Jasmonic acid facilitates flower opening and floral organ development through the upregulated expression of SlMYB21 transcription factor in tomato

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Abstract

Plants coordinate the timing of flower opening with pollen and gynoecium maturation to achieve successful pollination. However, little is known about how the coordination is executed. We found that flower bud development was paused immediately before flower opening in a jasmonic acid (JA)-insensitive tomato mutant, *jai1-1*. Phytohormone measurement and RNA analysis in flower buds revealed that newly synthesized JA peaked at two days before flower opening and the expression of a transcription factor gene *SlMYB21* delayed in *jai1-1*. Buds of transgenic tomato plants expressing an artificial repressor, *AtMYB24-SRDX*, which was expected to impede the function of *SlMYB21*, aborted flower opening and resembled those of *jai1-1*. Furthermore, the *AtMYB24-SRDX* plants produced abnormal pollen grains deficient in germination and pistils that did not support pollen tube elongation. We concluded that JA facilitates the expression of *SlMYB21*, which coordinates flower opening, pollen maturation, and gynoecium function in tomato.

Keywords: flower opening; jasmonic acid; male and female fertility; SlMYB21 transcription factor; *Solanum lycoperisicum*

Short title

JA coordinates fertility with flower opening through SlMYB21

Introduction

The flower is the most important reproductive organ in angiosperms. The flowers release mature pollen grains from anthers, attract pollinators by color and scent, attach the pollen grains to the pollinators, and accept pollen grains at stigmas. Therefore, coordination of flower opening with pollen maturation and gynoecium maturation is important for successful pollination and subsequent fertilization. Flower opening is regulated by both the internal environment such as pollen and ovule development and circadian rhythms and the external environment such as light, temperature, and humidity.[1, 2] Moreover, many phytohormones, such as auxin, gibberellin (GA), abscisic acid (ABA), ethylene, and jasmonic acid (JA), have been reported to be involved in the regulation of flower opening with relation to both internal and external cues.[1]

Involvement of JA in the regulation of flower opening was initially reported in the analysis of a JA-deficient mutant, *defective in anther dehiscence1* (*dad1*), in Arabidopsis.[3] The *dad1* flower buds developed normally until the end of the middle stage, which corresponded to two days before flower opening in the wild type (WT). In the later stage, however, the development of the mutant flower buds was retarded in comparison to WT until flower opening, and as a consequence, more unopened buds were clustered in the inflorescence of *dad1*.[3, 4] The *dad1* flowers also showed delayed anther dehiscence and pollen grain infertility, and all three defects including delayed flower opening were rescued by exogenous application of JA.[3] Similar phenotypes appeared in other JA biosynthesis mutants such as *allene oxide synthase*, *oxophytodienoate reductase3* (*opr3*), double mutants *lipoxygenase3 lipoxygenase4*,[5-8] and a JA-insensitive mutant, *coronatine insensitive1* (*coi1*).[9] COI1 is a JA receptor essential for all the JA responses including defense and stress responses in Arabidopsis.[10] COI1 is an F-box protein that forms a complex with a jasmonate

ZIM-domain protein, and the complex specifically binds to jasmonoyl-L-isoleucine (JA-Ile). Hence, JA-Ile is thought to be an active form of JA.[11, 12] These observations indicate that JA is an activator of flower opening, anther dehiscence, and pollen development in Arabidopsis.

Regulation of flower opening by JA was also reported in other plants. In *Brassica rapa*, flower opening was suppressed in the transformants expressing an antisense gene of *BrDAD1*, which is the putative ortholog of Arabidopsis *DAD1*, and the phenotype was restored by application of JA.[13] In wild tobacco, *Nicotiana attenuata*, a series of transgenic plants in which JA biosynthetic genes such as *ALLENE OXIDE CYCLASE* (*AOC*), or a JA perception gene, *COI1*, were silenced showed delayed flower opening or incomplete corolla expansion phenotypes.[14] In rice, opening of florets was induced by treatment of methyl jasmonate, and the floret opening as well as anther dehiscence was impaired in the dysfunctional mutants of *OsJAR1*, which encodes an enzyme forming JA-Ile.[15, 16] Enhanced flower opening by methyl jasmonate treatment was also observed in *Brassica napus*.[17]

Along with the defects in flower opening, JA-related mutants often affect the development of floral organs. Rice mutant *extra glume 1* (*eg1*), which is a mutation in a homolog of *DAD1*, changed floral organ identity and number.[18] Tomato mutant *jasmonic-acid insensitive1* (*jai1*), of which the causal gene is *SlCOI1*, is a unique ortholog of Arabidopsis *COI1* and showed reduced pollen viability and female sterility.[19, 20] Dobritzsch *et al.* proposed that the reduced pollen fertility in *jai1* is attributed to the premature tapetum degradation and premature anther dehiscence in the absence of normal JA signaling.[21] These observations revealed that JA is not only a common regulator of flower opening in many plants but also involved in the development of floral organs including male and female gametophytes in a species-dependent manner. However, their mechanisms are largely unknown.

A transcriptome analysis comparing JA-treated and not-treated stamens of Arabidopsis *opr3* mutants revealed that two R2R3-type MYB transcription factors, *AtMYB21* and *AtMYB24*, were strongly induced by JA.[22] T-DNA insertion mutants of *atmyb21* exhibited delayed flower opening, shorter anther filaments, delayed anther dehiscence, and reduced male fertility, resembling the phenotype of JA-deficient mutants.[22] Another transcriptome analysis identified three genes *AtMYB21*, *AtMYB24*, and *AtMYB57* as GA-dependent genes in flower buds.[23] All *atmyb21* phenotypes were enhanced in *atmyb21 atmyb24* double mutants and were further exacerbated in *atmyb21 atmyb24 atmyb57* triple mutants, suggesting overlapping functions of these three *R2R3-MYB* genes.[22, 24] The authors concluded that GA acts through JA to control expression of the *R2R3-MYB* genes and promote stamen filament elongation.[23]

Involvement of putative orthologs of *AtMYB21*/*24*/*57* in flower opening has also been reported in other plants. Transgenic torenia plants expressing the *Pro35S*:*AtMYB24-SRDX* gene, which consisted of the cauliflower mosaic virus 35S promoter (*Pro35S*) and the *AtMYB24* coding sequence fused to the sequence of an EAR motif repression domain (SRDX), exhibited a deficiency in flower opening.[25] The EAR motif was originally identified as a transcription repression domain of ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR in Arabidopsis and has the ability to change transcription activators to repressors when it is fused to the activators.[26, 27] In petunia and *Nicotiana attenuata*, suppression of the *EMISSION OF BENZENOIDS II* (*EOBII*) gene, a close homolog of *AtMYB21*/*24*, by RNA interference caused a failure of flower opening.[28] These findings indicate that the ortholog(s) of *AtMYB21*/*24*/*57* regulate flower opening in diverse plant species. However, the relationship between JA signaling and regulation of *AtMYB21*/*24*/*57* ortholog(s) has not yet been elucidated in plants other than Arabidopsis.

In this study, we found that tomato plants having *jai1-1*, a strong allele of *jai1*

mutation, showed delayed flower opening. Phytohormone measurement and transcript analysis revealed that JA stimulated the expression of the *SlMYB21* gene, a unique ortholog of *AtMYB21*/*24*/*57* in tomato. We showed that transgenic tomato expressing a chimeric transcription repressor for *SlMYB21* exhibited a more exacerbate phenotype than *jai1-1* particularly in male and female sterility. We will discuss the involvement of JA signaling and *SlMYB21* in the regulation of flower opening and floral organ development in tomato.

Materials and methods

Plant materials and growth conditions. Tomato cultivar Micro-Tom (*Solanum lycopersicum* L. cv. Micro-Tom) was used unless otherwise indicated. *jai1-1*, a deletion allele of the *SlCOI1* gene (Solyc05g052620) in a Micro-Tom background, was described previously.[19] Due to the strong sterility of *jai1-1* homozygotes, *jai1-1* was maintained as a heterozygous line, and homozygous *jai1-1* plants were identified by a PCR-based genotyping.[19] We established a WT line having a homozygous unmutated *SlCOI1* allele from the progeny of a *jai1-1* heterozygote and named it "MT-GH". MT-GH was used as the WT control in most experiments in this study. For RNA sequencing (RNA-seq), a line of Micro-Tom (MT-J, TOMJPF00001) obtained from the National Bio-Resource Project (http://tomato.nbrp.jp/indexEn.html) was used. Another cultivar of tomato (cv. Momotaro 8, Takii, Japan) was also used for some experiments. Seeds were sown on pre-fertilized soil (Nippi No. 1, Nippon Hiryo, Japan) and grown under fluorescent white light (170 μmol/s/m^2) of 16 h light and 8h darkness at 25°C.

RNA preparation and gene expression analysis. A whole flower bud or the floral organs of a bud were harvested and immediately frozen in liquid nitrogen. Total RNA was extracted using an RNeasy Plant Mini Kit (QIAGEN, www.qiagen.com). Extracted RNA was reverse transcribed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, www.toyobo-global.com). qRT-PCR was performed using the StepOne Real-Time PCR system (Thermo Fisher Scientific, www.thermofisher.com) and THUNDERBIRD SYBR qPCR Mix (Toyobo). RNA yields and reverse transcription efficiencies were normalized by the amount of *DNAJ* (a subset of tomato *DnaJ*/*Hsp40* homologs comprising Solyc04g009770, Solyc05g055160,

and Solyc11g006460) mRNA or *SAND* (Solyc03g115810) mRNA, as described in the literature.[29] The primers used in qRT-PCR are listed in Supplemental Table S1.

RNA-seq analysis was carried out as described previously.[30] Reference cDNA sequences were extracted from a whole genome sequence database (version SL2.40 of *Solanum lycopersicum* cv. Heinz 1706) by use of ITAG2.3 gene prediction information (Sol Genomics Network, solgenomics.net).

Measurement of phytohormones. Each flower bud was separately harvested from an inflorescence and immediately frozen in liquid nitrogen. Extraction and purification of phytohormones were performed as described previously.[31] Quantification of phytohormone species excepting JA-Ile were extracted and measured as described previously.[31, 32] For JA-Ile quantification, stable isotope-labeled JA-Ile ([$^2H_2$]N-[(-)-Jasmonyl]-isoleucine, OlChemim, www.olchemim.cz/) was used as the internal standard. Detection was performed by ultra-high performance liquid chromatography (UHPLC)-electrospray interface (ESI) and quadrupole-orbitrap mass spectrometer (UHPLC/Q-Exactive™; Thermo Fisher Scientific) with an AQUITY UPLC HSS T3 column (2.1 × 100 mm; 1.8 μm, Waters).

Construction of plasmids and transgenic plants. To construct the plasmid pBCKK-Pro35S:AtMYB24:SRDX expressing the *AtMYB24-SRDX* gene under the control of the cauliflower mosaic virus 35S promoter, the *Pro35S:MYB24-SRDX* fragment was transferred from a plasmid 35S:MYB24-SRDX/pEntry, which is a previously reported entry plasmid constructed on p35SSRDXG vector,[33] to the pBCKK binary vector [34] by a Gateway LR reaction (Thermo Fisher Scientific). Transformation of tomato plants was performed as described previously.[35]

187 *Microscopy.* An SZX12 stereo microscope (Olympus,
188 www.olympus-global.com/en/) was used for observation of the morphologies of flower
189 buds and flowers. To observe pollen tube growth *in vivo*, manually pollinated pistils
190 were harvested 24 h after pollination, fixed in ethanol:acetic acid (3:1) for 24 h, and
191 treated for 12 h with 5N NaOH. After being rinsed three times in water, the pistils were
192 stained in 0.1 % aniline blue dye solution in 0.1 M K-phosphate buffer (pH 11) for 1 to
193 4 h. The pistils were mounted on a slide with 80% (v/v) glycerol solution and observed
194 by a fluorescence microscope BX60 (Olympus) under UV light excitation (Filter set
195 WU, excitation 330-385 nm, emission >420 nm). To visualize the pollen nuclei, pollen
196 grains in dehisced anthers were suspended in 1 μg/ml 4',6-diamidino-2-phenylindole
197 (DAPI) solution in 1 x PBS (130 mM NaCl, 5.1 mM Na_2HPO_4, 1.6 mM KH_2PO_4) by
198 brief vortexing. The pollen suspension was mounted on a slide and observed by
199 fluorescence microscopy with UV excitation. To observe the female gametophytes,
200 ovules were collected from newly opening flowers, fixed in ethanol:acetic acid (9:1 v/v)
201 solution for 2 h, and cleared in a modified Hoyer's solution (7.5 g gum arabic, 100 g
202 chloral hydrate, 5 ml glycerol, 30 ml H_2O). Specimens were observed by differential
203 interphase contrast microscopy.

Results

Delayed flower opening in a JA-insensitive mutant

In our growth condition, most of the WT Micro-Tom plants produced nine flowers in the primary inflorescence. Fig. 1A shows a typical primary inflorescence in which the first (a) and second (b) flowers have opened and the next three buds (c to e) have petals longer than the sepals (hereafter called the petal-emerged phase). The other four buds (f to i) did not show their petals (Fig. 1A). We observed the daily developmental process of each flower bud in the inflorescence and found that one flower newly opened every day in basal to apical order, and once-opened flowers continued to open for two days before senescence. The duration of the petal-emerged phase (including the partially opened bud) was three days on average (Fig. 1D, F).

To analyze the roles of JA on the bud development and flower opening in tomato, we observed a JA-insensitive tomato mutant, *jai1-1*, which is a deletion allele of *jai1* lacking a whole exon 2 and exon 3 of the *SlCOI1* gene.[19, 20] Fig. 1B shows a primary inflorescence of *jai1-1* at the same developmental stage as that of the WT inflorescence shown in Fig. 1A. The size and shape of the four youngest flower buds (f to i) and the next three petal-emerged buds (c to e) of *jai1-1* were similar to those of the corresponding buds in WT. However, the two largest buds (a and b) in *jai1-1* had not yet opened, whereas the counterparts in WT had fully opened (Fig. 1A, B). Fig. 1C shows another inflorescence of *jai1-1* when the first two flowers (a and b) were partly (but not yet fully) opened. All the other buds showed the petals. Continuous observation of the inflorescence revealed that most of the *jai1-1* flower buds required four to five days for passing though the petal-emerged phase and an additional two days for the partially opened phase before complete opening (Fig. 1E, F). These results indicated that the *jai1-1* flower buds paused the development for three days in comparison to WT

before opening. Hence, we concluded that JA has an important role in facilitating flower opening in tomato.

Levels of phytohormones in developing WT and jai1-1 *flower buds*

The above observations prompted us to examine whether JA and other phytohormones are biosynthesized and accumulated in tomato flower buds prior to flower opening and whether the levels might be different between in WT and *jai1-1* flower buds. To describe the flower development of Micro-Tom, we used the bud age in days. We defined the age of the flower bud that attained 4 mm in length as a "day-1 bud" and larger flower buds on the following days as a "day-X bud" (Fig. 2A). Typically, the day-2 bud became longer than 5 mm but its calyx was still closed. On day 3, the calyx opened and petals could be observed. On day 4, petals became longer than the sepals. On day 5, petals enlarged, whereas petal color remained pale green. On day 6, petal color changed to light yellow. On day 7, petals began to elongate outward, which meant initiation of flower opening. On day 8, petals elongated completely and bent outward. The flower fully opened, and this morphology continued to the end of day 9, except petals partially closed during the night. Then, flower began to close on day 10 and was senesced on day 11. The size and morphology of *jai1-1* flower buds were equivalent to those in WT until day 5, but the development was delayed thereafter (Fig. 2B). The day-6 bud of *jai1-1* was indistinguishable from that on day 5. Petals began to turn yellow on day 7, but they were still tinged with green and never opened on day 8. The bud finally began to open on day 9 and was fully open on day 10. Petals closed on day 12 (Fig. 2B). Consistent with the observation shown in Fig. 1, bud development in *jai1-1* was retarded for two to three days at the petal-emerged phase, and subsequent flower opening and closing proceeded two days later than in WT.

We performed simultaneous measurement of phytohormones from the day-1 bud

256 to flower opening. Three flower buds were harvested for each stage (day 1 to day 7 for
257 WT and day 1 to day 9 for *jai1-1*), and an individual bud was separately used for
258 hormone extraction and measurement. It is an important advantage of this method that
259 the levels of various hormones in the same samples can be compared directly. First, we
260 analyzed JA and its biologically active form, JA-Ile, in WT. Both molecules began to
261 increase on day 3, reached a sharp peak on day 5, at which petal growth was nearly
262 completed but the color had not yet turned yellow, and decreased to the basal level
263 when the flower began opening on day 7 (Fig. 2A, C). In contrast, accumulation of JA
264 and JA-Ile in *jai1-1* was hardly detectable throughout flower bud development (Fig. 2B,
265 C), which confirmed a previously reported result.[21] These results suggested that the JA
266 and JA-Ile accumulated in the day-5 buds is important for facilitating the subsequent
267 flower opening.

268 Similar peak formation around day 5 was observed for the levels of
269 indole-3-acetic acid (IAA) and abscisic acid (ABA), whereas gibberellin A_1 (GA_1)
270 reached the peak slightly earlier at day 3 (Fig. 2C). However, there were only small
271 differences between WT and *jai1-1*. Two cytokinins, N^6-(Δ^2-isopentenyl) adenine (iP),
272 and *trans*-zeatin (tZ), temporary increased in the day-2 flower buds of both WT and
273 *jai1-1* (Fig. 2C). Intriguingly, tZ increased in the day-7 bud in WT and day-9 bud in
274 *jai1-1*, both of which corresponded to opening flowers. Although the difference on day
275 7 was not statistically significant due to large deviations in WT, all three measured
276 values for WT were larger than those for *jai1-1*. Hence, it indicated that the level of tZ
277 might be causally related to flower opening.

278 Salicylic acid (SA) increased gradually during bud development but not
279 differentially between WT and *jai1-1*, suggesting no relation to flower opening (Fig.
280 2C). GA_4 was possibly accumulated in young flower buds and the opening flower in
281 WT, though the reliability of this finding is low due to large deviations among samples

(Fig. 2C). In summary, simultaneous phytohormone measurement revealed that the levels of JA, JA-Ile, and tZ were affected by *jai1-1* mutation during flower bud development. In the following experiments, we focused on the expression of biosynthesis and downstream genes of JA.

Expression of JA-related genes during flower bud development in WT and jai1-1

Among genes encoding JA biosynthetic enzymes, putative orthologs of Arabidopsis *AOC* (hereafter *AtAOC*) and *OPR3* (*AtOPR3*) have been identified in tomato,[36, 37] and we refer to them as *SlAOC* (Solyc02g085730) and *SlOPR3* (Solyc07g007870), respectively. The expression levels of *SlAOC* and *SlOPR3* were measured by quantitative reverse transcription PCR (qRT-PCR). In WT, the *SlAOC* expression was low until day 2, rapidly increased to the highest level at day 3, and gradually decreased by flower opening at day 7 (Fig. 3A). *SlOPR3* was moderately expressed throughout development with a broad peak around day 4 (Fig. 3B). It was characteristic that these peaks slightly preceded (by one to two days) the accumulation of JA and JA-Ile. In contrast, the level of *SlAOC* expression in *jai1-1* was kept low throughout bud development and was significantly lower than that in WT during day 1 to day 6 (Fig. 3A). *SlOPR3* was expressed in the mutant at a level comparable to WT, but the peak was unclear (Fig. 3B). The reduced expression of JA biosynthetic genes, especially of *SlAOC*, was consistent with the deficient accumulation of JA and JA-Ile in the mutant.

The weak correlation between *SlOPR3* expression and JA accumulation prompted us to examine whether *AtOPR3* homologs other than *SlOPR3* in the tomato genome contribute to JA synthesis in developing flower buds. A BLAST search revealed six genes as being similar to *SlOPR3* in the tomato genome (Supplemental Fig. S1A) but no genes homologous to *SlAOC*. We analyzed an RNA-seq data set consisting of a series of

developing WT flower buds at days 1, 2, 3, 5, 6, and 7 and found that *SlOPR3* as well as *SlAOC* was highly expressed in unopened flower buds with a peak at day 3, whereas the expression levels of most other *SlOPR3* homologs in tomato, excepting Solyc10g086220, were much lower than that of *SlOPR3* (Supplemental Fig. S1, Supplemental Table S2). Solyc10g086220 showed moderate expression throughout bud development, but it has been shown that this gene, designated *LeOPR1* or *SlOPR1*, is not involved in JA synthesis.[38] Therefore, we concluded that *SlOPR3* is predominantly involved in JA synthesis in developing flower buds and the contribution of other *SlOPR3* homologs is small, if any.

In Arabidopsis, three *R2R3-MYB* genes, *AtMYB21*, *AtMYB24*, and *AtMYB57*, regulate flower opening and stamen elongation downstream of the JA signaling pathway.[22, 23, 39] To identify the orthologs of these genes in tomato, we performed a similarity analysis of a subset of tomato and Arabidopsis *R2R3-MYB* genes.[40] We found that only *SlMYB21* (Solyc02g067760) belonged to the same clade as *AtMYB21*/*24*/*57*, suggesting that *SlMYB21* is a unique ortholog of *AtMYB21*/*24*/*57* (Fig. 3D). The expression of *SlMYB21* was first detected in the day-3 bud, in which JA and JA-Ile accumulation began, and reached the maximum level on day 6, which was one day later than the peaks of JA and JA-Ile accumulation (Figs. 2A, C, 3C). The expression profile was consistent with the data of RNA-seq analysis (Supplemental Fig. S1D). The RNA-seq analysis also revealed that the expression level of *SlMYB21* in developing flower buds was much higher than those of the other nine genes shown in Fig. 3D (Supplemental Fig. S1D, Supplemental Table S2). In *jai1-1* buds, the first detection of *SlMYB21* expression was delayed to day 5, which was two days later than the timing in WT (Fig. 3C). The expression increased gradually and finally reached the maximum level on day 9, which was comparable to the WT level at day 6 (Fig. 3C). The results suggested that the expression of *SlMYB21* does not require, but is facilitated by,

activated JA signaling in flower buds. The delayed flower opening in *jai1-1* was coincident with the delayed expression of the *SlMYB21* gene.

Suppression of SlMYB21 *mimics the delayed flower opening phenotype of* jai1-1

In order to test whether *SlMYB21* is involved in flower opening in tomato, we constructed transformants of Micro-Tom containing the *Pro35S*:*AtMYB24-SRDX* gene, which expressed a chimeric repressor protein, AtMYB24-SRDX, under the control of the cauliflower mosaic virus 35S promoter.[33)] Because *AtMYB24* is one of the closest homologs of *SlMYB21* (Fig. 2F), the expression of *Pro35S*:*AtMYB24-SRDX* is expected to repress the transcription of *SlMYB21* target genes in tomato. Among independently obtained 12 transformants redifferentiated from transgenic calli, six plants showed a remarkable phenotype. Flower buds of the transformants showed severely retarded opening or never opened until senescence (Fig. 4A, B). This phenotype resembles those of transgenic torenia plants containing the same gene [25)] as well as of Arabidopsis *atmyb21 atmyb24* double mutants of strong alleles and *atmyb21 atmyb24 atmyb57* triple mutants.[22, 23)] Taken together with the uniqueness of *SlMYB21* in the clade of *AtMYB21*/*24*/*57* (Fig. 3D), we concluded that *AtMYB24-SRDX* acts as a repressor of *SlMYB21* in tomato.

We compared the bud and flower development of the *AtMYB24-SRDX* transformants to that of WT and *jai1-1* in the period from day 3 to day 10 (Fig. 4C–E). Until day 5, the bud morphologies of all three lines, in particular the size and color of organs, were indistinguishable. The first difference was observed at day 6, when the WT petals began to turn yellow. Similar to *jai1-1*, the color of *AtMYB24-SRDX* petals remained pale green at this stage (Fig. 4C–E). Flower buds of *AtMYB24-SRDX* plants were severely delayed in flower opening, and in extreme cases they never opened even when petals began to senesce on day 13 (Fig. 4E). Nevertheless, observation of the

360 inner organs revealed that the timings of anther color change (on the day 7) and anther
361 dehiscence (on the day 8) were not delayed in either *jai1-1* or *AtMYB24-SRDX* plants
362 (Fig. 4C-E). These observations indicated that the *SlMYB21* gene regulates the timing of
363 flower opening but not of anther dehiscence in tomato.
364
365 *Deficient male and female fertility in* AtMYB24-SRDX *flowers*
366 Flowers of *AtMYB24-SRDX* plants occasionally bore fruits but they contained few
367 seeds (Fig. 5A), suggesting that *SlMYB21* plays an important role in male and/or female
368 organs. A similar phenotype is observed in *jai1-1* (Fig. 5A), and the sterility has been
369 attributed to the deficiency in male and female organs, though the detailed mechanism
370 has not been elucidated.[19, 20)] qRT-PCR analysis revealed that the *SlMYB21* gene was
371 expressed in all floral organs of Micro-Tom flower buds immediately before flower
372 opening (day 6), and the levels in petals, anthers, filaments, and styles were more than
373 two times higher than that in sepals (Fig. 5B). Thus, we attempted to examine how
374 *SlMYB21* contributes to the fertility. We used transgenic *AtMYB24-SRDX* plants
375 generated in the background of commercial cultivar Momotaro 8, of which the large
376 organ size made dissection easier. Two out of 17 independent transformants of this
377 cultivar showed the obvious opening-deficient phenotype in flowers (Supplemental Fig.
378 S2) and were used for further analysis.
379 We manually pollinated *AtMYB24-SRDX* pollen grains onto the WT stigma and
380 tested pollen tube elongation by aniline blue staining. In contrast to the control
381 experiment, in which the WT pollen elongated many pollen tubes, a few pollen tubes
382 were observed in a style when *AtMYB24-SRDX* pollen was pollinated (Fig. 5C). The
383 result that only a small number of pollen grains remained on the stigma indicated that
384 most of the pollinated pollen grains dropped out during the process of aniline blue
385 staining, presumably because they did not anchor to the stigma by their germinated

386 pollen tubes. Defective pollen tube elongation was also observed for *jai1-1* pollen
387 grains. In this case, many pollen grains germinated but aborted their tube growth in the
388 styles (Fig. 5C). A morphological abnormality was also found in *AtMYB24-SRDX*
389 pollen grains. Fully developed pollen grains in tomato contain two nuclei, a large
390 vegetative nucleus and a small or elongated generative cell nucleus. But DAPI staining
391 of pollen grains collected from dehisced anthers revealed that the generative nuclei in a
392 considerable proportion (more than 10%) of *AtMYB24-SRDX* pollen grains had already
393 divided into two putative sperm cell nuclei (Fig. 5D, E). Such generative cell division in
394 ungerminated pollen grains was rarely observed in WT and *jai1-1* pollen grains (Fig.
395 5D, E), even though the flowers had senesced. Therefore, we concluded that *SlMYB21*
396 is required for the development and function of pollen grains.

397 To evaluate the female fertility of *AtMYB24-SRDX* flowers, we pollinated WT
398 pollen grains onto the *AtMYB24-SRDX* stigma and observed the pollen tube elongation.
399 Most pollen grains germinated, but the pollen tubes frequently terminated their growth
400 in the stigma or in the style, suggesting that *SlMYB21* is required for stigmas and styles
401 to facilitate pollen tube elongation (Fig. 5F). Pistils of *jai1-1* as well as WT did not
402 show such defects. The failure of pollen tube elongation might be attributed to the
403 developmental defects of ovules. However, we could not find any abnormalities among
404 the ovules of *AtMYB24-SRDX*, *jai1-1*, and WT plants. In conclusion, *SlMYB21* is
405 necessary for the functional development of both male and female organs.
406

Discussion

In our growth conditions, Micro-Tom plants open a new flower every day in their primary inflorescences. This means that an inflorescence meristem of a primary shoot produces one flower bud every day, so that the inflorescence consists of a series of flower buds the ages of which differ by one day from the neighbors. We found that *jai1-1* flower buds develop normally until day 5 of bud age, but subsequently delay the growth and require two to three extra days for opening. This phenotype resembles those of Arabidopsis JA-deficient and JA-insensitive mutants.[3-9] In WT plants, JA and JA-Ile peak on day 5. After day 5, the developmental delay of *jai1-1* buds appears, suggesting that activation of JA signaling is required to trigger the process of flower opening. The accumulated JA and JA-Ile levels in WT flower buds were higher than those in wounded tomato leaves,[41-43] suggesting that strong JA signaling is required for the flower opening. An RNA measurement showed that the level of *SlAOC* mRNA was temporally increased in the day-3 buds, which preceded the peak of JA accumulation by two days. The induction of *SlAOC* is dependent on the function of *SlCOI1*, the causal gene of *jai1-1*, which accounts for the failure of JA accumulation in *jai1-1* buds. Another JA biosynthetic gene, *SlOPR3*, was moderately expressed throughout bud development but was also weakly upregulated in an *SlCOI1*-dependent manner. Hence, it seems likely that the activation of a positive feedback loop of JA synthesis is necessary for the promotion of flower opening. Activated JA signaling accelerates the expression of *SlMYB21*, the expression of which coincides with flower opening. Furthermore, strong repression of *SlMYB21* caused a failure of flower opening in tomato, as reported in other plants such as torenia and petunia. These indicate that JA facilitates flower opening through the activation of *SlMYB21* expression.

With respect to the development of stamens in *jai1-1* flower buds, anther color

433 change and anther dehiscence normally occurred, whereas petal elongation was delayed.
434 This differential development in *jai1-1* was previously explained as premature anther
435 dehiscence and premature pollen release.[21)] However, based on our morphology and
436 gene expression analyses, we proposed that anther wall development was normal but
437 petal expansion was delayed in *jai1-1*.

438 Simultaneous phytohormone measurement revealed that GA_1 peaked at day 3 in
439 parallel with the expression of *SlAOC*. In Arabidopsis, GA is required for the induction
440 of JA biosynthetic genes such as *DAD1*, and JA controls the expression of
441 *AtMYB21*/*24*/*57* genes and promotes stamen filament elongation.[23)] Auxin was also
442 accumulated simultaneously with or slightly earlier than JA in the day-4 to day-5 flower
443 buds. Arabidopsis AUXIN RESPONSE FACTOR6 (ARF6) and ARF8 are required for
444 the activation of *DAD1* expression,[4)] and also *AtMYB21*/*24* expression, by both
445 JA-dependent and independent pathways.[39)] Our results suggested the existence of a
446 similar mechanism in tomato. We also found a temporal accumulation of cytokinins and
447 abscisic acid during flower bud development. In particular, an increased tZ level in
448 parallel with flower opening is characteristic, though their biological meanings have not
449 yet been explained.

450 Observation of *AtMYB24-SRDX* flowers revealed that *SlMYB21* is not only
451 required for flower opening but also plays important roles in male and female organ
452 development. However, the phenotypic characteristics of *AtMYB24-SRDX* tomato were
453 not identical to those of *atmyb21 atmyb24* double mutants in Arabidopsis. In addition to
454 the lack of petal expansion leading to unopened flowers, the *atmyb21 atmyb24* double
455 mutants with a combination of knockout alleles showed short stamen filaments,
456 undehisced anthers, and reduced pollen fertility, but female fertility was normal.[22, 39)] In
457 contrast, the *AtMYB24-SRDX* tomato showed abnormal pollen development, impaired
458 pollen tube elongation, and deficient stigma/style function, but anther dehiscence was

459 normal. The effect on filament elongation cannot be evaluated due to innate short
460 filaments in tomato. It is suggested that the functions of *SlMYB21* and *AtMYB21*/*24*
461 have varied in the course of morphological diversification between tomato and
462 Arabidopsis. This difference partly explains the phenotypic difference between
463 Arabidopsis *coi1* and tomato *jai1*. The former shows a failure of filament elongation
464 and anther dehiscence, which results in the production of dysfunctional pollen grains.[9)]
465 In contrast, the latter shows no defect in anther dehiscence but shows abnormal male
466 and female fertility.[19, 20)] We frequently found aborted pollen tube elongation of *jai1-1*
467 pollen grains, which is consistent with their reduced viability reported previously;[19)]
468 however, we have not found any reason for female sterility.

469 We also found that there were considerable differences between *jai1-1* mutants
470 and *AtMYB24-SRDX* plants in the phenotypes of male and female organs. Pollen grains
471 of *AtMYB24-SRDX* plants hardly germinated on the stigma, whereas *jai1-1* pollen
472 grains germinated but aborted pollen tube elongation. Precocious division of generative
473 cells in pollen grains frequently observed in *AtMYB24-SRDX* plants occurs only rarely
474 in *jai1-1* pollen grains. Furthermore, styles of *AtMYB24-SRDX* plants inhibit pollen tube
475 elongation, whereas *jai1-1* styles do not show such a defect. These observations indicate
476 that the *SlMYB21* gene is required for the functional development of both male and
477 female organs after the opening of flowers. Relatively weak defects in *jai1-1* mutants in
478 comparison to *AtMYB24-SRDX* plants might be explained by retarded but increased
479 expression of *SlMYB21* genes in *jai1-1* flowers.

480 In summary, JA synthesized in developing flower buds accelerates the expression
481 of the *SlMYB21* gene, which coordinately induces petal elongation and functional
482 development of male and female organs in tomato. JA might determine the timing of
483 flower opening by integrating the signals of other hormones such as GA and auxin.
484 Although the role of JA in inducing the expression of *AtMYB21*/*24* or *SlMYB21* genes is

485 common between Arabidopsis and tomato, the functions of these MYB genes have
486 diverged between the two species.

Author contributions

T.N., T.S., T.H., H.S., and S.I. designed and coordinated the experiments. T.N., T.S., Y.T., and R.I. carried out the experiments and analyzed the results. T.N. and S.I. wrote the manuscript. All the authors have read and approved the final manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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513

514 **Supplemental materials**

515 The supplemental materials for this paper are available at
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517

518 **Accession number**

519 FASTQ files of the RNA-seq reads are available with the accession number
520 DRA006324.

521

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649

650 **Figure Legends**

651 Fig. 1. Delayed flower opening in JA-insensitive *jai1-1* mutants.

652 Note: (A–C) Primary inflorescences of WT Micro-Tom (MT-GH) (A) and *jai1-1*
653 (B, C) plants. (A) and (B) were almost at the same developmental stage because the size
654 and shape of the young flower buds were equivalent. (C) was at a later stage around
655 three days after (B). Each flower and flower bud was labeled with a lowercase letter (the
656 largest one was a, followed in order by b, c, d...). A fully opened flower (white squares),
657 a partially opened flower (grey squares), a petal-emerged bud (black squares), and a
658 smaller bud (no square) are indicated, respectively. (D, E) Developmental progress of
659 flower buds in WT (D) and *jai1-1* (E). The shapes of all flower buds in a representative
660 primary inflorescence were continuously observed once daily at noon. A fully opened
661 flower (white bars), a partially opened flower (grey bars), a petal-emerged bud (black
662 bars), and a smaller bud (black lines) were indicated, respectively. (F) Average length of
663 the period from petal emergence (when petal exceeds sepal) to fully open, which is the
664 sum of the lengths of petal-emerged buds and partially opened flowers. An error bar
665 represents the S.D. *n* is indicated in each bar. Significance was calculated using an
666 unpaired Student's *t*-test. ***$P<0.001$.

667

668 Fig. 2. Development of WT and *jai1-1* flower buds toward opening and changes of
669 phytohormone levels in the flower buds. The levels of nine phytohormones in the same
670 bud sample were simultaneously measured.

671 Note: (A, B) Morphology of representative flower buds and flowers at indicated
672 ages. WT (MT-GH) (A) and *jai1-1* (B). Bars = 5 mm. (C) The means of individual
673 hormone levels in three samples of each age and their S.D. are indicated as pmol per
674 gram fresh weight (pmol/gFW). WT (solid line) and *jai1-1* (dashed line) were compared.

675 Asterisks indicate significant differences between WT and *jai1-1* at the same bud age
676 (Student's *t*-test, $P<0.05$). $n = 3$.
677
678 Fig. 3. Changes of gene expression in WT and *jai1-1* flower buds toward opening.
679 Note: (A−C) Comparisons among the changes of gene expressions of *SlAOC* (A),
680 *SlOPR3* (B), and *SlMYB21* (C) in developing flower buds/flowers. WT (solid lines) and
681 *jai1-1* (dashed lines) were measured. The highest level of each gene expression in WT
682 was arbitrarily set to 1. *DNAJ* was used as the internal control. Error bars indicate the
683 S.D. Asterisks indicate significant differences between WT and *jai1-1* at the same bud
684 age (Student's *t*-test, $P<0.05$). $n = 3$. (D) A neighbor-joining phylogenetic tree showing
685 amino acid sequence similarities among SlMYB21 and closely related R2R3-MYB
686 proteins in tomato and Arabidopsis. AtMYB21, AtMYB24, and AtMYB57 are
687 indicated by asterisks. Bootstrap values are indicated at the roots.
688
689 Fig. 4. Bud and flower development in *AtMYB24-SRDX* plants.
690 Note: (A, B) Inflorescences of WT (A) and *AtMYB24-SRDX* (B) plants. (C−E)
691 Morphology of representative flower buds and flowers at the indicated day number of
692 age. WT (C), *jai1-1* (D), and *AtMYB24-SRDX* (E) plants, all of which were in a
693 Micro-Tom (MT-GH) background, are compared. Lower panels are an enlargement of
694 the inner organs after removal of the front organs. Arrowheads indicate dehisced anthers.
695 Bars = 1 mm.
696
697 Fig. 5. Abnormal male and female fertility in *AtMYB24-SRDX* flowers.
698 Note: Abbreviations for the plant samples in this figure are as follows. M8,
699 Momotaro 8; *AtMYB24-SRDX*, *AtMYB24-SRDX* transgenic tomato in a Momotaro 8
700 background; MT, Micro-Tom (MT-GH); *jai1-1*, *jai1-1* mutant. (A) Cross sections of

701 developing fruits. Arrowheads, developing seed; arrows, placenta. Bars = 5 mm. (B)
702 Expression levels of the *SlMYB21* gene in various floral organs of day-6 Micro-Tom
703 flower buds. Se, sepals; Pe, petals; An, anthers; Fi, filaments; St, styles; Ov, ovaries.
704 *SAND* was used as the internal control. The level in petals was arbitrarily set to 1. Error
705 bars, SD. n = 3. (C) Pollen tube elongation visualized by aniline blue staining. WT
706 Micro-Tom pistils were manually pollinated with the pollen grains of indicated plant
707 samples. Arrows, aborted pollen tube. Bars = 100 μm. (D) DAPI-stained pollen grains
708 corrected from dehisced anthers. Arrowheads, generative cell nucleus; arrows,
709 vegetative cell nucleus; asterisks, tricellular pollen grain. Bars = 100 μm. (E)
710 Appearance rate of trinuclear pollen grains. More than 100 pollen grains were counted
711 per assay. Error bars, S.D. n = 3. ** $P < 0.01$ (Student's t-test). (F) Pollen tube
712 elongation visualized by aniline blue staining. Pistils of indicated plant samples were
713 manually pollinated with WT (M8) pollen grains. Arrows, aborted pollen tube. Bars =
714 100 μm. (G) Ovules isolated from opening or equivalent flower buds after treatment
715 with clearing solution. Brackets, female gametophyte; arrowheads, micropyle. Bars =
716 100 μm.
717

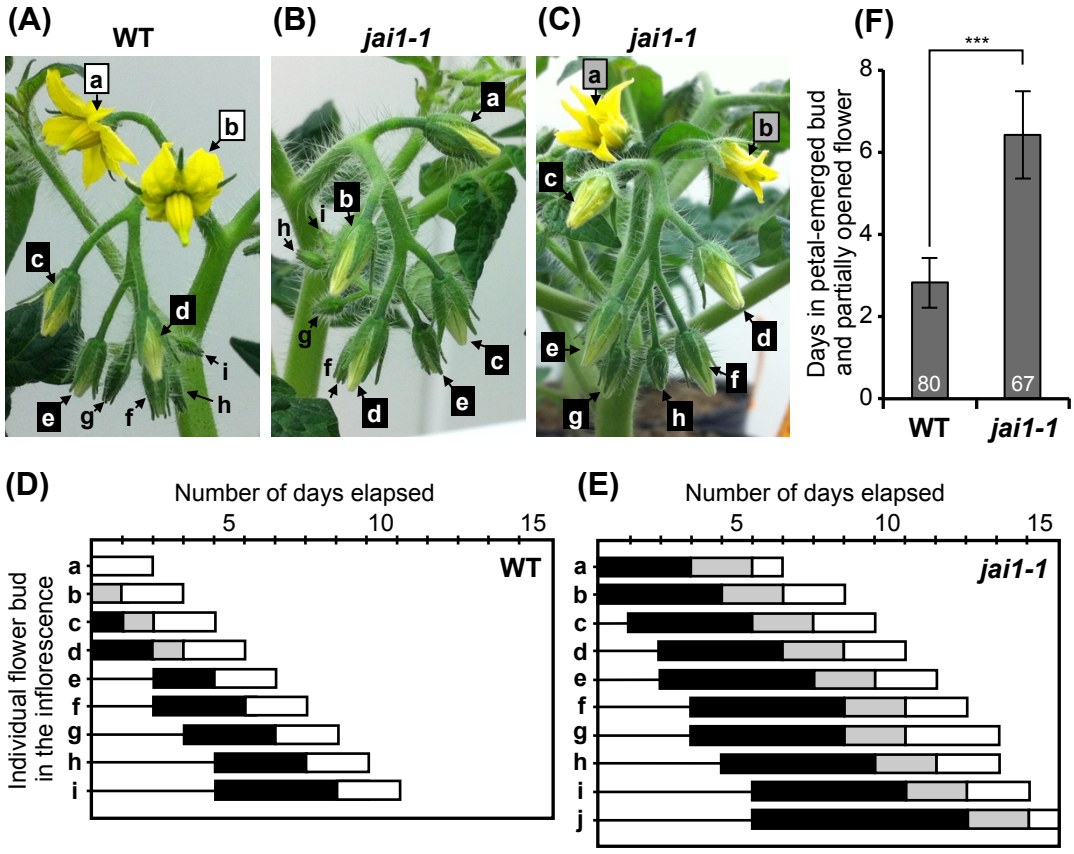


Fig. 1. Delayed flower opening in JA-insensitive *jai1-1* mutants.

Note: (A–C) Primary inflorescences of WT Micro-Tom (MT-GH) (A) and *jai1-1* (B, C) plants. (A) and (B) were almost at the same developmental stage because the size and shape of the young flower buds were equivalent. (C) was at a later stage around three days after (B). Each flower and flower bud was labeled with a lowercase letter (the largest one was a, followed in order by b, c, d...). A fully opened flower (white squares), a partially opened flower (grey squares), a petal-emerged bud (black squares), and a smaller bud (no square) are indicated, respectively. (D, E) Developmental progress of flower buds in WT (D) and *jai1-1* (E). The shapes of all flower buds in a representative primary inflorescence were continuously observed once daily at noon. A fully opened flower (white bars), a partially opened flower (grey bars), a petal-emerged bud (black bars), and a smaller bud (black lines) were indicated, respectively. (F) Average length of the period from petal emergence (when petal exceeds sepal) to fully open, which is the sum of the lengths of petal-emerged buds and partially opened flowers. An error bar represents the S.D. *n* is indicated in each bar. Significance was calculated using an unpaired Student's *t*-test. ***$P<0.001$.

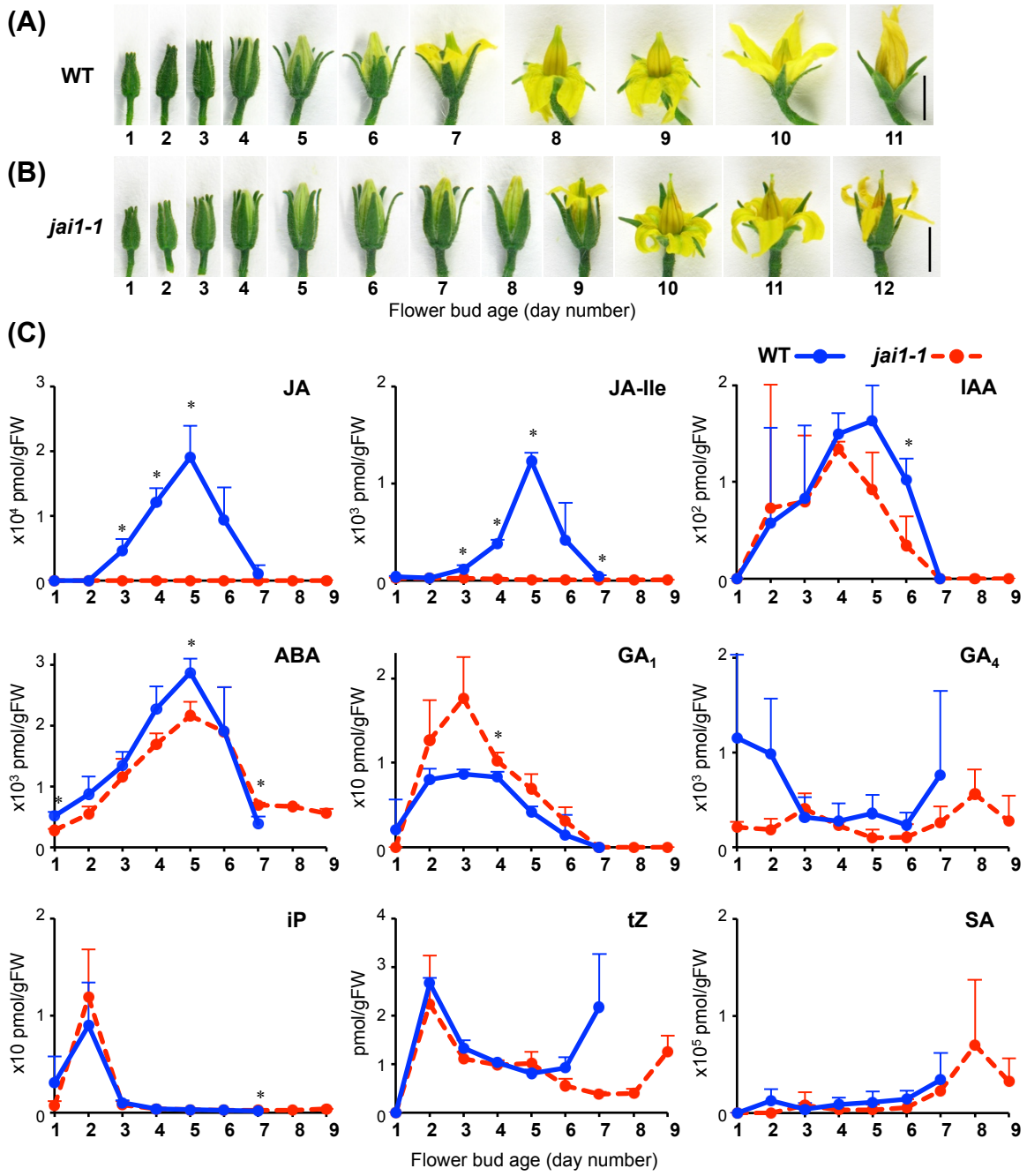
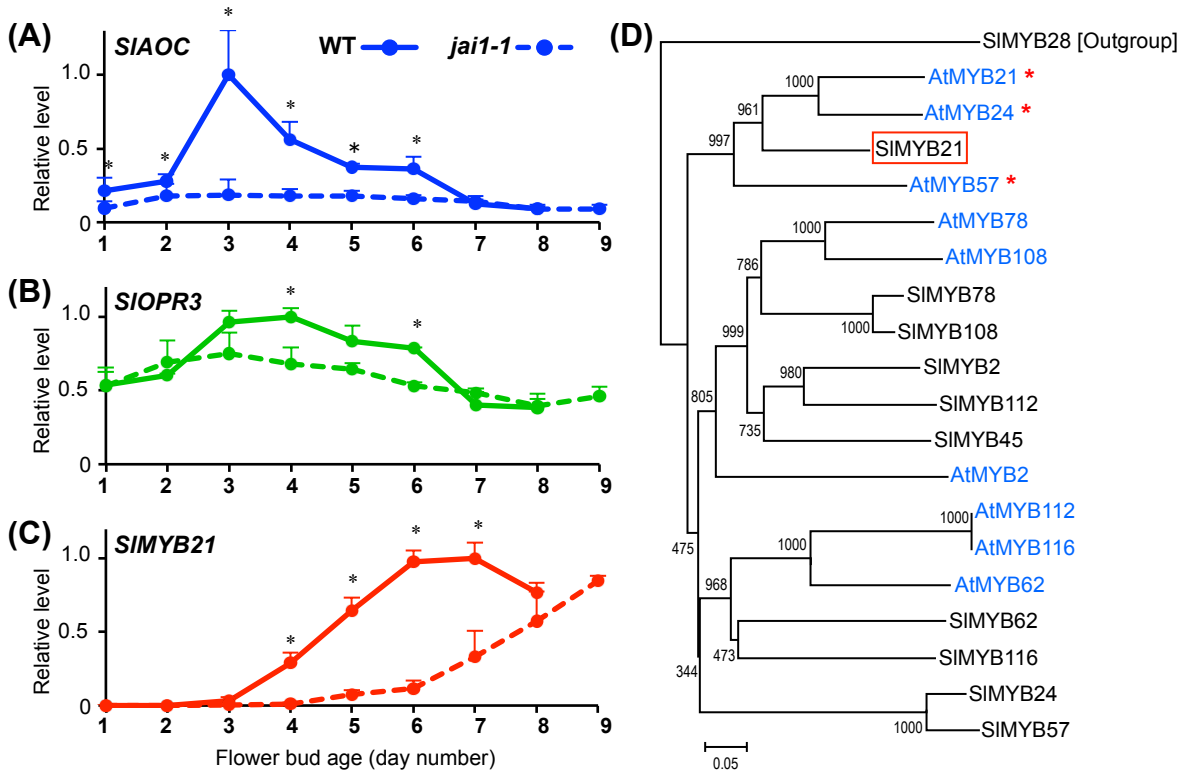


Fig. 2. Development of WT and *jai1-1* flower buds toward opening and changes of phytohormone levels in the flower buds. The levels of nine phytohormones in the same bud sample were simultaneously measured.

Note: (A, B) Morphology of representative flower buds and flowers at indicated ages. WT (MT-GH) (A) and *jai1-1* (B). Bars = 5 mm. (C) The means of individual hormone levels in three samples of each age and their S.D. are indicated as pmol per gram fresh weight (pmol/gFW). WT (solid line) and *jai1-1* (dashed line) were compared. Asterisks indicate significant differences between WT and *jai1-1* at the same bud age (Student's *t*-test, $P<0.05$). $n = 3$.



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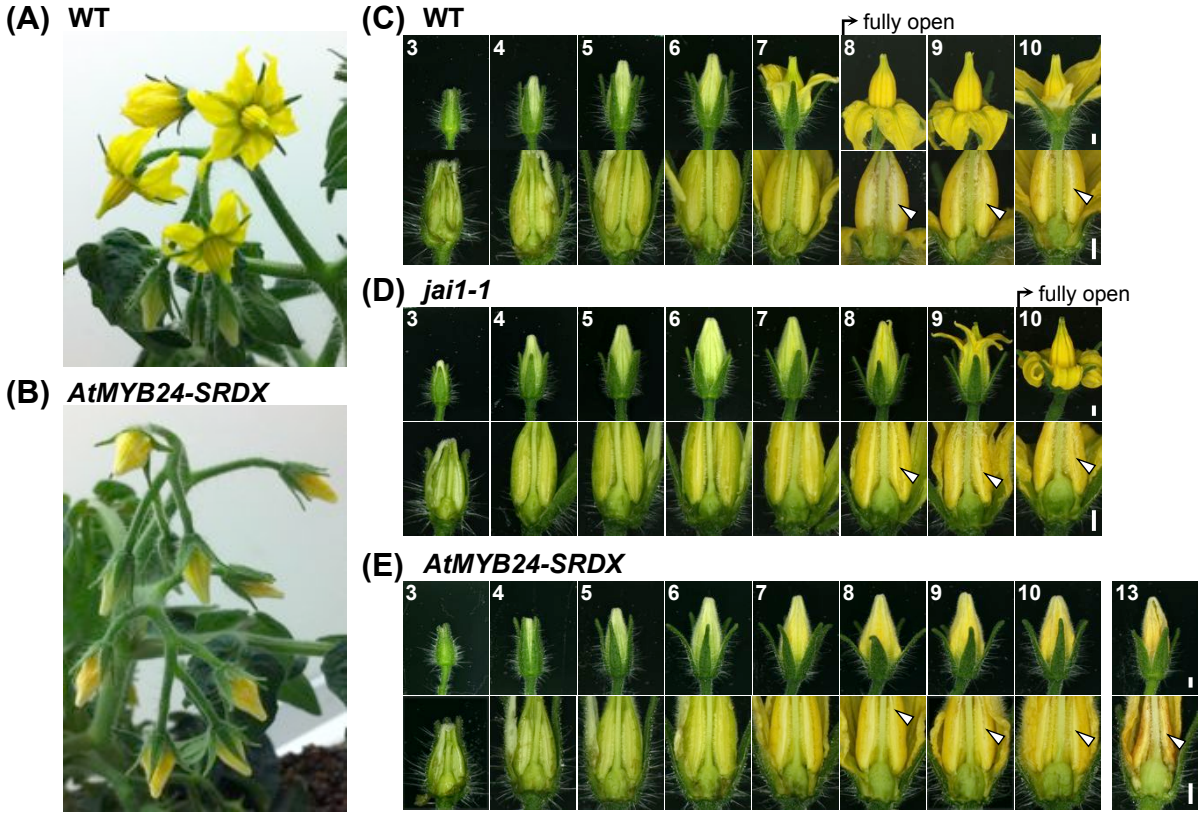

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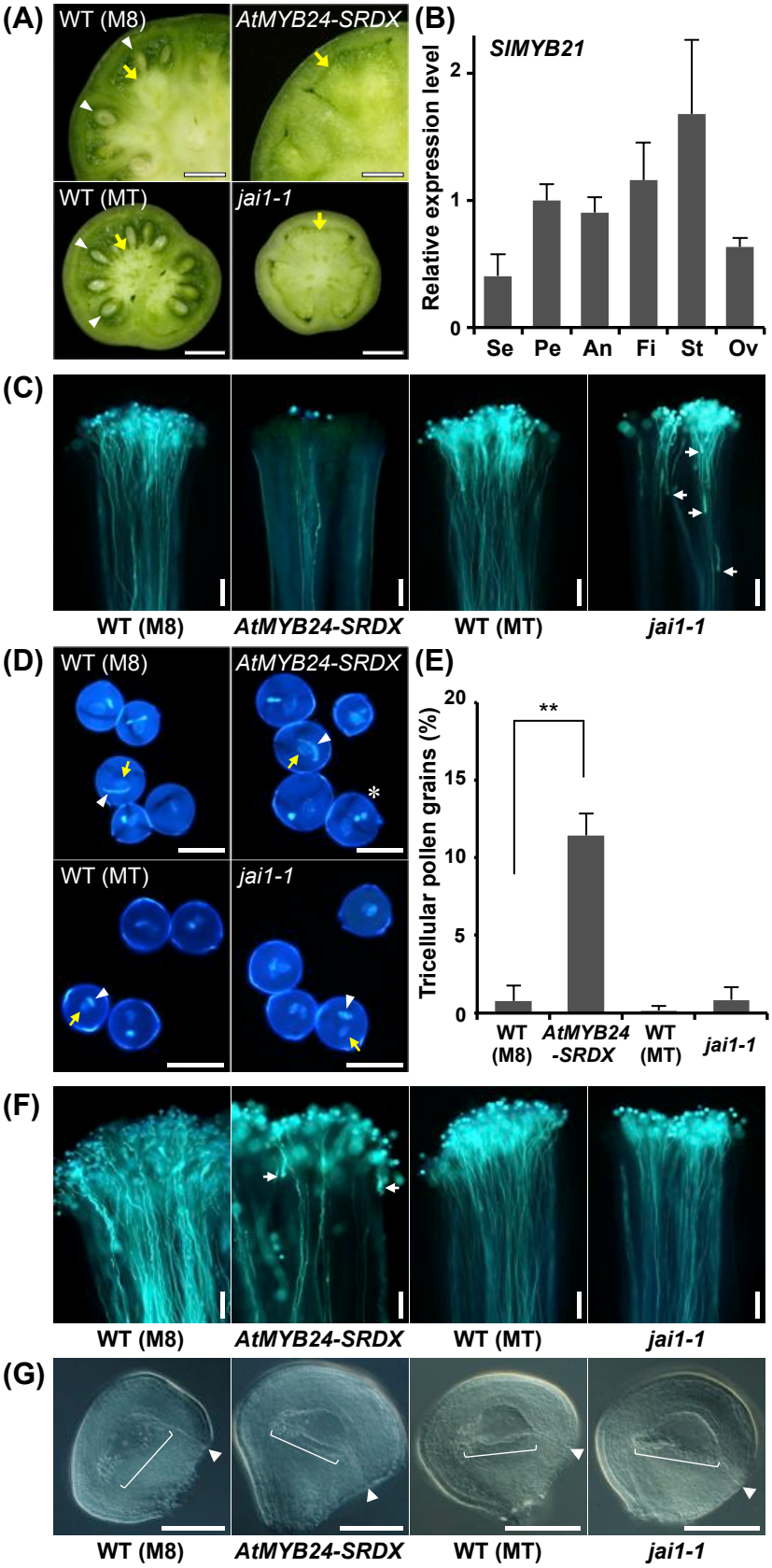

Fig. 5. Abnormal male and female fertility in *AtMYB24-SRDX* flowers.

Note: Abbreviations for the plant samples in this figure are as follows. M8, Momotaro 8; *AtMYB24-SRDX*, *AtMYB24-SRDX* transgenic tomato in a Momotaro 8 background; MT, Micro-Tom (MT-GH); *jai1-1*, *jai1-1* mutant. (A) Cross sections of developing fruits. Arrowheads, developing seed; arrows, placenta. Bars = 5 mm. (B) Expression levels of the *SlMYB21* gene in various floral organs of day-6 Micro-Tom flower buds. Se, sepals; Pe, petals; An, anthers; Fi, filaments; St, styles; Ov, ovaries. *SAND* was used as the internal control. The level in petals was arbitrarily set to 1. Error bars, SD. $n = 3$. (C) Pollen tube elongation visualized by aniline blue staining. WT Micro-Tom pistils were manually pollinated with the pollen grains of indicated plant samples. Arrows, aborted pollen tube. Bars = 100 μm. (D) DAPI-stained pollen grains corrected from dehisced anthers. Arrowheads, generative cell nucleus; arrows, vegetative cell nucleus; asterisks, tricellular pollen grain. Bars = 100 μm. (E) Appearance rate of trinuclear pollen grains. More than 100 pollen grains were counted per assay. Error bars, S.D. $n = 3$. ** $P < 0.01$ (Student's *t*-test). (F) Pollen tube elongation visualized by aniline blue staining. Pistils of indicated plant samples were manually pollinated with WT (M8) pollen grains. Arrows, aborted pollen tube. Bars = 100 μm. (G) Ovules isolated from opening or equivalent flower buds after treatment with clearing solution. Brackets, female gametophyte; arrowheads, micropyle. Bars = 100 μm.