

Conservation of the dehiscence zone gene regulatory network in dicots and the role of the SEEDSTICK ortholog of California poppy (*Eschscholzia californica*) in fruit development

Dominik Lotz

Justus-Liebig-University

Le-Han Rössner

Justus-Liebig-University

Katrin Ehlers

Justus-Liebig-University

Doudou Kong

Justus-Liebig-University

Clemens Rössner

Justus-Liebig-University

Oliver Rupp

Justus-Liebig-University

Annette Becker

`annette.becker@bot1.bio.uni-giessen.de`

Justus-Liebig-University

Research Article

Keywords:

Posted Date: December 6th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-5070850/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at EvoDevo on December 27th, 2024. See the published version at <https://doi.org/10.1186/s13227-024-00236-0>.

Abstract

Background

Fruits, with their diverse shapes, colors, and flavors, represent a fascinating aspect of plant evolution and have played a significant role in human history and nutrition. Understanding the origins and evolutionary pathways of fruits offers valuable insights into plant diversity, ecological relationships, and the development of agricultural systems. *Arabidopsis thaliana* (Brassicaceae, core eudicot) and *Eschscholzia californica* (California poppy, Papaveraceae, sister group to core eudictos) both develop dry dehiscent fruit, with two valves separating explosively from the replum upon maturation. This led to the hypothesis, that homologous gene regulatory networks direct fruit development and dehiscence in both species.

Results

Comprehensive phylogeny reconstructions using recently available reference genomes show that genes essential for *Arabidopsis* dehiscence zone formation show lineages-specific genes, specific to Brassicaceae or core eudicots. Transcriptome analysis of valve and replum tissue of California poppy yielded the *SEEDSTICK* (*STK*) ortholog as candidate for dehiscence zone regulation. Expression analysis of *STK* orthologs from other dry dehiscing fruits shows their involvement in fruit development of several species. Functional analysis using Virus-Induced Gene Silencing (VIGS) clarified the roles of *EscaSTK*: an evolutionary conserved role in seed filling and seed coat development, and a novel role in restricting cell divisions in the inner cell layer of the valve.

Conclusion

Our analysis shows that the gene regulatory network described in *Arabidopsis* is lacking to a large extent in other dicots, even if their fruits form a dehiscence zone at the valve margins. Orthologs of *STK*, known to be involved in ovule development and seed abscission in *Arabidopsis*, was recruited to a network regulating fruit wall proliferation in California poppy. There, *EcSTK* allows fruit maturation without premature capsule rupture.

Background

All flowering plants develop fruits that house the seeds and ensure dispersal of the mature seeds. The evolutionary innovation of carpels that, after fertilization mature into fruits, provided angiosperms with a competitive advantage, allowing them to colonize diverse habitats and establish complex ecological relationships with animals. Some fruits make use of wind, water or animals to move their fruits and seeds away from the maternal plant. Flowering plants utilize a large diversity of mechanisms to disperse their seeds, often involving intricate morphological adaptations, such as feathery appendages, floating

devices or hooks. Seed dispersal mechanisms are important traits when considering biodiversity conservation because it enables colonization of new habitats ensuring species survival (Lorts et al. 2008; Balanzà et al. 2016).

Dry dehiscent fruits shatter upon maturity to disperse the seeds, often explosively, requiring the coordination of fruit opening and seed detachment from the fruit (Balanzà et al. 2016). In *Arabidopsis* fruits, the two valves separate from the replum at the dehiscence zone (DZ) that runs along the full length of the fruit. The dehiscence zone is formed by a precise patterning of lignified and unlignified cells at the replum/valve margins. The fruits break open when the combination of those two cell types builds up tension resulting from the differential hygro-responsive properties upon drying out (Ballester & Ferrándiz 2017). This process also involves weakening of cell adhesion at the separation layer with tensions provided by the surrounding tissues or external agents. The mentioned tensions can come from pod walls, properties of lignified and non-lignified tissues or changes in turgor association (Ballester & Ferrándiz 2017; Huss & Gierlinger 2021). Many crops, such as *Brassica napus* (canola) or the protein rich legumes like soy (*Glycine max*), pea (*Pisum sativum*) or lentil (*Lens culinaris*) have dry dehiscent fruits and pre-harvest fruit dehiscence causes major yield loss (Parker et al. 2021).

The gene regulatory network (GRN) directing fruit tissue specification and DZ development in the dry dehiscent species *Arabidopsis* was elucidated previously (Fig. 1A): the formation of the DZ with its intricate lignin pattern is regulated by *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*), *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*). *ALC* is required for the formation of the separation layer, composed of small cells with only little cell wall lignification at the valve/replum border. *SHP1* and *SHP2* induce *IND*, which activates lignin biosynthesis genes in the dehiscence zone (Liljegren et al. 2000; Rajani & Sundaresan 2001; Liljegren et al. 2004; Ballester & Ferrándiz 2017).

FRUITFULL (*FUL*) specifies valve identity and *REPLUMLESS* (*RPL*) specifies replum identity (Fig. 1A), limiting the dehiscence zone region to the valve/replum margins (Ferrándiz et al. 2000; Roeder et al. 2003; Liljegren et al. 2004). Interestingly, indehiscence can be also achieved in the dry dehiscent species *Nicotiana benthamiana* when the *FUL* or *SHP* orthologs are knocked down suggesting some degree of functional conservation of these two genes among dicots (Ballester & Ferrándiz 2017). Interestingly, little is known about the extent of conservation of fruit dehiscence zone regulatory genes in dicots. Dry dehiscent fruits have in common that their fruit walls are comparatively thin, as the units of propagation are the released seeds. Thus, a tight regulation of fruit wall thickness is required as fruits with excessive internal tissue may rupture prematurely.

Interestingly, the GRN regulating lengthwise fruit expansion requires *STK* in *Arabidopsis*, which activates cytokinin-degrading enzymes and *FUL*, both required for fruit elongation. This deprives the developing fruit off cytokinin, which is required before fertilization for gynoecium formation. (Di Marzo et al. 2020). After fertilization, lengthwise cell divisions are infrequent and most of the valve elongation occurs via cell expansion and is limited to the regions harboring fertilized ovules, suggesting a mobile, seed derived signaling cascade (Ripoll et al. 2019). However, little is known about the genetic regulation of fruit

expansion along the horizontal plane in *Arabidopsis*, aside from those genes that are also involved in adaxial/abaxial polarity specification in lateral organs, such as *KANADI*, *GYMNOS*, or *YABBY* family genes. However, their mutant's phenotypes are not affected in the number of cell layers in cross sections (Eshed et al. 1999). Comparing dry and dehiscent fruits in the Solanaceae, Pabon-Mora and Litt (2011) documented that postfertilization fruit wall expansion can be driven by cell divisions of all layers of the fruit wall, exo-, endo-, and mesocarp, and phylogenetic aspects seem to play a more important role than fruit types in determining which layer expands most. The purpose of this study was to find candidate regulators of dry dehiscent fruit development in species outside the *Arabidopsis* that may help to elucidate how fruits of species develop whose genomes do not encode orthologs of the well-known *Arabidopsis* fruit development genes.

California poppy (*Eschscholzia californica*, Papaveraceae, Ranunculales) has been used as a genetic model system to compare gene functions within the dicots, mainly because of its phylogenetic position as a representative of the sister lineage to the core eudicots and its amenability to Virus-Induced-Gene Silencing (Becker et al. 2023; Becker et al. 2024). It has dry dehiscent fruits with a structure similar to *Arabidopsis*: two valves connected to the replum, with the valves explosively separating from the replum upon dehydration during maturity (Becker et al. 2005; Pabón-Mora et al. 2014).

This study address the following questions: (1) To which extent is the dehiscence zone GRN network conserved in angiosperms? (2) Which transcription factors may contribute to dehiscence zone formation in California poppy? (3) What is the role of *STK* in California poppy fruit development? (4) Are *STK* homologs differentially expressed in valve and dehiscence zones of fruits in other eudicots?

Results

Most homologs of *Arabidopsis* fruit dehiscence regulators are absent from the *E. californica* genome

The *Arabidopsis* dehiscence zone gene regulatory network is well documented (Fig. 1A) but we were interested to learn about its degree of conservation across angiosperms. In the likely case that the major regulators of dehiscence zone formation are absent in species outside the Brassicaceae, dehiscence zones in other species are patterned by different networks. These may only partly rely on orthologs known from *Arabidopsis*. We carried out phylogeny reconstructions including representatives of major angiosperm lineages with sequenced genomes for homologs of transcription factors constituting the core gene regulatory network of *A. thaliana* (Fig. 1A, Supplemental Fig. 1). These revealed only partial conservation, with dehiscence zone regulator homologs missing from many lineages (Fig. 1B).

Homologs of *RPL* are found in all angiosperms except for the poppies (California poppy and *Papaver somniferum*, opium poppy), and most species maintain more than one copy. In contrast, *IND* and *ALC* are found only in *Arabidopsis*, suggesting specificity to Brassicaceae. *ALC* results from a gene duplication of an *ALC/SPT*-like gene in the lineage leading to Brassicaceae giving rise to the *SPT* and *ALC*, with the *ALC* coding sequencing lacking several conserved segments, as also shown previously by others (Groszmann et al. 2011; Zumajo-Cardona et al. 2017) Except for *Triticum aestivum* (wheat), *ALC/SPT*-like genes can

be found in all species analyzed, most retaining more than one copy. *FUL* homologs are found in most species analyzed but are lacking in the Ranunculales and *Carica papaya* (papaya). *SHP* genes are present in the core eudicots only, but even in this lineage, they are absent from *Pisum sativum* (pea). In contrast, *STK* homologs are found in all species analyzed, with the exception of *Aquilegia coerulea* (columbine) (Fig. 1B). In summary, we found that *IND* and *ALC* are not even conserved throughout the Brassicales, and *SHP* genes have originated in the lineage leading to the core eudicots. The replum tissue of many species may be specified by *RPL*, which is present even in the non-dehiscent fleshy fruit bearing papaya. *FUL/AP1/CAL* homologs are present in all angiosperms, except for Papaya, but the *euFUL* genes, which are orthologs to Arabidopsis *FUL*, derived from a core eudicot specific duplication. Interestingly, *Amborella trichopoda* and *Nymphaea colorata*, from the ANA grade, have only a single homolog of Arabidopsis dehiscence zone regulator genes, as does papaya. In contrast, species with notoriously large genomes like wheat and soy bean have retained many copies of these genes, for example eight *FUL/AP1/CAL* genes in wheat. In California poppy, the dehiscence zone GRN lacks the orthologs of dehiscence zone and replum regulators, such as *RPL*, *IND*, and *SHP1/2*, while *FUL/AP1/CAL*, *IND/HEC3*, *ALC/SPT*, and *STK* homologs are present. Taken together, the specification of the California poppy dehiscence zone requires a set of regulators at least partially different from the ones characterized in *Arabidopsis*.

Fruit anatomy and differential gene expression of valve and replum tissue in California poppy fruits

The California poppy fruit anatomy is reminiscent of that from Arabidopsis: two congenitally fused carpels form the gynoecium, and the fruit is dry dehiscent, with a clear differentiation of valve, replum and dehiscence zone. However, the fruit is more rigid than that of Arabidopsis due to thick lignified lateral ridges along the valve (for a detailed description of fruit and dehiscence zone development, including a timeline for lignification, see Suppl. Material 1 and Suppl. Figure 2). Interestingly, its dehiscence zone architecture deviates strongly from that of *Arabidopsis*: shortly before dehiscence, around 32 days after pollination (dap), each valve shows five strongly lignified ridges abaxially of the vascular bundles. A sclerenchymatic ring of lignified cells loosely connects these ridges. The two lateral valve ridges are more elevated and part of the fully lignified dehiscence zone, which resides at the connection of the two valves (Fig. 2A). The replum is composed of a half-moon shaped sclerenchyma cap supported by a vascular bundle (Fig. 2B). The fruits will eventually dehisce explosively between the replum and the two lateral valve ridges (Fig. 2C and D).

To identify candidate genes for dehiscence zone regulation in California poppy, we hand-dissected fruits early in their development at two distinct developmental stages. Dehiscence zone (DZ) including replum and lateral valve ridges were collected separately from the valve tissue, while avoiding contamination with placenta tissue and developing ovules. At 2 dap (young stage, Suppl. Figure 2A, B), clusters of future sclerenchyma cells became visibly distinct from the surrounding parenchyma tissue at the future lateral and median valve ridges, and replum, but lignification has not commenced yet. At 6 dap (later stage,

Suppl. Figure 2C, D), the sclerenchyma patterning is completed and lignification will commence shortly. The transcriptome analysis was carried out in three biological replicates with between 48 and 62 Million reads per replicate. Figure 2E and Suppl. Figure 3 show expression of homologs of Arabidopsis dehiscence zone and other putative developmental regulators in the valve and replum region at two different stages. The two *FUL* paralogs, *EcFUL1* and *EcFUL2* show indeed stronger expression in the valves than in the dehiscence zone, with *EcFUL1* expressed at a higher level (Fig. 2E). The closest relatives of Arabidopsis dehiscence zone markers *SHP1* and *SHP2* in California poppy are *EscaAG1* and *EscaAG2*, and while *EscaAG1* is strongly expressed throughout all transcriptomes, *EscaAG2* shows stronger expression in the dehiscence zones than in the valve regions of both stages observed (Fig. 1E). However, the two *SPT* homologs of California poppy equally distant to *ALC* of Arabidopsis are not differentially expressed, as are the *AP2* homologs (Suppl. Figure 3).

In addition we find that several genes whose Arabidopsis orthologs are not known to be involved in dehiscence zone formation show expression differences between the developmental stages (Fig. 2E), such as *EscaMYB85*, which is expressed stronger at 6 dap, and *EscaPIDa* and *EscaAS1*, which are expressed stronger at 2 dap. *EscaZFP2* and *EscaMYB26* show strong expression in only the 6 dap dehiscence zone. Only few genes like *EscaNF-YB3L*, *EscaAG2*, *EscaYAB3*, *EcFUL1* and *2* are expressed differentially between dehiscence zone and valve region in both stages and they may be involved in the continuous regulation of dehiscence zone patterning. *EscaSTK* is specifically down regulated in young valves at 2 dap suggesting a role in fruit development. Taken together, we find substantial differences in the expression of transcriptional regulators between the two developmental stages and valve and dehiscence zone tissues.

Down regulation of *EscaSTK* affects floral organ identity and leads to premature fruit rupture

EscaSTK was then functionally characterized as a candidate gene for a fruit developmental regulator, based on its specific expression pattern. Virus-Induced-Gene Silencing (VIGS) was carried out to down regulate the expression of *EscaSTK*. 155 plants were treated with *EscaSTK*-VIGS and analyzed for phenotypic differences, together with with *EcPDS*-VIGS treated plants for visual control of the experiment, *mEGFP*-VIGS as negative (mock) control, and untreated plants which were all compared with each other (Fig. 3, Table 1, Suppl. Figure 4). The first flower bud of the VIGS treated plants was removed for RNA extraction and the three next formed flowers/fruits were analyzed. In 6.5% of all *EscaSTK*-VIGS treated plants, homeotic conversions of outer stamen to petals were observed (Fig. 3A, Table 1). Interestingly, also the *mEGFP*-VIGS treated plants were affected in their flower development, with petals growing narrower when compared to the untreated plants (Fig. 3A). Vegetative development of *EscaSTK*- and *mEGFP*-VIGS treated plants was not affected. All *EcPDS*-VIGS treated plants showed photobleaching in vegetative organs and flowers (Suppl. Figure 4).

VIGS treatment significantly reduces the growth rates of fruits in the rapid elongation phase between pollination and 9 dap, but also in the final, slower elongation phase between 10 and 16 dap (Fig. 3B and C). Fruits of *EscaSTK*- and *mEGFP*-VIGS treated failed to fully expand in length and width. Fruits of

untreated plants reach around 6 cm in length and 3.5 mm in width. In contrast, *EcSTK*- and *mEGFP*-VIGS treated plants grow only up to approximately 4.5 cm and 4.8 cm in width, respectively (Fig. 3B and C). While there is only little difference in fruit growth rate up to 9 dap, fruit growth of VIGS- treated plants differs significantly between dap 10 and 16 (Fig. 3C). Fruits of *mEGFP*-VIGS treated plants grow less than those of untreated plants and fruit growth rate of *EscaSTK*-VIGS treated plants becomes even negative after 10 dap. This may be due to a cessation of growth in combination with premature opening and dehydration.

Untreated and *mEGFP*-VIGS treated fruits open around 30 dap to explosively release the seeds (Fig. 3D and E). In 11.8% of the *EscaSTK*-treated plants, premature opening was observed (Table 1) already after on average 13 dap, resulting in fruits and seeds that dry out prematurely and failure to disperse the seeds (Fig. 3F). Our results suggest that *EscaSTK* contributes to a lesser extent to floral organ identity of stamens, with its main role is in prevent premature fruit rupture allowing for seed maturation and explosive seed dispersal.

***EscaSTK* down regulation causes phenotypic alterations of the endocarp and seed morphology**

EscaSTK-VIGS treated fruits show a high variance in length and width in addition to premature rupturing, and we were interested to learn if these phenotype observations depend on the degree of *EscaSTK* expression down regulation. qRT-PCR analysis with *EscaSTK*-specific primers not overlapping with the VIGS fragment was carried out in technical triplicates of three *mEGFP*- and 24 randomly selected *EscaSTK*-VIGS treated plants. One third of the plants showed downregulation of *EscaSTK* to less than 50% of the wild type expression, suggesting that variance in expression may be related to variance in phenotype (Fig. 4A).

As the *EscaSTK*-VIGS treated plants show premature rupture of fruits (Fig. 3), we were interested to learn how the fruit tissues are affected by the reduction of *EscaSTK* expression. Cross sections of *mEGFP*-VIGS treated plants showed similar morphologies in overall architecture (Fig. 4B), dehiscence zone patterning (Fig. 4C), and seed morphology (Fig. 4D) to untreated plants (Fig. 2A, B). Figure 4E-G and Fig. 4H-J show two independently *EscaSTK*-VIGS treated plants with prematurely ruptured fruits. The most striking observation in those plants is the altered shape and size of the endocarp and adjacent fruit cell layers (Fig. 4E, F, H, I). In these tissues, the cells grew larger and elongated in radial direction. These elongated cell layers emerged at the valve margins and tapered towards the valve centers. However, they did not cover the replum so that there was always a hollow slit between the elongated cells originating from the two valves (Fig. 4E, H, F and I). The elongated cells filled almost the entire space between the growing seeds in the capsule, i.e. the former locule (Fig. 4H and E). Further, in *EscaSTK*-VIGS treated plants, the endocarp layer sometimes underwent additional, tangential cell divisions such that additional parenchymatous cell layers developed at the adaxial side of the pericarp (Fig. 4E, H and Suppl. Figure 2N-P, white arrows). This additional adaxial cell material may force the capsules to break open in a premature state, leading to fruit and seed desiccation (Fig. 3F and 4E, H). Interestingly, the premature fruit rupture occurred at random sites along the pericarp, sometimes at the dehiscence zone, but also

between the median ridges of the valves (asterisks in Fig. 2N-P). The patterning of the dehiscence zone and the abaxial valve tissues, including the early lignification events, did not seem to be altered drastically in the *EscaSTK*-VIGS treated capsules.

Furthermore, *EscaSTK*-VIGS treated capsules often harbored seeds with irregular shape (Fig. 4E, G, H, J). We observed that endocarp development and seed filling was disturbed in *EscaSTK*-VIGS treated plants. However, the surface cells of those seeds had a turgescient shape as observed in seeds of untreated plants, indicating that seed developmental defects were not simply provoked by early desiccation (Suppl. Figure 5A-C).

In *mEGFP*-VIGS treated seeds as well as with phenotypically less conspicuous *EscaSTK*-VIGS treated seeds, the testa layers derived from the inner and outer integuments were clearly delimited (Fig. 4D, Suppl. Figure 5D, E). As a typical feature of wild type California poppy seeds, the homogenous outermost epidermis of the seed coat features stomata (Jernstedt & Clark 1979), as do the *EscaSTK*-treated seeds (Fig. 4J, inset). Epidermal cells of the seed coat are differentiated into flat facet cells and elongated, partially multilayered ridge cells (F and R in Fig. 4D, Suppl. Figure 5D), which had not yet collapsed in this developmental stage.

Conversely, we find that in severely affected *EscaSTK*-VIGS treated seeds, discrimination of the testa layers was ambiguous (Fig. 4G, J). A strikingly thick, uniform parenchyma, composed of mostly five to six layers of relatively large cells formed at the seed surface. In the three more intensely stained cell layers underneath, which presumably emanated from the inner integument, single cell layers could hardly be discriminated, as cells had flattened in radial direction during seed growth.

In summary, we find thick layers of parenchymatous cells lining the adaxial side of the fruit wall of *EscaSTK*-VIGS treated plants that may cause premature fruit rupture. Further, seeds of *EscaSTK*-VIGS treated plants seeds fail to expand and develop additional layers of voluminous cells on the surface. This suggests that *EscaSTK* is involved in restricting cell divisions in the endocarp layer of the capsules, seed filling, and seed coat formation.

STK orthologs are differentially expressed in the dry dehiscent fruits of legumes

Legumes are agriculturally important protein crops also producing dry dehiscent fruits, and we were interested if we can find differential expression of *STK* orthologs in the fruits of legumes. The soy bean genome encodes for two *STK* homologs, termed *GmSTK1* and *GmSTK2*. They show low or no expression in leaves, and substantial expression in buds and open flowers. Both *STK* homologs are expressed in dehiscence zones, but only *GmSTK2* is also expressed in early stage of dehiscence zone formation. Both genes show no expression in the valves (Fig. 5). The faba bean (*Vicia faba*) and pea (*Pisum sativum*) genomes each encode for a single *STK* ortholog, termed *VfSTK* and *PsSTK*, respectively. *VfSTK* expression is found in leaves, buds and flowers, but hardly in developing fruits (Fig. 5). *PsSTK* shows no expression in leaves and low expression level in buds. However, in open flowers and dehiscence zones, it is expressed at a high level. Taken together, our data show that *STK*

orthologs are expressed in the fruits of soy and pea but most likely not in faba bean, suggesting that their possible roles in fruit development may not be fully conserved throughout the legumes.

Discussion

Gene regulatory network for the dehiscence zone patterning

Dry dehiscent fruits require an intricate patterning mechanisms allowing for a coordinated fruit rupture in combination with mostly explosive seed dispersal. As dry dehiscent fruits originated independently in several angiosperms lineages, for example within the Solanaceae, transitions from fleshy fruits to dry dehiscent fruits are frequent (Pabón-Mora & Litt 2011). We were interested in learning if and how the underlying developmental program differs between *Arabidopsis* as a representative of the Brassicaceae and California poppy as a representative of the Papaveraceae, two lineages that split more than 150 Million years ago (Zuntini et al. 2024)

Our comprehensive phylogenetic analysis of the GRN involved in the patterning of the *Arabidopsis* dehiscence zone revealed that many regulators are absent in species outside the Brassicaceae, a finding also supported by Pabón-Mora et al. 2014. However, the genomes used for data mining in this analysis were of markedly high quality, such that the absence of a gene is not due to lack of sequence information but reflects either gene loss or lineage specific WGD not shared by all taxa in the dataset. Thus, based on our analysis, we can conclude that the GRN involved in regulating the patterning and development of dry dehiscent fruits in *Arabidopsis* is not conserved throughout angiosperms. More likely, genes regulating patterning of dry dehiscent fruits in the diverse angiosperm lineages were recruited independently.

Conservation of STK orthologs in seed coat development

Seeds are a major contributor to the colonization of terrestrial habitats by vascular plants. They are vectors for nutrients and water supporting initial seedling growth and provide protection against physical force. The seed coat acts as seed's interface with the environments and its chemical and physical properties determine the degree of protection and control dormancy and germination (North et al. 2014; Coen & Magnani 2018). During California poppy fruit development, seeds develop from anatropous, bitegmic ovules, and as such, they do not deviate in their development and morphology from the majority of seeds found in dicots. The seed coat is a maternal, diploid tissue derived from the two integuments surrounding the ovule. The *Arabidopsis* *STK* gene has an important role in ovule and subsequent seed development, as *stk shp1 shp2* mutants fail to develop both integuments and in their place a structure reminiscent of a carpeloid organ is formed (Pinyopich et al. 2003). However, during *Arabidopsis* seed development, the number of layers in the seed coat and their morphological differentiation is similar in wild type and single *stk* seeds, but the cells are smaller (Mizzotti et al. 2014). Only double mutant combinations of *STK* and *ARABIDOPSIS BSISTER* (*ABS*) show abnormalities in their cellular differentiation in that they lack the endothelium (Mizzotti et al. 2012). This is unlike in the *EscaSTK-VIGS* treated California poppy seeds, whose seed coat shows substantial deviations from control treated

plants in their state of differentiation, number and size of cells. While some layering of the seed coat tissue can be observed (Fig. 4G and J), the outer tissue layers of the *EscaSTK*-VIGS treated plants appear parenchymatous suggesting that seed coat cell identity may not have been fully established in these plants. Apparently, the role of *EscaSTK* as regulator of tissue differentiation in the seed coat is different from that of its Arabidopsis ortholog, and independent of the California poppy *ABS* gene.

The identity of this thick layer of parenchymatous tissue surrounding the seed in the *EscaSTK*-VIGS treated plants remains unclear, but a second hypothesis regarding its origin relates to the Arabidopsis *shp1 shp2 stk* ovules that develop carpel-like structures around their integument-free nucellus. In analogy, the tissue surrounding the *EscaSTK*-VIGS treated seeds may also be undifferentiated carpel wall, such that without *EscaSTK*, the ovule integuments undergo homeotic conversions and a carpel is formed instead. The absence of *SHP1* and *SHP2* in genomes outside the core eudicots (Fig. 1B) supports this hypothesis, and possibly, integument identity in California poppy relies on *EscaSTK* alone. However, further analyses are required to support this hypothesis, requiring earlier stages ovules from *EscaSTK* knock-down plants.

Further, Arabidopsis *stk* seeds are smaller, due to a smaller cell size in the seed coat, and it was shown previously that *STK* activates several target genes, for example the cell cycle regulator *E2Fa*, influencing cell cycle progression in the developing seed coat by directly binding to its promoter leading to histone modifications (Paolo et al. 2021). The *EscaSTK*-VIGS treated seeds are also smaller and malformed, but we cannot rule out that their size and shape difference is due to the pressure the massive endocarp is exerting on them. However, Fig. 4G and J show that the cell size of *EscaSTK*-VIGS treated seeds outer cell layers seems increased, suggesting that the involvement of *STK* orthologs in cell cycle control seems conserved between the two species, but not the specific molecular mechanism of cell size regulation, resulting in opposing phenotypes. Moreover, *EscaSTK* not only regulates cell size, but also cell divisions in the outer layers of the seed coat, as the parenchymatous tissue is five to six cell layers thick, providing an additional link of *EscaSTK* to cell cycle regulation.

Conservation of the gene regulatory network for endocarp development

In many dry dehiscent plant species, the differentiation of endocarp layer plays a major role in dehiscence. The endocarp also protects the seed and is involved in communication with the maturing seed (Dardick & Callahan 2014). In Arabidopsis, the endocarp is composed of two layers (*ena* and *enb*) which have contrasting functions: *enb*, together with the replum and valve margins accumulates lignin and solidifies, creating tension upon dehydration. *Ena* secretes cell wall degrading enzymes creating the separation layer from which the valves separate. While *IND* is directing the lignification of the *enb*, *ALC* activates the cell wall degrading enzymes in the separation layer *ena*. Both genes are prevented to act outside their expression domain by *FUL*, preventing their expression in the valve, and *RPL*, preventing their expression in the replum, as *FUL* and *RPL* target the *SHP1/2* genes which activate *IND* and *ALC* (Ferrándiz 2002; Liljegren et al. 2004; Ogawa et al. 2009). Within the Brassicaceae, the genetic pathway seems rather conserved, as mutants of the Arabidopsis dehiscence pathway orthologs in *Lepidium*

campestre have similar phenotypes than those from *Arabidopsis* (Lenser & Theißen 2013) and changes to dehiscence zone architecture may be due to expression changes rather than absence of regulators. In core eudicots outside the Brassicaceae, *IND* and *ALC*, the direct target genes of the *SHP* orthologs are missing, as those arose in a Brassicales-specific whole genome duplication. This suggests that the genetic regulation of the *ena/enb* layer patterning of wall loosening/lignification may be specific to the Brassicales. However, almost all angiosperm genomes included in our analysis encode for *HEC3/IND* genes and *ALC/SPT* genes, which are equidistant to the Brassicales *HEC3* and *IND* and the *ALC* and *SPT* orthologs, respectively. Unfortunately, their function is unknown in plants outside *Arabidopsis*, except for *PvIND*, the ortholog from common bean, which is a candidate locus for pod shattering, but its molecular mechanism or mutant phenotype is unknown (Gioia et al. 2013).

Only few examples exist so far reporting *STK* homolog expression in the endocarp: In *Prunus persica* (peach), expression of the homologs of *SHP* and *STK* in the endocarp suggest that they may be involved in endocarp formation and regulation of its lignification. Conversely, *FUL* homolog expression is found outside the endocarp, possibly to restrict endocarp and limit lignification to its margins (Dardick et al. 2010). In *Elaeis guineensis* (oil palm), the *STK* ortholog *SHELL* regulates endocarp thickness, and the naturally occurring mutant shows a reduced endocarp size in combination with much higher yield than in the homozygous wild type oil palms.

Generally, the pericarp of California poppy fruits is lignified in a pattern different to *Arabidopsis*, and it is most likely the lignified valve ridges intersperse by dehydrating parenchymatous mesocarp cells that create the tension required for detachment of the valves from the replum. Interestingly, the separation layer between the lateral valve ridges and the replum sclerenchyma is composed of a narrow layer of cells that are even slightly lignified. Possibly, cell wall degrading enzymes are not required to actively enforce dehiscence, rather, premature rupture is prevented by the formation of a lignified ring all around the fruit very late during fruit maturation (Fig. 2A, Suppl. Figure 2, Suppl. Material 1).

Possibly, the specialized role of the endocarp in being a major contributor to dehiscence has originated in the lineage leading to core eudicots, and in species outside the core eudicots, other parts of the pericarp are equally or more important for dehiscence. Dry dehiscent fruits have originated several times independently in several large taxonomical groups, including the Solanaceae, Apiceae, and Campanulaceae, with frequent transitions back to fleshy or dry, dehiscent fruits (Pabón-Mora & Litt 2011; Pramanik et al. 2023). It is thus possibly that in several of the fleshy to dehiscent fruit shifts, different parts of the pericarp provided specialized anatomies optimized for dehiscence. Interestingly, even though fruit formation is an important ecological and agronomical trait, research on the molecular evolution of fruit types is rare. More specifically, we lack functional studies documenting the transitions from dry dehiscent to fleshy fruits and vice versa. In most fleshy species, the fleshy part of the fruit is made up often by the parenchymatous mesocarp, but in several crop species, endocarp becomes fleshy, as seen in watermelon or mango (Dardick & Callahan 2014). Our study and the work by Pabon-Mora et al., (2012) on *EscaFUL1* and *EscaFUL2* are the first cases where reduction of gene function resulted in additional, and more fleshy cells in the endocarp layer. One could hypothesize this phenotype as a first

step towards a transition from dry dehiscent to fleshy fruits, involving two MADS-box transcription factors generally involved in flower and seed formation that may have gained a novel function in endocarp growth regulation. If then either *EscaFUL1/EscaFUL2* or *EscaSTK* is lost, capsules become fleshier.

Conclusions

Our analysis broadly started out to carry out a presence/absence analysis of the major regulators of dehiscence zone formation in Arabidopsis. Finding that many members of the dehiscence zone GRN are lacking from species outside the Brassicaceae, we intended to identify dehiscence zone regulators in California poppy, which, like Arabidopsis, produces dry dehiscent fruits. The California poppy ortholog of *STK*, *EscaSTK*, which is not involved in dehiscence zone formation in Arabidopsis, was differentially expressed between dehiscence zone and valve. Knock-down of *EscaSTK* causes premature rupture of the capsule due to excessive proliferation and elongation of the endocarp cells. Further, *EscaSTK* knock down leads to larger, more, and less differentiated cells in the seed coat, but changes in the lignification pattern of the dehiscence zone was not observed in the stages we analyzed. These functional analyses show the remarkable contributions to *EscaSTK* to reproductive development in California poppy: restriction of endocarp cell division and elongation as well as seed coat differentiation and growth limitation. Previous work in Arabidopsis demonstrated *STK*'s direct regulation of several pathways associated with seed development, cell cycle and seed specific metabolism. Our work shows that *STK* orthologs are being recruited to several GRNs directing sexual plant reproduction in evolution, such as regulating lignification in the funiculus of ovules, endothelium differentiation and cell size control in the developing seed coat, and activating metabolic pathways in the late stages of seed coat development in Arabidopsis. In California poppy, *EscaSTK* contributes to stamen identity, cell cycle control during endocarp and seed coat development. The lineages of Arabidopsis and California poppy split around 150 Million years ago, sufficient time for a MADS-box transcriptional regulator to diversify its functions, most likely by accumulation of its DNA-binding sites in the regulatory regions of novel target genes and shifts in protein interaction partners. *STK* orthologs may turn up as candidate genes in screens for diverse reproductive traits of traditional or novel crops to become species-specific breeding targets, e.g. for endocarp layer size, seed size or seed dehiscence.

Methods

Plant growth and histology

E. californica (cv. Aurantiaca Orance King, B&T World Seeds, Aigues-Vive, France) was grown from seeds at 22°C under long day conditions (16 hours of light, light intensity ~ 95 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Freshly opened flowers were hand pollinated to ensure fruit development.

Fruits were fixed in FAA (50% EtOH, 5% acetic acid, 10% formaldehyde) plus a drop of TWEEN20 on ice (4°C) and vacuum infiltrated for 1h. Fresh FAA was added and the samples were passed through a

dehydration series with 30 min in 50%, 70%, 85%, 90% and finally 100% EtOH respectively, with fresh 100% EtOH overnight incubation at 4°C. Then followed incubation in 100% EtOH for 1h, 50% EtOH with 50% Rotihistol for 2h, 100% Rotihistol for 2h, and again 100% Rotihistol for 2h. The fruits were then placed on solid Paraplast Plus chips, covered by Rotihistol and incubated for three days at 60°C, exchanging the molten Paraplast Plus every 8–12 h. Then the samples were poured into petri dishes allowing the Paraplast Plus to solidify. Samples were sectioned on a Leica RM2125RTS microtome to 5–7 µm thickness, dried, incubated for 20 min in 100% Rotihistol and then rehydrated (5 min steps in 100%, 95%, 85%, 70%, 50%, 30% EtOH, respectively) and dried.

For samples around dap 18 an alternative embedding and staining protocol was used, due to the brittleness of the fruits. The fruits were fixed in 1% PFA and 2% GA in 0.1 M phosphate buffer pH 7.2, followed by 30 min vacuum infiltration and 3 h incubation at RT. The solution was replaced for overnight incubation at 4°C with gentle shaking. Dehydration was performed as described above with the last step being EtOH:LR-White 1:1 (v/v). After infiltration in 100% LR-White, the samples were placed into gelatin capsules surrounded by resin, polymerized at 50°C for 24 hours and sectioned with a microtome (Leica Om U2, Wetzlar, Germany) to 1–1.5 µm thickness. Sections embedded with the Paraplast method were stained with a 2% (w/v) phloroglucinol-HCl solution (Images shown in Fig. 2A, Suppl. Figure 2A-D and K-M) (Pradhan Mitra & Loqué 2014). Due to the bad staining quality of the phloroglucinol-HCl staining method within LR-White sections, we analyzed these sections via one drop of fuchsin-astra blue diluted in water incubating for five minutes (Images shown in Suppl. Figure 2E-J). Alternatively, sections were stained with crystal violet staining solution for better tissue perceptibility. The sections were analyzed using a Leica DM5500B microscope. Images were manipulated for contrast and assembled using CorelDraw12.

RNA extraction, library preparation, sequencing

Material collection for RNAseq was carried out by manual dissection of 2 and 6 dap fruits to collect valve (v) and dehiscence zone (dz) tissues separately. RNA extraction of fruits of wild type plants for RNAseq and fruits of VIGS treated plants for qRT-PCR was carried out using the NucleoSpin® RNA Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Library preparation for RNAseq was carried out using the Illumina TruSeqmRNA (San Diego, CA, USA) kit according to the manufacturer's instructions and sequenced on Illumina NovaSeq6000 (San Diego, CA, USA) at the West German Genome Center (WGGC) Cologne, Germany.

Phylogeny reconstructions

Protein sequences of dehiscence genes were identified using BLAST (Altschul et al. 1990) to mine the JGI Phytozome database (Goodstein et al. 2012), in house databases (*P. somniferum*, *Vicia faba*, Guo et al. 2018; Jayakodi et al. 2023), sequences were aligned using MAFFT (--localpair --maxiterate 10000 --

reorder, Katoh & Standley 2013). Phylogenies (Maximum Likelihood 100 bootstraps) were generated using iqtree (-T AUTO -m TEST -b 100 -con) (Minh et al. 2020) with full length protein sequences.

Transcriptome expression analysis

We used the published *Eschscholzia californica* draft genome sequence (Hori et al. 2018) for transcriptome mapping. The read counts were estimated with Salmon (Patro et al. 2017, version 1.9.0). The expression levels of orthologs of published genes were used for generating the heatmap. The R package 'Pheatmap' was used to generate cluster heatmaps of log-normalized TPM matrices.

VIGS

A 379 bp fragment of *EScaSTK* including the K-box region was PCR amplified from floral bud cDNA using primers that include EcoRI and BamHI restriction sites, digested, cloned into the equally digested pTRV2 plasmid vector, and sequenced, creating the pTRV2-*EScaSTK* plasmid. The TRV2-mEGFP plasmid included an around 500 bp fragment of *mEGFP* and was prepared the same way. The pTRV1, pTRV2-*EScaSTK*, and pTRV2-mEGFP vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. Plant infiltration was carried out following the protocol of Wege et al. (2007) and Tekleyohans et al., (2013). 155 *E. californica* plants were inoculated with pTRV1 and pTRV2-*EScaSTK*, 45 plants remained untreated, 30 plants were inoculated with pTRV1 and pTRV2-*EScaPDS* (Wege et al. 2007), and 30 plants were inoculated with pTRV1 and pTRV2-mEGFP. Flowers of VIGS treated plants were hand pollinated. Every seven days, pictures of plants and capsules were taken (12MP 1/2.55" sensor, 26mm-equivalent f/1.8-aperture lens, PDAF, OIS), length and width of fruits were measured via ImageJ.

qRT-PCR

For qRT-PCR, the first floral bud of VIGS-treated plants was collected. cDNA was generated as described above. The relative expression level of *EScaSTK* was determined in untreated and VIGS-treated plants via qRT-PCR. *E. californica* GAPDH was used as a reference gene and all primers were tested to have an efficiency above 2. qRT-PCR was carried out using a LightCycler 480 II (Roche, Basel, Switzerland). List of used primers can be found in the Supplementary Table (Suppl. Tables). Three technical replicates were performed of each sample. qRT-PCR was performed in a total volume of 20 µl containing 10 µl Luna Universal qPCR Mix (NEB), 1 µl diluted cDNA (1:50), 1 µl of each primer and 3 µl water. PCR conditions were pre-incubation at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 sec and elongation at 72°C for 10 sec. The relative expression differences was analyzed relative to the wild-typ using the $\Delta\Delta C_t$ method (Pfaffl 2001).

Legume flowers at anthesis, leaves, buds, and fruit dehiscence zones and valves at two developmental stages were collected for qRT-PCR. *Pisum sativum* RNA was isolated using the NucleoSpin® RNA Plant

kit (Macherey-Nagel, Düren, Germany) for *P. sativum*; *Glycine max* and *Vicia faba* RNA was isolated using the the NucleoSpin® RNA Plant and Fungi kit (Macherey-Nagel, Düren, Germany). qPCR was used to detect the expression of *STK* orthologs in different tissues. For *G. max* and *P. sativum*, *ACTIN* was used as housekeeping gene and for *V. faba* *UBIQUITIN* (Gutierrez et al. 2011). Expression was related to these. qRT-PCR and phenotypic data were visualized using the R package 'ggplot2'.

Table 1 Numbers of VIGS treated plants and phenotype occurrence						
Treatment	No. of plants	No. of capsules	No. of plants not flowering	WT-like phenotype	Prematurely ruptured fruits	Flowers with homeotic conversions
untreated	30	83	1 3%	30 100%	0 0%	0 0%
pTRV2-mEGFP	59	254	3 5,1%	56 94,9%	0 0%	0 0%
pTRV2-EscaSTK	155	521	13 8,4%	114 73,5%	18 11,6%	10 6,5%

Declarations

Acknowledgements

We thank Dietmar Haffer and Claudia Jung-Blasini for help with growing the plants and Andrea Weisert for her support with wet lab experiments. The student Siwei Pang contributed towards the collection of data for this work. This work was supported by the de.NBI Cloud within the German Network for Bioinformatics Infrastructure (de.NBI) and ELIXIR-DE (Forschungszentrum Jülich and W-de.NBI-001, W-de.NBI-004, W-de.NBI-008, W-de.NBI-010, W-de.NBI-013, W-de.NBI-014, W-de.NBI-016, W-de.NBI-022).

Funding

none

Author information

Dominik Lotz¹, Le-Han Rössner¹, Katrin Ehlers¹, Doudou Kong¹, Clemens Rössner¹, Oliver Rupp², Annette Becker^{1*}

¹ Institute of Botany, Justus-Liebig-University, Heinrich-Buff-Ring 38, 35392 Giessen, Germany

² Institute of Systems Biology and Bioinformatics, Justus-Liebig-University, Heinrich-Buff-Ring 58, 35392 Giessen, Germany

Contributions

DL, investigation, visualization

L-HR, microscopic investigations, data analysis, visualization

KE, microscopic investigations and data analyses

DK, data visualization (heat maps, phenotypes, qPCR)

CR, dataset compilation and phylogeny reconstructions, visualization

OR, read count estimation

AB, conceived the study, analyzed and interpreted data, wrote largest part of the manuscript.

Corresponding author

Annette Becker

Ethics Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Not applicable.

References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of molecular biology* 215:403–410. doi: 10.1016/S0022-2836(05)80360-2
2. Balanzà V, Roig-Villanova I, Di Marzo M, Masiero S, Colombo L (2016) Seed abscission and fruit dehiscence required for seed dispersal rely on similar genetic networks. *Development* (Cambridge, England) 143:3372–3381. doi: 10.1242/dev.135202
3. Ballester P, Ferrándiz C (2017) Shattering fruits: variations on a dehiscent theme. *Current opinion in plant biology* 35:68–75. doi: 10.1016/j.pbi.2016.11.008
4. Becker A, Bachelier JB, Carrive L, Conde E Silva N, Damerval C, Del Rio C, Deveau Y, Di Stilio VS, Gong Y, Jabbour F, Kramer EM, Nadot S, Pabón-Mora N, Wang W (2024) A cornucopia of diversity-

- Ranunculales as a model lineage. *Journal of experimental botany* 75:1800–1822. doi: 10.1093/jxb/erad492
5. Becker A, Gleissberg S, Smyth DR (2005) Floral and Vegetative Morphogenesis in California Poppy (*Eschscholzia californica* Cham.). *International Journal of Plant Sciences* 166:537–555. doi: 10.1086/429866
 6. Becker A, Yamada Y, Sato F (2023) California poppy (*Eschscholzia californica*), the Papaveraceae golden girl model organism for evodevo and specialized metabolism. *Frontiers in plant science* 14:1084358. doi: 10.3389/fpls.2023.1084358
 7. Coen O, Magnani E (2018) Seed coat thickness in the evolution of angiosperms. *Cellular and molecular life sciences : CMLS* 75:2509–2518. doi: 10.1007/s00018-018-2816-x
 8. Dardick C, Callahan AM (2014) Evolution of the fruit endocarp: molecular mechanisms underlying adaptations in seed protection and dispersal strategies. *Frontiers in plant science* 5:284. doi: 10.3389/fpls.2014.00284
 9. Dardick CD, Callahan AM, Chiozzotto R, Schaffer RJ, Piagnani MC, Scorza R (2010) Stone formation in peach fruit exhibits spatial coordination of the lignin and flavonoid pathways and similarity to *Arabidopsis* dehiscence. *BMC biology* 8:13. doi: 10.1186/1741-7007-8-13
 10. Di Marzo M, Herrera-Ubaldo H, Caporali E, Novák O, Strnad M, Balanzà V, Ezquer I, Mendes MA, Folter S de, Colombo L (2020) SEEDSTICK Controls *Arabidopsis* Fruit Size by Regulating Cytokinin Levels and FRUITFULL. *Cell reports* 30:2846-2857.e3. doi: 10.1016/j.celrep.2020.01.101
 11. Eshed Y, Baum SF, Bowman JL (1999) Distinct mechanisms promote polarity establishment in carpels of *Arabidopsis*. *Cell* 99:199–209. doi: 10.1016/S0092-8674(00)81651-7
 12. Ferrándiz C (2002) Regulation of fruit dehiscence in *Arabidopsis*. *Journal of experimental botany* 53:2031–2038. doi: 10.1093/jxb/erf082
 13. Ferrándiz C, Liljegren SJ, Yanofsky MF (2000) Negative regulation of the SHATTERPROOF genes by FRUITFULL during *Arabidopsis* fruit development. *Science (New York, N.Y.)* 289:436–438. doi: 10.1126/science.289.5478.436
 14. Gioia T, Logozzo G, Kami J, Spagnoletti Zeuli P, Gepts P (2013) Identification and characterization of a homologue to the *Arabidopsis* INDEHISCENT gene in common Bean. *The Journal of heredity* 104:273–286. doi: 10.1093/jhered/ess102
 15. Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, Rokhsar DS (2012) Phytozome: a comparative platform for green plant genomics. *Nucleic acids research* 40:D1178-86. doi: 10.1093/nar/gkr944
 16. Groszmann M, Paicu T, Alvarez JP, Swain SM, Smyth DR (2011) SPATULA and ALCATRAZ, are partially redundant, functionally diverging bHLH genes required for *Arabidopsis* gynoecium and fruit development. *The Plant journal : for cell and molecular biology* 68:816–829. doi: 10.1111/j.1365-313X.2011.04732.x
 17. Guo L, Winzer T, Yang X, Li Y, Ning Z, He Z, Teodor R, Lu Y, Bowser TA, Graham IA, Ye K (2018) The opium poppy genome and morphinan production. *Science (New York, N.Y.)* 362:343–347. doi:

18. Gutierrez N, Giménez MJ, Palomino C, Avila CM (2011) Assessment of candidate reference genes for expression studies in *Vicia faba* L. by real-time quantitative PCR. *Molecular Breeding* 28:13–24. doi: 10.1007/s11032-010-9456-7
19. Hori K, Yamada Y, Purwanto R, Minakuchi Y, Toyoda A, Hirakawa H, Sato F (2018) Mining of the Uncharacterized Cytochrome P450 Genes Involved in Alkaloid Biosynthesis in California Poppy Using a Draft Genome Sequence. *Plant & cell physiology* 59:222–233. doi: 10.1093/pcp/pcx210
20. Huss JC, Gierlinger N (2021) Functional packaging of seeds. *The New phytologist* 230:2154–2163. doi: 10.1111/nph.17299
21. Jayakodi M, Golicz AA, Kreplak J, Fechete LI, Angra D, Bednář P, Bornhofen E, Zhang H, Boussageon R, Kaur S, Cheung K, Čížková J, Gundlach H, Hallab A, Imbert B, Keeble-Gagnère G, Koblížková A, Koblřová L, Krejčí P, Mouritzen TW, Neumann P, Nadzieja M, Nielsen LK, Novák P, Orabi J, Padmarasu S, Robertson-Shersby-Harvie T, Robledillo LÁ, Schiemann A, Tanskanen J, Törönen P, Warsame AO, Wittenberg AHJ, Himmelbach A, Aubert G, Courty P-E, Doležel J, Holm LU, Janss LL, Khazaei H, Macas J, Mascher M, Smýkal P, Snowdon RJ, Stein N, Stoddard FL, Stougaard J, Tayeh N, Torres AM, Usadel B, Schubert I, O'Sullivan DM, Schulman AH, Andersen SU (2023) The giant diploid faba genome unlocks variation in a global protein crop. *Nature* 615:652–659. doi: 10.1038/s41586-023-05791-5
22. Jernstedt JA, Clark C (1979) STOMATA ON THE FRUITS AND SEEDS OF ESCHSCHOLZIA (PAPAVERACEAE). *American Journal of Botany* 66:586–590. doi: 10.1002/j.1537-2197.1979.tb06261.x
23. Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution* 30:772–780. doi: 10.1093/molbev/mst010
24. Lenser T, Theißen G (2013) Conservation of fruit dehiscence pathways between *Lepidium campestre* and *Arabidopsis thaliana* sheds light on the regulation of INDEHISCENT. *The Plant journal : for cell and molecular biology* 76:545–556. doi: 10.1111/tpj.12321
25. Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, Yanofsky MF (2000) SHATTERPROOF MADS-box genes control seed dispersal in *Arabidopsis*. *Nature* 404:766–770. doi: 10.1038/35008089
26. Liljegren SJ, Roeder AHK, Kempin SA, Gremski K, Østergaard L, Guimil S, Reyes DK, Yanofsky MF (2004) Control of fruit patterning in *Arabidopsis* by INDEHISCENT. *Cell* 116:843–853. doi: 10.1016/s0092-8674(04)00217-x
27. Lorts CM, Briggeman T, Sang T (2008) Evolution of fruit types and seed dispersal: A phylogenetic and ecological snapshot. *Journal of Systematics and Evolution*:396–404. doi: 10.3724/SP.J.1002.2008.08039
28. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Haeseler A von, Lanfear R (2020) IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular biology and evolution* 37:1530–1534. doi: 10.1093/molbev/msaa015

29. Mizzotti C, Ezquer I, Paolo D, Rueda-Romero P, Guerra RF, Battaglia R, Rogachev I, Aharoni A, Kater MM, Caporali E, Colombo L (2014) SEEDSTICK is a master regulator of development and metabolism in the Arabidopsis seed coat. *PLoS genetics* 10:e1004856. doi: 10.1371/journal.pgen.1004856
30. Mizzotti C, Mendes MA, Caporali E, Schnittger A, Kater MM, Battaglia R, Colombo L (2012) The MADS box genes SEEDSTICK and ARABIDOPSIS Bsister play a maternal role in fertilization and seed development. *The Plant journal : for cell and molecular biology* 70:409–420. doi: 10.1111/j.1365-313X.2011.04878.x
31. North HM, Berger A, Saez-Aguayo S, Ralet M-C (2014) Understanding polysaccharide production and properties using seed coat mutants: future perspectives for the exploitation of natural variants. *Annals of botany* 114:1251–1263. doi: 10.1093/aob/mcu011
32. Ogawa M, Kay P, Wilson S, Swain SM (2009) ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are Polygalacturonases required for cell separation during reproductive development in Arabidopsis. *The Plant cell* 21:216–233. doi: 10.1105/tpc.108.063768
33. Pabón-Mora N, Ambrose BA, Litt A (2012) Poppy APETALA1/FRUITFULL orthologs control flowering time, branching, perianth identity, and fruit development. *Plant physiology* 158:1685–1704. doi: 10.1104/pp.111.192104
34. Pabón-Mora N, Litt A (2011) Comparative anatomical and developmental analysis of dry and fleshy fruits of Solanaceae. *American Journal of Botany* 98:1415–1436. doi: 10.3732/ajb.1100097
35. Pabón-Mora N, Wong GK-S, Ambrose BA (2014) Evolution of fruit development genes in flowering plants. *Frontiers in plant science* 5:300. doi: 10.3389/fpls.2014.00300
36. Paolo D, Rotasperi L, Schnittger A, Masiero S, Colombo L, Mizzotti C (2021) The Arabidopsis MADS-Domain Transcription Factor SEEDSTICK Controls Seed Size via Direct Activation of E2Fa. *Plants* (Basel, Switzerland) 10. doi: 10.3390/plants10020192
37. Parker TA, Lo S, Gepts P (2021) Pod shattering in grain legumes: emerging genetic and environment-related patterns. *The Plant cell* 33:179–199. doi: 10.1093/plcell/koaa025
38. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C (2017) Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods* 14:417–419. doi: 10.1038/nmeth.4197
39. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* 29:e45. doi: 10.1093/nar/29.9.e45
40. Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, Yanofsky MF (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* 424:85–88. doi: 10.1038/nature01741
41. Pradhan Mitra P, Loqué D (2014) Histochemical staining of Arabidopsis thaliana secondary cell wall elements. *Journal of visualized experiments : JoVE*. doi: 10.3791/51381
42. Pramanik D, Becker A, Roessner C, Rupp O, Bogarín D, Pérez-Escobar OA, Dirks-Mulder A, Droppert K, Kocyan A, Smets E, Gravendeel B (2023) Evolution and development of fruits of *Erycina pusilla* and

- other orchid species. PloS one 18:e0286846. doi: 10.1371/journal.pone.0286846
43. Rajani S, Sundaresan V (2001) The Arabidopsis myc/bHLH gene ALCATRAZ enables cell separation in fruit dehiscence. Current biology : CB 11:1914–1922. doi: 10.1016/S0960-9822(01)00593-0
 44. Ripoll J-J, Zhu M, Brocke S, Hon CT, Yanofsky MF, Boudaoud A, Roeder AHK (2019) Growth dynamics of the Arabidopsis fruit is mediated by cell expansion. Proceedings of the National Academy of Sciences of the United States of America 116:25333–25342. doi: 10.1073/pnas.1914096116
 45. Roeder AHK, Ferrándiz C, Yanofsky MF (2003) The role of the REPLUMLESS homeodomain protein in patterning the Arabidopsis fruit. Current biology : CB 13:1630–1635. doi: 10.1016/j.cub.2003.08.027
 46. Tekleyohans DG, Lange S, Becker A (2013) Virus-induced gene silencing of the alkaloid-producing basal eudicot model plant *Eschscholzia californica* (California Poppy). Methods in molecular biology (Clifton, N.J.) 975:83–98. doi: 10.1007/978-1-62703-278-0_7
 47. Wege S, Scholz A, Gleissberg S, Becker A (2007) Highly efficient virus-induced gene silencing (VIGS) in California poppy (*Eschscholzia californica*): an evaluation of VIGS as a strategy to obtain functional data from non-model plants. Annals of botany 100:641–649. doi: 10.1093/aob/mcm118
 48. Zumajo-Cardona C, Ambrose BA, Pabón-Mora N (2017) Evolution of the SPATULA/ALCATRAZ gene lineage and expression analyses in the basal eudicot, *Bocconia frutescens* L. (Papaveraceae). EvoDevo 8:5. doi: 10.1186/s13227-017-0068-8
 49. Zuntini AR, Carruthers T, Maurin O, Bailey PC, Leempoel K, Brewer GE, Epitawalage N, Françoso E, Gallego-Paramo B, McGinnie C, Negrão R, Roy SR, Simpson L, Toledo Romero E, Barber VMA, Botigué L, Clarkson JJ, Cowan RS, Dodsworth S, Johnson MG, Kim JT, Pokorny L, Wickett NJ, Antar GM, DeBolt L, Gutierrez K, Hendriks KP, Hoewener A, Hu A-Q, Joyce EM, Kikuchi IABS, Larridon I, Larson DA, Lirio EJ de, Liu J-X, Malakasi P, Przelomska NAS, Shah T, Viruel J, Allnutt TR, Ameka GK, Andrew RL, Appelhans MS, Arista M, Ariza MJ, Arroyo J, Arthan W, Bachelier JB, Bailey CD, Barnes HF, Barrett MD, Barrett RL, Bayer RJ, Bayly MJ, Biffin E, Biggs N, Birch JL, Bogarín D, Borosova R, Bowles AMC, Boyce PC, Bramley GLC, Briggs M, Broadhurst L, Brown GK, Bruhl JJ, Bruneau A, Buerki S, Burns E, Byrne M, Cable S, Calladine A, Callmander MW, Cano Á, Cantrill DJ, Cardinal-McTeague WM, Carlsen MM, Carruthers AJA, Castro Mateo A de, Chase MW, Chatrou LW, Cheek M, Chen S, Christenhusz MJM, Christin P-A, Clements MA, Coffey SC, Conran JG, Cornejo X, Couvreur TLP, Cowie ID, Csiba L, Darbyshire I, Davidse G, Davies NMJ, Davis AP, van Dijk K-J, Downie SR, Duretto MF, Duvall MR, Edwards SL, Eggli U, Erkens RHJ, Escudero M, La Estrella M de, Fabriani F, Fay MF, Ferreira PdL, Ficinski SZ, Fowler RM, Frisby S, Fu L, Fulcher T, Galbany-Casals M, Gardner EM, German DA, Giaretta A, Gibernau M, Gillespie LJ, González CC, Goyder DJ, Graham SW, Grall A, Green L, Gunn BF, Gutiérrez DG, Hackel J, Haevermans T, Haigh A, Hall JC, Hall T, Harrison MJ, Hatt SA, Hidalgo O, Hodgkinson TR, Holmes GD, Hopkins HCF, Jackson CJ, James SA, Jobson RW, Kadereit G, Kahandawala IM, Kainulainen K, Kato M, Kellogg EA, King GJ, Klejevskaia B, Klitgaard BB, Kloppe RR, Knapp S, Koch MA, Leebens-Mack JH, Lens F, Leon CJ, Lèveillé-Bourret É, Lewis GP, Li D-Z, Li L, Liede-Schumann S, Livshultz T, Lorence D, Lu M, Lu-Irving P, Luber J, Lucas EJ, Luján M, Lum M, Macfarlane TD, Magdalena C, Mansano VF, Masters LE, Mayo SJ, McColl K, McDonnell AJ, McDougall AE, McLay TGB, McPherson H, Meneses RI, Merckx VSFT, Michelangeli FA, Mitchell JD,

Monro AK, Moore MJ, Mueller TL, Mummenhoff K, Munzinger J, Muriel P, Murphy DJ, Nargar K, Nauheimer L, Nge FJ, Nyffeler R, Orejuela A, Ortiz EM, Palazzesi L, Peixoto AL, Pell SK, Pellicer J, Penneys DS, Perez-Escobar OA, Persson C, Pignal M, Pillon Y, Pirani JR, Plunkett GM, Powell RF, Prance GT, Puglisi C, Qin M, Rabaler RK, Rees PEJ, Renner M, Roalson EH, Rodda M, Rogers ZS, Rokni S, Rutishauser R, Salas MF de, Schaefer H, Schley RJ, Schmidt-Lebuhn A, Shapcott A, Al-Shehbaz I, Shepherd KA, Simmons MP, Simões AO, Simões ARG, Siros M, Smidt EC, Smith JF, Snow N, Soltis DE, Soltis PS, Soreng RJ, Sothers CA, Starr JR, Stevens PF, Straub SCK, Struwe L, Taylor JM, Telford IRH, Thornhill AH, Tooth I, Trias-Blasi A, Udovicic F, Utteridge TMA, Del Valle JC, Verboom GA, Vonow HP, Vorontsova MS, Vos JM de, Al-Wattar N, Waycott M, Welker CAD, White AJ, Wieringa JJ, Williamson LT, Wilson TC, Wong SY, Woods LA, Woods R, Worboys S, Xanthos M, Yang Y, Zhang Y-X, Zhou M-Y, Zmarzty S, Zuloaga FO, Antonelli A, Bellot S, Crayn DM, Grace OM, Kersey PJ, Leitch IJ, Sauquet H, Smith SA, Eiserhardt WL, Forest F, Baker WJ (2024) Phylogenomics and the rise of the angiosperms. *Nature* 629:843–850. doi: 10.1038/s41586-024-07324-0

Figures

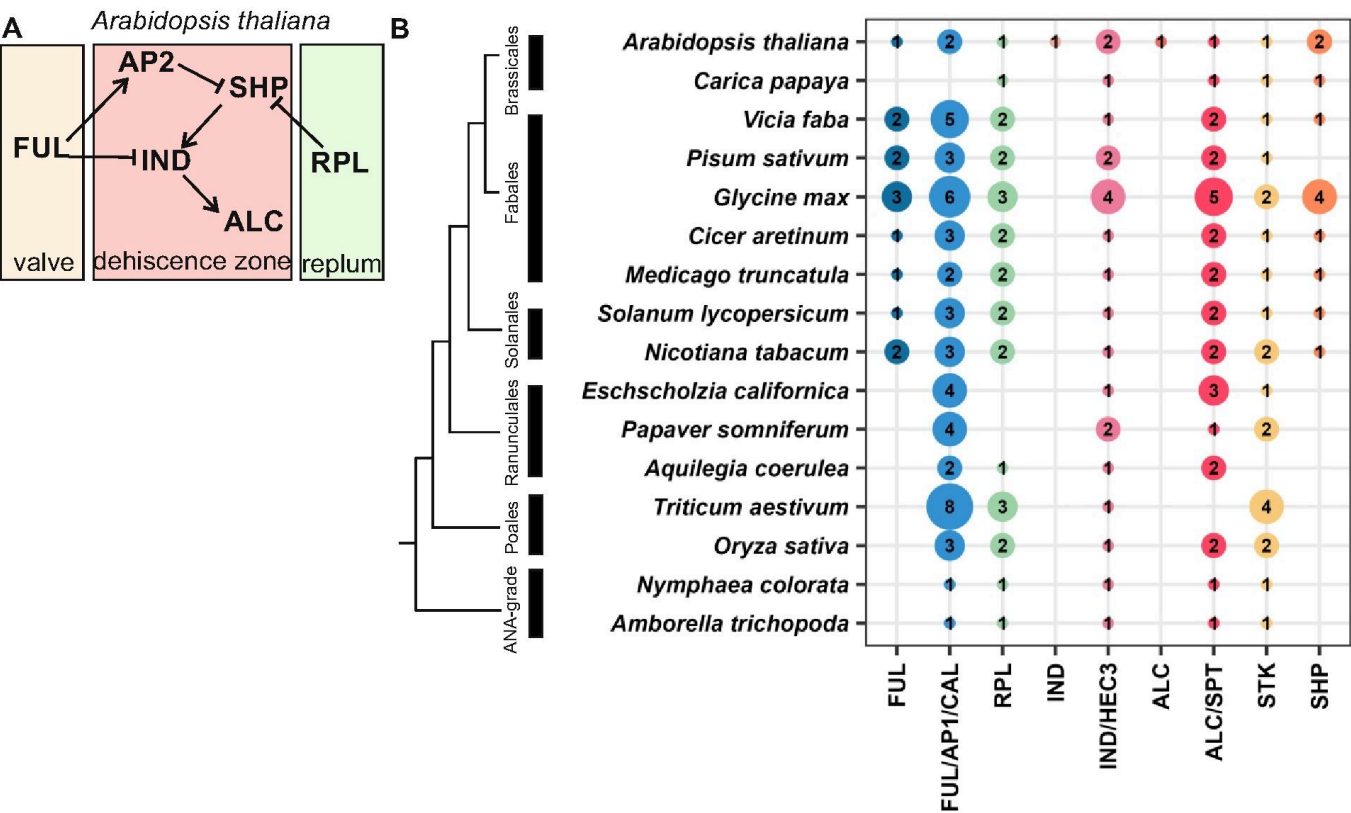


Figure 1

Regulatory network of dehiscence zone formation in *A. thaliana* and presence of orthologs of dehiscence zone regulatory transcription factors in other plant species. (A) Simplified representation of

the known transcription factors important for dehiscence zone formation in *Arabidopsis thaliana* based on Ballester and Ferrandiz, 2017. Gene activation is indicated by arrows, repression by bars. (B) Summary of the phylogeny reconstructions of a subset of transcription factor families shown in A (Suppl. Figure 1). On the left, a simplified phylogeny shows the relationship of the species included in our analysis. Numbers in colored bubbles represent copy numbers of the respective homologs. In addition to the single FUL and ALC column, a FUL/AP/CAL and ALC/SPT clades are generated due to an unclear phylogenetic separation between these genes (Suppl. Fig. 1).

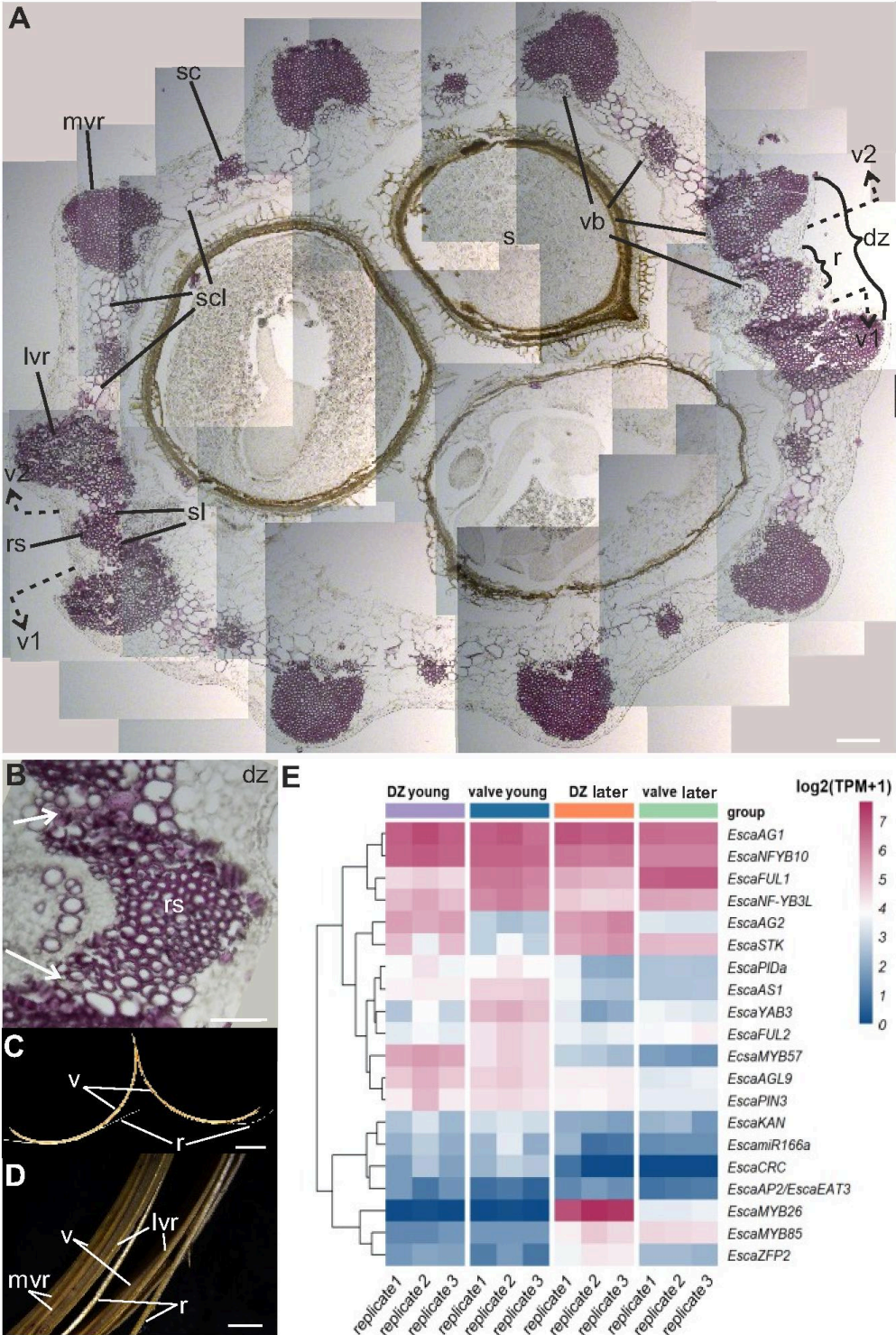


Figure 2

California poppy fruit lignification pattern, fruit opening and transcriptome analysis of dehiscence zone and valves. (A) Overview of the cross-sectioned mature poppy fruit 32 days after pollination (dap), composed of two valves (v1 and v2) separated by two repla (r). Each valve includes two sclerified lateral valve ridges (lvr), three sclerified median valve ridges (mvr), and intervening small sclerenchyma caps (sc), covering the adaxial side of the vascular bundles (vb). These sclerenchymatous valve tissues are interconnected via layers of sclereids (scl) traversing the radially arranged parenchyma cells which separate lvr, mvr and sc. Each dehiscence zone (dz) includes two lvr and the central r, which contains a vb with a lignified replum sclerenchyma cap (rs) and is bordered by narrow, radially orientated lignified separation layers (sl). Ridges, sc, and rs together with sclereids and sl form a closed ring of lignified sclerenchymatous elements, which likely provides mechanic stability to the fruit. Seeds (s) are located within the capsules and are attached to the fruit wall via the placenta tissue at the valve margins. (B) Enlargement of the dehiscence zone showing the replum sclerenchyma and lignified cell files that will separate upon rupture (white arrows). (C) Split fruit after rupture with valves (V) and replum fragments (r) indicated. (D) Enlargement of ruptured valves. (E) Transcriptome analysis of 2 dap (young) and 6 dap (later) stage of fruit development showing dehiscence zones separated from valve regions. Calculation were done using Euclidean distance. Scale bars represent 200nm in A, 100nm in B, 1cm in C and 2mm in D .

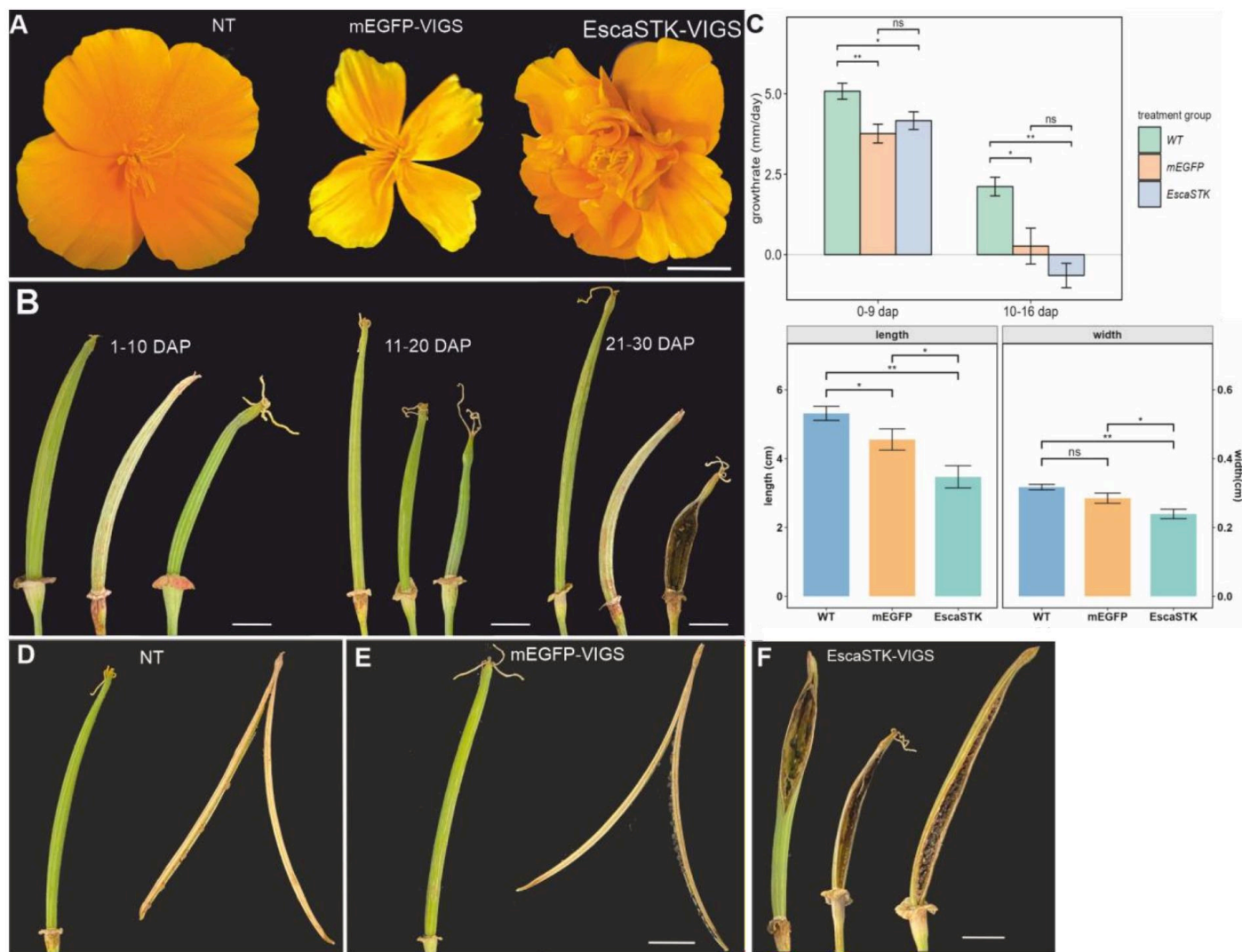


Figure 3

Effects of Virus-induced gene silencing of *EscaSTK* on flower and fruit development. (A) Effect of VIGS on flower morphology, with (left) untreated plants, (center) *mEGFP*-VIGS treated plants, (right), *EscaSTK*-VIGS treated plants. (B) Quantification of growth rates in the VIGS treated plants. (C) Fruit morphology during different collection intervals with untreated (left), *mEGFP*-VIGS treated (middle) and *EscaSTK*-VIGS treated (right) plants. (D) Maximum fruit size of VIGS treated plants. (E) Untreated fruit before (left) and after (right) dehydration and dehiscence. (F) *mEGFP*-VIGS treated fruit before (left) and after (right) dehydration and dehiscence. (G) *EscaSTK*-VIGS treated fruit before (left) and after (right) dehydration and dehiscence. (H) Fruit rupture at dap. Scale bar represents 1cm.

Since the phenotypic data did not conform to a normal distribution, the significance of the difference was tested using the Wilcoxon signed-rank test in R. Ns: not significant, * $p < 0.05$, ** $p < 0.01$

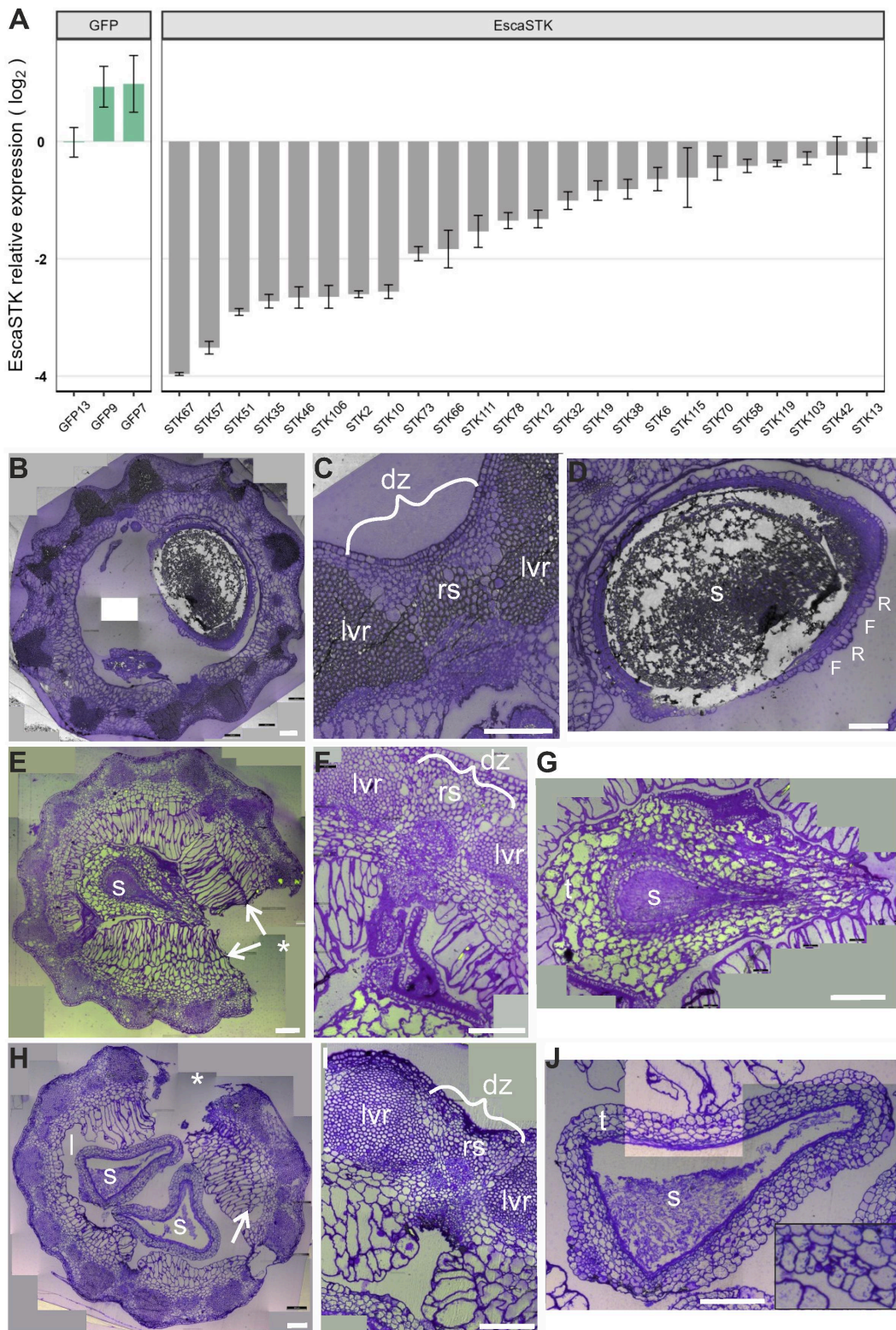


Figure 4

Downregulation of *EscaSTK* by VIGS and effects of *EscaSTK*-VIGS on tissue development of fruits and seeds. (A) qRT-PCR analysis of the first buds of VIGS treated plants. Log₂ normalized expression relative to untreated plants is shown, with *GAP-DH* as internal standard. (B-D) show a cross-sectioned *mEGFP*-VIGS treated capsule as negative control with a WT-like histology. (B) Overview of the capsule, (C) detailed view of the dehiscence zone, and (D) detailed view of the seed. The respective images of

prematurely opened *EscaSTK* capsules with intermediate down regulation (50-75 % of remaining *EscaSTK* expression, E-G) or with severe down regulation (25-50 % of remaining *EscaSTK* expression, H-J) are shown. Histology of the dehiscence zone and the abaxial valve tissues, as well as the early lignification events in the fruit wall, remain largely unaltered in *EscaSTK* capsules (E, F, H, I). White arrows in E and H denote the thick-walled tangential boundary between endocarp layers and underlying tissues. The asterisks in H and E indicated premature rupture of the capsule either at the dehiscence zone (H) or between the median valve ridges (E). The inset in J shows stomata in the seed epidermis. Size bars are 200 μ m. dz: dehiscence zone, F: facet cells, l: locule, mvr: median valve ridge, lvr: lateral valve ridge, p: parenchyma at seed coat surface, R: ridge cells, rs: replum sclerenchyma, s: seed, sc: small sclerenchyma caps; scl: sclereids, sl: separation layer, t: testa.

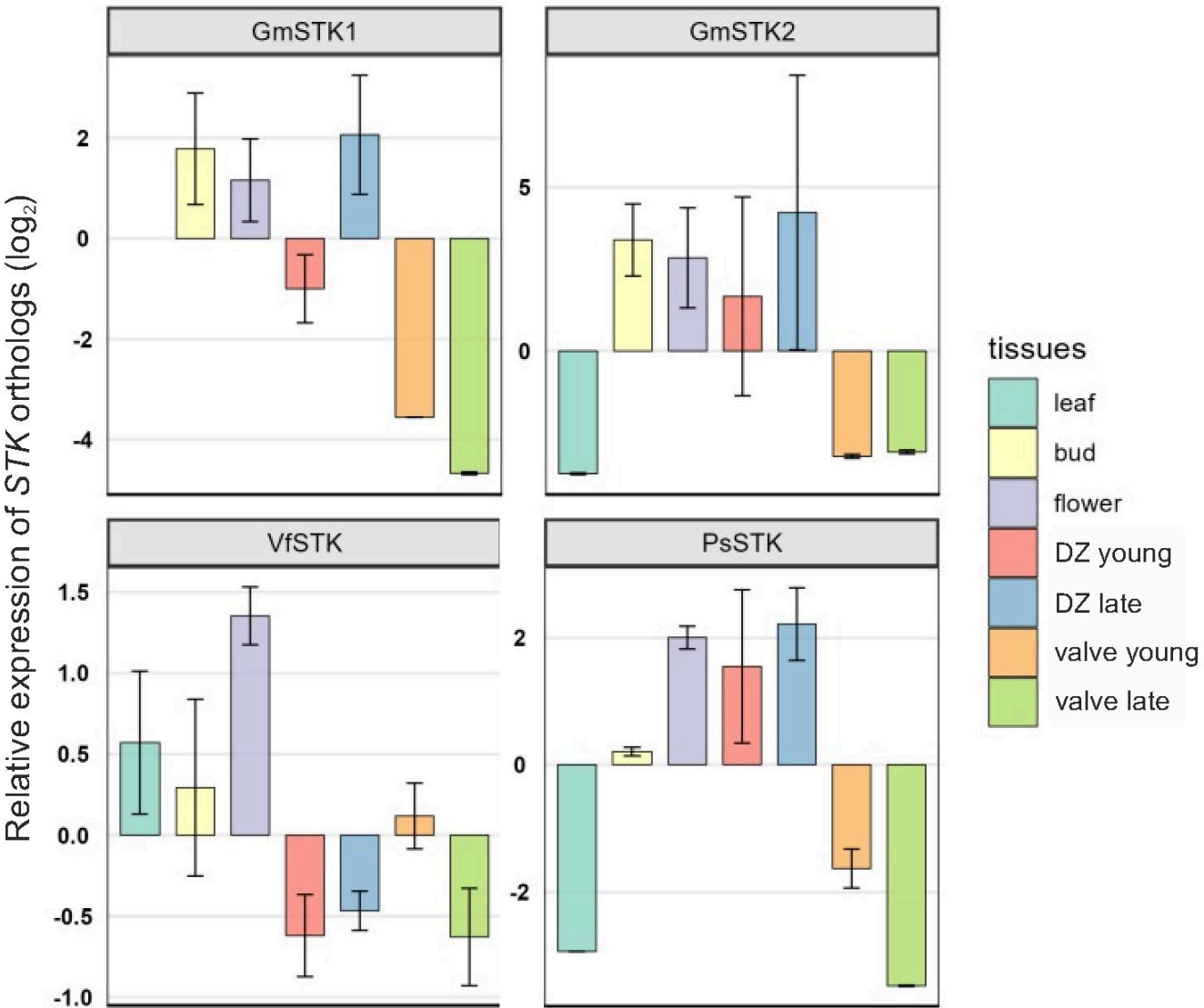


Figure 5

Expression of legume *STK* orthologs

qRT-PCR analysis of legume flowers at anthesis, leaves, buds, and fruit dehiscence zones and valves at two developmental stages. Log2 normalized expression relative to housekeeping genes are shown. On the top are two *STK* orthologs in *Glycine max*, *GmSTK1* and *GmSTK2*. The bottom are two orthologs of *STK* in *Vicia faba* and *Pisum sativum*, *VfSTK* and *PsSTK* respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Suppl.Fig.1Phylogenies1.pdf](#)
- [Suppl.Fig.2DehiscenceZoneDevelopment1.pdf](#)
- [Suppl.Fig.3heatmap.pdf](#)
- [Suppl.Fig.4PDS.tif](#)
- [Suppl.Fig.5histologyfruitwall.pdf](#)
- [SupplementalMaterial1KE.docx](#)
- [SupplementalTable1dominikTPMmatrix.csv](#)