Temperature Modulates Tissue-Specification Program to Control Fruit Dehiscence in Brassicaceae

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ABSTRACT

Plants respond to diurnal and seasonal changes in temperature by reprogramming vital developmental pathways. Understanding the molecular mechanisms that define environmental modulation of plant growth and reproduction is critical in the context of climate change that threatens crop yield worldwide. Here, we report that elevated temperature accelerates fruit dehiscence in members of the Brassicaceae family including the model plant Arabidopsis thaliana and important crop species. Arabidopsis fruit development is controlled by a network of interacting regulatory genes. Among them, the INDEHISCENT (IND) gene is a key regulator of the valve-margin tissue that mediates fruit opening, hence facilitating fruit dehiscence. We demonstrated that the valve-margin development is accelerated at higher temperature and that IND is targeted for thermosensory control. Our results reveal that IND upregulation is facilitated via temperature-induced chromatin dynamics leading to accelerated valve-margin specification and dispersal of the seed. Specifically, we show that temperature-induced changes in IND expression are associated with thermosensory H2A.Z nucleosome dynamics. These findings establish a molecular framework connecting tissue identity with thermal sensing and set out directions for the production of temperature-resilient crops.

Key words: Brassicaceae, seed dispersal, fruit dehiscence, temperature response, gene regulation, nucleosome dynamics


INTRODUCTION

Efficient and timely dispersal of seeds in tune with the prevailing seasonal conditions is essential for maximum reproductive success and underlies biodiversity and crop productivity. Seed dispersal is a key trait that must be controlled when domesticiating plants for food production (Konishi et al., 2000). Indeed, control of seed dispersal is crucial in maintaining yields of some crops such as oilseed rape (Brassica napus), in which yield loss due to preharvest seed dispersal (fruit dehiscence) is a major issue aggravated by adverse weather conditions (Price et al., 1990). In some species of the Brassicaceae family, including Arabidopsis thaliana and B. napus, dehiscence happens as the fruits dry out and the valves (pod walls) detach from a central septum promoted by precocious lignin deposition and cell separation along the valve margins (Spence et al., 1996). Impressive advances in understanding the molecular mechanisms and genetic networks involved in fruit patterning and dehiscence have long been achieved (Gu et al., 1998; Ferrándiz et al., 2000; Lijigren et al., 2000, 2004; Rejani and Sundaresan, 2001; Roeder et al., 2003; Sorefan et al., 2009).

However, despite the prospect of climate change affecting crop performance (Battisti and Naylor, 2009), it is not yet known how environmental signals such as temperature affect the process. Here we show that changes in ambient temperature influence fruit dehiscence in Brassicaceae through modulation of a tissue-specification program. We find that IND expression is regulated by temperature, which is in turn controlled by thermosensory chromatin dynamics.

RESULTS

Higher Temperature Accelerates Pod Shattering in Brassicaceae

To study the effect of temperature on fruit dehiscence, we monitored fruit development and dehiscence from Arabidopsis plants grown at 17°C, 22°C, and 27°C (Figure 1A–1C). Plants were grown at 22°C until flowering and subsequently transferred to
17°C, 22°C, or 27°C. We initially observed that mature fruits at 27°C (developmental stage 19) (Smyth et al., 1990; Roeder and Yanofsky, 2006) were shorter than those at 17°C and 22°C (Figure 1A). Intriguingly, at 27°C fruits dehisced earlier than those at 22°C, while they remained closed longer at 17°C (Figure 1A). This raises the possibility that the dehiscence process per se is under thermal control, although these effects could also be due to accelerated maturation at the higher temperature. To test this, we compared fruits at the same developmental stage (stage 17b when embryos have entered the early bent-cotyledon stage) but grown at different temperatures. External examination of the valve margins by scanning electron microscopy suggested that they were more pronounced at the higher temperature, especially at the base of the fruit (Figure 1B). To test this in more detail, we stained cross-sections of stage-17b fruits with toluidine blue O and found that lignified cells in the valve margin were more pronounced at higher temperature (Figure 1C). To quantify the lignification levels, we stained cross-sections with crystal violet (Smith et al., 2017). After staining, fluorescence from the lignified cell walls was captured by confocal microscopy (Figure 1D) and the fluorescence intensity was measured from confocal images (Figure 1E). As shown in Figure 1D and 1E, the lignin fluorescence intensity of the valve-margin cells increased with the rising temperature. These results demonstrate that lignification at the valve margin is enhanced by increasing temperature.

To quantify the effect of temperature on Arabidopsis fruit opening directly, we carried out random impact test (RIT) assays (Morgan et al., 1998; Arnaud et al., 2010). For this experiment, we used fruits grown at 17°C, 22°C, and 27°C until stage 18, thereby eliminating any effect of differential maturation speed. The results of the RIT demonstrated that resistance to dehiscence decreased with increasing temperature at which the fruits were grown (Figure 1F). Together these data show that the process of fruit dehiscence in Arabidopsis is accelerated by higher temperature.

Arabidopsis is closely related to many important Brassicaceae crops, such as oilseed rape (B. napus), in which premature seed dispersal in the field (pod shatter) is a serious agricultural problem (Price et al., 1998). To test whether the temperature effect on fruit dehiscence extends to other members of the Brassicaceae family, we performed the RIT assay on fruits of Capsella rubella, Lepidium campestris, and B. napus cv. Topas and B. napus cv. Westar grown at 22°C and 27°C. In all these species, fruit dehiscence is increased at 27°C compared with 22°C (Figure 1G and Supplemental Figure 1), consistent with the data in Arabidopsis (Figure 1F). These results therefore suggest that acceleration of fruit dehiscence at higher temperature is conserved across members of the Brassicaceae family.

Increased INDE Expression Promotes Fruit Dehiscence in Arabidopsis

Key regulators of fruit opening have been identified in Arabidopsis (Liljegren et al., 2000, 2004; Roeder et al., 2003). The Molecular Plant 11, 588–606, April 2018 © The Author 2018.
SHATTERPROOF1 (SHP1) and SHP2 genes encode two closely related MAOS-box proteins (Lijigren et al., 2000) that function redundantly to control valve-margin specification. The INDEHISCENT (IND) gene acts downstream of SHP1 and SHP2 and encodes a member of the basic helix-loop-helix (bHLH) family of transcription factors. In ind mutants, valve-margin development is abolished including the specification of separation layer cells and lignin deposition (Supplemental Figure 2A and 2B), and as a consequence the fruits fail to open (Lijigren et al., 2004). The FRUITFULL (FUL) gene is expressed in the valves of the fruit where it functions to restrict the expression of IND and SHP genes to the valve margins (Gu et al., 1998; Ferrándiz et al., 2000; Lijigren et al., 2004). Since the functions of these factors are conserved across the Brassicaceae family (Ostergaard et al., 2006; Girin et al., 2010; Mülhauzen et al., 2013), we used Arabidopsis to test whether members of this genetic network are under temperature control to facilitate efficient fruit dehiscence. By comparing the expression levels of FUL, SHP1, SHP2, and IND at 17°C, 22°C, and 27°C, we found that IND was upregulated with increasing temperature whereas the other genes tested were unaffected (Figure 2A and Supplemental Figure 2C). Expression of HSP70, an output of the thermosensory pathway (Kumar and Wigge, 2013), was upregulated with elevated temperature (Supplemental Figure 2), verifying that our assays were under precise temperature control. Since SHP1 and SHP2 are expressed in other tissues during fruit development (Savidge et al., 1995; Flanagan et al., 1996), we cannot rule out that these genes are also under temperature control at the valve margin. However, since the IND gene functions downstream of the redundant SHP genes (Ferrándiz et al., 2000) and showed a significant induction by temperature, we initially explored the role of IND in temperature-controlled valve-margin formation. To further analyze the expression of IND in the valve margin, we generated a reporter line IND:3xVENUS-NLS containing a single T-DNA insertion. Consistent with the tissue specificity of IND expression, VENUS signal was specifically detected in the valve margin and, importantly, the signal intensity was enhanced when temperature increased (Figure 2B and 2C), further supporting the gene expression data (Figure 2A). Similar results were obtained using a different reporter for IND expression (IND::IND:HYFP) (Supplemental Figure 2D). These results suggest that thermosensory regulation of IND expression underlies temperature-modulated dehiscence. We therefore reasoned that increased IND function at elevated temperature could define the control of fruit dehiscence in response to temperature. In further support of this hypothesis, we found that the ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADP31) gene, which functions downstream of IND and mediates cell separation to allow fruit dehiscence (Ogawa et al., 2003), is also induced at higher temperature (Supplemental Figure 2E).
expression system that can be induced by addition of dexamethasone (Dex) (Craft et al., 2003). In this system, expression of the LhGR gene is driven by a tissue-specific promoter. Upon Dex treatment, the cytoplasmic LhGR protein is translocated into the nucleus where it binds to a bidirectional inducible promoter (pCP6). The pCP6 promoter simultaneously drives the expression of a β-glucuronidase (GUS) reporter gene and the gene of interest, allowing induction to be verified by monitoring expression of the GUS reporter (Supplemental Figure 3). To ensure valve-margin specificity, we chose 2.5 kb of the native IND promoter to drive the IND gene after Dex treatment (IND-GR×IND/GUS) (Craft et al., 2005, Grin et al., 2010), and the construct was transformed into the ind-2 mutant (Supplemental Figure 3). As shown in Figure 2D, no GUS signal was detected before Dex treatment at both 17°C and 22°C. When treated with increasing concentrations of Dex, the GUS signal in the valve margin increased gradually, indicating a concentration-dependent induction of IND expression by Dex. Notably, the GUS signal was enhanced at 22°C compared with 17°C when treated with Dex concentrations of 25 μM and 100 μM (Figure 2D), reflecting a difference in LhGR protein abundance due to the temperature-sensitivity of the IND promoter. Therefore, reduced levels of LhGR protein at 17°C would limit the induction of GUS and IND even with higher concentrations of Dex. To test whether the temperature-dependent difference in IND abundance in this system translates to tissue differentiation, we studied lignification of valve-margin cells using crystal violet-stained sections. While no lignified valve-margin cells were observed in untreated IND-GR×IND/GUS ind-2 fruits, the effect of IND after Dex treatment was reflected by the increase in lignification of valve-margin cells (Figure 2E). After treatment with increasing concentrations of Dex, the valve-margin defects in ind-2 mutants were gradually rescued (Figure 2E). Consistent with the GUS-staining results (Figure 2D), lignin fluorescence was stronger at 22°C than at 17°C when treated with the same concentration of Dex (Figure 2E), indicating increased IND protein levels at 22°C. RTI assays revealed that fruits treated with Dex were increasingly dehiscient and that fruits at 22°C were more dehiscient than those at 17°C (Figure 2F). Taken together, our findings show that elevated temperature enhances IND expression in the valve margin, which in turn leads to accelerated lignin production and dehiscence.

Regulation of H2A.Z Occupancy at the IND Locus by Temperature

In Arabidopsis, chromatin dynamics defined by the variant histone H2A.Z has been identified as a key mediator of thermosensitive responses and has been shown to modulate temperature responses through the regulation of gene expression (Kumar and Wigge, 2010). H2A.Z nucleosomes have a high occupancy at lower temperature whereby it functions on the majority of its target genes as a repressor of transcription. H2A.Z occupancy declines with an increase in temperature, which therefore primarily facilitates the induction of temperature-responsive gene expression (Kumar and Wigge, 2010). We have shown above that IND is expressed in a temperature-dependent manner. It is therefore possible that IND is controlled by changes in H2A.Z occupancy, providing a possible mechanism for temperature-responsive modulation of fruit dehiscence. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) assays to analyze temperature-dependent H2A.Z nucleosome dynamics at the IND gene at 17°C, 22°C, and 27°C in stage-16 fruits. Firstly, confocal microscopy verified that the H2A.Z:GFP reporter used (Kumar and Wigge, 2010) is expressed in the valve margin tissue where IND is expressed (Figure 3A). The ChIP analysis revealed a high H2A.Z occupancy at the +1 nucleosome of IND at 17°C and the occupancy declined at 22°C and 27°C (Figure 3B), demonstrating that temperature affects H2A.Z occupancy at the IND locus. Consistent with precise temperature control in this experiment, the occupancy of H2A.Z at the +1 nucleosome of HSP70 decreases in response to temperature (Figure 3C), while the expression of HSP70 increases (Supplemental Figure 4A) (Kumar and Wigge, 2010). Notably, H2A.Z occupancy at IND at 27°C showed no significant difference from that at 22°C (Figure 3B), indicating that chromatin at the IND locus is already in an "open" state at 22°C. Since our analysis revealed that fruits at 27°C are more dehiscient than those grown at 22°C (Figure 1F) and that the levels of IND transcripts are higher at 27°C (Figure 2A), it is possible that other factors in addition to H2A.Z dynamics are also involved in temperature-controlled IND expression. Over the broad temperature range (17°C to 27°C), H2A.Z nucleosomes therefore show robust dynamics at the IND locus, suggesting that H2A.Z-mediated thermosensitive chromatin dynamics indeed
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Figure 4. arp6-1 Mutant Fruits Display Enhanced Dehiscent Phenotype due to the Increased Expression of IND.

(A) Comparison of fruit dehiscence between Col-0 and arp6-1 siliques at 16 DAF grown at 22°C. Scale bar, 1 mm.

(B) Scanning electron microscopy images of the bases of stage-17b fruits in Col-0 and arp6-1 plants grown at 22°C. Scale bars, 100 µm.

(C) Crystal violet-stained cross-sections of stage-17b fruits in Col-0 and arp6-1 plants. Scale bars, 10 µm.

(D) Shattering quantification of Col-0 and arp6-1 fruits at 22°C. The measurement of Col-0 fruits is set as 1.0. The data are from three biological repeats (n = 3), 20 silique each. Error bars denote SD. **P < 0.01 (Student's t-test).

(E) Analysis of IND expression in Col-0 and arp6-1 stage-19 flowers by qPCR. Relative levels of indicated transcripts are normalized to UbQ10. The level of IND transcripts in Col-0 fruits is set as 1.0. The data are from three biological repeats (n = 3). Error bars denote SD. **P < 0.01 (Student's t-test).

(F) z-Stack projection images of IND:3xVENUS-NLS in Col-0 and arp6-1 mutant fruits at stage 16. Scale bars, 50 µm.

(G) Conceptual model of temperature-accelerated fruit dehiscence. When the temperature rises, H2A.Z occupancy at the IND gene declines, relieving the repression of IND. Increased IND expression driven by H2A.Z dynamics and potentially other mechanisms accelerates fruit dehiscence at higher temperature.

plays an important role in controlling fruit dehiscence. A recent study has revealed that chromatin dynamics of ~35% of genes in Arabidopsis is controlled by thermosensitive H2A.Z binding (Cortijo et al., 2017). This is in agreement with previous suggestions that H2A.Z dynamics provides a conducive chromatin environment for gene upregulation, while gene-specific factors will be required to promote expression (Kumar et al., 2012). For example, while FUL, SHP1, and SHP2 gene expression was unaffected by temperature (Supplemental Figure 2C), we still found that H2A.Z occupancy at the +1 nucleosomes of these genes decreased with temperature (Figure 3D; Supplemental Figure 4B and 4C). This suggests that the regulatory framework required to induce FUL, SHP1, and SHP2 expression is not available or able to take advantage of the open chromatin state in response to increased temperature.

**arp6-1 Mutant Fruits Display Enhanced Expression of IND and Accelerated Dehiscence**

The Arabidopsis SWR1 complex, composed of several subunits including ACTIN-RELATED PROTEIN6 (ARP6) and PHOTOPERIOD-DEPENDENT EARLY FLOWERING1 (PE1), catalyzes the incorporation of H2A.Z into nucleosomes (Choi et al. 2007). Mutants such as arp6-1, which are defective in incorporating H2A.Z into nucleosomes, show enhanced temperature response and phenocopy plants grown at higher ambient temperature (Kumar and Wigge, 2010). Therefore, based on our results we would predict that the arp6-1 mutant exhibits accelerated dehiscence compared with wild-type and that IND is expressed at a higher level. To test the first prediction, we analyzed the development and dehiscence of arp6-1 mutant fruits. Consistent with an earlier report (Deal et al., 2005), arp6-1 fruits were shorter than wild-type fruits (Figure 4A) and reminiscent of wild-type fruits grown at elevated temperature (Figure 1A). Moreover, when grown under the same conditions, fruit dehiscence of arp6-1 is accelerated compared with wild-type (Figure 4A), which is supported by the valve-margin morphology (Figure 4B and 4C) with more pronounced indentations (Figure 4B) and stronger lignin fluorescence in the arp6-1 valve-margin cells (Figure 4C). This phenotype thereby resembles wild-type fruits at higher temperature (Figure 1B and 1D) and those with enhanced IND expression (Figure 2B). Dehiscence was quantified using the Rit assay, which further confirmed that arp6-1 grown at 22°C exhibits enhanced fruit dehiscence (Figure 4D) similar to wild-type fruits grown at 27°C (Figure 1F). Besides arp6-1, mutations in PE1 and the H2A.Z-coding genes HTA8, HTA9, and HTA11 also exhibited accelerated fruit dehiscence (Supplemental Figure 5). These results therefore support the conclusion that H2A.Z plays a key role in temperature-dependent fruit dehiscence. Since our results presented above show that elevated temperature-induced IND expression is associated with H2A.Z dynamics and that increased IND expression accelerates fruit dehiscence, we analyzed IND expression in arp6-1 mutant fruits by quantitative RT-PCR (qRT-PCR). This experiment revealed increased IND expression in arp6-1 fruits compared with wild-type fruits (Figure 4E), while the expression of the valve-identity gene, FUL, was unchanged (Supplemental Figure 6). Consistent with previous data, HSP70 expression is enhanced in arp6-1 fruits (Supplemental Figure 6) (Kumar and Wigge, 2010). These data
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were supported at the tissue-specific level by crossing the IND: 3xVenus:ALS reporter line into the apr6-1 mutant, revealing a stronger Venus signal in the valve margins of the apr6-1 mutant (Figure 4P). Interestingly, expression of the SHP1 and SHP2 genes were also modestly but significantly induced in the apr6-1 mutant fruits (Supplemental Figure 6). As mentioned above, it is therefore possible that SHP gene expression could indeed be temperature induced and that the lack of observed induction may be due to expression in different tissue masking that increased expression in the valve margin.

Taken together, our data show that the regulation of the Arabidopsis IND gene is a direct output of the thermosensory response and that perturbed temperature response leads to altered fruit dehiscence. Our results have established an unprecedented link between fruit tissue identity and thermosensory signaling, demonstrating that temperature-induced chromatin dynamics governed by H2A.Z nucleosomes could influence IND expression and fruit dehiscence in Arabidopsis (Figure 4G).

DISCUSSION

Plants have an extraordinary ability to adjust their life cycle to the environment and optimize phenological processes such as seed germination and reproductive transition to suit the proper seasonal conditions such as changes in temperature, thus ensuring reproductive success. We show that similar to other phenological traits, fruit dehiscence is strongly influenced by changes in temperature, indicating a possible seasonal timing for better reproductive fitness. Moreover, we show that this temperature-responsive modulation of fruit dehiscence is widely conserved across members of the Brassicaceae family, including major oilseed crops, adding to the significance of this study beyond fundamental biology.

Temperature effects on fruit dehiscence can be a combination of temperature-responsive general growth and development combined with the specific effects on the processes that underlie fruit opening. Our experiments were therefore carefully designed to minimize the confounding effects of temperature-induced developmental timing to specifically study the fruit dehiscence process. The data show that ambient temperature particularly affects the tissue-specification program that underlies pod shatter. A more pronounced valve margin and enhanced lignification of the valve-margin cells at elevated temperature reveals an additional layer of environmental regulation on the well-established valve-margin specification program. Central to this program is the temperature-responsive modulation of IND expression. Through a controllable expression system, we have further shown that IND is indeed sufficient to modulate tissue specification and fruit opening in a dose-dependent manner and in response to temperature.

Importantly, our data demonstrate that temperature-responsive IND expression is associated with the previously described thermosensory chromatin dynamics governed by H2A.Z nucleosomes. We observed robust H2A.Z dynamics at the IND locus over a wide temperature range (17°C–27°C). Interestingly, an open chromatin structure with respect to H2A.Z nucleosomes was already achieved at 22°C, although IND expression continued to increase at 27°C. It is not surprising that chromatin conformation dynamics on its own cannot fully account for changes in gene expression in response to temperature. While being a key component controlling temperature-responsive gene expression, H2A.Z does not act as a thermosensor by itself (Kumar and Wigge, 2010). Temperature-responsive H2A.Z nucleosome dynamics creates a conducive chromatin environment for the control of gene expression by appropriate regulatory proteins. This has been exemplified in the thermosensory modulation of flowering whereby H2A.Z dynamics and activity of the PIF4 transcription factor cooperatively accelerate flowering in response to increasing temperature (Kumar et al., 2012). The possibility of such a multilayered framework for the thermosensory control of fruit dehiscence offers a robust mechanism to fine-tune seed dispersal. The H2A.Z occupancy and expression data in apr6-1 presented here raises the possibility that SHP1 and SHP2 are also induced by increasing temperature. Since this was not the case for FUL, it is therefore plausible that not only IND, but the valve-margin identity pathway in general is under temperature control while valve formation is unaffected. Future work will focus on this aspect.

In summary, our results show that fruit dehiscence is a robustly plastic developmental trait that can be fine-tuned by the prevailing environmental conditions. Our data show that the temperature influence on the tissue-specification program is achieved through the integration of developmental signaling with that of thermosensory responses. This offers an elegant mechanism for a physical factor such as temperature to influence key traits. Interestingly, this is in agreement with a recent study from the Santalaceae family showing that thermogenesis induces explosive seed dispersal in dwarf mistletoe (deBruyn et al., 2015). This suggests that the dispersal mechanism across systems has a built-in thermosensitivity, which could respond to either a developmentally regulated thermogenesis or seasonal temperature changes to control seed dispersal. We therefore speculate that such mechanisms have evolved to facilitate proper seasonal timing of dispersal to ensure that seeds are released under conditions that are both timely and climatically optimal for germination. We also show that the control of fruit dehiscence by temperature is widely conserved in the Brassicaceae family. In the past few years, significant progress has been made in translating knowledge on fruit development in Arabidopsis to address the problem of pod shatter in important crops, such as oilseed rape (Oestergaard et al., 2008; Grish et al., 2013). By revealing the fundamental mechanisms that modulate fruit dehiscence in response to temperature, our findings introduce an environmental factor to the current knowledge, which provides alternative avenues for crop improvement in the face of climate change.

METHODS

Plant Materials and Growth Conditions

Arabidopsis ecotype Columbia-0 (Col-0) was used as the wild-type. The ind-2 and apr6-1 mutants were as previously reported (Ljungren et al., 2004; Choi et al., 2007). Plants were grown under long-day conditions (16 h light/8 h dark) at 22°C until flowering. The plants were then transferred separately to the short-day conditions (8 h light/16 h dark) at 17°C, 22°C, and 27°C.
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Random Impact Test

The RT assays were modified from previous reports (Morgan et al., 1996; Arnaud et al., 2010). Fruits of Arabidopsis, Lepidium, and Capsella at stage 18 (stages defined previously by Smyth et al., 1990; Roeder and Yanofsky, 2000) were mature, dry pods of B. napus were harvested, and placed at room temperature for at least 3 days. For Arabidopsis, Lepidium, and Capsella, 20 undamaged fruits were placed in a 60-mm-diameter glass Petri dish together with four to six steel balls of 2-mm diameter. The Petri dish was then attached to an Eppendorf Thermostat and was shaken at 1000 rpm for successive 5-s intervals until all the fruits had shattered. For B. napus, 20 dry pods were placed together with six 4-mm steel balls in a 20-cm-diameter cylindrical container. The container was shaken at a frequency of 4.98 Hz and a stroke length of 51 mm for 8-s intervals until all the pods had desheathed. After each interval, fruits were examined and those with at least one of the valves detached were considered broken. The percentage of broken fruits was recorded each time, from which a curve was plotted showing the time that 50% of fruits had shattered.

qRT–PCR

Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized by M-MuLV Reverse Transcriptase (Promega). qPCR was performed using SYBR Green Jumpstart Taq ReadyMix (Sigma) on a Chromo4 Real-Time PCR detector (Bio-Rad). All the experiments were performed using three biological and three technical repeats. USB2.10 was used as an internal control.

Scanning Electron Microscopy

Fruits were fixed in FAA (50% ethanol, 5% glacial acetic, and 3.7% formaldehyde) for 4 h at room temperature and overnight at 4°C. Samples were then dehydrated through an ethanol series (15 min each in 50%, 60%, 70%, 80%, 90%, 95%, 100%, and 100% and dry ethanol). After critical point drying, samples were coated with gold and examined using a Zeiss Supra 55VP field emission scanning electron microscope.

Histology

Fruits were fixed in FAA solution and dehydrated through an ethanol series. The samples were then embedded in the resin Technovit 7100 (Electron Microscopy Sciences) according to the manufacturer’s instructions. Sections (5 µm) were prepared and stained with 0.01% toluidine blue O before taking images on a Leica DMI6000 microscope. Visualization of lignified cells was performed by staining the sections with 0.02% crystal violet (Sigma). Images were taken on a Zeiss LSM 780 confocal microscope. Fluorescence of lignified cell walls was excited by the 561-nm excitation and collected between 660 and 758 nm. Images were analyzed using ImageJ (Schneider et al., 2012). The lignified valve-margin cell walls were outlined and mean intensity was measured. Mean fluorescence intensity of non-lignified cell wall was also recorded as background. The difference between the two values was calculated as the final mean fluorescence intensity.

Reporter Constructs

For the IND::3xVENUS::ALS construct, a 2.7-kb IND promoter was amplified from the vector pCRCRY769/GW/TOPO (Invitrogen) as a level 0 module. The modules of 3xVENUS (IE10429) and NLS (EC25118) were obtained from Giles Oldroyd’s laboratory (John Innes Centre). The OCS terminator was amplified from the vector pICH86988 (Weber et al., 2011) and cloned into the vector pCRCRY769/GW/TOPO as a level 0 module. The modules of IND::3xVENUS::ALS, the cloned level 0 modules of IND::3xVENUS::ALS, and OCS terminator directly into the level 2 vector pICH86969 (Weber et al., 2011) by Golden Gate assembly.

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For the IND::IND::EFVP construct, a 2.7-kb IND native promoter in frame with the 510-bp IND coding sequence was amplified from genomic DNA. The amplicon was cloned into the destination vector pRLV16, which comes with EFVP.

For the ADPG1::ADPG1::GFP construct, the ADPG1 promoter and ADPG1 coding sequence were amplified from genomic DNA and cloned into the vector pCRCRY769/GW/TOPO (Invitrogen) as a level 0 module. The level 0 modules of ADPG1::GFP and OCS were obtained from Giles Oldroyd’s laboratory, and OCS terminator was then cloned directly into the level 2 vector pICH86969 (Weber et al., 2011) by Golden Gate assembly to generate ADPG1::ADPG1::GFP.

Confocal Microscopy

Fluorescent images were taken on a Zeiss LSM 780 confocal microscope. For the imaging of IND::3xVENUS::ALS and IND::IND::EFVP, VENUS, EFVP, and chloroplast autofluorescence were excited by the 514-nm excitation. Fluorescence emissions were recorded from 519 to 659 nm for VENUS and EFVP signals and from 628 to 735 nm for autotransfluorescence. The images were obtained by z-stack scanning, ensuring that all the nuclear VENUS and EFVP signals were collected. ImageJ (Schneider et al., 2012) was used to project the z-stacks to 2D images and the VENUS intensities from the 2D images were measured with ImageJ. For the imaging of H2A-Z::GFP and ADPG1::ADPG1::GFP, GFP and chloroplast autofluorescence were excited by the 488-nm excitation. GFP emission spectra were collected between 499 and 526 nm and autotransfluorescence signals were collected between 626 and 695 nm.

Construct of IND::GR::IND::GUS

A 2.5-kb IND promoter fragment was amplified from Arabidopsis genomic DNA and cloned into the Gateway donor vector pDONR207, then recombined into the vector pB4N-LR-LhG4R2 (Craft et al., 2009) to produce IND::LhG4R2. The IND coding sequence was amplified from Arabidopsis genomic DNA (single exon) and cloned into the Gateway donor vector pDONR207, then recombined into the vector pOp6p promoter (Craft et al., 2009), to produce pOp6p::IND. Finally, the IND::LhG4R2 fusion fragment was digested by Ascl and inserted into the vector of pOp6p::IND to generate IND::LhG::pOp6::pOp6::IND (IND::GR::IND::GUS) (Supplemental Figure S). The construct was introduced into Agrobacterium tumefaciens strain AGL1 for transformation into ind-2 mutant plants.

Dexamethasone Treatment

Dex (10 mM) (Sigma-Aldrich) stock solution dissolved in ethanol was prepared and stored at -20°C. Dex solutions at 1 μM, 5 μM, and 25 μM with 0.015% Silwet L-77 were freshly made as working solutions. For the treatment, inflorescence and siliques were dipped in the solutions for 5 s and treated every 2 days until siliques reached stage 18.

GUS Assay

Fruits at stage 18 were placed in Eppendorf tubes containing X-Gluc solution [1 mg/ml X-Gluc [s-brom-o-choro-3-indoly glucuronide. Melford] dissolved in DMSO, 100 mM sodium phosphate buffer, 10 mM EDTA, 0.5 mM K3Fe(CN)6, 3 mM K4Fe(CN)6, and 0.01% Triton X-100] and vacuum infiltrated for 30 s, then incubated at 37°C in the dark for 16 h. The fruits were then treated in 70% ethanol to destain for 2-3 days before taking images on a Leica M205 FA stereo microscope.

Chromatin Immunoprecipitation

ChIP was carried out essentially as described by Kumar et al. (2012) with minor modifications. There are three H2A.Z-coding genes (HTHA, HTA3, and HTA11) in Arabidopsis and HTA11::HTA11::GFP (Kumar and Wigge, 2010) (referred to as H2A.Z::GFP) was used to carry out the ChIP assay. Stage-16 fruits from HTA11::HTA11::GFP (H2A.Z::GFP) plants grown at 17°C, 22°C, and 27°C were collected and immediately frozen in liquid
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Approximately 0.5 g of tissue was ground in liquid nitrogen and chromatin was prepared as described earlier and subsequently cross-linked with 1% formaldehyde. ChIP was performed using paramagnetic μMACS beads coated with monoclonal anti-GFP antibody (Miltenyi Biotec) according to the manufacturer’s instructions. Beads were washed four times with the immunoprecipitation buffer followed by two washes with TE. Reverse crosslinking was done by boiling at 65°C for 10 min in presence of 10% Chelex (Bio-Rad) followed by Proteinase K treatment at 50°C. Similarly, input DNA was prepared after reversing the crosslinks at 95°C for 10 min in presence of 10% Chelex, followed by Proteinase K and RNase treatment. DNA was ethanol precipitated following phenol/chloroform extraction. qPCR was performed using a Roche LightCycler.

SUPPLEMENTAL INFORMATION
Supplemental Information is available at Molecular Plant Online.

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AUTHOR CONTRIBUTIONS
X.-R.L., S.V.K., and L.O conceived the experiments. X.-R.L performed the experiments except the H2A.Z ChIP assays, which were performed by S.V.K. J.D. constructed the IND:IND:EYFP construct. X.-R.L., S.V.K., and L.O. analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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