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Control of Fruit Patterning in *Arabidopsis* by INDEHISCENT

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Summary

The *Arabidopsis* seedpod opens through a spring-loaded mechanism known as pod shatter, which is essential for dispersal of the seeds. Here, we identify INDEHISCENT (IND), an atypical bHLH protein, that is necessary for fruit opening and is involved in patterning each of the three fruit cell types required for seed dispersal. Previous studies suggested that FRUITFULL (FUL), a member of the MADS-domain transcription factor family, is required for fruit growth since *ful* mutant fruit fail to undergo the dramatic enlargement that normally occurs after fertilization. Here we show, however, that FUL is not directly required for fruit elongation and instead is required to prevent ectopic activity of IND. Our molecular and genetic studies suggest a model for the regulatory interactions among the genes that control fruit development and the mechanism that results in the expression of *IND* in a narrow stripe of cells.

Introduction

Understanding the developmental origins of complex patterns remains one of the fundamental challenges facing contemporary scientists. What are the mechanisms by which distinct cell types become specified to carry out diverse functions? While extensive research over the past decade has revealed how entire floral organs are specified, the next great endeavor will be to determine how particular cell types within these floral organs are established.

The fertilized flower produces the fruit, perhaps the most intricate plant organ, which is composed of many distinct cell types. The *Arabidopsis* fruit forms a seedpod that encloses and protects the seeds as they are maturing, then dries and opens to disperse the seeds at maturity. Patterning genes partition the developing *Arabidopsis* fruit into three major regions: the valves, the replum, and the valve margins (Figures 1, 2A, and 2D). The valves are the seedpod walls that encircle the developing seeds and connect to the replum, which forms the middle ridge that attaches the fruit to the plant. The valve margins (or margins for short) form at the boundary between the valves and the replum and are specialized for seed dispersal. When the fruit matures and dries, the valves detach from the replum along the margins in a process called dehiscence or pod shatter. Three specialized cell types contribute to the opening of the fruit: the two layers of the margin—the separation layer (or dehiscence zone) and the lignified margin layer—as well as the lignified valve layer (endocarp *b*) (Figures 1, 2D, and 2F). The valves detach through cell-cell separation within the dehiscence zone that occurs following the secretion of hydrolytic enzymes (Meakin and Roberts, 1990a, 1990b). The stiffening of cell walls through lignification of the lignified margin layer (Figures 1 and 2F) and the internal lignified valve layer (Figures 1B and 2F) has been proposed to contribute mechanically to fruit opening (Spence et al., 1996). As the fruit dries, differential shrinkage of the remaining thin-walled valve cells relative to the rigid lignified margin and valve layers is thought to create internal tension, causing the shattering that is characteristic of fruit dehiscence.

Previous studies of *Arabidopsis* fruit development have identified a few of the transcriptional regulators involved in margin specification (Liljegren et al., 2000; Rajani and Sundaresan, 2001). The functionally redundant MADS-domain factors SHATTERPROOF1 (SHP1) and SHATTERPROOF2 (SHP2) are required for both separation layer differentiation and to promote lignification of the lignified margin layer. Consequently, when *shp1 shp2* fruit are mature, they fail to open, and the seeds are trapped inside. The ALCATRAZ (ALC) basic helix-loop-helix (bHLH) transcription factor, which is also required for fruit dehiscence, specifies the differentiation of the separation layer.

In *Arabidopsis*, as in all flowering plants, the fruit grows after fertilization, expanding to accommodate the developing seeds. The *fruitfull* (*ful*) mutant produces tiny fruit that fail to elongate because the valves do not differentiate correctly (Gu et al., 1998). Therefore, it has been thought that FUL is required for valve cell development and consequently the elongation of the fruit. *FUL* encodes another MADS-domain transcription factor and acts in part by repressing expression of *SHP1* and *SHP2*, which are normally expressed at the valve margin but are ectopically expressed throughout *ful* mutant valves (Ferrándiz et al., 2000b). However, removal of the ectopic SHP activity from the *ful* mutant valves does not restore fruit elongation.

Here, we report the discovery of the *INDEHISCENT* (*IND*) gene, which encodes a member of an atypical class of eukaryotic bHLH proteins and is required for seed dispersal. IND is involved in the differentiation of all three cell types required for fruit dehiscence and acts as the key regulator in a network including SHP and ALC that controls specification of the valve margin. Furthermore, we have discovered that IND, ALC, SHP, and FUL interact to allow differentiation of the lignified valve layer, the spring-loaded mechanism of *Arabidopsis* fruit that promotes opening. Finally, we show that FUL is not

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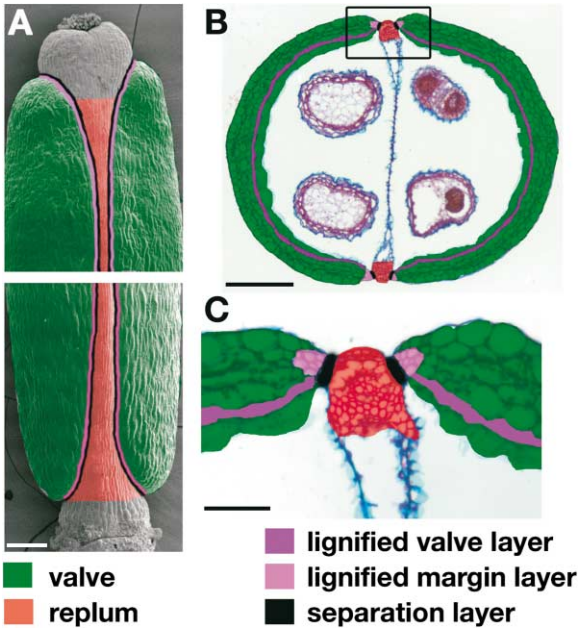

Figure 1. Wild-Type Fruit

(A) Scanning electron micrograph (SEM) of the apex and base of a mature wild-type fruit (stage 17), with the regions of the fruit colorized as indicated.

(B) Transverse section of a wild-type fruit (stage 17) with the cell types colorized as in (A). The valve margin region shown in (C) is boxed and approximates those shown throughout the paper.

(C) Close up of the valve margin region of the transverse section boxed in (B).

Scale bars in (A) and (B) represent 200 μm. Scale bar in (C) represents 50 μm.

directly required for fruit elongation as was previously thought. Instead FUL is required to negatively regulate *IND*, restricting *IND* expression to the valve margin. We show that ectopic IND, ALC, and SHP activity throughout the valves is largely responsible for the failure of valve cell expansion and differentiation in *ful* mutant fruit. Therefore, the primary role of FUL is not to specify valve development and fruit elongation, but instead to limit valve margin development.

Results

Mutations in *IND* Prevent Seed Dispersal

To uncover additional loci required for margin development, mutagenesis screens of adult *Arabidopsis* plants were carried out upon senescence (Liljegren et al., 2000). At this stage, wild-type fruit shatter at a slight touch, and mutants affecting seed dispersal can easily be identified. Four of the mutants found that produce indehiscent fruit represent a unique complementation group: *indehiscent* (*ind*). A fifth mutant allele of *IND* was kindly provided by Cristina Ferrándiz.

Examination by scanning electron microscopy showed that fruit from three of the *ind* mutant alleles bear a striking resemblance to *shp1 shp2* fruit, with lack of margin definition particularly apparent at the fruit bases (Figure 2B; Liljegren et al., 2000). Fruit from a weaker allele, *ind-1*, although also indehiscent, are more similar to *alc* fruit in appearance, with defined margins (Figure 2C; Rajani and Sundaresan, 2001). Further characterization of *ind* fruit was carried out using *ind-2* as the representative allele. We observed that margin development and seed dispersal are more severely affected in *ind* compared to *shp* fruit, especially at the fruit apex where the *ind* margin is less distinct (Supplemental Figures S1A, S1E, and S1G available at http://www.cell.com/cgi/content/full/116/6/843/DC1). While *shp* fruit sometimes open slightly at the apical margin, this was not seen in *ind* fruit (data not shown).

To examine more closely how cells at the fruit margin are affected by mutations in *IND*, sections of mature *ind* fruit (stage 17) were compared to wild-type (Figures 2D and 2E). Both the small cells that typify the separation zone and the adjacent lignified cell layers at the wild-type margin are not apparent in *ind* fruit, resulting in markedly less constriction of the margins than is seen in wild-type.

IND Controls Margin Lignification

To determine whether lignified margin layer cells are also affected by mutations in *IND*, we examined the lignification pattern of *ind* fruit compared to wild-type (Figures 2F and 2G). While lignification of the vascular bundles and lignified valve layer appear unaffected in *ind* fruit, we observed no lignified cells throughout the margins of *ind-2* fruit (Figure 2G). As margin lignification is only partially affected in *shp* fruit and unaffected in *alc* fruit (Liljegren et al., 2000, Rajani and Sundaresan, 2001), these results indicate that IND is primarily responsible for controlling the lignification of margin cells. Interestingly, the margins of *ind-1* fruit, like *alc* fruit, are lignified (Figure 2H), suggesting that the role of IND in separation layer specification can be distinguished genetically from its role(s) in margin constriction and lignification.

IND Regulates Expression of the YJ80 Margin Marker

To further monitor the effect of mutations in *IND* on cellular differentiation at the margin, expression of molecular markers derived from an enhancer trap screen (Eshed et al., 1999) were examined in *ind* fruit compared to wild-type. We discovered that the expression pattern of one marker, YJ80, is dramatically affected by mutations in *IND* (Figures 2I and 2J). In wild-type fruit, YJ80 is expressed in stripes at the margin, in the guard cells scattered throughout the valves, and in the seed abscission zone (Figure 2I and data not shown). Mutations in *IND* completely disrupt expression of this marker throughout the margins, whereas the other fruit expression domains are unaffected.

Since we could detect differences between the margin defects of *ind* and *shp1 shp2* fruit, and the phenotype of *alc* fruit is clearly distinct from that of *ind* and *shp1 shp2*, we expected that the phenotypes of these three mutants might be further distinguished with margin markers. We found that the YJ80 marker is still present at the apical fruit margins of *shp1 shp2* and *alc* mutants (Figures 2K and 2L), although expression of YJ80 is disrupted at the basal margins of *shp1 shp2* fruit (data not shown), and at the central margins of *alc* fruit (Figure

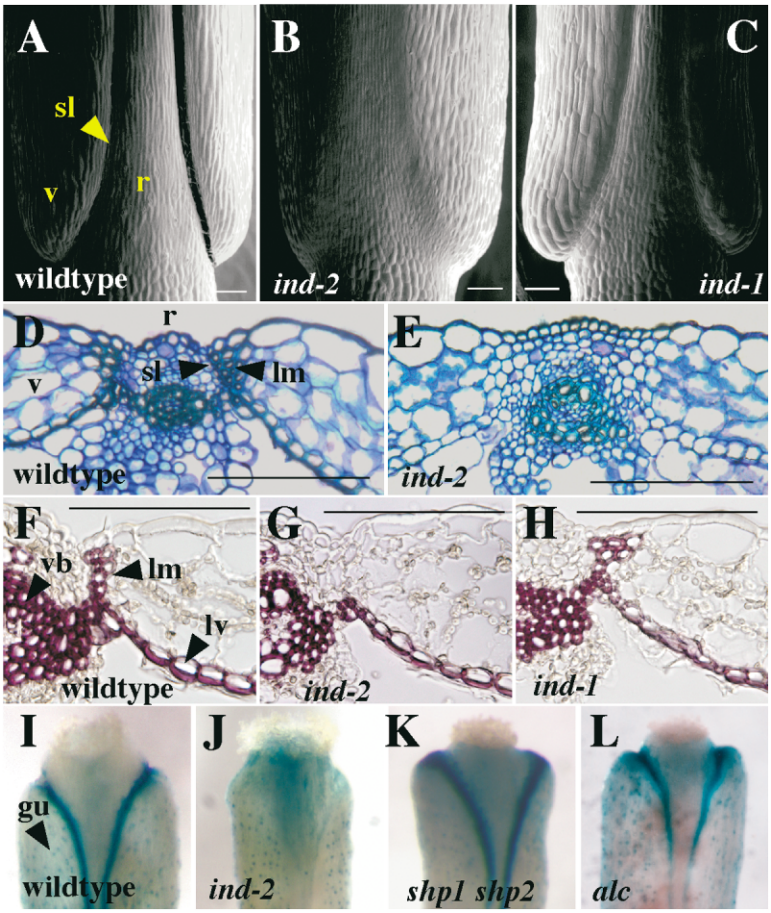

Figure 2. IND Controls Margin Development in *Arabidopsis* Fruit

(A) SEM of a mature wild-type fruit base (stage 17). Cells at the margin between the valve (v) and the replum (r) form the separation layer (sl).

(B) Fruit from the strong *ind-2* mutant, showing less constriction of the valve margins and lacking a separation layer.

(C) Fruit from the weaker *ind-1* mutant (in a *shp1* background, which has no phenotype on its own). Seed dispersal does not occur despite the normal appearance of the margin.

(D) Transverse section of a wild-type fruit (stage 17) stained with toluidine blue. Cells in the separation and lignified margin (lm) layers expand more slowly after fertilization, resulting in marked constriction at the margin.

(E) Section from an *ind-2* fruit, showing increased expansion of margin cells and the absence of separation layer differentiation.

(F) Transverse section of a wild-type fruit (late stage 17) stained with phloroglucinol. Patches of margin cells adjacent to the valve are lignified (lm), in addition to the inner lignified valve cell layer (lv) and the vascular bundles (vb) of the replum.

(G) In *ind-2* fruit, patches of lignified cells are not detected at the margin.

(H) Margin lignification is unaffected in *ind-1* fruit.

(I) β-glucuronidase expression of the YJ80 margin marker occurs at the margins and in guard cells (gu) of young wild-type fruit (stage 16).

(J) Expression of YJ80 is not observed throughout the margins of *ind-2* fruit.

(K) YJ80 expression at the apical margins of *shp1 shp2* fruit is not disrupted.

(L) Expression of YJ80 at the apical (and basal) margins of *alc* fruit is not affected.

All scale bars represent 100 μm.

2L). These results correspond with our observations that apical margin development is more severely affected in *ind* fruit than in *shp1 shp2* fruit, and further suggest that IND may be the key regulator of the gene corresponding to YJ80.

IND Represents a Unique Class of Eukaryotic bHLH Proteins

Through our previous studies, we identified a margin-specific marker, GT140, that is largely absent from the margins of both *shp1 shp2* and *35S::FUL* indehiscent fruit (Sundaresan et al., 1995, Liljegren et al., 2000, Ferrándiz et al., 2000b). Since these results suggested that the gene corresponding to GT140 could be involved in margin development, we isolated genomic sequence flanking the *Ds* transposon using TAIL/PCR (Tsugeki et al., 1996). The insertion site was found to be on chromosome 4 between two predicted ORFs, At4g00120 and At4g00130 (Figure 3A). Subsequent analysis of At4g00120 demonstrated that a genomic fragment containing 2.6 kb from the promoter region directed expression of β-glucuronidase in the same margin-specific pattern as GT140 (Figure 3B). Furthermore, approximately 25% of the transgenic lines failed to show significant GUS activity and produced indehiscent fruit (data not shown), suggesting that At4g00120 was cosuppressed in these lines and could be required for fruit dehiscence.

At4g00120, an open reading frame with no introns, encodes a protein with a basic helix-loop-helix (bHLH) domain (Figure 3A). To investigate whether this gene might be affected by mutations at the *ind* locus, we sequenced the coding region in each of our mutant alleles. All five alleles were found to contain single nucleotide changes within the coding region (Figure 3A), and three, including *ind-2*, encode truncated proteins without the bHLH domain. Complementation using a 3.4 kb genomic fragment spanning At4g00120 rescues the *ind* mutant phenotype (data not shown), further confirming that IND is the GT140 bHLH factor. The protein encoded by *IND* is predicted to be 169 amino acids in length (Figure 3C).

Transcriptional regulators with a bHLH domain bind DNA through residues in the basic region while the helix-loop-helix domain promotes dimerization, allowing family members to form hetero- or homodimers (Murre et al., 1989). The majority of the predicted 147 *Arabidopsis* bHLH proteins contain a critical glutamic acid residue (E) at site 9 within the basic region that is critical for DNA binding, whereas IND and thirteen other predicted *Arabidopsis* bHLH sequences have an alanine residue

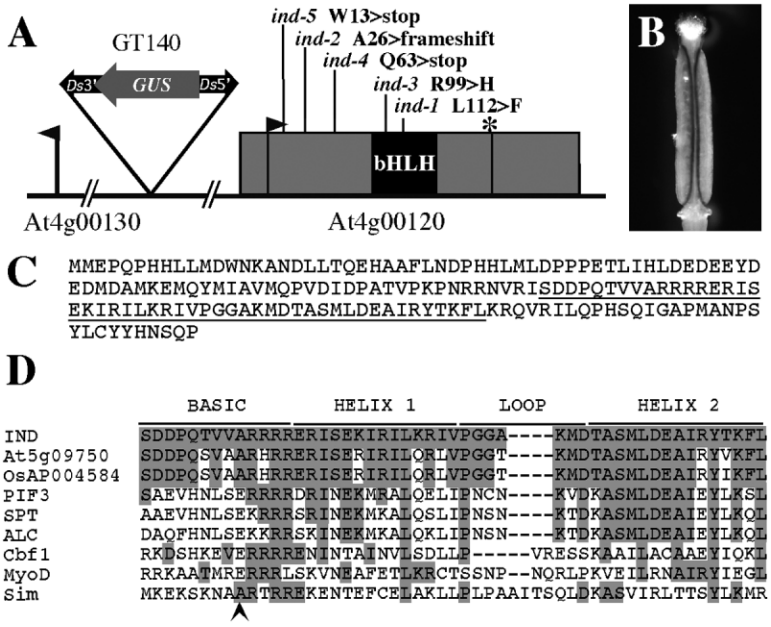

Figure 3. *IND* Encodes an Atypical bHLH Protein

(A) Schematic drawing of the At4g00120 locus, showing the site of the GT140 transposon insertion and the sequence alterations of the characterized *ind* mutant alleles. Predicted translational start sites of At4g00120 and its neighbor, At4g00130, are indicated by arrowheads, the single exon of At4g00120 is shown as a box, the position of the bHLH domain is marked in black, and the predicted site of the stop codon is indicated by an asterisk.

(B) The margin-specific pattern of β-glucuronidase expression directed by the At4g00120 regulatory region.

(C) Amino acid sequence of the predicted IND protein. The location of the bHLH domain is underlined.

(D) Sequence alignment of the bHLH region from IND and related proteins from plants, yeast and animals. Characterized proteins include the B-class members PHYTOCHROME INTERACTING FACTOR (PIF3), SPATULA (SPT), ALCATRAZ (ALC) from *Arabidopsis* (Ni et al., 1998; Heisler et al., 2001; Rajani and Sundaresan, 2001) and centromere binding factor (Cbf1) from yeast (Cai and Davis, 1990); A-class MyoD from human (Pearson-White, 1991), and C-class single-minded (Sim) from fly (Nambu et al., 1991). Amino acids conserved between IND and other proteins are shaded. An arrowhead marks the atypical alanine residue found in the basic region of IND and Sim. Uncharacterized proteins with IND-like atypical basic regions include At5g09750 and OsAP004584 predicted from *Arabidopsis* and *Oryza sativa* sequences, respectively.

(A) instead (Figure 3D; Fisher and Goding, 1992; Buck and Atchley, 2003; Toledo-Ortiz et al., 2003). Sequence conservation between IND and the closest *Arabidopsis* relatives is primarily restricted to the bHLH domain (Figure 3D). ALC, which is also required for fruit dehiscence, shares only 42% identity with IND in the bHLH domain (Figure 3D).

Expression of *IND* Expands throughout the Valves of *ful* Fruit

To determine the pattern of *IND* expression in wild-type and mutant fruit, we performed antisense in situ hybridization with an *IND*-specific probe. After fertilization, *IND* is expressed in stripes about four cells wide at the margins of developing wild-type fruit (Figure 4A). We also detected *IND* expression in the inner valve layer (Figure 4A), which becomes lignified later in fruit development. Like *SHP1* and *SHP2* (Ferrándiz et al., 2000b), expression of *IND* expands throughout the valves of *ful* mutant fruit (Figure 4B), indicating that FUL is required in the valves to restrict *IND* expression to the margins.

Expanded IND Activity in *ful* Fruit Inhibits Growth and Causes Ectopic Lignification

Mutations in *FUL* cause severe defects in fruit growth, primarily due to lack of valve cell expansion after fertilization of the gynoecium (Gu et al., 1998). Previously, we have found that the ectopic expression of *SHP1* and *SHP2* in *ful* fruit does not account for their reduced growth, as *shp1 shp2 ful* fruit are only slightly longer than *ful* fruit (Ferrándiz et al., 2000b; see also Figures 4C and 4D). To determine whether ectopic IND activity could instead be primarily responsible for the expansion defects of *ful* fruit, we constructed the *ind ful* double mutant. Remarkably, we discovered that fruit growth is considerably restored in *ind ful* fruit compared to *ful* fruit (Figures 4C and 4D). Whereas mature *ful* fruit (2.5 ± 0.2 mm) are 25% the length of wild-type fruit (10.1 ± 0.7 mm), *ind ful* fruit (6.8 ± 0.4 mm) are significantly longer—more than twice the length of *ful* fruit and 67% the length of wild-type. Scanning electron micrographs of *ful* and *ind ful* fruit compared to wild-type demonstrate the restoration of valve epidermal cell expansion due to loss of IND activity (Figures 4G to 4I). Furthermore, differentiation of some epidermal cells into guard cells is seen in *ind ful* fruit (Figure 4I), and is never observed in *ful* fruit (Figure 4H).

In addition to growth defects, *ful* fruit also show ectopic lignification of several valve cell layers (Ferrándiz et al., 2000b). During wild-type fruit development, lignification of a single inner valve layer (Figure 2F) is thought to contribute to fruit opening. In *ful* fruit, lignification of three additional valve layers occurs (Figure 4E). Because we found that IND is required for lignification of the wild-type fruit margin, we suspected that expanded IND activity might be the cause of ectopic lignification of *ful* fruit. Indeed, as lignification of only the correct inner valve layer is observed in *ind ful* fruit (Figure 4F), expanded IND activity is not only largely responsible for the lack of valve expansion, but also causes the ectopic valve lignification of *ful* fruit.

Since *SHP1*, *SHP2*, and *IND* are each expressed at the valve margins of wild-type fruit, we have interpreted their expression throughout the valves of *ful* fruit as suggestive of an expansion of valve margin identity (Ferrándiz et al., 2000b; this work). The notable suppression of the *ful* fruit phenotype conveyed by loss of IND activity provides experimental validation of this hypothesis. Furthermore, the phenotypic differences between *ind ful*

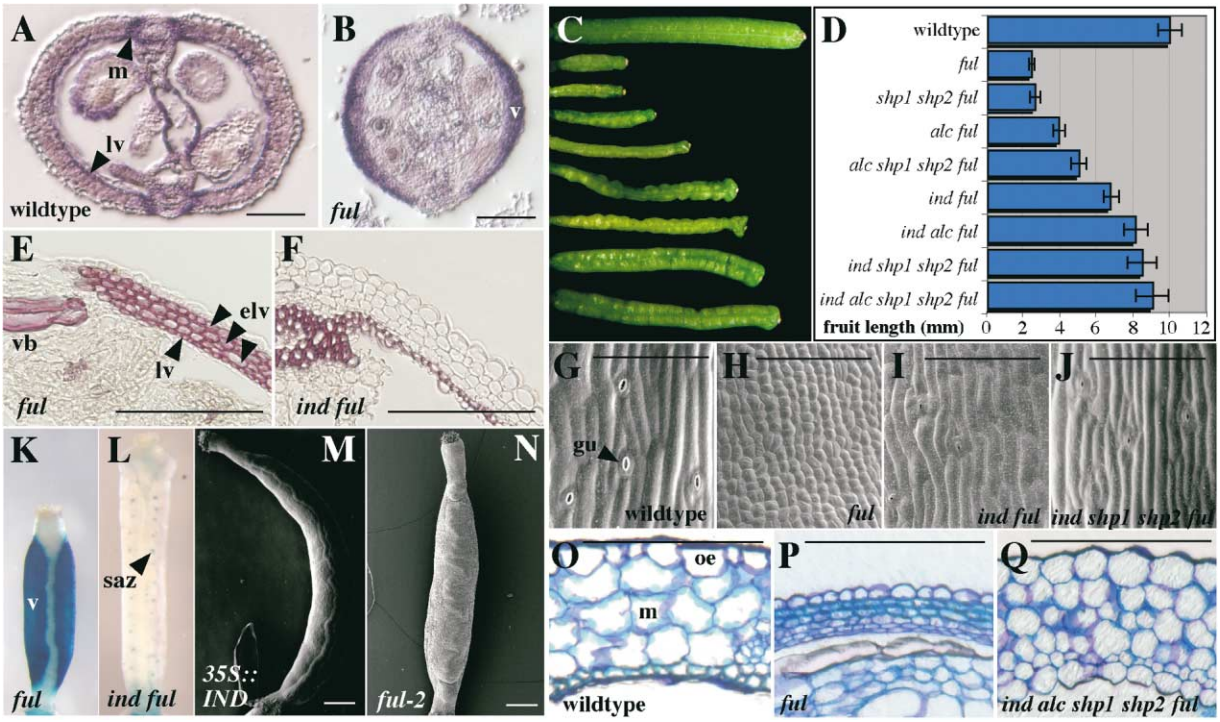

Figure 4. Expanded IND, SHP and ALC Activity in *ful* Mutant Fruit Inhibits Fruit Growth

(A) Transverse section of a young wild-type fruit (stage 16) probed with *IND* antisense RNA. *IND* is expressed in stripes at the margin (m), and is also detected in the lignified valve layer (lv).

(B) Section of a *ful* fruit (stage 15), showing expansion of *IND* expression throughout the valves (v).

(C) Comparison of wild-type and mutant mature fruit size (stage 17). From top to bottom, wild-type, *ful*, *shp1 shp2 ful*, *alc ful*, *alc shp1 shp2 ful*, *ind ful*, *ind alc ful*, *ind shp1 shp2 ful*, and *ind alc shp1 shp2 ful*.

(D) Measurement of wild-type and mutant fruit length. For each genotype, n = 100.

(E) Transverse section of a *ful* fruit (late stage 17) stained with phloroglucinol. Additional cell layers of the valve become ectopically lignified (elv).

(F) In *ind ful* fruit, ectopic valve lignification does not occur.

(G) Scanning electron micrograph of wild-type valve epidermal cells. Cells are elongated and differentiated guard cells (gu) are present.

(H) Valve epidermal cells in *ful* fruit do not expand after fertilization, and guard cell differentiation is never observed.

(I) Valve cell expansion and some guard cell differentiation occur in *ind ful* fruit.

(J) Expansion and differentiation of the valve epidermis in *ind shp1 shp2 ful* fruit appears nearly like wild-type.

(K) β-glucuronidase expression of the YJ80 margin marker expands throughout the valves of *ful* fruit (stage 16).

(L) YJ80 expression in *ind ful* fruit is absent from the margins and valves. Expression in the seed abscission zone (saz) is unaffected.

(M) Fruit (stage 17) from a weak *35S::IND* transgenic line, which shows reduced valve cell expansion, zigzag growth of the replum, and increased style elongation.

(N) Fruit produced by moderate *ful-2* mutant.

(O) Transverse section of a wild-type fruit valve stained with toluidine blue. Valve cell expansion is particularly apparent in the outer epidermis (oe) and meosphyll (m) cell layers.

(P) Valve cell expansion defects are evident throughout the layers of *ful* fruit.

(Q) Cell expansion is largely restored throughout the valves of *ind alc shp1 shp2 ful* fruit.

All scale bars represent 100 μm, except in (M) and (N), which represent 500 μm.

and *shp1 shp2 ful* fruit (Figure 4C) constitute compelling genetic evidence that IND expression and/or activity is not simply regulated by SHP, as suggested by the reduced, yet persistent expression of the GT140 marker for IND in *shp1 shp2 ful* fruit (Ferrándiz et al., 2000b).

An interesting lead to follow in the search for additional factors, which inhibit margin cell expansion, or promote their subsequent lignification, is YJ80. Like *IND*, expression of the YJ80 marker at the margin (Figure 2I) expands throughout the valves of *ful* fruit (Figure 4K), and, with the exception of the few guard cells, is completely absent in the valves of *ind ful* fruit (Figure 4L). As expected from analysis of YJ80 in *shp1 shp2* and *alc* fruit (Figures 2K and 2L), expression of YJ80 persists throughout the valves of *shp1 shp2 ful* and *alc ful* fruit (data not shown), strongly suggesting that the gene corresponding to this marker is specifically regulated by IND.

Plants with Ectopic *IND* Expression Produce *ful*-Like Fruit

To further explore the developmental effects of ectopic IND activity, we generated transgenic plants expressing *IND* under control of the constitutive cauliflower mosaic virus 35S promoter (Benfey and Chua, 1990). Phenotypic analysis revealed that 17 of 101 *35S::IND* T1 plants produced *ful*-like fruit with severe growth defects (data not shown). Furthermore, a significant number of *35S::IND* T1 plants exhibited weaker *ful*-like fruit phenotypes (Figures 4M and 4N) (Ferrándiz et al., 2000a), much like the

fruit produced by plants constitutively expressing *SHP1* and *SHP2* (Liljegren et al., 2000). These results correspond well with our discovery that mutations in *IND* significantly suppress the *ful* fruit phenotype, and demonstrate that ectopic IND activity is sufficient to inhibit fruit growth.

Loss of IND, SHP, and ALC Activity Largely Suppresses the *ful* Fruit Phenotype

Since mutations in *IND* not only have the most severe effect on margin development, but also suppress the *ful* phenotype more dramatically than mutations in *ALC*, or *SHP1* and *SHP2* (Figure 4C*)*, we wondered if ALC or SHP regulate any aspects of margin development independently of IND. To address this question, we conducted a systematic genetic analysis to uncover the relative contributions of IND, ALC, and SHP to margin development and to determine the extent their ectopic activities have on the *ful* fruit phenotype.

By comparing *ind shp1 shp2*, *ind alc*, and *ind* fruit, we observed an enhanced loss of apical margin definition in *ind shp1 shp2* fruit compared to *ind* fruit, but did not detect any morphological differences between *ind alc* and *ind* fruit (Supplemental Figure S1 available on *Cell* website). A smaller, but similar loss of margin definition was also evident in our examination of *alc shp1 shp2* fruit compared to *shp1 shp2* fruit (data not shown). These results suggest that SHP1 and SHP2 do regulate some aspects of margin development independently of IND and ALC, and that ALC activity is primarily encompassed by IND.

The IND-independent activity of SHP is much more apparent when comparing *ind shp1 shp2 ful* to *ind ful* fruit (Figures 4C and 4D). Fruit length in *ind shp1 shp2 ful* fruit (8.5 $\pm$ 0.8 mm) is largely restored (84%) to wild-type, and the overall appearance of the fruit, while rumpled, is more like wild-type, due to increased lateral valve cell expansion. Furthermore, scanning electron micrographs of *ind shp1 shp2 ful* valve cells (Figure 4J) compared to wild-type, *ful*, and *ind ful* fruit (Figures 4G to 4I) demonstrate the extensive restoration of guard cell differentiation due to the combined loss of ectopic IND and SHP activity. Support for the ALC-independent activity of SHP is also more evident when comparing *alc shp1 shp2 ful* to *alc ful* fruit (Figures 4C and 4D). Although the fruit length (5.1 $\pm$ 0.4 mm) of *alc shp1 shp2 ful* fruit is only partially restored (51%) compared to wild-type, it is significantly longer than that of *alc ful* fruit (4.0 $\pm$ 0.3 mm). Taken together, these results indicate that SHP regulates factors involved in margin development and cell expansion independently of IND and ALC.

Although our initial observations of *ind alc* fruit suggested that ALC might not play any roles in fruit development independent of IND, analysis of *ind alc ful* and *ind alc shp1 shp2 ful* fruit has revealed that ALC does possess both IND- and SHP-independent roles (Figures 4C and 4D). Fruit produced by the *ind alc ful* mutant are significantly longer (8.2 $\pm$ 0.6 mm) than *ind ful* fruit (6.8 $\pm$ 0.4 mm). Furthermore, a slight increase in length is also observed in comparing *ind shp1 shp2 ful* (8.5 $\pm$ 0.8 mm) to *ind alc shp1 shp2 ful* (9.1 $\pm$ 0.9 mm) fruit (Figures 4C and 4D).

Thus, while our genetic analysis has confirmed that IND does indeed play the dominant role in establishing the fruit margin, it has also revealed that SHP and ALC have distinct activities at the margin. Furthermore, the remarkable restoration of fruit growth observed in *ind alc shp1 shp2 ful* fruit compared to *ful* fruit (90 and 25% the length of wild-type fruit, respectively), highlights the roles of ectopic IND, ALC, and SHP activity in inhibiting *ful* fruit growth (Figures 4C and 4D). Loss of ectopic IND, ALC, and SHP activity in *ful* fruit largely restores the appearance and size of cells within the mesophyll and outer epidermal layers to that of wild-type fruit (Figures 4O to 4Q). These results suggest that the primary function of FUL in promoting fruit growth is to restrict IND, ALC, and SHP activities to the fruit margin.

IND, SHP, ALC, and FUL Activities Contribute to Differentiation of the Lignified Valve Layer

In addition to finding that SHP and ALC have IND-independent roles in margin development, we also discovered that together with IND and FUL these factors are involved in specifying lignification of the lignified valve layer. Examination of *ind alc shp1 shp2* fruit compared to wild-type (Figures 5A and 5B) revealed that a few cells in the lignified valve layer adjacent to each valve margin fail to lignify (see asterisks in Figure 5B). A similar, but less penetrant, retraction of lignified valve layer cells from the replum was also observed in *ind shp1 shp2* fruit (data not shown). The appearance and size of these nonlignified cells is most like those found in the neighboring mesophyll cell layers (Figure 5B).

In wild-type fruit, *FUL* is expressed throughout the valves (Figure 5D) (Gu et al., 1998). Previously, we have found that the expression of *FUL* retracts slightly from the valve margin in *shp1 shp2* mutant fruit (Ferrándiz et al., 2000b). In *ind* mutants, we also observe a slight retraction of the *FUL* from the margin (Figure 5E). The retraction of *FUL* from the margin is more dramatic in *ind alc shp1 shp2* quadruple mutant fruit (Figure 5F), and correlates with the absence of lignified cells near the margin in the lignified valve layer (Figure 5B). When FUL activity is removed in the *ind alc shp1 shp2 ful* quintuple mutant, lignification of the lignified valve layer is completely absent (Figure 5C) except for a few cells at the base of the fruit. The observation that lignification of this layer is reduced but not eliminated in *ind shp1 shp2 ful* quadruple mutant fruit (data not shown) indicates that ALC also plays a role in specifying this cell type. Since the lignified valve layer is completely eliminated only when all five transcription factors—IND, ALC, SHP1, SHP2, and FUL—are inactivated, it is evident that each factor contributes to lignification of this layer.

Discussion

The fruit, a complex structure unique to flowering plants, facilitates seed maturation and dispersal. We have identified an atypical bHLH gene, *IND*, which specifies an essential pattern element of dehiscent fruit, the valve margin. We show here that IND is required for differentiation of margin cells into adjacent separation and lignification layers (Figures 6A and 6C). IND acts largely downstream of the SHP1 and SHP2 MADS-domain transcription factors, which are also involved in specifying

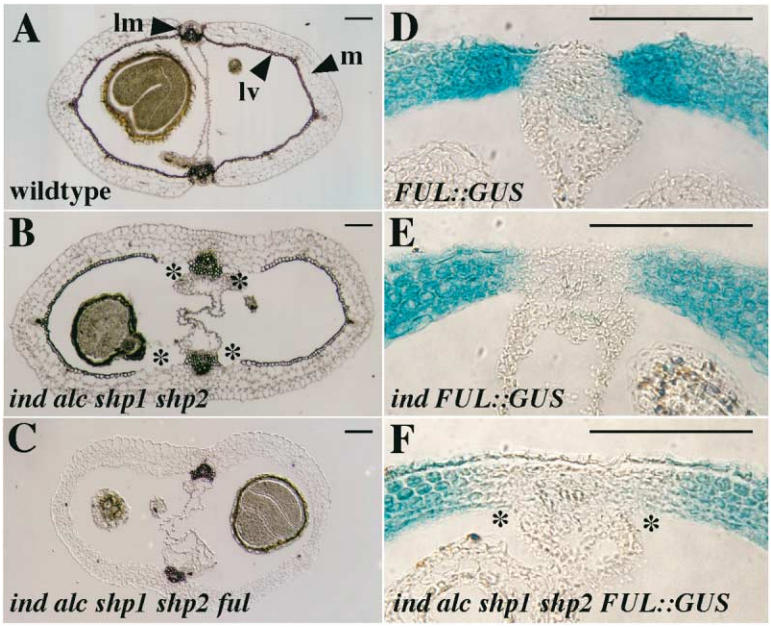

Figure 5. IND, SHP, ALC, and FUL Control Differentiation of the Lignified Valve Layer

(A) Transverse section of a wild-type fruit (stage 17) stained with phloroglucinol.

(B) In addition to the absence of lignified margin cells, *ind alc shp1 shp2* fruit also show broad regions next to the margin (marked with asterisks) that are missing lignified valve cells. Unlignified cells are larger than the lignified valve cells in the same layer.

(C) Lignification of the entire lignified valve layer does not occur throughout *ind alc shp1 shp2 ful* fruit.

(D) β-glucuronidase expression of the *FUL* valve marker in transverse sections of *ful-1* heterozygous fruit (Gu et al., 1998). *FUL* expression occurs throughout the valves and margin, and is absent from the replum.

(E) The domain of *FUL* expression shows a small movement away from the replum in *ind ful-1/+* fruit.

(F) In *ind alc shp1 shp2 ful-1/+* fruit, the *FUL* valve expression domain shrinks significantly and its boundary is more diffuse.

All scale bars represent 100 μm.

valve margin development (Figure 6A). Another bHLH transcription factor, ALC, is specifically involved in the differentiation of the margin separation layer (Rajani and Sundaresan, 2001). Together IND, ALC, and SHP form a regulatory network that orchestrates the differentiation of the valve margin, allowing seed dispersal to take place (Figure 6A). Mutations in the *FUL* MADS-box gene prevent the postfertilization elongation of the fruit. The valve cells of *ful* mutant fruit fail to grow and differentiate normally and become ectopically lignified late in fruit development (Gu et al., 1998; Ferrándiz et al., 2000b). We have shown that expansion of IND activity throughout the valves is primarily responsible for the severe growth defects and ectopic lignification of *ful* fruit, and that together, mutations in *IND*, *ALC*, *SHP1*, and *SHP2* largely suppress the *ful* mutant phenotype. Thus, FUL is not directly required for fruit expansion, but instead negatively regulates *IND*, *ALC*, and *SHP* to ensure that that valve margin differentiation occurs only at the edge of the valve (Figure 6A). We also show that IND, SHP, ALC, and FUL activities control differentiation of the other fruit-specific pattern element important for seed dispersal, the lignified valve layer, as lignification of this cell type fails to occur throughout *ind alc shp1 shp2 ful* fruit (Figures 6B, 6C, and 5C). Therefore, IND is involved in patterning all three cell types of the fruit that allow the spring-loaded, pod shatter mechanism.

IND Encodes an Atypical bHLH Family Member that May Interact with ALC

IND is a member of a unique group of eukaryotic bHLH proteins with an atypical basic region. Like the *Drosophila* bHLH-PAS domain proteins single-minded (Sim) and Trachealess (Trh) (Nambu et al., 1991; Wilk et al., 1996), IND and 13 other predicted *Arabidopsis* bHLH sequences contain an alanine rather than a glutamate at position 9 within the basic region (Buck and Atchley, 2003). Following the bHLH nomenclature system and recent identification of additional eukaryotic groups (Ledent and Vervoort 2001; Toledo-Ortiz et al., 2003), this unique set of plant bHLH proteins represents group G.

Since bHLH proteins with atypical basic regions such as Sim are unable to bind the canonical E-box bHLH binding site alone (Swanson et al., 1995), it will be important in future studies to characterize the heterodimer specificity and DNA binding capacity of IND and other group G plant bHLH proteins. Like Sim and Trh, which each form heterodimers with the bHLH-PAS protein Tango and bind an asymmetric target sequence (Wharton et al., 1994; Sonnenfeld et al., 1997), IND and other IND-like bHLH proteins may only bind DNA as heterodimers with other bHLH proteins.

One candidate for a heterodimeric partner with IND is ALC, and together, IND and ALC may regulate the differentiation of the separation layer. Such a pairing is supported by our finding that IND and ALC interact in yeast (Supplemental Figure S2 available on *Cell* website). However, since ALC does not have any apparent role in margin lignification, we speculate that IND may interact with additional bHLH proteins and/or other factor(s) to direct the lignification of margin cells.

Dissecting the Relative Contributions of IND, SHP, and ALC to Margin Differentiation

The process of valve margin specification requires the activities of three factors: IND, SHP1 (or SHP2), and ALC. While we might expect that these factors work in a linear cascade of gene activity, instead we found that they form a nonlinear regulatory network. Although IND appears to play the most significant role in this process, we have discovered that all three activities have distinct as well as overlapping roles in directing margin formation (Figure 6A). The inability to detect *IND* expression in the margins of *shp1 shp2* fruit (Liljegren et al., 2000; data not shown) suggests that IND acts largely downstream of SHP (Figure 6A). However, several lines of

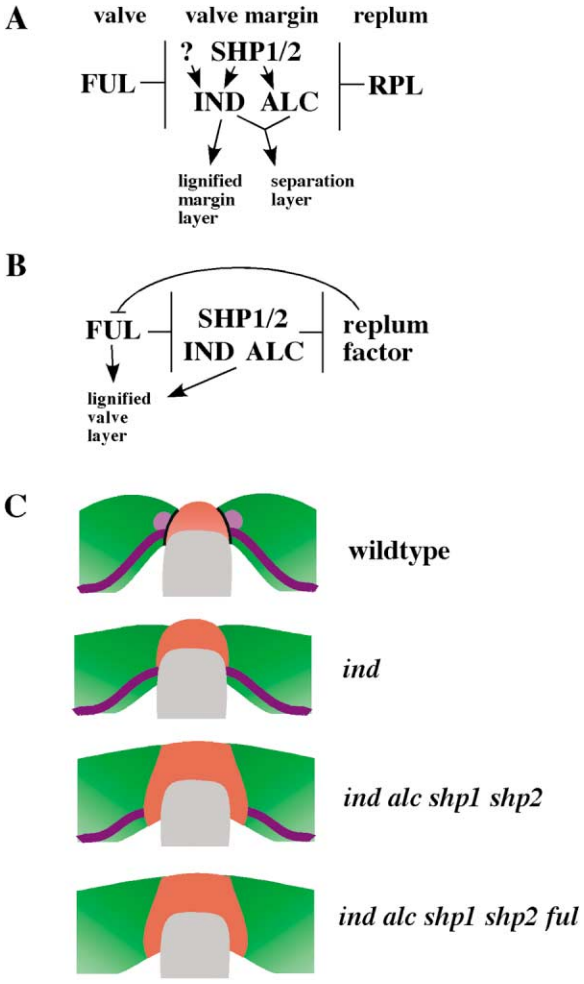

Figure 6. Setting the Valve Margin

(A) A model of the regulatory network specifying valve margin development.

(B) A model for the regulation of the valve lignified layer.

(C) Representation of wild-type, *ind*, *ind alc shp1 shp2*, and *ind alc shp1 shp2 ful* fruit cross-sections depicting the replum (red), valves (green), lignified margin layer and lignified valve layer (magenta), and separation layer of the margin (black).

evidence indicate that IND also has key activities at the margin which are independent of SHP. First, the loss of IND activity has a greater effect on margin development than does loss of SHP activity. We have found IND to be the primary regulator of margin lignification, which is absent in *ind* fruit but only reduced in *shp1 shp2* fruit (Figure 2G; Liljegren et al., 2000). Furthermore, IND is required for the valve margin expression of the YJ80 marker, and this marker is still expressed in the apical margin of *shp1 shp2* fruit (Figures 2J and 2K). Second, since *IND* is expressed at low levels in the valves of *shp1 shp2 ful* fruit, we know that *IND* expression is not completely dependent on SHP (Ferrándiz et al., 2000b). Third, loss of IND activity enhances the phenotype of *shp1 shp2* fruit (Supplemental Figures S1E, S1F, S1K, and S1L available on *Cell* website). Finally, mutations in *IND* (but not in *SHP*) dramatically suppress the growth defects of *ful* fruit (Figure 4C). Together, these data indicate that IND must have margin activities that are mediated by low levels of SHP-independent expression, and that while SHP1 and SHP2 are important transcriptional activators of *IND*, one or more additional factor(s) must also be involved in activating *IND* expression (Figure 6A).

Conversely, the role of SHP in margin differentiation is not only to activate *IND* since the *ind* mutant phenotype is enhanced by the loss of SHP activity in *ind shp1 shp2* fruit (Supplemental Figures S1G, S1H, S1K, and S1L available on *Cell* website). One of the additional roles of SHP is to activate *ALC* (Supplemental Figures S3A and S3B available on *Cell* website), and together IND and ALC activity may account for most of the roles of SHP in margin development (Figure 6A). However, SHP clearly has additional roles since the *ind alc shp1 shp2* quadruple mutant shows a more dramatic loss of valve margin definition than the *ind alc* double mutant (Figure 5B, Supplemental Figures S1I, S1J, S1M, and S1N available on *Cell* website). Moreover, loss of SHP activity further suppresses the growth defects of *ind alc ful* fruit (Figure 4C) and SHP, IND, and ALC appear to play redundant roles (see below) in promoting lignification of the lignified valve layer (Figures 5B and 5C).

ALC appears to have a more limited role in margin development than either SHP or IND, as *alc* mutations affect differentiation of the separation layer, but not the lignified margin layer. We propose that a heterodimeric complex of IND and ALC may specify the separation layer of the valve margin (Figure 6A), which is supported by the finding that ALC and IND interact in yeast (Supplemental Figure S2 available on *Cell* website). Consistent with this hypothesis is the observation that margin specification in *ind* fruit is not visibly enhanced by the additional loss of ALC activity in *ind alc* fruit (Supplemental Figures S1G, S1H, S1I, and S1J available on *Cell* website). However, at the molecular level, *ind alc* fruit can be distinguished from *ind* fruit. In addition to GT140, expression of another valve margin marker, YJ36, is dependent on SHP activity (Liljegren et al., 2000). YJ36 expression, which is found in the outer and inner epidermal cells at the margins of wild-type fruit, is unaffected in *ind* and *alc* fruit but largely absent in the margins of *ind alc* fruit (data not shown). These results suggest that while ALC may primarily specify the margin separation layer together with IND as a heterodimeric complex, certain functions of ALC in margin development are independent of IND, as ALC and IND appear to redundantly regulate the expression of YJ36.

FUL Restricts Margin Differentiation to the Valve Edge

Mutations in *FUL* cause dramatic alterations in fruit development, preventing fruit elongation after fertilization (Gu et al., 1998). The valve cells of *ful* mutant fruit appear to be mispecified as valve margin cells and consequently fail to expand. Moreover, three cell layers of *ful* mutant valves become ectopically lignified during the late stages of fruit development (Figure 4E), as if they have adopted the fate of the lignified margin layer. Whereas SHP plays an important role in activating *IND* expression, FUL negatively regulates *IND* expression in the valves, limiting *IND* to the valve margin in wild-type fruit (Figure 6A). Remarkably, it is the ectopic expression

of *IND* in the *ful* mutant valves that is largely responsible for both the failure of *ful* valve cells to expand and their ectopic lignification (Figures 4B, 4C, and 4F). This indicates that IND is the key regulator of the lignified margin layer. Since certain valve cell types, such as the guard cells in the outer epidermis and the lignified valve layer, can correctly differentiate in *ind ful* fruit (Figures 4F and 4I), FUL is not required to direct the specification of all valve cell types per se, but rather to prevent valve cells from erroneously adopting a valve margin cell fate.

FUL also negatively regulates the expression of *SHP* and *ALC* (Ferrándiz et al., 2000b; Supplemental Figure 3C available on *Cell* website). Although loss of either SHP or ALC ectopic activity slightly mitigates the severity of the *ful* phenotype (Figure 4C; Ferrándiz et al., 2000b), valve development is not rescued to the dramatic extent as with removal of IND. However, the additional removal of both SHP and ALC activities from *ind ful* fruit further restores their elongation compared to wild-type fruit (Figures 4C and 4D). Therefore, the primary role of FUL is to restrict IND, ALC, and SHP activity to the valve edge, which allows normal growth and differentiation elsewhere throughout the valve (Figure 6A). On the other hand, our results also suggest that FUL has additional roles in fruit cell type specification, as indicated by the lack of proper differentiation of the inner valve epidermis in both *ful* and *ind alc shp1 shp2 ful* mutant fruit (Figures 4P and 4Q, data not shown).

While FUL acts to limit margin differentiation to the valve edge, it has recently been shown that margin development is restricted to the replum border by the REPLUMLESS (RPL) homeodomain protein (Roeder et al., 2003). Just as the valve growth defects of *ful* fruit can be largely rescued by the removal of ectopic IND, SHP, and ALC activities, replum development in *rpl* mutant fruit can be largely restored by removal of ectopic SHP activity. This implies that there is a preestablished pattern underlying valve and replum development, which is subsequently overlaid by the valve margin pattern elements. Thus, we propose that the mechanism responsible for efficient fruit dehiscence involves negative regulation of the valve margin identity genes by FUL in the valves, and by RPL in the replum, ensuring that margin differentiation occurs within a narrow band of cells at the valve/replum boundary (Figure 6A). This would allow precise stripes of *IND* expression to form at the valve margin through activation of *IND* by SHP within the margin and restriction by FUL and RPL on the valve and replum sides, respectively.

Specification of the Lignified Valve Cell Layer

One of the key components of the spring-loaded mechanism for seed dispersal is the formation of a single cell layer of lignified valve cells, and yet the genes that specify this cell layer have remained largely mysterious. The only gene thus far known to be involved in the initial specification of this cell layer is *AGAMOUS*, which encodes a MADS-domain transcription factor that positively regulates *SHP* expression (Yanofsky et al., 1990; Savidge et al., 1995; Flanagan et al., 1996; Alvarez and Smyth, 1999). We propose that IND, SHP, ALC, and FUL all contribute to the later differentiation of this cell layer since lignification is only absent when the activities of all five factors are eliminated in the *ind alc shp1 shp2 ful* quintuple mutant (Figure 5C). These data suggest a model in which IND, SHP, ALC and FUL act redundantly to specify the lignified valve layer (Figures 6B and 5C). An alternative possibility is that FUL is the primary determinant of this cell layer, and that its presence in *ful* mutant fruit is due to the activity of IND, SHP, and ALC. It was surprising to find that the same factors that are responsible for fruit opening at the valve margin are also required for the development of the lignified valve layer—revealing that differentiation of the three cell types necessary for seed dispersal is largely controlled by the same suite of genes.

Further insights have come from inspection of the loss of IND, ALC, and SHP activity on the *FUL* expression domain (Figures 5E and 5F). As noted above (Figure 6A), RPL restricts valve margin development from the replum (Roeder et al., 2003), and we have previously hypothesized that SHP activity in the margin might reciprocally restrict replum development (Ferrándiz et al., 2000b; Figure 6B). Consistent with this idea is the observation that the valve domain of *FUL* expression retracts in both *ind* and *shp1 shp2* fruit, pulling slightly away from the margins (Figure 5E; Ferrándiz et al., 2000b). One explanation for this observation is that IND and SHP normally restrict the replum domain of a hypothetical factor from the valve margin (Figure 6B). Expansion of such a factor in *ind* and *shp1 shp2* fruit could lead to the observed retraction of the *FUL* expression domain. Interestingly, loss of IND, SHP, and ALC activity together leads to an even further retraction of *FUL* expression from the valve margin in *ind alc shp1 shp2* fruit (Figure 5F), which correlates with the loss of lignification of the lignified valve layer in the cells closest to the replum (Figures 5C and 6C). Thus, we hypothesize that IND, ALC, and SHP are all involved in negatively regulating a replum factor (Figure 6B), the expansion of which leads to the retraction of *FUL* and the partial loss of lignification in the lignified valve layer of *ind alc shp1 shp2* fruit (Figures 6B and 6C). Whether all five factors act redundantly or FUL is the primary determinant in specifying this cell layer, both models are supported by the hypothesis that a replum factor is restricted by IND, SHP, and ALC activity. In future studies, it will be interesting to explore the possibility that RPL may act as part of this hypothetical replum factor.

Agricultural Importance of Pod Shatter

Controlling seed dispersal remains an important challenge for growers of many agricultural crops worldwide. Efforts to optimize harvest yield are particularly vital for oilseed crop plants such as canola (*Brassica napus*, *B. rapa*), where pod shatter causes average annual losses of 20% and up to 50% under adverse weather conditions (Child et al., 1998; MacLeod 1981). Recent studies have demonstrated that downregulation of the *IND* ortholog in canola leads to a complete loss of fruit dehiscence (Guy Vancanneyt, personal communication), suggesting that a detailed understanding of the genes controlling fruit dehiscence in *Arabidopsis* should lead to improved strategies for controlling seed dispersal in important crop plants.

Now that we are beginning to understand the regula-

tory networks underlying the development of the three fruit cell types important for dehiscence, it is also possible to investigate the extent to which differences in IND, SHP, ALC, and FUL activity account for natural variation in pod shatter, as well as to trace the origins of these characteristic elements of dry, dehiscent fruit in evolutionary history.

Experimental Procedures

Plants

Mutant alleles of *IND* and *ALC* were obtained through ethyl methanesulphonate mutagenesis as previously described (Liljegren et al., 2000). The *ind-2* allele contains a single nucleotide deletion within codon 26, which results in a frameshift and production of a truncated protein of 35 amino acids. The *ind-1* and *ind-3* alleles contain nucleotide substitutions within codons 112 and 99, which change a leucine to a phenylalanine and an arginine to a histidine, respectively. The *ind-4* and *ind-5* alleles contains nucleotide substitutions within codons 63 and 13, which change a glutamine and a tryptophan to stop codons, causing production of truncated proteins of 62 and 12 amino acids, respectively. The *alc-2* mutation contains a nucleotide substitution at the splice donor site of the third intron, which should disrupt splicing of the transcript region encoding the second helix of the bHLH domain. The *ind-2* and *alc-2* alleles were backcrossed three times to Ler and used for subsequent genetic analyses, along with the *shp1-1*, *shp2-1*, and *ful-5* alleles (Liljegren et al., 2000, Kempin et al., 1997, Ferrándiz et al., 2000a).

Mutant Genotyping

Plants homozygous for the *ind-2* and/or *alc-2* alleles were detected with CAPS (cleaved amplified polymorphic sequence; Konieczny and Ausubel, 1993) markers based on an AluI site abolished by the *ind-2* mutation and an AseI site introduced by the *alc-2* mutation. The *shp1-1* and *shp2-1* mutations were detected as described previously (Liljegren et al., 2000).

cDNA Analysis

To examine the transcripts produced at the *IND* locus, 5′ and 3′ RACE-PCR (Roche) were performed as described by the manufacturer using total or polyA RNA, respectively, as template. For 5′ RACE, 5′-GAGTTGTGGTAATAACAAAGGTAAG-3′ was used in the reverse transcriptase reaction, and additional nested oligos 5′-GGCTTCGTCGAGCATGGAAGC-3′ and 5′-GAGCAACCACCGTCT GAGGATCG-3′ were used in subsequent rounds of PCR. For 3′ RACE, oligo dT was used in the reverse transcriptase reaction, and the nested primer 5′-CCCTGCCACGGTCCCTAAGC-3′ in a subsequent round of PCR. The resulting fragments were cloned into pCR2.1 (Invitrogen) and sequenced. Analysis of *IND* cDNA clones derived from 5′ and 3′ RACE-PCR suggests that the *IND* transcript is 751 nucleotides (nt), with a 510 nt open reading frame, and 5′ and 3′ untranslated regions of 40 and 201 nt, respectively. Further support for the assigned open reading frame is provided by an *IND* EST (AF488578).

Marker Analyses

To isolate flanking sequence from the GT140 marker (Sundaresan et al., 1995), TAIL (Thermal Asymmetric Interlaced)/PCR was performed using nested oligos specific for the left and right transposon borders and degenerate primers as described previously (Tsugeki et al. 1996). The transposon insertion was detected 2782 nucleotides 5′ of the predicted ATG of At4g00120, and creates a duplication of 8 bp (GTATTTGC) flanking the insertion site.

The YJ80 enhancer trap line was generated by *Agrobacterium*-mediated transformation with the plasmid pOCA-28-15-991 (Eshed et al., 1999). Transgenic plants containing YJ80, GT140, YJ36 (Liljegren et al., 2000), or a *FUL* marker (Gu et al., 1998) were crossed into mutant plants. For β-glucuronidase expression analyses, fruit from wild-type and mutant plants were fixed, sectioned, and stained as described (Blázquez et al., 1997) with minor modifications.

Generation of Transgenic Plants

Using genomic DNA from the GT140 insertion line as a template, a 2.9 kb region spanning from 180 nucleotides upstream of the predicted At4g00120 translational start site and extending into the Ds insertion element was PCR amplified. This fragment was cloned into pCR2.1 (Invitrogen), then excised as a SalI/BamHI fragment and cloned into the plant transformation vector, pBI101.3. 17 of 38 transgenic T1 lines produced indehiscent fruit.

A 3.4 kb genomic region of *IND*, extending 2740 bases 5′ and 480 bases 3′ of the coding region, was PCR amplified using Columbia DNA as a template. This fragment was cloned into pCR2.1, then excised as an XbaI fragment and cloned into the pEL112 plant transformation vector (Eric Lam). Basta-resistant transgenic plants exhibiting a complemented phenotype were PCR analyzed to confirm that they were homozygous for the *ind-2* allele.

A full-length *IND* cDNA was PCR amplified with the oligos (5′-CGTCGACGATGAAAATGGAAAATGGTATGTATA-3′ and 5′-CGGAT CCGTTCATCAGGGTTGGGAGTTGTG-3′) using Columbia DNA as a template. After cloning this product into pCR2.1, a SalI/BamHI fragment containing the *IND* cDNA was cloned into the pBIN-JIT vector (Ferrándiz et al., 2000b). The resulting construct placed *IND* under the control of a tandem repeat of the 35S promoter.

Microscopy and Histology

Wild-type (Landsberg erecta ecotype), mutant, and transgenic fruit and flowers were fixed, prepared, and analyzed by scanning electron microscopy as previously described (Liljegren et al., 2000). Tissue fixation and phloroglucinol staining of paraplast sections (8 or 10 μm) from late stage 17 fruit were done as described (Liljegren et al., 2000). Plastic sections (3 μm) were prepared with JB4 resin (Electron Microscopy Sciences) as described (Roeder et al., 2003) from the tenth stage 17 fruit on wild-type and mutant influorescences.

In Situ Hybridization

Wild-type and mutant sections were hybridized with antisense or sense RNA as described (Ferrándiz et al., 2000b). The *IND* probe was synthesized with T7 RNA polymerase from a SalI-digested pINDAS template to generate a 328 nucleotide antisense transcript encompassing the 5′ region through part of the first helix of the bHLH domain. pINDAS was created by ligating the *IND* product PCR amplified from Colombia DNA with 5′-GAGCAACCACCGTCTGAGG ATCG-3′ and 5′-CGTCGACGATGAAAATGGAAAATGGTATGTATA-3′ into the pCR2.1 vector.

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