Current Biology 21, 1215–1219, July 26, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.06.008

Report

The Same Regulatory Point Mutation Changed Seed-Dispersal Structures in Evolution and Domestication

Nicolas Arnaud,[1] Tom Lawrenson,[1] Lars Østergaard,[2,*] and Robert Sablowski[1,*]

[1]Department of Cell and Developmental Biology

[2]Department of Crop Genetics

John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

Summary

It is unclear whether gene regulatory changes that drive evolution at the population and species levels [1–3] can be extrapolated to higher taxonomic levels [4, 5]. Here, we investigated the role of *cis*-regulatory changes in fruit evolution within the Brassicaceae family. *REPLUMLESS* (*RPL*, At5g02030) controls development of the replum, a structure with an important role in fruit opening and seed dispersal [6]. We show that reduced repla resembling the *Arabidopsis rpl* mutant correlated across the Brassicaceae with a point mutation in a conserved *cis*-element of *RPL*. When introduced in *Arabidopsis*, this nucleotide change specifically reduced *RPL* expression and function in the fruit. Conversely, *Brassica RPL* containing the *Arabidopsis* version of the *cis*-element was sufficient to convert the *Brassica* replum to an *Arabidopsis*-like morphology. A mutation in the same nucleotide position of the same *cis*-element in a *RPL* ortholog has been independently selected to reduce seed dispersal during domestication of rice [7], in spite of its very different fruit anatomy. Thus, single-nucleotide regulatory mutations at the same position explain developmental variation in seed-dispersal structures at the population and family levels and suggest that the same genetic toolkit is relevant to domestication and natural evolution in widely diverged species.

Results and Discussion

Brassica and *Arabidopsis* RPL Show Variation at a Nucleotide Position Previously Implicated in the Regulation of Seed Shattering in Rice

The regulatory network that controls fruit opening is at least partly conserved between *Arabidopsis thaliana* and domesticated plants, in which the control of seed dispersal is a key feature [8, 9]. Replum morphology, however, differs between *Arabidopsis* and its closest crop relatives (*Brassica spp*): whereas *Arabidopsis* has a prominent replum, with approximately ten cell files separating the valves, the outer replum of *Brassica* is much reduced, leaving the valves in close contact (Figures 1A–1C). This morphology is reminiscent of the *Arabidopsis replumless* (*rpl*) mutant [6] (Figure 1C), although the meristem and stem defects of *rpl* [10–12] are not found in *Brassica*. This prompted us to investigate whether differences in fruit morphology between *Arabidopsis* and *Brassica* could be due to *cis*-regulatory changes causing loss of *RPL* expression specifically in the *Brassica* fruit.

We used phylogenetic footprinting to identify *RPL* regulatory sequences. *Arabidopsis thaliana RPL* (*AtRPL*) was compared with *RPL* from *Arabidopsis lyrata* and *Capsella rubella*, whereas the three *RPL* homeologs identified in the *B. rapa* genome [13] were compared with each other. These three *B. rapa* genes were named *BraA.RPL.a*, *BraA.RPL.b*, and *BraA.RPL.c* according to the standard gene nomenclature of the *Brassica* genus [14] but will for simplicity be referred to here as *BrRPLa*, *BrRPLb*, and *BrRPLc*, respectively. Within noncoding sequences with a high conservation score in either *Arabidopsis* or *B. rapa* (see Figure S1 available online), we looked for known *cis*-elements that differed between these species. A 13 bp sequence was of particular interest because it matched a *cis*-element found in the rice *RPL* ortholog and has been implicated in the reduction of seed shattering during rice domestication [7] (Figure 1D). Within the *cis*-element in domesticated rice, a C-A nucleotide change reduced *RPL* expression specifically at the base of the grain, preventing the formation of the abscission zone necessary for seed shattering [7]. Strikingly, the *Arabidopsis* and *B. rapa* sequences showed a single-nucleotide polymorphism at the same position in this *cis*-element: like shattering rice, *Arabidopsis* had a cytosine in this position, whereas a thymine was present in *Brassica*. By analogy with the effect of the rice mutation, we hypothesized that mutation of this *cis*-element might have altered *RPL* expression in the fruit and consequently replum development in *Brassica*. We henceforth call this *cis*-regulatory sequence *Shl* for *Shattering element-like* and refer to the *Arabidopsis* and *Brassica* versions of the *cis*-element as C-*Shl* and T-*Shl*, respectively.

The Nucleotide Change in the Shl Element Specifically Alters RPL Expression and Function during *Arabidopsis* Fruit Development

To test whether the nucleotide change in *Shl* affected *RPL* expression, we generated *Arabidopsis* transgenic lines with the β-glucuronidase reporter (GUS) directed by the *RPL* promoter from *Arabidopsis* containing either C-*Shl* or T-*Shl*. The C-*Shl* promoter drove expression in the inflorescence meristem, developing flowers and in the developing fruit (stage 16 as defined in [15]) including the replum, reproducing the previously described *RPL* expression pattern [6]. The T-*Shl* promoter directed comparable expression in the inflorescence meristem and developing flowers, but expression in the stage 16 fruit was much reduced (Figure 2A). Thus, mutation of C-*Shl* to T-*Shl* in *Arabidopsis* reduced *RPL* expression specifically during fruit development.

To test the functional relevance of the *Shl* element in *Arabidopsis*, we transformed the loss-of-function *rpl-3* mutant with the complete *AtRPL* gene containing either C-*Shl* or T-*Shl*. *rpl* mutants, including *rpl-3*, have pleiotropic phenotypes including phyllotaxis defects, irregular internode elongation, and a reduced replum [6, 10, 11]. In multiple independent transformants, C-*Shl AtRPL* rescued all *rpl-3* defects. In contrast, although T-*Shl AtRPL* complemented the phyllotaxis and internode defects to the same extent as C-*Shl*

*Correspondence: lars.ostergaard@bbsrc.ac.uk (L.Ø.), robert.sablowski@bbsrc.ac.uk (R.S.)

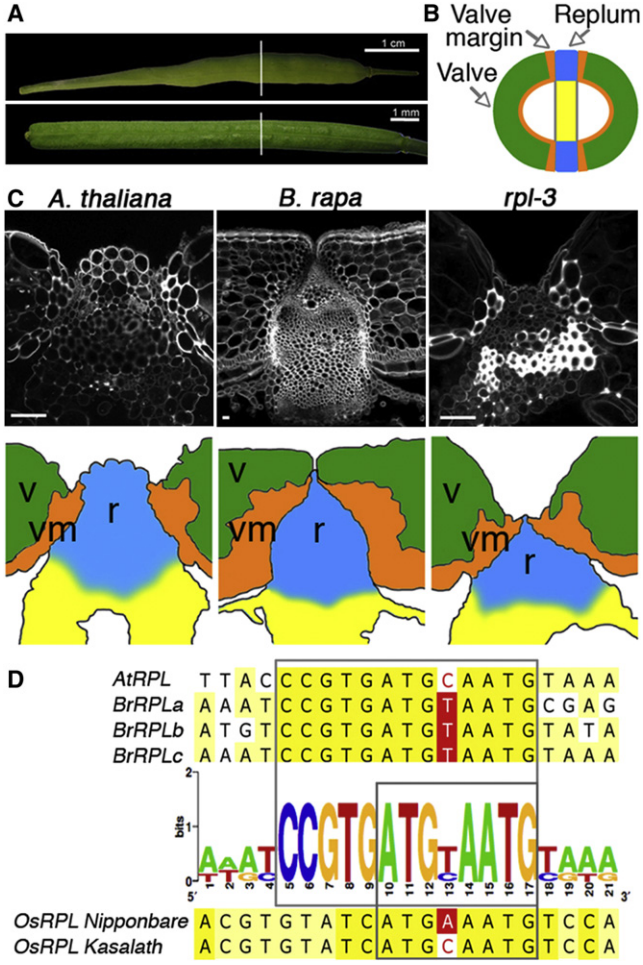

Figure 1. *Brassica* Has a Reduced Replum Like the *Arabidopsis rpl* Mutant and Carries a Mutation in a *RPL cis*-Element that Controls Seed Dispersal in Rice

(A) Mature fruits of wild-type *B. rapa* (top) and *Arabidopsis* (bottom); a white line indicates the region sectioned in (B) and (C).

(B) Schematic cross-section showing fruit tissues involved in seed dispersal.

(C) Upper panels: mPS-PI [27] confocal sections through the replum of *Arabidopsis thaliana*, *B. rapa*, and *Arabidopsis rpl-3* mutant. Lower panels: diagrams based on the pictures above, with the tissues in (B) indicated: valve (green), valve margins (orange), and replum (blue).

(D) Alignment and consensus sequence (WebLogo) of the *Shl* element from *RPL* in *Arabidopsis*, *B. rapa*, and rice; the position of the polymorphism found between *Arabidopsis* and *Brassica* and between shattering (*Kasalath*) and nonshattering (*Nipponbare*) rice cultivars is marked in red.

AtRPL, replum development was only partially rescued (Figures 2B–2E). We conclude that a single nucleotide change in *Arabidopsis RPL*, changing C-*Shl* to T-*Shl*, is sufficient to reduce *RPL* expression in the fruit and consequently to convert the *Arabidopsis* replum to resemble that of *Brassica*.

The Converse Nucleotide Change in the Shl Element Is Sufficient to Convert the *Brassica* Replum to an *Arabidopsis*-Like Morphology

We next tested whether changing T-*Shl* to C-*Shl* in the context of *B. rapa RPL* would be sufficient to convert the *Brassica* replum to an *Arabidopsis*-like morphology. As seen in rice [7], the C-*Shl* allele was dominant over the T-*Shl RPL* in *Arabidopsis* (data not shown); therefore, it should be possible to detect the function of transgenic C-*Shl RPL* against the background of endogenous *Brassica RPL* homeologs containing T-*Shl*. For ease of transformation, in these experiments we used *B. oleracea*, which has the same replum morphology as *B. rapa* and also contains *RPL* with T-*Shl* (Figure 3; Figure S2). We generated transgenic lines with *BrRPLb* containing either C-*Shl* or T-*Shl*, as a control for gene dosage effects. As expected, T-*Shl BrRPLb* did not change fruit morphology compared to the wild-type control. In contrast, multiple C-*Shl BrRPLb* lines showed an enlarged replum, which was most obvious near the base of the fruit (9 out of 10 C-*Shl-BrRPLb* but none of 8 T-*Shl-BrRPLb* lines; Fisher's exact test p value = 0.0004) (Figure 3A). In the central region of the fruit, where the replum is narrowest, transversal sections and scanning electron microscopy also showed that *C-Shl BrRPLb* lines had a prominent replum made of thin, elongated cells as described in *Arabidopsis* (6 out of 10 C-*Shl BrRPLb* but none of 8 T-*Shl BrRPLb* lines; Fisher's exact test p value = 0.0128) (Figure 3B). Taken together, our data demonstrate that a single nucleotide change in a conserved *cis*-regulatory element of *RPL* is sufficient to reproduce the difference in replum morphology observed between *Arabidopsis* and *Brassica*.

The Nucleotide Change in Shl Has Preceded Domestication and Correlates with Replum Morphology in Multiple Members of the Brassicaceae Family

The precedent set by the *Shl* mutation in rice domestication raises the question of whether the C-T nucleotide substitution in *Shl* could also have been selected during *Brassica* domestication. However, this seemed unlikely, because it would require independent selection of the same nucleotide change for all three *BrRPL* homeologs. To confirm that T-*Shl* was present in *Brassica* before domestication, we analyzed *RPL* sequences in *B. atlantica*, which is a wild relative of *B. oleracea* with traits usually not associated with domestication, such as perenniality and self-incompatibility. *B. atlantica RPL* contained T-*Shl* (Figure S2), showing that T-*Shl RPL* evolved in *Brassica* independently of domestication. Furthermore, the correlation between replum morphology and T-*Shl* or C-*Shl* extended to multiple Brassicaceae species. Species closer to *Arabidopsis*, such as *Capsella rubella* and *Lepidium campestre*, had C-*Shl* and prominent *Arabidopsis*-type repla, whereas members of the Brassiceae tribe, including *Brassica nigra* and *Sinapis alba*, had T-*Shl* and narrow *Brassica*-like repla (Figure 4). Considering that C-*Shl* is found in distantly related plants such as *Brachypodium distachyon*, soybean (*Glycine max*), and the wild rice relative *Oryza rufipogon* [7] (Figure 4A), C-*Shl* is likely the ancestral version of the conserved *Shl* element, which mutated to T-*Shl* after the split between the *Arabidopsis* and *Brassica* ancestors some 43 million years (Myr) ago and before the triplication of the *Brassica* genome approximately 22.5 Myr ago [16].

In our analysis there was, however, one exception to the correlation between narrow repla and T-*Shl*: *Cardamine hirsuta* had T-*Shl* and a wide replum (data not shown). Therefore, although the C-T change in *Shl* is sufficient to account for the difference in replum morphology between *Arabidopsis* and *Brassica*, there are alternative ways of producing a wide replum that do not depend on C-*Shl-RPL*. One possibility is through changes in *INDEHISCENT* (*IND*) gene activity, which is suppressed by *RPL* in the replum during *Arabidopsis* fruit

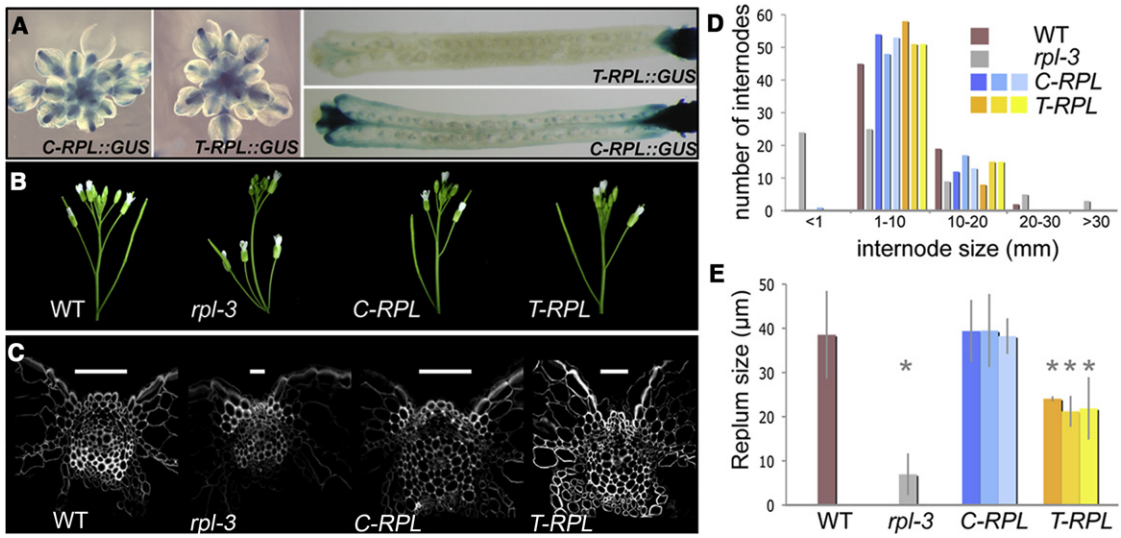

Figure 2. A *Brassica*-Like Mutation in *Shl* Reduced *AtRPL* Expression and Function Specifically in the Fruit

(A) β-glucuronidase expression driven by the *AtRPL* promoter containing either C-*Shl* (C-*RPL*) or T-*Shl* (T-*RPL*) in the inflorescence meristem and the developing fruit (stage 16 [15]).

(B and C) Rescue of defects in phyllotaxis (B) and replum development (C) (mPS-PI/confocal sections) in *Arabidopsis rpl-3* mutants transformed with the complete *AtRPL* gene containing either C-*Shl* (C-*RPL*) or T-*Shl* (T-*RPL*).

(D) Quantification of the rescue of the stem development defects of *rpl-3*; the histogram shows the distribution of internode sizes of wild-type (WT), *rpl-3*, and three independent transgenic lines with C-*RPL* or T-*RPL*, n = 66.

(E) Replum size (mean and standard deviation) measured in mPS-PI/confocal cross-sections of WT, *rpl-3*, and three independent transgenic lines with C-*RPL* or T-*RPL*; asterisks indicate statistically significant differences from WT (two-tailed Student's t test; normal distribution according to Shapiro-Wilk test; p values 7.65879E-07 for *rpl3*, 0.00126 for *T-RPL-1*, 0.00031 for *T-RPL-2*, and 0.00327 for *T-RPL-3*; n = 6).

development [6, 17]: loss of *IND* function in both *Arabidopsis* and *Brassica* also results in expanded repla without the need to modify *RPL* function [9].

Conclusions

We show that a single nucleotide change in a conserved *cis*-element of *RPL* is sufficient to explain evolutionary variation in a morphological trait within Brassicaceae and that this nucleotide change coincides with a mutation previously implicated in rice domestication [7]. It is striking that mutations at the same nucleotide position within the same *cis*-element of *RPL* change the development of structures involved in seed dispersal in both Brassicaceae and rice, which are separated by 140 Myr of evolution [18]. Although at first sight the anatomy of the seed-dispersal structures in rice and Brassicaceae is very different, both cases involve developmentally regulated cell separation. Hence, the conserved regulatory input that acts through *Shl* may connect *RPL* activity with the development of tissues involved in separation processes such as abscission and dehiscence. In rice, the *Shl* element contains a RY repeat [19], suggesting that *Shl* could be targeted by B3-domain transcription factors [7]. In Brassicaceae, however, the core RY sequence (CATG) is not present within *Shl*. Determining the identity and functional conservation of the one or more transcription factors that bind to *Shl* during fruit development in both rice and Brassicaceae remain important challenges for the future. Another future challenge will be to determine the possible adaptive value of the morphological variation caused by the change in *RPL* expression during *Brassica* evolution.

The key role of mutations in gene regulatory sequences in evolution [20] has been supported by genetic analyses of variation between closely related species or populations [1–3], including studies of the genetic basis of plant domestication [21]. However, the functional analysis of gene regulatory changes over larger evolutionary distances has been more challenging. A recent study has shown that replacement of an enhancer in the mouse *Prx1* gene with the corresponding bat enhancer increased forelimb length in mouse, but the cumulative effect of multiple, unknown genetic differences needs to be invoked to explain the large difference in forelimb length between mouse and bat [4]. In plants, *cis*-regulatory changes in KNOTTED-like homeobox (KNOX) genes have been implicated in differences in leaf morphology between different genera, but these changes have not been characterized at the molecular level [22]. We reveal a well-defined *cis*-regulatory change with a causal role in morphological variation at higher taxonomic levels; further examples will be needed to show whether the simplicity of the regulatory change seen in our case is exceptional. In addition, our work suggests that domestication and natural evolution can use the same genetic toolkit and highlights the potential of plant evo-devo and breeding to inform each other.

Experimental Procedures

Plant Growth and Culture Conditions

Arabidopsis plants were grown on soil in long-day conditions (16 hr light/8 hr dark). The *rpl-3* mutant was in *Wassilewskija* background (*WS*). Transgenic *Brassica* plants were grown in a glasshouse at 18°C with 16 hr light.

Constructs and Sequences

Constructs were generated according to standard techniques and sequenced. Primer sequences used in this work are listed in Table S1. An *Arabidopsis RPL* 9.2 kb fragment was excised from pAR33 [6] using BamHI and cloned into pPZP222 [23] to create pC-RPL. Directed mutagenesis to introduce *T-Shl* instead of *C-Shl* within the *RPL* gene was performed using

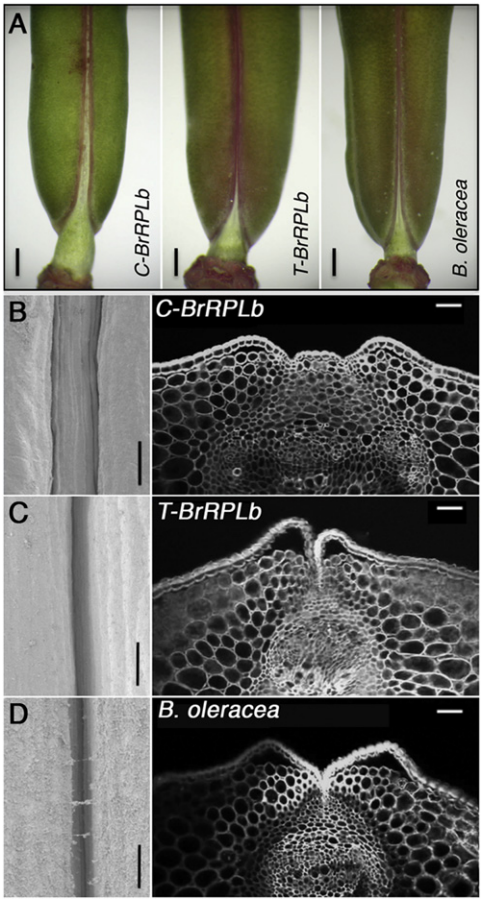

Figure 3. *BrRPLb* with an *Arabidopsis*-Type Mutation in *ShI* Changes the Replum of *B. oleracea* to an *Arabidopsis*-Like Morphology

(A) Basal region of phloroglucinol-stained stage 17 fruits of *B. oleracea* transformed with *C-BrRPLb*, *T-BrRPLb*, or untransformed control; scale bar represents 1 mm, a quantitative analysis of the replum morphology in multiple transgenic lines is shown in Figure S3.

(B–D) Scanning electron micrographs (left) and corresponding mPS-PI/confocal sections (right) of the midregion of stage 17 fruits of *B. oleracea* transformed with *C-BrRPLb* (B), *T-BrRPLb* (C), or untransformed control (D); scale bar represents 50 μm.

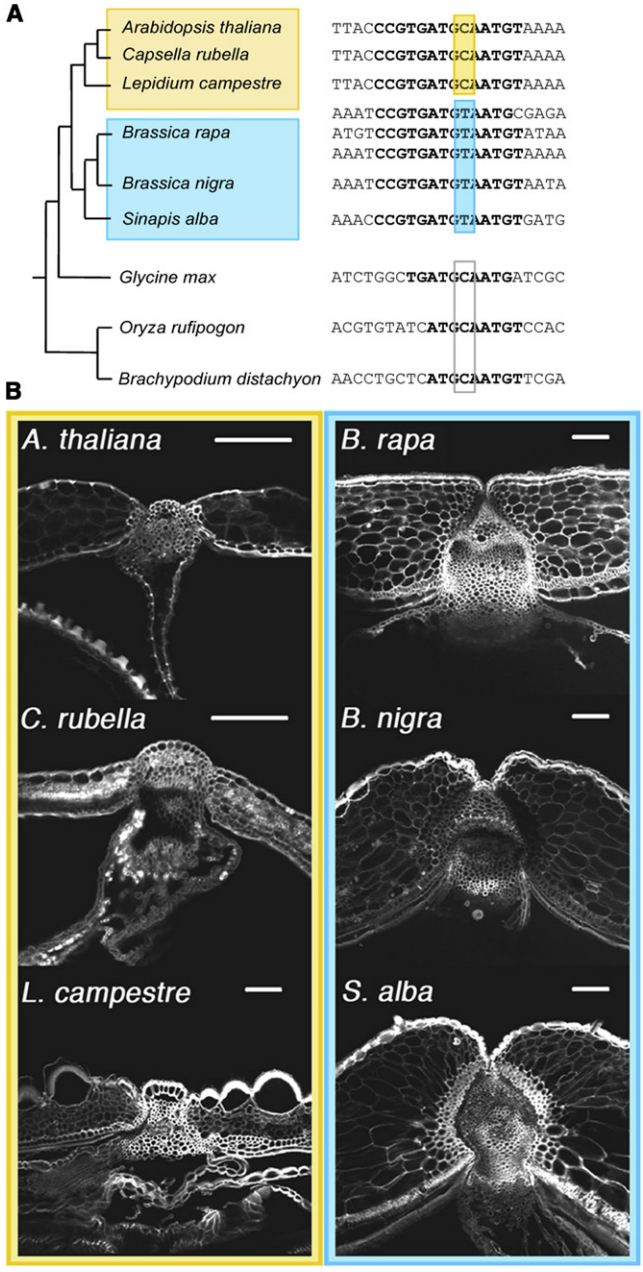

Figure 4. Correlation between the C-T Change in the ShI Element of RPL and Replum Morphology in Brassicaceae Plants

(A) Phylogenetic relationship (modified from [28]) between Brassicaceae species (highlighted in yellow and blue boxes) and distant relatives (*G. max*, *O. rufipogon*, *B. distachyon*), along with corresponding *ShI* sequences; the position of the C-T polymorphism is indicated.

(B) mPS-PI/confocal cross-sections showing the replum morphology of the Brassicaceae species in (A); scale bar represents 100 μm.

a two-step PCR protocol using primers oNA43/B1 and oNA44/C1 and pAR33 as template. The resulting fragment harboring *T-ShI* was exchanged with the wild-type PstI fragment using PstI to generate pT-RPL. To generate the construct with the wild-type RPL promoter fused to GUS, we excised a genomic fragment from pAR43 [6] and cloned it into pBluescript KS$^-$ using BamHI/XhoI. The coding sequence was then removed and replaced with a GUS-NOS fragment using NcoI/SphI to generate C-RPL::GUS. The T-RPL::GUS mutated version was obtained using a two-step PCR protocol to generate the mutation within *ShI* using M13/B1 and GUS-REV/C1. The resulting fragment was then exchanged with the wild-type fragment using *Asp718*/HindIII. These two constructs were moved to the pCGN1547 binary vector using *Asp718*/BamHI.

A BrRPLb 7.5 kb fragment was amplified from BAC KBrH113P03 using oNA39/oNA42 and cloned into pGEMT Easy Vector (Promega) according to the manufacturer's instructions to create the T-BrRPLb construct. A mutagenized fragment was generated by a two-step PCR protocol using RA/RD and RC/RB. This fragment was introduced instead of the wild-type fragment into BrRPLb in pGEMT using BlpI/BmtI to generate the C-BrRPLb construct. These two constructs were moved to the pCGN1547 binary vector using BamHI to create C-BrRPLb and T-BrRPLb.

ShI sequences were PCR amplified from *Brassica nigra*, *Sinapis alba*, and *Brassica atlantica* genomic DNA using primer oNA46 and oNA48 and from *Lepidium campestre* genomic DNA using oNA103 and oNA48. Resulting PCR products were cloned into pGEMT Easy Vector and sequenced. For each species, ten clones have been sequenced using the oNA48 primer. *ShI* sequences from *Brassica oleracea*, *Capsella rubella*, and *Brachypodium dystachyon* were obtained by blasting *AtRPL* genomic sequence against sequence databases (http://www.phytozome.net/).

Phylogenetic Footprinting

The phylogenetic footprinting analysis was performed using rVISTA 2.0 server (http://rvista.dcode.org/). Conserved modules were further analyzed using the FootPrinter 3.0 server in order to identify precise motifs [24].

Microscopy

Modified pseudo-Schiff propidium iodide (mPS-PI) staining of fruits was performed as previously described [25]. Confocal microscopy was performed using a Zeiss Axo Imager M1 upright microscope. PI was excited using a 488 nm argon ion laser and collected between 600 and 656 nm. Images were analyzed using Zeiss LSM 510 software.

For GUS staining, tissues were fixed in 90% acetone on ice for 20 min, then rinsed with a rinse buffer containing 0.5 mM K-ferrocyanide (Sigma, P-8131) and 0.5 mM K-ferricyanide (Sigma, P-9387) in 50 mM sodium phosphate buffer (pH 7.2). Samples were then incubated for 16 hr at 37°C in rinse buffer containing 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (Melford, MB1121). Samples were dehydrated in ethanol series and then rehydrated prior to the clearing using a chloral hydrate:water:glycerol solution (8:3:1).

For valve margin staining, fruits were stained for 2 min in a 2% phloroglucinol solution in 95% ethanol and then imaged in 50% hydrochloric acid.

For scanning electron microscopy, fruits were fixed at 4°C overnight in FAA (3.7% of formaldehyde, 5% acetic acid, and 50% ethanol), dehydrated through an ethanol series, critical point dried in liquid CO_2, sputter-coated with gold, and analyzed and photographed with a Philips XL 30 FEG SEM.

Quantitative image analysis was done with ImageJ (http://rsb.info.nih.gov/ij/index.html), and image processing (brightness, contrast, cropping) was applied in parallel with the relevant controls using Adobe Photoshop CS4.

Plant Transformation

Arabidopsis plants were transformed using the floral-dip method [26]. *Brassica oleracea* genotype DH1012 was transformed using the *Agrobacterium tumefasciens* strain AGL1 as described previously [9].

Supplemental Information

Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.cub.2011.06.008.

Acknowledgments

We thank Thomas Girin, Adrien Sicard, Scott Boden, Ian Bancroft, and Jan Lohmann for sequences; Rachel Wells and Judith Irwin for DNA samples; Elisabeth Wischnitzki for help with phylogenetic analysis; Penelope Sparrow and Cassie Goldsack for help with *Brassica* transformation; and Enrico Coen, Pauline Haleux, and Sophien Kamoun for critical comments. This work was funded by grants from the Biotechnology and Biological Sciences Research Council (BB/E024807/1) and the European Union (PIEF-GA-2009-236568).

Received: May 18, 2011
Revised: June 3, 2011
Accepted: June 3, 2011
Published online: July 7, 2011

References

1. McGregor, A.P., Orgogozo, V., Delon, I., Zanet, J., Srinivasan, D.G., Payre, F., and Stern, D.L. (2007). Morphological evolution through multiple cis-regulatory mutations at a single gene. Nature *448*, 587–590.
2. Clark, R.M., Wagler, T.N., Quijada, P., and Doebley, J. (2006). A distant upstream enhancer at the maize domestication gene tb1 has pleiotropic effects on plant and inflorescent architecture. Nat. Genet. *38*, 594–597.
3. Chan, Y.F., Marks, M.E., Jones, F.C., Villarreal, G., Jr., Shapiro, M.D., Brady, S.D., Southwick, A.M., Absher, D.M., Grimwood, J., Schmutz, J., et al. (2010). Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. Science *327*, 302–305.
4. Cretekos, C.J., Wang, Y., Green, E.D., Martin, J.F., Rasweiler, J.J., 4th, and Behringer, R.R. (2008). Regulatory divergence modifies limb length between mammals. Genes Dev. *22*, 141–151.
5. Cooper, K.L., and Tabin, C.J. (2008). Understanding of bat wing evolution takes flight. Genes Dev. *22*, 121–124.
6. Roeder, A.H., Ferrándiz, C., and Yanofsky, M.F. (2003). The role of the REPLUMLESS homeodomain protein in patterning the Arabidopsis fruit. Curr. Biol. *13*, 1630–1635.
7. Konishi, S., Izawa, T., Lin, S.Y., Ebana, K., Fukuta, Y., Sasaki, T., and Yano, M. (2006). An SNP caused loss of seed shattering during rice domestication. Science *312*, 1392–1396.
8. Østergaard, L., Kempin, S.A., Bies, D., Klee, H.J., and Yanofsky, M.F. (2006). Pod shatter-resistant Brassica fruit produced by ectopic expression of the FRUITFULL gene. Plant Biotechnol. J. *4*, 45–51.
9. Girin, T., Stephenson, P., Goldsack, C.M.P., Kempin, S.A., Perez, A., Pires, N., Sparrow, P.A., Wood, T.A., Yanofsky, M.F., and Østergaard, L. (2010). Brassicaceae INDEHISCENT genes specify valve margin cell fate and repress replum formation. Plant J. *63*, 329–338.
10. Byrne, M.E., Groover, A.T., Fontana, J.R., and Martienssen, R.A. (2003). Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. Development *130*, 3941–3950.
11. Smith, H.M., and Hake, S. (2003). The interaction of two homeobox genes, BREVIPEDICELLUS and PENNYWISE, regulates internode patterning in the Arabidopsis inflorescence. Plant Cell *15*, 1717–1727.
12. Bhatt, A.M., Etchells, J.P., Canales, C., Lagodienko, A., and Dickinson, H. (2004). VAAMANA—a BEL1-like homeodomain protein, interacts with KNOX proteins BP and STM and regulates inflorescence stem growth in Arabidopsis. Gene *328*, 103–111.
13. Stephenson, P., Baker, D., Girin, T., Perez, A., Amoah, S., King, G.J., and Østergaard, L. (2010). A rich TILLING resource for studying gene function in Brassica rapa. BMC Plant Biol. *10*, 62.
14. Ostergaard, L., and King, G.J. (2008). Standardized gene nomenclature for the Brassica genus. Plant Methods *4*, 10.
15. Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in Arabidopsis. Plant Cell *2*, 755–767.
16. Beilstein, M.A., Nagalingum, N.S., Clements, M.D., Manchester, S.R., and Mathews, S. (2010). Dated molecular phylogenies indicate a Miocene origin for Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA *107*, 18724–18728.
17. Liljegren, S.J., Roeder, A.H., Kempin, S.A., Gremski, K., Østergaard, L., Guimil, S., Reyes, D.K., and Yanofsky, M.F. (2004). Control of fruit patterning in Arabidopsis by INDEHISCENT. Cell *116*, 843–853.
18. Chaw, S.M., Chang, C.C., Chen, H.L., and Li, W.H. (2004). Dating the monocot–dicot divergence and the origin of core eudicots using whole chloroplast genomes. J. Mol. Evol. *58*, 424–441.
19. Bäumlein, H., Nagy, I., Villarroel, R., Inzé, D., and Wobus, U. (1992). Cis-analysis of a seed protein gene promoter: the conservative RY repeat CATGCATG within the legumin box is essential for tissue-specific expression of a legumin gene. Plant J. *2*, 233–239.
20. Carroll, S.B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell *134*, 25–36.
21. Doebley, J.F., Gaut, B.S., and Smith, B.D. (2006). The molecular genetics of crop domestication. Cell *127*, 1309–1321.
22. Hay, A., and Tsiantis, M. (2006). The genetic basis for differences in leaf form between Arabidopsis thaliana and its wild relative Cardamine hirsuta. Nat. Genet. *38*, 942–947.
23. Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol. Biol. *25*, 989–994.
24. Fang, F., and Blanchette, M. (2006). FootPrinter3: phylogenetic footprinting in partially alignable sequences. Nucleic Acids Res. *34* (web server issue), W617–W620.
25. Arnaud, N., Girin, T., Sorefan, K., Fuentes, S., Wood, T.A., Lawrenson, T., Sablowski, R., and Østergaard, L. (2010). Gibberellins control fruit patterning in Arabidopsis thaliana. Genes Dev. *24*, 2127–2132.
26. Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. *16*, 735–743.
27. Truernit, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthélémy, J., and Palauqui, J.C. (2008). High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of Phloem development and structure in Arabidopsis. Plant Cell *20*, 1494–1503.
28. Johnston, J.S., Pepper, A.E., Hall, A.E., Chen, Z.J., Hodnett, G., Drabek, J., Lopez, R., and Price, H.J. (2005). Evolution of genome size in Brassicaceae. Ann. Bot. (Lond.) *95*, 229–235.