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RESEARCH ARTICLE

Seed abscission and fruit dehiscence required for seed dispersal rely on similar genetic networks

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ABSTRACT

Seed dispersal is an essential trait that enables colonization of new favorable habitats, ensuring species survival. In plants with dehiscent fruits, such as *Arabidopsis*, seed dispersal depends on two processes: the separation of the fruit valves that protect the seeds (fruit dehiscence) and the detachment of the seeds from the funiculus connecting them to the mother plant (seed abscission). The key factors required to establish a proper lignin pattern for fruit dehiscence are SHATTERPROOF 1 and 2 (SHP1 and SHP2). Here, we demonstrate that the SHP-related gene *SEEDSTICK* (*STK*) is a key factor required to establish the proper lignin pattern in the seed abscission zone but in an opposite way. We show that STK acts as a repressor of lignin deposition in the seed abscission zone through the direct repression of *HECATE3*, whereas the SHP proteins promote lignin deposition in the valve margins by activating *INDEHISCENT*. The interaction of STK with the SEUSS co-repressor determines the difference in the way STK and SHP proteins control the lignification patterns. Despite this difference in the molecular control of lignification during seed abscission and fruit dehiscence, we show that the genetic networks regulating these two developmental pathways are highly conserved.

KEY WORDS: Lignin, *Arabidopsis*, Seed abscission, Fruit dehiscence, MADS-box genes

INTRODUCTION

The mechanisms by which plants disperse seeds have been widely described and are intimately linked to the type of fruit produced. For many plant species, seed dispersal entails the physical separation of the seed from the mother plant in a process named seed abscission. Abscission is a common developmental process that allows the separation of two tissues or organs in plants (Addicott, 1982; Lewis et al., 2006). As a developmental process, abscission requires the differentiation of one or few cell layers with thin cell walls (abscission or separation layer) that will degenerate at the end of the process, and an adjacent cell layer characterized by the presence of thick and lignified cell walls (lignified layer) that contributes to confer the mechanical force needed for the separation.

Despite the importance of this process for plant fitness, in seed crops such as cereals and brassica species, seed shattering is an unfavorable agricultural trait that causes important economic losses (Philbrook and Oplinger, 1989; Price et al., 1996). Therefore, in these crops, mutations that prevent shattering have been selected during their domestication (Arnaud et al., 2011; Konishi et al., 2006; Li et al., 2006; Lin et al., 2012; Yoon et al., 2014; Zhou et al., 2012).

In many species, seed dispersal is dependent on two separation events: fruit dehiscence and subsequent seed abscission. The molecular mechanisms controlling fruit dehiscence have been well characterized in *Arabidopsis thaliana*: after fertilization, the fruit elongates and differentiation of the valve margins between the valves and the replum takes place. The valve margin consists of two narrow cell strips: the separation layer and the lignified layer (Fig. 1A). Both layers contribute to fruit opening in a process known as dehiscence that is mediated by mechanical forces generated as the fruit dries (Spence et al., 1996). Separation takes place between the lignified layer and the adjacent non-lignified cells of the replum (separation layer), without (or with minimal) cell rupture (Ogawa et al., 2009; Roberts et al., 2002; Spence et al., 1996). This process leaves the mature seeds exposed, which allows them to easily fall from the mature plant to be dispersed by different vectors such as rain or wind (Spence et al., 1996).

Several genes have been described to have key regulatory roles in valve margin formation and therefore in fruit dehiscence. Two closely related MADS-box transcription factor-encoding genes, *SHATTERPROOF 1* (*SHP1*) and *SHP2*, redundantly control valve margin identity. In the double *shp1 shp2* mutant, the valve margin does not develop and the fruit becomes indehiscent (Fig. 1B). SHP proteins regulate the expression of three basic helix-loop-helix (bHLH) genes: *INDEHISCENT* (*IND*), *ALCATRAZ* (*ALC*) and *SPATULA* (*SPT*). *IND* has shown to be necessary for the specification of both the lignified and the separation layer of the valve margin, whereas *ALC* and *SPT* are only involved in the formation of the separation layer (Groszmann et al., 2011; Heisler et al., 2001; Liljegren et al., 2000, 2004; Rajani and Sundaresan, 2001) (Fig. 1A). IND activates the expression of *ALC* and *SPT*, and at the same time promotes its own heterodimerization with them through DELLA protein degradation (Arnaud et al., 2010; Girin et al., 2011). Finally, ALC and SPT are able to repress *IND* expression (Lenser and Theissen, 2013). This complex regulation allows the proper establishment and differentiation of the valve margin tissues to ensure proper fruit dehiscence.

While the molecular and genetic network involved in silique shattering has been described, little is known about the factors controlling seed abscission. The *seedstick* (*stk*) mutant was characterized in *Arabidopsis* showing a defect seed abscission (Pinyopich et al., 2003). This mutant presents a thicker and longer funiculus, with seeds that do not fall from the mature silique (Fig. 1B). Interestingly, *SHP1*, *SHP2* and *STK* are closely related, and functional redundancy has been demonstrated regarding the determination of ovule identity (Favaro et al., 2003; Pinyopich et al.,

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2003). Moreover, the three genes share similar expression patterns in ovule development, whereas in the silique valve margin tissue, only the two SHP genes are expressed (Ferrándiz et al., 2000; Mizzotti et al., 2012, 2014; Pinyopich et al., 2003).

Recently it has been suggested that *HECATE 3* (*HEC3*) may also play a role in seed abscission, as seeds of *hec3* mutants do not separate from the funiculus (Gremski et al., 2007; Ogawa et al., 2009). HEC3 is a bHLH transcription factor like IND. Interestingly, both HEC3 and IND interact with SPT and ALC (Girin et al., 2011; Gremski et al., 2007; Groszmann et al., 2011) and share several downstream targets, for instance, the two polygalacturonases (PGs) *ADPG1* and *ADPG2*, which participate in cell separation mechanisms (Ogawa et al., 2009).

This work describes for the first time the lignification pattern of the funiculus-seed junction, which is required for seed abscission. We demonstrate the role of STK and HEC in the molecular control of this process, showing that ectopic lignin accumulation as well as the absence of lignification disrupt seed abscission. Furthermore, we show that several of the transcription factors involved in fruit dehiscence also participate in the regulation of seed abscission, indicating that conserved genetic and molecular mechanisms regulate the two processes that ultimately control seed dispersal.

RESULTS

STK regulates lignification patterning in the seed abscission zone

It has been reported that *shp1 shp2* and *stk* mutants are impaired in separation processes, being defective in fruit dehiscence and seed abscission, respectively (Liljegren et al., 2000; Pinyopich et al., 2003) (Fig. 1B). SHP1 and SHP2 participate in the establishment and differentiation of the valve margin tissue, conferring a characteristic lignification pattern necessary for valve opening. In *shp1 shp2* mutants no lignin deposition is observed in the valve margin, correlating with an indehiscent phenotype (Liljegren et al., 2000). By contrast, the role of lignin and the mechanisms by which *STK* controls seed detachment are unknown. Therefore, we decided to perform a detailed analysis of the wild-type abscission zone at the end of stage 17B (according to Smyth et al., 1990). At this stage, lignin was detected in the vascular bundle of the funiculus, in the seed coat close to the funiculus (hilum) and in a delimited region of the seed coat over the embryo radicle that we named the 'spur' (Fig. 2A,B). We also observed a monolayer ring of lignified cells surrounding the vascular bundle at the edge of the funiculus, close to the seed coat (Fig. 2B,C).

To investigate if *stk* could modify the lignification pattern in the seed abscission zone (SAZ) we characterized lignin deposition in the SAZ in the wild type compared with the *stk* mutant at different stages of fruit development. When ovules are fertilized and fruit starts growth (stage 13-14), lignin depositions are not observed at the SAZ of the wild type or the *stk* mutant, except for the vasculature in the funiculus (Fig. 2D,G). At stages 16-17A, in the wild type, lignin was only detected in the vasculature (Fig. 2E), whereas in the *stk* mutant, lignification was also observed in the cells surrounding the vasculature in the region that will develop the SAZ (Fig. 2H). At stage 17B, the lignification ring/layer colocalizes with the abscission zone, delimiting the breaking point that allows seed separation (Fig. 2J). In contrast to the wild type, the *stk* mutant showed ectopic lignification of the cells surrounding the vascular bundle of the funiculus (Fig. 2H,I,K) instead of a single layered ring (Fig. 2F,J,L).

To quantify this phenotype, we measured the lignified area in the SAZ of 10 images each for the wild type and *stk* mutant (Fig. 2L).

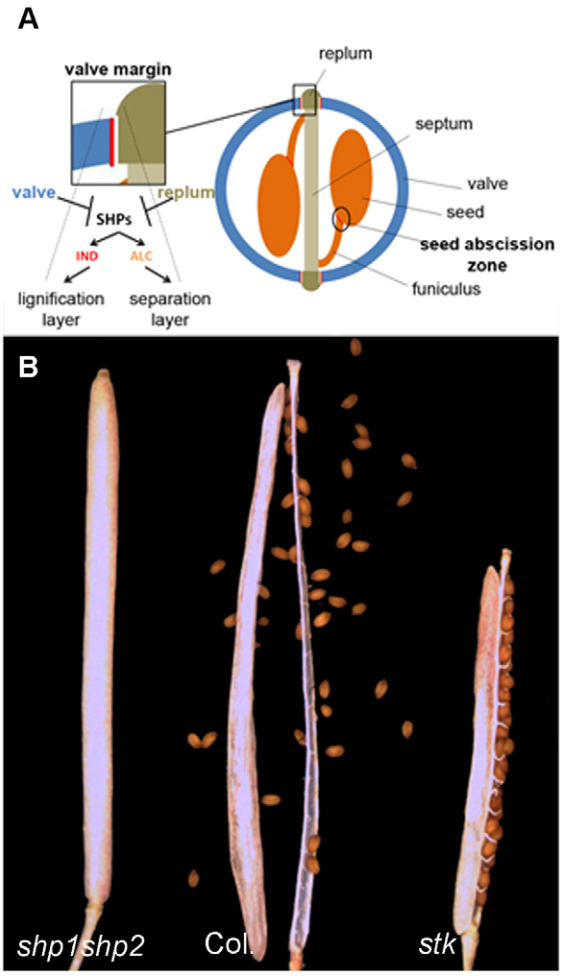

Fig. 1. *Arabidopsis* wild-type and mutant fruit phenotypes. (A) Schematic representation of a transversal section of an *Arabidopsis* fruit. Valve margin and SAZ are indicated, as well as the simplified genetic regulatory network that controls fruit dehiscence. (B) Separation phenotypes of wild-type Columbia (*Col-0*), *stk* single mutant and *shp1 shp2* double mutant fruits. *Col-0* mature fruits show valve separation (fruit dehiscence) and seed abscission (center), whereas the *shp1 shp2* double mutant is not able to dehisce (left) and the *stk* mutant cannot abscise the seeds (right).

This analysis clearly reflected a more highly lignified SAZ in the *stk* mutant with respect to wild-type plants (*stk*, 1580.62±245.56 μm^2 versus WT, 523.49±75.58 μm^2; $P<0.05$, Student's *t*-test). Our results suggest that the altered lignification pattern of the *stk* mutant produces an abscission zone that is more resistant to mechanical forces, explaining the lack of seed abscission observed in the *stk* mutant.

HEC3, a direct target of STK, mediates the ectopic lignification observed in *stk*

The formation of the lignification layer at the valve margin depends mainly on *IND*, a downstream target of the SHP proteins (Liljegren et al., 2004). We therefore investigated if *IND* was also involved in the establishment of the lignified ring in the SAZ. The *ind-2* mutant did not show any defect in the SAZ and the *IND::GUS* reporter line showed no activity in the funiculus or SAZ (Fig. S1A-C). Interestingly, it has been proposed that *HEC3*, a gene closely related to *IND* and involved in transmitting tract development (Gremski et al., 2007), could be also defective in seed abscission (Ogawa et al., 2009). Therefore, we characterized the lignification

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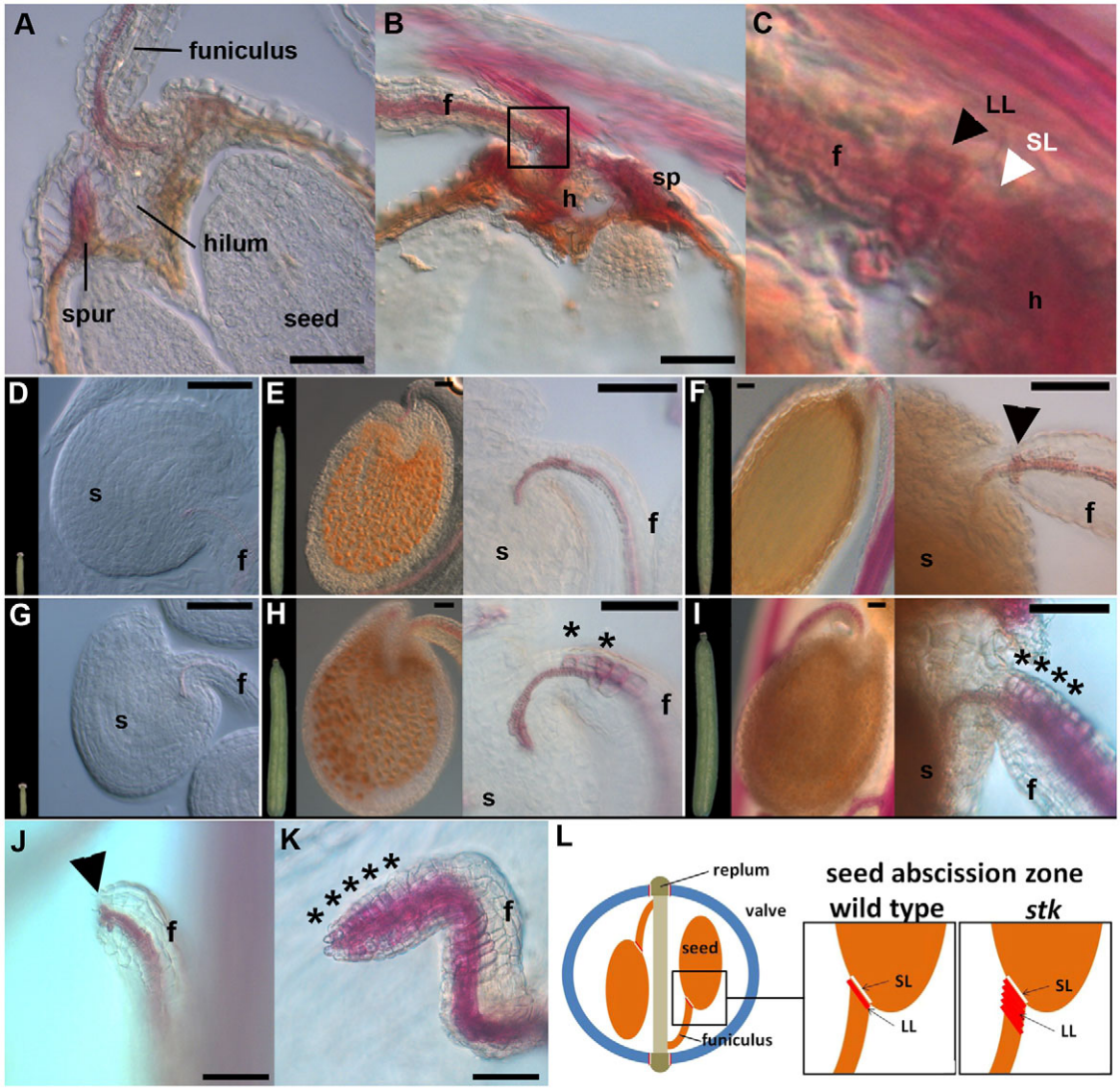

Fig. 2. Lignification pattern in wild-type and *stk* mutant plants. Fruits stained with phloroglucinol; magenta staining indicates lignin deposition. (A) Section of the apical side of a wild-type seed from a stage 18 fruit. Lignin is detected at the vasculature, the center of the funiculus, the hilum and in the spur. (B) Section of the apical side of a wild-type seed from a stage 18 fruit showing a lignified cell ring where the funiculus reaches the hilum. (C) Magnification of the boxed region in B. (D-I) Time course of the SAZ lignification in wild-type (D-F) and *stk* plants (G-I). For each panel, a picture of the fruit at the corresponding stage of development (left), and a detail of the position of the SAZ (right) are shown. For late 16 and 17B stages, a picture of the developing seed is also shown (center). At stage 13, lignin is weakly detected on the vasculature of the funiculus (D,G). At late stage 16, the vasculature of the funiculus in the wild type is completely lignified (E). At the same stage, the *stk* mutant (H) starts to accumulate ectopic lignification around the vasculature in the junction between the seed and the funiculus, where the SAZ will develop. At stage 17B, the SAZ is evident in the wild-type plants (F), with the formation of a clear lignification ring at the edge of the funiculus. At the same stage, *stk* presents a completely lignified SAZ (I). (J) The lignified layer at the SAZ in a wild-type funiculus after seed abscission. (K) The extensive lignification at the SAZ of the *stk* mutant (seed abscised manually). (L) Schematic representation of the phenotypes observed in the SAZ of wild-type and *stk* plants. Sections are 10 µm thick in A and 20 µm in B,C. Black arrowhead indicates the lignified ring/lignification layer and white arrowhead indicates the position of the separation layer. Asterisks indicate ectopic lignification. Images in D-K show whole-mount staining. Scale bars: 50 µm. f, funiculus; h, hilum; LL, lignification layer; SL, separation layer; s, seed; sp, spur.

pattern in this mutant and observed that the lignified ring at the SAZ was absent in the *hec3-1* mutant (Fig. 3A,B), correlating with a smaller lignified area with respect to the wild type (*hec3*, 328.61±71.02 µm^2 versus WT, 523.49±75.58 µm^2; n=10; P<0.05, Student's t-test) and the absence of seed abscission (Fig. 3E). This result suggests that, like IND in the valve margin, HEC3 is a regulator for establishing the lignification pattern in the SAZ and it is a good candidate to act downstream of STK.

Thus, to assess if *HEC3* acts downstream of STK we generated the *stk hec3* double mutant and analyzed the lignification pattern at the SAZ. The *stk hec3* double mutant showed no seed abscission (Fig. 3E) and a lignified area in the SAZ similar to the *hec3* single mutant (*stk hec3*, 374.06±83.00 µm^2 versus *hec3*, 328.61±71.02 µm^2; n=10; P<0.05, Student's t-test). Like the *hec3* mutant, the double *stk hec3* mutant did not develop any lignified ring, completely suppressing the ectopic lignification observed in the *stk* single mutant (Fig. 3B-D). The fact that *hec3* was epistatic to *stk* suggests that the SAZ phenotype observed in *stk* depends largely on the activity of HEC3. On the other hand, the ectopic lignification of the funiculus, as observed in the *stk* mutant, was still present in the *stk hec3* double mutant, suggesting that *HEC3* plays a specific role in the determination of the SAZ (Fig. S1D-G).

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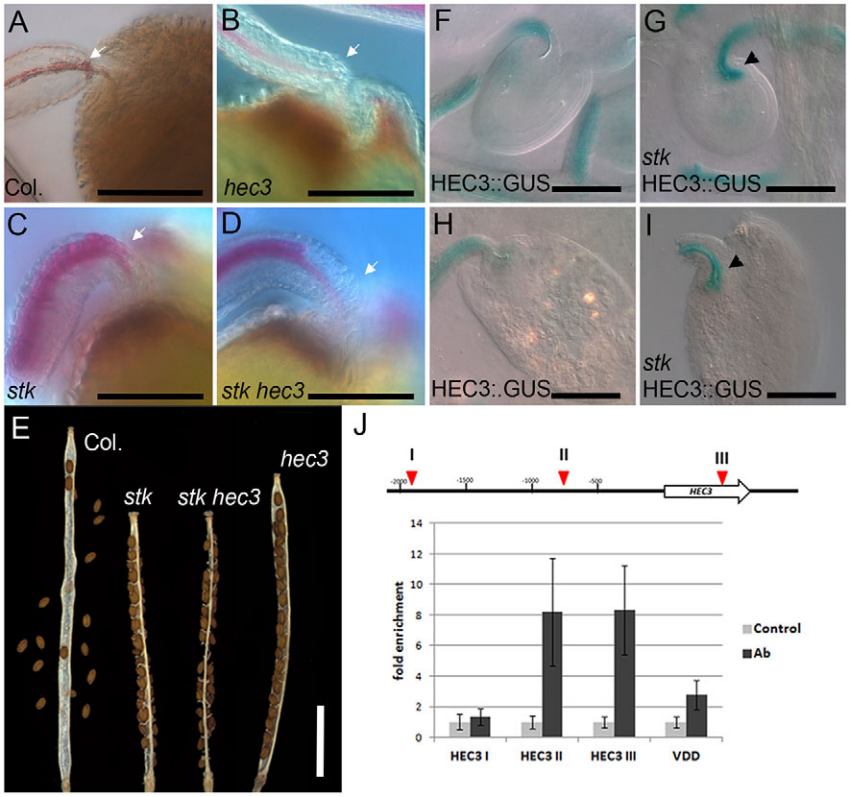

Fig. 3. *STK* acts upstream of *HEC3*. (A-D) Cleared stage 17 fruits stained with phloroglucinol; magenta staining indicates lignin deposition. Black arrowheads indicate the expected position of the lignified ring. (A) Wild-type SAZ showing the lignified ring at the end of the funiculus. (B) *hec3* mutant SAZ. No lignified ring was observed. (C) *stk* mutant SAZ showing ectopic lignification at the end of the funiculus. (D) *stk hec3* mutant SAZ. Although ectopic lignification of vasculature tissue was detected, neither ectopic lignification nor a lignified ring was observed at the SAZ. (E) Seed abscission phenotypes of wild-type, *stk*, *stk hec3* and *hec3* plants. (F-I) *HEC3::GUS* activity analysis in the SAZ of fruits at stage 15 (F,G) and 16 (H,I). (F,H) Signal detected in wild-type; (G,I) signal detected in the funiculus and ectopically in the hilum region (black arrows) in the *stk* mutant. (J) (Top) Schematic diagram of the *HEC3* genomic region. The *HEC3* gene is represented by a white arrow, while the upstream and downstream genomic regions are represented by a black line. The red arrowheads indicate the regions amplified in the ChIP analysis, identified by roman numbers. (Bottom) ChIP enrichment tests showing the binding of STK-GFP to the HEC3 II and HEC3 III regions and to a region on the *VERDANDI* (*VDD*) promoter used as positive control (Matias-Hernandez et al., 2010). Bars represent the ratio of amplified DNA (*STK:GFP*/*Col.*) in the INPUT starting genomic DNA (Control) or in the immunoprecipitated DNA with the GFP antibody (Ab). Error bars represent the propagated error value using three replicates. Scale bars:100 µm (A-I), 2 mm (B).

To clarify whether *HEC3* was acting downstream of STK, we analyzed the expression pattern of the *HEC3::GUS* reporter line in the *stk* mutant background. As described previously (Gremski et al., 2007), in the wild-type background, GUS activity was clearly detected in the transmitting tract and in the funiculus. In the *stk* mutant, in contrast to wild-type plants, ectopic GUS activity was detected in the region where the funiculus attaches the seed (Fig. 3F-I).

As several MADS-box binding sites (CArG-boxes) are present in the *HEC3* genomic region, we decided to perform chromatin immunoprecipitation (ChIP) using the *pSTK:STK:GFP* line (Mizzotti et al., 2014). We found that STK was able to bind two CArG-boxes in the *HEC3* genomic region, one at 763 bp upstream of the ATG, and another inside the gene coding sequence at 475 bp downstream of the ATG (Fig. 3J). This result strongly suggests that STK directly regulates *HEC3*, probably by acting as a repressor during SAZ formation to ensure the correct lignification pattern and thereby facilitating seed abscission.

ALC and *SPT* participate in seed abscission, but do not affect the lignification pattern

Since *ALC* (Rajani and Sundaresan, 2001) and *SPT* (Heisler et al., 2001) play important roles in the determination of the valve dehiscence zone (Girin et al., 2011; Groszmann et al., 2011), we investigated if they also have a function in seed abscission. We analyzed two independent *ALC* mutant alleles: *alc-1* (Rajani and Sundaresan, 2001) and *alc-10* (T-DNA insertion mutant). Both alleles showed a normal lignification ring (Fig. S2B,G) and apparently a normal seed abscission phenotype. However, a detailed analysis of the SAZ revealed that the separation of the seed from the funiculus was different in the *alc* mutant compared with the wild type. Some of the collected seeds from *alc* plants retained a small part of the funiculus attached to the seed (Fig. S1H). Further analysis of the funiculus after seed detachment revealed three different phenotypes in *alc*. First, several layers of cells remained attached to the lignification ring at the edge of the funiculus (Fig. 4B); second, the separation occurred in the funiculus, before the lignification ring (Fig. 4C); and last, the separation was as in wild-type plants (Fig. 4A,D) (35.6%, 42.1% and 22.3%, respectively; n=233). These results indicated that separation occurs by the unspecific rupture of the SAZ, suggesting that ALC could participate in the differentiation of the SAZ, and as in the valve margin, could be essential for the specification of the separation layer.

The *alc-1* mutant contains a GUS insertion in the coding region (Sundaresan et al., 1995). The analysis of the GUS activity in *alc-1*/+ plants confirmed the expression in the funiculus and SAZ (Fig. 4F). As the SHP genes control *ALC* expression in the valve margin, we decided to check if *ALC* expression was controlled by STK. We crossed *alc-1* and *stk* single mutants to analyze both the double mutant phenotype as well as the expression pattern of *ALC* in the *stk alc-1*/+ plants. The GUS signal was detected along the funiculus in a similar pattern in both the wild type and the *stk* mutant (Fig. 4F,G). Analysis of the lignification pattern of the SAZ of the double mutant *stk alc-1* showed no variation from the described *stk* pattern (Fig. S2A,B,D,F,G and I) and consequently, seeds of the double mutant showed the same phenotype as the *stk* mutant.

In valve margin specification, *SPT* is required for the proper development of both the separation and the lignification layer (Girin et al., 2011). To assess possible roles for SPT in the differentiation of the SAZ, we analyzed a *SPT::GUS* reporter line. In agreement with the previously described expression pattern of *SPT* (Groszmann et al., 2010), we detected GUS activity in the funiculus during ovule development (Fig. 4H) that was specifically restricted to the SAZ at stage 17 (Fig. 4I).

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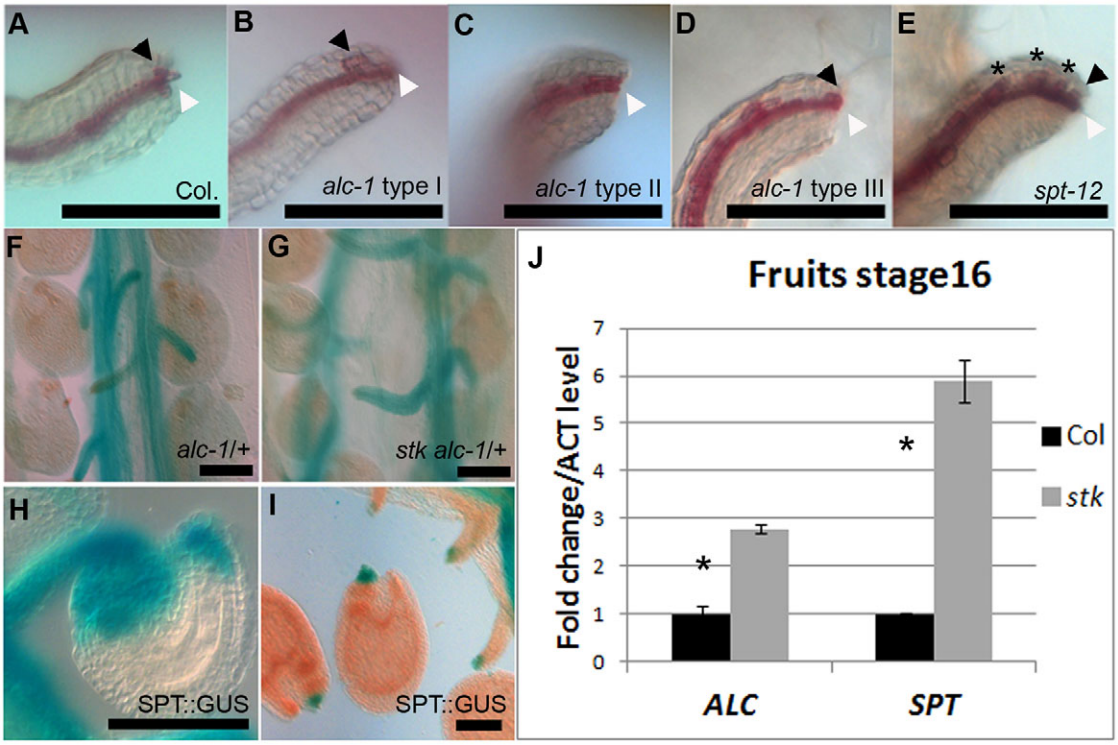

Fig. 4. *ALC* and *STP* act downstream of *STK*. Magenta staining indicates lignin deposition. (A-E) Funiculi after seed abscission. Separation points and lignification layers are indicated by white and black arrowheads, respectively. (A) *Col-0*. (B-D) The three different phenotypes observed in *alc-1*: separation several cell layers below the lignified ring in the funiculus (B); separation above the lignified ring (C) and separation at the lignified ring as in the wild type (D) and *spt-12* mutant (E). Asterisks indicate the ectopic lignification points observed in this mutant. (F) GUS signal in *alc-1*/+ plants is detected in the entire funiculus at stage 17 fruits. (G) GUS signal in *stk alc-1*/+ plants showed no changes in expression pattern with respect to the *alc-1*/+ controls. (H) *SPT::GUS* activity at anthesis is detected in the entire funiculus, the chalazal and apical regions of the ovule. (I) *SPT::GUS* activity at stage 17 fruit seeds was restricted to the SAZ. (J) qRT-PCR expression analysis of *ALC* and *SPT* in the wild-type and *stk* mutant backgrounds. The mean of three independent biological replicates is shown. Error bars represent s.d. **P*<0.05 (Student's *t*-test). Scale bars: 100 µm.

The characterization of the *spt-12* mutant showed no differences at the SAZ with respect to the wild type (Fig. S2C,H). The seeds do not have abscission defects; however, ectopic lignified areas were found along the funiculus, close to the vascular bundle (Fig. 4A,E), suggesting that *SPT* might be involved in the control of lignin deposition in the funiculus.

As both *ALC* and *SPT* genes seem to affect seed abscission and lignin deposition, respectively, we analyzed their expression levels in the *stk* background by qRT-PCR using fruits at stage 16 (Smyth et al., 1990), in order to see if the *stk* phenotype could be explained, at least in part, by changes in their expression levels. We found that both *SPT* and *ALC* were upregulated in the *stk* mutant (Fig. 4J), suggesting that both genes might act downstream of STK. The *stk spt-12* double mutant showed the same ectopic lignification observed in the *stk* single mutant (Fig. S2A,C,E,F,H,J). This result also suggests that the ectopic lignification observed in *stk* might be independent of *SPT*.

STK regulates lignification of the seed abscission zone by interacting with SEU

Our results indicate that *STK* is a master regulator of seed shattering by controlling the lignification pattern in the SAZ similar to the function of the SHP genes in the dehiscence zone. However, *STK* and SHP genes seem to have opposite roles in the lignification of the two tissues: STK represses lignification, whereas the SHP proteins promote lignification. A possible explanation for this divergence might be that the closely related SHPs and STK factors interact with different proteins in the different tissues, and thereby modify their activities. In this way, SHPs could be working as transcriptional activators in the valve margin while STK as a repressor in the SAZ. To further investigate this possibility, we focused on two known co-repressors that interact with MADS-domain transcription factors: SEUSS (SEU) and LEUNIG (LUG) (Gonzalez et al., 2007; Gregis et al., 2006; Sridhar et al., 2004, 2006). We analyzed the lignification pattern of the *seu* and *lug* single mutants, as well as the *seu lug*/+ double mutant (as the homozygous double mutant is sterile and never produces seeds) in the SAZ. The *seu* and the *seu lug*/+ mutants, like the *stk* mutant, presented an ectopically lignified SAZ (Fig. 5A,B,D), while the *lug* single mutant showed a normal SAZ phenotype (Fig. 5A,C). Interestingly, no defects were observed regarding the funiculus lignification in the *seu* and *lug* single and double mutants, suggesting a specific role for them in the differentiation of the SAZ. In agreement with this, the *seu* mutant showed a larger lignified area in the SAZ than the wild-type plants (*seu*, 1001.06±199.37 µm^2 versus WT, 565.05±82.00 µm^2; n=10; P<0.05, Student's *t*-test) and presented a pronounced but milder resistance to seed abscission with respect to *stk* (Fig. 5E).

The similarity between *seu* and *stk* phenotypes suggested a possible interaction between those factors. To investigate whether STK and SEU interact, we performed a bimolecular fluorescence complementation (BiFC) experiment. No fluorescence complementation was observed, although complementation occurred in all the positive controls (Fig. 5F; Fig. S3A). However, since MADS-domain transcription factors bind DNA as dimers, we

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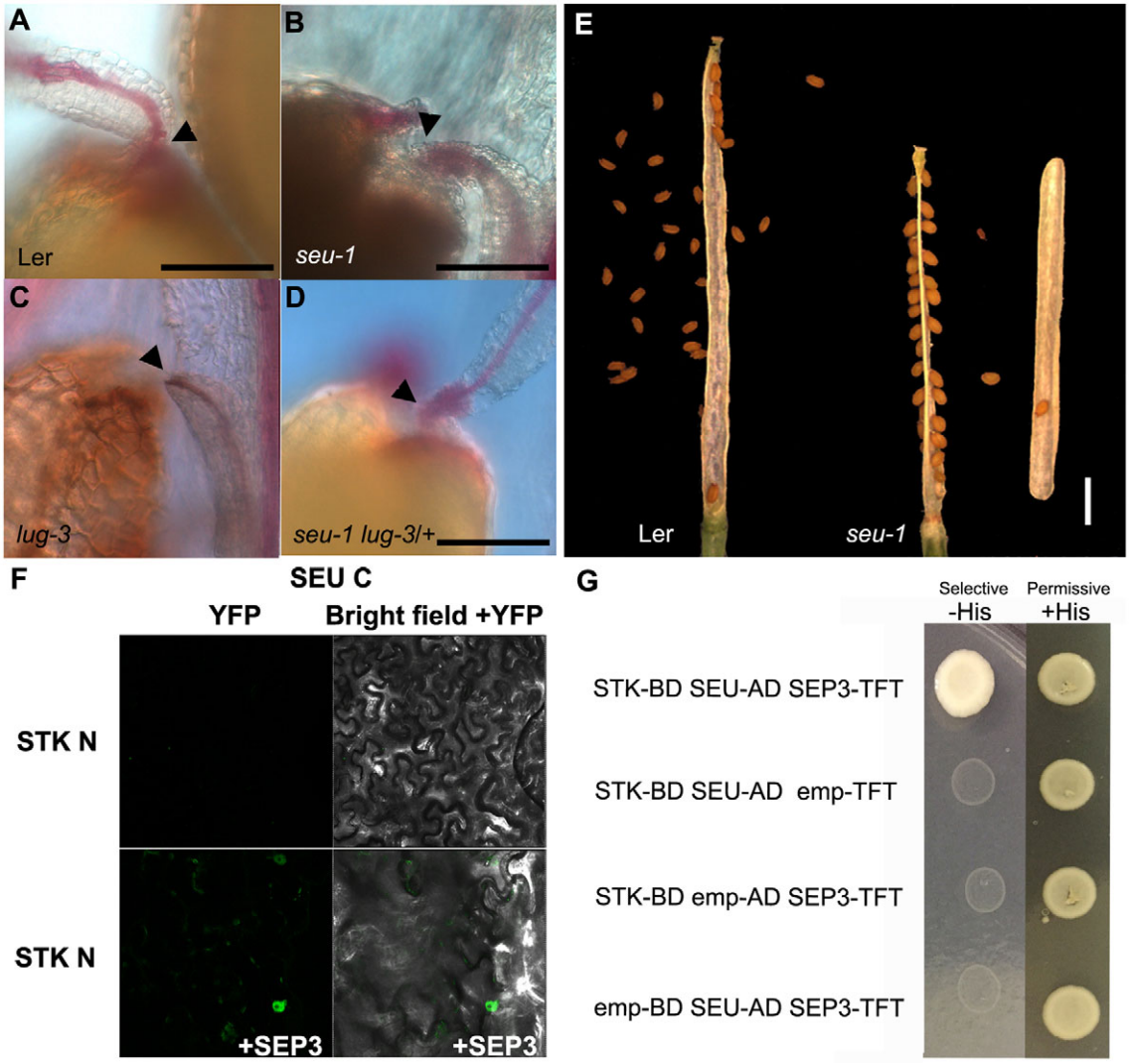

Fig. 5. *SEU* participates in the control of seed abscission zone development together with STK. (A) Wild-type Landsberg *erecta* (L*er*) SAZ showing the lignified ring at the end of the funiculus. (B) *seu-1* mutant SAZ showing ectopic lignification at the end of the funiculus. (C) *lug-3* mutant showing a normal SAZ. (D) *seu-1 lug-3*/+ mutant SAZ showing a strong ectopic lignification at the end of the funiculus. Arrows indicate separation points. (E) Comparison of the seed abscission phenotype between wild-type L*er* and *seu-1* mutant. The *seu-1* mutant shows seeds resistant to abscission. (F) BiFC experiment showing the interaction of STK with SEU. The interaction is mediated by the formation of a MADS-box dimer as the YFP signal only is detected in the presence of SEP3, a known interactor of STK. STK N, STK phusion with the N-terminal part of the split YFP; SEU C, SEU phusion with the C-terminal part of the split YFP. (G) Yeast three-hybrid assay showing STK-SEP3-SEU interaction. Yeast strains were grown on either selective (without tryptophan, leucine, uracil and histidine) or permissive (without tryptophan, leucine, uracil) medium. Emp-AD, empty vector containing the GAL4 activation domain; emp-BD, empty vector containing the GAL4 binding domain; emp-TFT, empty pTFT. Scale bars: 100 µm (A-D), 1 mm (E).

hypothesized that STK might only interact with SEU as a MADS-domain dimer. STK is not able to form homodimers (de Folter et al., 2005), but it forms heterodimers with the MADS-domain protein SEPALLATA 3 (SEP3) to exert its function during ovule development. Thus, we decided to test the interaction between STK and SEU in the presence of SEP3, expressed in its native form. Under these conditions, fluorescence complementation was observed (Fig. 5F), indicating that STK can interact physically with SEU when it forms a heterodimer with SEP3. This result was also validated by yeast three-hybrid experiments (Fig. 5G).

STK and SHP gene expression profiles determine their sub-functionalization in the lignification processes

Our analyses indicate that STK acts in the abscission zone as a repressor of the lignification pathway by forming a complex with SEU, suggesting that its transcriptional repression activity might be due to the interaction with SEU. Since STK and SHP proteins are closely related it might well be that their difference in function in the fruit dehiscence and SAZ is only dependent on the proteins with which they may interact locally. *SEU* is not expressed in the valve margin (Bao et al., 2010). To test this, we introduced the *pSHP2::STK* construct in the *shp1 shp2* double mutant and tested if STK was able to complement the indehiscence phenotype. As a control, the *pSHP2::SHP2* construct was used. The obtained results were similar for both constructs (Fig. 6A-C): 65% of the plants (13 out of 20) transformed with the control line (*pSHP2::SHP2*) complemented the fruit indehiscence phenotype, while with the *pSHP2::STK* construct, we observed that the phenotype was complemented in 55% of the plants (11 out of 20). These results indicate that SHP and STK are functionally exchangeable, since *STK* was able to play the same role as *SHP*s when expressed at the valve margin tissue.

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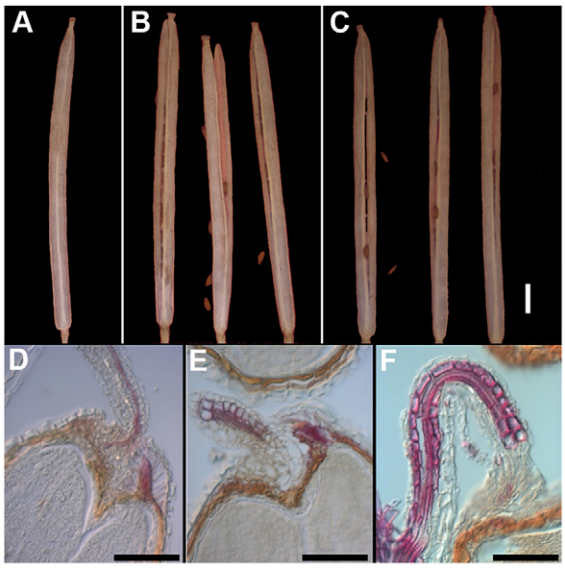

Fig. 6. STK and SHP proteins work identically. (A) Indehiscent phenotype of a *shp1 shp2* mutant fruit. (B) *shp1 shp2* mutant fruits complemented with the *pSHP2:SHP2* construct. (C) *shp1 shp2* mutant fruits complemented with the *pSHP2:STK* construct. (D-F) Phloroglucinol staining on 10 µm thick longitudinal sections of stage 17 fruits. Magenta staining indicates lignin deposition. (D) Wild-type seed showing the vasculature lignification along the funiculus. (E) *stk* mutant seed showing ectopic lignification along the funiculus. (F) *stk shp1 shp2* mutant seed showing stronger lignification along the funiculus than *stk*. Scale bars: 1 mm (C, for A-C), 100 µm (D-F).

Supporting the hypothesis that STK and SHPs have redundant functions when expressed in the same tissues, we observed that the funiculus (where the three genes are normally co-expressed) of the *stk shp1 shp2* triple mutant presented an enhanced lignin accumulation phenotype (Fig. 6F) when compared with the *stk* single mutant (Fig. 6E), and the wild type (Fig. 6D). This suggests that in the funiculus the SHPs act, like STK, as inhibitors of the lignification pathway, which is the opposite to what they do in the valve margin. In fact, the SHP2 protein was, like STK, also able to interact with SEU (Fig. S3).

DISCUSSION

Seed abscission requires a specific lignification pattern

Controlled lignin deposition plays important roles in plant separation processes (Roberts, 2000; Roberts et al., 2002). Besides fruit dehiscence in *Arabidopsis* (Liljegren et al., 2000, 2004), examples in other species have also been described: leaf abscission in citrus (Agustí et al., 2008), fruit and flower abscission in tomato (Iwai et al., 2013; Nakano et al., 2012) and seed shattering in rice (Yoon et al., 2014). All the separation processes have in common the formation of a lignified layer close to a non-lignified layer of cells that define the separation or abscission zone. Both ectopic accumulation and absence of lignification disrupt the separation process as we have shown in the *stk* and *hec3* mutants (Fig. 3E).

In *Arabidopsis*, lignification patterns of the valve margin and SAZ are similar, but not identical. The lignified layer of the valve margin has continuity with the endocarp layer b (end b) of the valve and it is also lignified. At the same time, the vascular region of the replum is also connected to the end b by some small cells with thin lignified walls, just below the separation layer. The rupture of these small cells, as a continuation of the separation layer, allows valve separation. At the SAZ, the lignified layer is formed around the vascular bundle of the funiculus. This vascular bundle continues deep inside the hilum region of the seed. The separation point in the SAZ, the separation layer, is the cell layer adjacent to the lignified layer, but no differences in the lignification of the vascular bundle were observed in this position. This observation might indicate that seed detachment needs the mechanical rupture of this vascular bundle.

We have also identified another lignified structure – the spur. Its proximity to the lignified layer suggests that the last contact between these two lignified structures could provide, together with the force of the wind or other external factors, the mechanical force needed to break the vascular bundle at the weaker region of the funiculus, allowing seed abscission.

Fruit dehiscence and seed abscission share similar control mechanisms

The presence of a similar lignification pattern in different tissues of the plant (Liljegren et al., 2004; this work) and even in unrelated species (Agustí et al., 2008; Iwai et al., 2013; Yoon et al., 2014) strongly suggests that similar genetic networks could control different kinds of separation processes. We show that this is the case, at least in *Arabidopsis*. Our study reveals that fruit dehiscence and seed abscission mechanisms are highly conserved, with the SHPs and STK factors acting as key regulators. Downstream genes that are controlled by these MADS-domain factors, such as *SPT*, *ALC*, *IND* and *HEC3* are also conserved, and their mutants present similar defects both at the valve margin and the SAZ (Fig. 7).

We demonstrate that the role exerted by *IND* in the valve margin is adopted by its closely related gene *HEC3* in the SAZ. In both mutants, the lignification layer is absent in the valve margin (*ind*) or the SAZ (*stk*). These results are in agreement with previous publications showing that both factors are able to interact with the same proteins

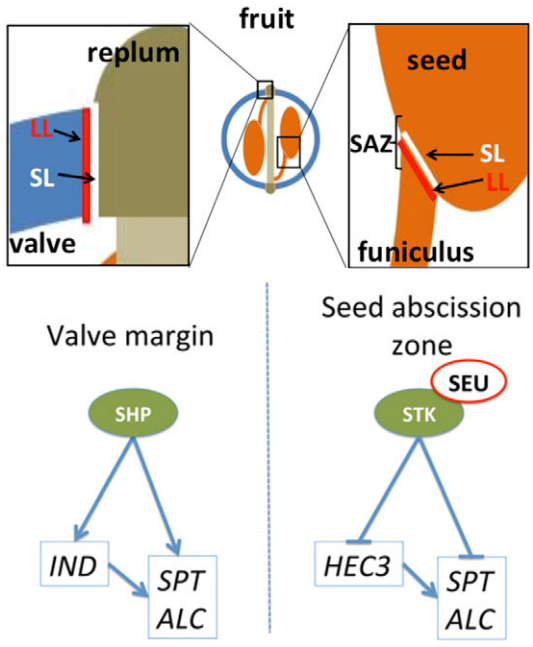

Fig. 7. Mechanistic models for the establishment of the valve margin and the SAZ. The valve margin and SAZ development are controlled by the redundant MADS-box factors SHP and STK, respectively. These proteins control the expression of identical or similar genes – *IND*/*HEC3*, *SPT* and *ALC* – that trigger the final differentiation of the tissue, but in opposite ways, working as activators in the valve margin and as repressors in the SAZ. The repressor activity in the funiculus depends on the specific interaction with the SEU co-repressor factor. LL, lignification layer; SL, separation layer.

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and regulate the same downstream targets (Arnaud et al., 2010; Girin et al., 2011; Gremski et al., 2007; Groszmann et al., 2011; Kay et al., 2013; Ogawa et al., 2009). We also show that, as in the valve margin, *spt* and *alc* single mutants do not show changes in the lignification pattern of the SAZ (Girin et al., 2011; Rajani and Sundaresan, 2001) but they are affected in seed abscission, probably as a result of defects in the specification of the separation layer. This is more evident for the *alc* mutant: these plants produce indehiscent fruits because of the absence of the separation layer. In the SAZ, this layer could also be affected. In *alc* mutants most of the seeds abscised the fruit by the rupture of the funiculus outside the separation layer, suggesting a failure in the specification of this layer. In the case of *spt* mutants, fruit dehiscence and seed abscission are not severely affected. Interestingly, the *SPT* expression pattern showed similarities between the fruit and the funiculus/seed, being widely expressed at early developmental stages and becoming specifically restricted to the separation zone at later steps of development. To better assess the role of SPT and ALC in the SAZ, it would be interesting to analyze the phenotype of this region in the double *spt alc* mutant, because recently, a partial redundancy between these genes has been described (Groszmann et al., 2011).

While in the valve margin SHPs have been described to activate the expression of *IND/HEC3*, *ALC* and *SPT*, we have found that those genes are repressed by STK in the SAZ. We propose that this change in activity (activation versus repression) is exerted by the interaction of STK with the transcriptional co-repressor SEU (Fig. 7). It has been shown that *SEU* is expressed in the ovule and funiculus, but not in the valve margin (Bao et al., 2010). This specific expression pattern might explain why *STK* is able to rescue the *shp1 shp2* indehiscent phenotype when expressed in the valve margin. In the absence of SEU, STK could function as an activator of the downstream targets recovering valve margin identity. In agreement with this, we have found that the *seu* mutant has seed abscission defects, which could be related to the ectopically lignified SAZ observed, indicating that repression of specific targets is needed to establish this zone correctly.

Our work shows that *LUG* is probably not involved in the regulation of STK targets in the SAZ, but like *SEU*, *LUG* expression is predominantly detected in ovule and funiculus in mature flowers and not in the valve margin (Conner and Liu, 2000). It has been shown that SEU usually works together with LUG, but also with LEUNIG HOMOLOG (LUH), a partially redundant *LUG* gene (Franks et al., 2002, 2006; Gregis et al., 2006; Grigorova et al., 2011; Sitaraman et al., 2008; Sridhar et al., 2004). It will be interesting to study the role of LUH in this process, as well as its interaction with LUG in order to investigate a possible redundancy between them.

Fruit dehiscence and seed abscission may have evolved from a unique ancient separation mechanism

The AG clade in *Arabidopsis*, composed of *STK*, *AG*, *SHP1* and *SHP2* (Parenicova et al., 2003), has been proposed to originate from the duplication of a common ancestor that produced the *AG* and *STK* lineages, and after this, a second duplication event in the *AG* lineage gave rise to the actual *AG* and SHP lineages (Kramer et al., 2004; Moore and Purugganan, 2005; Parenicova et al., 2003). Despite the functional redundancy described for this group of genes, the role of *SHP1*/*2* in valve margin differentiation (Pinyopich et al., 2003) has been proposed to be an example of neo-functionalization (Airoldi and Davies, 2012; Moore and Purugganan, 2005). However, we suggest another possibility: our work indicates that STK controls seed abscission through the direct regulation of *HEC3*, which finally controls lignin deposition. This mechanism is very similar to the mechanism accepted for valve margin establishment and fruit dehiscence where SHPs control the expression of *IND*, which finally controls lignin deposition. Together, this parallelism and the results shown here indicate that SHP and STK still conserve a function that may be present in the common ancestor of the AG clade. As fruit dehiscence is a recent adaptive trait specific to Brassicaceae (Eames and Wilson, 1930), we suggest that the mechanism controlling fruit dehiscence may have evolved from a previously established mechanism controlling seed abscission. Supporting this, *IND*, a gene only present in the Brassicaceae (Kay et al., 2013; Pabon-Mora et al., 2014), might have been recruited for valve margin specification, as no apparent roles have been described in other tissues. However *HEC3*, which has a key role in seed abscission, is present in all angiosperms. Those data suggest that the link between STK and HEC3 could have been present before the acquisition of fruit dehiscence. With the emergence of the *SHP1/2* and *IND* genes, the SHP-IND module could have evolved independent of the STK-HEC3 module to direct fruit dehiscence. The study in other species to assess a possible functional conservation of the role of HEC3, as well as the STK-HEC3 module, could bring deeper insights to the understanding of the separation processes in plants, providing new ways to improve the yield in many crops.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana seeds were stratified for 2 days at 4°C after sowing. Plants were grown at 22°C under long-day conditions (LD). All mutant plants and marker lines used were in Columbia background except GT140 line, *lug-3*, *seu-1* and *alc-1* that were in Landsberg *erecta* (L*er*). *alc-1* was backcrossed twice into Columbia. All mutant and marker lines used have been described previously: *alc-1* (Rajani and Sundaresan, 2001), *GT140* (*IND::GUS*) (Ferrándiz et al., 2000; Liljegren et al., 2004), *hec3-1* (Gremski et al., 2007), *HEC3:GUS* (Gremski et al., 2007), *ind-2* (Liljegren et al., 2004), *lug-3* (Liu and Meyerowitz, 1995), *seu-1* (Franks et al., 2002), *shp1 shp2* (Liljegren et al., 2000), *spt-12* (Ichihashi et al., 2010), *SPT:GUS* (Groszmann et al., 2010), *stk-2* (Pinyopich et al., 2003), *pSTK:STK:GFP* (Mizzotti et al., 2014). *alc-10* seeds were obtained from the European *Arabidopsis* Stock Center (NASC ID N603775).

Constructs and plant transformation

STK and *SHP2* coding sequences were amplified and cloned through BP recombination into pDONR 207 (Invitrogen). To clone the *SHP2* promoter, a genomic fragment of 2154 bp upstream of the ATG of *SHP2* was amplified, digested (*Aat*II) and ligated into pBGW0 previously modified to generate the GATEWAY destiny vector *pSHP2::GW* (Ceccato et al., 2013; Karimi et al., 2002). The cloned coding sequences were introduced into the *pSHP2::GW* vector by LR recombination (Invitrogen). *Agrobacterium* strain GV3101was used to transform *Arabidopsis* (Clough and Bent, 1998). Transgenic lines carrying a single transgene insertion were selected. See Table S1 for the primer sequences used.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from fruits at stage 16 with the RNeasy Plant Mini kit (Qiagen). cDNA synthesis was performed with 2 µg total RNA using a First-Strand cDNA Synthesis kit (Invitrogen). The qPCR master mix was prepared using the iQTM SYBR Green Supermix (Bio-Rad). Results were normalized to the expression of the *ACTIN 8* (*ACT8*) reference gene. PCR reactions were performed in a Bio-Rad iCycleriQ optical system (v.3.0a). Three technical and three biological replicates were performed for each sample. See Table S1 for primer sequences.

β-Glucuronidase (GUS) staining

For GUS histochemical detection, samples were treated for 15 min in 90% ice-cold acetone and then washed for 5 min with washing buffer (25 mM sodium phosphate, 5 mM ferrocyanide, 5 mM ferricyanide and 1% Triton X-100) and

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incubated for 4-16 h at 37°C with staining buffer (washing buffer+1 mM X-Gluc). Following staining, plant material was fixed, cleared in chloral hydrate and mounted for bright-field microscopy. Observations were performed using a Zeiss Axiophot D1 microscope equipped with differential interface contrast (DIC) optics. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (v.4.1).

Fixation, sectioning and phloroglucinol staining

Siliques were fixed in FAA (3.7% formaldehyde, 5% acetic acid and 50% ethanol). After fixation, the tissues were dehydrated in a series of increasing ethanol concentrations. Subsequently, the ethanol was replaced by Histoclear and the tissue was embedded in paraffin. Sections (10 µm and 20 µm) were placed on poly-lysine coated slides, deparaffinized and washed twice with 100% ethanol. Slides were stained with 2% phloroglucinol solution in 96% ethanol for 2 min and then 50% HCl was applied for 30 s. Samples were analyzed by DIC microscopy.

Fixation of whole fruits, clearing and phloroglucinol staining

Stage 17 fruits were fixed in ethanol-acetic fixative (9:1) under vacuum for 10 min, and then kept overnight at 4°C. One 70% ethanol wash (30 min) was applied before the clearing step. Samples were maintained in clearing solution [chloral hydrate:glycerol:H_2O (8 g:1 ml:2 ml; w/v/v)] for a minimum of 24 h. Samples were then dehydrated in a series of increasing ethanol concentrations. Fruits were dissected under a stereoscope, and treated as described above.

Quantification of lignification

Ten representative pictures of each mutant and their respective controls were analyzed with Fiji software (Schindelin et al., 2012). The lignified cells in the SAZ were selected (we considered the last seven cellular layers at the end of the funiculus) for calculating the lignified area. The statistical significance of the differences was assessed using Student's *t*-test ($P<0.05$).

Seed abscission phenotype

Valves of dry fruits were removed under a stereomicroscope in order to make the seeds visible. After valve removal, the fruit support was shaken/beaten several times to force seed separation. Images were recorded after most of the wild-type seeds were detached.

Chromatin immunoprecipitation (ChIP)

Genomic regions located between the flanking genes of *HEC3* were analyzed bioinformatically to identify CArG-box sequences with up to one base mismatch. Columbia wild-type and *pSTK:STK:GFP* inflorescences containing young fruits were collected for analysis. ChIP experiments were performed as described by Mizzotti et al. (2014) using a commercial antibody against GFP (Living Colors polyclonal antibody raised in rabbit; Clontech, 632460).

Bimolecular fluorescence complementation (BiFC)

STK, *SHP2*, *SEP3*, *SEU* and *LUG* CDS were cloned into pYFPN43 and pYFPC43 (http://www.ibmcp.upv.es/FerrandoLabVectors.php), and BiFC experiments were conducted as previously described (Belda-Palazon et al., 2012). Interactions were monitored with a Leica TCS SP2 confocal microscope (Leica Microsystems).

Yeast hybrid assays

Three-hybrid assays were performed in the yeast strain PJ69-4A (James et al., 1996). *STK*, a truncated version of *SEP3* (Brambilla et al., 2007) and *SEU* were cloned into pGBKT7 (Clontech), pGADT7 (Clontech) and pTFT1 (Gregis et al., 2006). Yeast colonies were selected on medium lacking leucine, tryptophan, adenine and/or uracil. Three-hybrid interactions were assayed on selective YSD medium lacking leucine, tryptophan, adenine and histidine.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

V.B. and I.R.-V. designed, performed and interpreted experiments and wrote the paper, S.M. and M.D.M. performed and interpreted experiments and L.C. interpreted experiments and wrote the paper.

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Data availability

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: ALC (At5g67110), HEC3 (At5g09750), IND (At4g00120), LUG (At4g32551), SEP3 (At1g24260), SEU (At1g43850), SHP1 (At3g58780), SHP2 (At2g42830), SPT (At4g36930), STK (At4g09960).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.135202.supplemental

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