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Thomas Girin, Pauline Stephenson, Cassandra M P Goldsack, Sherry A Kempin, Amandine Perez, et al.. Brassicaceae INDEHISCENT genes specify valve margin cell fate and repress replum formation. The Plant Journal, 2010, 63 (2), pp.329-338. 10.1111/j.1365-313X.2010.04244.x . hal-01204205

HAL Id: hal-01204205

<https://hal.science/hal-01204205v1>

Submitted on 30 May 2020

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Brassicaceae *INDEHISCENT* genes specify valve margin cell fate and repress replum formation

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Received 12 February 2010; revised 9 April 2010; accepted 21 April 2010; published online 26 May 2010.

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SUMMARY

Members of the Brassicaceae family, including *Arabidopsis thaliana* and oilseed rape (*Brassica napus*), produce dry fruits that open upon maturity along a specialised tissue called the valve margin. Proper development of the valve margin in *Arabidopsis* is dependent on the *INDEHISCENT* (*IND*) gene, the role of which in genetic and hormonal regulation has been thoroughly characterised. Here we perform phylogenetic comparison of *IND* genes in *Arabidopsis* and *Brassica* to identify conserved regulatory sequences that are responsible for specific expression at the valve margin. In addition we have taken a comparative development approach to demonstrate that the *BraA.IND.a* and *BoIC.IND.a* genes from *B. rapa* and *B. oleracea* share identical function with *Arabidopsis IND* since ethyl methanesulphonate (EMS) mutant alleles and silenced transgenic lines have valve margin defects. Furthermore we show that the degree of these defects can be fine-tuned for crop improvement. Wild-type *Arabidopsis* produces an outer replum composed of about six cell files at the medial region of the fruits, whereas *Brassica* fruits lack this tissue. A strong loss-of-function *braA.ind.a* mutant gained outer replum tissue in addition to its defect in valve margin development. An enlargement of replum size was also observed in the *Arabidopsis ind* mutant suggesting a general role of Brassicaceae *IND* genes in preventing valve margin cells from adopting replum identity.

Keywords: Brassicaceae, *INDEHISCENT*, fruit development, tissue patterning, promoter element conservation, tissue specificity.

INTRODUCTION

During evolution, flowering plants have acquired a range of ways by which to effectively disperse their seeds and in this way optimise survival of the next generation. These mechanisms are often achieved by the formation of specific tissues that allow the fruit to efficiently release the seeds at maturity. *Arabidopsis* and *Brassica* species are members of the Brassicaceae family and produce inflorescences with cruciform flowers. Brassicaceae fruits develop as siliques in the centre of the flowers and elongate upon fertilisation to form a number of specialised tissues including the valves (or seed pod walls), a central replum and valve margins that form at the valve/replum borders where fruit opening will take place in a process known as dehiscence or pod shatter. This process is the result of highly coordinated processes,

which allow the separation of the valves from the replum and lead to the timely dispersal of mature seeds (Roberts *et al.*, 2002; Balanzá *et al.*, 2006; Østergaard, 2009; Girin *et al.*, 2009). Upon fruit maturation, the valve margins mediate fruit opening through degradation of a pectin-rich separation layer by secreted cell wall-degrading enzymes such as polygalacturonases (Petersen *et al.*, 1996; Spence *et al.*, 1996; Degan *et al.*, 2001; Ogawa *et al.*, 2009).

Setting up the overall patterning programme of the Brassicaceae fruit is a prerequisite for proper development, and several of the key genetic factors from *Arabidopsis* have been identified (Dinneny and Yanofsky, 2005; Alonso-Cantabrana *et al.*, 2007) (Fig. 1). Replum tissue is specified by the *REPLUMLESS* (*RPL*) gene, which acts by limiting the

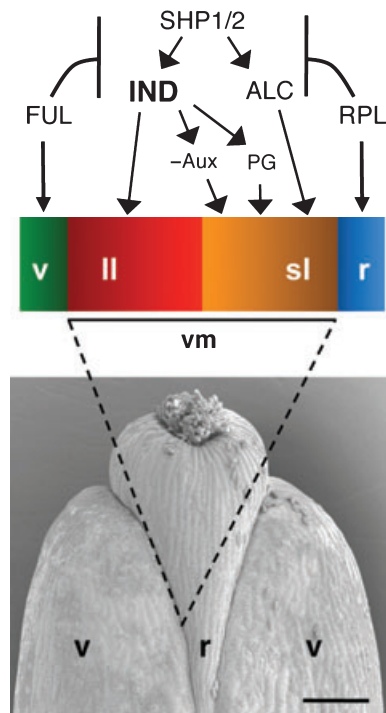


Figure 1. Overview of current knowledge on valve margin formation in *Arabidopsis*.

Top: Schematic outline of genetic and hormonal regulation of development of the lignified layer (red) and separation layer (orange). Valve and replum are indicated in green and blue, respectively.

Bottom: Scanning electron micrograph of the apical parts of a fruit from *Arabidopsis thaliana* ecotype *Landsberg erecta*. FUL, FRUITFULL; SHP1/2, SHATTERPROOF 1 and 2; RPL, REPLUMLESS; IND, INDEHISCENT; ALC, ALCATRAZ; PG, Polygalacturonase; -Aux, auxin minimum; v, valve; ll, lignified layer; sl, separation layer; r, replum; vm, valve margin. Bar = 200 μ m.

expression of valve margin identity genes *SHATTERPROOF1* and *-2* (*SHP1/2*), *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) to the narrow stripes where valve margins will form (Liljegren *et al.*, 2000, 2004; Roeder *et al.*, 2003; Rajani and Sundaresan, 2001). In the valves, the *FRUITFULL* (*FUL*) gene is required for post-fertilisation elongation of the fruit (Gu *et al.*, 1998) and acts similarly to *RPL* by repressing *SHP1/2* and *IND* gene activity (Ferrández *et al.*, 2000).

It was recently found that this genetic network controls distribution of the phytohormone auxin to ensure accurate fruit patterning (Sorefan *et al.*, 2009). In wild-type fruits, local depletion of auxin at the valve margin is required for specification of the separation layer where fruit opening takes place. *IND* mediates the formation of this auxin minimum by regulating auxin transport (Sorefan *et al.*, 2009).

Although efficient seed dispersal is an advantage for plants growing in the wild, asynchronous pod shatter presents a serious problem for oilseed rape production, with annual losses of 11–25% (Price *et al.*, 1996). The elucidation of the genetic interactions during *Arabidopsis*

fruit development has provided some clear directions on how to reduce asynchronous pod shatter in oilseed rape. We have previously shown in *B. juncea* that ectopic expression of the *Arabidopsis FRUITFULL* (*FUL*) gene under control of the Cauliflower Mosaic Virus 35S promoter results in completely pod shatter-resistant fruits similar to *35S::FUL* transgenic *Arabidopsis* fruits (Østergaard *et al.*, 2006; Ferrández *et al.*, 2000). This result confirmed that knowledge gained on modulating traits from *Arabidopsis* fruit development can be successfully transferred to oilseed rape. Nevertheless, a total block of shattering is not desirable since this will lead to significant seed damage when opening the fruits, and it is clear that careful fine-tuning is required to optimise this trait.

Here we compare the function of *IND* genes identified in *Brassica* species with the *IND* gene of *Arabidopsis* (abbreviated *AtIND* hereafter). In addition to a universal role in promoting valve margin development, analysis of strong loss-of-function *braA.ind.a* and *atind* mutants revealed a function for Brassicaceae *IND* genes in repressing replum formation. We also demonstrate that the activity of these genes can be modified to either completely or partially inhibit valve margin formation with potential for oilseed rape breeding, and we identify a region upstream in the promoter of *Arabidopsis* and *Brassica* genes that is sufficient for valve margin-specific expression of the *IND* genes in the fruit.

RESULTS

Diploid *Brassica* genomes contain one copy of a putative *INDEHISCENT* orthologous gene

Based on synteny between *Arabidopsis* and *B. rapa* genomes, we identified 12 bacterial artificial chromosome (BAC) clones from *B. rapa* ssp. *pekinensis* (variety Chiifu) that were likely to contain a potential orthologue of the *AtIND* gene. Presence of an *AtIND* homologous gene was confirmed in all the clones by PCR, and sequencing of several independent PCR products revealed only one copy of the gene. Furthermore, only this gene was identified by sequencing of PCR products obtained with degenerate primers on genomic DNA and cDNA. This sequence was subsequently used to design primers for direct BAC sequencing to obtain the entire gene sequence including the predicted open reading frame (ORF) of 516 bp, 3110 bp of promoter sequence and 1345 bp of sequence downstream of the predicted stop codon (Fig. 2a). The gene was named *BraA.IND.a* in accordance with the standardised *Brassica* gene nomenclature (Østergaard and King, 2008) and the sequence has been deposited in GenBank (accession number GU386357). The *B. rapa* genome is called the A genome and consists of 10 chromosomes (A1–A10). Based on the mapping of BAC clones, we found that the *BraA.IND.a* gene is located on *B. rapa* chromosome A3 corresponding to the

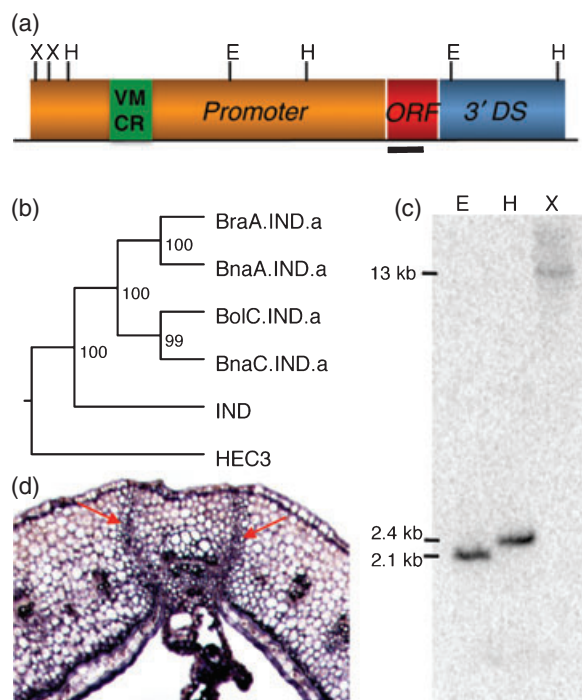


Figure 2. Sequence, copy number and expression analysis of *Brassica* and *Arabidopsis* *IND* genes.

(a) Gene structure of *AtIND* and *BraA.IND.a*. The promoter region is shown in orange, the open reading frame (ORF) is in red and the 3' downstream sequence (3' DS) of the gene is indicated in blue. The green region in the promoter, annotated VMCR (valve margin *cis* region), indicates the conserved region between *Arabidopsis* and *Brassica* *IND* genes containing *cis* regulatory sequences. The line below the ORF indicates the position of the probe used for the Southern blot in (c) and *in situ* hybridisation in (d).

(b) Phylogenetic tree showing the sequence relationship between *Brassica* and *Arabidopsis* *IND* ORFs. The *IND*-related *Arabidopsis* *HECATE3* (*HEC3*) gene (Gremski *et al.*, 2007) was used as an outgroup.

(c) Southern blot analysis using gDNA from *B. rapa* variety R-o-18. Three restriction enzymes were used (E, *EcoRI*; H, *HindIII*; X, *XbaI*). Positions of restriction sites for these enzymes are shown in (a).

(d) *In situ* hybridisation showing expression at the valve margin of a *B. rapa* R-o-18 gynoecium at fertilisation (stage 12/13).

syntenic region of *Arabidopsis* chromosome IV where *AtIND* resides (Parkin *et al.*, 2005). As for *AtIND*, the ORF of the *B. rapa* homologue is predicted to be contained within a single exon (Fig. 2a). The encoded BraA.IND.a protein shares 98% amino acid identity with the *AtIND* protein in the DNA-binding bHLH domain and 66% overall (Fig. S2 in Supporting Information).

After divergence of the *Arabidopsis* and *Brassica* genera about 20 million years ago (Yang *et al.*, 1999; Koch *et al.*, 2000; Parkin *et al.*, 2005), the diploid *Brassica* genome underwent a triplication event (Lysak *et al.*, 2005). Consequently, it is often the case that single-copy *Arabidopsis* genes exist as three paralogues in diploid *Brassica* genomes. To determine the number of *BraA.IND* genes, we performed a Southern blot analysis on genomic DNA from *B. rapa* using three restriction enzymes. Only one band

was observed in each lane at the expected size, which suggests that *BraA.IND.a* is present as a single-copy gene in the *B. rapa* genome (Fig. 2c). This observation is in agreement with the sequencing data, and it therefore suggests that *B. rapa* has lost two copies of this gene since the genome triplication event.

To ascertain whether this apparent gene loss is conserved among other members of the *Brassica* genus, we performed PCR with conserved oligos and isolated a sequence from *B. oleracea* genomic DNA with a high level of identity to the ORFs of *AtIND* (77%) and *BraA.IND.a* (95%). This gene was named *BolC.IND.a* (GenBank accession number GU386356), and similar to the situation in *B. rapa*, only a single sequence was identified in *B. oleracea* when the PCR products were sequenced.

B. napus (oilseed rape) was formed by hybridisation between *B. rapa* and *B. oleracea*. As a consequence *B. napus* is allotetraploid consisting of two genomes; the A genome from *B. rapa* and the C genome from *B. oleracea* (U. N., 1935). In the GenBank database, we identified two putative *IND* sequences from *B. napus* (HB416515 and HB416517). A phylogenetic analysis revealed that instead of being most closely related to each other, the two *B. napus* *IND*-like sequences cluster with *BraA.IND.a* and *BolC.IND.a* (Figs 2b and S2). This analysis furthermore shows that the *Brassica* *IND* genes identified here more closely resemble *AtIND* than the closest *Arabidopsis* homologue, *HECATE3* (*HEC3*) (Pires and Dolan, 2009).

We subsequently mapped the chromosomal location of the two *B. napus* *IND* homologues in the Tapidor/Ningyou7 doubled haploid mapping population (Qiu *et al.*, 2006). This analysis showed that the gene most closely related to *BraA.IND.a* is positioned on *B. napus* chromosome A3 (HB416515, named *BnaA.IND.a*) whilst the one most closely related to *BolC.IND.a* is positioned on *B. napus* chromosome C3 (HB416517, named *BnaC.IND.a*). Together, these results demonstrate that the two *IND* sequences identified in *B. napus* originate from each of the two diploid progenitors (*B. rapa* and *B. oleracea*).

Conserved upstream sequence in the *IND* promoter is sufficient for valve margin expression and regulation by *SHP* and *FUL* genes

In *Arabidopsis*, *AtIND* is required for valve margin formation and both *in situ* hybridisation and reporter lines have demonstrated that *AtIND* is indeed expressed in this tissue (Liljegren *et al.*, 2004; Sorefan *et al.*, 2009). In accordance with this, we performed *in situ* hybridisation on *B. rapa* fruits and found that the *BraA.IND.a* gene was expressed in valve margin cells (Fig. 2d). This result suggests that the function of the *Arabidopsis* and *Brassica* *IND* genes is conserved.

By aligning the promoter regions of *AtIND* with *BraA.IND.a*, we identified a 400-bp region about 1.8–2.2 kb upstream of the start codon with 86% sequence identity (Fig. S3). This is

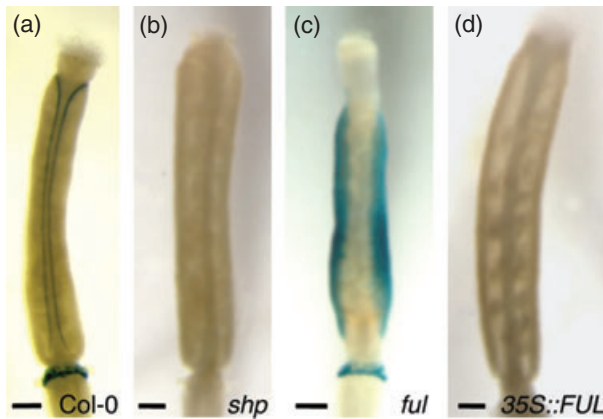


Figure 3. A 400-bp fragment of the *AtIND* promoter is sufficient for valve margin expression. p400-m35S::GUS expression in wild-type Col-0 (a), *shp1/2* double mutant (b), *ful-2* mutant (c) and 35S::FUL transgenic line (d). Bar = 500 µm.

remarkably high for a non-coding region and suggests either that it in fact does encode transcribed sequence or that it harbours regulatory sequences that are responsible for its expression pattern. By northern blot analysis using RNA from Arabidopsis fruit we were unable to detect any signal indicative of expression of upstream regions including the 400-bp sequence, although a clear signal was observed when using a probe in the ORF (Fig. S4).

From Arabidopsis genomic DNA, we cloned the 400-bp region in front of a minimal 35S promoter driving the GUS reporter gene in the pDW294 vector (Busch *et al.*, 1999). This construct, p400-m35S::GUS, was transformed into Arabidopsis and 28 out of 37 lines showed expression in the valve margin region (Fig. 3a) whereas the m35S::GUS alone did not (pDW294 in Fig. S5a). Staining in nectaries was occasionally observed, but this was also the case for the pDW294 without any *IND* promoter sequence (Fig. S5a).

Expression of *AtIND* at the valve margin was previously demonstrated to depend on the *SHATTERPROOF1* and *SHATTERPROOF2* (*SHP1/2*) genes, whereas *AtIND* expression in the valves is prevented by the activity of the *FRUITFULL* (*FUL*) gene (Liljegren *et al.*, 2000; Ferrándiz *et al.*, 2000). These data were obtained with an enhancer trap line, GT140, in which the GUS reporter is inserted 3 kb upstream of the *AtIND* gene (Liljegren *et al.*, 2000). Crosses of the p400-m35S::GUS line to the *shp1/2* double mutant, *ful* single mutant and the 35S::FUL transgenic line revealed that regulation of this construct is indistinguishable from the regulation of GT140 expression (Fig. 3b–d). These results confirm that regulation of *AtIND* expression by *SHP1/2* and *FUL* occurs via elements in this 400-bp region.

Positioned close to the centre of the 400-bp region is a CArG box element, which is conserved among the Arabidopsis and *Brassica IND* genes (Fig. S3). CArG boxes are binding sites for the transcription factor family of MADS box

proteins (Riechmann *et al.*, 1996). Since both *SHP1/2* and *FUL* belong to this family, we speculated whether this sequence is required for the valve margin expression of *AtIND*. To this end we mutated the CArG box sequence, but found that it had no effect on the valve margin specificity (Fig. S5d). Moreover, a 20-bp probe containing this element was not bound by either *SHP1/2* or *FUL* protein in an *in vitro* gelshift assay (data not shown).

Overlapping fragments containing the 5' half or the 3' half of the 400-bp region in front of m35S::GUS showed no valve margin expression (Fig. S5b and c) suggesting that interactions between factors that bind different elements in the 400-bp region are involved in regulating tissue-specificity.

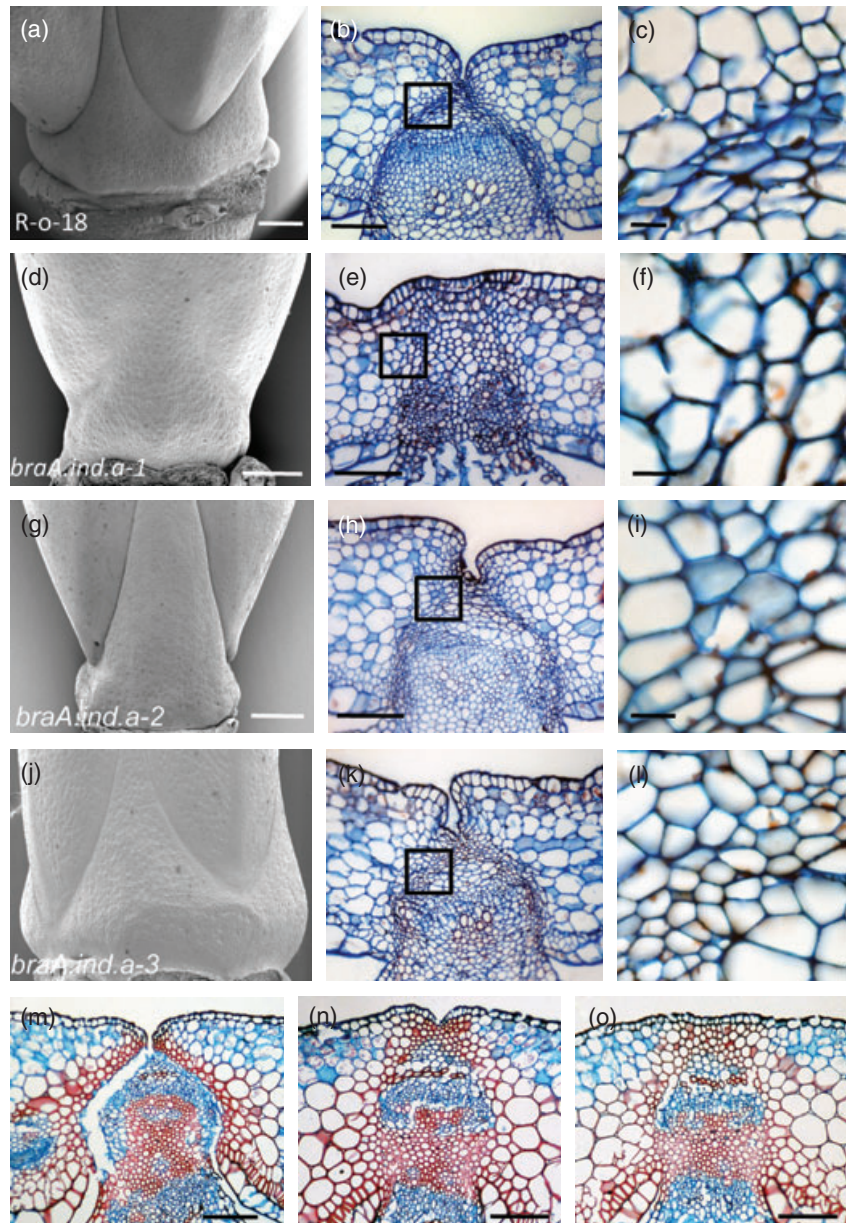
***Brassica IND* genes function to specify valve margin and repress replum formation**

Similarities in sequences and expression patterns of genes from different species are good indications that they have similar function. However, the strongest evidence for such conservation comes from analysis of loss-of-function mutants. We used the recently developed reverse genetics platform for TILLING (targeting induced local lesions in genomes) in *B. rapa* variety R-o-18 (Stephenson *et al.*, 2010) to identify a number of lines with point mutations in the *BraA.IND.a* gene. Three alleles were selected for further analysis and grown to homozygosity. The *braA.ind.a-1* allele has a premature stop codon at the beginning of the bHLH domain (Q102X) expected to completely disrupt the function of the gene. *braA.ind.a-2* has a mis-sense mutation in the *IND*-specific N-terminal region changing serine to phenylalanine (S37F), while *braA.ind.a-3* has a mis-sense mutation in a region sharing homology with the *HEC* genes (Gremski *et al.*, 2007) that leads to a change of glutamic acid to lysine (E82K).

Homozygous *braA.ind.a* mutants showed a dramatic loss of valve margin formation compared with wild-type R-o-18 (Fig. 4a–f), which was visible both by scanning electron microscopy (SEM) and tissue-staining of cross sections. It was not possible to identify a valve margin defect in the two mis-sense mutant alleles, *braA.ind.a-2* and *braA.ind.a-3*, by SEM (Fig. 4g,j); however, close examination of the valve margin region in cross sections revealed a lack of small cells that are characteristic of this tissue, thus suggesting that both of these alleles have partially lost valve margin identity (Fig. 4h,i,k,l).

To test if the *BolC.IND.a* gene has the same function as *BraA.IND.a* and *AtIND*, we adopted an RNA silencing approach. The *BolC.IND.a* ORF was cloned as inverted tandems with an intron sequence in between and expressed behind the 35S promoter. This construct was transformed into *B. oleracea* genotype DH1012 by *Agrobacterium tumefaciens*-mediated transformation and transgenic shoot regeneration. Out of 13 primary transgenic plants, seven exhibited a strong defect in fruit opening compared with

Figure 4. Mutations in the *Brassica IND* gene lead to defects in valve margin formation. Analysis of wild-type *B. rapa* var. R-o-18 (a–c), *braA.ind.a-1* (d–f), *braA.ind.a-2* (g–i) and *braA.ind.a-3* (j–l) mutant alleles at developmental stage 17A by scanning electron microscopy (a, d, g, j) and Alcian Blue/Saffranin O-staining of cross sections (b, c, e, f, h, i, k, l). (m)–(o) Cross sections of stage-17C fruits from *B. oleracea* genotype DH1012 untransformed (m) and from two independent lines transformed with a *BoIC.IND.a* silencing construct (n, o) stained by Alcian Blue/Saffranin O. Bars = 500 μm (a, d, g, j), 100 μm (b, e, h, k, m, n, o), 20 μm (c, f, i, l).



both the untransformed tissue culture-regenerated controls and wild-type *B. oleracea* DH1012 fruits. The identical phenotype was observed in progeny of the primary transgenic lines at a 3:1 ratio. We characterised two transgenic lines in more detail by tissue staining of cross sections, and found that fruits from these lines had lost the ability to form valve margin tissue (Fig. 4m–o). *BoIC.IND.a* RNA levels were indeed significantly reduced in these lines (Fig. S6), and the results therefore demonstrate that *IND* gene function is conserved between members of the *Brassica* genus.

When analysing the strong *braA.ind.a-1* mutant allele, we observed that in addition to valve margin defects these fruits had gained an outer replum, which is not observed in the wild type (Fig. 5a,b,e,f) or in the weaker mutant alleles. A

similar gain-of-replum effect was observed in one of the two *BoIC.IND.a* RNA interference (RNAi) lines compared with untransformed DH1012 (Fig. 4m and o). This observation shows that *Brassica IND* genes are repressors of replum formation, and to test if a similar function could be revealed for *AtIND* in *Arabidopsis*, we analysed replum morphology of *ind* versus wild-type *Arabidopsis* fruits. SEM photographs and measurements of replum width and counting number of cell files (Figs 5c,d and S7) revealed a significant enlargement of replum tissue with additional cell files in the *ind* mutant background. Together these results demonstrate that *IND* genes from *Brassica* and *Arabidopsis* function to promote valve margin development and repress replum formation.

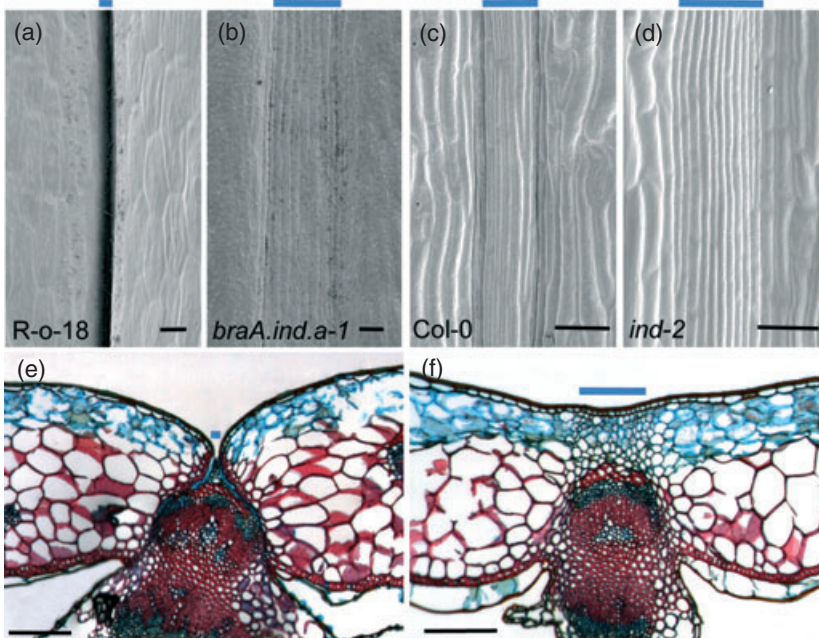


Figure 5. Replum formation occurs in the *braA.ind.a-1* mutant reminiscent of *Arabidopsis* wild-type fruits.

Scanning electron micrographs of replum/valve margin regions of stage-17A fruits from *B. rapa* R-o-18 (a), *braA.ind.a-1* (b), *Arabidopsis thaliana* Col-0 (c) and *ind-2* mutant (d). Alcian Blue/Saffranin O-staining of cross sections (e, f) of stage-17C fruits from *B. rapa* R-o-18 (e) and *braA.ind.a-1* (f). Blue lines indicate the width of the replum in (a)–(d). Bars = 200 μ m (e, f), 20 μ m (a–d).

DISCUSSION

Fruits from members of the Brassicaceae encapsulate and nurture the seeds from fertilisation until maturation. At maturity, Brassicaceae fruits open along a specific tissue called the valve margin, allowing the separation of the valves from the replum. This dehiscence process is carefully controlled so that opening of the fruit will only take place when the seeds are fully developed. In *Arabidopsis*, it has previously been demonstrated that the bHLH transcription factor IND is required for valve margin formation (Liljegren *et al.*, 2004; Sorefan *et al.*, 2009), and here we show that IND orthologous genes in members of the *Brassica* genus also have this function. We also show an additional function of IND from both *Arabidopsis* and *B. rapa* in repressing the formation of an adjacent tissue, the replum.

The *BraA.IND.a* gene in *B. rapa* was completely sequenced including the single-exon ORF, more than 3 kb of sequence upstream of the predicted start codon and around 1.3 kb downstream of the predicted stop codon. Expression of *BraA.IND.a* was found at the valve margin of *B. rapa* fruits, in agreement with a role in specifying this tissue.

Southern blot analysis indicated that *BraA.IND.a* exists as a single-copy gene in *B. rapa*. Since the diploid *Brassica* genome underwent a triplication event after diverging from the common ancestor of *Arabidopsis* and *Brassica*, it is most likely that the *Brassica* diploid genome has lost two copies of the gene. Previous work has shown the dramatic developmental effects that ectopic expression of *AtIND* has in *Arabidopsis* (Sorefan *et al.*, 2009). Perhaps accumulation of mutations leading to mis-expression of *BraA.IND* genes provided a substantial disadvantage for such individuals

and was therefore selected against by inactivation/deletion of the genes. In contrast, a high selective pressure on the expression pattern of the remaining gene may have ensured its specific activity at the valve margin.

Brassica napus (oilseed rape) originates from a cross between the diploid progenitors *B. rapa* and *B. oleracea* (U. N., 1935) which is likely to have taken place during human cultivation approximately 10 000 years ago. A phylogenetic analysis of *Brassica IND* cDNA sequences suggests that each of the two diploid genomes in *B. napus* contains one *IND* orthologue. This is consistent with *Brassica IND* genes existing as single copies in diploid *Brassica* species. Since the chromosomal location of the two *Brassica IND* genes identified here occur in equivalent regions of the respective genomes (chromosomes A3 and C3), the data suggest that *Brassica IND* gene loss may have happened soon after the genome triplication event and prior to diversification of *B. rapa* and *B. oleracea* about 3.7 million years ago (Inaba and Nishio, 2002).

We identified a 400-bp region in the promoters of *Arabidopsis* and *Brassica IND* genes that is highly conserved and sufficient for valve margin expression and regulation by factors that control *AtIND* expression in *Arabidopsis* (Fig. 3). Our results suggest that there may be at least two interacting *cis*-regulatory elements within this 400-bp sequence, both necessary for valve margin expression of the gene. It is likely that the valve margin-specific expression of *IND* genes is particularly sensitive to mutations in this region, as suggested by the high level of sequence conservation between Brassicaceae genera.

Since the *IND* gene is regulated by MADS box transcription factors (Liljegren *et al.*, 2000; Ferrández *et al.*, 2000), it

was intriguing that a MADS box recognition motif (CARG box) was identified in this region. However, reporter gene analysis showed that this sequence is not required for valve margin expression. A scan of the region for other known *cis*-regulatory sequences using the PLACE database (Higo *et al.*, 1999) did not reveal the presence of any obvious candidate elements, except for a non-canonical CARG box, which is, however, not conserved in *B. rapa*. Hence additional studies will be required to elucidate the sequences involved and the factors binding to them.

The function of the *BraA.IND.a* gene was unequivocally established by the identification of loss-of-function mutations in this gene in *B. rapa*. Fruits from the strong *braA.ind.a-1* mutant allele had completely lost valve margin formation, whereas weaker alleles had partial defects (Fig. 4). Likewise, silencing of the *BoIC.IND.a* gene by RNA silencing also led to fruits with strong defects in valve margin specification.

Fruits from the Brassicaceae family produce a replum at the medial plane of the fruit that connects to the valves via the valve margin tissue. At the interior the replum contains a large vascular bundle, and at the exterior Arabidopsis fruits produce an outer replum of approximately six files of highly elongated cells that are narrower than valve cells (Fig. 5c). This is in contrast to fruits from *Brassica* species that have little or no outer replum cell files (Fig. 5). However, in the strong *braA.ind.a-1* mutant allele and in a *BoIC.IND.a* RNAi line, we observed formation of outer replum tissue in addition to the defect in valve margin development, suggesting that *BraA.IND.a* is a repressor of replum formation. This observation was supported by the development of enlarged repla with additional cell files in an Arabidopsis *ind* mutant compared with wild type, and our data therefore show that *IND* genes from Brassicaceae are repressors of replum tissue formation. It may be possible to expand this statement to valve margin identity genes in general, since mutations in the *SHP* genes rescues the replum defect of a *rpl* mutant in Arabidopsis (Roeder *et al.*, 2003). The absence of replum tissue in the *braA.ind.a-2* and *braA.ind.a-3* mutants as well as in a *B. oleracea* RNAi line suggests that valve margin development is more sensitive to a reduction in *IND* activity than repression of replum cell identity.

Control of fruit dehiscence (or pod shatter) is of considerable agronomic importance as oilseed rape farmers lose 11–25% of their harvest each year due to asynchronous fruit opening and seeds falling to the ground in the field (Price *et al.*, 1996). We have previously shown that fruit-opening in *B. juncea* can be prevented by expressing the Arabidopsis *FUL* gene under control of the 35S promoter (Østergaard *et al.*, 2006). Although completely sealed *Brassica* fruits such as the 35S::*FUL* plants and the *braA.ind.a-1* mutant allele presented here are unsuitable for agronomic purposes, our data show that partial dehiscence can be

achieved by fine-tuning the level of *BraA.IND.a* activity. This finding may have important implications for future strategies for control of pod shatter either by introgression of the alleles presented here or by screening for similar mutations in existing *B. napus* TILLING populations (Wang *et al.*, 2008).

Over the last few decades, work in the model plant Arabidopsis has led to astounding leaps in our understanding of plant growth and development, and has in some instances pointed out clear directions for crop improvement, particularly in the closely related *Brassica* crop species. The results presented here demonstrate that the direction of this flow of information may be reversed, and that work in *Brassica* can reveal fundamental aspects of plant development which are unlikely to have been obtained by restricting the studies to Arabidopsis.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col-0) and Landsberg *erecta*, mutants and transgenics were grown in the glasshouse under long-day conditions (16 h light) at 21°C. Developmental stages of Arabidopsis flowers and fruits were defined as in Smyth *et al.* (1990).

Seeds of *B. rapa* var. R-o-18 wild type (Rusholme *et al.*, 2007) and *braA.ind.a* mutant alleles were sown in John Innes No. 2 compost and kept at 7°C with no light for 3 days before being transferred into the glasshouse at 18°C with 16 h light. The *braA.ind.a* mutants were obtained by TILLING in a *B. rapa* population described in Stephenson *et al.* (2010). Developmental stages for *B. rapa* flower and fruit developmental stages were defined based on Smyth *et al.* (1990), but slightly modified with the division of stage 14 into 14A and 14B and stage 17 into stage 17A, 17B and 17C (Fig. S1).

Identification of BACs containing the *BraA.IND.a* gene and verification by direct BAC DNA sequencing

A database of BAC end sequences from *B. rapa* ssp. *Pekinensis* (variety Chiifu) was searched at <http://atidb.org> using the accession number for the *AtIND* gene (*At4g00120*) to identify BAC clones that, based on alignment of their end sequences on the Arabidopsis genome, may contain potential orthologues of *AtIND*. Some of the BAC end sequences were highly similar to the *AtIND* sequence, and were used in combination with the Arabidopsis sequence and two putative *B. napus* *IND* sequences available in the GenBank database (HB416515 and HB416517) to design primers in conserved regions of the gene. These primers were then used to obtain the whole *BnaA.IND.a* sequence by sequencing directly, using both BAC DNA as template and PCR products obtained from BAC clones, genomic DNA and cDNA from *B. rapa*.

BnaA.IND.a and *BnaC.IND.a* mapping in a Tapidor/Ningyou7 population

BnaA.IND.a and *BnaC.IND.a* were mapped in the Tapidor/Ningyou 7, doubled haploid, *B. napus* population using single-strand conformation polymorphism (SSCP) analysis according to Bertin *et al.* (2005). Briefly, PCR products were generated using primers BIND-F (5'-TTGCCAACGTTGAGGTAGCT-3') and BIND-R (5'-CGCTGTGCTCATATCTGACC-3') and denatured immediately prior to resolution in

a non-denaturing gel system; 0.5 × MDE gel solution, 0.6 × TBE (Lonza Rockland, <http://www.lonza.com/>). After electrophoresis, bands were visualised by silver staining as described in Bassam *et al.* (1991). Genetic linkage mapping was performed using JOINMAP v.3.0 software (Stam, 1993).

Generation of GUS reporter lines

AtIND promoter fragments were cloned into the *HindIII* restriction site of the pDW294 plasmid (Busch *et al.*, 1999), thereby placing these fragments in front of a minimal Cauliflower Mosaic Virus 35S promoter driving expression of the β-glucuronidase gene (GUS). In p400-m35S::GUS the fragment was generated using primers IND125 (5'-AAGCTTTGGCAATACCTTAATGTCG-3') and IND126 (5'-AAGCTTGCCATAAGGAAGCATAAGTCG-3'); in p5VMCR primers IND125 and IND124 (5'-AAGCTTCTAAACCCACGGTTTCTTCG-3') were used whereas primers IND117 (5'-AAGCTTGTAAGAAAATGTGAAG-3') and IND116 (5'-AAGCTTTTTCGCTAACAAATTC-3') were used to generate the fragment for the p3VMCR construct. The pΔCARG construct was created by overlapping PCR, first generating two fragments using primers IND19 (5'-AAAAGGCC TTATGTTAATATCACCGTAGG-3') and INDNCO2 (5'-CCATGGCAAT TTAACATCATTGTTCTTC-3') together and INDNCO1 (5'-CCATGGTG TATAGGAGTTGTCAAAG-3') and IND110 (5'-AAAACCGGTGGGTT GGGAGTTGTGG-3') together. The entire fragment without the CARGbox was then generated using primers IND19 and IND116 (5'-AAGCTTTTTCGCTAACAAATTC-3') in the presence of the two partially overlapping fragments and subsequently cloned into to the *HindIII* site of pDW294 in the sense orientation with respect to the minimal 35S promoter. Transgenic plants were selected by kanamycin resistance after *A. tumefaciens*-mediated transformation into Arabidopsis (Col-0 ecotype).

Construct for *BolC.IND.a* silencing in *B. oleracea*

A DNA fragment containing the entire ORF of *BolC.IND.a* was amplified using primers BIND3 (5'-AAAAGCAGGCTATGTCTGGCT CAAAAGCAGATGC-3') and BIND4 (5'-AGAAAGCTGGGTTCCAGACA TAGGAGCTCCAAGC-3'). The resulting fragment was Gateway cloned into the pBRACT507 vector (<http://www.bract.org/constructsavailable/pBractVectors/Constructs/constructs.html>) to generate the pRNAi-IND plasmid. pBRACT507 is designed for RNA silencing, containing the inserts as inverted repeats with a short intron in between and expression driven by the 35S promoter.

Brassica oleracea transformation

A genetically uniform doubled haploid *B. oleracea* genotype DH 1012 (Sparrow *et al.*, 2004) was transformed using the *A. tumefaciens* strain *AGL1* (Lazo *et al.*, 1991) harbouring the pRNAi-IND plasmid. The transformation protocol was as described in Sparrow *et al.* (2006) and on the BRACT website (<http://www.bract.org>) with the following modifications: Explants were initially selected on a low kanamycin concentration (5 mg/L) to compensate for use of the weaker NOS promoter driving the *nptII* gene. Regenerated shoots were later isolated and maintained on 25 mg/L kanamycin. Several lines exhibited strong fruit opening defects, and four lines were chosen for phenotypic analysis of hemizygous primary transgenics and of homozygous individuals in the following generation.

Quantitative RT-PCR

RNA extraction and reverse transcription (RT) reaction from stage-12 gynocelia were performed as described in Girin *et al.* (2007). The quantitative (Q)-PCR reaction was carried out using SYBR Green JumpStart *Taq* ReadyMix (Sigma, <http://www.sigmaldrich.com/>) in a Chromo4 thermocycler (Bio-Rad, <http://www.bio-rad.com/>).

Specific primers used were: 5'-AACCGCCGTAACGTAAGGGTAAG-3' and 5'-TGTGTAATAACAAAGGCGAGAAGG-3' for *BolC.IND.a*, and 5'-TTCAATGTCCCTGCCATGTA-3' and 5'-GAGACGGAGGATAG CGTGAG-3' for the internal control *ACT2/7* (Marmagne *et al.*, 2010). Relative mRNA accumulation was calculated using the standard curve method (Rutledge and Côté, 2003), with all amplification efficiencies being above 1.8. Values in Fig. S6 correspond to the average of three biological repeats ± SD.

GUS staining

Fruits were pre-fixed in 90% acetone (4°C) for 20 min at room temperature. Samples were subsequently rinsed with the reaction buffer [50 mM Na₂PO₄ (pH 7.2), 0.2% Triton X-100, 5 mM ferrocyanide, 5 mM ferricyanide] and incubated overnight in reaction buffer with 2 mM X-Gluc at 37°C. To clear the samples, a series of ethanol washes was performed (50–95% in multiple steps) after incubation. GUS-stained tissue was examined under light microscopy.

Alcian blue/Safranin-O staining

Tissues were fixed for 6 h in an FAA solution (3.7% formaldehyde, 5% acetic acid, 50% ethanol) and subsequently dehydrated through an ethanol series. The tissues were cleared with Histoclear (National Diagnostics, <http://www.nationaldiagnostics.com/>) and embedded in paraffin. An RM2255 rotary microtome (Leica, <http://www.leica.com/>) was used to make 8 μm transverse stem sections. After deparaffinisation, sections were stained with an Alcian Blue 8Gx/Safranin-O solution [0.05% Alcian Blue 8Gx and 0.01% Safranin-O in 0.1 M acetate buffer (pH 5.0)] as described in Østergaard *et al.* (2006). Sections were examined under light microscopy.

Light microscopy

Light microscopy images were obtained using a MZ16 stereomicroscope (Leica) and the images were captured with a DFC280 digital camera (Leica). Measurements were performed on captured images using Leica Application Suite (LAS) software.

Scanning electron microscopy

Fruit were fixed for approximately 6 h at 25°C in FAA and subsequently dehydrated through an ethanol series. After critical point drying, tissues were coated with gold and examined in a Philips XL30 FEG microscope using an acceleration voltage of 3 kV.

Southern blot

Ten micrograms of genomic DNA from R-o-18 was completely digested with each restriction enzyme indicated in Fig. 2c and run on a 0.5% agarose gel. The Southern blotting protocol was as in Sambrook and Russell (2001). A ³²P-labelled probe was generated using a fragment of the *BraA.IND.a* ORF (positions 138–538) and the Random Prime Labelling System from Amersham Biosciences (<http://www.gelifsciences.com>). After hybridisation with this probe overnight at 65°C, the filter was washed in 0.2 × SSC at 65°C and exposed to a Storage Phosphor screen (Molecular Dynamics) for 7 days before visualisation in a Typhoon 9200 phosphorimager (Amersham Biosciences, <http://www.gelifsciences.com>).

Northern blot

Five micrograms of total RNA from Arabidopsis fruits was loaded on 1.5% agarose formaldehyde gel. Northern blotting was performed as described by Sambrook and Russell (2001). Preparation of probes, hybridisation and subsequent washes was as described above for Southern blotting.

In situ hybridisation

In situ hybridisation was performed essentially as described (Long *et al.*, 2002) on tissue from *B. rapa* var. R-o-18 fruits at developmental stage 12. Tissue fixation, embedding and sectioning was carried out as described above. For hybridisation, an antisense ribonucleotide probe of *BraA.IND.a* was synthesised (positions 538–138) by cloning the fragment in the pCR2.1 vector (Invitrogen, <http://www.invitrogen.com/>) and selection for antisense orientation with respect to the T7 promoter. The resulting plasmid was linearised by *SpeI* digestion and the antisense probe was prepared using T7 RNA polymerase.

Phylogenetic analysis

The nucleotide sequences of ORFs were manually aligned. The most parsimonious tree was found using the DNAPENNY programme implemented in the PHYLIP package (Felsenstein, 1989) and bootstrapped 100 times. The tree was rooted with *HEC3* (*At5g09750*).

ACKNOWLEDGEMENTS

We thank Kim Findlay at the John Innes Centre for assistance on SEM analysis and the John Innes Centre horticultural staff for their valuable and skilled help with general plant husbandry. We also wish to thank Nicolas Arnaud, George Averell and Sara Fuentes for discussions on the manuscript. This work was supported by grants BB/E006965/1 and BB/D018005/1 from the Biotechnology and Biological Sciences Research Council (BBSRC) to LØ and by a grant from the National Science Foundation to MFY.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Developmental stages of *B. rapa* R-o-18 flowers and fruits.

Figure S2. CLUSTALW alignment of the *Brassica* and *Arabidopsis* IND and the *Arabidopsis* HEC3 amino acid sequences based on the open reading frame sequences used for the phylogeny shown in Figure 2b.

Figure S3. Alignment of the 400-bp promoter region of *AtIND* and *BraA.IND.a*.

Figure S4. Northern blot analysis to test for expression of regions in the upstream region of the *IND* gene.

Figure S5. GUS reporter staining of fruit from various lines.

Figure S6. Expression analysis of *BoIC.IND.a* in stage-12 gynoecia.

Figure S7. Replum from *ind-2* mutant fruits are wider than wild-type fruits both in absolute width and in number of replum cell files.

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GenBank accession number: GU386357 (*BraA.IND.a*) and GU386356 (*BoIC.IND.a*).