with more than the typical two to three locules (Barrero et al., 2006; Munos et al., 2011). Increases in locule number often lead to a flat fruit of a larger size and the mutation is common in beefsteak tomato and tomatoes on the vine (Munos et al., 2011; Rodriguez et al., 2011) (**Figure 1C**). Since carpel primordia arise early in floral development, it is likely this gene functions in regulating meristem size and/or in the initiation of organ primordia. The locus was fine mapped to a 1608 bp region located between a putative ortholog of *WUSCHEL* (*WUS*) (annotated gene ID Solyc02g083950, available at http://solgenomics.net/) and a WD40 motif containing protein (Solyc02g083940). Further association mapping led to the identification of two single nucleotide polymorphisms located 1080 bp downstream of the putative tomato ortholog of *WUS* (Munos et al., 2011) (**Figure 3**). *WUS* encodes a homeodomain transcription factor that is required for maintaining the stem cell identity in the SAM (Mayer et al., 1998; Clark, 2001). The WD40

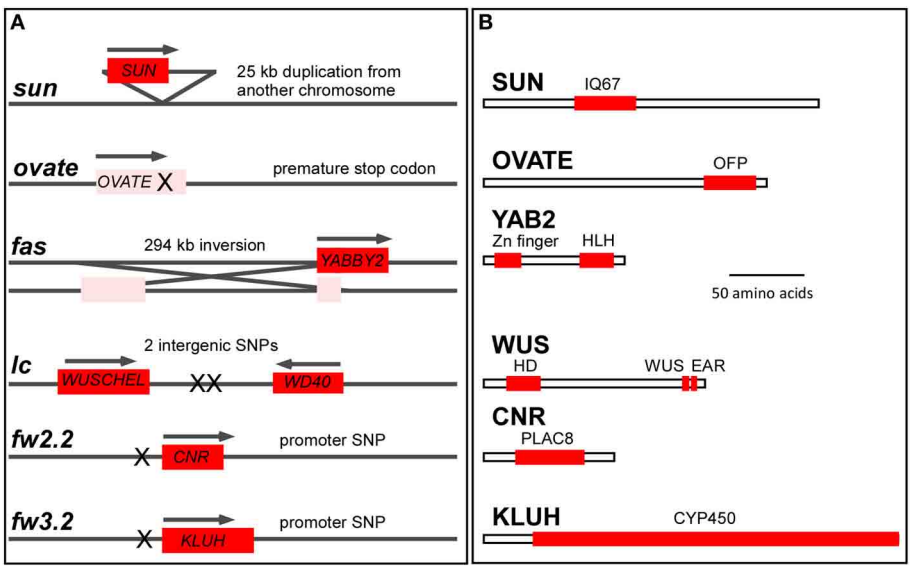

FIGURE 3 | The molecular basis of tomato fruit shape and weight variation. (A) Genome structure of the fruit shape and weight loci and the underlying mutations. Red box indicates the coding region of a functional gene whose regulation is altered by the mutation (denoted by X). Pink indicates a loss-of-function mutation of the gene. The size of the loci are not drawn to scale. **(B)** Protein features of the fruit shape and weight proteins. The box represents the coding region. The most important domains are listed as red boxes. IQ67, CaM binding domain of 67 amino acid and containing IQ; OFP, Ovate Family Protein motif of unknown function; HLH, YABBY type of DNA binding domain featuring a helix-loop-helix structure; HD, DNA binding homeodomain of the helix-loop-helix-turn-helix structure; WUS, essential for proper functioning of WUSCHEL; EAR, transcriptional repressor function; PLAC8, similarity to the placenta-specific gene 8 protein; CYP450, cytochrome P450. Size bar = 50 amino acids.

containing motif protein belongs to a large family involving in diverse functions ranging from signal transduction to transcriptional regulation (Ullah et al., 2008). Increased expression of *WUS* in Arabidopsis leads to increased floral organ number, which is similar to the phenotype found in the *lc* mutant (Mayer et al., 1998; Clark, 2001). Therefore, based on the predicted function *SlWUS* is the most likely candidate to underlie *lc*, impacting the first phase that regulates the final dimension of the tomato fruit (**Figure 2A** and **Tables 1**, **2**). Similar to Arabidopsis, *SlWUS* is expressed in the youngest floral buds and the shoot apex and virtually undetectable in other tomato tissues (**Figure 4A**). Its expression is also high in the IM/FM tissues, decreasing very rapidly as floral development progresses (**Figure 4B**).

WUS is critical in the regulation of the stem cell population size in all meristems, yet the *lc* mutation itself does not lead to dramatic changes in *SlWUS* gene expression compared to wild type (Munos et al., 2011). Therefore, the high locule number phenotype is likely due to subtle changes in expression that were not captured by the method of gene expression quantification. WUS positively regulates the expression of the MADS box transcription factor *AGAMOUS* (*AG*) (Lenhard et al., 2001; Lohmann et al., 2001) and AG is critical in determining stamen and gynoecium identity (Yanofsky et al., 1990). Therefore, WUS-induced expression of *AG* links meristem activities to organ identity processes. AG in turn down-regulates expression of *WUS* providing the mechanism for changing stem cell identity of the remaining FM to carpel identity (Lohmann et al., 2001; Liu et al., 2011). In Arabidopsis, *WUS* down-regulation is mediated by two downstream CArG cis-regulatory elements to which AG binds, resulting in the epigenetic silencing of *WUS* (Tilly et al., 1998; Liu et al., 2011). Intriguingly, the two SNPs located downstream of tomato *WUSCHEL* are located in a putative tomato CArG cis-regulatory element (**Figure 5A**). This suggests that the *lc* mutation causes a loss-of-function regulatory element permitting higher expression of *SlWUS* and maintenance of a larger stem cell population resulting in increased locule numbers. Furthermore, this finding implies that the *lc* mutation acts at the transition from stem cell identity to carpel identity acting just prior to the stage shown in **Figure 2B**. Other critical components of the WUS signaling pathway are provided by the CLAVATA (CLV) proteins (Clark, 2001; Brand et al., 2002; Lenhard and Laux, 2003). In particular, the WUS and CLV3 feedback loop is tightly linked to the regulation of meristem size in Arabidopsis (Schoof et al., 2000), suggesting that members of the CLV pathway may be involved in the regulation of tomato meristem size and its organization

Table 2 | List of genes controlling fruit weight and shape variation in tomato.

Locus/QTL	Underlying gene ID	Putative cellular/molecular function and length of the protein	Timing of the impact on morphology	Most likely cause of allelic variation	References
fw2.2	*Cell number regulator (CNR)* Solyc02g090730	Increased expression is associated with reduced cell division. May permit transport across membranes. Protein may be located at the plasmamembrane and contains a PLAC8 domain including two putative transmembrane motifs. 163 aa	Phase 1 or 2 (**Figures 2A,B**)	SNP in the promoter of the gene	Frary et al., 2000; Guo et al., 2010
fw3.2	*KLUH* Solyc03g114940	A cytochrome P450 of the 78A class and the likely ortholog of *AtKLUH*. Hypothesized to synthesize a mobile signal. Substrate unknown. 516 aa	Phase 5, (**Figure 2G**)	SNP in the promoter of the gene	Anastasiou et al., 2007; Chakrabarti et al., 2013
lc	*WUSCHEL* Solyc02g083950	Homeobox domain protein. Required to maintain stem cell identity in meristems. 73 aa	Phase 1, (**Figure 2A**)	Two SNP located downstream of *WUSCHEL*	Mayer et al., 1998; Munos et al., 2011
fasciated	*YABBY2* Solyc11g071810	Transcription factor involved in organ polarity and meristem organization. 177 aa	Phase 1 or 2, (**Figures 2A,B**)	Gene knock out by a 294 kb inversion with a breakpoint in the first intron of *YAB2*	Cong et al., 2008; Huang and Van Der Knaap, 2011; Huang et al., 2013
ovate	*OVATE* Solyc02g085500	Increased expression is associated with shorter plants and plant organs. May be a repressor of transcription. Contains the OFP domain. 352 aa	Phase 3, (**Figures 2B,C**)	Premature stop codon in an exon associated with a mutant phenotype	Liu et al., 2002; Hackbusch et al., 2005; Huang et al., 2013
sun	*SUN* Solyc10g079240	Increased expression is associated with elongated fruit. Positive regulator of growth. Contains the IQ67 motif that binds calmodulin. 421 aa	Phase 3 and 6, (**Figures 2B,C,G**)	Interchromosomal gene duplication mediated by the transposon *Rider*	Abel et al., 2005; Xiao et al., 2008; Huang et al., 2013

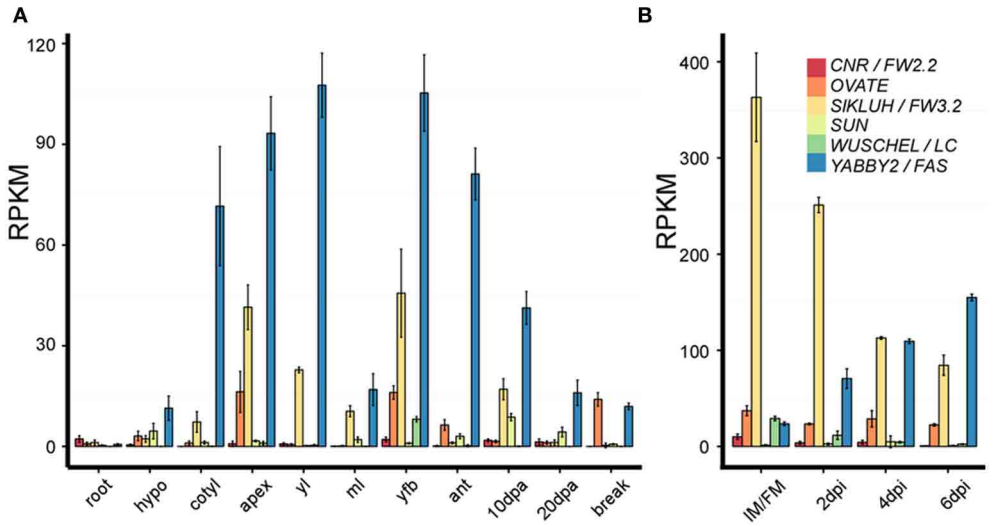

FIGURE 4 | Expression analysis of the six fruit shape and weight genes in different tissues and at different developmental stages. Samples were collected from the *S. pimpinellifolium* accession LA1589. The data was obtained from 3 to 4 biological replicate RNA samples that were sequenced using the HiSeq2000 Illumina sequencing technology (Huang et al., 2013) (http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi). The expression was normalized using the reads per kilobase per million mapped reads of each gene model (RPKM). **(A)** The root, hypocotyl (hypo), cotyledon (cotyl) and shoot apex including the SAM (apex) were collected from the same seedlings germinated in petri dishes. All other tissues were collected from mature plants grown in the greenhouse (Huang et al., 2013). yl, young leaves; ml, mature leaves; yfb, young floral buds from 10 dpi and younger; ant, whole flower at anthesis; 10 and 20 dpa, developing fruit 10–20 days after anthesis; break, breaker stage fruit which is immediately before turning color. **(B)** IM/FM, 2, 4, 6 dpi flower buds that were fixed in RNAlater solution. The tissues were hand-dissected using a dissecting scope prior to RNA isolation. Each replicate out of 3 is represented by 100–150 samples that were pooled prior to RNA extraction.

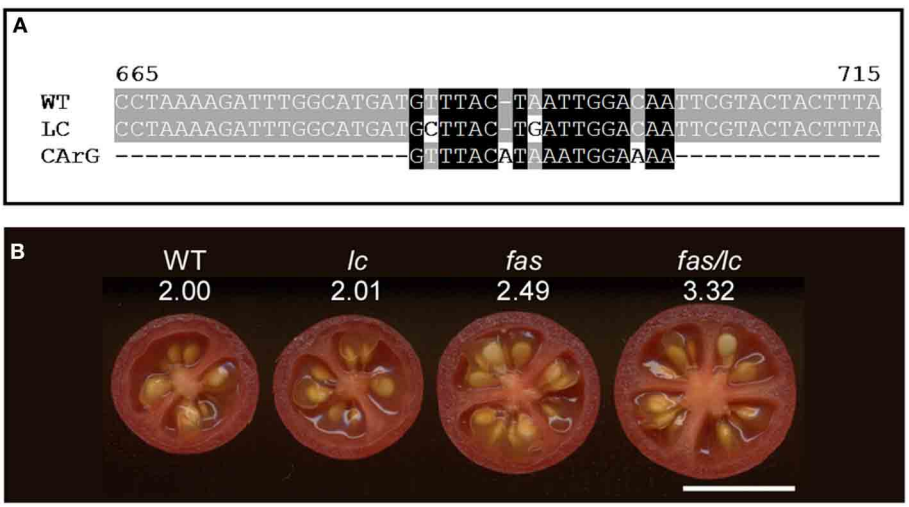

FIGURE 5 | The effect of *lc* and *fas* loci on locule number in tomato. **(A)** Alignment of the wild type (accession JF284938) and mutant (JF284939) *LC* allele sequences with the canonical MADS box transcription factor CArG1 binding sequence (Tilly et al., 1998). The two mutations in *LC* reduce the alignment to the consensus sequence. **(B)** The effect on locule number in the *fas*, *lc* and the double *fas/lc* NIL compared to wild type (WT). The number below the NIL indicates the average locule number from over 40 fruit evaluated each from 5 plants. The increase in locule number in the double NIL indicates synergistic interactions of the two mutations. Size bar = 1 cm.

leading to changes in locule number and the final shape of the fruit.

FASCIATED

The mutation in *FASCIATED* (*f* or *fas*) leads to increases in locule number with more pronounced effects on locule number than *lc* (Lippman and Tanksley, 2001). *fas* is found in certain heirloom tomatoes and a few commercially grown beefsteak varieties (Rodriguez et al., 2011) (**Figures 1B,C**). In addition to increased locule number, the *fas* mutation results in increased number of all floral organs (Lippman and Tanksley, 2001; Barrero and Tanksley, 2004). Significant epistatic interactions have been

detected between *lc* and *fas* (Lippman and Tanksley, 2001; Barrero and Tanksley, 2004), suggesting that both genes act together by co-regulating a core pathway that controls locule number. *FAS* was fine mapped to the bottom of chromosome 11 and, contrary to previously reported results, the mutation resulted from a 294 kb inversion with one of the breakpoints in the first intron of a member of the *YABBY* family creating a null mutation (Huang and Van Der Knaap, 2011). This *YABBY* member, *SlYABBY2* (*YAB2*) is considered to underlie *fas* (Cong et al., 2008) (**Figure 3**). Compared to any other fruit shape or weight gene, *YAB2* expression is very high in cotyledons, shoot apex, young leaves, young floral buds, and anthesis stage flowers (**Figure 4A**). In IM/FM and developing floral buds, its expression is relatively low in the meristem but increases in flower buds 6 days after initiation (**Figure 4B**).

The *YABBY* family of transcription factors is known to control the abaxial-adaxial polarity of SAM, IM, and FM, while also specifying the cell fate of the abaxial region in lateral organs. YABBY proteins function redundantly with other polarity proteins and are required to establish the proper boundaries within the meristem and developing organ primordia (Bowman and Smyth, 1999; Bowman et al., 2002). Moreover, YABBYs have been shown to impact the signaling from lateral organs to the meristem and coordinately maintain the normal growth of meristem in Arabidopsis and rice (Goldshmidt et al., 2008; Tanaka et al., 2012). Because of the function of YABBY family proteins and its expression pattern, we consider that *FAS* is controlling the second stage of final fruit size and shape regulation by impacting meristem organization and boundary information (**Figure 2B**, **Table 2**). However, because of its epistatic interaction with *LC*, it is also possible that *FAS* impacts meristem size as well as organization (**Figure 2A**). The details of how YAB2 impacts locule number are not well understood.

Of the two loci controlling locule number, *lc* and *fas*, the former mutation is much more widespread in the tomato germplasm than the latter while the latter has a more dramatic effect on locule number resulting in up to countless locules per fruit (Munos et al., 2011) (**Figures 1B,C**). In near-isogenic lines (NILs) using the wild species LA1589 as the background, the impact of these two genes on locule number is much less dramatic (**Figure 5B**), supporting the notion that in the cultivated background modifiers of these mutations exist. Further genetic analyses would reveal the molecular nature of those modifiers. The epistatic interaction between the two loci is clearly evident in the wild species background as locule number increase in the double NIL is higher than the sum of locule number found in the single NILs (**Figure 5B**).

OVATE

The shape of many ellipsoid and obovoid varieties such as those found in grape tomato is controlled by the gene that regulates fruit elongation, *OVATE* (Ku et al., 1999; Liu et al., 2002; Rodriguez et al., 2011) (**Figure 1A**). The gene was fine mapped to chromosome 2 and the mutation resulted in a premature stop codon in a newly defined class of plant proteins, Ovate Family Proteins (OFP) (Liu et al., 2002; Hackbusch et al., 2005) (**Figure 3** and **Table 2**). The expression of wild type OVATE is the highest in the shoot apex, youngest floral buds and breaker stage fruit (**Figure 4A**). Additionally, even though *OVATE* expression is the highest in the IM/FM, expression is reduced by only ∼30% in flower buds 2, 4, and 6 days after initiation (**Figure 4B**); the latter stage corresponds to the stage shown in **Figure 2C**. Not all tomato varieties that carry the *ovate* mutation display an elongated shape which led to the mapping of two suppressor loci, *sov1* and *sov2*, on chromosomes 10 and 11, respectively (Rodriguez et al., 2013). These suppressors are thought to play important roles in the regulation of shape mediated by the OVATE pathway. OVATE does neither affect floral organ identity, FM organization nor floral organ number (Liu et al., 2002). Instead, OVATE appears to have a specific role in the regulation of anisotropic growth along the proximal-distal axis at the proximal end of the fruit (**Figure 6**). Near-isogenic lines carrying the *ovate* mutation show that shape is already determined at anthesis (Van Der Knaap and Tanksley, 2001) (**Figure 6A**) and obovoid shape gradually decreases during the development of the fruit (**Figures 6B,C**).

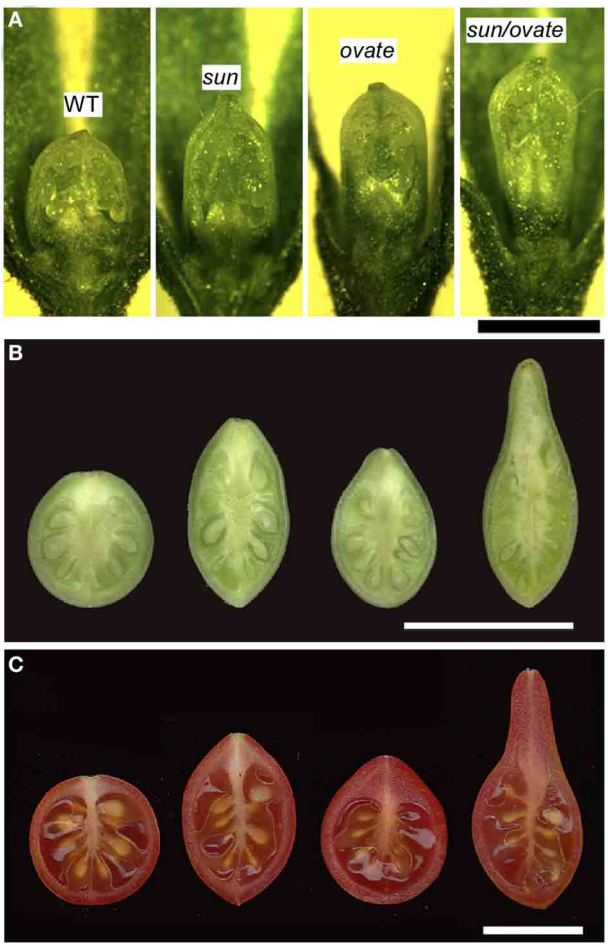

FIGURE 6 | The effect of the *sun* and *ovate* loci on fruit elongation. (A) Effect of wild type (WT), *sun*, *ovate* and *sun/ovate* on ovary shape at anthesis. Size bar = 1 mm. **(B)** Effect of WT, *sun*, *ovate*, and *sun/ovate* on fruit shape 10 days post anthesis. **(C)** Effect of WT, *sun*, *ovate* and *sun/ovate* on mature fruit shape. The shape in the double NIL indicates synergistic interactions of the two mutations. Size bar in B and C = 1 cm.

The molecular function of OVATE and its family members are not well understood. Yeast two Hybrid (Y2H) screens using Arabidopsis KNOX and BELL transcription factors as bait led to the identification of OFP members, lending support for the notion that OVATE interacts with patterning genes that impact fruit shape at the early stages of gynoecium development (Hackbusch et al., 2005; Wang et al., 2010). OFP members have also been shown to repress transcription (Wang et al., 2007, 2011) and overexpression of *AtOFP1* leads to dwarf phenotypes in Arabidopsis and tobacco, in part by negatively regulating the transcription of *GA20ox1*, a key gene in the gibberellin biosynthesis pathway (Hackbusch et al., 2005; Wang et al., 2007). Contrary to findings in Arabidopsis, Y2H of the tomato OVATE protein as bait did not lead to the identification of transcription factors including KNOX or BELL. Instead, 11 out of 26 members of the TONNEAU1 Recruiting Motif (TRM) superfamily were identified including the putative ortholog of AtTRM17/20 (**Figure 7** and **Table 3**). Of all interacting clones obtained, 63.8% belonged to the TRM family. The TRM clones identified from the screen were partial clones and the overlap between interacting clones of the same gene is highlighted in orange (**Figure 7**). TRMs interact with TONNEAU1a (TON1a), TON1b and TON2/FASS proteins, which play critical roles in preprophase band formation and microtubule array organization (Camilleri et al., 2002; Azimzadeh et al., 2008; Spinner et al., 2010, 2013; Drevensek et al., 2012). This finding suggests that OFPs interact with TRMs and microtubules in addition to acting as transcriptional repressors, and thus could provide a mechanistic link between organ patterning and growth. TON1a, TON1b and TON2 interact with the TRM via the M2 and M3 motifs, respectively whereas the TRM motif that recognizes OVATE has not yet been identified. Most single knockouts of Arabidopsis *OFP*s exhibit no or mild phenotypes (Pagnussat et al., 2007; Li et al., 2011; Wang et al., 2011). On the contrary, the premature stop codon mutation found in tomato *OVATE* causes a dramatic morphological change in ovary shape, suggesting it may be a unique member of the family. These findings together suggest that OVATE acts early in carpel development, possibly during phase 3 corresponding to the link between primordia initiation and positioning to growth of the developing carpels.

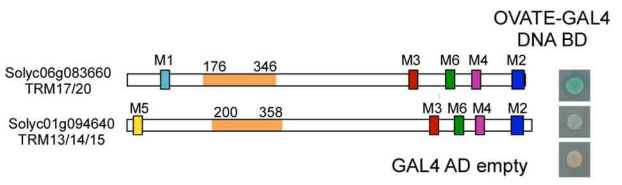

FIGURE 7 | Protein structure of two TONNEAU1 Recruiting Motif (TRM) proteins that interact with tomato OVATE in a Y2H study. Full length OVATE was used as bait and a truncated form of tomato TRM proteins were used as prey. The clones were grown on medium selecting for the bait and prey plasmids whereas the X-α-Gal staining highlights the strength of the interaction. The most likely Arabidopsis ortholog is listed below the gene annotation number. Solyc06g083360 interacts strongly and Solyc01g094640 interacts weakly with OVATE. The colored boxes designate the M1-M6 motifs defining the TRM family (Drevensek et al., 2012). The orange domain in the protein is the overlapping region of different prey fragments identified in the Y2H screen and the numbers indicate amino acid positions.

SUN

SUN controls fruit elongation, including those found in commercially grown plum tomatoes, the very long and tapered shaped heirloom and oxheart tomatoes (Rodriguez et al., 2011) (**Figure 1B**). *SUN's* effect on fruit elongation is much more pronounced than the effect of *OVATE* (**Figures 1A,B**, **6**). The locus was fine mapped to the short arm of chromosome 7 and found to encode a member of the IQD family of calmodulin-binding proteins (Van Der Knaap et al., 2004; Xiao et al., 2008). The mutation arose from a highly unusual 24.7 kb duplication event from chromosome 10 to chromosome 7 (Jiang et al., 2009) (**Figure 3**, **Table 2**). This transposition was mediated by the retrotransposon *Rider*, which has also been found to underlie mutations at a few other loci in cultivated tomato unrelated to fruit shape (Jiang et al., 2012). Expression of wild type *SUN* is found in 10 days post anthesis fruit but in general is extremely low in all tissues examined (**Figure 4**). The duplication placed *SUN* in a new genome environment leading to much higher expression throughout floral and fruit development and an extremely elongated fruit (Xiao et al., 2008, 2009).

The effect of *SUN* on fruit shape is noticeable at anthesis albeit that the effect of the gene is more pronounced immediately following fertilization (Van Der Knaap and Tanksley, 2001; Xiao et al., 2009; Wu et al., 2011) (**Figures 6A,B**). The results suggest that *SUN* sets up the patterning before anthesis during gynoecium development whereas the execution of the patterning plan occurs in part after fertilization. Interestingly, *SUN* also controls sepal and terminal leaflet shape and high expression leads to twisted stems and leaf rachises (Wu et al., 2011) implying a role for this gene in lateral (leaf and sepal) as well as terminal (fruit) organ development. Epistatic interaction of *SUN* and

Table 3 | Tomato TONNEAU1 Recruiting Motif proteins (TRM) that interact with OVATE in the Y2H screen.

Tomato Gene ID	Arabidopsis Ortholog[1]	PBS[2]	Number of clones	Percentage of total
Solyc07g008670.2.1	TRM5	A	31	16.8
Solyc09g005750.2.1	TRM19	A	27	14.6
Solyc06g083660.2.1	TRM17/20	A	16	8.7
Solyc03g115000.2.1	TRM3/4	A	8	4.3
Solyc02g082680.2.1	TRM26	A	8	4.3
Solyc09g063080.1.1	TRM17/20	B	7	3.8
Solyc01g094640.2.1	TRM13/14/15/33	B	7	3.8
Solyc07g032710.2.1	TRM30/34	B	5	2.7
Solyc03g006840.2.1	TRM25	C	6	3.2
Solyc08g081160.2.1	TRM13/14/15/33	C	2	1.1
Solyc12g007140.1.1	TRM30/34	D	1	0.5

[1] The most likely ortholog(s) in Arabidopsis were determined based on BLAST search against TAIR10 Arabidopsis proteins.

[2] PBS, Predicted Biological Score, which is computed to assess the reliability of the interaction. A denotes strong and reliable interaction and D denotes weak and/or questionable interaction.

OVATE is likely with respect to growth of the proximal part of the fruit (**Figure 6C**). The degree of obovoid (pear) shape is much pronounced in the double NIL than in the sum of the single NILs.

SUN changes fruit shape by redistributing fruit mass; an increase in cells in the proximal-distal direction is accompanied by a decrease in cell number in the columella and septum in the medio-lateral direction throughout the entire fruit (Wu et al., 2011) (**Figures 6A–C**). This suggests that alterations in cell division patterns are critical for fruit shape changes mediated by SUN. Yet, how SUN accomplishes changes in cell division patterns is poorly understood. The IQD members share a common central motif of 67 conserved residues named the IQ67 domain that binds calmodulin (CaM) (Abel et al., 2005; Levy et al., 2005; Huang et al., 2013). High expression of the first identified member of the family, *AtIQD1*, leads to increases in glucosinolates (Levy et al., 2005), a class of secondary metabolites involved in plant defense that is absent from Solanaceous plants. How increases in glucosinolate levels in Arabidopsis relate to fruit shape changes in tomato is therefore, not clear. High expression of *SUN* leads to phenotypes associated with auxin homeostasis, yet direct links with auxin through signaling and hormone levels have not been established (Wu et al., 2011; Clevenger, 2012). A recent Y2H study demonstrated that Arabidopsis IQD1 interacts with CaM/CMLs and kinesin light chain-related protein-1 (KLCR1), the latter acts as a motor for transport of vesicles, organelles, mRNA-protein complexes within the cytoplasm along microtubules (Burstenbinder et al., 2013). The directional transport of cargo by kinesins could involve the regulation of cell division patterns (Hirokawa et al., 2009; Akhmanova and Hammer, 2010; Verhey et al., 2011). The association of AtIQD1 with microtubules suggests that it acts as a scaffold protein to recruit cargo to kinesin motors for directional transport along microtubules (Burstenbinder et al., 2013). Whether SUN plays a similar role as AtIQD1 by interaction with KLCR1 proteins is unknown. However, the possible involvement in transport of cargo and the regulation of cell division patterns would suggest that the mutant version of *SUN* that is highly expressed in developing flowers may act as early as stage 3 in organ development, similarly to *OVATE* (**Figures 2B,C**, **Table 1**).

CNR/FW2.2

The first fruit weight QTL that was cloned from vegetables and fruit crops was *FW2.2* (Frary et al., 2000). The locus was fine mapped to the bottom of chromosome 2 and found to encode a member of a novel family of cysteine-rich proteins that share the PLAC8 motif (Guo et al., 2010). The family is known to regulate cell number, hence the new name for *FW2.2-like* genes: *Cell Number Regulator* (*CNR*) (Guo et al., 2010; Guo and Simmons, 2011) (**Figure 3**). The underlying mutation to cause changes in fruit weight was predicted to be in the promoter as there were no polymorphisms in the coding region of the gene (Frary et al., 2000). Association mapping led to the identification of a putative promoter mutation that underlies the fruit weight changes (**Figure 3**). Expression of *CNR/FW2.2* is in general low, except in the root, young flower buds and developing fruit (**Figure 4A**). Its expression is also the highest in the IM/FM reducing to nearly undetectable levels in the floral buds 6 days after initiation (**Figure 4B**). The allele increasing fruit weight causes the enlargement of the placenta and columella regions of the fruit (Cong et al., 2002; Gonzalo et al., 2009). Previous studies suggested that the members of the CNR family are localized to the membrane facilitating the transport of ions such as cadmium (Song et al., 2004) and calcium (Nakagawa et al., 2007) across membranes (Guo et al., 2010; Libault et al., 2010). Very little additional information is known about the function of CNR/FW2.2 and how regulation of ion transport would lead to changes in cell division. Ovary size is different at anthesis, implying that CNR/FW2.2 acts early during development of the gynoecium. Based on expression profile, the promoter mutation may result in fruit weight changes as early as phase 1 or 2 (**Table 1**).

TOMATO FRUIT WEIGHT AND SHAPE GENES ACTING POST-ANTHESIS

SUN

SUN clearly impacts the patterning of the fruit prior to anthesis (see above). However, the most dramatic effect of *SUN* on shape is manifested after anthesis, during phase 5, which is the cell division stage of fruit development (Van Der Knaap and Tanksley, 2001; Xiao et al., 2009) (**Figure 6B**). As a result of *SUN* expression, cell number was much higher along the proximal-distal axis and lower along the medio-lateral axis at 7 days post anthesis compared to anthesis (Wu et al., 2011) which are likely due to the changes in cell division rates in one direction over another and not the duration of cell division since fruit ripening time is not altered (data not shown). The proposed changes in cell division rates in different tissues of the developing fruit is likely because fruit weight is not altered and thus SUN appears to result in a redistribution of mass. This change in shape is accompanied by changes in gene expression profiles that are specific to the developing pericarp and columella, especially for genes related to cell division (Clevenger, 2012). These findings suggest that the differences in growth along the various axes after anthesis are accompanied by differential gene expression to achieve the final fruit shape. These differences in gene expression in the different tissue types cede at the time when fruit shape mediated by *SUN* is final which is around 10 days post-anthesis (Clevenger, 2012).

SLKLUH/FW3.2

The second fruit weight QTL identified from vegetable and fruit crops is *FW3.2* (Chakrabarti et al., 2013). The gene was fine mapped to the bottom of chromosome 3 encoding a cytochrome P450 of the CYP78A class and the likely ortholog of Arabidopsis *KLUH* (Zhang et al., 2012; Chakrabarti et al., 2013) (**Figure 3**). Based on association mapping and additional segregation experiments, a mutation in the promoter of *SlKLUH* is proposed to underlie the change in tomato fruit weight. This mutation is located 512 bp upstream of the predicted start of *SlKLUH* transcription in a putative *cis*-element that is known as an organ-specific element found in nodulin and leghemoglobin genes (Stougaard et al., 1990; Chakrabarti et al., 2013) (**Figure 3**). Expression of tomato *KLUH* is high in young growing tissues containing meristems or developing seeds (**Figure 4A**). Also, its expression is particularly high in the IM/FM and decreases in the developing flower buds (**Figure 4B**). Moreover, within the fruit,

KLUH is very highly expressed in the developing seeds and much lower in the developing pericarp (Chakrabarti et al., 2013).

The mutant allele of *SlKLUH*, found in many cultivated tomato accessions, does not impact ovary size at anthesis; rather its effect on fruit weight becomes apparent 3 weeks post-anthesis (Zhang, 2012). Yet, transgenic down regulation of *SlKLUH* led to shorter plants and leaves, smaller flowers in addition to reduced fruit weight (Chakrabarti et al., 2013). This result implies that the role of KLUH in plant development is broader than the differences in the function of the natural KLUH alleles demonstrate. The increase in fruit weight arises primarily from increased pericarp and septum areas, resulting from additional number of cells. The increases in cell number is likely the result of a change in duration of cell division and not the rate since fruit ripening is delayed as well. In addition to fruit weight, *SlKLUH* has a pleiotropic effect on branching behavior. The large fruit allele of *SlKLUH* causes reduced branch number and length as well as fewer fruits. This leads to comparable yields from NIL plants carrying the wild type or the mutant *SlKLUH* allele (Chakrabarti et al., 2013).

It has been hypothesized that KLUH generates a mobile growth promoting signal different from the known phytohormones. However, the exact molecular and biochemical nature of the "mobile" signal remains elusive and the substrate for this subfamily of P450 enzymes is also yet to be deciphered (Anastasiou et al., 2007; Adamski et al., 2009).

DO ORTHOLOGS OF TOMATO FRUIT WEIGHT AND SHAPE GENES IMPACT FRUIT MORPHOLOGY IN OTHER DOMESTICATED PLANTS?

The domestication of fruit and vegetable crops was likely driven by selections for increases in fruit weight and shape in many incipient crop species. Thus, the question arises whether any of the tomato genes or members of their families are associated with fruit weight and shape in other species. Of the fruit weight genes, other members of the CYP78A class to which *SlKLUH/FW3.2* belongs are known to regulate floral organ and fruit size, leaf and seed size, embryo and endosperm size, apical dominance and plastochron length in Arabidopsis, moss and rice (Ito and Meyerowitz, 2000; Miyoshi et al., 2004; Anastasiou et al., 2007; Adamski et al., 2009; Katsumata et al., 2011; Fang et al., 2012; Nagasawa et al., 2013). More intriguingly, in *Capsicum spp* (chile pepper), *Cucumis melo* (melon) and *Vitis vinifera* (grape), the putative ortholog of *KLUH* and members of the same CYP78A class were associated with larger fruit, suggesting a possible role of this small and largely unknown cytochrome P450 family in parallel domestication processes in fruit and vegetable crops (Chakrabarti et al., 2013; Doligez et al., 2013; Monforte et al., 2014). Collectively, these findings point toward an evolutionarily highly conserved function for this subfamily of P450s in regulating plant organ size. For *CNR/FW2.2*, members of the family regulate plant growth and biomass as well as ear length and kernel number per row in maize (Guo et al., 2010) and the number of nitrogen-fixing nodules in soybean (Libault et al., 2010). QTL studies into the regulation of fruit weight in chile pepper, melon and cherry have also implied a possible role for *FW2.2/CNR-like* genes to control weight in a range of crop species (Paran and Van Der Knaap, 2007; De Franceschi et al., 2013; Monforte et al., 2014).

Of the fruit elongation genes, down regulation of a member of the OFP family in pepper led to a longer shaped fruit (Tsaballa et al., 2011), whereas in melon several OFP members mapped to fruit shape QTLs (Monforte et al., 2014). This suggests that the OFP family is likely to control shape of other fruit and vegetables. Of the locule number genes, a weakly overexpressed *WUSCHEL-like* gene in soybean showed an enlarged gynoecium (Wong et al., 2011) which also implies that natural alleles of *WUS* could impact the size of fruits and vegetables in other crops.

CONCLUSIONS

Recent discoveries have started to shed light on the regulation of fruit shape and weight, and the molecular mechanisms underlying this diversity found in cultivated germplasms. However, these six genes are unlikely to represent the entire repertoire of genes acted on by domestication and diversification. The identification of suppressors of *ovate* (Rodriguez et al., 2013) and the effects of genetic background on the severity of the *lc* and *fas* mutants both provide evidence for the existence of other genes that interact with these major regulators of fruit shape and size. In addition, the identification of additional fruit weight QTLs (Huang and Van Der Knaap, 2011) will result in the identification of new regulators in fruit weight. Further, the exploitation of TILLING mutants that impact shape and weight may also significantly augment the resources available in the fruit morphology tool kit (Okabe et al., 2011). The molecular and biochemical characterization of the genes and encoded proteins in the future will greatly add to our understanding into the pathways regulating the final dimensions of the fruit.

Advancing the research into the function of fruit morphology proteins is going to lead to fundamental insights into plant developmental processes. Especially processes that regulate cell proliferation and enlargement patterns, as well as its rate and duration are of particular importance since they pertain to growth of all plant organs and eventually yield. In all, the discoveries made using tomato fruit morphology as a model will undoubtedly support fundamental and applied research that is applicable to many other plant systems.

AUTHOR CONTRIBUTIONS

All authors contributed critically to the writing and editing of the manuscript, agree to be accountable for the data presented and approve the version of the manuscript. Esther van der Knaap wrote the manuscript and constructed **Figure 3**, **Table 1**. Manohar Chakrabarti contributed the section about KLUH. Yi Hsuan Chu and Zejun Huang contributed to the section about LC and FAS and **Figures 1**, **5**. Josh P. Clevenger, Liang Sun, and Yanping Wang contributed to the section about SUN. Eudald Illa-Berenguer and Qi Mu contributed to the section about CNR, **Table 2**, and **Figure 4**. Neda Keyhaninejad and Shan Wu contributed to **Figure 7** and **Table 3**. Shan Wu contributed to the section about OVATE and **Figures 2**, **6**.

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Genetic regulation and structural changes during tomato fruit development and ripening

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Fruits are an important evolutionary acquisition of angiosperms, which afford protection for seeds and ensure their optimal dispersal in the environment. Fruits can be divided into dry or fleshy. Dry fruits are the more ancient and provide for mechanical seed dispersal. In contrast, fleshy fruits develop soft tissues in which flavor compounds and pigments accumulate during the ripening process. These serve to attract animals that eat them and disseminate the indigestible seeds. Fruit maturation is accompanied by several striking cytological modifications. In particular, plastids undergo significant structural alterations, including the dedifferentiation of chloroplasts into chromoplasts. Chloroplast biogenesis, their remodeling in response to environmental constraints and their conversion into alternative plastid types are known to require communication between plastids and the nucleus in order to coordinate the expression of their respective genomes. In this review, we discuss the role of plastid modifications in the context of fruit maturation and ripening, and consider the possible involvement of organelle-nucleus crosstalk via retrograde (plastid to nucleus) and anterograde (nucleus to plastid) signaling in the process.

Keywords: tomato, fruit development, ripening, plastid, retrograde and anterograde signaling

FLOWERS ARE THE KEY FACTOR IN THE EVOLUTIONARY SUCCESS OF ANGIOSPERMS

Angiosperms are seed-producing vascular plants, in which the ovules – the precursors of the seeds – develop within the ovary. Developing seeds are enclosed inside the fruits, as also indicated by the term angiosperm, which derives from two Greek words: *angeion*, meaning "vessel" and *sperma*, meaning "seeds." Estimates of the number of angiosperm species so far described range between 250,000–270,000 and 400,000 (Soltis et al., 2008; Magallón and Castillo, 2009), and they have established themselves in every type of terrestrial and aquatic (fresh and saltwater) habitat. In a letter to J. D. Hooker, written in July 1879 (Darwin and Seward, 1903), Charles Darwin referred to the sudden rise and rapid diversification of angiosperms as "an abominable mystery." Researchers since have pointed to the innovative aspects of their mode of reproduction – their short reproductive cycles, flower formation, the development of closed carpels and the small size of the male and female gametophytes. The phenomenon of double fertilization, leading to formation of the diploid zygote, and the polyploid endosperm, is also thought to have contributed to the evolutionary success of the angiosperms (Haig and Westoby, 1989; Donoghue and Scheiner, 1992).

A dicot flower can be divided into four concentric but distinct whorls. The sepals of the first or outermost whorl form the calyx, while the corolla, consisting of the petals, lies in the second whorl; in the third whorl is the androecium and the gynoecium develops in the central (fourth) whorl. The female reproductive organ the gynoecium, include the carpels. Carpels are structures that are made up of an ovary and a stigma and contain one or more ovules. One or more carpels are combined into the pistil (ovary, style, stigma), forming the gynoecium as a whole. In the majority of flowering plants, fertilization is required to initiate the transition from ovule to seed, whereas the surrounding carpel(s) and, in some species, other floral organs differentiate into the fruit (Coombe, 1975). Furthermore, fruits represent a major evolutionary innovation, are essential for plant reproduction and adaptation, and greatly enhance the efficiency of seed dispersal. The ability to germinate and grow far away from the parent plant allows angiosperms to colonize new areas, reducing the risk of inbreeding and sibling competition (Willson and Traveset, 2000).

CLASSIFICATION OF ANGIOSPERM FRUITS

According to Brooks, fruits are "matured carpels with or without accessory structures and/or seeds" (Coombe, 1976). Nitsch, on the other hand, defined them as "the tissues which support the ovules and whose development is dependent upon the events occurring in these ovules." Nitsch's definition thus includes the "false" fruits, so called because extracarpellary tissues give rise to much of the fleshy tissue that bears or encloses the true fruits. Examples include pomes and strawberries, which form by the expansion and proliferation of the receptacle (Perkins Veazie, 1995; Velasco et al., 2010).

Based on their texture, fruits are mainly divided into two major groups: fleshy and dry. At maturity, dry fruits are characterized by dry pericarp (Simpson, 2011) and they can be further classified into dehiscent or indehiscent fruits. Dehiscent fruits open and release the mature seeds, while the indehiscent fruits do not disperse the seeds. It has been proposed that dry dehiscent fruits, found in all major clades of angiosperms, correspond to the ancestral type (Knapp, 2002), whereas the *Arabidopsis* silique with its specialized dehiscence zone may be a more recent evolutionary invention (Mühlhausen et al., 2013).

With regard to fleshy fruits Darwin writes "this (a fruit's) beauty serves merely as a guide to birds and beasts in order that the fruit may be devoured and the manured seeds disseminated." Darwin recognized that seeds protected by a fleshy fruit become more attractive for animals, which in turn play an essential role in their dispersal. The attractiveness and juiciness of fleshy fruits originate in the important cytological modifications which the parenchymal tissue undergoes during ripening – including chlorophyll degradation, accumulation of carotenoids and flavonoids, development of an aroma and flavors, and softening of the pulp (Willson and Whelan, 1990; Rodríguez et al., 2013).

Nevertheless, the specific biochemical programs that result in ripening phenomena vary among species, as highlighted by the fact that fleshy fruits can be further divided into two categories: climacteric and non-climacteric. The term "climacteric" was initially proposed to emphasize the dramatic increase in fruit respiration – marked by a burst of CO_2 production (Biale, 1964). However, climacteric fruit ripening is actually stimulated by ethylene (Razali et al., 2013), although ethylene-dependent and -independent genes have been identified both in climacteric and non-climacteric fruits (Barry and Giovannoni, 2007). Intriguingly, recent data indicates that also the dry *Arabidopsis* silique shows a climacteric behavior as suggested by the patterns of ethylene production and respiration, and by its response to ethylene exogenous application (Kou et al., 2012).

DEVELOPMENT AND MATURATION OF THE TOMATO FRUIT

Among climacteric fleshy fruits, the tomato proved attractive to early inhabitants of the Americas, who initiated its domestication by selecting varieties with fruits larger than those of the wild ancestor *Lycopersicon esculentum* cv. *cerasiforme* (Tanksley, 2004; Peralta et al., 2006; Cong et al., 2008) – a process which has gone on up to the present day, as shown by the large collection of cultivars now in use, characterized by fruits with different sizes and shapes (Tanksley et al., 1996; Grandillo et al., 1999; Tanksley, 2004; Bai and Lindhout, 2007). Moreover, tomato fruits contribute more nutrients to the diet than any other fruit or vegetable, since they contain relatively large amounts of lycopene (Chalabi et al., 2004), vitamins C and A, potassium, folic acid and many other metabolites. Lycopene, for instance, has a strong antioxidant capacity because of its great ability to trap peroxyl radicals. Epidemiological studies recommend the consumption of foods containing high concentrations of lycopene, since it reduces the risk for certain types of cancer, including prostate cancer (Gann et al., 1999; Giovannucci et al., 2002; Jian et al., 2005).

From a botanical point of view, the tomato fruit is a berry, which can be bi- or multilocular (**Figure 1**). The septa of the carpels divide the ovary and the fruit into two or more locules. Seeds develop attached to the placenta, a parenchymatous tissue, which becomes gelatinous and fills the locular cavities during fruit development and maturation (Grierson and Kader, 1986; Ho and Hewitt, 1986; Bertin, 2005; Mintz-Oron et al., 2008).

After fertilization, the ovary wall is transformed into the pericarp, which can be divided into three different structures: exocarp, mesocarp, and endocarp. The external exocarp consists of a cuticle layer that thickens as the fruit ages, and the skin, which includes an epidermal cell layer and three to four layers of a collenchymatous tissue, in which starch accumulates and few plastids are retained (Esau, 1953; Varga and Bruinsma, 1986; Joubès et al., 2000; Lemaire-Chamley et al., 2005; Mintz-Oron et al., 2008). The mesocarp, the intermediate layer, is a parenchymatous tissue formed by big cells with large vacuoles (Joubès et al., 2000; Lemaire-Chamley et al., 2005; Mintz-Oron et al., 2008). The cells of the mesocarp commonly undergo six to eight rounds of DNA duplication (endocycles) reaching ploidy levels of up to 512C (Bourdon et al., 2010) and are reminiscent of the palisade cells of leaves (Gillaspy et al., 1993) since they contain several chloroplasts, the organelle where photosynthesis occurs and produces up to 20% of fruit photosynthate, whereas the rest of photoassimilates are imported from source leaves (Hetherington et al., 1998). Nevertheless, the role of fruit photosynthesis in fruit metabolism and development is not fully understood. Early shading experiments (Tanaka et al., 1974) as well as the fruit-specific antisense inhibition of the chloroplastic Fructose 1,6-bisphosphatase (FBPase) indicated an important contribution of fruit photosynthesis to fruit yield, as shown by the reduction in weights of ripe fruits with reduced photosynthetic performance (Obiadalla-Ali et al., 2004). On the contrary, tomato lines exhibiting a fruit-specific reduction in the expression of glutamate 1-semialdehyde aminotransferase (GSA) and, as a consequence, lowered chlorophyll levels and photosynthetic activity, displayed almost no differences in fruit size and weight (Lytovchenko et al., 2011). However, these lines were characterized by a striking reduction in the rate of seed set as well as an altered seed morphology, which displayed a much reduced embryo-to-seed ratio, indicating that fruit photosynthesis is an important source of carbone assimilate for proper seed set and establishment under normal growth conditions.

Finally, the endocarp, the innermost structure, consists of a single cell layer adjacent to the locular region (Mintz-Oron et al., 2008; Xiao et al., 2009; de Jong et al., 2010).

Fertilization (stage 0) normally initiates the development of the tomato fruit, which proceeds through several major stages (Picken, 1984; Gillaspy et al., 1993; **Figure 1**). The first stage, immediately after fertilization, is characterized by rapid cell division, leading to a progressive increase in pericarp cell number. The end of this stage – around two weeks after pollination – is marked by a sharp fall in the rate of cell division, when the fruit is about 0.8–1 cm in diameter. During the second stage, fruit growth relies on cell expansion and leads to a significant increase in weight. Cell expansion coincides with endoreduplication (Bergervoet et al., 1996). By the end of this stage fruits have a diameter of around 2 cm. During the third phase, the fruit enters the mature green (MG) stage (Ho and Hewitt, 1986; Giovannoni, 2004; Czerednik et al., 2012) and attains its final size, which varies greatly among cultivars and is very susceptible to environmental influences (Chevalier, 2007).

Roughly 2 days after reaching the MG stage, the tomato fruit undergoes an extensive metabolic reorganization, which marks the beginning of the fruit ripening process (Ho and Hewitt, 1986). Two main phases can be distinguished, which are referred to as the breaking (BR) and the ripening (RR) stages. Conversion of chloroplasts into chromoplasts signals the BR phase, as indicated by the change in color to yellow-orange, owing to

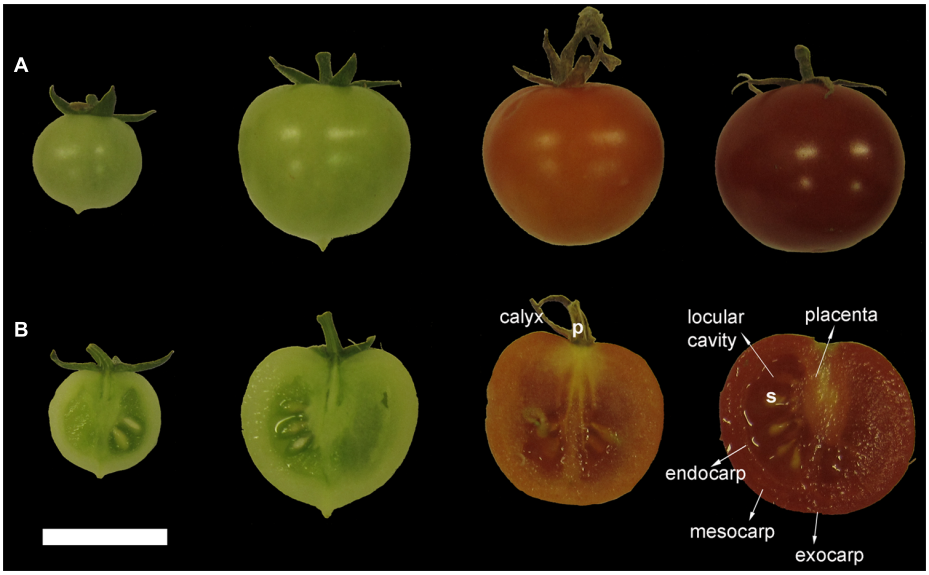

FIGURE 1 | Different stages of tomato fruit development and anatomical details. (A) Tomato fruit development can be divided into different stages: IG, immature green; MG, mature green; BR, orange-breaker; and RR, red ripening stages are shown. **(B)** Transverse sections of fruits corresponding to the developmental stages shown in **(A)**. p, pedicel; s, seed. Scale bar: 2 cm.

carotenoid accumulation, and concomitant chlorophyll degradation (**Figure 1**). Interestingly, proper ripening in tomato occurs only if fruits are harvested after having completed at least 40% of their normal growth: even exogenous application of ethylene fails to induce ripening in undeveloped locules.

At the end of the ripening process the abscission zone (AZ) is formed in the pedicel (Szymkowiak and Irish, 1999; Mao et al., 2000) to allow fruit to fall once mature. AZs differentiate at predetermined positions and contain a group of small cells lacking large vacuoles; in tomato, differentiation of the pedicel AZ is controlled by the MADS-box transcription factor JOINTLESS (Szymkowiak and Irish, 1999; Mao et al., 2000).

TOMATO: A MODEL SPECIES FOR FLESHY FRUIT STUDIES

Tomato is an ideal model plant for studying climacteric fruit ripening. Several tomato gene banks have been established and more than 75,000 accessions of tomato are maintained (Larry and Joanne, 2007; Minoia et al., 2010; Okabe et al., 2011; Saito et al., 2011). In addition, several mutants affected in fruit size, shape, development, and ripening have been isolated (Liu et al., 2002; Tanksley, 2004; Xiao et al., 2009; Rodríguez et al., 2011). Recently, the genome of *Solanum lycopersicon* cv. "Heinz 1706" (Tomato Genome Consortium, 2012; Aoki et al., 2013) has been fully sequenced and made publicly available. The predicted size of its diploid genome is approximately 900 megabases (Mb), distributed on 12 chromosomes, more than 75% of which is heterochromatin and largely devoid of genes. Around 33,000 genes have been predicted and some 5000 genes are preferentially expressed in fruits (Tikunov et al., 2013). With its short generation time, and the availability of a routine transformation technology, mapping populations, and microarrays of mapped DNA markers, tomato is a highly tractable experimental system. Several "omics" tools (transcriptomics, proteomics, and metabolomics) have been used to explore fruit formation and development (Alba et al., 2004; Fei et al., 2004; Fernie et al., 2004; Rose et al., 2004; Alba et al., 2005; Moore et al., 2005), leading to the genetic characterization of several important traits that have been selected during tomato domestication.

For instance several loci, named *FRUIT WEIGHT* (*FW*), have been recognized as key regulators of fruit mass (Grandillo et al., 1999; Paran and van der Knaap, 2007). Thus the *FW2.2* allele increases *FW* by up to 30% and is found in commercial cultivars (Frary et al., 2000), whilst the small-fruited allele is present in wild tomato species. *FW2.2* encodes a plasma membrane-localized protein that inhibits cell division; therefore low levels of *FW2.2* mRNA promote cell cycling, leading to bigger fruits containing more and larger cells (Nesbitt and Tanksley, 2001).

Tomato fruit size is also influenced by locule number. Two loci, *fasciated* (*f* or *fas*) and *locule number* (*lc*), affect floral meristem size and organ/carpel number. *FAS* encodes a YABBY transcription factor, and it is down-regulated in the high-locule-number mutant (Barrero and Tanksley, 2004). The *lc* locus seems to correspond to two single nucleotide polymorphisms (SNPs) that map close to the tomato homolog of the *WUSCHEL* gene in *Arabidopsis thaliana*; however, no deregulation of *WUS* has been observed in low- or high-locule cultivars (Muños et al., 2011). In *Arabidopsis* the WUS protein is involved in stem cell maintenance, and its up-regulation leads to the formation of extra carpels (Carles et al., 2004).

GENETIC AND HORMONAL REGULATION OF FRUIT DEVELOPMENT: A TOMATO PERSPECTIVE

THE GENETICS OF FRUIT FORMATION

Fruit formation requires intimate exchange of developmental information between ovules and carpels. Signals that stimulate

fruit development may be produced by pollen grains (O'Neill, 1997; O'Neill and Nadeau, 1997) and in ovules once fertilization has successfully occurred (Gillaspy et al., 1993), leading to alteration of the developmental fate of pistils from senescence to fruit set (Vercher et al., 1984; van Doorn and Woltering, 2008).

Since fruits are mature gynoecia, carpel patterning anticipates fruit architecture. Carpel identity is in turn controlled by the homeotic genes of class C, which includes all the members of the AGAMOUS sub-clade (AG; Dreni et al., 2011), named for the first member identified, in *A. thaliana* (Yanofsky et al., 1990; Becker and Theißen, 2003). Several comparative studies indicate that the functions of members of the AG sub-clade are conserved from monocots to basal core eudicots (Bowman et al., 1989; Bradley et al., 1993; Pnueli et al., 1994; Mena et al., 1996; Davies et al., 1999; Pan et al., 2010; Yellina et al., 2010; Dreni et al., 2011).

In tomato, as in snapdragon (Mizzotti et al., 2014), there are two *AG*-like genes (**Figure 2**), *TAGL1* and *TAG* (*TOMATO AG-LIKE 1* and *TOMATO AG*). Silencing of *TAGL1* influences fruit ripening, without affecting floral organ specification (Vrebalov et al., 2009; Giménez et al., 2010; Pan et al., 2010). In particular, *tagl1* fruits are characterized by a thinner pericarp, reduced firmness at the BR stage, and the maintenance of plastids in the collenchyma cells of the pericarp; consequently *tagl1* fruits accumulate more chlorophyll and lutein than wild-type fruits (Itkin et al., 2009; Vrebalov et al., 2009; **Table 1**).

Recently, the semi-dominant insertion mutation *Arlequin (Alq)* has been mapped and found to correspond to an altered form of the *TAGL1* gene (Giménez et al., 2010). In *Alq* plants, sepals are transformed into fruits which undergo a ripening process, like the true fruits originated by the pistils. Thus this mutant phenocopies transgenic lines that overexpress *TAGL1* (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010).

TAG1, on the other hand, has been shown to be necessary for determination of stamens and carpels, as revealed by the effects of its down-regulation using antisense and RNAi approaches (Pnueli et al., 1994; Pan et al., 2010). Indeed, pistils are replaced by a reiteration of flowers in transgenic plants expressing *TAG1* antisense (Pnueli et al., 1994) – just as in the *Arabidopsis ag* mutant (Yanofsky et al., 1990). In contrast, virtually complete silencing of *TAG1* by RNAi does not affect pistil fate in this way instead, pistils develop into red fruits, indicating a loss of determinacy (Pan et al., 2010).

Besides *AG* genes, several other MADS-box transcription factors are involved in fruit formation and maturation (**Figure 2**). Vrebalov et al. (2002) showed that the classical mutation *rin* disrupts the function of RIN-MADS. RIN-MADS lies very close to another MADS-box gene, *MACROCALYX* (*MC*), which is also silenced in *rin* plants. However, antisense repression of *RIN-MADS* and *MC* confirmed that only RIN-MADS is necessary for tomato ripening (**Table 1**).

Several independent groups have described a plethora of direct targets for RIN-MADS (Ito et al., 2008; Fujisawa et al., 2011, 2013; Martel et al., 2011). Thus RIN-MADS binds to regulatory regions of several genes, whose products are involved in fruit metabolism and ripening, and transcriptionally regulates enzymes involved in cell wall (*Polygalacturonase, PG*; β-*Galactosidase 4, TBG4*; *Endo-(1,4)-β-mannanase 4, LeMAN4*; α-*Expansin 1, LeEXP1*), and carotenoid metabolism. RIN-MADS is also a master regulator of ethylene biosynthesis in developing fruits, acting via the control regions of the genes *LeACS2* (*1-aminocyclopropane-1-carboxylic acid synthase 2*), *LeACS4*, *LeACO1* (*ACC oxidase 1*). Moreover, RIN-MADS stimulates the transcription of *Lipoxygenase* (*Lox*), the product of which catalyzes the dioxygenation of 1,4 pentadiene *cis*-polyunsaturated fatty acids to their hydroperoxide derivatives (HPO), resulting in the production of volatile compounds that contribute to fruit flavor and aroma (Yilmaz, 2001).

RIN-MADS also binds and activates the promoter of *NEVER RIPE* (*NR*; Lanahan et al., 1994; Yen et al., 1995), which encodes

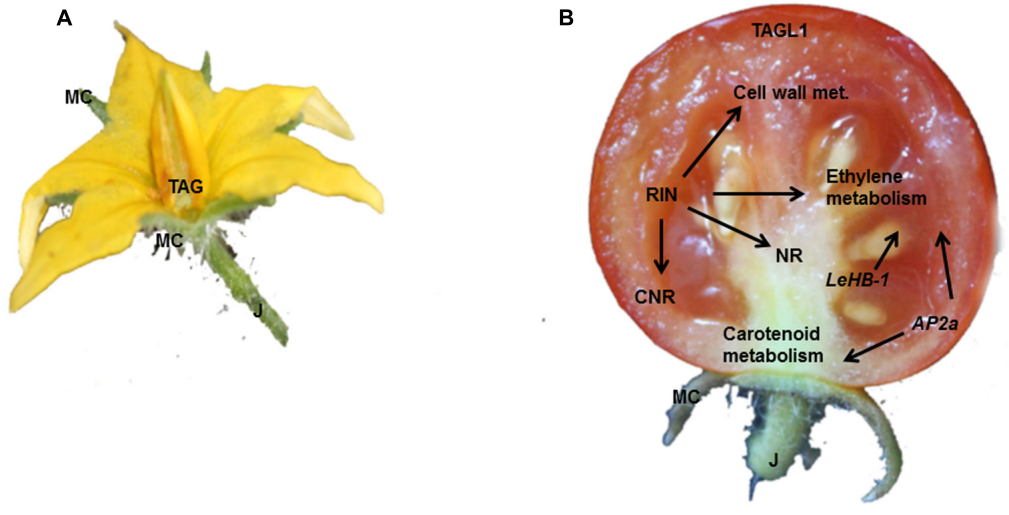

FIGURE 2 | The key regulators involved in fruit commitment, formation and ripening. (A) The MADS-box transcription factors TAG controls pistil identity, whist J regulates the pedicel abscission zone differentiation. **(B)** TAGL1, RIN, AP2a, and LeHB-1 all impact the ethylene metabolism; although RIN controls also the transcription of genes involved in cell wall modification.

Table 1 | Mutations associated with defects in fruit maturation and ripening.

Tomato fruit mutants			
Mutant	**Phenotype/tissues affected**	**Reference**	**Gene product**
tag	Flower meristem and inner whorl fate determination	Pnueli et al. (1994), Pan et al. (2010)	MADS-box transcription factor
tagl1	Chlorophyll and carotenoid accumulation	Vrebalov et al. (2009), Giménez et al. (2010), Pan et al. (2010)	MADS-box transcription factor
	Plastids present in the collenchyma of the exocarp		
rin	Ripening delay	Vrebalov et al. (2002)	MADS-box transcription factor
mc	Sepal development	Vrebalov et al. (2009)	MADS-box transcription factor
j	Abscission zone formation	Szymkowiak and Irish (1999), Mao et al. (2000)	MADS-box transcription factor
Nr	Ripening delay	Lanahan et al. (1994), Yen et al. (1995)	Ethylene receptor
CNR	Ripening delay	Manning et al. (2006)	SBP transcription factor
AP2a	Regulation of carotenoid and chlorophyll metabolism	Chung et al. (2010), Karlova et al. (2011)	AP2 transcription factor
slarf7 (RNAi)	Parthenocarpy	de Jong et al. (2009)	Auxin-responsive factor 7
gr	Ripening delay	Barry and Giovannoni (2006)	RTE-like proteins

an ethylene receptor protein. The loss of this protein/DNA interaction explains the delay in ripening seen in *rin* mutants (Klee and Tieman, 2002).

RIN-MADS also positively stimulates the transcription of *colorless non-ripening* (*CNR*), which codes for a SQUAMOSA-PROMOTER BINDING PROTEIN (Cardon et al., 1999; Manning et al., 2006) whose absence causes delay in fruit ripening and softening as a consequence of reduced ethylene production (Thompson et al., 1999). The interaction between *CNR* and RIN-MADS has been shown to be regulated by a complex mechanism. The *CNR* promoter is progressively demethylated during ripening, but in *cnr* mutants the promoter remains hypermethylated, which prevents RIN-MADS from binding to it (Zhong et al., 2013). Zhong et al. (2013) observed that the methylation states of several RIN-MADS targets change during ripening, indicating that progressive demethylation is necessary for RIN-MADS binding. Indeed, these authors showed that tens of thousands of sites in the tomato epigenome undergo modification during fruit development.

Transcriptomic studies suggest that many more transcription factors are involved in the regulation of ripening (Vriezen et al., 2008; Pascual et al., 2009) and recently members of the APETALA2 family have been shown to play a role in the process. For instance, the tomato *APETALA2a* gene (Karlova et al., 2011) participates in the control of fruit ripening by regulating genes involved in ethylene and auxin signaling, and in the differentiation of chloroplasts into chromoplasts. Down-regulation of *AP2a* in transgenic fruits is associated with the accumulation of β-carotene at the expense of lycopene (Chung et al., 2010). Ethylene metabolism is also controlled by the transcription factor *Lycopersicum esculentum Homeobox-1* (*LeHB-1*), which binds to control regions of *ACO1* (Lin et al., 2008).

HORMONES AND FRUIT DEVELOPMENT

Fruit development and maturation is tightly controlled by hormone homeostasis (Pandolfini, 2009). Indeed, several findings indicate that manipulation of hormone homeostasis is able to induce fruit development and ripening in the absence of fertilization – a phenomenon known as parthenocarpy.

Thus treatment of unpollinated flowers with auxins is sufficient to stimulate fruit growth in tomato and other horticultural plants, indicating that administration of the hormone can substitute for the signals provided by pollination and fertilization (Nitsch, 1952). Auxin homeostasis can be altered by manipulating its synthesis, perception or signaling. For example, AtARF8 (*Arabidopsis thaliana* auxin response factor 8) and tomato ARF7 have both been implicated in fruit initiation. *atarf8* mutants develop parthenocarpic fruits, while tomato fruits that express the *arf8-4* allele are seedless (Wang et al., 2005). Parthenocarpy can also be induced by silencing *ARF7* in tomato via RNA interference (de Jong et al., 2009).

Besides auxins, gibberellins (GA) play an important role in coordinating fruit growth and seed development. Active GA induce fruit set in crop plants and in *Arabidopsis* (Gillaspy et al., 1993; Dorcey et al., 2009), in agreement with transcriptomic analyses showing that GA biosynthesis genes are highly expressed in pollinated ovaries (Lemaire-Chamley et al., 2005). Inhibition of GA production by paclobutrazol has negative effects on fruit growth and seed set in tomato (Serrani et al., 2007), while the transgenic tomato lines *pat2* and *pat3/4* show overexpression of GA biosynthetic genes in their parthenocarpic fruits (Rotino et al., 2005). This is consistent with the finding that silencing of *DELLA* genes (Hauvermale et al., 2012), which code for negative regulators of GA signaling, results in the development of parthenocarpic fruit in both tomato and *Arabidopsis* (Martì et al., 2007; Dorcey et al., 2009; Fuentes et al., 2012).

Tomato is a climacteric fruit and its ripening is dependent on an ethylene burst. Conversely, in several tomato mutants in which ripening is delayed (including *rin, cnr* and *nr*), ethylene production is compromised. Synthesis of ethylene depends on the action of two enzymes, ACC synthase (ACS) and ACC oxidase (ACO). ACS converts S-adenosylmethionine

into 1-aminocyclopropane-1-carboxylate, which is subsequently transformed into ethylene by ACO. ACC synthases in tomato are encoded by a multigene family (Zarembinski and Theologis, 1994; Oetiker et al., 1997), but only *LeACS2* and *LeACS4* are up-regulated during climacteric fruit ripening (Olson et al., 1991; Barry et al., 1996, 2008; Baldwin et al., 2000; Alba et al., 2005; Barry and Giovannoni, 2006), and the down-regulation of *LeACS2* and *ACO* delays ripening and the transgenic tomato fruits increase their shelf life (Xie et al., 2006).

NEVER RIPE is a semi-dominant mutation that affects one of the seven ethylene receptors (*Lycopersicum esculentum ethylene receptor*, *LeETR1-7*) present in the tomato genome. Of these seven genes, however, only *LeETR4, LeETR6* and *Nr* (*LeETR3*) are strongly expressed during fruit ripening.

Green-ripe (*Gr*) is also a dominant non-ripening mutant (Barry and Giovannoni, 2006; Xie et al., 2013), whose phenotype is due to misexpression of the *Gr* gene in developing fruits and organs, where it is normally not active. GR codes for a homolog of the *Arabidopsis* RTE1 protein (Barry and Giovannoni, 2006), a factor that is able to bind to and modify ethylene receptors, although how it affects receptor function remains unclear.

THE CHLOROPLAST TO CHROMOPLAST TRANSITION AND NUCLEUS-PLASTID COMMUNICATION

The onset of fruit ripening and the consequent reprogramming of cellular metabolism is most strikingly reflected in the conversion of fully developed chloroplasts into chromoplasts, a type of plastid that accumulates massive amounts of colorful carotenoids to attract insects and mammals that facilitate the dispersal of the seeds contained in fleshy fruits (Egea et al., 2010).

The chloroplast to chromoplast transition involves various structural modifications, including changes in the density and size of the organelle (Rosso, 1968; Spurr and Harris, 1968; Harris and Spurr, 1969), breakdown of chlorophylls, disruption of the thylakoid membrane and the aggregation of carotenoids into crystals (Egea et al., 2011). Scanning confocal microscopy analyses indicate that at the MG stage of tomato development only chloroplasts are present, mainly located in the mesocarp cells.

During the breaker stage (BR), plastids begin to accumulate carotenoids, with the rate of accumulation of lycopene being three- to fourfold higher than that of chlorophyll decline (Trudel and Ozbun, 1970; Wu and Kubota, 2008; Egea et al., 2011).

From a structural point of view, the dedifferentiation of chloroplasts into chromoplasts begins with the breakdown of starch granules and the lysis of thylakoid membranes (Ljubesić et al., 1991; Egea et al., 2010). Concomitantly, new membranes are formed, which are derived from the plastid inner envelope and become sites of carotenoid accumulation and crystal formation (Simkin et al., 2007). During the transition plastoglobules and stromules increase in size and number (Harris and Spurr, 1969; Gray et al., 2001; Kwok and Hanson, 2004; Egea et al., 2010). Plastoglobules serve to sequester lipids and carotenoids (Klee and Giovannoni, 2011; Nogueira et al., 2013), whereas the stromules provide extra surface area for the import of novel plastid proteins (Kwok and Hanson, 2004).

The situation just described is typical for immature chromoplasts at early stages of differentiation. At the full ripening stage, the plastids in fruits are almost exclusively chromoplasts. Interestingly, using real-time recording of the transition occurring in the mesocarp tissues, Egea et al. (2011) were able to demonstrate that the transition from chloroplasts to chromoplasts occurs more synchronously within individual cells than between different cells of the fruit tissue. Moreover, since these authors found no evidence for *de novo* formation of plastids, they concluded that all chromoplasts originate from pre-existing chloroplasts, as previously suggested (Pyke and Howells, 2002; Waters et al., 2004; Egea et al., 2011).

Over 95% of the ∼3000 proteins found in the chloroplast are encoded in the nuclear genome, translated in the cytoplasm and then imported into the organelle (Richly and Leister, 2004; Li and Chiu, 2010). Therefore, the transition from chloroplast to chromoplast must involve extensive exchange of information between the nucleus and the plastids, in order to regulate the plastid proteome and ensure that the organelle can meet the changing metabolic and energy demands of the cell (Chi et al., 2013). This notion is supported, for example, by the fact that mutation of the tomato *lutescent2* locus (*l2*), encoding a chloroplast-targeted zinc metalloprotease, delays the onset of fruit ripening, which implies the existence of a chloroplast-derived signal that stimulates ripening (Barry et al., 2012).

Communication between plastids and the nucleus, and the nature of plastid-derived signals, have been widely studied in model organisms such as *A. thaliana*, and this has led to the identification of several key factors that are essential for chloroplast biogenesis (biogenic control) and adaptation to physiological and environmental conditions (operational control; for a review see Woodson and Chory, 2008; Chi et al., 2013).

Interestingly, the expression of these factors is maintained in *Arabidopsis* and tomato fruits at different developmental stages, suggesting a possible involvement of anterograde (nucleus-to-plastid) and retrograde (plastid-to-nucleus) signaling pathways in fruit maturation and ripening.

THE ANTEROGRADE PATHWAY

During anterograde regulation, nucleus-encoded transcriptional and post-transcriptional regulators convey information about cell type to the plastid, and nuclear genes direct the synthesis and delivery of proteins that are appropriate for the organelle's development, division and differentiation into chloroplasts, amyloplasts, chromoplasts, and other plastid types (Leon et al., 1998; Raynaud et al., 2007). In general, nucleus-encoded post-transcriptional regulators, such as proteins of the tetratricopeptide-repeat (TPR) and pentatrico-peptide-repeat (PPR) families (D'Andrea and Regan, 2003; Nakajima et al., 2012), bind to specific chloroplast mRNAs, and control their maturation and/or stabilization by acting as adaptors for enzymes of chloroplast RNA metabolism. Alternatively, they regulate protein synthesis initiation and/or elongation by recruiting the chloroplast translation machinery to specific mRNAs (Blatch and Lässle, 1999; Shikanai and Fujii, 2013). Through these processes, TPR, PPR, and other types of imported proteins mediate subtle regulatory changes, such as the assembly and abundance of specific protein

complexes in response to developmental and environmental stimuli.

Conversely, large-scale developmental switches, such as the reprogramming that takes place during the chloroplast-to-chromoplast transition (Leon et al., 1998), lead to a general increase in transcription and in differential transcript accumulation. The plastome of higher plants is transcribed by two quite different transcription systems that originate from a cyanobacterial- and proteobacterial-like endosymbiont respectively (Maliga, 1998; Liere et al., 2011). The cyanobacterial ancestor of chloroplasts provided a eubacterial-type RNA polymerase (PEP) whose four-subunit core, comprising α, β, β′, and β″ proteins, is encoded by the plastid genes *rpoA*, *rpoB*, *rpoC1,* and *rpoC2*. The PEP plays a prominent role in the expression of photosynthesis-related genes in leaf chloroplasts, but it is also present in dry seeds and is active during germination. The activity and specificity of PEP is regulated by nucleus-encoded sigma-like transcription factors (SIGs). In *Arabidopsis* six such sigma factors (SIG1-6) have been identified, and they appear to have distinct roles during embryonic photosynthesis (SIG5), seed maturation and germination (SIG3) and very early plant development (SIG2 and SIG6).

Two nuclear genes encode the plastid proteobacterial-like RNA polymerases (NEPs), named RPOTp and RPOTmp, the latter being targeted to and active in mitochondria also (Liere et al., 2011). NEPs are active in the expression of housekeeping genes in plastids, and they play an important role in the build-up of the plastid transcriptional and translational apparatus during stratification, germination and early seedling development.

Putative homologs of *Arabidopsis* anterograde signaling factors can be identified in tomato, using BLAST queries of transcript (cDNA ITAG release 2.31) and protein databases (ITAG release 2.31) available on the SGN website[1]. In addition, the expression patterns of the corresponding mRNAs in leaves and in tomato fruits at different times during maturation (1-cm fruit, 2-cm fruit and MG fruit, BR, and RR stages) can be assessed with the aid of the Tomato eFP browser[2] (**Table 2**).

As expected, all putative homologs of sigma factors appear to be down-regulated in fruit with respect to leaves, confirming their predominant role in the PEP-mediated expression of photosynthesis-related genes. The only exception is represented by the *SIG5* homolog (*Solyc03g007370*), which is expressed at slightly higher levels in ripening tomato fruits than in leaves.

Conversely, the three putative tomato homologs of plastid proteobacterial-like RNA polymerases (Solyc07g005930; Solyc02g089340; Solyc05g010660) display intriguing expression patterns in developing fruits. The closest homolog of RPOTp (*Solyc07g005930*) is down-regulated in fruit, while the other two (*Solyc02g089340; Solyc05g010660*), which are more similar to the RPTOPmp form found in both mitochondria and plastids, show higher expression levels relative to leaves in all the different fruit stages. In particular, their expression levels follow very similar patterns, with a first peak occurring at the MG fruit stage and a second at the ripening stage. These observations imply a very strict coordination of mitochondrial and plastid transcription activities during fruit formation and maturation.

[1]http://solgenomics.net/tools/blast/

[2]http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi

THE RETROGRADE PATHWAY

The term retrograde signaling refers to the regulation of nuclear gene expression in response to the developmental stage and functional state of the plastids, including plastid differentiation (Enami et al., 2011). In the classical scenario, the retrograde signal is generated in the plastids, then exported, and traverses the cytosol to act in the nucleus. Several metabolites have been proposed to act as messengers during retrograde signaling. These include (1) tetrapyrroles (Mg-protoporphyrin IX or heme; Strand et al., 2003; Woodson et al., 2011); (2) 3-phosphoadenosine-5-phosphate (PAP; Estavillo et al., 2011); and (3) methylerythritolcyclodiphosphate (MEcPP; Xiao et al., 2012).

The involvement of tetrapyrroles in retrograde signaling in *Arabidopsis* was revealed by the identification of *g*enome *un*coupled (*gun*) mutants that, unlike wild type, continue to express photosynthesis-related nuclear genes including *ribulose bisphosphate carboxylase small subunit* (*RBCS*) and *light harvesting complex of photosystem II* (*Lhcb*s) even when chloroplasts have been photobleached by exposure to the herbicide norflurazon (Susek et al., 1993). In particular, GUN2, GUN3, and GUN6 are involved in the iron branch of tetrapyrrole biosynthesis leading to heme and phytochromobilin, and code for the enzymes heme oxygenase 1, phytochromobilin synthase, and Fe-chelatase 1, respectively. GUN4 and GUN5, on the other hand, operate in the magnesium branch that leads to chlorophylls, and form part of the Mg-chelatase enzymes together with CHLH, CHLD, CHLI-1, and CHLI-2 subunits (for a review, see Chi et al., 2013). However, the role of Mg-protoporphyrin IX (Mg-ProtoIX) as a plastid signal has been questioned, since its accumulation following norfluorazon treatment has not been observed in two independent studies (Mochizuki et al., 2008; Moulin et al., 2008). Consequently, it was suggested that either rapid changes in the flux through the tetrapyrrole pathway, or the accumulation of Mg-ProtoIX in a specific cellular compartment could be the origin of the plastid signal (Mochizuki et al., 2008; Moulin et al., 2008); however, these aspects deserve further investigations.

A novel role as a retrograde signaling messenger was recently assigned to PAP (Estavillo et al., 2011). PAP accumulates in the chloroplast under drought conditions or upon exposure to excess light, and functions as a mobile signal that alters nuclear RNA metabolism by inhibiting exoribonucleases (XRNs). Evidence for PAP-mediated chloroplast-to-nucleus communication came with the identification of the *alx8* mutant, which exhibits constitutive up-regulation of genes normally induced by high-light stress. The *alx8* phenotype is caused by a lesion in SAL1, a phosphatase that regulates PAP levels by dephosphorylating PAP to adenosine monophosphate (AMP).

Recently, a role as a retrograde signaling metabolite has been also reported for MEcPP, a precursor of isoprenoids produced by the plastidial methylerythritol phosphate (MEP) pathway (Xiao et al., 2012). This finding came from the observation that *Arabidopsis* plants showing constitutive expression of selected stress-responsive nuclear genes also accumulated high levels of MEcPP, as a consequence of a lesion in the enzyme HDS, which

Table 2 | Relative expression levels of the putative homologs of *Arabidopsis* anterograde and retrograde factors in developing tomato fruit.

		Gene	*Arabidopsis*	Tomato						
Anterograde pathway	Sigma Factors	SIG1	ATIG64860	Solyc03g097320	0,07	0,09	0,10	0,12	0,05	0,01
		SIG2	ATIG08540	Solyc01g081490	0,30	0,28	0,22	0,19	0,25	0.48
		SIG3	AT3G53920	Solyc08g065970	0,37	0,41	0,41	0,31	0,20	0,02
		SIG4	AT5G13730	Solyc01g087690	0,02	0,00	0,00	0,00	0,00	0,00
		SIG5	AT5G24I20	Solyc03g007370	0,19	0,46	0,26	0,52	0,58	1,27
		SIG6	AT2G36990	Solyc09g008040	0,21	0,18	0,18	0,17	0,20	0,22
	NEPs	RPOTp	AT2G24I20	Solyc07g005930	0,57	0,43	0,76	0,72	0,40	0,65
		RPOTmp	AT5G15700	Solyc02g089340	1,41	1,14	1,38	2,14	0,93	3,52
				Solyc05g010660	1,45	1,60	2,53	2,63	1,50	2,52
Retrograde pathway	genome unclopled (gun)	GUN1	AT2G31400	Solyc06g009520	0,92	1,03	0,95	0,85	0,84	1,64
		GUN2	AT2G26670	Solyc12g009470	0,38	0,43	0,42	0,26	0,38	0,45
		GUN3	AT3G09150	Solyc01g008930	1,90	1,36	0,97	0,93	0,50	0,34
		GUN4	AT3G59400	Solyc06g073290	0,28	0,23	0,14	0,03	0,01	0,01
		GUN5	AT5G13630	Solyc04g015750	0,21	0,38	0,16	0,11	0,13	0,61
	Mg Chelatase	CHLH	AT5G13630	Solyc04g015750	0,21	0,38	0,16	0,11	0,13	0,61
		CHLD	AT1G08520	Solyc04g015490	0,70	0,47	0,25	0,24	0,14	0,20
		CHLI-1	AT4G18480	Solyc10g008740	0,47	0,32	0,31	0,11	0,12	0,05
	Fe Chelatase	FC1 (GUN6)	AT5G26030	Solyc10g084140	1,08	1,60	2,49	3,18	3,16	3,71
				Solyc08g065480	1,39	2,16	2,28	4,92	3,80	4,77
		FC2	AT2G30390	Solyc05g018650	0,34	0,54	0,93	0,61	0,62	0,44
	Stress-related	SAL1	AT5G63980	Solyc05g056490	1,92	1,99	1,46	1,64	2,40	3,27
		XRN2	AT5G42540	Solyc04g049010	1,94	2,57	4,49	5,35	5,32	6,76
		XRN3	AT1G75660	Solyc04g081280	1,46	1,45	2,27	1,73	1,73	1,93
				Solyc12g089280	1,57	1,10	1,05	1,20	0,80	1,24
		HDS	AT5G60600	Solyc11g069380	0,99	0,90	0,61	0,66	0,95	1,43
	Redox- and ROS-mediated	STN 7	AT1G68830	Solyc12g021280	0,23	0,29	0,26	0,30	0,26	0,38
		EX1	AT4G33630	Solyc01g105990	0,69	0,58	0,81	0,59	0,71	0,72
		EX2	AT1G27510	Solyc06g071430	0,40	0,42	0,63	0,58	0,82	1,04
		PRIN2	ATIG10522	Solyc05g006110	0,39	0,24	0,20	0,07	0,03	0,01
		PTM	AT5G35210	Solyc10g081470	0,33	0,26	0,19	0,18	0,20	0,37
		GLK1	AT2G20570	Solyc07g053630	0,00	0,00	0,00	0,01	0,00	0,00
		GLK2	AT5G44190	Solyc08g077230	1,77	1,29	0,43	0,39	0,79	0,46
		ABI4	AT2G40220	Solyc05g052410	0,65	0,79	1,63	0,93	4,03	5,06
		HY5	AT5G11260	Solyc08g061130	1,23	1,80	0,70	2,07	7,47	3,41
		HSP90	AT1G04130	Solyc09g064390	2,74	2,99	3,16	4,59	6,13	12,82
				Solyc09g064380	3,12	4,57	2,63	4,48	6,31	11,68

>8,00, 7,01–8,00, 6,01–7,00, 5,01–6,00, 4,01–5,00, 3,01–4,00, 2,01–3,00, 1,01–2,00, 0,80–0,99, 0,60–0,79, 0,40–0,59, 0,20–0,39, 0–0,19

Putative homologs of known Arabidopsis anterograde and retrograde signaling factors in tomato were identified by BLAST screening of the transcript and protein databases available on the SGN website (http://solgenomics.net/tools/blast/).

The tomato genes selected for further analysis were those most closely related to the query sequence, as indicated by their E-values. In some cases, more than one homologous gene was identified. For instance, three putative homologs of the NEP genes can be identified in tomato, as well as two putative homologs each for FC1 and HSP90, and three each for the XRN2 and -3 genes, while query sequences for homologs of Arabidopsis CHLI-1 (AT4G18480) and CHLI-2 (AT5G45930, not shown) both identified the same tomato gene (Solyc10g008740).

The absolute expression levels of the selected genes in leaves and in tomato fruits at different developmental stages (1-, 2- and 3-cm fruits, mature green fruits, breaker fruits, and ripening fruits) were obtained using the Tomato eFP Browser.

The expression level of each gene at each stage of tomato fruit development was normalized with respect to the corresponding expression level in leaves. Values and colors (see color scale) in each box refer to the normalized expression level of that gene.

is responsible for the conversion of MEcPP to HMBPP in the plastid-specific, non-mevalonate MEP pathway.

Changes in chloroplast homeostasis are also closely associated with changes in the redox state of the thylakoid electron transport chain (Baier and Dietz, 2005), particularly the redox state of the plastoquinone pool (PQ) and increases in the levels of reactive oxygen species (ROS), which also trigger retrograde signaling processes (Apel and Hirt, 2004; Asada, 2006). Components of the redox and ROS signaling circuits have been identified by genetic analysis in *A. thaliana*. They include STN7, a dual-function thylakoid protein kinase required for state transitions and photosynthetic acclimation (Bonardi et al., 2005; Pesaresi et al., 2009), Executor 1 (EX1) and Executor 2 (EX2; Lee et al., 2007) – both required for $^1O_2$-dependent nuclear gene expression changes and stress responses – and PRIN2, which has been shown to be part of the plastid RNA polymerase (PEP) machinery (Kindgren et al., 2012a).

A further retrograde signaling pathway appears to originate from perturbation of plastid gene expression (PGE) both at the level of transcription and translation (Sullivan and Gray, 1999; Woodson et al., 2013). *Arabidopsis* mutants defective in SIG2 and SIG6 factors have been, indeed, shown to be the source of plastid retrograde signals (Woodson et al., 2013). Moreover, based on transcriptomic analyses, the transcription-, translation- and tetrapyrrole-mediated pathways seem to converge, within the chloroplast, at the level of the GUN1 protein (Koussevitzky et al., 2007; Woodson et al., 2013). Unlike the other *GUN* genes, *GUN1* encodes a plastid-located PPR protein that is part of the transcriptionally active plastid chromosome (pTAC). However, the molecular details of GUN1 function remain elusive.

Generally speaking, the majority of tomato proteins that share homology with *Arabidopsis* retrograde signaling factors are encoded by genes that show reduced expression (with respect to leaves) in the fruits at the MG, breaker and ripening stages (**Table 2**). This is true of the tomato homologs of GUN2 (Solyc12g009470) and GUN3 (Solyc01g008930), and the subunits of the Mg-chelatase enzymes GUN4 (Solyc06g073290), GUN5 (Solyc04g015750), CHLD (Solyc04g015490), CHLI-1, and CHLI-2 (Solyc10g008740). In contrast, *GUN6* transcripts (Solyc08g065480 and Solyc08g065480) encoding ferrochelatase 1 (FC1) accumulate to relatively high levels in all fruit stages, suggesting that FC1-dependent heme synthesis might play a key role as a source of messenger molecules to coordinate plastid and nucleus activities during fruit ripening. At all events, stress-related retrograde signals like PAP and MEcPP do not appear to have a major role in fruit formation, as shown by the leaf-like levels of *HDS* transcripts (Solyc11g069380) and the increased accumulation of both SAL1 (Solyc05g056490) and XRN2 (Solyc04g049010) and XRN3 (Solyc04g081280) mRNAs in all stages of fruit differentiation and maturation. Similarly, all factors involved in redox- and ROS-mediated retrograde signals are encoded by genes that are only weakly transcribed in fruits, such as STN7 (Solyc12g021280), EX1 (Solyc01g105990), EX2 (Solyc06g071430), and PRIN2 (Solyc05g006110), further supporting the inference that stress-related pathways are not involved in the chloroplast-chromoplast transition.

Once retrograde signals have been generated, they must be exported to the nucleus and interact with transcription factors to regulate gene expression. Hence, the discovery in *Arabidopsis* of a mechanism for the transduction of a retrograde signal in the nucleus represents a major breakthrough. The GUN1-dependent retrograde pathway has recently been shown to be mediated by N-PTM, an N-terminal fragment of the transcription factor PTM that is associated with the chloroplast envelope membrane. Once formed, N-PTM is translocated to the nucleus, where it activates the expression of ABI4, an AP2-type transcription factor reported to have a general role in plastid retrograde signaling (Sun et al., 2011). This pathway, however, does not seem to play a key role during fruit maturation and ripening, as indicated by the low accumulation of Solyc10g081470 transcripts, which code for the putative homolog of PTM in tomato, at all stages of fruit development.

GLK1 and GLK2 (Golden 2-like 1 and Golden 2-like 2) are MYB-GARP transcription factors that also act downstream of plastid retrograde signaling to regulate a large set of genes encoding photosynthetic thylakoid membrane proteins (Rossini et al., 2001). Two GLK genes are found in the tomato genome (*GLK1, Solyc07g053630; GLK2, Solyc08g077230*; Powell et al., 2012), and *GLK2* accumulates during the earliest stages of fruit maturation, when new chloroplasts are needed to keep pace with cell division and expansion. Breeders have selected tomato varieties carrying light-green fruit before ripening, and Powell et al. (2012) have demonstrated recently that the light-green trait is due to the presence of a truncated version of *GLK2/Solyc08g077230*. These varieties produce fruits with a reduced sugar content, as a consequence of the reduced photosynthetic performance of the mesocarp cells. In agreement with that, overexpression of either *GLK1* and *GLK2* resulted in dark green tomato fruit with high chlorophyll and chloroplast levels in addition to more stacked thylakoid grana and elevated starch in the fruit (Nguyen et al., 2014).

Furthermore, the decrease in accumulation of *GLK2/Solyc08g077230* transcripts at later stages in fruit development agrees with the increased accumulation, at breaker and ripening stages, of *ABI4* (Koussevitzky et al., 2007), *HY5* and *HSP90* genes (Kindgren et al., 2012b), which are known to inhibit photosynthesis-related gene expression. This indicates that they are part of the genetic program leading to the dismantling of the thylakoid membrane and its associated photosynthetic machinery.

CONCLUSION

In this survey we have explored the genetic and the hormonal regulation of fruit formation and development in tomato. Many players in the regulation of ripening have been identified, and their action clarified. However, the exchange of information between plastids and the nucleus has not been satisfactorily explored with regard to fruit ripening, despite the fact that the dedifferentiation of chloroplasts into chromoplasts is such a spectacular aspect of the whole process. Indeed comparative analyses reveal that several genes encoding protein involved in the retrograde and anterograde signaling undergo to transcriptional regulation and these waves can be associated to important developmental checkpoints. Indeed a better comprehension of these signaling pathways will provide

new molecular tools to be used in breeding programs finalized to important applicative improvements, such as increase tomato fruit quality and tomato shelf-life.

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Evolution of the fruit endocarp: molecular mechanisms underlying adaptations in seed protection and dispersal strategies

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Plant evolution is largely driven by adaptations in seed protection and dispersal strategies that allow diversification into new niches. This is evident by the tremendous variation in flowering and fruiting structures present both across and within different plant lineages. Within a single plant family a staggering variety of fruit types can be found such as fleshy fruits including berries, pomes, and drupes and dry fruit structures like achenes, capsules, and follicles. What are the evolutionary mechanisms that enable such dramatic shifts to occur in a relatively short period of time? This remains a fundamental question of plant biology today. On the surface it seems that these extreme differences in form and function must be the consequence of very different developmental programs that require unique sets of genes. Yet as we begin to decipher the molecular and genetic basis underlying fruit form it is becoming apparent that simple genetic changes in key developmental regulatory genes can have profound anatomical effects. In this review, we discuss recent advances in understanding the molecular mechanisms of fruit endocarp tissue differentiation that have contributed to species diversification within three plant lineages.

Keywords: fruit development, endocarp, dehiscence, lignification, fruit evolution

INTRODUCTION

In general, fruits can be divided into two classes; dry fruits and fleshy fruits. Dry fruits are thought to predate their fleshy counterparts and are typically dispersed by physical forces (Scutt et al., 2006). Once the seeds mature, they are ejected by pod shattering, swept up by the wind, or adhere to animal surfaces for transport (epizoochory). In contrast, seed dispersal in fleshy fruits most often depends on animals consuming the fruit and dispersing the seeds after ingesting or discarding them. Whether it is a dry or fleshy fruit, all fruits contain tissue layers derived from the carpel ovary which are collectively called the pericarp (**Figure 1**). The pericarp can often be further differentiated into additional layers called endocarp (innermost layer), mesocarp (intermediate layer), and exocarp (skin or surface layer). Pericarp differentiation in dry fruits is often difficult to discern as each layer sometimes only contains a few rows of cells. In most fleshy fruits, the mesocarp comprises the soft edible portion of the fruit but in some exceptions the fleshy portion is formed from tissues other than the ovary (**Figure 1**). These are sometimes known as false fruits. For example, apple produces a pome fruit in which the core represents the true ovary derived fruit and the edible portion originates from the hypanthium; formed from the fused base of petals and sepals. In contrast, the fleshy portion of the strawberry is formed from the flower receptacle.

The endocarp is differentiated from the inner layer of the ovary and is the tissue layer immediately adjacent to the seed. It plays diverse roles in fruit function and can be fleshy as found in watermelon, fibrous like in mango, or extremely hard and durable as in a peach. Fruits with a hardened endocarp are called drupes. Drupes include a number of economically important crops such as peach, cherry, plum, almond, coffee, mango, olive, coconut, pistachio, date, raspberry, oil palm, and walnuts (**Figure 2**). The hardened endocarp provides a physical barrier around the seed protecting it from disease and herbivory (Doster and Michailides, 1999). The seeds of drupes are dispersed by animals either after consumption (blackberries) or upon being discarded (peaches). Once dispersed the seeds escape their woody enclosure via cracking and splitting of the endocarp shell due to environmental exposure.

In dry fruits the endocarp plays a primary role in seed dispersal. Dry fruits are generally categorized as either dehiscent or indehiscent depending on whether or not the pericarp splits open at maturity. Dehiscence is a mechanism of seed dispersal whereby the pod is forcibly opened by internal physical tension which builds during fruit maturation, causing the seeds to be suddenly discharged. Wisteria represents an extreme case in which the pods are explosive, ejecting the seeds very long distances. Other examples of dehiscent fruits include sweet pea, soybean, alfalfa, milkweed, mustard, cabbage, and poppy. Dry indehiscent fruits do not undergo this process and include a number of nuts, sunflowers, and windborn seed types such as the winged seeds found in maple and ash or cypsela-type structures produced by dandelions.

In dehiscent fruits, differentiation of the endocarp and specialized adjoining tissue layers from the mesocarp regulates pod shatter. This process has been extensively studied in *Arabidopsis thaliana* which is in the family *Brassicaceae* (reviewed by Ferrándiz, 2002; Dinneny and Yanofsky, 2004; Dinneny et al., 2005; Lewis et al., 2006). *Arabidopsis* fruits form as a bivalved silique

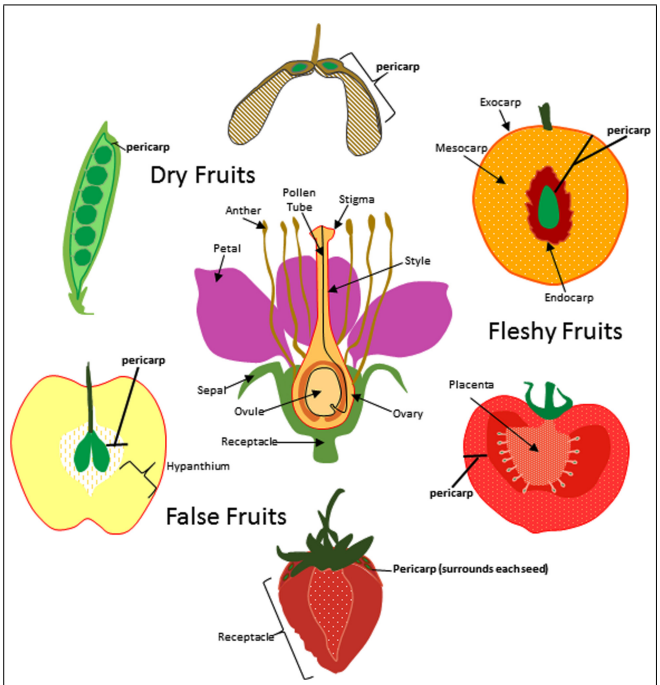

FIGURE 1 | Origin of fruit tissue layers in dry, fleshy, and false fruits. For simplicity a flower with a single carpel is shown at center though it is important to note that many of the flowers that give rise to the fruits depicted here produce multiple carpels. The ovary and other floral tissues are indicated and the carpel is outlined in red. Pericarp (bold) is indicated for pea, maple, peach, tomato, strawberry and apple fruits. Exocarp, mesocarp, and endocarp are indicated for peach.

FIGURE 2 | Examples of lignified endocarps in drupes after removal of exocarp and mesocarp. Seeds are contained inside and not shown (lower row is magnified for visibility). (1) Coconut, (2) mango, (3) Walnut, (4) Peach, (5) Apricot, (6) Olive, (7) Date, (8) Pistachio, (9) Blackberry, and (10) Cherry.

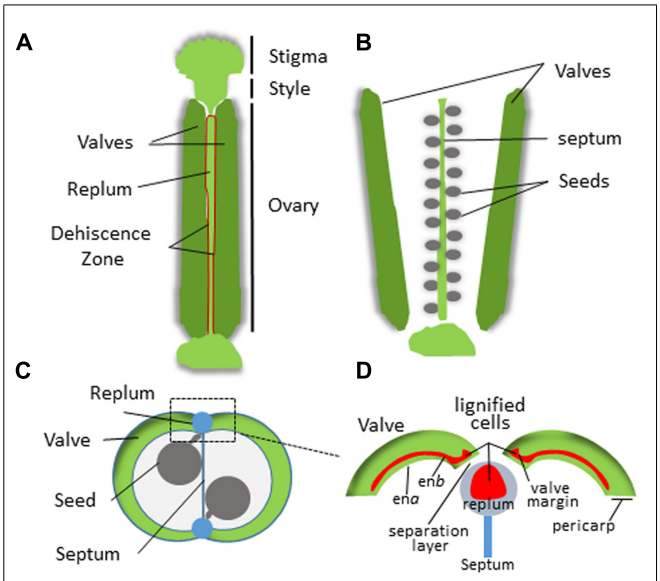

FIGURE 3 | Structure of the *Arabidopsis* silique. (A) Intact silique prior to dehiscence. Dehiscence zone is highlighted in red. **(B)** Valve separation after dehiscence revealing the seeds attached to the septum. **(C)** Cross section of the silique. **(D)** Magnified view of the replum region. The pericarp tissue is indicated and lignification zones are shown in red.

containing regularly arranged seeds (**Figure 3**). The pericarp in each silique forms two valves that sandwich a thin papery tissue called the septum onto which the seeds are attached. The valves are connected to the septum on two sides by an external part of the septum called the replum. The endocarp is sub-divided into two layers; endocarp A (en*a*) and endocarp B (en*b*) that line the inner surface of the valves. A distinct tissue layer referred to as the valve margin forms a hinge on either side of the replum at the tip of the silique. Upon maturation, cells within the en*a* layer secrete cell wall degrading enzymes while cells within the en*b* layer, vascular bundles within the replum, the valve margins, and patches of neighboring mesocarp lignify and harden (Ferrándiz, 2002; Liljegren et al., 2004; Ogawa et al., 2009). This simultaneous separation and hardening of the en*b*, valve margin, inner replum, and adjoining mesocarp tissues creates tension forces that eventually cause the pod to split open along a distinct separation layer that divides the valve margins from the replum. Silique dehiscence is a highly coordinated process that is tightly coupled to complex differential pericarp tissue patterning of the mesocarp, endocarp, valve, valve margins, separation layer, and replum.

In addition to seed protection and dispersal, the endocarp also plays an important role in sustaining and communicating with developing seeds. Seeds are connected to the maternal fruit tissue via an umbilical structure called the funiculus. The funiculus initiates from the seed coat and attaches to the placenta on the ovary wall. As the fruit matures, the placental layer of the ovary often becomes part of, or is fused to the endocarp.

A hallmark of both drupes and dehiscent fruits is the hardening of the endocarp as the fruit matures. Hardening occurs via secondary cell wall formation and lignification. The process of

secondary wall formation in fruit tissues has not been studied to any great extent. However, based on the structural similarities between endocarp tissue and wood, information about this process can be inferred from studies on wood formation. In plant stems, xylem cells undergo a series of changes as they transform from fleshy to woody tissue. These include cell elongation, cell expansion, secondary cell wall deposition, programmed cell death, and finally heartwood formation (Dejardin et al., 2010). Secondary walls are comprised of multiple layers made up of cellulose, hemi-cellulose, and lignin with smaller amounts of pectin and proteins.

Lignin provides a matrix within secondary cell walls for polymerization of cellulosic and hemi-cellulosic polymers which together contribute to providing tissue rigidity and tensile strength (Novaes et al., 2010). Most of the genes for the major enzymes in the pathway and the potential regulatory points have been identified (**Figure 4**; Boerjan et al., 2003). Lignin is formed from the phenylpropanoid pathway, the end products of which are coniferyl and sinapyl alcohols. These lignin monomers serve as the basis for lignification which is the process of producing the lignin polymer via oxidative reactions aided by peroxidases and laccases. Radical coupling of the monomers, particularly cross-coupling with the growing polymer, is a multi-step process that produces the complex lignin polymer.

The mechanism of endocarp hardening in peach has been investigated to a limited extent examining only one or two components or enzymes in the composition and formation of the stone tissue (Ryugo, 1963; Abeles and Biles, 1991; Alba et al., 2000; Hayama et al., 2006) Ryugo documented in the early 1960s that peach stones are rich in lignin, the seasonal pattern of lignin accumulation, and the presence of lignin biosynthesis intermediates (Ryugo, 1961, 1963). These studies and others have shown an increase in stone dry weight and lignification that begins in the second stage of fruit development until maturity (Ryugo, 1961; Nakano and Nakamura, 2002). More recently, biochemical analysis of drupes including olive, black walnut, peach, and coconut indicate they contain nearly twice as much lignin as wood, suggesting that the process of secondary wall formation can occur to a relatively extreme degree in fruit endocarp tissues (Mendu et al., 2011).

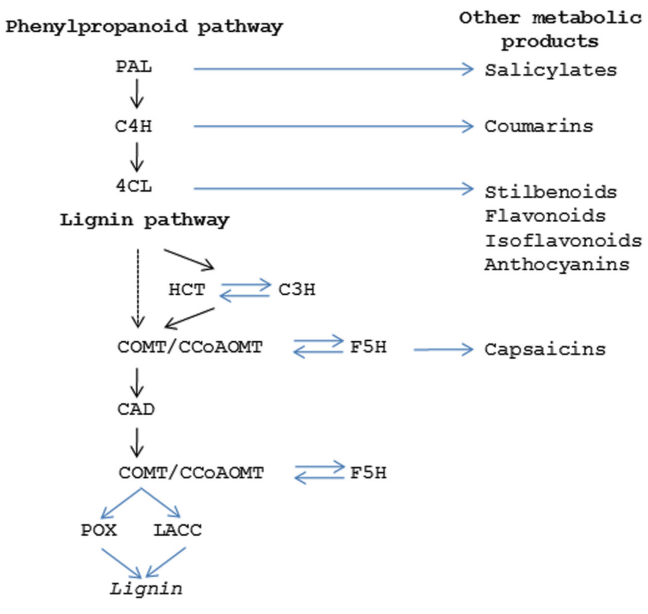

FIGURE 4 | Secondary metabolic pathways. Diagram showing the enzymes in the phenylpropanoid pathway (PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL,4-coumarate:coenzyme A ligase) which produces the precursor products for lignin (HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; C3H, 4-coumarate 3 –hydroxylase; COMT, caffeic acid O-methyltransferase; CCoAOMT, Caffeoyl CoA O-methyltransferase; F5H, ferulate 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; POX, peroxidase; LACC, laccase). Steps in phenylpropanoid and ligin pathways that give rise to other secondary metabolism products are indicated.

In addition to lignin, the phenylpropanoid pathway produces other secondary metabolic products that play important roles in fruit function (**Figure 4**). In some cases these compounds are critical for conferring seed protection and specifying seed dispersal. Coumarins, stilbenes, flavonols, and isoflavonoids have anti-microbial properties that limit bacterial and fungal disease (Dixon and Paiva, 1995). Other compounds contribute to fruit flavor and aroma; either attracting or deterring herbivores (Smith, 1982; Biggs and Northover, 1988; Peters and Constabel, 2002; Vom Endt et al., 2002). Herbivores are also strongly influenced by fruit coloration which is often attributable to anthocyanins and confer red or purple colorations. While this topic will not be extensively covered here, the fact that many of these functions arise from modifications of the same core enzymatic pathway highlights how relatively small changes in the control of secondary metabolic products can have large impacts on fruit phenotypes.

GENETIC BASIS FOR ENDOCARP SPECIFICATION

Advances in genetics and genomics technologies are speeding identification of the underlying genes and signaling pathways that control differentiation of ovarian tissues into endocarp, mesocarp, and exocarp. *Arabidopsis* is leading the way and the information gained is now being translated to numerous other crops. While our current knowledge is still limited, it is becoming apparent that the same or very similar cellular programs contribute to pericarp tissue differentiation in a variety of species. Here, we review and discuss the developments regarding this emerging field of study in the Brassicaceae, Rosaceae, and Solanaceae families.

BRASSICACEAE

Brassicaceae includes a number of economically important plants such as mustard, cabbage, radish, broccoli, and turnips. The model plant *Arabidopsis thaliana* is also a member of this family. Most Brassica species have a dehiscent pod-like fruit called a silique (long and narrow) or silicle (short and wide) and contain a distinctive replum tissue that separates the two valve margins. Mutagenesis screens in *Arabidopsis* have generated a large number of fruit morphology mutants. Some of these were found to contain defects in the dehiscence process and were named according to their phenotypes including *indehiscent* (*ind*), *shatterproof* (*shp*), *alcatraz* (*alc*), *spatula* (*spt*), *fruitfull* (*ful*), and *replumless* (*rpl*; reviewed by Dinneny and Yanofsky, 2004; Dinneny et al., 2005; Lewis et al., 2006). The identification and cloning of the underlying genes

has provided insight into the molecular mechanisms of dehiscence and how pericarp tissues differentiate and lignify during development.

Specific zones within the pericarp are controlled by a coordinated set of transcription factors (TF) that specify tissue fate (**Figure 5**). The lignified valve margin layer responsible for pod shatter is determined by two partially redundant genes, *SHP1* and *SHP2*, which encode MADS-BOX TFs. *SHP* is closely related to the class C gene *Agamous* (*AG*) that regulates flower carpel and stamen identity (Liljegren et al., 2000). Siliques in *shp1*/*shp2* double mutants do not lignify within the valve margin layer and fail to dehisce. Specification of valve cell fate by *SHP1* and *SHP2* is delimited by another MADS-BOX TF called FUL. *FUL* is expressed throughout the valves and negatively regulates *SHP1* and *SHP2*, restricting their activity to the valve margin. Arabidopsis *ful* mutants produce siliques where the entire valve mesocarp lignifies while FUL over-expression leads to conversion of valve margins and outer replum into non-lignified valve tissue; resulting in indehiscent siliques (Ferrándiz et al., 2000). SHP1 and SHP2 positively regulate a basic helix loop helix (bHLH) TF called IND (Liljegren et al., 2004). *IND* is also negatively regulated by FUL and has been shown to prevent valve margin cells from adopting a valve identity

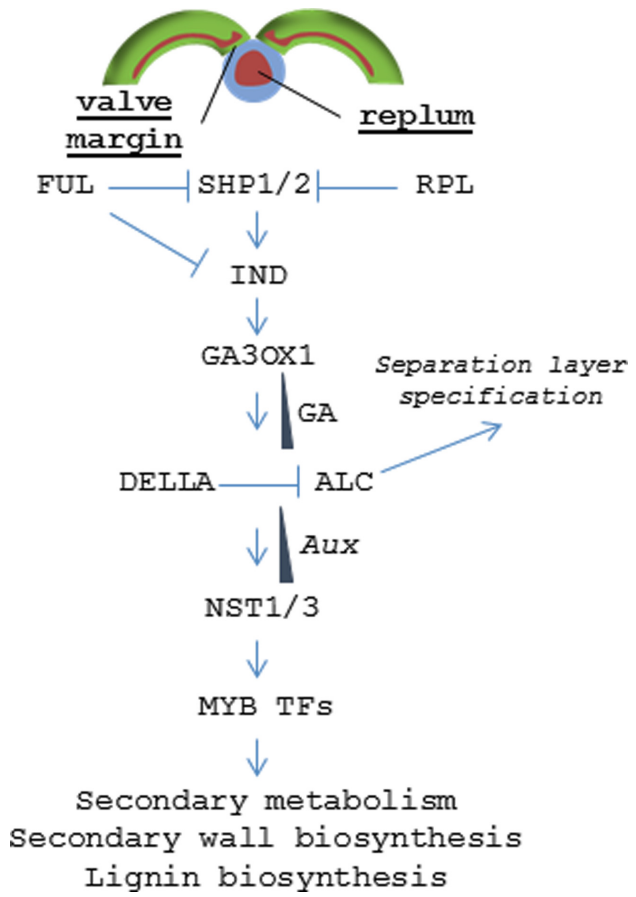

FIGURE 5 | Signaling pathway regulating dehiscence in *Arabidopsis*. Model depicting the hierarchy of transcription factors that control tissue patterning in the valve margin and replum.

(Girin et al., 2010). It does this by coordinating an auxin gradient in the separation layer cells resulting in the formation of lignified valve margin tissues required for valve separation (Sorefan et al., 2009).

The non-lignified valve margin layer is determined by ALC and SPT, two partially redundant bHLH class TFs (Rajani and Sundaresan, 2001; Groszmann et al., 2011). *alc* mutants develop relatively normal siliques but lack the non-lignified layer (separation layer) that separates the lignified valve margin from the replum lignification zone. This blocks valve separation resulting in an indehiscent phenotype. ALC is negatively regulated by gibberellic acid (GA) through the DELLA repressor. IND induces expression of a gene encoding a GA activating enzyme (*GA3OX1*) resulting in GA accumulation in the separation layer and subsequent dissociation of the DELLA protein from ALC (Arnaud et al., 2010).

In the replum, RPL serves a similar function as FUL and prevents those non-lignifying cells from adopting a valve margin cell fate by inhibiting *SHP2* expression in the replum (Roeder et al., 2003). In the more severe *rpl* mutant phenotypes, the lignified valve margin layer intrudes into the replum lignification layer resulting in a partially indehiscent silique. *RPL* encodes a BELL1 family homeodomain TF (Roeder et al., 2003). BELL1 had been previously known to negatively regulate *AG* (Yanofsky et al., 1990; Western and Haughn, 1999).

SHP1 and SHP2 have retained some class C function and are marginally redundant with AG in ovule and floral organ differentiation and appear to be regulated by AG (Pinyopich et al., 2003). Ectopic expression of SHP resulted in conversion of sepals to carpel-like structures even in the absence of *AG* (Favaro et al., 2003). Thus, sub-functionalization of *AG* genes has resulted in overlapping and partially redundant pathways controlling different stages of flower and fruit development (Savidge et al., 1995; Colombo et al., 2010). This is also evident for *STK*, another *AG*-like MADS-BOX gene that resides in a distinct clade. STK controls funiculus development and seed release and shares partial redundancy with AG, SHP1 and SHP2 in specifying ovule cell fate but does not have a class C function (Pinyopich et al., 2003; Zahn et al., 2006). Still, STK has retained the capacity for class C function as ectopic STK expression can complement carpel formation in an *agamous* mutant (Favaro et al., 2003). All four members of the AG lineage are known to function in multi-meric MADS-BOX protein complexes with proteins encoded by members of the *SEPALLATA* (*SEP*) floral organ identity genes that together modulate downstream transcriptional activation (Davies et al., 1996; Pelaz et al., 2000; Favaro et al., 2003). The finding that *AG*-like genes independently control both dehiscence and seed release imply that this closely related family of transcriptional regulators has evolved to control distinct fruit development processes (Pinyopich et al., 2003).

While the mechanisms regarding valve margin and replum specification are known in Arabidopsis, signaling associated with en*b* determination is less clear. Each of the dehiscence mutants *shp1*, *shp2*, *ind*, *alc*, *ful*, and *rpl* show relatively normal endocarp development with the exception of the quintuple mutant *ind alc shp1 shp2 ful* that displays a complete loss of pericarp lignification (Liljegren et al., 2004). This finding suggests that en*b* cell fate requires this same pathway but there may be significant

redundancy and/or signaling feedback loops that are not fully understood. But once pericarp tissue identity has been established, at least one downstream pathway leading to tissue differentiation and lignification is known. Two NAC (NO APICAL MERISTEM) family TFs called NST1 and NST3 (NO SECONDARY WALL THICKENING) [also known as SND (SECONDARY WALL-ASSOCIATED NAC DOMAIN)] were found to regulate secondary wall formation and lignification within the endocarp layers (Mitsuda and Ohme-Takagia, 2008). *nst1 nst3* double mutants show little or no lignin accumulation and were found to be required for expression of genes involved in cell wall biosynthesis and secondary metabolism (Mitsuda and Ohme-Takagia, 2008). *NST1* was also shown to regulate anther dehiscence and lignification of woody and vascular tissues (Mitsuda et al., 2005; Zhong et al., 2007). NST1 acts upstream of a series of MYB (myeloblastosis) TFs that, in turn, directly regulate the expression of genes encoding key enzymes in the phenylpropanoid pathway that drives lignin biosynthesis (Mitsuda et al., 2007; Zhong et al., 2007, 2008). Orthologs of *NST1* have similar functions in *Medicago* and poplar vascular tissues; suggesting that lignification in endocarp tissues occurs via the same pathway as that in vegetative tissues and wood (Zhao et al., 2010; Zhong et al., 2010). Still, there is a gap in our understanding of how *NST1* and *NST3* become activated in a tissue specific fashion. The finding that *IND* controls auxin patterning may hold the key as wood formation is also known to be regulated by the establishment of local auxin gradients (Nilsson et al., 2008; Sorefan et al., 2009).

Based on the knowledge gained in Arabidopsis, a number of researchers have evaluated whether these same genetic pathways are conserved in other Brassica species. The valve margins of dehiscent fruit in *Lepidium campestre* are very similar to that of Arabidopsis and expression of *ALC*, *IND*, *SHP1*, and *SHP2* was likewise found to be limited to the valve margins (Mummenhoff et al., 2009). Lenser and Theißen (2013) showed that RNAi knockdown or over-expression of *IND*, *ALC*, *SHP*, or *FUL* resulted in the anticipated indehiscent phenotypes and mimicked those observed in Arabidopsis with only minor differences. The regulatory interactions among these genes in *FUL* or *IND* lines were also conserved as ALC was found to be a negative regulator of *IND* in both *L. campestre* and Arabidopsis (Lenser and Theißen, 2013). In contrast, gene expression of *ALC*, *IND*, *SHP1*, and *SHP2* was found to be abolished in the tissue corresponding to the valve margins in *Lepidium appelianum*, a Brassica species that produces an indehiscent fruit lacking the separation layer (Mummenhoff et al., 2009). The authors concluded that the evolution of indehiscence in this species likely involved changes in an upstream regulator of the pathway. Expression and functional studies ruled out known regulators including orthologs of *FUL*, *RPL*, and *APETALA2* (*AP2*; Mühlhausen et al., 2013). Some dehiscent *Brassica* species vary with respect to the development of valve margins. *Erucaria erucarioides* and *Cakile lanceolata* produce heteroarthrocarpic fruits where only the proximal segment of the silique dehisces while the distal portion remains indehiscent (Avino et al., 2012). Expression of the valve margin identify genes *ALC*, *FUL*, *IND*, *RPL*, *SHP1*, and *SHP2* was largely conserved in the proximal dehiscent part of the fruit but absent in the distal indehiscent portion. Collectively, these studies indicate that evolutionary adaptations in *Brassica* siliques are, in part, driven by changes in the expression of a single coordinated developmental pathway that helps define the valve, valve margin, separation layer, and replum lignification zones.

ROSACEAE

In contrast to the Brassicaceae, plants in the family Rosaceae encompass an extremely wide range of fruiting types including drupes, pomes, achenes, as well as a number of dry dehiscent and indehiscent fruits. The genus *Prunus* exclusively contains drupes including peaches, plums, apricots, almonds, and cherries which produce a large lignified endocarp that surrounds the seed; commonly called the stone. These fruits grow in a sigmoidal pattern and display a pause in growth that coincides with endocarp hardening. This may be a consequence of the increased carbon and energy demands associated with lignification (Callahan et al., 2009). Recent studies on the pattern and timing of endocarp lignification reveal it is a highly coordinated process that occurs over a 2- to 3-week period (Tani et al., 2007; Dardick et al., 2010; Hu et al., 2011; Lombardo et al., 2011; **Figure 6**). While the timing can vary between cultivars, lignin is often first detectable approximately 35-45 days after bloom in a thin endocarp layer along the fruit suture and in the funiculus. But after several days the entire endocarp begins to lignify. Hardening appears to follow the same pattern as lignin accumulation since the tissue in which lignin is first detectable is also the first to harden.

While functional studies are still lacking, expression profiling data suggests that many of the same genes that control dehiscence in Brassica species also control endocarp development in peach (*Prunus persica*; Dardick et al., 2010; **Figure 7**). The peach homologs of *SHP* and *STK* were found to be up-regulated in the endocarp shortly after pollination. *SHP* and *STK* expression were restricted to the endocarp and seed but gradually decline near the onset of lignin accumulation. Likewise, *FUL* expression remained higher in the mesocarp and exocarp but was constitutively low in the endocarp. This is consistent with a possible role in delimiting endocarp lignification margins. Upon the decline of *SHP* and *STK*, the expression of a peach *NST1* homolog rapidly accumulated along with secondary metabolism and cell wall biosynthesis genes. While clear homologs of *ALC* and *IND* were not found in peach, the two most similar genes were not endocarp specific

FIGURE 6 | Pattern of lignin production in plum endocarp. Shown is a plum fruit series sectioned parallel to the suture line and stained with Phloroglucinol-HCL which turns red in the presence of lignin. After 53 days the endocarp begins to harden such that it can no longer be cut with a scalpel.

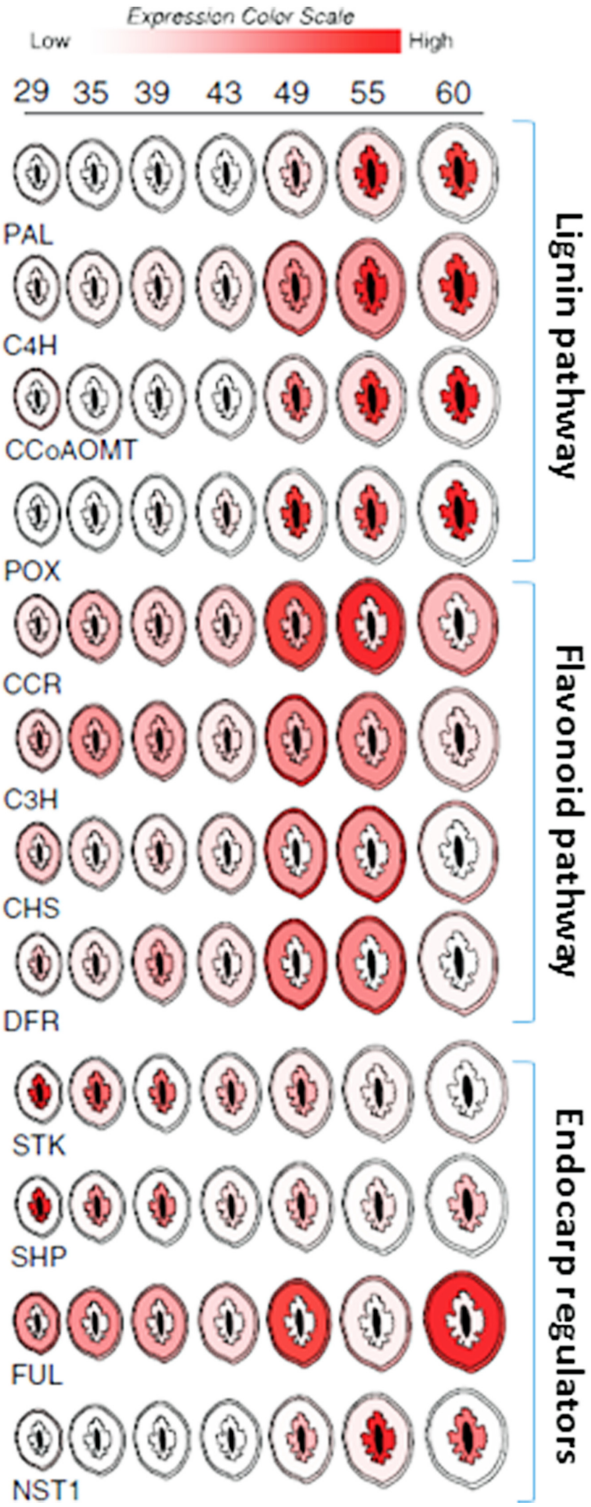

FIGURE 7 | Spatial/temporal pattern of gene expression in developing peach fruit. Cross sections of peach fruits from 29 to 60 days after bloom are depicted. Relative gene expression levels within the exocarp (outer skin), mesocarp (fleshy middle), and endocarp (inner stone) sections are color coded (scale bar at top). Expression in seed (black center) is not shown. Target gene abbreviations are listed below each series and the relevant pathways are delimited by brackets and labeled (right).

(Dardick et al., 2010). *ALC* was previously shown to be specific to *Brassica* species and evolved as a recent duplication of another bHLH TF called *SPATULA* (*SPT*; Groszmann et al., 2008). Tani et al., 2011 showed that the expression patterns of peach *SPT* were consistent with a role in specifying endocarp margins. Collectively, these data imply that highly similar pathways likely control pericarp development in both *Prunus* and Brassica fruits.

Peach mesocarp and exocarp tissues accumulate other secondary metabolic compounds including flavonoids. Flavonoids are an important class of compounds found in nearly all fruit. They provide resistance against disease and pests and contribute to fruit flavor and color. Well known examples include the anthocyanins which are commonly responsible for the orange, red, and purple colorations found in many fruits. Like lignin, flavonoids are also synthesized via secondary metabolism pathways which are thought to be competitive with lignin since both draw on the same precursors of the phenylpropanoid pathway. Peach fruit showed simultaneous activation of the lignin and flavonoid pathways during early fruit development (Dardick et al., 2010; Hu et al., 2011). These events were spatially coordinated such that phenylpropanoid pathway genes were induced in all three pericarp layers; endocarp, mesocarp, and exocarp (though to a much greater degree in endocarp). But in the endocarp this upregulation was accompanied by lignin pathway induction and concomitant flavonoid pathway repression while in the mesocarp and exocarp flavonoid pathway genes were induced and lignin genes were repressed (**Figure 7**). Presumably this coordination allows the fruit to accumulate defense compounds, flavor, and color development in the mesocarp and exocarp while simultaneously enabling endocarp lignification. Thus, seed protection via endocarp lignification appears to be coordinated with the production of compounds necessary for defense, herbivore attraction, and seed dispersal.

There is tremendous variation in *Prunus* endocarp phenotypes which have been selected through breeding. For example, almond shells vary with respect to endocarp thickness, hardness, and brittleness. These agronomic qualities are critical for processing almonds and other types of nuts. Some peach varieties suffer from a phenotype called "split pit" where the endocarp does not seal along the suture leaving the seed vulnerable to pests and disease. Peach cultivars that resume rapid fruit growth before the stone has completely hardened are more likely to have split pits. Tani et al. (2007) found that SHP expression in a split pit resistant variety was lower during the lignification stage while FUL expression was significantly elevated in the sensitive variety during later stages of fruit growth.

"Stoneless" is a naturally occurring phenotype first found in a wild-type plum (*Prunus domestica*) species from France, Sans Noyau (Callahan et al., 2009). "Stoneless" does not completely develop the endocarp layer resulting in a partially naked seed that sits within an empty fruit cavity (**Figure 8**). We have observed that the "Stoneless" phenotype is strongly influenced by the environment since in years with hot spring temperatures fruit tend to contain a more complete stone while in cooler years very little stone is present (Callahan et al., 2009). The hardened tissue that remains in "Stoneless" appears to coincide with the

FIGURE 8 | Naturally occurring *prunus* mutant phenotype.
(A) Transverse section of a wild-type plum (left) and "Stoneless" (right). The endocarp is visible as a whitish tissue layer surrounding the seed. In "Stoneless" a cavity is visible where endocarp tissue is normally found. **(B)** Same fruit stained with phloroglucinol-HCL to visualize lignin production (red color). Little or no lignin staining is found in "Stoneless."

funiculus and a portion of the plancental endocarp wall (Callahan et al., 2009). Expression studies show that the lignification process likely functions normally in "Stoneless" since secondary metabolism genes are still induced. The lack of endocarp tissue suggests that this mutant does not contain a complete endocarp layer.

A handful of studies have also been carried out in other Rosaceous genera. Hawthorn (*Crataegus* spp.) produces a pome fruit that often contains a hardened endocarp like a drupe, however, some species are known to produce soft, edible endocarps. Expression profiling studies revealed that unlike that observed in those with a hardened endocarp, the lignin pathway was not upregulated in the endocarp of soft hawthorns (Dai et al., 2013). In another pome fruit, japanese pears (*Pyrus pyrifolia*), examination of the gene expression patterns of *SHP* and *FUL* among numerous other MADS-box genes showed that *SHP* expression was limited to the fruit core during early fruit development and was largely absent in the fruit cortex and skin, consistent with the ovarian origin of the core (Ubi et al., 2013). In contrast, *FUL* expression was more uniform and was present in skin, cortex, and core regions. While the pear core itself does not lignify, the adjoining layer is lined by disorganized stone cells or schlereids that can be found scattered throughout the hypanthium resulting in a gritty flesh texture (Tao et al., 2009). In strawberry (*Fragaria ananassa*), Daminato et al. (2013) found that silencing or over-expression of *SHP* did not appreciably alter fruit form. This may be expected since the flesh of strawberry is derived from the flower receptacle and not the pericarp. However, *SHP* transgenic lines did show significant changes in ripening time. These results are consistent with similar experiments in tomato where *SHP* was also shown to be a key regulator of fruit ripening (Itkin et al., 2009). Due to availability of whole genome sequences for a number of Rosaceous species including strawberry, apple, and peach along with established transformation systems, this family offers an excellent opportunity to further study the diversification of fruit development (Velasco et al., 2010; Shulaev et al., 2011; Verde et al., 2013).

SOLANACEAE

The family Solanaceae also contains a wide variety of both dry and fleshy fruit types which have repeatedly undergone a number of berry-to-capsule and capsule-to-berry transitions. A detailed developmental analysis by Pabón-Mora and Litt (2011) showed that early developmental stages are similar among capsular and berry type fruits. Later developmental stages were marked by differentiation of endocarp including changes in cell number, cell expansion, and sclerification.

Tomato (*Solanum lycopersicum*) has long served as a model for fleshy fruit development and ripening. The role of a tomato *SHP* homolog called *TOMATO AGAMOUS-LIKE 1* (*TAGL1*) has been extensively studied (Vrebalov et al., 2009; Itkin et al., 2009). Silencing of *TAGL1* resulted in both a thinner pericarp layer and impaired ripening. Pericarp thickness was reduced by approximately 50% in *TAGL1* silenced lines compared to wild-type which was attributed to fewer numbers of cell layers (Vrebalov et al., 2009). This same effect on pericarp thickness was not observed in the small fruited MicroTom tomato variety which has a naturally thin pericarp (Pan et al., 2010). Ripening in *TAGL1* silenced lines or lines expressing a chimeric dominant TAGL1 repressor displayed reduced carotenoids (the pigments responsible for fruit coloration in tomato), lower levels of ethylene, and repression of ripening associated genes including those associated with ethylene biosynthesis and signaling (Itkin et al., 2009; Vrebalov et al., 2009; Pan et al., 2010). The role of *TAGL1* in ripening was distinct from the previously described MADS-BOX gene *RIPENING INHIBITOR* (*RIN*), however, these two MADS-BOX genes may overlap in their ability to induce ethylene as TAGL1 protein was shown to bind the *ACC synthase2* (*ASC2*) promoter in a transient assay (Itkin et al., 2009). In contrast, TAGL1 overexpression led to increased fruit fleshiness, fruit-like sepals that ripened, and increased accumulation of carotenoids (Itkin et al., 2009; Vrebalov et al., 2009). When transformed into an *Arabidopsis shp1 shp2* double mutant, *TAGL1* did not rescue the indehiscent phenotype suggesting that *TAGL1* and *SHP1* may have functionally diverged. In contrast, over-expression of the peach *SHP* homolog (also called *PpPLENA*) in tomato gave rise to a phenotype reminiscent of that observed for *TAGL1* (Tadiello et al., 2009). Experiments to test whether peach *SHP* can complement *Arabidopsis* mutants have not yet been reported. It was shown, however, that ectopic expression of *SHP* derived from the Rosaceous species *Taihangia rupestris* led to conversion of sepals to carpelloid structures and promoted premature pod shatter (Lü et al., 2007). Collectively, these findings suggest that *SHP*-like genes have conserved functions but may have differentiated during the evolution of new fruiting structures and seed dispersal strategies.

Tobacco species are also members of the Solanaceae and produce dry capsular fruits that dehisce upon maturation. Overexpression of a *Nicotiana tobacum* homolog of *FUL* led to indehiscent phenotypes in both *N. tobacum* and *N. sylvestris* that was attributed to reduced lignification along the carpel midrib

(Smykal et al., 2007). Knock down of *SHP* through virus induced gene silencing (VIGS) led to a complete loss of dehiscence and lack of lignified layers lining the dehiscence zones (Fourquin and Ferrándiz, 2012). In addition, *SHP* silencing caused significant alterations in flower development marked by incomplete carpel fusion and shortened styles. Meristem specification was also altered leading to additional carpel and stamen abnormalities. Simultaneous silencing of *SHP* and *AG* led to further loss of stamen and carpel identity as did silencing *AG* alone, suggesting that *SHP* plays only a minor role in C-function (Fourquin and Ferrándiz, 2012). The data imply a limited sub-functionalization of *SHP* from the progenitor C-class TF *AG* in *N. benthamiana*. Similar C-function overlap between *AG* homologs in snapdragon (called *FARINELLI* and *PLENA*, respectively) and in Petunia (called *PETUNIA MADS-BOX GENE 3* (*PMADS3*) and *FLORAL BINDING PROTEIN 6* (FBP6)) was also observed (Causier et al., 2005; Heijmans et al., 2012).

BROADER PERSPECTIVES

As our knowledge of fruit development expands beyond model crops, some of the genes responsible for natural variation in fruit forms are beginning to emerge. A recent report on *Medicago* showed that the coiled pod morphology unique to some members of that genus was likely the result of amino acid changes with a *SHP* homolog that promotes increased valve margin lignification (Fourquin et al., 2013). The loss of the hardened endocarp in commercial oil palm varieties was recently traced to mutations in the DNA binding domain of SHELL, a *STK* homolog, that were shown to prevent association with SEP (Singh et al., 2013). This stands in contrast to Arabidopsis where *STK* does not appear to play a role in endocarp differentiation (Pinyopich et al., 2003; Zahn et al., 2006). Findings such as these provide our first glimpse into how plants have evolved such a dizzying array of fruiting structures and seed dispersal strategies. It is now becoming clear that rapid conversions of fruit form and function are possible through changes in the expression patterns and/or activity of sub-functionalized *AG*-like genes or their associated regulators. These changes can lead to spatial/temporal shifts in cell fate determination accompanied by modifications in secondary metabolic activities that mediate downstream events such as lignification, coloration, and/or generation of herbivore attractants/repellents.

The current emphasis on Arabidopsis as a model system has undoubtedly introduced some level of bias into our current level of knowledge and there is a clear need for plant biologists to expand molecular developmental studies to other crops. For example, the degree to which *AG*-like genes and their known partners have played a role in natural selection of plant species remains to be seen. New sequencing technologies that enable gene mapping through genome-wide association studies (GWAS) along with a growing genomic toolkit promise to address these questions. Ongoing experiments to unveil the specific changes that have allowed different fruit forms to emerge within the same plant lineage will help shed light on the identity of key developmental pathways, the degree of plasticity of these regulatory systems, and how specific plants have adapted to occupy new niches.

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Evolution of fruit development genes in flowering plants

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The genetic mechanisms regulating dry fruit development and opercular dehiscence have been identified in *Arabidopsis thaliana*. In the bicarpellate silique, valve elongation and differentiation is controlled by *FRUITFULL* (*FUL*) that antagonizes *SHATTERPROOF1-2* (*SHP1/SHP2*) and *INDEHISCENT* (*IND*) at the dehiscence zone where they control normal lignification. *SHP1/2* are also repressed by *REPLUMLESS* (*RPL*), responsible for replum formation. Similarly, *FUL* indirectly controls two other factors *ALCATRAZ* (*ALC*) and *SPATULA* (*SPT*) that function in the proper formation of the separation layer. FUL and SHP1/2 belong to the MADS-box family, IND and ALC belong to the bHLH family and RPL belongs to the homeodomain family, all of which are large transcription factor families. These families have undergone numerous duplications and losses in plants, likely accompanied by functional changes. Functional analyses of homologous genes suggest that this network is fairly conserved in Brassicaceae and less conserved in other core eudicots. Only the MADS box genes have been functionally characterized in basal eudicots and suggest partial conservation of the functions recorded for Brassicaceae. Here we do a comprehensive search of *SHP*, *IND*, *ALC*, *SPT,* and *RPL* homologs across core-eudicots, basal eudicots, monocots and basal angiosperms. Based on gene-tree analyses we hypothesize what parts of the network for fruit development in Brassicaceae, in particular regarding direct and indirect targets of *FUL,* might be conserved across angiosperms.

Keywords: AGAMOUS, INDEHISCENT, FRUITFULL, Fruit development, REPLUMLESS, SPATULA, SHATTERPROOF

INTRODUCTION

Fruits are novel structures resulting from transformations in the late ontogeny of the carpels that evolved in the flowering plants (Doyle, 2013). Fruits are generally formed from the ovary wall but accessory fruits (e.g., apple and strawberry) may contain other parts of the flower including the receptacle, bracts, sepals, and/or petals (Esau, 1967; Weberling, 1989). For purposes of comparison we will discuss fruits that develop from the carpel wall only. Fruit development generally begins after fertilization when the carpel wall (pericarp) transitions from an ovule containing, often photosynthetic vessel, to a seed containing dispersal unit. The fruit wall will differentiate into endocarp (1-few layers closest to developing seeds, often inner to the vascular bundle), mesocarp (multiple middle layers, including the vascular bundles and outer tissues), and exocarp (for the most part restricted to the outermost layer, and only occasionally including hypodermal tissues) (Richard, 1819; Sachs, 1874; Bordzilowski, 1888; Farmer, 1889; Roth, 1977; Pabón-Mora and Litt, 2011). Fruits are classified by their number of carpels, whether multiple carpels are free or fused, texture (dry or fleshy), how the pericarp layers differentiate and whether and how the fruits open to disperse the seeds contained inside (Roth, 1977).

There is a vast amount of fruit morphological diversity and fruit terminology that corresponds to this diversity (reviewed in Esau, 1967; Weberling, 1989; **Figure 1**). For example, fruits made of a single carpel include follicles or pods (e.g., *Medicago truncatula;* **Figure 1D**) and sometimes drupes (e.g., *Ascarina rubricaulis*; **Figure 1K**). Follicles and pods both have thick walled exocarp and thin walled parenchyma cells in the mesocarp. However, follicles also have thin walled parenchyma cells in the endocarp while many pods have a heavily sclerified endocarp with 2 distinct layers with microfibrils oriented in different directions (Roth, 1977). When follicles mature the parenchyma and schlerenchyma cell layers dry at different rates causing the fruit to open at the carpel margins (adaxial suture) while pods open at the carpel margin and the median bundle of the carpel due to additional tensions in the endocarp (Roth, 1977; Fourquin et al., 2013). Fruits that are multicarpellate but not fused can include follicles that are free on a receptacle (e.g., *Aquilegia coerulea;* **Figure 1H**). Fruits that are multi-carpellate and fused include berries (e.g., *Solanum lycopersicum, Carica papaya,* and *Vitis vinifera*; **Figures 1B,C,E**), capsules (e.g., *Arabidopsis thaliana*, *Eschscholzia californica*, *Papaver somniferum*; **Figures 1A,F,G**), caryopses (grains of *Oryza sativa* and *Zea mays*; **Figures 1I,J**), and drupes (e.g., peach). These multicarpellate fruits differ by the differentiation of the pericarp

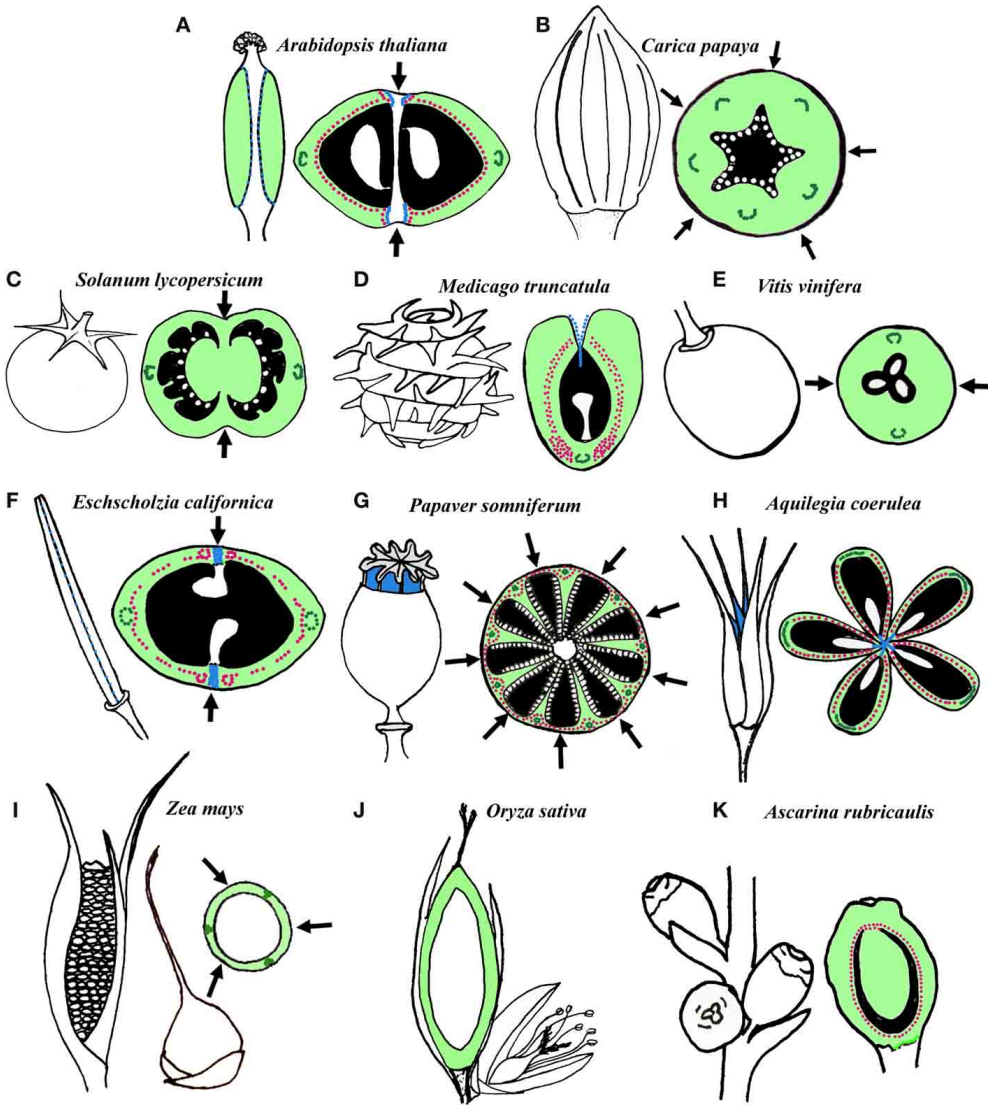

FIGURE 1 | Schematic representation and transverse/longitudinal sections of several fruits. (A–E) Examples of fruits in core eudicots. **(A)** Operculate capsule of *Arabidopsis thaliana* (Brassicaceae) derived from a bicarpellate and bilocular syncarpic gynoecium. **(B)** Berry of *Carica Papaya* (Caricaceae) derived from a pentacarpellate and unilocular syncarpic gynoecium. **(C)** Berry of *Solanum lycopersicum* (Solanaceae) derived from a bicarpellate and bilocular gynoecium. **(D)** Dehiscent pod of *Medicago truncatula* (Fabaceae) derived from a recurved single carpel. **(E)** Berry of *Vitis vinifera* (Vitaceae) derived from a bicarpellate and unilocular gynoecium. **(F–H)** Examples of fruits in basal eudicots. **(F)** Longitudinally dehiscent capsule of *Eschscholzia californica* (Papaveraceae) derived from a bicarpellate and unilocular syncarpic gynoecium. **(G)** Poricidal capsule of *Papaver somniferum* (Papaveraceae) derived from an 8- to 10-carpellate syncarpic gynoecium with numerous incomplete locules**. (H)** Longitudinally dehiscent follicles of *Aquilegia coerulea* (Ranunculaceae) derived from a pentacarpellate apocarpic gynoecium. **(I–J)** Caryopsis of Poaceae **(I)** *Zea mays* and **(J)** *Oryza sativa*. In both species the fruit is derived from 3 carpels. **(K)** Drupe of *Ascarina rubricaulis* (Chloranthaceae) derived from a unicarpellate gynoecium. (Black, locules; light green, carpel wall; dark green, main carpel vascular bundles; pink, Lignified tissue; blue, dehiscence zones; white, seeds; arrows, fusion between carpels).

and their dehiscence mechanisms. Berries and drupes tend to be indehiscent and the pericarp of berries is often fleshy and composed mainly of parenchyma tissue (Richard, 1819; Roth, 1977). The endocarp and mesocarp of drupes is also fleshy, however, the endocarp is composed of highly sclerified tissue termed the stone (Richard, 1819; Sachs, 1874). Caryopses are also indehiscent and have a thin wall of pericarp fused to a single seed (Roth, 1977). Capsules can have few to many cells in the pericarp and the different layers of the pericarp can be composed of parenchyma tissue in most layers and sclerenchyma tissue in the mesocarp and/or endocarp. Capsules can dehisce at various locations including at the carpel margins (septicidal), at the median bundles (loculicidal) or through small openings (poricidal) (Roth, 1977). The extreme fruit morphologies found across angiosperms, even in closely related taxa suggest that fruits are an adaptive trait, thus, homoplasious seed dispersal forms and

transformations from berries to capsules or drupes and vice versa are common in many plant families (Pabón-Mora and Litt, 2011).

The molecular basis that underlies fruit diversity is not well-understood. However, the fruit molecular genetic network in *Arabidopsis thaliana* (Arabidopsis), necessary to specify the different components of the fruit including the sclerified (lignified) tissues necessary for the controlled opening (dehiscence) of the fruit are well-characterized (Reviewed in Ferrándiz, 2002; Roeder and Yanofsky, 2006; Seymour et al., 2013). Arabidopsis fruits develop from two fused carpels and are specialized capsules called siliques, which open along a well-defined dehiscence zone (Hall et al., 2002: Avino et al., 2012). The siliques are composed of two valves separated by a unique tissue termed the replum present only in the Brassicaceae. The valves develop from the carpel wall and are composed of an endocarp, mesocarp and exocarp. The replum and valves are joined together by the valve margin. The valve margin is composed of a separation layer closest to the replum and liginified tissue closer to the valve. The endocarp of the valves becomes lignified late in development and plays a role, along with the lignified layer and separation layer of the valve margin, in fruit dehiscence (Ferrándiz, 2002).

Developmental genetic studies in *Arabidopsis thaliana* have uncovered the genetic network that patterns the Arabidopsis fruit. FRUITFULL (FUL) is necessary for proper valve development and represses SHATTERPROOF 1/2 (SHP 1/2) (Gu et al., 1998; Ferrándiz et al., 2000a). SHP1/2 are necessary for valve margin development (Liljegren et al., 2000). REPLUMLESS (RPL) is necessary for replum development and represses SHP1/2 (Roeder et al., 2003). The repression of SHP1/2 by FUL and RPL keeps valve margin identity to a small strip of cells. SHP1/2 activate INDEHISCENT (IND) and ALCATRAZ (ALC), which are both necessary for the differentiation of the dehiscence zone between the valves and replum (Girin et al., 2011; Groszmann et al., 2011). IND is important for lignification of cells in the dehiscence zone while IND and ALC are necessary for proper differentiation of the separation layer (Rajani and Sundaresan, 2001; Liljegren et al., 2004: Arnaud et al., 2010). SPATULA (SPT) also plays a minor role, redundantly with its paralog ALC in the specification of the fruit dehiscence zone (Alvarez and Smyth, 1999; Heisler et al., 2001; Girin et al., 2010, 2011; Groszmann et al., 2011).

FUL, SHP1/2, RPL, IND, SPT, and ALC all belong to large transcription factor families. FUL and SHP1/2 belong to the MADS-box family (Gu et al., 1998; Liljegren et al., 2000), IND, SPT, and ALC belong to the bHLH family and RPL belongs to the homeodomain family (Heisler et al., 2001; Rajani and Sundaresan, 2001; Roeder et al., 2003; Liljegren et al., 2004). Some of these transcription factors are known to be the result of Brassicaceae specific duplications, others seem to be the result of duplications coinciding with the origin of the core eudicots (Jiao et al., 2011). For instance *SHP1* and *SHP2* are *AGAMOUS* paralogs and Brassicaceae-specific duplicates belonging to the C-class gene lineage (Kramer et al., 2004). FUL is a member of the *AP1/FUL* gene lineage unique to angiosperms (Purugganan et al., 1995). FUL belongs to the euFULI clade, that together with euFULII and euAP1 are core-eudicot specific paralogous clades. Nevertheless, pre-duplication proteins are similar to euFUL proteins, hence they have been named FUL-like proteins and are present in all other angiosperms (Litt and Irish, 2003). Likewise, *ALC* and *SPT* and *IND* are the result of several duplications in different groups of the bHLH family of transcription factors, but the exact duplication points have not yet been identified (Reymond et al., 2012; Kay et al., 2013). Hence, it is unclear whether this gene regulatory network can be extrapolated to fruits outside of the Brassicaceae. Functional evidence from *Anthirrhinum* (Plantaginaceae) (Müller et al., 2001), *Solanum* (Solanaceae) (Bemer et al., 2012; Fujisawa et al., 2014), and *Vaccinium* (Ericaceae) (Jaakola et al., 2010) in the core eudicots, as well as *Papaver* and *Eschscholzia* (Papaveraceae, basal eudicots) (Pabón-Mora et al., 2012, 2013b) suggest that at least *FUL* orthologs have a conserved role in regulating proper fruit development even in fruits with diverse morphologies. *euFUL* and *FUL-like* genes control proper pericarp cell division and elongation, endocarp identity, and promote proper distribution of bundles and lignified patches after fertilization. However, functional orthologs of *SHP*, *IND*, *ALC*, *SPT*, or *RPL* have been less studied and it is unclear whether they are conserved in core and non-core eudicots. The limited functional data gathered suggests that at least in other core eudicots *SHP* orthologs play roles in capsule dehiscence (Fourquin and Ferrandiz, 2012) and berry ripening (Vrebalov et al., 2009). Likewise, *SPT* orthologs have been identified as potential key players during pit formation in drupes, likely regulating proper endocarp margin development (Tani et al., 2011). *RPL* orthologs have not been characterized in core eudicots, but an *RPL* homolog in rice is a domestication gene involved in the non-shattering phenotype, suggesting that the same genes are important to shape seed dispersal structures in widely divergent species (Arnaud et al., 2011; Meyer and Purugganan, 2013). At this point, more expression and functional data are urgently needed to test whether the network is functionally conserved across angiosperms, nevertheless, all these transcription factors are candidate regulators of proper fruit wall growth, endocarp and dehiscence zone identity, and carpel margin identity and fusion (Kourmpetli and Drea, 2014). In the meantime, another approach to study the putative conservation of the network is to identify how these specific gene families have evolved in flowering plants as duplication and diversification of transcription factors are thought to be important for morphological evolution. Although, based on gene analyses no functions can be explicitly identified, the presence and copy number of these genes will provide testable hypothesis for future studies in different angiosperm groups. Thus, to better understand the diversity of fruits and the changes in the fruit core genetic regulatory network we analyzed the evolution of these transcription factor families from across the angiosperms. We utilized data in publicly available databases and performed phylogenetic analyses. We found different patterns of duplication across the different transcription factor families and discuss the results in the context of the evolution of a developmental network across flowering plants.

MATERIALS AND METHODS

CLONING AND CHARACTERIZATION OF GENES INVOLVED IN THE FRUIT DEVELOPMENTAL NETWORK

For each of the gene families, searches were performed by using the Arabidopsis sequences as a query to identify a

first batch of homologs using Blast tools (Altschul et al., 1990) through Phytozome (http://www.phytozome.net/; Joint Genome Institute, 2010) from all plant genomes available from Brassicaceae and other core eudicots, *Aquilegia coerulea* (basal eudicot) and monocots. To better understand the evolution of the fruit developmental network we have extended our search to other core eudicots, basal eudicots, monocots, basal angiosperms, and gymnosperms using the 1 kp transcriptome database (http://218.188.108.77/Blast4OneKP/home.php). This is a database that comprises more than1000 transcriptomes of green plants and therefore represents a large dataset for blasting orthologous genes of the core fruit gene network outside of Brassicaceae. It is important to note that the oneKP public blast portal does not have the complete transcriptomes publicly available yet for many species and that often the transcriptomes available are those from leaf tissue, reducing the possibilities to blast fruit specific genes in some taxa. In addition we used two additional databases: The Ancestral Angiosperm Genome Project (AAGP) http://ancangio.uga.edu to search specific sequences in *Aristolochia* (Aristolochiaceae, basal angiosperms) and *Liriodendron* (Magnoliaceae, basal angiosperms) and Phytometasyn (http://www.phytometasyn.ca) to search specific sequences from basal eudicots. The sampling was specifically directed to seed plants, therefore outgroup sequences included homologs of ferns and mosses of the targeted gene family (when possible) in addition to closely related gene groups (Supplementary Tables 1–5). Outgroup sequences used for the *APETALA1*/*FRUITFULL* genes include *AGAMOUS Like-6* genes from several angiosperms (Litt and Irish, 2003; Zahn et al., 2005; Viaene et al., 2010). For *AGAMOUS/SEEDSTICK* genes the outgroup includes *AGAMOUS Like-12* sequences from several angiosperms (Becker and Theissen, 2003; Carlsbecker et al., 2013). For *HECATE3*/*INDEHISCENT* genes outgroup sequences include the closely related AtbHLH52 and AtbHLH53 from *Arabidopsis* as well has *HECATE1* and *HECATE2* from other angiosperms (Heim et al., 2003; Toledo-Ortiz et al., 2003). For *SPATULA/ALCATRAZ* outgroup sequences include *HEC3*/*IND* from *Arabidopsis* and other angiosperms (Heim et al., 2003; Toledo-Ortiz et al., 2003; Reymond et al., 2012), and finally for *REPLUMLESS/POUND-FOOLISH* genes the outgroup sequences include *AtSAW1*, *AtSAW2*, and *AtBEL1*, as well as *SAW1* and *SAW2* angiosperm homologs (Kumar et al., 2007; Mukherjee et al., 2009). Vouchers of all sequences and accession numbers are supplied in Supplementary Tables 1–5.

PHYLOGENETIC ANALYSES

Sequences in the transcriptome databases were compiled using Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), where they were cleaned to keep exclusively the open reading frame. Nucleotide sequences were then aligned using the online version of MAFFT (http://mafft.cbrc.jp/alignment/server/) (Katoh et al., 2002), with a gap open penalty of 3.0, an offset value of 0.8, and all other default settings. The alignment was then refined by hand using Bioedit taking into account the protein domains and amino acid motifs that have been reported as conserved for the five gene lineages (alignments shown in **Figures 2**, **4**, **6**, **8**, **10**) Maximum Likelihood (ML) phylogenetic analyses using the nucleotide sequences were performed in RaxML-HPC2 BlackBox (Stamatakis et al., 2008) on the CIPRES Science Gateway (Miller et al., 2009). The best performing evolutionary model was obtained by the Akaike information criterion (AIC; Akaike, 1974) using the program jModelTest v.0.1.1 (Posada and Crandall, 1998). Bootstrapping was performed according to the default criteria in RAxML where bootstrapping stopped after 200–600 replicates when the criteria were met. Trees were observed and edited using FigTree v1.4.0. Uninformative characters were determined using Winclada Asado 1.62.

RESULTS

APETALA1/*FRUITFULL* GENE LINEAGE

APETALA1 (AP1) and FRUITFULL (FUL) are members of the *AP1/FUL* gene lineage. Thus, they belong to the large MADS-box gene family present in all land plants (Gustafson-Brown et al., 1994; Purugganan et al., 1995; Gu et al., 1998; Alvarez-Buylla et al., 2000; Becker and Theissen, 2003). Sequences of AP1 and FUL recovered by similarity in the transcriptomes generally span the entire coding sequence, although some are missing 20–30 amino acids (AA) from the start of the 60 AA MADS domain. The alignment includes the conserved MADS (M) and K domains, approximately with 60 AA and 70–80 AA, respectively, an intervening domain (I) between them with 30 and 40 AA and the C-terminal domain of approximately 200 AA. The alignment of the ingroup consists of a total of 180 sequences (i.e., 29 sequences from 25 species of basal angiosperms, 12 sequences from 4 species of monocots, 44 sequences from 22 species of basal eudicots, and 95 sequences from 35 species of core eudicots). Predicted amino acid sequences of the entire dataset reveal a high degree of conservation in the M, I, and K regions until position 222. The C-terminal domain is more variable, but four regions of high similarity can be identified: (1) a region rich in tandem repeats of polar uncharged amino acids (PQN) up until position 285 in the alignment (Moon et al., 1999); (2) a highly conserved, predominantly hydrophobic motif between positions 290 and 310; (3) a negatively charged region rich in glutamic acid (E) that includes the transcription activation motif in euAP1 proteins (Cho et al., 1999) and (4) the end of the protein that includes a farnesylation motif (CF/YAA) for euAP1 proteins (Yalovsky et al., 2000) and the FUL motif (LMPPWML) for euFUL and FUL-like proteins (Litt and Irish, 2003) (**Figure 2**).

A total of 1715 characters were included in the matrix, of which 1117 (65%) were informative. Maximum likelihood analysis recovered five duplication events, two affecting monocots, particularly grasses resulting in *FUL1*, *FUL2*, and *FUL3* genes (Preston and Kellogg, 2006), another occurring early in the diversification of the Ranunculales in the basal eudicots resulting in the *RanFL1* and *RanFL2* clades (Pabón-Mora et al., 2013b) and two coincident with the diversification of the core-eudicots (Litt and Irish, 2003; Shan et al., 2007) resulting in the *euFULI*, *euFULII,* and *euAP1* clades (**Figure 3**). Bootstrap supports (BS) for those clades is above 80 except for the *RanFL1* and *RanFL2* clades, however within each clade, gene copies from the same family are grouped together with strong support (Pabón-Mora et al., 2013b), and the relationships among gene clades are mostly consistent with the phylogenetic relationships of the sampled taxa (Wang et al., 2009). Another duplication occurred concomitantly

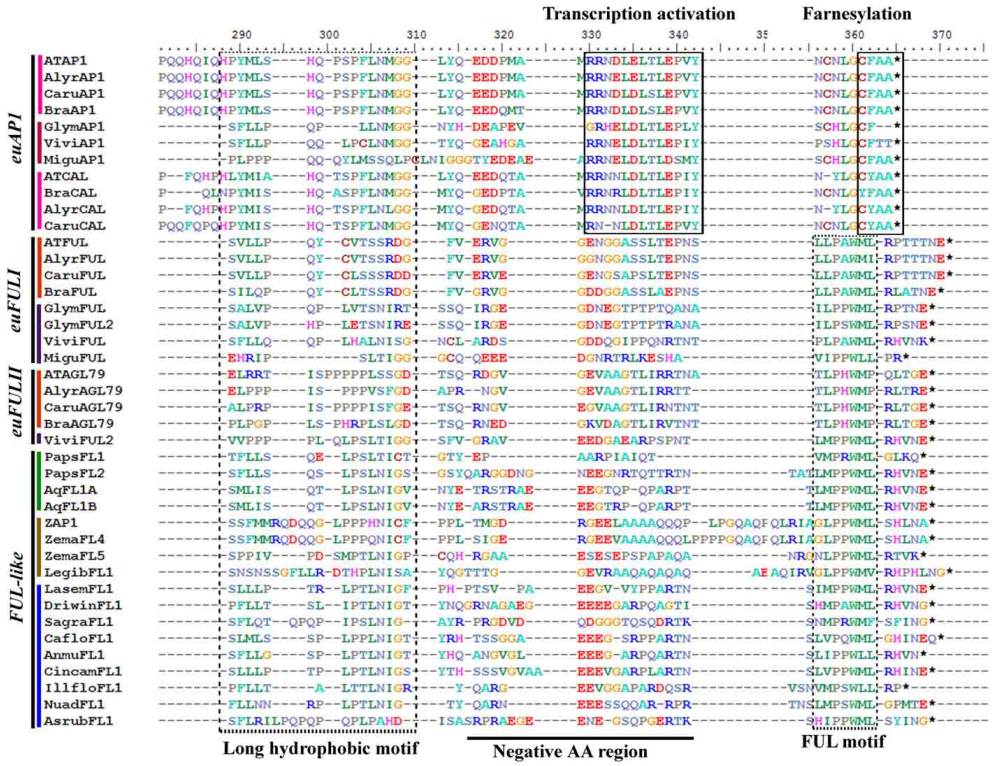

FIGURE 2 | Alignment of the end of the K and the complete C-terminal domain of APETALA1/FRUITFULL proteins (labeled with the clade names they belong to). Colors to the left of the sequences indicate the taxon they belong to as per color key in **Figure 3**. The box to the left shows a conserved long hydrophobic motif, previously identified, but with unknown function, followed by a region variable but consistently with negatively charged amino acids [i.e., rich in glutamic acid (E) particularly in euFULI, euFULII, and FUL-like proteins, and in arginine (R), particularly in euAP1 proteins]. The transcription activation and the farnesylation motifs (boxed) distinguish the euAP1 proteins. The FUL-motif (boxed) is typically found in FUL-like and euFUL proteins.

with the core-eudicot diversification and resulted in the *euAP1* and *euFUL* gene clades (90 BS), followed by another duplication in the *euFUL* clade resulting in the *euFULI* and *euFULII* clades (**Figure 3**; Litt and Irish, 2003; Shan et al., 2007). The duplication itself has low BS, but the *euFULI* and *euFULII* clades have high support with 81 and 74, respectively. Within Brassicaceae another duplication occurred within the euAP1 clade resulting in the *AP1* and *CAL* Brassicaceae gene clades (100 BS) (**Figure 3**; Lowman and Purugganan, 1999; Alvarez-Buylla et al., 2006). Major sequence changes are linked with the core-eudicot duplication. Whereas euFUL proteins retain the characteristic FUL-like motif present in FUL-like pre-duplication proteins present in basal angiosperms, monocots and basal eudicots, the euAP1 proteins acquired, due to a frameshift mutation, a transcription activation and a farnesylation motif at the C-terminus (Cho et al., 1999; Yalovsky et al., 2000; Litt and Irish, 2003; Preston and Kellogg, 2006; Shan et al., 2007), that is very conserved in CAL proteins as well Kempin et al. (1995); Alvarez-Buylla et al. (2006).

Taxon-specific *euFUL* duplications have occurred in *Solanum* (Solanaceae), *Theobroma*, *Gossypium* (Malvaceae), *Eucalyptus* (Myrtaceae), *Glycine* (Fabaceae), *Populus* (Salicaceae) *Portulaca* (Portulacaceae), *Silene* (Caryophyllaceae), and *Malus* (Rosaceae) (**Figure 3**). On the other hand, *euFUL* homologs are likely to be pseudogenized in *Manihot* (Euphorbiaceae), and *Carica* (Caricaceae), where searches on the available genomic sequences, did not retrieve any *euFUL* orthologs. Taxon-specific *euAP1* duplications have occurred in *Malus* (Rosaceae), *Solanum* (Solanaceae), *Manihot* (Euphorbiaceae), and *Citrus* (Rutaceae). *euAP1* homologs seem to be lacking for *Eucalyptus* (Myrtaceae), as sequences previously reported as *EAP1* and *EAP2* by Kyozuka et al. (1997) are members of the *euFULI* and *euFULII* clades. *euAP1* Homologs were also not found in *Fragaria* (Rosaceae) but have been previously reported (Zou et al., 2012) suggesting that the sequence may be divergent enough that is not found through the phytozome blast search. Similarly, *euAP1* sequences were not found in the transcriptomic sequences available for *Silene* (Caryophyllaceae), but have been found before (SLM4, SLM5; Hardenack et al., 1994). In addition, they are likely missing or silent (not expressed) in *Portulaca* (Portulacaceae) but these data will have to be reevaluated as more transcriptomic data from these species becomes publicly available.

AGAMOUS/SEEDSTICK GENE LINEAGE

The SEEDSTICK (STK), AGAMOUS (AG), SHATTERPROOF1 (SHP1) and SHP2 proteins belong to the C and D class of the large MADS-box transcription factor family (Yanofsky et al., 1990; Purugganan et al., 1995; Becker and Theissen, 2003;

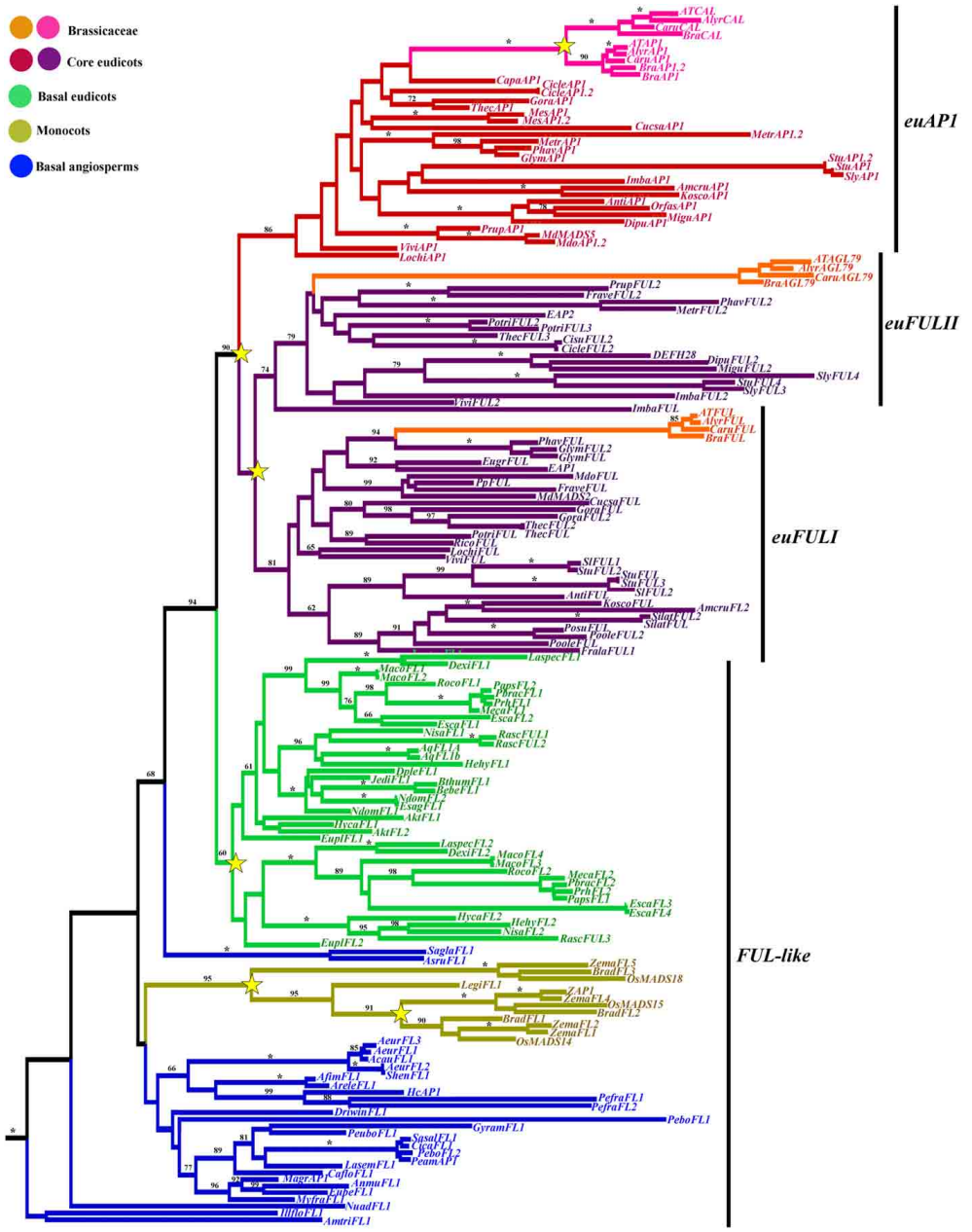

FIGURE 3 | ML tree of *APETALA1/FRUITFULL* genes in angiosperms showing five duplication events (yellow stars). Two duplications in Poaceae, resulting in three distinct monocot *FUL-like* clades; one duplication in basal eudicots resulting in two Ranunculiid *FUL-like* clades; two duplications in the core eudicots resulting in the *euFULI*, *euFULII*, and *euAP1* clades and one additional duplication specific to Brassicaceae resulting in the *CAL* clade. Branch colors denote taxa as per the color key at the top left; BS values above 50% are placed at nodes; asterisks indicate BS of 100.

Colombo et al., 2008). Sequences recovered by similarity in the transcriptomes generally span the entire coding sequence, although some are missing 20–30 amino acids (AA) from the start of the 60 AA MADS domain. The alignment includes the conserved MADS and K domains, approximately with 60 AA and 60–80 AA, respectively, an intervening domain between them with 25 and 30 AA and the C-terminal domain expanding ca. 200 AA. The alignment of the ingroup consists of a total of 185 sequences (i.e., 14 sequences from 14 species of gymnosperms, 13 sequences from 11 species of basal angiosperms, 24 sequences from 18 species of monocots, 35 sequences from 18 species of basal eudicots, and 89 sequences from 40 species of core eudicots). Predicted amino acid sequences of the entire dataset reveal a high degree of conservation in the M, I, and K regions until position 228. A few positions conserved that distinguish the STK from the AG/SHP clade such as the typical Q105 always present in the

STK proteins (with the exception of ChlspiSTK) (Kramer et al., 2004; Dreni and Kater, 2014). Others that distinguish between the AG and the PLE/SHP clades are the GI or IS in positions 105/106 in euAG proteins vs. the conserved RD in the same positions in PLE/SHP proteins. The C-terminal domain is more variable, but two regions of high similarity can be identified: (1) The AG Motif I and (2) The AG Motif II both with predominantly acidic or hydrophobic amino acids. These two motifs are conserved in both the *AGAMOUS/SHATTERPROOF* and the *SEEDSTICK* gene clades in angiosperms as well as in the pre-duplication gymnosperm homologous genes (**Figure 4**) (Kramer et al., 2004; Dreni and Kater, 2014). Only Poaceae *AG/SHP* and *STK* homologs present noticeable divergence in those motifs (**Figure 4**; Dreni and Kater, 2014).

A total of 1720 characters were included in the matrix, of which 915 (53%) were informative. Maximum likelihood analysis recovered five duplication events. The most important one occurred concomitantly with the origin of angiosperms and resulted in the *AG/SHP* and the *STK* gene clades (**Figure 5**). BS for this duplication is low (<50), and the position of the *AG/SHP* monocot clade is variable (retested in parsimony analyses, data not shown), nevertheless the two main resulting clades have BS of 82 and within each clade, relationships among genes are mostly consistent with the phylogenetic relationships of the sampled taxa (APG, 2009). This contrasts with the single copy C and D class genes found in gymnosperms (Kramer et al., 2004; Carlsbecker et al., 2013). They appear to be paraphyletic with respect to the angiosperm C and D lineages, but the three clades that they form have strong supports (**Figure 5**). Both angiosperm gene lineages underwent additional duplications in the grasses that for the most part have two *AG/SHP* gene clades and two *STK* gene clades (Dreni et al., 2013). The *STK* genes have remained mostly single copy in all other angiosperms including basal angiosperms and basal and core eudicots, with only two exceptions. In monocots the radiation of the Poaceae seems to be associated with a duplication in the *STK* genes (BS 98), and in the core eudicots, taxon specific duplications seem to have affected independently *Gossypium* (Malvaceae) and *Glycine* (Fabaceae), each with two *STK* paralogs (**Figure 5**). In addition, our data supports the idea that *STK* genes have been lost or are not expressed in the Eupteleaceae and the Ranunculaceae (basal eudicots), as *STK* homologs were not retrieved from the transcriptomic data available for *Euptelea* or the *Aquilegia* genome. This is consistent with the findings of Liu et al. (2010) and Kramer et al. (2004).

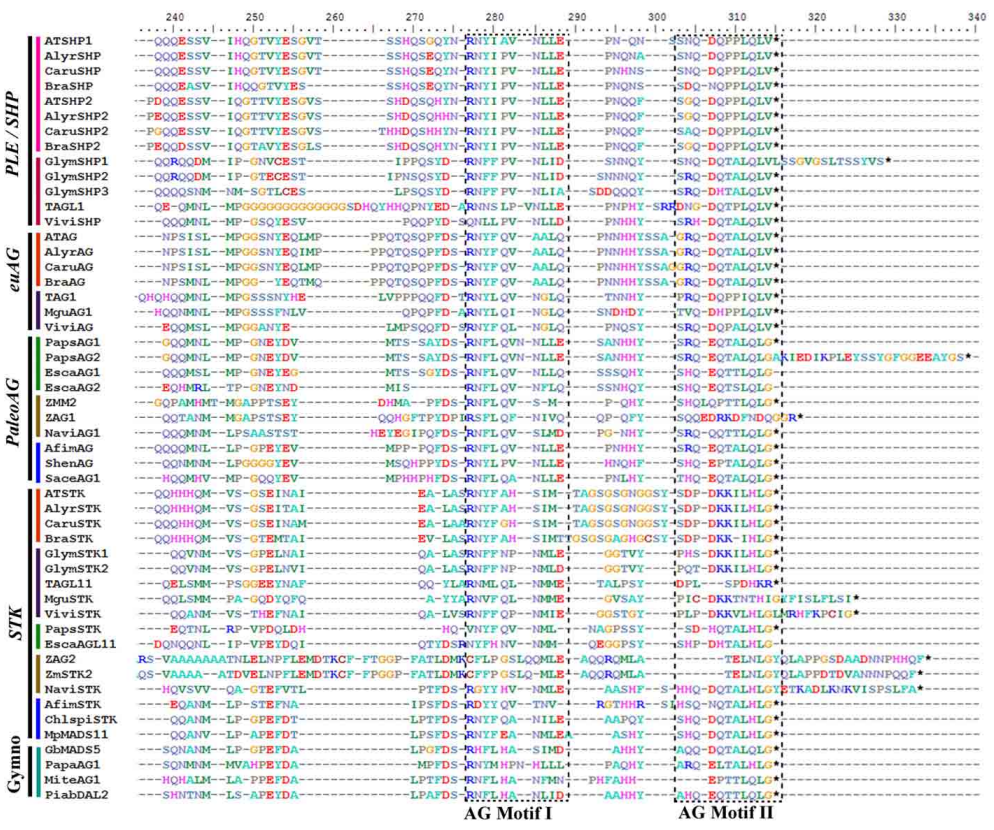

FIGURE 4 | Alignment of the end of the K and the complete C-terminal domain of AGAMOUS/SEEDTICK proteins (labeled with the clade names they belong to). Colors to the left of the sequences indicate the taxon they belong to as per the color key in **Figure 5**. Previously identified conserved AG Motifs I and II in both protein clades are boxed; note that sequences in between the motifs are very different between the AGAMOUS and the SEEDSTICK orthologous proteins, and there appears to be a GS/GN repeat in this region exclusive to Brassicaceae STK sequences; note also the divergence at the end of the K-domain between the closely related paralogous SHP1 and SHP2 in the Brassicaceae. The alignment also includes the atypical paleoAGAMOUS proteins in *Papaver* (PapsAG1, PapsAG2) due to alternative splicing.

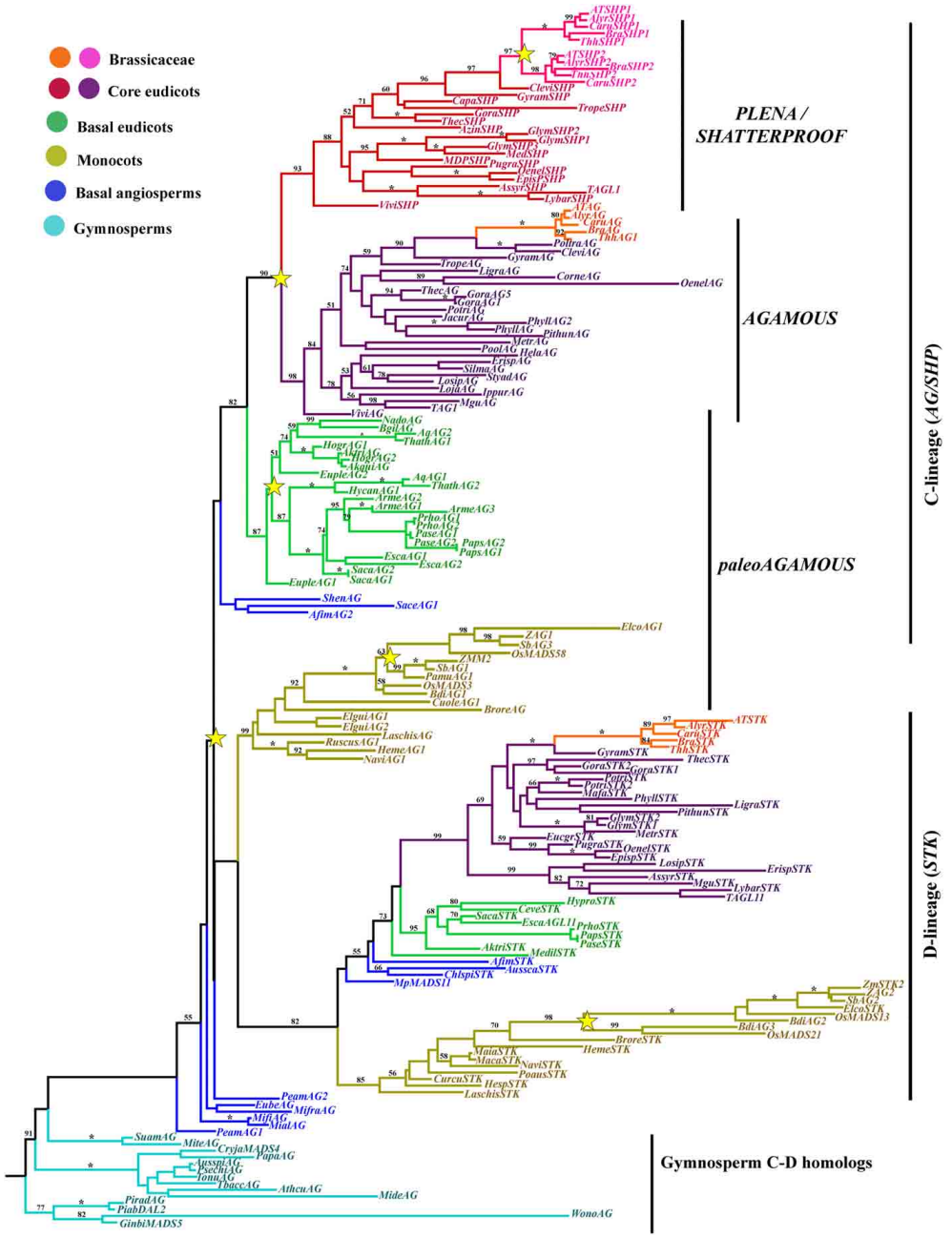

FIGURE 5 | ML tree of *AGAMOUS/SEEDSTICK* genes in seed plants showing a number of duplication events (yellow stars). A duplication coincident with the diversification of the angiosperms, resulting in the D-lineage and the C-lineage clades (also known as *AGL11* and *AG* lineage, respectively). The D-lineage underwent a duplication in Poaceae but for the most part has been kept as single copy in angiosperms (see text for exceptions). The C-lineage duplicated independently in Poaceae, resulting in two paleoAG grass clades, in basal eudicots, resulting in two Ranunculaceae specific clades, and in the core eudicots, resulting in the *euAG* and the *PLE/SHP* gene lineages. An additional duplication occurred with the diversification of the Brassicaceae resulting in the *SHP1* and *SHP2* clades. Branch colors denote taxa as per color key at the top left; BS above 50% are placed at nodes; asterisks indicate BS of 100.

The *AG/SHP* genes have undergone additional duplications during angiosperm diversification. One such duplication seems to have occurred in basal eudicots, before the diversification of the Ranunculaceae, that has two gene clades with strong support (100BS) however, the exact time is unclear as sampling is limited (**Figure 5**; Yellina et al., 2010). Members of the Papaveraceae, also have two paralogous *AG* genes, however, at least in *Papaver* species and the closely related *Argemone*, the two transcripts seem to be the result of alternative splicing, identical to the case reported in *P. somniferum* by Hands et al. (2011). Two additional duplications

occurred in the *AG/SHP* genes, one connected with the diversification of the core eudicots resulting in the *euAG* and the *PLE/SHP* clades (90BS), and the second one in the *PLE/SHP* clade in Brassicaceae resulting in the *SHP1* and *SHP2* gene clades (97BS; **Figure 5**; Kramer et al., 2004; Zahn et al., 2006).

Taxon-specific *euAG* duplications have occurred in *Gossypium* (Malvaceae) and *Phyllanthus* (Euphorbiaceae). Likewise, *PLE/SHP* specific duplications have affected *Glycine* (Fabaceae) and *Brassica* (Brasicaceae). On the other hand, *euAG* homologs are likely to be pseudogenized or have diverged dramatically in sequence in *Malus* (Rosaceae), *Glycine* (Fabaceae), and *Carica* (Caricaceae), as an exhaustive search in their available genomic sequences did not result in any significant hit. Similarly, *PLE/SHP* homologs have diverged considerably or have been lost in *Populus* (Salicaceae) and *Mimulus* (Phrymaceae). Our analysis did not find any *PLE/SHP* homologs in *Lonicera* (Caprifoliacaeae), *Lobelia* (Campanulaceae), *Stylidium* (Stylidiaceae), *Sylibum*, *Erigeron* (Asteraceae), *Coriaria* (Coriariaceae), *Heracleum* (Asteraceae), *Polansia* (Capparaceae), *Ipomoea* (Colvolvulaceae), and *Linum* (Linaceae). Some of the same cases were also noticed by Dreni and Kater (2014) (i.e., loss of *euAG* in *Carica*, and loss of *PLE/SHP* in *Populus* and *Mimulus*), suggesting that pseudogenization likely happened in *PLE/SHP* genes of many core eudicots after the duplication event, however these data would have to be confirmed as a larger set of transcripts from these species becomes publicly available. This scenario is very different in Brassicaceae, where additional duplications occurred as a result of a Whole Genome Duplications (WGD) (Barker et al., 2009; Donoghue et al., 2011) but functional paralogs only remained in the *PLE/SHP* clade with two *SHP* homologs. The Brassicaceae specific copies resulting from this duplication in the *euAG* and the *STK* clades have been likely pseudogenized.

ALCATRAZ/SPATULA GENE LINEAGE

ALCATRAZ (ALC) and SPATULA (SPT) belong to the large bHLH transcription factor family (Toledo-Ortiz et al., 2003; Reymond et al., 2012). Sequences recovered by similarity in the transcriptomes generally span the entire coding sequence. Alignment of the ingroup consists of a total of 139 sequences (i.e., 7 sequences from 7 species of gymnosperms, 5 sequences from 5 species of basal angiosperms, 16 sequences from 13 species of monocots, 14 sequences from 14 species of basal eudicots, and 97 sequences from 53 species of core eudicots). Predicted amino acid sequences of the entire dataset reveal a high degree of conservation in the M, I, and K regions until position 222. The alignment includes a first region extremely variable of 310 AA, where only a few local blocks of conserved amino acids (AA) are observed in closely related species. A second region follows this from 311 to 349 AA with a largely conserved motif DDLDCESEEGG/QE rich in hydrophobic and negative amino acids, in all members of the SPT/ALC proteins in gymnosperms and angiosperms. The exceptions are: The SPT-like2 grass clade with the sequence E/Q H/QLDLVMRHH/Q and the ALC Brassicaceae clade with the sequence VAETS/AQE/DKYA that have more polar uncharged amino acids accompanying the hydrophobic and negatively charged ones (not shown; this region is located immediately before the N-flank shown in **Figure 6**). Right after this region and before the bHLH domain there is a region from 350 to 357 AA in the alignment, rich in polar uncharged and positively charged amino acids fairly conserved across angiosperms and gymnosperms (R/PS/PRSSS/L) with the exception of the SPT-like1 paralogous grass genes that have instead Glycine (G) repeats in this region, labeled as N-flank in reference to the bHLH domain (**Figure 6**). Within the bHLH domain that goes from AA 359 to 410, the SPT/ALC proteins as most other AtbHLH proteins have on average 9 positively charged (K, R, and H) amino acids, in the basic motif that spans 17 AA (**Figure 6**). This is followed by the completely conserved helices interrupted by a loop (HLH), responsible for homodimerization and heterodimerization (Murre et al., 1989; Ferre-D'Amare et al., 1994; Nair and Burley, 2000; Toledo-Ortiz et al., 2003). SPT/ALC share with most other bHLH proteins studied to date, from both animals and plants, the positions H9, E13, R16, L27, K39, L56 (**Figure 6**). The presence of E13 and R16 makes SPT/ALC proteins E-box binders (CANNTG), as these residues are critical to contact the CA in the E-box and confers the DNA binding activity of SPT/ALC proteins (Fisher and Goding, 1992; Ellenberg et al., 1994; Shimizu et al., 1997; Fuji et al., 2000). Furthermore, the E13 residue is essential for DNA binding. SPT/ALC proteins can be further classified into G-box (CACGTG) binders within the E-box binders category, as they possess the H9, E13, R17 positions (Toledo-Ortiz et al., 2003). This binding, specifically to G-boxes, has been demonstrated *in vitro* for SPT (Reymond et al., 2012). After the end of the second helix there is a conserved motif LQLQVQ completely conserved in all sequences, followed by a fairly conserved motif MLS/TMRNGLSLH/N/PPL/MGLPG, both are included at the C-flank of the bHLH motif. This last motif is once again more variable in the ALC Brassicaceae paralogs and in the gymnosperm SPT/ALC homologs (**Figure 6**). From the position 438 until the end of the alignment there are no other regions that seem to be conserved across all SPT/ALC homologs, nevertheless there are some small regions that can be confidently aligned, particularly among closely related plant groups. In this region, there is a very noticeable increase in variation and shortening of the coding sequence in the Brassicaceae ALC homologs suggesting a faster sequence mutation rate. This is likely linked with divergent functions in this gene clade compared with other angiosperm and gymnosperm SPT/ALC proteins.

Because the beginning of the proteins was extremely variable and the homologous nucleotides in the alignment were not clear, we only used the AA from the beginning of the bHLH domain until the end of the proteins for the phylogenetic analysis. A total of 703 characters were included in the matrix, of which 224 (32%) were informative. Maximum likelihood analysis recovered two duplication events. The most important is correlated with the diversification of the core eudicots, resulting in the SPATULA and the ALCATRAZ gene clades (**Figure 7**). Nevertheless, support for this duplication is extremely low (<50), likely because the bHLH motif has little variation, and positional homology cannot be assigned confidently outside this region (Toledo-Ortiz et al., 2003; Pires and Dolan, 2010). This contrasts with the single copy SPT/ALC homolog present in basal eudicots, most monocots, basal angiosperms and gymnosperms. Another duplication is again correlated with the diversification of the Poaceae

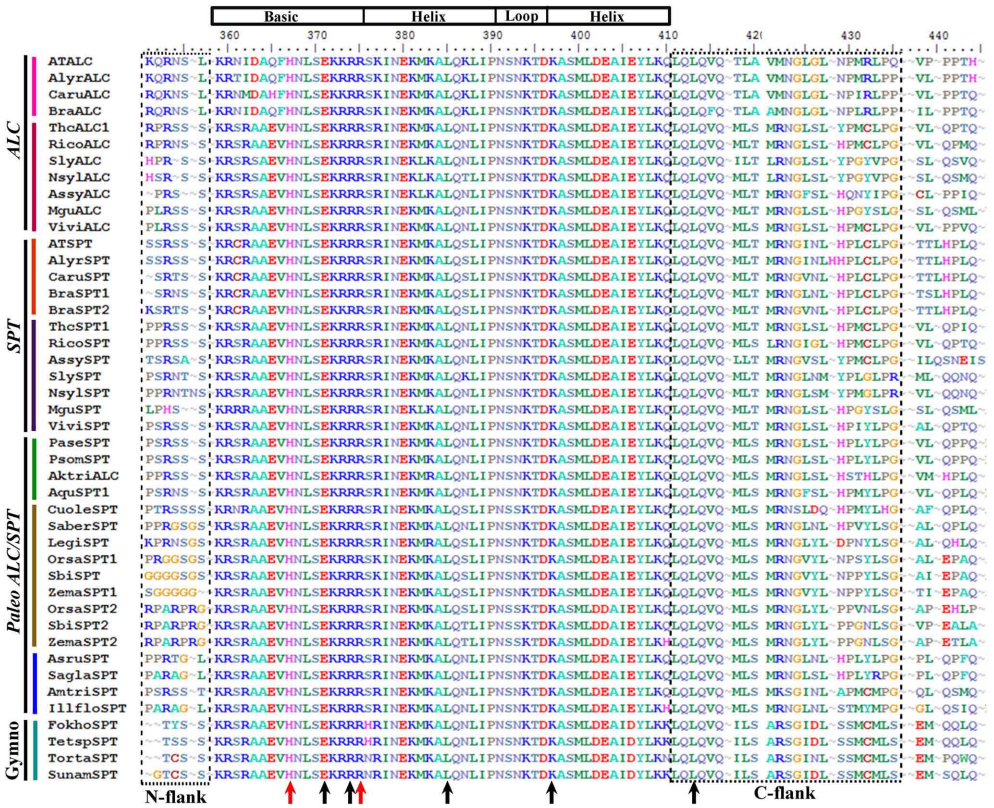

FIGURE 6 | Alignment of the bHLH domain of SPATULA/ALCATRAZ proteins (labeled with the clade names they belong to). Colors to the left of the sequences indicate the taxon they belong to as per color conventions in **Figure 7**. The bHLH was drawn based on Toledo-Ortiz et al. (2003) and in our alignment corresponds with positions K359-Q410. The alignment shows an N-flank before the start of the bHLH domain rich in Serine (S). Within the bHLH domain, black arrows indicate positions E13, R16, L27, K39, L56, which are conserved in all bHLH plant and animal genes. E13 provides the SPT/ALC proteins with E-box binding (CANNTG) activity. The H9 and R17 positions (red arrows) show aminoacids that provide the SPT/ALC proteins with G-box (CACGTG) binding activity. The alignment also shows the conserved motif LQLQVQ in the C-flank of the bHLH motif followed by a fairly conserved motif MLS/TMRNGLSLH/N/PPL/MGLPG (boxed).

(**Figure 7**), that also has low BS (**Figure 7**). However, clades resulting from this duplication have BS100. Most core eudicots had at least two copies, one belonging to the SPT and the other to the ALC clades, however, taxon-specific duplications of *SPT* genes were observed in *Gossypium, Theobroma* (Malvaceae), *Digitalis* (Plantaginaceae), *Solanum tuberosum* (Solanaceae), *Apocynum* (Apocynaceae), and *Brassica* (Brasssicaceae). Our analysis also detected taxon-specific duplications of *ALC* genes in *S. tuberosum* (Solanaceae), *Manihot* (Euphorbiaceae), *Populus* (Salicaceae), and *Cleome* (Cleomaceae).

Although gene losses are harder to confirm, *SPT* homologs were not found in the genome assemblies of *Manihot* (Euphorbiaceae), *Carica* (Caricaceae), and *Mimulus* (Phrymaceae), or the transcriptomic sequences available for: *Urtica* (Urticaceae), *Celtis* (Ulmaceae), *Ficus* (Moraceae), *Cleome* (Cleomaceae), *Strychnos* (Loganiaceae), *Azadirachta* (Meliaceae). On the other hand *ALC* homologs were not found in the genomic sequences available for *Medicago* (Fabaceae), *Eucalyptus* (Myrtaceae), and *Gossypium* (Malvaceae) and the transcriptomes of *Castanea* (Fagaceae), *Digitalis* (Plantaginaceae), *Punica* (Lythraceae), *Oenothera* (Oenotheraceae), *Lobelia* (Campanulaceae), *Cavendishia* (Ericaceae), and *Fouquieria* (Fouquieriaceae).

INDEHISCENT/*HECATE3* GENE LINEAGE

INDEHISCENT (IND) and HECATE3 (HEC3) also belong to the large bHLH transcription factor family (Heim et al., 2003; Toledo-Ortiz et al., 2003). Sequences recovered by similarity in the transcriptomes generally span the entire coding sequence. The alignment of the ingroup consists of a total of 56 sequences (i.e., 5 sequences from 5 species of gymnosperms, 2 sequences from 2 species of basal angiosperms, 14 sequences from 10 species of monocots, 5 sequences from 5 species of basal eudicots, and 30 sequences from 23 species of core eudicots). The alignment includes a first region extremely variable of 415 AA, where there are very few regions of conserved amino acids and no evident conserved motifs, even in closely related taxa. This is followed by a short region rich in DE (negatively charged amino acids) until AA 430. Immediately after there is the N flank of the bHLH domain with a large region of hydrophobic amino acids from AA 430 to 449, identified previously as the HEC domain, and present only in IND/HEC3 genes when compared to other HEC

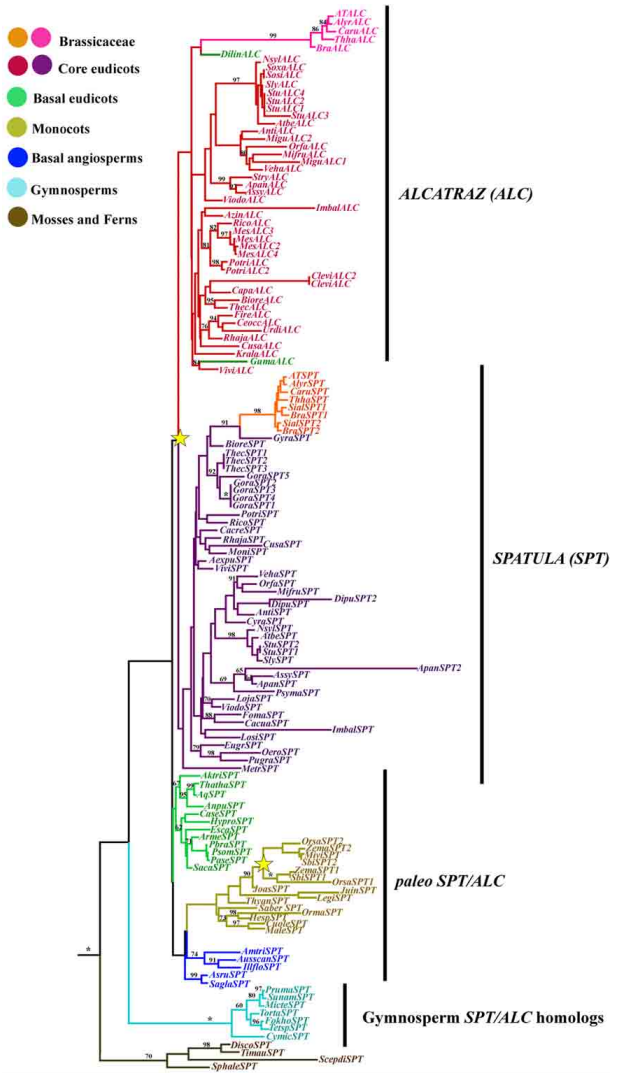

FIGURE 7 | ML tree of *SPATULA/ALCATRAZ* genes in seed plants showing two duplication events (yellow stars). One duplication in the Poaceae, resulting in two *SPATULA-like* clades, and a second independent duplication coincident with the diversification of the core eudicots resulting in the *SPT* and the *ALC* clades. Most sequence changes are linked with the *ALC* genes, particularly in Brassicaceae. Branch colors denote taxa as per color key at the top left; BS above 50% are placed at nodes; asterisks indicate BS of 100.

genes (like HEC1 and 2) (Heim et al., 2003; Gremski et al., 2007; Pires and Dolan, 2010). This region also includes a small motif identified as conserved for all members of bHLH group VIIb called Domain 17 by Pires and Dolan (2010) (**Figure 8**). The end of this domain overlaps with the beginning of the basic region of the bHLH domain. Within the bHLH domain, that goes from AA 462 to 515, the IND/HEC3 proteins, as most other AtbHLH proteins, have on average 9 positively charged (K, R, and H) amino acids, in the basic motif (**Figure 8**) that spans 17 AA. This is followed by the completely conserved helices interrupted by a loop (HLH), responsible for homodimerization and heterodimerization (Murre et al., 1989; Ferre-D'Amare et al., 1994; Nair and Burley, 2000; Toledo-Ortiz et al., 2003; Girin et al., 2010, 2011). Unlike most other bHLH proteins studied to date, the IND/HEC3 proteins have changes in some of the key amino acids, and they possess Q9 instead of H9, A13 instead of E13, they have R16 and R17 and they also conserve L27, A39, Q56 (**Figure 8**). The lack of H9 and E13 suggests that IND and HEC3 are not E-box binders (CANNTG) (Fisher and Goding, 1992; Ellenberg et al., 1994; Shimizu et al., 1997; Fuji et al., 2000; Toledo-Ortiz et al., 2003). After the end of the second helix there is the C flank without any regions obviously conserved (**Figure 8**). From the position 530 until the end of the alignment at AA 655 there are no other regions that seem to be conserved across all *IND/HEC3* homologs. In this region, there is a very noticeable increase in the variation and shortening of the coding sequence in the Brassicaceae IND homologs suggesting a faster sequence change likely linked with divergent functions in this gene clade compared with other angiosperm and gymnosperm IND/HEC3 proteins.

Similar to the SPT/ALC proteins the IND/HEC3 presented very variable 5′and 3′ sequence proteins, nevertheless the IND/HEC3 are smaller and the regions with uncertainty in the alignment were short so we decided to use the entire alignment for phylogenetic analysis. A total of 2127 characters were included in the matrix, of which 997 (47%) were informative. Maximum likelihood analysis recovered a single duplication event concordant with the origin of the Brassicaceae (**Figure 9**). Although BS is low, the clades resulting from this duplication have 100BS. This contrasts with the single copy IND/HEC3 homologs present in the rest of the core eudicots, basal eudicots, most monocots (with the exception of *Zea mays* that has four HEC3 paralogs), basal angiosperms and gymnosperms. Because of similarity sequences with HEC3, more noticeable before the HEC domain (data not shown) they have been called HEC3-like (Kay et al., 2013). Most core eudicots that have genomic sequences available had a single HEC3 copy with the exception of *Populus* (Salicaceae) with three paralogs. From those species with available genomic sequences we could not find homologs in *Eucalyptus* (Myrtaceae), *Manihot* (Euphorbiaceae), or *Glycine* (Fabaceae).

REPLUMLESS/POUND-FOOLISH GENE LINEAGE

REPLUMLESS (RPL) and POUNDFOOLISH (PNF) belong to the TALE group of homeodomain protein (Kumar et al., 2007; Mukherjee et al., 2009) Sequences recovered by similarity in the transcriptomes generally span the entire coding sequence. The alignment of the ingroup consists of a total of 132 sequences (i.e., 11 sequences from 11 species of gymnosperms, 7 sequences from 6 species of basal angiosperms, 14 sequences from 10 species of monocots, 17 sequences from 15 species of basal eudicots, and 83 sequences from 46 species of core eudicots). The alignment includes a first region extremely variable of 544 AA with almost no similarity except sometimes in short regions between closely related taxa. Between positions 545 and 579 AA a first region of high similarity is found. This region includes a previously undescribed G/VPLF/LGPFTGYAS/TI/VLKG/SAT motif. From 560 to 575 AA a SKY motif (SKYLKPAQQ/MV/LLEEFCD/S/N) follows (Mukherjee et al., 2009), however, a true SKY motif is only present

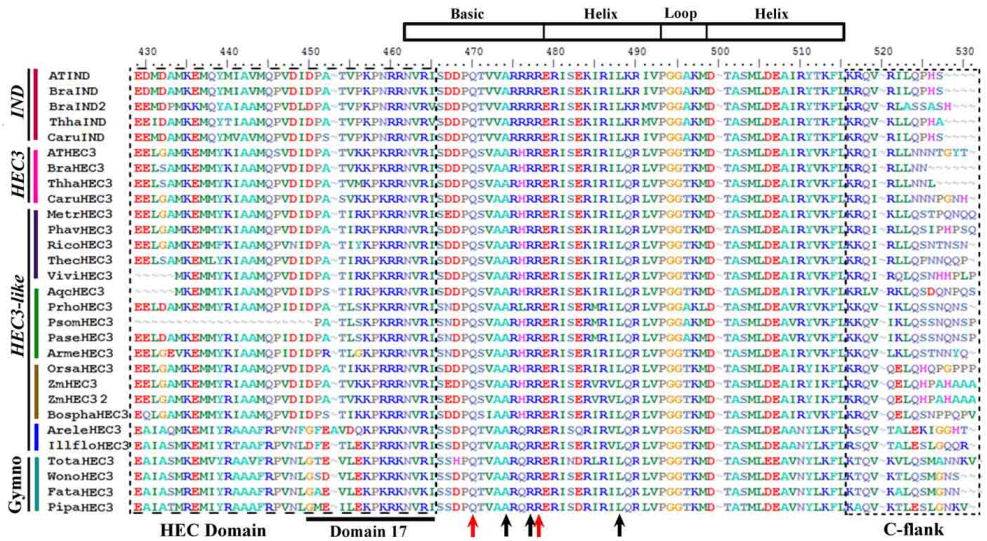

FIGURE 8 | Alignment of the bHLH domain of *HECATE3/INDEHISCENT* proteins (labeled with the clade names they belong to). Colors to the left of the sequences indicate the taxa they belong to as per color key in **Figure 9**. The bHLH was drawn based on Toledo-Ortiz et al. (2003) and in our alignment corresponds with positions N462-L515. Boxed to the left is the N-flank of the bHLH domain rich in hydrophobic aminoacids (called the HEC domain by Kay et al. (2013) and includes domain 17 by Pires and Dolan (2010); note that to Kay et al. (2013) the bHLH domain starts at S462 right after the end of the HEC domain). Black arrows in the bHLH domain indicate key aminoacids for E-box binding activity. Although R16 and L27 are conserved, position E13 (see **Figure 6**) is replaced by a hydrophobic A13 suggesting that HEC3/IND proteins lack this activity. Note that R17 (red arrow) is still conserved but due to the lack of E13 is unclear whether this amino acid conferring specificity plays any role in binding on its own. Additionally, the classic G-box recognition motif is not present in this proteins as the critical H/K positively changes aminoacids are replaced by Q9 with polar and uncharged side chains. Boxed to the right is the poorly conserved C flank of the bHLH motif.

in the gymnosperm RPL/PNF proteins as in the angiosperm RPL and PNF proteins this motif is replaced by SK/RF, with the only exception being *Ascarina* (Chloranthaceae) lacking the entire motif (not shown). There is another region of high variability from AA 576 to 659 before the beginning of the 60AA BELL-domain (from AA 660 to 729) that is highly conserved across gymnosperm and angiosperm RPL/PNF proteins (**Figure 10**). Between the BELL-domain and the homeodomain, there is a region spanning AA 730–792 with high variability where no clear motifs can be identified. This is immediately followed by the 63AA homeodomain spanning the AA 793–856 (**Figure 10**). From AA 857 to 1143 there are some regions that show enough similarity to be confidently aligned, nevertheless, it is clear that there has been increased divergence in the PNF angiosperm proteins when compared to the RPL and RPL/PNF homologs in angiosperms and gymnosperms, respectively. Within this final portion of the protein the only other motif that is invariant across all RPL/PNF proteins is the "ZIBEL" motif (G/A VSLTLGL; Mukherjee et al., 2009), in our alignment located between positions 1055 and 1063 AA, at the C-terminal portion after the homeodomain. There was however no evidence in our alignment of the presence of another "ZIBEL" motif between the SKY motif and the BELL-domain, unlike what is reported in AtBEL1 and other BEL-like homeodomain proteins (Mukherjee et al., 2009).

A total of 2149 characters were included in the matrix, of which 757 (35%) were informative. Maximum likelihood analysis recovered a major duplication event concordant with the diversification of angiosperms resulting in the *RPL* clade and the *PNF* clade (BS 93 for the duplications and 100BS for each clade) (**Figure 11**). In addition a second duplication event within the *RPL* clade is evident in grasses (Poaceae). Thus, most angiosperms, except grasses, have two homologs one in each clade contrasting with the single copy *RPL/PNF* present in gymnosperms (**Figure 11**). Taxon-specific duplications in the *RPL* clade have occurred in *Populus* (Salicaceae), *Gossypium, Theobroma* (Malvaceae), *Solanum* (Solanaceae), *Malus* (Rosaceae), and *Glycine* (Fabaceae). On the other hand, taxon-specific duplications in the *PNF* clade include those seen in *Populus* (Salicaceae), *Glycine* (Fabaceae), *Manihot* (Euphorbiaceae), *Malus* (Rosaceae), and *Gossypium* (Malvaceae).

Although gene losses are harder to confirm, *PNF* homologs were not found in the genome assemblies of *Mimulus* (Phrymaceae), *Eucalyptus* (Myrtaceae), *Medicago* (Fabaceae), *Solanum tuberosum* and *S. lycopersicum* (Solanaceae), or the transcriptomic sequences available for the core eudicots: *Ipomoea* (Convolvulaceae), *Asclepia* (Asclepiadaceae), *Thymus, Melissa, Pogostemon, Scutellaria* (Lamiaceae), *Moringa* (Moringaceae). *RPL* homologs were not found in the transcriptomes of several basal eudicots including: *Argemone, Hypecoum, Ceratocapnos* (Papaveraceae), *Nandina* (Berberidaceae), and *Akebia* (Lardizabalaceae). *One* thing to note is that no *PNF/RPL* homologs were found in *Papaver*, *Eschscholzia* (Papaveraceae), or *Aquilegia* (Ranunculaceae). In these taxa the similarity searches resulted in gene homologs more closely related to the outgroup sequences *SAW-like1* and *SAW-like2* than to *RPL/PNF*, although specific losses are hard to assess it is clear that at least in the

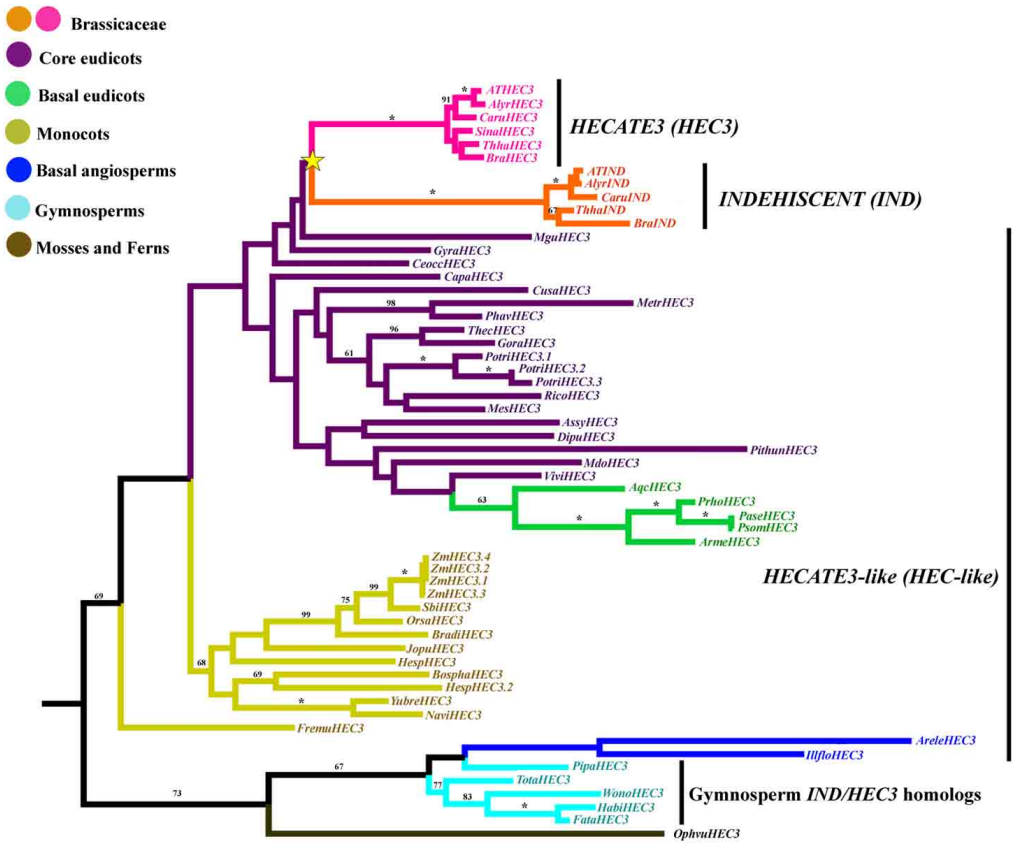

FIGURE 9 | ML tree of *INDEHISCENT/HECATE3* genes in seed plants showing a duplication in Brassicaceae (yellow star). This duplication resulted in the *INDEHISCENT* Brassicaceae specific genes from a HECATE3-like ancestral single copy in most core and basal eudicots, monocots and basal angiosperms. Most sequence changes are linked with the *IND* genes. Branch colors denote taxa as per color key at the top left; BS above 50% are placed at nodes; asterisks indicate BS of 100.

Aquilegia genome there are no other sequences that show more similarity to *RPL/PNF* suggesting that there has been a specific loss of these genes. In the other taxa it is possible that as more transcriptomic sequences become available, *RPL/PNF* copies can be found.

DISCUSSION

Our data, which includes sampling from all genomes available through Phytozome and transcriptomes available in the oneKP, and the phytometasyn public blast portals allowed us to identify major duplications and losses in *AP1/FUL*, *STK/AG*, *SPT/ALC*, *HEC3/IND*, and *RPL/PNF* genes. Based on our analyses we have also extrapolated how the fruit developmental network as we know it from *Arabidopsis thaliana* may have evolved and been co-opted across angiosperms. Our data shows that major duplications in all gene lineages studied here coincide with paleo-polyploidization events that have been previously identified at different times in land plant evolution, namely, ε mapped to have occurred before the diversification of the angiosperms, two consecutive events known as the σ and the ρ, that occurred before the diversification of the Poaceae (Jiao et al., 2011), an independent genome-wide polyploidization event in the Ranunculales (Cui et al., 2006), the γ event at the base of the core eudicots (Jiao et al., 2011; Zheng et al., 2013), and the taxa-specific α and β duplications in lineages like the Brassicaceae, Fabaceae, and Salicaceae (Blanc et al., 2003; Bowers et al., 2003; Barker et al., 2009; Abrouk et al., 2010; Donoghue et al., 2011). Taxa-specific duplications were found frequently (in at least two of the five gene families) in *Eucalyptus* (Myrtaceae), *Glycine* (Fabaceae), *Gossypium* (Malvaceae), *Malus* (Rosaceae), *Populus* (Salicaceae), *Solanum* (Solanaceae), and *Theobroma* (Malvaceae). This is likely the result of taxon specific recent WGD as these are well-known polyploids with diploid sister groups that have retained single copy genes (Sterck et al., 2005; Sanzol, 2010; Schmutz et al., 2010; Argout et al., 2011; Grattapaglia et al., 2012; Tomato Genome Consortium, 2012). Some groups show additional gene duplications in a single gene family but not in others, for example *Manihot* (with 4 *ALC* copies), *Portulaca* and *Silene* (with 2 *euFUL* copies). These cases suggest that at least some copies may have originated by tandem repeats or retrotransposition instead of WGD or alternatively that heterogeneous diploidization events can be occurring after polyploidization (Fregene et al., 1997; Olsen and Schaal, 1999: Abrouk et al., 2010), however, assessing taxa specific duplications and losses at the family level (and

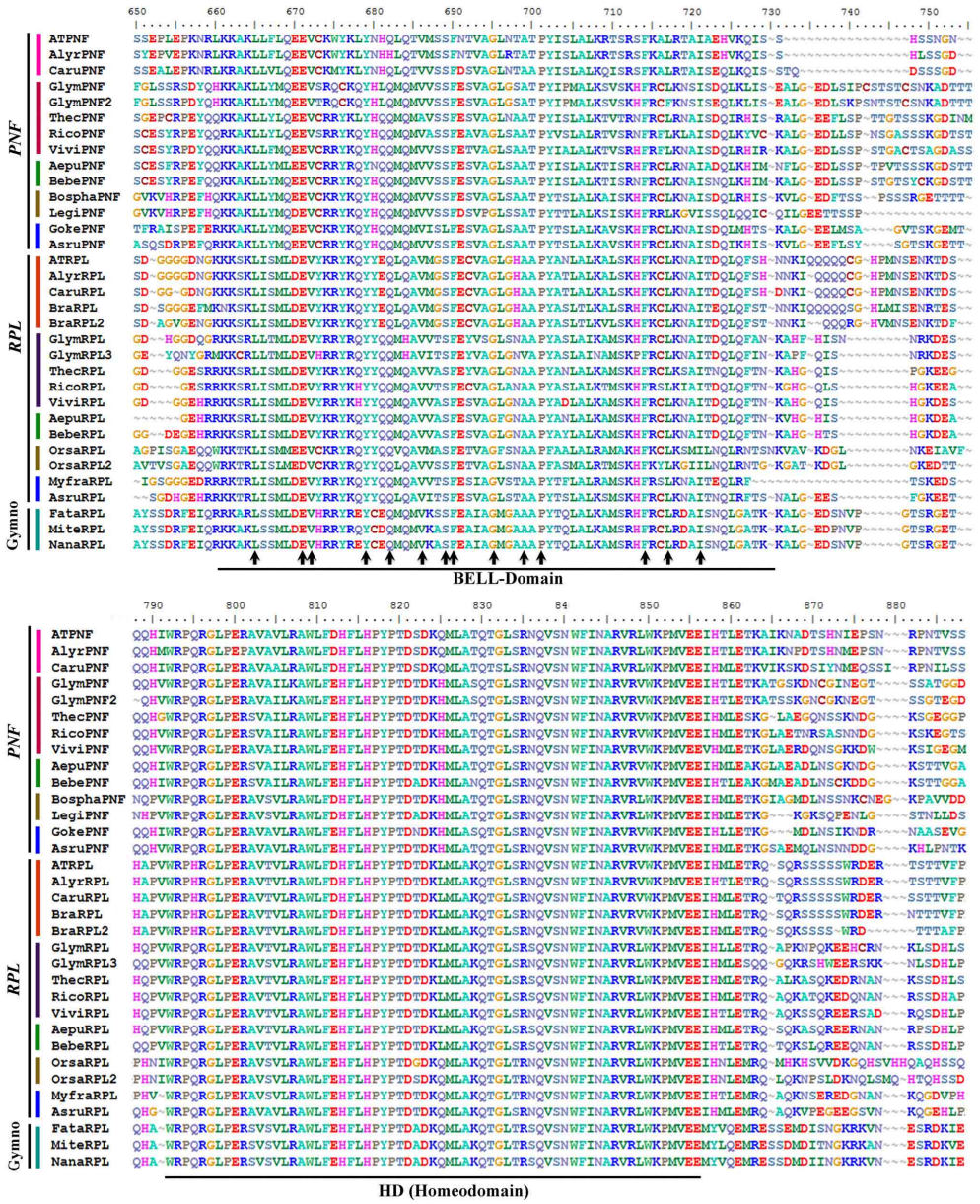

FIGURE 10 | Alignment of the BELL-domain and the Homeodomain of *REPLUMLESS/POUNDFOOLISH* proteins (labeled with the clade names they belong to). Colors to the left of the sequences indicate the taxa they belong to as per color key in **Figure 11**. Two domains are shown: the BELL domain (also called the MEINOX domain by Smith et al., 2002) has some invariant amino acids (arrows) in all gymnosperm and angiosperm RPL/PNF, important for dimerization that include L5, E11, V12, Y19, Q22, V26, S29, F30, G35, A40, P42, F55, L58, I62. The Homeodomain (HD) is very conserved (85%) with 53 AA conserved in seed plants out of 62 aminoacids total in the domain. Domains were drawn based on Mukherjee et al. (2009).

infra-familial levels) will require a more comprehensive search utilizing all available EST databases as well as targeted cloning efforts.

THE MADS–BOX GENES HAVE UNDERGONE INDEPENDENT AND OVERLAPPING DUPLICATION EVENTS AT DISTINCT TIMES DURING PLANT EVOLUTION

The MADS-box genes, greatly diversified in plant evolution have been well-studied in terms of their duplications during land plant evolution (Becker and Theissen, 2003). The *AP1/FUL* lineage for instance, appeared together with the radiation of angiosperms and has duplicated independently twice in monocots (specifically Poaceae; Preston and Kellogg, 2006), once in basal eudicots (Pabón-Mora et al., 2013b) and twice in core eudicots and one additional time in Brassicaceae (Figure 3; Litt and Irish, 2003; Shan et al., 2007). All of these duplications coincide with polyploidization events previously mentioned (Blanc et al., 2003; Bowers et al., 2003; Cui et al., 2006; Barker et al.,

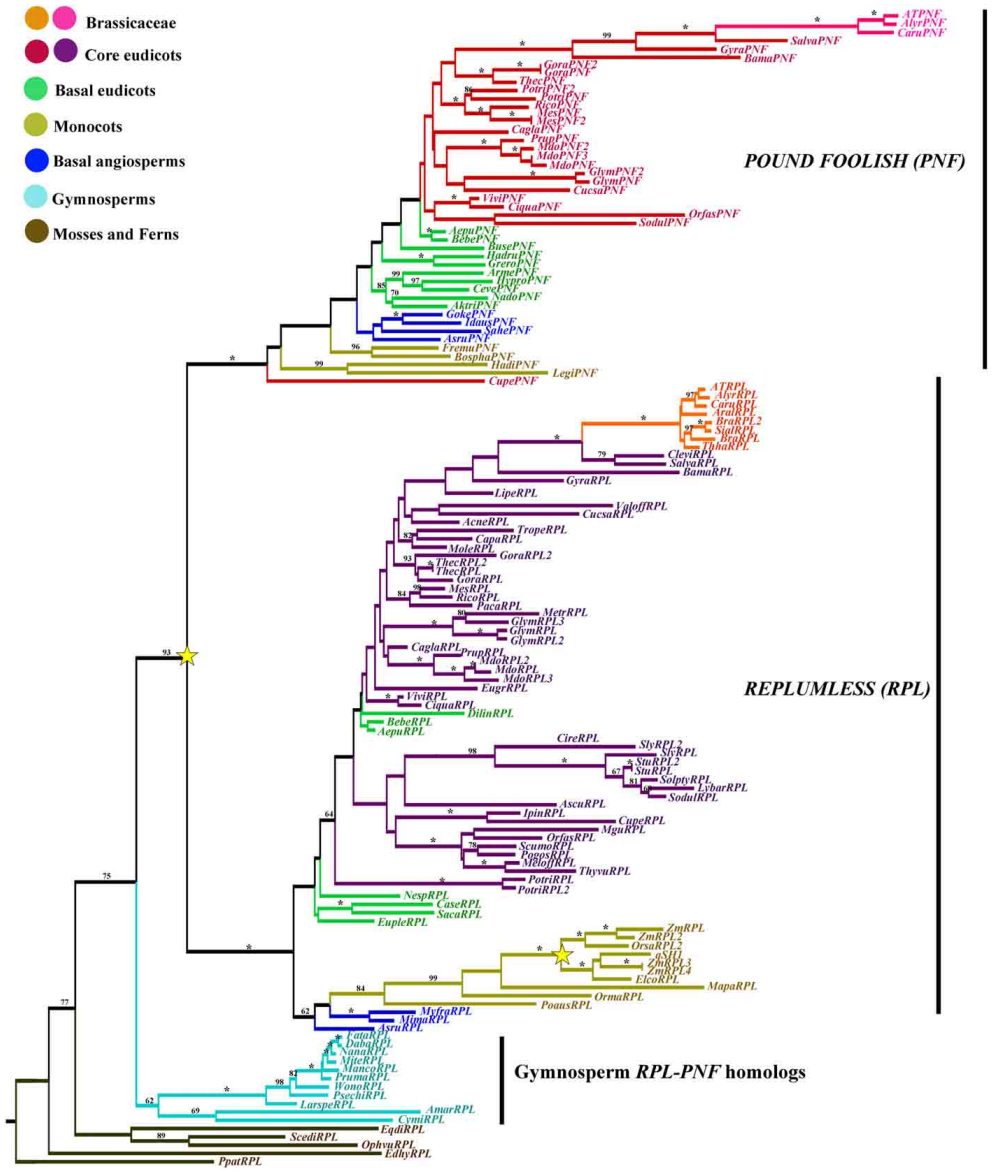

FIGURE 11 | ML tree of *REPLUMLESS/POUNDFOOLISH* genes in seed plants showing two duplications (star). One coinciding with the origin of the flowering plants, resulting in the *RPL* and the *PNF* clades. A second one occurring before the diversification of Poaceae. Branch colors denote taxa as per color key at the top left; BS above 50% are placed at nodes; asterisks indicate BS of 100.

2009; Donoghue et al., 2011; Jiao et al., 2011; Zheng et al., 2013). As a consequence of the numerous duplications, *Arabidopsis* has four gene copies: *APETALA1*, *CAULIFLOWER*, *FRUITFULL* functioning redundantly in flower meristem identity (Ferrándiz et al., 2000b), and independently in floral organ identity, specifically sepal and petal identity (*AP1*, *CAL*) (Coen and Meyerowitz, 1991; Bowman et al., 1993; Kempin et al., 1995; Mandel and Yanofsky, 1995) and fruit wall development (*FUL*) (Gu et al., 1998). The fourth copy, *AGAMOUS-like79* (*AGL79*) likely functioning in root development (Parenicová et al., 2003). Other core eudicots have *euAP1* genes often controlling floral meristem identity and sepal identity (Huijser et al., 1992; Berbel et al., 2001; Benlloch et al., 2006), *euFULI* genes controlling fruit wall patterning, in dry and fleshy fruits (Müller et al., 2001; Jaakola et al., 2010; Bemer et al., 2012), and *euFULII* genes (*AGL79* orthologs) playing roles in inflorescence architecture (Berbel et al., 2012). In addition some *euFULI* genes also control branching, flowering time and leaf morphology (Immink et al., 1999; Melzer et al., 2008; Berbel et al., 2012; Burko et al., 2013). Basal eudicots and monocots have a single type of gene, also referred to as the pre-duplication genes more similar to euFUL proteins, hence called *FUL-like* (Litt and Irish, 2003; Pabón-Mora et al., 2013b). Those perform a wide array of functions from leaf morphogenesis, to flowering time and transition to reproductive

meristems, to sepal and sometimes petal development, to fruit wall development (Murai et al., 2003; Pabón-Mora et al., 2012, 2013a,b).

Overall, the role of *AP1/FUL* homologs in fruit development, has been recorded for many *euFUL* genes in the core eudicots and some *FUL-like* genes in basal eudicots. These analyses suggest that *euFUL* genes control proper identity and development of the fruit wall in dry fruits like that of *Antirrhinum* (Müller et al., 2001), *Nicotiana* (Smykal et al., 2007), *Arabidopsis* (Gu et al., 1998), and *Brassica* (Østergaard et al., 2006), as well as proper firmness, coloration, and ripening in fleshy fruits like that of tomato (Bemer et al., 2012; Fujisawa et al., 2014), Bilberry (Jaakola et al., 2010), peach (Tani et al., 2007; Dardick et al., 2010), and even fruits resulting from fusion of accessory organs like apple (Cevik et al., 2010). The roles in fruit development are conserved in the pre-duplication *FUL-like* genes in Papaveraceae, in the basal eudicots, where *FUL-like* genes control proper fruit wall growth, vascularization, and endocarp development (Pabón-Mora et al., 2012). Altogether the available data suggest that euFUL and FUL-like proteins act as major regulators in late fruit development that control both dehiscence and ripening and seem to have acquired these roles early on in the evolution of the angiosperms, at least before the diversification of the eudicots (see also Ferrándiz and Fourquin, 2014). Our gene tree analyses show that FUL-like proteins are present in basal angiosperms, nevertheless, because of the lack of means to down-regulate genes in basal angiosperms, there are no known roles of *FUL-like* genes in this plant group. Expression patterns are similar to those reported in basal eudicots (unpublished data), suggesting that fruit development roles are likely to be conserved in early diverging angiosperms, together with pleiotropic roles in leaf and flower development, similar to those observed in basal eudicots (Pabón-Mora et al., 2012, 2013a).

The *AG/STK* lineage is present in seed plants and duplicated at the base of flowering plants resulting in the *STK* and the *AG/SHP* clades (**Figure 5**; Kramer et al., 2004; Zahn et al., 2006). This duplication coincides with the ε ancestral whole genome duplication before the diversification of the angiosperms (Jiao et al., 2011). Independently, each gene clade has duplicated in monocots (Dreni and Kater, 2014). Additionally the *AG/SHP* genes (also called C-lineage or AG lineage) underwent duplications in basal eudicots (at least in Ranunculaceae), core eudicots, and the Brassicaceae, the last two coincident with the same polyploidization events γ and α/β described before (**Figure 5**; Blanc et al., 2003; Bowers et al., 2003; Barker et al., 2009; Donoghue et al., 2011; Jiao et al., 2011). The *STK* gene clade (also called D lineage or AGL11 lineage) has remained as single copy in all angiosperms, with the exception of grasses.

Consequently, *Arabidopsis* has four gene copies: *SEEDSTICK*, *AGAMOUS, SHATTERPROOF1* (*SHP1*) and *SHP2*. All four paralogs function redundantly in ovule development in *Arabidopsis* (Favaro et al., 2003; Pinyopich et al., 2003) with *SEEDSTICK* controlling also proper fertilization and seed development (Mizzotti et al., 2012). *AGAMOUS*, represents the canonical C-function of the ABC model of flower development, and thus has specific roles in stamen and carpel identity. Finally *SHATTERPROOF* genes antagonize *FUL* and give identity to the dehiscence zone during fruit development. Functional studies in homologous genes in core eudicots and monocots have identified conserved roles in ovule development for *STK* orthologs (Colombo et al., 2008). In fact, the D-class genes involved in ovule identity were postulated based on the role of *FLORAL BINDING PROTEIN 7* (*FBP7*) in Petunia, and seem to be conserved in monocots as the *osmads13* shows defects in ovule identity (Dreni et al., 2007; Colombo et al., 2008). Additionally, *SHELL,* the *STK* homolog in oil palm (*Elaeis guineensis*) has been recently linked with oil yield, produced in the outer fibrous ring surrounding the seed, likely seed derived (Singh et al., 2013). Likewise, *STK* homologs across other non-grass monocots like *Hyacinthus* shows a restricted expression to developing ovules (Xu et al., 2004). Our gene tree analyses confirms that the *STK* or D lineage has remained predominantly unduplicated during angiosperm evolution, suggesting conserved roles in ovule identity and seed development in all angiosperms. Because these genes are also present in gymnosperms, this role is likely to be the ancestral role for the gene lineage, nevertheless more expression and functional data is needed to support this hypothesis.

On the other hand, *AG/SHP* homologs have undergone different patterns of functional evolution. Many core eudicot *euAG* and *PLE/SHP* genes have overlapping early roles in reproductive organ identity (Davies et al., 1999; Causier et al., 2005; Fourquin and Ferrandiz, 2012; Heijmans et al., 2012) and only SHP genes retain late functions in fruit development, specifically in dehiscence (Fourquin and Ferrandiz, 2012) and ripening (Vrebalov et al., 2009; Giménez et al., 2010). This is likely due to overlapping spatial and temporal expression patterns of paralogous genes (see for instance Fourquin and Ferrandiz, 2012), shared protein interactions (Leseberg et al., 2008), and lower protein sequence divergence (0.7–0.87 similarity) when compared to STK proteins (0.45–0.6) (**Figure 4**).

Basal eudicots and monocots have only one type of *AG* genes, known as the *paleoAG* genes, that in general only play early roles in stamen and carpel identity (Dreni et al., 2007, 2013; Yellina et al., 2010; Hands et al., 2011). Interestingly the basal eudicot paralogous genes that have been characterized in *Eschscholzia* and *Papaver,* are the result of a taxon-specific duplication in *Eschscholzia* and alternative splicing in *Papaver*. Both strategies seem to be common across basal eudicots, for instance, our sampling suggests that early diverging Papaveraceae and Lardizabalaceae have taxon-specific duplications producing two *AGAMOUS-like* copies, whereas subfamily Papaveroideae (*Papaver* and relatives including the polyploid *Argemone*) express alternative transcripts. There are also duplications that seem to have occurred before the diversification of other families, such as the Ranunculaceae (**Figure 5**). Functional characterization of these copies show that the two paralogs have overlapping and unique roles. For instance, in *Papaver somniferum* (Papaveraceae) one of the transcripts is largely involved in stamen and carpel identity whereas the second one becomes restricted to the carpel (Hands et al., 2011). Similar subfunctionalization scenarios have reported in Poaceae where paralogous copies in *Zea mays* and *Oryza sativa* have become functionally divergent, one largely involved in reproductive organ identity (*ZMM2* and *OsMADS3*) and the other mostly restricted to controlling carpel identity and floral meristem determinacy (*ZAG1* and *OsMADS58*) (Mena

et al., 1996; Dreni et al., 2007, 2011). Nonetheless, the functional impact of taxon specific duplications will have to be discussed case by case, and will likely provide insights on the redundancy vs. sub- and neo-functionalization patterns in *AGAMOUS-like* paralogous copies. The lack of fruit defects in basal eudicot paleoAG mutants suggest that fruit development roles are unique to core eudicot copies and have become completely fixed in SHP duplicates in the Brassicaceae (Fourquin and Ferrandiz, 2012).

Expression patterns of *paleoAG* genes in basal angiosperms include stamens and carpels, and occasionally inner tepals (Kim et al., 2005) and suggest conserved roles in reproductive organ identity but do not exclude roles in late fruit development. Although comparative studies, are needed to understand the role of *AGAMOUS* homologs in early diverging flowering plants, the conserved expression of *AG/STK* homologs in gymnosperms (Jager et al., 2003; Carlsbecker et al., 2013) suggest that the ancestral role of the gene lineage includes ovule identity. Such a role was then kept as part of the functional repertoire in *STK* genes, and *AG* genes were likely recruited first for carpel identity in early diverging angiosperms and later on for fruit development in core eudicots (Kramer et al., 2004).

DUPLICATION OF ALCATRAZ AND SPATULA OCCURRED AT THE BASE OF THE CORE EUDICOTS

ALCATRAZ (ALC) belongs to the large bHLH transcription factor family (Pires and Dolan, 2010). In Arabidopsis, the most closely related bHLH protein to ALC is SPATULA (SPT). SPT orthologs have been identified across the seed plants (Groszmann et al., 2008). However, previous studies have been unable to identify additional ALC orthologs outside of the Brassicaceae (Groszmann et al., 2011). Therefore, the SPT and ALC duplication was thought to have occurred during a whole genome duplication event in the lineage leading to the Brassicaceae (Groszmann et al., 2011). Here we identified a duplication at the base of the core eudicots that led to the evolution of specific ALC and SPT lineages in the core eudicots. This duplication coincides with the γ duplication event (Jiao et al., 2011; Zheng et al., 2013). The presence of ALC orthologs across the core eudicots is surprising since it is necessary for differentiation of the separation layer in the dehiscence zone, which has been thought to be specific to the Brassicaceae (Eames and Wilson, 1928; Rajani and Sundaresan, 2001).

However, recent studies in Arabidopsis have shown that ALC and SPT are partially redundant in carpel and valve margin development (Groszmann et al., 2011). These proteins are thought to have undergone subfunctionalization as ALC has a more prominent role in the differentiation of the dehiscence zone and SPT has a more prominent role in carpel margin development. We identified paleo SPT/ALC orthologs in basal eudicots, basal angiosperms and monocots, that all have more than 6 basic residues in the basic region, which indicates that, these all have DNA binding activities (**Figures 6**, **7**) (Toledo-Ortiz et al., 2003). In addition, the paleo SPT/ALC orthologs have conserved residues in the basic region that indicates that these recognize E-boxes in other proteins and specifically G-boxes (**Figure 6**) (Toledo-Ortiz et al., 2003). This indicates that paleo SPT/ALC may have similar downstream targets as Arabidopsis SPT and ALC.

Differences in SPT and ALC function may be due to different protein–protein interactions in the fruit developmental network. In Arabidopsis, SPT can interact with SPT, ALC, IND, and HEC, which are all bHLH proteins and are all generally involved in carpel margin development (Gremski et al., 2007; Girin et al., 2011; Groszmann et al., 2011). All of the SPT, ALC, and paleo SPT/ALC and gymnosperm SPT/ALC orthologs that we identified have a conserved Leu residue at position 27 that has been shown to be fundamental for dimer formation in mammals (**Figure 6**) (Toledo-Ortiz et al., 2003). In addition, there is a high level of conservation in the HLH domain of all the SPT, ALC and paleo SPT/ALC orthologs we identified and bHLH proteins are thought to form dimers with other members that have highly similar HLH domains. In species where only a single SPT/ALC ortholog was identified, it may form homodimers similar to SPT in Arabidopsis (Groszmann et al., 2011). SPT proteins have a conserved acidic domain and amphipathic helix N terminal to the bHLH domain, which is thought to be integral to its function in early gynoecium development (Groszmann et al., 2008, 2011). The amphipathic helix but not the acidic domain has been identified in ALC (Groszmann et al., 2008, 2011; Tani et al., 2011). We found the acidic domain to be conserved across angiosperms and gymnosperms except for the SPT-like2 grass genes and the Brassicaceae ALC genes. Functional analyses of ALC orthologs outside of the Brassicaceae will be necessary to understand how this gene acquired a role in dehiscence zone formation and to understand the evolution of the fruit network.

Both *SPT* and *ALC* share conserved atypical E-box elements in their cis-regulatory sequences (Groszmann et al., 2011). This sequence is required for SPT expression in the valve margin and dehiscence zone, however, similar expression studies are lacking in ALC. The expression of ALC in the valve margin is regulated by SHP1/2 and FUL in Arabidopsis (Liljegren et al., 2004). Although there are few functional analyses of SPT or ALC outside of Arabidopsis, recent studies in peach (*Prunus persica*) have indicated a role for the peach SPT ortholog (PPERSPT) in fruit development (Tani et al., 2011). PPERSPT was found to be expressed in the perianth, ovary and later in the margins of the endocarp where the carpels meet. PPERSPT is expressed in the region where the pit will later split. Further analyses of pre-duplication paleo SPT/ALC genes in angiosperms and SPT/ALC homologs in gymnosperms will be necessary to determine the ancestral function of these genes but it is likely these have roles in ovule development.

INDEHISCENT ORTHOLOGS ARE CONFINED TO THE BRASSICACEAE

INDEHISCENT (IND) is important for the development of the lignified layer and the separation layer in the valve margin of Arabidopsis fruits (Liljegren et al., 2004). IND belongs to the large family of bHLH transcription factors and is most closely related to HECATE3 (HEC3) in Arabidopsis (Bailey et al., 2003; Heim et al., 2003; Toledo-Ortiz et al., 2003). Our analyses across land plants show that the duplication of HEC3 and IND occurred in the lineage leading to the Brassicaceae as previous results indicated (**Figure 9**) (Kay et al., 2013). This duplication likely

coincides with α and β genome duplications identified at the base of the Brassicaceae (Blanc et al., 2003; Bowers et al., 2003; Jiao et al., 2011). We found HEC3-like genes not only in angiosperms (Kay et al., 2013) but also in gymnosperms and ferns (**Figure 9**). These HEC3-like genes also share the N terminal domain, HEC, atypical bHLH and C terminal domains previously identified in angiosperms (**Figure 8**) (Kay et al., 2013). It is likely that the duplication resulting in HEC3 and IND in the Brassicaceae was integral for the evolution of the tissues specific to Brassicaceae fruits.

Evolution of the fruit developmental network involving IND may be due to changes in IND protein–protein interactions or to cis-regulatory changes affecting IND expression. IND interacts with both SPT and ALC to promote valve margin development (Liljegren et al., 2004; Girin et al., 2011). IND has not acquired new interactions with SPT as HEC1/2/3 can also interact with SPT (Gremski et al., 2007). However, it is not known if HEC1/2/3 can interact with ALC.

Expression of IND is found early in carpel marginal tissues and throughout the replum (Girin et al., 2011). HEC1/2/3 are also expressed in carpel marginal tissues (Gremski et al., 2007). Expression of IND later becomes restricted to the valve margin where it has a prominent role in lignification and separation layer development necessary for dehiscence (Liljegren et al., 2004; Girin et al., 2011). Sequence analyses of *Brassica rapa* IND (BraA.IND.a) and Arabidopsis IND identified a shared 400 bp sequence in the cis-regulatory regions with high similarity (Girin et al., 2010). This region was able to direct expression in the valve margin and its expression was regulated by FUL and SHP1/2 (Liljegren et al., 2000, 2004; Ferrándiz et al., 2000a; Girin et al., 2010). It is likely that this 400 bp region in the cis-regulatory region of Brassicaceae INDs was integral for the neofunctionalization of IND in dehiscence zone development.

REPLUMLESS ORTHOLOGS DIVERSIFIED IN THE ANGIOSPERMS

REPLUMLESS (RPL) belongs to the TALE class of homeodomain proteins closely related to BELL (Roeder et al., 2003; Hake et al., 2004). This group of proteins has been termed BELL-Like homeodomain (BLH) proteins and have a homeodomain near the C terminus and a MEINOX INTERACTING DOMAIN (MID) near the N terminus (Hake et al., 2004; Hay and Tsiantis, 2009). The MID domain is composed of the SKY and BEL domains, which has also been largely defined as a bipartite BEL domain (**Figure 10**; Mukherjee et al., 2009). The MID domain, as its name indicates, is important for interacting with the MEINOX domain of the other class of TALE homeodomain proteins, KNOX. Heterodimers between KNOX and BLH are thought to give them specificity in their developmental roles. There are 13 BLH proteins in Arabidopsis and the most closely related paralog to RPL in Arabidopsis is PNF (Hake et al., 2004).

We identified PNF and RPL orthologs throughout the angiosperms indicating that a duplication occurred at the base of the angiosperms before they diversified (**Figure 11**). RPL is integral for replum formation in the Arabidopsis fruit and represses SHP1/2 (Roeder et al., 2003). However, RPL [also called PENNYWISE (PNY), BELLRINGER (BLR), and VAAMANA] has multiple roles in Arabidopsis development including meristem development, inflorescence, and fruit development (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Hake et al., 2004). Therefore, it is difficult to extrapolate possible roles for the RPL orthologs that we identified. In Arabidopsis, RPL represses SHP1/2 to keep valve margin identity to a few cell layers (Roeder et al., 2003). These cell layers later become lignified and are important for fruit dehiscence. Interestingly, a RPL ortholog in rice (qSH1) is responsible for seed shattering. Grains have a lignified layer at the base where the grains will abscise at maturity. In rice, qSH1 is mutated and this is correlated with a loss of seed shattering in domesticated rice (Konishi et al., 2006; Arnaud et al., 2011). In Arabidopsis, RPL represses SHP1/2, which are the paralogous lineage of AGAMOUS (AG) (Roeder et al., 2003; Kramer et al., 2004; Zahn et al., 2006). In addition, BLR (RPL) represses AG in inflorescences and floral meristems (Bao et al., 2004). This may be an ancient regulatory module that was co-opted for carpel development in angiosperms. Analyses of RPL orthologs and their interacting KNOX proteins outside of the Brassicaceae are necessary to understand the role of RPL in fruit development and how the Arabidopsis network evolved to include RPL.

EVOLUTION OF THE FRUIT DEVELOPMENTAL NETWORK

We have shown that the proteins involved in the Arabidopsis fruit regulatory network, namely FRUITFULL, SHATTERPROOF, REPLUMLESS, ALCATRAZ, and INDEHISCENT have undergone independent duplication events at distinct times during plant evolution. As a result the main regulators have changed in number, coding sequence and likely in protein interactions across angiosperms (**Figure 12**). Based on the reconstruction of all these gene lineages we were able to identify the presence of homologs of these genes across angiosperms. From our results it is clear that most core eudicots have a gene complement nearly similar to that present in the Brassicaceae, except for the lack of IND, and the presence of only one copy of SHP genes and not two as in Brassicaceae (**Figure 12**). Basal eudicots, monocots and basal angiosperms seem to have a narrower set of gene copies, as many duplications, coincide with the diversification of the core eudicots. Nevertheless, taxon specific duplications have occurred, and the effect of local duplicates may provide these lineages with some functional flexibility and opportunities for neofunctionalization and or subfunctionalization to occur.

We propose that a core developmental module consists of FUL-like, AG, RPL, HEC3, and SPTlike-1 and these were co-opted to play roles in basic fruit patterning and lignification. This is supported by the fact that many of the derived MADS box proteins retain early roles in carpel development, for example SHP1/2 are also involved in carpel fusion and transmitting tract development (Colombo et al., 2010). Similarly, the bHLH proteins, are important for carpel meristem development, for the development of common carpel structures such as the transmitting tract, septum and style (Groszmann et al., 2008, 2011; Girin et al., 2011). In addition, RPL is also known to have pleiotropic effects in plant development particularly in various plant meristems (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Hake et al., 2004; Smith et al., 2004). Many of the MADS-box protein homologs present in basal angiosperms, monocots, and

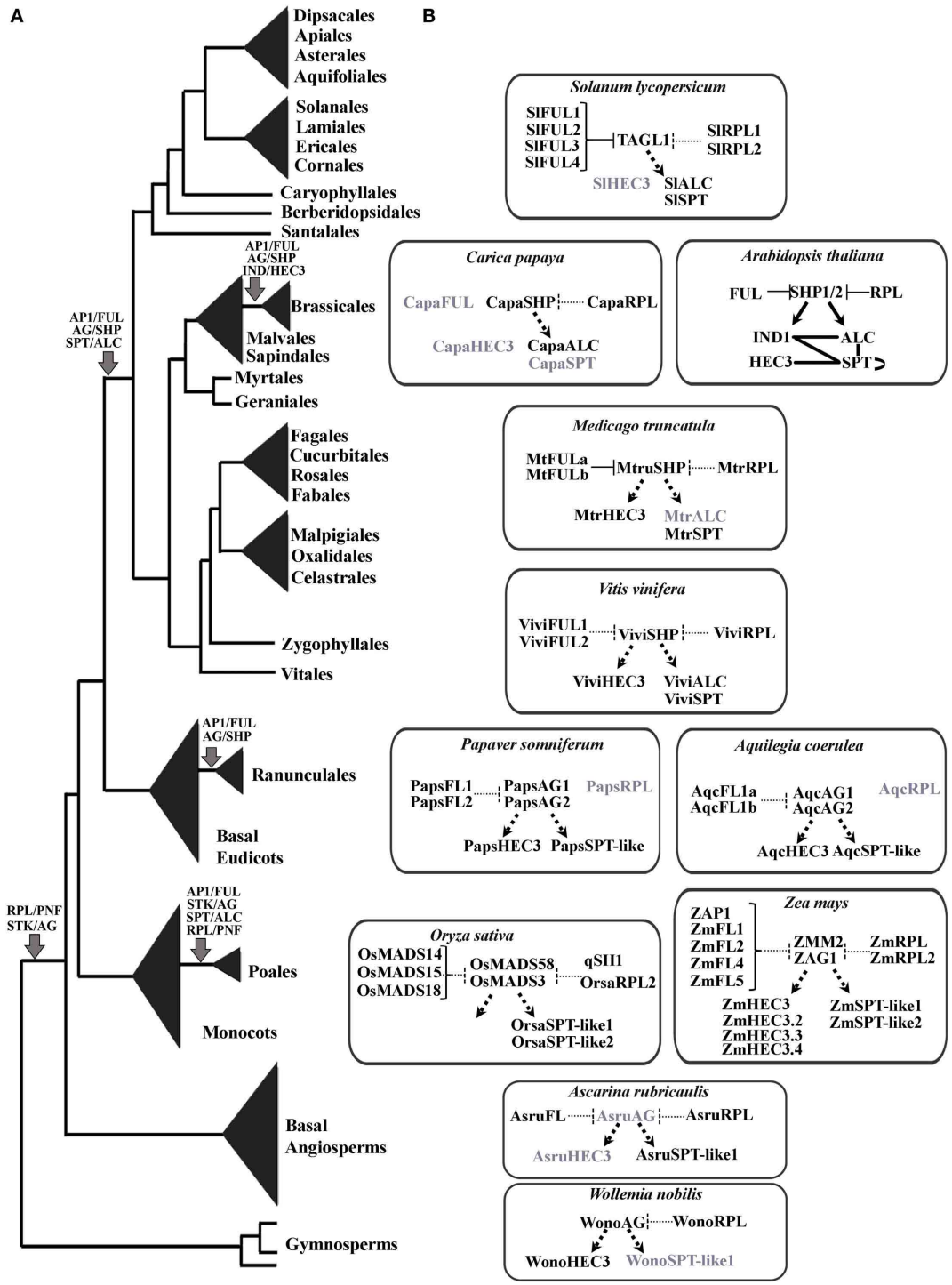

FIGURE 12 | Overview of the fruit developmental gene network. (A) Seed plant phylogeny with the time points for the AP1/FUL, STK/AG, SPT/ALC, HEC3/IND, and RPL/PNF gene lineages duplications. **(B)** Reconstruction of the fruit developmental network across selected angiosperms. The only network functionally characterized is that of Brassicaceae where FUL and RPL repress SHP1/2 to shape the fruit wall, and SHP1/2 activate IND, SPT, and ALC to form the dehiscence zone. All other networks are extrapolated from *Arabidopsis*. Functional and protein–protein interaction data are necessary to validate these hypothetical interactions. Proteins in black are those previously identified or recovered in our analyses. Proteins in gray were not recovered from databases and may have been lost in the respective taxa. Solid black lines, validated protein–protein interactions; solid black arrows, validated activation; solid T-bars, validated repression; dashed lines, putative protein–protein interactions; dashed arrows, putative activation interactions; dashed T-bars, putative repression.

basal eudicots play pleiotropic functions that include floral meristem and perianth identity (e.g., AP1/FUL proteins; Bowman et al., 1993; Gu et al., 1998; Ferrándiz et al., 2000b; Berbel et al., 2001, 2012; Murai et al., 2003; Pabón-Mora et al., 2012, 2013b), ovule, stamen, and carpel identity (STK/AG proteins; Jager et al., 2003; Yellina et al., 2010; Hands et al., 2011; Carlsbecker et al., 2013).

Unraveling the evolution of the fruit developmental network may provide some insight into the evolution of the carpel, which is of great interest. Our sampling shows that basal angiosperms have the simplest network with only one gene in each gene lineage, resembling fruitless seed plants in this respect. Gymnosperms have at least one member of each gene lineage with the exception of AP1/FUL proteins. It is possible that the evolution of the AP1/FUL proteins in angiosperms was integral to the evolution of the carpel. In addition, given the pleiotropy of the core fruit module genes, comparative molecular genetic analyses of these core genes will be necessary in basal angiosperms and gymnosperms to better understand their potential roles in carpel and fruit evolution in angiosperms.

One key element to better understand the evolution of the network will be the assessment of the interactions, a poorly studied aspect, yet critical, as changes in partners between pre-duplication and post-duplication proteins may have provided core eudicots with a more robust fruit developmental network. For example, it is clear that FUL and FUL-like share a number of floral and inflorescence protein partners but it is unclear how they interact with fruit proteins (Moon et al., 1999; Ciannamea et al., 2006; Leseberg et al., 2008; Liu et al., 2010); the same has been reported for AG and SHP proteins (Leseberg et al., 2008). In addition, the bHLH proteins are known to interact with each other to regulate downstream targets (Groszmann et al., 2008, 2011; Girin et al., 2011). However, SPT is known to also form homodimers and it may be that species that we have identified with a single SPT/ALC ortholog are able to form homodimers as well but may be limited in the regulation of diverse downstream targets (Groszmann et al., 2011). The expression of ALC in the valve margin is regulated by SHP1/2 and FUL. There are shared E box elements in ALC and SPT, which are known to be important for SPT expression in valve margin (Groszmann et al., 2011). Therefore, it is likely that differences in protein interactions and their downstream targets are important for evolution of fruit network.

We have analyzed the evolution of protein families known to be the core network controlling fruit development in Arabidopsis and by doing so we have been able to identify three main lines of urgent research in fruit development: (1) The functional characterization of fruit development genes other than the MADS box members, as there are nearly no mutant phenotypes for bHLH or RPL genes outside of Arabidopsis. (2) Assessing the regulatory network by testing interactions among putative protein partners in all major groups of flowering plants to understand how the core of the ancestral fruit developmental network evolved to build fruits with diverse morphologies and (3) The morpho-anatomical detailed characterization of closely related taxa with divergent fruit types across angiosperms, to better understand what mechanisms are responsible for changes in fruit development and result in homoplasious seed dispersal syndromes, and to postulate proteins from the network likely controlling such changes.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00300/abstract

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