INDUCER OF CBF EXPRESSION 1 (ICE1) is a male fertility regulator impacting anther dehydration in Arabidopsis

Donghui Wei1, Mingjia Liu1, Hu Chen1, Ye Zheng1, Yuxiao Liu1, Xi Wang2, Shuhua Yang2, Mingqi Zhou1*, Juan Lin1*

1 State Key Laboratory of Genetic Engineering, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai, China, 2 State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing, China

* 082023064@fudan.edu.cn (MZ); linjuan@fudan.edu.cn (JL)

Abstract

INDUCER OF CBF EXPRESSION 1 (ICE1) encodes a MYC-like basic helix-loop-helix (bHLH) transcription factor playing a critical role in plant responses to chilling and freezing stresses and leaf stomata development. However, no information connecting ICE1 and reproductive development has been reported. In this study, we show that ICE1 controls plant male fertility via impacting anther dehydration. The loss-of-function mutation in ICE1 gene in Arabidopsis caused anther indehiscence and decreased pollen viability as well as germination rate. Further analysis revealed that the anthers in the mutant of ICE1 (ice1-2) had the structure of stomium, though the epidermis did not shrink to dehisce. The anther indehiscence and influenced pollen viability as well as germination in ice1-2 were due to abnormal anther dehydration, for most of anthers dehisced with drought treatment and pollen grains from those dehydrated anthers had similar viability and germination rates compared with wild type. Accordingly, the sterility of ice1-2 could be rescued by ambient dehydration treatments. Likewise, the stomatal differentiation of ice1-2 anther epidermis was disrupted in a different manner compared with that in leaves. ICE1 specifically bound to MYC-recognition elements in the promoter of FAMA, a key regulator of guard cell differentiation, to activate FAMA expression. Transcriptome profiling in the anther tissues further exhibited ICE1-modulated genes associated with water transport and ion exchange in the anther. Together, this work reveals the key role of ICE1 in male fertility control and establishes a regulatory network mediated by ICE1 for stomata development and water movement in the anther.

Author summary

INDUCER OF CBF EXPRESSION 1 (ICE1) is a basic helix-loop-helix transcription factor playing multiple roles in Arabidopsis. It was initially identified as the activator of C-Repeat Binding Factor 3 (CBF3), a core modulator triggering cold acclimation. ICE1 also activates Flowering Locus C (FLC), a major repressor of floral transition, to delay flowering
under fluctuating environmental stimuli. In normal conditions, ICE1 participates in control of stomatal development in leaves and endosperm breakdown in seeds. Here we describe a role of ICE1 in male fertility development of Arabidopsis. We provide evidence that ICE1 controls stomatal differentiation in the anther epidermis and thereby anther dehiscence and pollen viability as well as germination. Consequently, fertility of ice1 mutant can be rescued by ambient dehydration. ICE1 regulates FAMA, one key regulator of guard cell differentiation, through direct binding to MYC-recognition elements in FAMA promoter. Moreover, we perform transcriptomic analysis using anther tissues and identify ICE1-regulated genes involved in water transport. These findings reveal a novel role of ICE1 in male fertility regulation through affecting water movement in the anther, which deepens our understanding of coordination between plant development and stress response, and potentially contributes to the pollination controls in crop breeding.

Introduction

The stamen is the male reproductive organ of flowering plants and at a gross level comprises the filament and the anther [1, 2]. The late phase of stamen development including filament elongation, anther dehiscence, and pollen maturation, is an essential process in which mature pollen grains are released from locules in the dehiscent anthers, thus enabling pollination and fertilization [3]. Successful fertilization relies on the production and effective release of viable pollen [4]. Failure of anther opening (dehiscence) results in male sterility, although the pollen itself can be fully functional [5]. Anther dehiscence is a complex process involving multiple aspects, such as cellular differentiation and degradation, combined with tissue structure alteration as well as dehydration in anthers, which are also regulated by phytohormones [5–6]. A variety of mutants with disturbed anther development in the late stages have been identified in Arabidopsis and the corresponding genes are characterized. The genes characterized so far are categorized into two major functional groups. One is a set of regulators controlling anther structure dynamics including the anther cell layers formation (e.g., middle layer [6], tapetum [5], septum [7] and stomium [8–11]), secondary thickening in the endothecium [12–20], programmed cell death in sporophyte tissues of anthers (e.g., tapetum, septum and stomium) [4, 21], and cell wall degradation (e.g., degradation of cell wall components, such as cellulose, hemicellulose and pectin, in anther dehiscence zones catalyzed by cell wall-degrading enzymes) [22]. The other group includes genes affecting the anther physiological changes, such as water influx [23], ion homeostasis [24, 25] and carbohydrate metabolism [26–28]. Notably, most of the genes belonging to this functional group are closely related to anther dehydration. Young anthers take up water for growth during early developmental stages, while at later stages anthers and pollen undergo dehydration before dehiscence [29, 30]. The dehydration caused by evaporation through stomata and water transport in the vascular bundle promotes pollen grains maturation, anther dehiscence and filament elongation [31–33]. In addition, these two groups of genes are regulated by phytohormones. Studies on jasmonic acid (JA) biosynthetic genes [32–36], JA signaling components including COI1 [37], MYC and MYB genes [38–43], and a JA transporter GTR1 [44] have demonstrated that JA plays essential roles in the control of timing of anther dehiscence and pollen maturation. JA positively affects stomium opening [45] and anther dehydration by regulating water transport from anther to filament [32, 46]. Auxin, generally known as a negative regulator of endothecium lignification, also functions essentially at late anther developmental stages [47–54]. Mutants with disrupted auxin biosynthetic genes or auxin responsive transcription factors are deficient in anther
dehiscence, pollen maturation or filament elongation [55–58]. During the modulation of stomium opening in anther dehiscence and pollen maturation, auxin negatively controls the biosynthesis of JA [52, 56–59]. Deficiency of genes participating in any of these processes can cause anther indehiscence, which is mediated and coordinated by cell layers development and anther dehydration. In comparison, the studies with respect to genes involved in anther dehydration remain relatively limited.

INDUCER OF CBF EXPRESSION 1 (ICE1), also known as SCREAM (SCRM1), is a MYC-like basic helix-loop-helix (bHLH) transcription factor regulating plant responses to chilling and freezing stress and leaf stomata development in normal conditions. Under cold stress, ICE1 is subjected to cold-activated modification [60–63] and subsequently binds to promoters of C-REPEAT BINDING FACTOR (CBF3) [64] to enhance cold tolerance. The identified modification of ICE1 protein includes sumoylation and phosphorylation. In cold exposure, a small ubiquitin-related modifier (SUMO) E3 ligase, SAP and Miz 1 (SIZ1), facilitates SUMO conjugation to ICE1 [60] and a protein kinase, OPEN STOMATA 1 (OST1), phosphorylates ICE1 to enhance its stability and transcriptional activity [61]. Meanwhile, mitogen-activated protein kinase 3 and 6 (MPK3/6) also phosphorylates but destabilizes ICE1 in response to cold [62, 63]. ICE1 can be degraded through E3 ubiquitin ligases, high expression of osmotically responsive genes 1 (HOS1) [65] and constitutive photomorphogenic 1 (COP1) [66]. These established a well-characterized regulatory network of ICE1 in low temperature. In ambient temperature, ICE1 directly interacts with three bHLH transcription factors, SPCH, MUTE, and FAMA, to regulate stomatal differentiation in the leaf epidermis [67]. Previous studies also demonstrated that the loss-of-function mutation of ICE1 caused early-flowering with elevated Flower Locus C (FLC) gene expression [68] and seed endosperm persistence phenotype that was also observed in the mutant of an endosperm breakdown regulator, ZHOUPI (ZOU) [69]. Thus, ICE1 functions in multiple organs at different developmental stages of plants in responses to environmental variations.

Here, we illuminate a novel role for ICE1 as a male fertility modulator in Arabidopsis. In the ice1 mutant, the anther wall could not shrink to complete a sufficient anther dehiscence and anthers failed to conduct pollen release. Pollen grains from those indehiscent anthers also showed less viability and lower germination rate. Phenotypic and transcriptomic evidences indicate that the deficient anther dehiscence and pollen germination are associated with water movement and dehydration of anther wall due to the impaired stomatal differentiation as well as altered water transport and ion exchange related genes. Our work brings a new member to anther dehiscence regulators and implicates a potential link among the regulation of environmental responses, vegetative growth, floral transition and fertility development.

Results and discussion

Loss-of-function mutation of ICE1 impairs fertility in Arabidopsis

In the previously characterized null mutant SALK_003155 in the Columbia (Col-0) background with a T-DNA insertion in the third exon of the ICE1 gene (Fig 1A) (named as ice1-2) [67], we observed reduced fertility (Fig 1B), nevertheless no information with respect to the function of ICE1 in reproductive development has been reported. The extremely low expression level of ICE1 was verified in inflorescences of the ice1-2 (Fig 1C). To investigate the function of ICE1 gene involved in plant fertility, we generated ICE1pro:ICE1 ice1-2 lines, named as c-ice1-2. Complementation of ICE1 expression and phenotype of reproductive development were confirmed (Fig 1B and 1C). Further characterization revealed that the ice1-2 developed significantly shorter siliques with fewer seeds in each, while c-ice1-2 plants showed restored phenotypes (Fig 1D–1F). In addition, ice1-2 pistils artificially pollinated with Col-0 pollen
Fig 1. Characterization of the sterile phenotype in ice1-2. (A) Structures of the ICE1 gene in ice1-2 mutant (SALK_003155). The scaled linear map depicts four exons as boxes and three introns as bold lines between boxes. The positions of qRT-PCR primers (indicated by arrows) and T-DNA insertion are shown. (B) Morphology of reproductive growth of Col-0, ice1-2 and c-ice1-2 plants. (C) Relative expression of ICE1 gene in inflorescences. The ACTIN2 gene (AT3G18780) was an internal control. SE, n = 3, ** p < 0.001. (D) Comparison of seed numbers per silique of each genotype. SE, n = 32, *** p < 0.001. (E) Morphology of siliques from Col-0, ice1-2 and c-ice1-2 fresh plants. (F) Comparison of silique length of each genotype. SE, n = 14.

https://doi.org/10.1371/journal.pgen.1007695.g001
grains were able to develop into normal siliques, while pollination using *ice1-2* pollen was failed in either Col-0 or *ice1-2* plants (S1A Fig), demonstrating that the mutant is female-sterile. Together, *ICE1* is involved in plant male fertility development and controls seed productivity. Intriguingly, another well characterized mutant *ice2-1/scrm2-1* (SAIL_808_B10) disrupting *ICE2/SCRM2*, the paralog of *ICE1* functioning similarly in cold response and leaf stomata development [70, 71], did not show any phenotype in fertility (S1B Fig), which could be due to functional redundancy or the different roles of *ICE1*-like transcription factors in developmental regulation.

### The *ice1* mutant is defective in anther dehiscence

After a closer examination of flower anatomy using scanning electron microscopy (SEM), we observed very few pollen grains around the style or on the stigma in *ice1-2* (S2B Fig) compared with Col-0 (S2A Fig) and *c-ice1-2* (S2C Fig), thus stigmas of *ice1-2* typically were unpollinated. Besides, anthers were only occasionally open while most of them remained indehiscent in *ice1-2*. We then compared the floral development in Col-0, *ice1-2*, and *c-ice1-2* plants using light microscopy across flower development stages [45, 72]. At stage 12, no difference of anther morphology was observed in Col-0, *ice1-2* and *c-ice1-2* (Fig 2Aa, 2Ae and 2Ai). In Col-0 and *c-ice1-2*, anthers started to dehisce at stage 13, with concomitant pollen release from the locules after the full expansion of the stigmatic papilla (stage 13) (Fig 2Ab and 2Aj) and shriveling of the anther epidermis cell wall (stage 14) (Fig 2Ac and 2Ak), followed by initial stages of siliques expansion and floral senescence (stage 15) (Fig 2Ad and 2Al) [1]. In contrast, most of *ice1-2* anthers did not dehisce at flower stage 13 and later stages (Fig 2Af–2Ah). Majority of the mutant anthers did not dehisce and release pollen grains until the initiation of floral senescence (stage 15) (Fig 2Ah). Based on the flower developmental series, we quantitatively analyzed the process of anther dehiscence in single inflorescences. The youngest flower with visible petals within a flower cluster was labeled as flower 1 and the next elder flower was labeled as flower 2, and so on [45] (Fig 2B). In Col-0 and *c-ice1-2* plants, more than 95% of anthers had dehisced in flower 3 (5.72 of 6 in Col-0 and 5.87 of 6 in *c-ice1-2*) and elder ones, while the dehisced anther number was significantly lower in *ice1-2* in flowers 3–5 (7.7%, 0.46 of 6 for flower 3). Even in the oldest flower 5 only 27% (1.62 of 6) of anthers were dehisced (Fig 2C). In fact, even for dehisced anthers in *ice1-2*, most of them were still not fully open like that in Col-0. Therefore, *ICE1* is required for dehiscence of anther and the decrease of fertility in *ice1-2* is related to indehiscent anthers. Further characterization of anther adaxial surface using SEM provided a closer insight into this phenotype. At stage 12 of anther development in Col-0 and *c-ice1-2* flowers, the anthers had locules filled with liquid and an indentation (stomium region) in epidermis [72] (Fig 2Da and 2Dd). From stages 12 to 13, the dehiscence program was initiated from the apical toward basal parts. A stomium emerged at the apical of anther and the epidermis cells started to shrink (Fig 2Db and 2Dj). The slit on the stomium begins to widen, resulting in release of pollen at stages 14 (Fig 2Dc and 2Dk) and stages 15 (Fig 2Dd and 2Dl). In contrast, in *ice1-2* anthers the stomium slit was visible at stage 13 and stage 14 (Fig 2Df and 2Dg). However, the stomium did not rupture sufficiently even at stage 15 and epidermis cells failed to shrink to release pollen from individual anther locules to the stigma (Fig 2Db). Hence, the *ice1* mutation disrupts the shrinkage of anther wall and prevent the release of pollen at the proper stage of pollination. Previous studies have shown that failure of anther dehiscence can be elicited by abnormal cell organization and differentiation of anther tissues [4]. The key processes affecting dehiscence include development of cell layers of the anther [6, 73], endothecium secondary thickening [12, 14], degradation of middle layer and tapetum [6, 74], septum breakdown [33, 75–77], and stomium opening [78]. To determine if
Fig 2. Stamen morphology and anther dehiscence in ice1-2. (A) Developmental series of flowers at flower developmental stage 12–15 within a single inflorescence from Col-0, c-ice1-2 and ice1-2. A, anther; F, filament; Ov, ovary; Pa, stigmatic papilla; Sg, stigma; Sy, style; Pe, Petal; Pg, Pollen grain. (B) Flower cluster showing the developmental series used to quantitatively describe anther dehiscence. The number 0 indicates the beginning of flower stage 12; 2 indicates the end of stage 12; 1 (stage 12); 3 (stage 13); 4 (stage 14); 5 (stage 15); -1 (stage 11); -2 (stage 11); -3 (stage 10); -4 (stage 9). (C) The number of dehiscent anthers in plants (SE, n = 15–28 flowers, one inflorescence per plant was used, ***, p < 0.001). (D) Scanning electron micrographs of the anther adaxial surface from flower stage 12–15. St, stamium; En, epidermis; L, locule, StR, stamium region; Pg, Pollen grain.

https://doi.org/10.1371/journal.pgen.1007695.g002
there was morphological abnormality in the anther tissues, we observed transverse sections of Col-0 and ice1-2 anthers from the emergence of dehiscence to senescence during stamen development. In both Col-0 and ice1-2, tapetum was visible and started to break down at anther developmental stage 10; at stage 11 endothecium started the lignification for secondary thickening, tapetum was degraded, and septum started to break down; at stage 12 the septum was degraded through a programmed cell death-like lysis to form a single locule (S3 Fig). In Col-0, stomium was open and epidermis started to shrink to release pollen grains at stage 13, and epidermis kept shrinking and releasing pollen at stage 14a. Until stage 14b all pollen grains were dispersed. In ice1-2, although stomium was ruptured, epidermis did not shrink and pollen grains were still covered inside the locules until stage 14b (S3 Fig). The auramine O staining in both semi-thin sections and fresh anthers at anther stage 13 also showed that no obvious difference was between Col-0 and ice1-2 for endothecium secondary thickening that was occurred from stage 11 (S4A and S4B Fig). Whereas at stage 14 very few pollen grains were still inside anthers of Col-0 (S4Be Fig), while the ice1-2 anthers were full of pollen (S4Bf Fig). Taken together, ICE1 may not influence formation of anther cell layers but regulates epidermis shrinkage at the stage of pollen dispersal.

Further, the sizes of stamen and pistil tissues were also investigated using light microscopy. The filaments were fully elongated to position the anthers at the height of the stigma at flower developmental stage 14 in Col-0 and c-ice1-2 (S5Aa and S5Ac Fig). In ice1-2, the stamen and style lengths were slightly shorter and the stamen/style length ratio was smaller (S5B and S5C Fig). The reduced elongation of stamen tissues is also commonly observed in mutants interrupting anther dehiscence [4]. But in ice1-2, the shorter stamen and pistil may not be the main reason of sterility, since the filaments were able to elongate and allowed anthers to reach stigma (S5Ab Fig).

The ice1 mutant shows decreased pollen viability and germination rate

During the dehiscence of the anther, one of the key forces that open the anther comes from the swelling of pollen grains [79]. In mutants such as apy6/7 [80], yuc6 [81] and ams [82], delay or lack of anther dehiscence is due to abnormal pollen exine formation or absence of pollen. Here, the pollen development in Col-0 and ice1-2 was examined. Similar with Col-0, ice1-2 anthers enveloped fully differentiated pollen grains (Fig 3A). The microspores developed into tricellular pollen and the exine structure was normally formed, suggesting an intact meiotic division process and completed trinucleate stage. However, viability of ice1-2 pollen grains was obviously lower than Col-0 and c-ice1-2 shown by fluorescein diacetate (FDA) staining (living cell emits blue-green light [40]) at anther stage 13 (Fig 3B and 3C), indicating that the pollen maturation was influenced at the final phase. Moreover, ice1-2 pollen grains showed a significantly lower in vitro germination rate compared with Col-0 at stage 13, and the germination remained poor until stage 15 (Fig 3D). Consistently, the in vivo germination capacity determined through pollination on Col-0 pistils also demonstrated that ice1-2 pollen was deficient in germination (Fig 3E). Most of ice1-2 anthers were manually opened or enlarged for collection of pollen grains. Interestingly, we noticed that when we selected the small proportion of ice1-2 anthers with obviously open stomium and pick pollen grains exposed at the stomium area to do the pollination, the germination was rescued at both stage 13 and stage 15 (Fig 3E). Notably, even for those ice1-2 anthers with open stomium, most of them were still half-dehiscent (Fig 3F). In the in vitro germination assay, hundreds of pollen grains including ones exposed at the stomium area and those enveloped inside epidermis were pooled on media. Thus, it was not surprising to see that pollen grains from ice1-2 anthers possessing open stomium still showed low in vitro germination rate, which was higher than typical ice1-2 anthers.
though (Fig 3D). Given the fact that pollen structure was intact and pollen grains exposed at the stomium area could germinate in pollination, the impaired pollen viability and germination in ice1-2 might be related to abnormal anther dehiscence and dehydration.

**The impaired anther dehiscence, pollen viability and pollen germination in ice1 mutant are due to deficiency in anther dehydration**

Water status is critical for development of pollen grains and anthers. Pollen maturation and anther dehiscence are coordinated processes involving water absorbance and dehydration
of anther tissues including endothecium and epidermal cells [4, 83]. Desiccation of the anther leading to shrinkage of the outer wall provides the final force for anther opening [31]. During pollen development, pollen water content will decrease to a minimum at maturity before dispersal, and rehydrate after pollination [83]. To confirm whether the defects of anther dehiscence and pollen maturation in ice1-2 were due to the issue of dehydration, we examined the anther dehiscence rate in different relative humidity (RH) conditions. The 80% RH environment was the normal growth condition of Arabidopsis plants and 40% RH was used as the dehydration treatment. The anther dehiscence rates and phenotypes were recorded at flower stage 13 that is the key stage for anther dehiscence and pollination [1]. Under 80% RH Col-0 showed higher anther dehiscence rate than ice1-2, while under 40% RH the ice1-2 anther dehiscence rate was significantly increased (Fig 4A and 4B). Moreover, the deficiency of ice1-2 in the pollen viability (Fig 4C and 4E), pollen germination (Fig 4D and 4F), and pollen function indicated by pollination on Col-0 pistils (Fig 4G and 4H) were all rescued by 40% RH treatment. Especially for pollen, ice1-2 reached wild type levels in all three indices. As a consequence, the sterility phenotypes of ice1-2 could be rescued by drought treatment as well (Fig 5A–5C). These further demonstrated that in ice1-2 the anther indehiscence and impaired pollen function are due to deficiency in dehydration of anther tissues such as anther wall, which can be derived from abnormal water allocation within the stamen. These are also consistent with the previous studies showing that pollen maturation and anther dehiscence are co-regulated during water movement associated processes [83].

**ICE1 is expressed in anther stomata and multiple flower vascular bundles**

It has been suggested that water moves out of the anther via the transport in the vascular bundle and evaporation of epidermis stomata [28, 31]. The dehydration of endothecium, connective, and locules can be partially attributable to the evaporation of water through the stomata on the abaxial surface of anthers [31]. Previous studies indicated that ICE1 was expressed in leaf guard cells [67]. We investigated the promoter activity of ICE1 at the stages of floral development involving anther dehiscence program events using β-glucuronidase (GUS) report system. Three independent ICE1pro::GUS transgenic lines were assayed and exhibited consistent patterns. The ICE1 promoter showed a strong activity in the inflorescence and floral organs (S6A Fig). At approximately flower stage 10 (the petals reach the lateral stamens) [1], the style, sepals, and filaments showed strong staining, whereas no obvious GUS staining was observed in the anther tissues (S6B Fig). As the flowers developed to stage 12–15, the GUS staining remained in sepals (S6C–S6E Fig), especially vascular tissues of sepals (S6F Fig), as well as the style (S6G Fig), and turned to be much stronger in connective of anthers (S6H Fig), filaments (S6I Fig), pedicels (S6J Fig), and vascular tissues of petals (S6K Fig). In immature siliques, GUS staining was restricted to the septum, the silique tip, and the base (S6L Fig). Remarkably, although the GUS signal in the adaxial side of anthers was weak in flowers at stage 12–15, a strong staining was observed in guard cells of stomata in the abaxial side of anthers (Fig 6A), where the ICE1 protein was accordingly accumulated (Fig 6B). The water transport from anther locules to filaments and petals is essential for pollen maturation and anther dehiscence [32]. Multiple genes involved in anther dehiscence were found to be specific expressed in anther guard cells [25, 45, 84, 85], filaments [6, 32, 49], anthers and filaments junction tissues [27, 50], anther wall and vascular bundle [23]. DAD1 strictly expressed in filaments controlling JA biosynthesis and likely water transport also regulates anther dehiscence and pollen maturation [32]. Consistent with the fact that sterile phenotype of ice1-2 can be rescued by dehydration, the high activity of ICE1 promoter in anther stomata and flower vascular bundles suggest...
Fig 4. The pollen inviability, low pollen germination rate and anther indehiscence in ice1-2 can be rescued when grown in low humidity. (A) Flowers and anthers from Col-0 and ice1-2 plants grown under 40% or 80% relative humidity (RH), respectively. The insets (top left corner) exhibit magnification of anther phenotypes. (B) Comparison of dehisced anther numbers between Col-0 and ice1-2 per flower at flower stage 13 under 40% and 80% RH, respectively. (SE, n = 25–292 flowers, *** p < 0.001). (C) FDA (fluorescein diacetate) staining of pollen from Col-0 and ice1-2 at stage 13 under 40% RH. (D) The in vitro germination of pollen from Col-0 and ice1-2 at stage 13 under 40% RH (upper row). The aniline blue-stained pistils of Col-0 flowers at 2 h after pollination with pollen from Col-0 and ice1-2 at stage 13 under 40% RH are also shown (lower row). (E-G) Comparison of pollen viability (E) (SE, n = 5), pollen germination rates (F) (SE, n = 3), and seed numbers per silique (G) (SE, n = 20) between Col-0 and ice1-2 grown under
40% RH. (H) Manual pollination on the Col-0 plants grown in the normal condition with pollen from Col-0 and ice1-2 under 40% RH, respectively. Arrows indicate the normal siliques generated using ice1-2 pollen under 40% RH.

https://doi.org/10.1371/journal.pgen.1007695.g004

a connection of ICE1 function in particular with appropriate dehydration of pollen and/or anthers.

**ICE1 regulates the stomatal differentiation in the anther**

At anthesis, endothecium and epidermal cells in anther wall lose most of water via evaporation of stomata on the abaxial side of anthers [86] and osmotic retraction of water through
Fig 6. Stomata development of the anther is controlled by ICE1. (A) GUS activity driven by ICE1 promoter is determined in the anther of flower stage 8 and 12. Strong GUS staining is shown in guard cells of stomata in the anther of flower stage 12 (indicated by red arrows). (B) Confocal images showing GFP-ICE1 accumulation (indicated by blue arrows) in the anther of flower stage 12. (C) Stomata numbers of anthers in flower stage 9–12. SE, n = 7–42 anthers. (D) Mode pattern of stomatal development in anthers. Diagram shows cell-state transitional steps within stomatal cell lineages. A subset of protodermal cells (white) assumes meristematic mother cell (MMC) identity and executes an asymmetric entry division that creates meristemoids (M) (blue) and a sister cell, called stomatal-lineage ground cell (SLGC) (white). The meristemoids reiterate asymmetric amplifying division, but eventually differentiate into the guard

ICE1 regulates anther dehydration

PLOS Genetics | https://doi.org/10.1371/journal.pgen.1007695 October 4, 2018 12 / 32
filaments and connective tissue surrounding the vasculature [27]. Actually, in *Arabidopsis* not much information focused on stomatal development in anthers has been reported, and little attention has been paid to the role of anther stomata in anther dehiscence. Not all plant species possess stomatal pores in anther epidermis and developmental process of anther stomata depends on species [87]. In order to systematically describe the stomata development in the anther of *Arabidopsis*, we counted the number of anther stomata in flowers at stages from 9 to 12 in Col-0. The anther stomata increased from 1.57 to 5.89 at stage 9 to 11, while at stage 12 much more stomata (22.38) were identified in the anther (Fig 6C). According to the stomatal lineage model in *Arabidopsis* leaves [88], stomata differentiate via a series of cell transitions. A group of protodermal cells called meristemoid mother cells can produce meristemoids (Ms) through asymmetric divisions. Meristemoids reiterate asymmetric divisions to generate surrounding stomatal lineage ground cells (SLGCs) and eventually differentiate into guard mother cells (GMCs). One guard mother cell undergoes one time of symmetric division to produce a pair of guard cells (GCs) (Fig 6D). We used scanning electron microscopy (SEM) to perform more detailed characterization for stomata lineage in Col-0 anthers of flowers from stage 8 (before generation of stomatal lineage cells) to stage 14 (after anther dehiscence). No stomata were observed in the adaxial side of anther epidermis. In the abaxial side, cell number started to increase but no stomatal lineage cells or mature GCs appeared yet at flower stage 8 (Fig 6Ea). At stage 9, cell types were destined and stomatal lineage cells as well as few mature guard cells within top area were identified (Fig 6Eb). After that, the epidermal cells gradually expanded and more stomata turned to mature. At stage 10 and 11, mature GCs kept increasing (Fig 6Ec and 6Ed). At stage 12 with a longer duration, the number of mature GCs significantly increased, and most of stomata matured completely at the end of stage 12 (Fig 6Ee). At this moment, the anther shape was changed from oval to round and stomata gradually matured from the top to the bottom. Mature GCs were concentrated in the middle lengthways of the abaxial side in the anther epidermis (Fig 6Ee). From stage 13 to 14, the enhancing shrinkage of anther wall prompted the rupture in the adaxial side and the pollen dispersed (Fig 6Ef and 6Eg). Stomata were not present in filaments. The accumulation of matured stomata in stage 12 from the top toward the bottom in epidermis coincided the stage at which the anther wall started to shrink and then opened from the top, suggesting the role of stomata in anther dehydration and dehiscence in *Arabidopsis*.

*ICE1* has been reported as a regulator of stomatal differentiation at the surface of leaves [67], but it is unclear whether *ICE1* is involved in stomatal differentiation in anthers. Since in mature stomata of anthers *ICE1* promoter was strongly active and ICE1 protein was highly accumulated (Fig 6A and 6B), we therefore examined how *ice1-2* mutation affected stomatal development in anthers. At flower stage 12, Col-0 and *c-ice1-2* plants. Cells colored in pink show distribution of mature stomata. Blue arrows indicate M and SLGC; green arrows indicate GMC; red arrows indicate GC. (G) Comparison of stomata numbers in Col-0, *ice1-2* and *c-ice1-2* plants at flower stage 12. SE, n = 30–42 anthers, ***p < 0.001).
the stomata development in \textit{ice1-2} leaves, in which stomata clusters, GMC-like tumors aligned in parallel, and some differentiated GCs expressing mature guard cell marker E994 were present [67]. Consistently, we observed that in \textit{ice1-2} leaves more than one third of stomata showed differentiated GCs and nearly half were immature stomata including GMC-like tumors. Stomata clusters were also recorded (S7A and S7B Fig). In comparison, \textit{ice1-2} leaves resemble \textit{fama} leaves in stomata development phenotype showing excessive GMC symmetric divisions and defective terminal differentiation of GCs [67], but the phenotype in \textit{ice1-2} leaves is weaker for they can still form some differentiated GCs [67, 89] (S7A Fig). Whereas \textit{ice1-2} anthers do not exhibit structures indicating unrestricted GMC symmetric divisions and hardly possess differentiated GCs. Thus, \textit{ICE1} prompts stomatal differentiation in the anther in a different manner compared with that in leaves, and therefore can regulate anther dehydration to allow the dehiscence.

The stamen-expressed and the guard cell-expressed genes were highly overlapped within ICE1-regulated gene sets

Besides evaporation through stomata, many factors, such as signal of phytohormones, nutrient metabolism and transporters, also influence anther dehydration [23, 27, 32]. At present the direct data with respect to water content in the anther remain limited. To further investigate the effect of ICE1 underlying the phenotypes observed, we collected anthers at flower stage 9–13 covering critical time points for dehiscence and performed RNA-Seq to analyze ICE1-regulated genes in anthers. There were 1165 genes differentially expressed in the anther of \textit{ice1-2} compared to Col-0, with 732 up-regulated genes (UGs) (LogFC > 1, FDR < 0.05) and 433 down-regulated genes (DGs) (LogFC < -1, FDR < 0.05) (Fig 7A and S1 Table). For corroboration of the transcriptome data, three up-regulated genes and three down-regulated genes were subjected to qRT-PCR and these expression changes showed a good agreement between RNA-seq and qRT-PCR data (S8 Fig).

Among these differentially expressed genes (DEGs), 574 UGs and 205 DGs were identified as guard cell-expressed genes according to the gene expression database (http://www.arabidopsis.org/servlets/TairObject?type=keyword&id=19990 [90] and previously published transcriptome data of the leaf stomatal lineage [91]. Meanwhile, 452 UGs and 146 DGs were detected as stamen-expressed genes through stamen gene expression database (http://www.arabidopsis.org/servlets/TairObject?type=keyword&id=20328 [92] (Fig 7A and S1 Table). There were 429 UGs and 114 DGs expressed in both the guard cell and the stamen, indicating the significantly strong overlap between genes expressed in these two tissues for ICE1-regulated DEGs (p < 8.405e-44 for UGs and p < 1.560e-20 for DGs by hypergeometric test). The overrepresentation of guard cell-expressed genes within ICE-regulated genes in the anther reflects the key role of ICE1 in the regulatory network of stomata development of the stamen, which is in line with the phenotyping results.

\textbf{ICE1 specifically binds to FAMA promoter to activate its transcription}

Eight of these 543 guard cell & stamen DEGs play key roles in leaf stomatal development, including four UGs (\textit{TMM}, \textit{SPCH}, \textit{MUTE}, \textit{bHLH93}) and four DGs (\textit{FAMA}, \textit{EPF1}, \textit{MPK12}, and \textit{MPK14}) [93]. The results of qRT-PCR also confirmed that the expression of these genes was differentially regulated at flower developmental stage 10–13 of \textit{ice1-2} compared with Col-0 [83] (Fig 7B). \textit{FAMA} and \textit{EPF1} controlling guard cell differentiation [67, 94] were significantly down-regulated, which was in line with the impaired terminal differentiation of anther guard cells in \textit{ice1-2}. In leaves the \textit{ice1-2} phenotype was close to \textit{fama}, but for anthers we could not gain \textit{fama} materials due to its severe developmental defects [89]. The up-regulation of \textit{TMM},
Fig 7. Guard cell expressed genes are overrepresented within ICE1-regulated genes in the anther. (A) Number of down- and up-regulated genes (DG and UG) in anthers at flower stage 9–13 from ice1-2 compared with that in Col-0. The guard cell-expressed genes and the stamen-expressed genes are shown in circles of light green and dark green, respectively. (B) Heat map showing expression patterns of eight leaf stomatal development genes at the flower stage 10–13 in anthers from Col-0 and ice1-2 measured by qRT-PCR. The gene expression profiles were normalized with ACTIN2 gene (AT3G18780) and were plotted using Heatmapper (http://www2.heatmapper.ca). (C) Regulatory network of stomatal development in the anther. Red shows up-regulated genes and blue shows down-regulated genes in the anther of ice1-2. When ICE1 is knocked-out, the differentiation from guard mother cells (GMCs) to guard cells (GCs) is blocked.

https://doi.org/10.1371/journal.pgen.1007695.g007
SPCH, MUTE and bHLH93 in ice1-2 can also be due to feedback effects (Fig 7C). Using FAMA-pro::FAMA-GFP plants, we observed specific accumulation of FAMA in anther guard cells (Fig 8A). Moreover, while EPF1 promoter does not contain E-box motif (CANNTG) that is a typical binding motif of bHLH transcription factors [63], there are nine E-box elements in the FAMA promoter (2.5 kb from the transcription start site) (Fig 8B and S9A Fig). The in vivo dual-LUC assay with transient expression of ICE1 driven by 35S promoter (used as the effector) and LUC driven by truncated FAMA promoter fragments (used as reporters) demonstrated that in addition to protein interaction, ICE1 activated the FAMA transcription (Fig 8C and 8D). Further investigation using electrophoretic mobility shift assay (EMSA) showed two E-box elements located at -582 to -613 bp (labeled as P3) and -629 to -664 bp (labeled as P4) upstream from transcription start site specifically interacted with ICE1 (S9A and S9B Fig, Fig 8E and 8F). P4 exhibited an obviously higher in vitro binding affinity than P3 (Fig 8G). Another E-box element located at -1569 to -1600 bp (labeled as P7) also showed a weak binding with ICE1 but no competitive binding of cold probe was observed (S9B and S9C Fig), suggesting that the shift was due to a non-specific binding or the binding affinity was extremely low. P7 contains the same core sequences with P3 (S9A Fig), thus the flanking sequences may also play an important role in the ICE1 binding affinity.

The direct interaction between ICE1 and FAMA promoter is a novel interplay in the regulatory network of guard cell differentiation. It has been reported that FAMA also plays a positive role for ICE1 expression in young seedlings but does not bind to ICE1 promoter [95]. When FAMA is associated with its promoter, it is not necessary for its own expression [89]. Given the weaker developmental defects in ice1 than fama, ICE1 is unlikely necessary for FAMA expression. Rather, ICE1 may enhance the transcription of FAMA with other activators in a redundant manner, which can be a part of the regulatory network in the stomatal lineage development. However, the identification of a novel direct target of ICE1 can be potentially beneficial for breeding application.

ICE1 regulates genes involved in water movement in the anther

Gene ontology (GO) analysis using singular enrichment provided by agriGO [96] showed that a number of ion transporters, hydrolases and dehydration associated genes were positively regulated by ICE1 in anthers (Fig 9A and S2 Table). Ion gradients or currents are critical for active water movement in the anther and they regulate the anther dehiscence and pollen germination [6, 24, 85, 97, 98]. Some mutants affecting cation homeostasis, such as mia deficient in a P-type ATPase cation pump [99] and nhx1 nhx2 null in two Na\(^+\)/H\(^+\) antiporters [24, 25], also failed in sufficient release of pollen from mature anthers. Twelve transporter genes, in particular genes of sugar transporters, metal transporters as well as ATPases, were down-regulated in ice1-2 anthers (Fig 9A). Among them, STP1 [100], STP4 [101], CAX3 [102] and ACA12 [103] were expressed in leaf stomatal guard cells. The number of seeds per silique of aca12 mutant was significantly less than that in the wild type, indicating that ACA12 impacts plant fertility [103]. Accordingly, we observed wilted flower buds in old ice1-2 plants, which resembled the phenotype of nhx1 nhx2 under osmotic stress [25] (Fig 9C), suggesting that ICE1 modulates the ion exchange affecting water movement in flowers. Three glucosinolates hydrolysis related genes, TGG1, TGG2, and TGG3, as well as several glucosinolates biosynthesis genes, were also positively regulated by ICE1 (S2 Table). The glucosinolates are a group of secondary metabolites involved in ABA-regulated stomatal opening [104] and floral development in drought conditions [105]. The tgg1 tgg2 mutant showed stomata with closed aperture in leaves resembling plants in the face of drought stress [106]. Thus, carbohydrate hydrolysis can also be involved in ICE1-regulated anther dehydration.
Fig 8. ICE1 directly binds to the promoter of FAMA to activate its expression. (A) Confocal images of FAMA protein accumulation (indicated by arrows) in the anther at flower stage 12 in FAMApro::FAMA-GFP plants. (B) The upstream region of 2.5 kb from transcription start site and ORF sequences of FAMA are shown with a black line and a blackish green box, respectively. The vertical lines indicate the E-box positions. Eight probes (P1 to P8) containing E-boxes are also exhibited. P6 contains two E-boxes. (C) Dual-LUC Assays in tobacco leaves. ICE1 driven by 35S promoter was served as the effector and LUC under control of FAMA promoter (2.5 kb upstream from transcription start site) was the reporter. (D) The relative activity (LUC/REN) is shown. The reporter co-transformed with pC1302 vector was used as the
Besides, genes responding to water deprivation and auxin-mediated signaling pathways were enriched (Fig 9B, S3 Table). Two ABA-induced dehydrin genes affecting water use efficiency, RAB18 and LTI30 [107, 108], were remarkably repressed in ice1-2 mutant. RAB18 is highly expressed in guard cells, suggesting a role in stomatal function [109]. The downregulated auxin-mediated signaling genes included SAUR41, GH3.5, GH3.6, BT2, BT5, IAA 32, and MPK12. BT family proteins are essential during later stages of male gametophyte development [110, 111]. MPK12 is a MAP kinase that is preferentially expressed not only in leaves but also in other guard cells [112], and positively regulates ABA [112], JA [113] and SA signaling [114] in leaf guard cells of Arabidopsis. It has been shown that auxin represses JA biosynthesis to control the timing of stomium opening and prevent early anther dehiscence [52]. The genes negatively regulated by ICE1 were categorized into two biological processes including JA biosynthesis and response, and flavonoids associated pathway. In the stamens and petals, RA is mainly accumulated in the filaments to regulate water transport, which sequentially triggers flower opening and anther dehiscence [32]. The JA biosynthesis or signaling deficiency can cause profoundly male sterile [4, 45]. The null mutant of COI1, a JA receptor, exhibited delayed anther dehiscence and produced sterile pollen [37, 45]. JA-synthesis related genes, such as LOX2, AOS and OPR3, affect water movement in flowers as well [45, 84] (Fig 9B and S3 Table). The interrupted transport of flavonoids leads to abnormal dehydration and dehiscence of anthers [84]. High amounts of flavonoids are also considered as endogenous auxin transport regulators that affect plant growth [115]. Here, the down-regulation of auxin signaling genes and up-regulation of JA and flavonoid related genes in ice1-2 can be due to either active balance in regulation of water allocation or compensatory feedback consequences of failed stomium enlargement caused by abnormal water movement in the anthers and/or other floral tissues.

All the identified enriched pathways in GO analysis of ICE1-regulated genes are related to water transport (Fig 10). The stomatal differentiation influencing evaporation is also controlled by ICE1. Together with the fact that dehydration rescued sterility in ice1, it can be demonstrated that ICE1 participates in the interaction between ambient environmental stimuli and water regulation in the anther tissues. At the same time, it has been reported that CBF3, a main target of ICE1, functions in early response to drought in flowers [105]. These can suggest a dual role of ICE1 in water-associated stress resistance and dynamic developmental processes in floral tissues. In summary, ICE1 is identified as a novel male fertility regulator in Arabidopsis and can be a promising target for application of molecular engineering in crop breeding.

Materials and methods

Plant materials

All Arabidopsis thaliana plants used were in the Columbia (Col-0) background. The seeds of ice1-2 (SALK_003155) were obtained from the Arabidopsis Biological Resource Center at Ohio State University (ABRC, http://abrc.osu.edu), as previously described [67]. The ICE1pro::GFP-ICE1 (SCRMP::GFP-SCRM) transgenic line is a generous gift from Pro. Keiko Torii (Department of Biology, University of Washington). The FAMApro::FAMA-GFP transgenic line is a generous gift from Ph. D. Xiaolan Chen (School of Life Sciences, Yunnan University).
Fig 9. Pathway enrichment and functional category of genes regulated by ICE1 in the anther. (A-B) The enrichment analysis of down-regulated genes (A) and up-regulated genes (B) that are expressed in both of the guard cell and the stamen. The key clusters in the three categories identified by GO are shown in columns. The ratios of each cluster to the total gene number are shown with percentage. (C) Phenotype of impaired ion exchange in the ice1-2 mutant. Arrows indicate the wilted flower buds.

https://doi.org/10.1371/journal.pgen.1007695.g009
To generate ICE1pro::GUS lines, a 2578bp upstream region from the start codon was amplified by PCR from Arabidopsis Col-0 genomic DNA and cloned into T-vector pMD-19T (TaKaRa). After the DNA sequences were confirmed, the promoter region was cloned into pCAMBIA1301 (CAMBIA, Australia) using the method as previously described [63]. Primers were AtPICEF-PstI (5'-TActgcagGGACCA CCGTCAATAACATCG-3'); AtPICER-NcoI (5'-TTccatggGCCAAAGTTGACA CCTTTACC-3'). The ICE1pro::GUS plasmid was electroporated into Agrobacterium tumefaciens strain GV3101 (WEIDI), which was used to transform the Col-0 plants by the floral dipping method [116].

For complementation of ice1-2 mutant, the ICE1 upstream region and open reading frame were amplified and subcloned into pCAMBIA1302 vector using primers AtPICEF-PstI; AtPICER-Ncol; AtICE1F-Spel (5'-ATactagtGATCATACCAGCATAACCCTGC-3'); AtICE1-BstEII (5'-TTgtaaccTCAGATCTACCAGCATAACC-3'). The ICE1pro: ICE1 fusion construct was then introduced into ice1-2/+ plants by the floral dipping method [116].

**Plant growth and drought treatment**

Plants were grown in greenhouses under long day conditions (16 h light/8 h dark) at 22˚C. The dehydration experiments were performed as previously described with some changes [105]. In brief, two treatments were carried out. One was the standard condition with 80% soil moisture and 80% air relative humidity. The other was drought condition with 40% soil moisture and 40% air relative humidity. Pots were arranged according to a randomized design and their positions were changed daily. Seeds were stratified in a cold room for 2 d at 4˚C in the dark. Plants were grown in standard condition until the moment just after bolting (the main
shoot was about 1 cm high). When the drought treatment was started, plants were transferred into the growth chamber (RXZ-436B-LED, Ningbo Jiangnan instrument factory, China). The soil moisture was maintained by daily weigh and watering until harvest.

**Pollen germination tests**

Pollen germination analysis was conducted mainly as previously described [32]. The *in vitro* assay was performed on pollen germination media using pollen isolated from flowers at designed stages. For pistil pollination, pollen grains from flowers at designed stages were hand-pollinated on Col-0 pistils. The pollinated pistils were subjected to aniline blue staining or kept growth for characterization of siliques and seeds. For *ice1-2* mutant the stomium was manually enlarged for releasing pollen or picking the pollen grains using dissecting needles.

**Semi-thin sectioning and staining**

Inflorescences of Col-0 and *ice1-2* mutant plants were collected, fixed and dehydrated as previously described [117]. The Technovit resin-embedded blocks were sectioned to a thickness of 1.0 μm slice using a motorized RM2265 rotary microtome (Leica) with a glass knife, and then heat-fixed on glass slides. After staining with 0.05% Toluidine Blue for 15–30 min, the sections were photographed under the Microscope Axio Scope.A1 (Carl Zeiss MicroImaging) with bright field after rinsing and drying. Lignin in tissue was visualized with 0.01% fluorescent brightener (Sigma) for 30s, then mounted with 0.001% auramine O (BBI Life Sciences) and observed by Microscope Axio Scope.A1 (Carl Zeiss MicroImaging) under GFP channel.

**Light and fluorescence microscopy**

Fluorescence microscopy was performed using a Leica confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems, Wetzlar, Germany) equipped with a 10× Leica HC PL APO objective. The lignified cells and GFP fusion protein were observed with 488 nm excitation/ 510-540nm emission.

Inflorescences and anthers were collected and photographed under a SteREO Discovery V8 dissecting microscope (Carl Zeiss MicroImaging) using a SPOT FLEX digital camera (Diagnostic Instruments). Pollen from anthers stage 13–14 [72] were collected and incubated in Fluorescein Diacetate (FDA) (Solarbio) solution (FDA (5mg/ml) in acetone and diluted by 20% sucrose to 0.1mg/ml) for 5min [118], and photographed under Microscope Axio Scope. A1 (Carl Zeiss MicroImaging) under DAPI channel with an Axio Cam HRc camera (Carl Zeiss MicroImaging).

**Scanning electron microscopy (SEM)**

For SEM analysis, tissues were dissected under anatomical lens (SMZ-161-BLED, Motic, China) if needed, then immediately mounted on aluminum stubs for SEM. For leaf tissues, small pieces (d = 8 mm) of leaves from about 5-week-old plants were cut, fixed, dehydrated and coated as previously described [106]. These images were taken with scanning electron microscope TM3000 (TM3000 Tabletop Microscope, HITACHI, Japan).

**GUS assay**

For histochemical GUS activity analysis, tissues were immersed in GUS staining buffers with vacuum infiltration and destained with 75% ethanol as previously described [119]. The GUS activity was observed with Microscope Axio Scope.A1 (Carl Zeiss MicroImaging).
**Transient transcription dual-luciferase assays**

Coding regions of ICE1 were cloned into the pCAMBIA1302. The promoter sequences of FAMA were PCR amplified and inserted into the pGreenII 0800-LUC vector, using primer pFAMAF-PstI 5’-TGCACTGCACTTTGGAAAATTTGATTGGGA-3’ and pFAMAR-SacII 5’-TCCCCGCGGAGTAAGCATCACC-3’. After sequencing, all the constructs were transformed into GV3101 Agrobacteria, while the pGreenII-0800 constructs were co-transformed with pSoup-P19. The mixture of cells containing constructs with protein and promoter was infiltrated according to the published method [120]. The luciferase activity of *Nicottiana benthamiana* extracts was determined using the dual-luciferase assay kit (Promega) and then detected by a Synergy 2 multimode microplate (BioTek) as described previously [120]. All tests were performed with three biological replicates and five technical replicates per assay.

**Electrophoretic mobility shift assay**

The electrophoretic mobility shift assay (EMSA) was performed as previously described [61]. In brief, the His-ICE1 recombination protein was expressed in E. coli induced by 1 mM IPTG at 37°C for 3 h and purified through sonication and His sepharose beads (Amersham Biosciences). EMSA was conducted using the Lightshift Chemiluminescent EMSA Kit (Pierce) with biotin-labeled and cold probes. Probe sequences were listed in S9A Fig.

**Quantitative RT-PCR**

Total RNA was extracted by RNApure Plant Kit (CWBIO) according to the manufacturer’s protocol. cDNA was reverse-transcribed using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa). SYBR Premix Ex Taq II (TaKaRa) was used for qPCR on a ABI StepOne Plus real-time system (Life Technologies). qRT-PCR was performed in triplicate and data were collected and analyzed with ABI STEPONETM software version 2.1 [121]. Various gene specific signal was normalized relative to *ACTIN2* gene (At3G18780) expression. The primer sequences were listed as follows:

- **ACTIN2-Forward**, 5’-CTTGCACCAAAGCAGCATGAA-3’
- **ACTIN2-Reverse**, 5’-CCGATCCAGACACTGTACTTCCTT-3’
- **ICE1_q3-Forward**, 5’-CAACCTCTCAAGCTTCCATCCGTT-3’
- **ICE1_q3 Reverse**, 5’-GCTGTATCGAACAGACCTTCAAGTTAA-3’
- **TGG1-1-Forward**, 5’-TCCTCAGTAAGATCATCAAGGAGA-3’
- **TGG1-1-Reverse**, 5’-AGACGCTTGAACGGAGTATA-3’
- **TGG2-1-Forward**, 5’-TCCGCAAGGCCATCAACCA-3’
- **TGG2-1-Reverse**, 5’-AACGACTGGTACCATAAGCCA-3’
- **CYP83B1-Forward**, 5’-GAGACGCAAGCACTTTGGA-3’
- **CYP83B1-Reverse**, 5’-TAGGGCGTGGTAGGCTCAAGA-3’
- **GSTF9-1-Forward**, 5’-GTTCCTGCTTGTTGACGG-3’
- **GSTF9-1-Reverse**, 5’-AGTGGTGCCTTTCCACATCAA-3’
- **SOT17-1-Forward**, 5’-TTCTTCTGCTGAGCTACC-3’
- **SOT17-1-Reverse**, 5’-AACGCTTTGGAAACCGGA-3’
ABI2_1-Forward, 5’-TGCAACGGTGAATCTAGGGT-3’
ABI2_1-Reverse, 5’-CCGTTGATTTCATCTCCGAC-3’
MUTE-1-Forward, 5’-CCAGACAATCGAGCCATCCA-3’
MUTE-1-Reverse, 5’-CCCACGATTCGCCTAGAGAC-3’
TMM-1-Forward, 5’-CAGTCTTCGGGTCCTTCACC-3’
TMM-1-Reverse, 5’-TCTCGAAGGGTACTGGTCTT-3’
SPCH-1-Forward, 5’-CTCCGACAGCTGACTCTACA-3’
SPCH-1-Reverse, 5’-TTCTCCGTTACGTCCACG-3’
FAMA-1-Forward, 5’-TTTCAAGAAGAAGGCCGGAC-3’
FAMA-1-Reverse, 5’-CCAGGTTAGAGCTTCCAGATATGTT-3’
EPF1-1-Forward, 5’-CCAACTATCTCCATCCAAGT-3’
EPF1-1-Reverse, 5’-CGTGCTAGCAATCTGGCAAC-3’
MPK12-1-Forward, 5’-TCTGTGTTGCTGCATCTCGG-3’
MPK12-1-Reverse, 5’-CGATAGCCGTAGTGGGCAAT-3’
MPK14-1-Forward, 5’-GGCATGTGAGAACGAAAACG-3’
MPK14-1-Reverse, 5’-TCGCCGATGAAGGTGTTTC-3’
bHLH93-1-Forward, 5’-TCGCCATGCAGCTCCAAA-3’
bHLH93-1-Reverse, 5’-TCCTCGTCTCTACGATCTATTTCA-3’

RNA sequencing and data analysis

Anthers at flower stages 9–13 from Col-0 and ice1-2 plants were collected and immediately frozen in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Around 2 μg of total RNA with an A260/280 value of 1.8–2.0 was used to prepare the libraries, which were subjected to paired-end (2 x 100 bp) sequencing in the Illumina Hi-seq 2000 system (Illumina Inc.). The RNA-seq analysis was performed as previously described with modifications [121]. In brief, raw reads were cleaned up with Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and mapped to the Arabidopsis genome (TAIR10) by TopHat2 [122], then further assembled using StringTie and Cufflinks-CuffMerge [123]. The read counts for each gene was calculated by HTSEQ v.0.6.0 [124] and the expression level was normalized as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). The differential expression analysis was performed using DEGseq2 [125]. Differentially expressed genes (DEGs) were selected when Log2 Fold-Change (Log2FC) > 1 or < -1, and False Discovery Rate (FDR, Benjamini-Hochberg adjusted P-value) < 0.05. The RNA-Seq data have been uploaded to the National Center for Biotechnology Information Sequence Read Archive under accession numbers GSE107260.

GO analysis

Gene ontology annotation and enrichment analysis was performed on agriGO, a publicly accessible analysis tool and database (http://bioinfo.cau.edu.cn/agriGO). Genes that express in
Supporting information

S1 Fig. The female fertility of ice1-2 and phenotype of ice2-1. (A) Manual pollination on Col-0 or ice1-2 pistils using Col-0 or ice1-2 pollen. Arrows indicate the normal siliques generated by pollination on ice1-2 pistils with Col-0 pollen. (B) Structures of the ICE2 gene in the ice2-1 mutant (SAIL_808_B10). Normal fertility was observed in ice2-1 plants under normal growth conditions.

S2 Fig. Characterization of the stamen in ice1-2. Scanning Electron Microscope (SEM) of flowers from Col-0 (A), ice1-2 (B) and c-ice1-2 (C) at flower stage 14. The pollen grains were released from the dehisced anther locules in Col-0 and c-ice1-2. The ice1-2 pollen grains failed to be released to receptive papillae on the stigma. A, Anther; F, filament; Ov, ovary; Pg, pollen grain; S, sepal; Sg, stigma; Sy, style.

S3 Fig. Anther developmental process in ice1-2. Semi-thin cross sections of anthers from Col-0 and ice1-2 at anther stage 10-14b were stained with toluidine blue. Ep, Epidermis; En, Endothecium; T, Tapetum; SrR, stomium region; St, stomium; Sm, septum; Fb, fibrous bands; C, Connective; V, Vascular bundle; Pg, pollen grains.

S4 Fig. The endothecium lignification of anthers in ice1-2. (A) Transverse sectioning of anthers at anther stage 10–13 with auramine O staining. Arrows indicate the positions of endothecium lignification. (B) Fresh anthers at stage 14 with auramine O staining. Secondary thickening is visible in the endothecium (arrows indicated). (a) The anther from Col-0; (b) the anther from ice1-2; (c) Close-up of (a); (d) Close-up of (b); (e) Photographed by bright-field microscopy of (a); (f) Photographed by bright-field microscopy of (b). Ep, Epidermis; En, endothecium; Pg, pollen grains.

S5 Fig. Characterization of length of the stamen and the style in ice1-2. (A) Phenotypes of the stamen and style in Col-0 (a), ice1-2 (b) and c-ice1-2 (c) at flower developmental stage 14. (B) Stamen and style lengths were measured from microscopy pictures (SE, n = 30–39 styles and 119–146 stamens, ***p < 0.001). (C) Ratio of filament/pistil according to length data shown in (B) (SE, n = 119–146, ***p < 0.001).

S6 Fig. ICE1 promoter-driven GUS expression pattern in flower tissues. (A) Inflorescence. (B) Flower at flower stage 10. (C) Flower at stage 12. (D) Flower at stage 14. (E) Flower at stage 15. (F) Sepal at stage 14. (G) Pistil at stage 14. (H) Adaxial side of the anther at flower stage 12. (I) Filament at stage 14. (J) Pedicel at stage 14. (K) Petal at stage 14. (L) Silique.

S7 Fig. Stomatal development of ice1-2 in leaves. (A) Scanning electron micrographs of stomata from abaxial leaf surface. (a) Mature stomata in Col-0. Yellow brackets show stomatal
cluster (b), paired differentiated guard cells (c), and immature stomata (d) in ice1-2. The differentiated guard cells in Col-0 (e) and ice1-2 (f) are also shown. (B) Comparison of proportions of different stomatal types in leaves between Col-0 and ice1-2.

**S8 Fig. The qRT-PCR verification of RNA-seq data.** Six genes were selected for comparison of RNA-seq and qRT-PCR results. For RNA-seq data, **FDR < 0.01, ***FDR < 0.001. For data of qRT-PCR, SE, n = 3, * p < 0.05, ** p < 0.01, *** p < 0.001. Three independent experiments were carried out with similar results.

**S9 Fig. Electrophoretic mobility shift assay (EMSA) showing interaction of ICE1 with nine E-box elements in 2.5 kb upstream from transcription start site of FAMA.** (A) Probe sequences containing nine E-box elements are listed. P6 contains two E-boxes. (B) Binding results of ICE1 to eight probes. P3, P4 and P7 showed binding activity. P3 and P4 exhibited competition by cold probes while P7 did not show competition. (C) P7 did not show competition by cold probes with high concentration.

**S1 Table.** Full list of genes that were differentially expressed with statistical significance (FDR < 0.05) by at least 2-fold in comparison of ice1-2 vs Col-0 in the anther at flower stage 9–13. Genes expressed in the guard cell and the stamen are labeled.

**S2 Table.** Down-regulated genes that are enriched in GO annotations.

**S3 Table.** Up-regulated genes that are enriched in GO annotations.

**Acknowledgments**

The authors would thank Dr. Keiko Torii (Department of Biology, University of Washington) for providing the SCRMpro::GFP-SCRM transgenic line and Dr. Xiaolan Chen (School of Life Science, Yunnan University) for providing the FAMAPro::FAMA-GFP transgenic line. We are grateful to Dr. Hong Ma (Fudan University), Dr. Fang Chang (Fudan University), and Dr. Zhongnan Yang (Shanghai Normal University) for helpful discussion.

**Author Contributions**

**Conceptualization:** Mingqi Zhou, Juan Lin.

**Data curation:** Mingqi Zhou, Juan Lin.

**Formal analysis:** Donghui Wei, Mingjia Liu, Mingqi Zhou, Juan Lin.

**Funding acquisition:** Juan Lin.

**Investigation:** Donghui Wei, Mingjia Liu, Hu Chen, Ye Zheng, Yuxiao Liu, Xi Wang, Mingqi Zhou.

**Methodology:** Donghui Wei, Mingjia Liu, Hu Chen, Ye Zheng, Mingqi Zhou, Juan Lin.

**Project administration:** Juan Lin.

**Resources:** Donghui Wei, Mingqi Zhou, Juan Lin.
Software: Donghui Wei, Ye Zheng.

Supervision: Shuhua Yang, Mingqi Zhou, Juan Lin.

Validation: Donghui Wei, Hu Chen, Mingqi Zhou, Juan Lin.

Visualization: Donghui Wei, Mingjia Liu, Yuxiao Liu.

Writing – original draft: Mingqi Zhou, Juan Lin.

Writing – review & editing: Mingqi Zhou, Juan Lin.

References

1. Smyth DR, Bowman JL, Meyerowitz EM. Early flower development in Arabidopsis. Plant Cell. 1990; 2: 755–767. https://doi.org/10.1105/tpc.2.8.755 PMID: 2152125

2. Irish VF. The flowering of Arabidopsis flower development. Plant J. 2010; 61: 1014–1028. https://doi.org/10.1111/j.1365-313X.2009.04065.x PMID: 20409275

3. Goldberg R, Beals T, Sanders P. Anther development: basic principles and practical applications. Plant Cell. 1993; 5: 1217–1229. https://doi.org/10.1006/pcel.1993.1073 PMID: 8281038

4. Wilson ZA, Song J, Taylor B, Yang C. The final split: the regulation of anther dehiscence. J Exp Bot. 2011; 62: 1633–1649. https://doi.org/10.1093/jxb/err014 PMID: 21325605

5. Kim SG, Lee S, Kim YS, Yun DJ, Woo JC, Park CM. Activation tagging of an Arabidopsis SHI-RELATED SEQUENCE gene produces abnormal anther dehiscence and floral development. Plant Mol Biol. 2010; 74: 337–351. https://doi.org/10.1007/s11103-010-9677-5 PMID: 20706774

6. Mizuno S, Osakabe Y, Maruyama K, Ito T, Osakabe K, Sato T, et al. Receptor-like protein kinase 2 (RPK2) is a novel factor controlling anther development in Arabidopsis thaliana. Plant J. 2007; 50: 751–766. https://doi.org/10.1111/j.1365-313X.2007.03083.x PMID: 17419837

7. Ge X, Dietrich C, Matsuno M, Li G, Berg H, Xia Y. An Arabidopsis aspartic protease functions as an anti-cell-death component in reproduction and embryogenesis. EMBO Rep. 2005; 6: 282–288. https://doi.org/10.1038/sj.embor.7400357 PMID: 15723040

8. Lane DR, Wiedemeier A, Peng L, Höfte H, Verna ettes S, Desprez T, et al. Temperature-sensitive alleles of RSW2 link the KORRIGAN endo-1,4-beta-glucanase to cellulose synthesis and cytokinesis in Arabidopsis. Plant Physiol. 2001; 126: 278–288. PMID: 11351091

9. Gorguet B, Schipper D, van Lammeren A, Visser RG, van Heusden AW. ps-2, the gene responsible for functional sterility in tomato, due to non-dehiscent anthers, is the result of a mutation in a novel polygalacturonase gene. Theor Appl Genet. 2009; 118:1199–1209. https://doi.org/10.1007/s00122-009-0974-9 PMID: 19219598

10. Ogawa M, Kay P, Wilson S, Swain SM. ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURO- NASE1 (ADPG1), ADPG2, and QUARTET2 are polygalacturonases required for cell separation during reproductive development in Arabidopsis. Plant Cell. 2009; 21: 216–233. https://doi.org/10.1105/tpc.108.063768 PMID: 19168715

11. Dawson J, Sozen E, Vizir I, Van Waeyenberge S, Wilson ZA, Mulligan J. Characterization and genetic mapping of a mutation (ms35) which prevents anther dehiscence in Arabidopsis thaliana by affecting secondary wall thickening in the endothecium. New Phytol. 1999; 144: 213–222.

12. Steiner-Lange S, Unie US, Eckstein L, Yang C, Wilson ZA, Schmelzer E, et al. Disruption of Arabidopsis thaliana MYB26 results in male sterility due to non-dehiscent anthers. Plant J. 2003; 34: 519–528. PMID: 12753990

13. Yang C, Xu Z, Song J, Conner K, Vizcay Barrena G, Wilson ZA. Arabidopsis MYB26/MALE STERILE35 regulates secondary thickening in the endothecium and is essential for anther dehiscence. Plant Cell. 2007; 19: 534–548. https://doi.org/10.1105/tpc.106.046391 PMID: 17329564

14. Yang C, Song J, Ferguson AC, Kisch D, Simpson K, Mo R, et al. Transcription factor MYB26 is key to spatial specificity in anther secondary thickening formation. Plant Physiol. 2017; 175: 333–350. https://doi.org/10.1104/pp.17.00719 PMID: 28724622

15. Mitsuoda N, Seki M, Shinazaki K, Ohme-Takagi M. The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell. 2005; 17: 2993–3006. https://doi.org/10.1105/tpc.105.036004 PMID: 16214898
17. Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, et al. NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. Plant Cell. 2007; 19: 270–280. https://doi.org/10.1105/tpc.106.047043 PMID: 17237351

18. Thévenin J, Pellet B, Letarnec B, Saulnier L, Gissot L, Maia-Guérard A, et al. The simultaneous repression of CCR and CAD, two enzymes of the lignin biosynthetic pathway, results in sterility and dwarfism in Arabidopsis thaliana. Mol Plant. 2011; 4: 70–82. https://doi.org/10.1093/mp/ssq045 PMID: 21083584

19. Thangasamy S, Guo CL, Chuang MH, Lai MH, Chen J, Jauh GY. Rice SIZ1, a SUMO E3 ligase, controls spikelet fertility through regulation of anther dehiscence. New Phytol. 2011; 189: 869–882. https://doi.org/10.1111/j.1469-8137.2010.03538.x PMID: 21083584

20. Huang C, Zhang R, Gui J, Zhong Y, Li L. The receptor-like kinase AtVRLK1 regulates secondary cell wall thickening. Plant Physiol. 2018; 177: 671–683. https://doi.org/10.1104/pp.17.01279 PMID: 29678858

21. Senatore A, Trobacher CP, Greenwood JS. Ricinosomes predict programmed cell death leading to anther dehiscence in tomato. Plant Physiol. 2009; 149: 775–790. https://doi.org/10.1104/pp.104.056408 PMID: 15734911

22. He H, Bai M, Tong P, Hu Y, Yang M, Wu H. CELLULASE6 and MANNASE7 affect cell differentiation and silique dehiscence. Plant Physiol. 2018; 176: 2186–2201. https://doi.org/10.1104/pp.17.01494 PMID: 22438021

23. Bots M, Vergeldt F, Wolters-Arts M, Weterings K, van As H, Mariani C. Aquaporins of the PIP2 class are required for efficient anther dehiscence in tobacco. Plant Physiol. 2005; 137: 1049–1056. https://doi.org/10.1104/pp.104.056408 PMID: 15734911

24. Basal E, Tajima H, Liang YC, Ohno MA, Ushijima K, Nakano R, et al. The Arabidopsis Na+/H+ antipor ters NHX1 and NHX2 control vacuolar pH and K+ homeostasis to regulate growth, flower development, and reproduction. Plant Cell. 2011; 23: 3482–3497. https://doi.org/10.1105/tpc.111.089581 PMID: 21954467

25. Barragán V, Leidi E, André Z, Rubio L, De Luca A, Fernández JA, et al. Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in Arabidopsis. Plant Cell. 2012; 24: 1127–1142. https://doi.org/10.1105/tpc.111.095273 PMID: 22438021

26. Clément C, Audran JC. Anther wall layers control pollen sugar nutrition in Lilium. Protoplasma. 1995; 187: 172–181.

27. Stadler R, Truernit E, Gahrtz M, Sauer N. The AtSUC1 sucrose carrier may represent the osmotic driving force for anther dehiscence and pollen tube growth in Arabidopsis. Plant J. 1999; 19: 269–278. PMID: 10476074

28. Bonner LJ, Dickinson HG. Anther dehiscence in Lycopersicon esculentum. New Phytol. 1990; 115: 367–375.

29. Goldberg RB. Plants: novel developmental processes. Science. 1988; 240: 1460–1467. PMID: 3287622

30. Franchi GG, Nepi M, Dafni A, Pacini E. Partially hydrated pollen: taxonomic distribution, ecological and evolutionary significance. Plant Syst Evol. 2002; 234: 211–227.

31. Keijzer CJ. The process of anther dehiscence and pollen dispersal. I. The opening mechanism of longitudinally dehiscent anthers. New Phytol. 1987; 105: 487–498.

32. Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K. The DEFECTIVE IN ANther DEHIScENCE1 gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell. 2001; 13: 2191–2209. PMID: 11595796

33. Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, et al. The Arabidopsis DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid biosynthesis pathway. Plant Cell. 2000; 12: 1041–1061. PMID: 10899973

34. von Malek B, van der Graaff E, Schnitzl K, Keller B. The Arabidopsis male-sterile mutant dde2-2 is defective in the ALLENE OXIDE SYNTHASE gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta. 2002; 216: 187–192. https://doi.org/10.1007/s00035-002-0906-2 PMID: 12430030

35. Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R. A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant. 2002; 31: 1–12. PMID: 12100478
36. Stintzi A, Browse J. The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A. 2000; 97: 10625–10630. https://doi.org/10.1073/pnas.190264497 PMID: 10973494

37. Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG. COI1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science. 1998; 280: 1091–1094. PMID: 9582125

38. Qi T, Huang H, Song S, Xie D. Regulation of jasmonate-mediated stamen development and seed production by a bHLH-MYB complex in Arabidopsis. Plant Cell. 2015; 27: 1620–1633. https://doi.org/10.1105/tpc.15.01116 PMID: 26028669

39. Yang XY, Li JG, Pei M, Gu H, Chen ZL, Qu LJ. Over-expression of a flower-specific transcription factor gene AtMYB24 causes aberrant anther development. Plant Cell Rep. 2007; 26: 851–862. https://doi.org/10.1105/tpc.107.057570 PMID: 18628351

40. Mandaokar A, Browse J. MYB108 acts together with MYB24 to regulate jasmonate-mediated stamen maturation in Arabidopsis. Plant Physiol. 2009; 149: 851–862. https://doi.org/10.1104/pp.108.132597 PMID: 19091873

41. Cheng H, Song S, Xiao L, Soo HM, Cheng Z, Xie D, et al. Gibberellin acts through jasmonate to control the expression of MYB21, MYB24, and MYB57 to promote stamen filament growth in Arabidopsis. PLoS Genet. 2009; 5: e1000440. https://doi.org/10.1371/journal.pgen.1000440 PMID: 19325888

42. Song S, Qi T, Huang H, Ren Q, Wu D, Chang C, et al. The jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect jasmonate-regulated stamen development in Arabidopsis. Plant Cell. 2011; 23: 1000–1013. https://doi.org/10.1105/tpc.111.083089 PMID: 21447791

43. Huang H, Gao H, Liu B, Qi T, Tong J, Xiao L, et al. Arabidopsis MYB24 regulates jasmonate-mediated stamen development. Front Plant Sci. 2017; 8: 1525. https://doi.org/10.3389/fpls.2017.01525 PMID: 28928760

44. Saito H, Oikawa T, Hamamoto S, Ishimaru Y, Kanamori-Sato M, Sasaki-Sekimoto Y, et al. The jasmonate-responsive GTR1 transporter is required for gibberellin-mediated stamen development in Arabidopsis. Nat Commun. 2015; 6: 6095. PMID: 25648767

45. Jewell JB, Browse J. Epidermal jasmonate perception is sufficient for all aspects of jasmonate-mediated male fertility in Arabidopsis. Plant J. 2016; 85: 634–647. https://doi.org/10.1111/tpj.13131 PMID: 26833563

46. Ma H. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. Annu Rev Plant Biol. 2005; 56: 393–434. https://doi.org/10.1146/annurev.arplant.55.031903.141717 PMID: 15862102

47. Cecchetti V, Pomponi M, Altamura MM, Pezzotti M, Marsillo S, D’Angeli S, et al. Expression of rolB in tobacco flowers affects the coordinated processes of anther dehiscence and style elongation. Plant J. 2004; 38: 512–525. https://doi.org/10.1111/j.0960-7412.2004.02064.x PMID: 15086797

48. Yassou H, Abu-Abied M, Belausov E, Madmony A, Sadot E, Riov J, et al. Glycophosphate-induced anther dehiscence in cotton is partially temperature dependent and involves cytoskeleton and secondary wall modifications and auxin accumulation. Plant Physiol. 2006; 141: 1306–1315. https://doi.org/10.1104/pp.106.081943 PMID: 16766672

49. Cecchetti V, Altamura MM, Falasca G, Costantino P, Cardarelli M. Auxin regulates Arabidopsis anther dehiscence, pollen maturation, and filament elongation. Plant Cell. 2008; 20: 1760–1774. https://doi.org/10.1105/tpc.107.057570 PMID: 18628351

50. Noh B, Murphy AS, Spalding EP. Multidrug resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development. Plant Cell. 2001; 13: 2441–2454. https://doi.org/10.1105/tpc.010350 PMID: 11701880

51. Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, et al. Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell. 2005; 9: 109–119. https://doi.org/10.1016/j.devcell.2005.05.014 PMID: 15992545

52. Cecchetti V, Altamura MM, Brunetti P, Petrocelli V, Falasca G, Ljung K, et al. Auxin controls Arabidopsis anther dehiscence by regulating endothecium lignification and jasmonic acid biosynthesis. Plant J. 2013; 74: 411–422. https://doi.org/10.1111/tpj.12130 PMID: 23410518

53. Cardarelli M, Cecchetti V. Auxin polar transport in stamen formation and development: how many actors? Front Plant Sci. 2014; 5: 333. https://doi.org/10.3389/fpls.2014.00333 PMID: 25076953

54. Cecchetti V, Brunetti P, Napoli N, Fattorini L, Altamura MM, Costantino P, et al. ABCB1 and ABCB19 auxin transporters have synergistic effects on early and late Arabidopsis anther development. J Integr Plant Biol. 2015; 57: 1089–1098. https://doi.org/10.1111/jipb.12332 PMID: 25626615
55. Cheng Y, DaI X, Zhao Y. Auxin biosynthesis by the YUCCA flavin monoxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes Dev. 2006; 20: 1790–1799. https://doi.org/10.1101/gad.1415106 PMID: 16818609

56. Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, et al. Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development. 2005; 132: 4107–4118. https://doi.org/10.1242/dev.01955 PMID: 16107481

57. Tabata R, Ikezaki M, Fujibe T, Aida M, Tian CE, Ueno Y, et al. Arabidopsis auxin response factor6 and ARF8 promote jasmonic acid production and flower maturation. Development. 2005; 132: 4107–4118. https://doi.org/10.1242/dev.01955 PMID: 20007966

58. Ghelli R, Brunetti P, Napoli N, De Paolis A, Cecchetti V, Tsuge T, et al. A newly identified flower-specific splice variant of AUXIN RESPONSE FACTOR8 regulates stamen elongation and endothecium lignification in Arabidopsis. Plant Cell. 2018; 30: 620–637. https://doi.org/10.1105/tpc.17.00840 PMID: 29514943

59. Reeves PH, Ellis CM, Ploense SE, Wu MF, Yadav V, Tholl D, et al. A regulatory network for coordinated flower maturation. PLoS Genet. 2012; 8: e1002506. https://doi.org/10.1371/journal.pgen.1002506 PMID: 22346763

60. Miura K, Jin JB, Lee JY, Yoo CY, Stirn V, Miura T, et al. Auxin response factor6 and 8 regulate jasmonic acid biosynthesis and floral organ development via repression of class 1 KNOX genes. Plant Cell Physiol. 2010; 51: 164–175. https://doi.org/10.1093/pcp/pcp176 PMID: 20007966

61. Dong CH, Agarwal M, Zhang Y, Xie Q, Zhu JK. The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc Natl Acad Sci U S A. 2006; 103: 8281–8286. https://doi.org/10.1073/pnas.0602874103 PMID: 16702557

62. Lee JH, Jung JH, Park CM. Light inhibits COP1-mediated degradation of ICE transcription factors to induce stomatal development in Arabidopsis. Plant J. 2015; 84: 29–40. https://doi.org/10.1111/tpj.12956 PMID: 26248809

63. Denay G, Creff A, Moussu S, Wagnon P, Thevenin J, Gerentes MF, et al. Endosperm development in Arabidopsis requires heterodimers of the basic helix-loop-helix proteins ZOHUPI and INDUCER OF CBP EXPRESSION 1. Development. 2014; 141: 1222–1227. https://doi.org/10.1242/dev.103531 PMID: 24552885

64. Funsova OV, Pogorelko GV, Tarasov VA. Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in Arabidopsis thaliana. Gene. 2009; 429: 98–103. https://doi.org/10.1016/j.gene.2008.10.016 PMID: 19026725

65. Kuribidaeva A, Ezhova T, Novokreshchenova M. Arabidopsis thaliana ICE2 gene: phylogeny, structural evolution and functional diversification from ICE1. Plant Sci. 2014; 229: 10–22. https://doi.org/10.1016/j.plantsci.2014.08.011 PMID: 25443829

66. Sanders PM, Bui AQ, Weterings K, McIntire KN, Hsu YC, Lee PY, et al. Anther developmental defects in Arabidopsis thaliana male-sterile mutants. Sex Plant Reprod. 1999; 11: 297–322.

67. Vernoud V, Laigle G, Rozier F, Meeley RB, Perez P, Rogowsky PM. The HD-ZIP IV transcription factor OCL4 is necessary for trichome patterning and anther development in maize. Plant J. 2009; 59: 883–894. https://doi.org/10.1111/j.1365-313X.2009.03916.x PMID: 19453441
74. Scott RJ, Spielman M, Dickinson HG. Stamen structure and function. Plant Cell. 2004; 16(Suppl): S46–S60.
75. Lashbrook CC, Gonzalez-Bosch C, Bennett AB. Two divergent endo-beta-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscising flowers. Plant Cell. 1994; 6: 1485–1493. https://doi.org/10.1105/tpc.6.10.1485 PMID: 7994180
76. Kuriyama H, Fukuda H. Developmental programmed cell death in plants. Curr Opin Plant Biol. 2002; 5: 568–573. PMID: 12393021
77. Sanders PM, Bui AQ, Le BH, Goldberg RB. Differentiation and degeneration of cells that play a major role in tobacco anther dehiscence. Sexual Plant Reproduction. 2005; 17(5): 219–241.
78. Beals TP, Goldberg RB. A novel cell ablation strategy blocks tobacco anther dehiscence. Plant Cell. 1997; 9: 1527–1545. https://doi.org/10.1105/tpc.9.9.1527 PMID: 9398959
79. Matsu T, Omasa K, Horie T. Mechanism of anther dehiscence in rice (Oryza sativa L.). Ann Bot. 1999; 84: 501–506.
80. Yang J, Wu J, Romanovicz D, Clark G, Roux SJ. Co-regulation of exine wall patterning, pollen fertility and anther dehiscence by Arabidopsis apyrase 6 and 7. Plant Physiol Biochem. 2013; 69: 62–73. https://doi.org/10.1016/j.plaphy.2013.04.022 PMID: 23728389
81. Torii KU. Stomatal differentiation: the beginning and the end. Curr Opin Plant Biol. 2015; 28: 16–22. https://doi.org/10.1016/j.pbi.2015.08.005 PMID: 26344486
82. Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T. The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. Genes Dev. 2007; 21: 1720–1725. https://doi.org/10.1101/gad.1550707 PMID: 17639078
83. Hachez C, Ohashi-Ito K, Dong J, Bergmann DC. Differentiation of Arabidopsis guard cells: analysis of the networks incorporating the basic helix-loop-helix transcription factor, FAMA. Plant Physiol. 2011; 155: 1458–1472. https://doi.org/10.1104/pp.110.167718 PMID: 21245191
84. Du Z, Zhou X, Ling Y, Zhang Z, Su Z. agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res. 2010; 38: W64–W70. https://doi.org/10.1093/nar/gkq310 PMID: 20435677
97. Bock KW, Honys D, Ward JM, Padmanaban S, Nawrocki EP, Hirschi KD, et al. Integrating membrane transport with male gametophyte development and function through transcriptomics. Plant Physiol. 2006; 140: 1151–1168. https://doi.org/10.1104/pp.105.074708 PMID: 16607029

98. Rehman S, Yun SJ. Developmental regulation of K accumulation in pollen, anthers, and papillae: anther dehiscence, papillae hydration, and pollen swelling leading to pollination and fertilization in barley (Hordeum vulgare L.) regulated by changes in K concentration? J Exp Bot. 2006; 57: 1315–1321. https://doi.org/10.1093/jxb/erj106 PMID: 16531463

99. Jakobsen MK, Poulsen LR, Schulz A, Fleurat-Lessard P, Möller A, Husted S, et al. Pollen development and fertilization in Arabidopsis is dependent on the MALE GAMETOPHYSIS IMPAIRED ANTHERS genome encoding a type V P-type ATPase. Genes Dev. 2003; 15: 528–537. https://doi.org/10.1101/gad.357305 PMID: 12691648

100. Stadler R, Büttner M, Ache P, Hedrich R, Iwashikina N, Melzer M, et al. Diurnal and light-regulated expression of AISTP1 in guard cells of Arabidopsis. Plant Physiol. 2003; 133: 528–537. https://doi.org/10.1104/pp.103.024240 PMID: 12972665

101. Truernit E, Schmid J, Epple P, Iillig J, Sauer N. The sink-specific and stress-regulated Arabidopsis STP4 gene: enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. Plant Cell. 1996; 8: 2169–2182. https://doi.org/10.1105/tpc.8.12.2169 PMID: 8989877

102. Hocking B, Conn SJ, Manohar M, Xu B, Athman A, Stancombe MA, et al. Heterodimerization of Arabidopsis calcium/proton exchangers contributes to regulation of guard cell dynamics and plant defense responses. J Exp Bot. 2017; 68: 4171–4183. https://doi.org/10.1093/jxb/erx208 PMID: 28645169

103. Yu H, Yan J, Du X, Hua J. Overlapping and differential roles of plasma membrane calcium ATPases in Arabidopsis growth and environmental responses. J Exp Bot. 2018; 69: 2693–2703. https://doi.org/10.1093/jxb/ery073 PMID: 29506225

104. Islam MM, Tani C, Watanabe-Sugimoto M, Uraji M, Jahan MS, Masuda C, et al. Myrosinanases, TGG1 and TGG2, redundantly function in ABA and MeJA signaling in Arabidopsis guard cells. Plant Physiol. 2009; 150: 1171–1175. https://doi.org/10.1109/tpc.8.12.2169 PMID: 19433491

105. Su Z, Ma X, Guo H, Sukiran NL, Guo B, Assmann SM, et al. Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in Arabidopsis. Plant Cell. 2013; 25: 3785–3807. https://doi.org/10.1109/jxb.113.115428 PMID: 24179129

106. Ahuja I, de Vos RC, Rohloff J, Stoopen GM, Halle KK, Ahmad SJ, et al. Arabidopsis myrosinanases link the glucosinolate-myrosinase system and the cuticle. Sci Rep. 2016; 6: 38990. PMID: 27976683

107. Lâng V, Palva ET. The expression of a rab-related gene, rab18, is induced by abscisic acid during the cold acclimation process of Arabidopsis thaliana (L.) Heynh. Plant Mol Biol. 1992; 20: 951–962. PMID: 1463831

108. Shi H, Chen Y, Qian Y, Chan Z. Low temperature-induced 30 (LTI30) positively regulates drought stress resistance in Arabidopsis: effect on abscisic acid sensitivity and hydrogen peroxide accumulation. Front. Plant Sci. 2015; 6: 883. https://doi.org/10.3389/fpls.2015.00893 PMID: 26593925

109. Nylander M, Svensson J, Palva ET, Wein BV. Stress-induced accumulation and tissue-specific localization of dehydrins in Arabidopsis thaliana. Plant Mol Biol. 2001; 45: 263–279. PMID: 11292073

110. Mandadi KK, Misra A, Ren S, McKnight TD. BT2, a BTB protein, mediates multiple responses to nutrients, stresses, and hormones in Arabidopsis. Plant Physiol. 2009; 150: 1930–1939. https://doi.org/10.1104/pp.109.139220 PMID: 19525324

111. Robert HS, Quint A, Brand D, Vivian-Smith A, Offringa R. BTB and TAZ domain scaffold proteins per-form a crucial function in Arabidopsis development. Plant J. 2009; 58: 109–121. https://doi.org/10.1111/j.1365-313X.2008.03764.x PMID: 19054356

112. Jammes F, Song C, Shin D, Munemasa S, Takeda K, Gu D, Cho D, et al. MAP kinases MPK9 and MPK12 are preferentially expressed in guard cells and positively regulate ROS-mediated ABA signaling. Proc Natl Acad Sci U S A. 2009; 106: 20520–20525. https://doi.org/10.1073/pnas.0907205106 PMID: 19910530

113. Khokon MA, Salam MA, Jammes F, Ye W, Hossain MA, Uraj M, et al. Two guard cell mitogen-activated protein kinases, MPK9 and MPK12, function in methyl jasmonate-induced stomatal closure in Arabidopsis thaliana. Plant Biol. 2015; 17: 946–962. https://doi.org/10.1111/plb.12321 PMID: 25703019

114. Khokon MA, Salam MA, Jammes F, Ye W, Hossain MA, Okuma E, et al. MPK9 and MPK12 function in SA-induced stomatal closure in Arabidopsis thaliana. Biosci Biotechnol Biochem. 2017; 81: 1394–1400. https://doi.org/10.1080/09168451.2017.1308244 PMID: 28387156

115. Besseau S, Hoffmann L, Geoffroy P, Lapière C, Pellet B, Legrand M. Flavonoid accumulation in Arabi-dopsis repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell. 2007; 19: 148–162. https://doi.org/10.1105/tpc.106.044495 PMID: 17237352
116. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 1998; 16: 735–743. PMID: 10069079

117. Zhu E, You C, Wang S, Cui J, Niu B, Wang Y, et al. The DYT1-interacting proteins bHLH010, bHLH089 and bHLH091 are redundantly required for Arabidopsis anther development and transcriptome. Plant J. 2015; 83: 976–990. https://doi.org/10.1111/tpj.12942 PMID: 26216374

118. Heslop-Harrison J, Heslop-Harrison Y, Shivanna KR. The evaluation of pollen quality and a further appraisal of the fluorochromatic (FCR) test procedure. Theor Appl Genet. 1984; 67: 367–375. https://doi.org/10.1007/BF00272876 PMID: 24258660

119. Wu LH, Zhou MQ, Shen C, Liang J, Lin J. Transgenic tobacco plants over expressing cold regulated protein CbCOR15b from Capsella bursa-pastoris exhibit enhanced cold tolerance. J Plant Physiol. 2012; 169: 1408–1416. https://doi.org/10.1016/j.jplph.2012.05.016 PMID: 22795746

120. Hellens RP, Allan AC, Friel EN, Bolitho K, Grafton K, Templeton MD, et al. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods. 2005; 1: 13. PMID: 16359558

121. Li A, Zhou M, Wei D, Chen H, You C, Lin J. Transcriptome profiling reveals the negative regulation of multiple plant hormone signaling pathways elicited by overexpression of C-repeat binding factors. Front Plant Sci. 2017; 8: 1647. https://doi.org/10.3389/fpls.2017.01647 PMID: 28983312

122. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 2013; 14: R36. PMID: 23618408

123. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc. 2016; 11: 1650–1667. PMID: 27560171

124. Anders S, Pyl PT, Huber W. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015; 31: 166–169. https://doi.org/10.1093/bioinformatics/btu638 PMID: 25260700

125. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014; 15: 550. PMID: 25516281