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Research Article

Modulating auxin response stabilizes tomato fruit set

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Abstract

Fruit formation depends on successful fertilization and is highly sensitive to weather fluctuations that affect pollination. Auxin promotes fruit initiation and growth following fertilization. Class A auxin response factors (Class A ARFs) repress transcription in the absence of auxin and activate transcription in its presence. Here, we explore how multiple members of the ARF family regulate fruit set and fruit growth in tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana*, and test whether reduction of SlARF activity improves yield stability in fluctuating temperatures. We found that several tomato *Slarf* mutant combinations produced seedless parthenocarpic fruits, most notably mutants deficient in *SlARF8A* and *SlARF8B* genes. Arabidopsis *Atarf8* mutants deficient in the orthologous gene had less complete parthenocarpy than did tomato *Slarf8a Slarf8b* mutants. Conversely, *Atarf6 Atarf8* double mutants had reduced fruit growth after fertilization. AtARF6 and AtARF8 likely switch from repression to activation of fruit growth in response to a fertilization-induced auxin increase in gynoecia. Tomato plants with reduced *SlARF8A* and *SlARF8B* gene dosage had substantially higher yield than the wild type under controlled or ambient hot and cold growth conditions. In field trials, partial reduction in the *SlARF8* dose increased yield under extreme temperature with minimal pleiotropic effects. The stable yield of the mutant plants resulted from a combination of early onset of fruit set, more fruit-bearing branches and more flowers setting fruits. Thus, ARF8 proteins mediate the control of fruit set, and relieving this control with *Slarf8* mutations may be utilized in breeding to increase yield stability in tomato and other crops.

Introduction

Fruits are major sources of flavor, nutrition, and fibers in the human diet and in the food industry. Fruits develop from the ovary, which contains the ovules. Following its growth and patterning during flower development, the gynoecium pauses growth until fertilization (Gasser and Robinson-beers 1993). Upon fertilization, ovules differentiate into seeds, and the surrounding maternal ovary resumes growth and develops into a fruit, a process termed fruit set. Normally, fruit set occurs only upon fertilization, and in the absence of fertilization the flower aborts (Gillaspy et al. 1993; Giovannoni 2004; Ariizumi et al. 2013; McAtee et al. 2013; Fenn and Giovannoni 2021). As a consequence, fruit set is compromised under nonoptimal temperatures

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that prevent fertilization, limiting the growing season, yield, and fruit quality (Charles and Harris 1972; Abad and Monteiro 1989; Peet et al. 1998; Sato et al. 2002, 2006; Firon et al. 2006; Klap et al. 2016). These limitations are likely to increase as the climate warms and heat waves become more frequent. Many lines of research have led to yield increase (Rodríguez-Leal et al. 2017; Soyk et al. 2017; Eshed and Lippman 2019). However, even when the yield potential is high, unfavorable conditions can lead to severe yield loss. Genetic relaxation of the coupling between fertilization and fruit set may contribute to the realization of yield potential by increasing yield stability in unfavorable environments.

Uncoupling fruit set from fertilization results in the formation of parthenocarpic fruits, which develop without seeds. Parthenocarpic fruits can result from genetic, environmental or hormonal alterations (Ariizumi et al. 2013; Joldersma and Liu 2018; Sharif et al. 2022). The signals for fruit set include auxin and other hormones produced by the embryo and/or endosperm within the developing seeds (Hu et al. 2008; Dorcey et al. 2009; De Jong, Mariani, et al. 2009; De Jong, Wolters-Arts, et al. 2009; Serrani et al. 2010; Figueiredo and Köhler 2018). Parthenocarpic fruits form as a result of altered auxin response (Abad and Monteiro 1989; Vivian-smith and Koltunow 1999; Kang et al. 2013; Joldersma and Liu 2018; Fenn and Giovannoni 2021; Sharif et al. 2022); when auxin is overproduced in ovaries of transgenic plants (Rotino et al. 1997, 2005; Acciarri et al. 2002; Mezzetti et al. 2004; Pandolfini et al. 2007); in response to inhibition of auxin transport (Serrani et al. 2010; Mounet et al. 2012; Pattison and Catalá 2012); or in transgenic plants with perturbed auxin physiology (Carmi et al. 2003; Molesini et al. 2009; Kim et al. 2020). Thus, local alteration of auxin response is sufficient to promote fruit development in plants with diverse fruit biology. This effect of auxin has been exploited in tomato (*Solanum lycopersicum*) cultivation: auxin applications promote fruit production in cool conditions when pollination is inefficient (Abad and Monteiro 1989).

Class A auxin response factors (Class A ARFs) are central components of the nuclear auxin signal transduction pathway (Chandler 2016; Weijers and Wagner 2016; Kato et al. 2017, 2020). In the presence of auxin, these transcription factors activate expression of auxin-responsive genes. Conversely, in the absence of auxin, these ARFs can repress gene expression when complexed with auxin/indole acetic acid (Aux/IAA) transcriptional repressors. Auxin switches Class A ARF activity from repression to activation by promoting Aux/IAA protein turnover (Chandler 2016; Weijers and Wagner 2016; Kato et al. 2017, 2020; Leyser 2018). There are 5 Class A ARFs in Arabidopsis and 7 in tomato, and these act in a partially overlapping manner to regulate growth in various tissues. For example, Arabidopsis *AtARF6*, *AtARF8*, and *AtNPH4/ARF7* could promote or inhibit hypocotyl elongation, depending on the growth conditions, and genetic background (Reed et al. 2018). Negative feedback loops as well as inputs from other signals may contribute to the nonlinear gene dosage responses in this and other contexts (Oh et al. 2014; Israeli et al. 2020). In tomato, fine-tuning the activity of Class A ARFs and their Aux/IAA repressor SlIAA9/ENTIRE caused a phenotypic continuum of leaf complexity (Israeli et al. 2019).

Several Class A ARF proteins affect flower and fruit development. Arabidopsis Class A ARFs AtARF6 and AtARF8, which are negatively regulated by the microRNA miR167, promote growth in hypocotyls, leaves, inflorescence stems, and flower organs (Nagpal et al. 2005; Wu et al. 2006; Tabata et al. 2010; Crawford and Yanofsky 2011; Reeves et al. 2012; Reed et al. 2018). Flowers of *Atarf6 Atarf8* (*Atarf6,8*) double mutants are largely male and female-sterile, as are *p35S:AtMIR167a* plants that overproduce miR167 to silence both *AtARF6* and *AtARF8* (Nagpal et al. 2005; Wu et al. 2006; Tabata et al. 2010). Female sterility of these plants results from a combination of defects in stigma growth, style growth and maturation, and transmitting tract differentiation that together limit the ability of pollen to germinate, grow, and fertilize mutant ovules (Nagpal et al. 2005; Crawford and Yanofsky 2011; Reeves et al. 2012). Of note, *Atarf8* single mutants are parthenocarpic, having excess gynoecium growth in the absence of fertilization (Vivian-smith et al. 2001; Goetz et al. 2006, 2007).

All tomato Class A ARFs interact physically and genetically with the Aux/IAA protein SlIAA9/ENTIRE (E) (Zouine et al. 2014; Hu et al. 2018; Israeli et al. 2019). *Sliaa9/entire* (*e*) mutants make fruit in the absence of fertilization (Wang et al. 2005, 2009), indicating that SlARF proteins likely regulate tomato fruit set. Indeed, altered activity of SlARF5/SlMONOPTEROS/SlMP, SlARF7, SlARF8, and/or SlARF2 led to partial parthenocarpy (De Jong, Wolters-Arts, et al. 2009; Hao et al. 2015; Breitel et al. 2016; Du et al. 2016; Hu et al. 2018). Similarly, downregulation of eggplant (*Solanum melongena*) *SmARF8* led to parthenocarpy (Du et al. 2016). In strawberry (*Fragaria* × *ananassa*), loss of *FvARF8* function increased fruit growth (Zhou et al. 2020). Transgenic eggplant overexpressing *ARF8* also have increased fruit growth (Du et al. 2016). These discoveries in multiple species suggest that ARF8 orthologs may have a broadly conserved role in fruit set. However, it is not clear which SlARFs are the central regulators of tomato fruit set.

Here, we show that tomato *Slarf8a Slarf8b* mutants form parthenocarpic fruits. In Arabidopsis, *AtARF6* and *AtARF8* inhibited fruit growth of emasculated flowers but promoted growth of pollinated gynoecia, consistent with these ARFs mediating the fruit growth response to auxin after fertilization. Several *Slarf8* mutant combinations more than doubled the yield under extreme temperatures. Partial reduction of *SlARF8* dose resulted in increased yield stability with minimal pleiotropic effects. The increased yield resulted from several developmental effects, including an early onset of fruit set, increased number of fruit-bearing branches and increased number of flowers that set fruit. The results therefore suggest that SlARF8 mediates the control of fruit set, and that fine-tuning ARF activity can be utilized to increase yield stability in fluctuating environments.

Results

Tomato Class A *SlARFs* are differentially expressed in multiple flower organs

To explore the contribution of tomato Class A ARFs to the control of fruit set and development, we first examined their expression in ovary and fruit tissues. Analysis of RNAseq data revealed that all class A *SlARFs* are expressed in young ovaries 5 d before anthesis, with *SlARF8A* and *SlARF8B* expressed at the highest relative levels (Supplemental Fig. S1A and Table S1). In a public transcriptomic dataset obtained from micro-dissected ovary tissues at the day of anthesis, *SlARF5/MP*, *SlARF8A*, and *SlARF8B* were the most highly expressed Class A ARFs (Shinozaki et al. 2018). Of these, *SlARF8A* was expressed in the placenta and pericarp, and *SlARF8B* was particularly highly expressed in the placenta (Supplemental Fig. S1, B and C). *SlARF5/SlMP* and *SlARF7* were expressed mainly in ovules, and *SlARF19A* and *SlARF19B* expression was relatively low and uniform throughout the ovary. *SlARF6A* was expressed most strongly in the placenta but at a much lower level than *SlARF8A* and *SlARF8B*. These expression patterns suggest that different class A SlARFs may regulate distinct aspects of fruit set and seed development, and that SlARF8A and SlARF8B may be particularly important for growth and development of the placenta, septum, and pericarp, which grow substantially when fruits form.

Slarf8a Slarf8b double mutants produce parthenocarpic fruits

To elucidate the function of specific tomato Class A ARFs in the control of fruit set, we examined fruit development in single mutants or mutant combinations in this family. We generated several mutant alleles in *SlARF8A* and *SlARF8B* (Supplemental Fig. S2; see Materials and methods), and used the previously described mutants in *SlARF5/MP*, *SlARF7*, *SlARF19A*, and *SlARF19B* (Israeli et al. 2019). *Slarf5/Slmp* mutants and mutant combinations that contained it rarely developed fruits when allowed to self-pollinate, and these fruits were small, seedless, elongated, and ovate-like (van der Knaap et al. 2014; Israeli et al. 2019) (Supplemental Fig. S3, B, H, I, O, Q, and T). Other single *Slarf* mutants did not have an apparent defect in fruit development. Of the double mutants, 2 combinations, *Slarf8a Slarf8b* and *Slarf19a Slarf19b* consistently produced seedless fruits when allowed to self-pollinate (Fig. 1; Supplemental Fig. S3, L to N). *Slarf19a Slarf19b* mutants grew very slowly and were not characterized in detail here. *Slarf8b* and *Slarf8a Slarf8b* fruits were slightly smaller than wild-type fruits (Fig. 1, A to E; Supplemental Fig. S3). While single *Slarf8a* and *Slarf8b* mutants showed low percentages of seedless fruit formation, all the fruits produced by *Slarf8a Slarf8b* double mutant plants were seedless, and mostly lacked locular gel and placenta (Fig. 1, A to D and F; Supplemental Fig. S3, D, E, and L).

Previously described *Slarf* mutations affect different aspects of vegetative phenotypes during plant development (Israeli et al. 2019). We asked whether *Slarf8a* and *Slarf8b* mutation combinations also affect vegetative development. *Slarf8* mutant plants were smaller than wild-type plants, with smaller and slightly less compound leaves (Supplemental Fig. S4, A to H). The hypocotyls of *Slarf8a Slarf8b* were slightly shorter compared to the wild-type (Supplemental Fig. S4, I and J). Thus, SlARF8A and SlARF8B promote vegetative growth, in contrast to their effect on unpollinated ovary growth.

The specific placenta expression and the mutant phenotypes suggested that SlARF8A and SlARF8B are particularly relevant for fruit set in tomato. We therefore focused further analysis mainly on *Slarf8a* and *Slarf8b* mutant combinations. The formation of seedless fruits is not always linked to the ability to form fruits independently of fertilization, termed parthenocarpy. To test for parthenocarpic fruit formation, we therefore emasculated flowers before anthesis. In contrast to the wild type, *Slarf8a Slarf8b* flowers produced parthenocarpic fruits following emasculation (Fig. 1, G to K). To understand the developmental basis for this parthenocarpy, we followed ovary development from early stages of flower development. The flower bud stages were classified according to bud and gynoecia size, opening of the sepals, color of the petals, and opening of the flowers, according to Dobritzsch et al. (2015). The youngest stage, designated stage 1 (S1) represented a small bud completely enclosed by sepals, 7 d before anthesis. S2 to S4 were Buds 5, 3, and 1 d before anthesis, respectively, where S2 corresponds to Stage 9 to 11 in Brukhin et al. (2003). S5 was at the time of anthesis (flower opening), and S6 represented an open flower with bright yellow petals, 1 d after anthesis and pollination. Developing *Slarf8a Slarf8b* ovaries started growing earlier and grew faster than wild-type ovaries, starting from S2, and unlike the wild type, did not pause growth at the S4 stage (Fig. 2A). Therefore, SlARF8A and SlARF8B appear to repress fruit set in unpollinated flowers from a very early stage of flower development.

To better understand the role of SlARF8A and B in ovary growth and inhibition of fruit set, we compared gene expression between developing ovaries of wild-type and *Slarf8a Slarf8b* plants. Young S2 gynoecia, before major changes in weight and size could be observed, were used for RNAseq (Supplemental Table S1). This experiment was repeated 2 times. Among the genes commonly overexpressed in both experiments was the GA biosynthesis gene *SlGA20ox-1* (Solyc03g006880), in agreement with GA acting downstream of auxin in fruit formation (Dorcey et al. 2009; Serrani et al. 2010), and with the finding that overexpression of *SlGA20ox-1* leads to parthenocarpic fruit formation (García-Hurtado et al. 2012) (Supplemental Fig. S5). The cytokinin degradation gene *CKX2* (Solyc10g017990) was also overexpressed in *Slarf8a Slarf8b* (Supplemental Fig. S5). Cytokinin was shown to promote fruit development (Bartrina et al. 2011; Joldersma and Liu 2018), and the upregulation of *CKX2* could result from feedback regulation or from a dual role for cytokinin in different

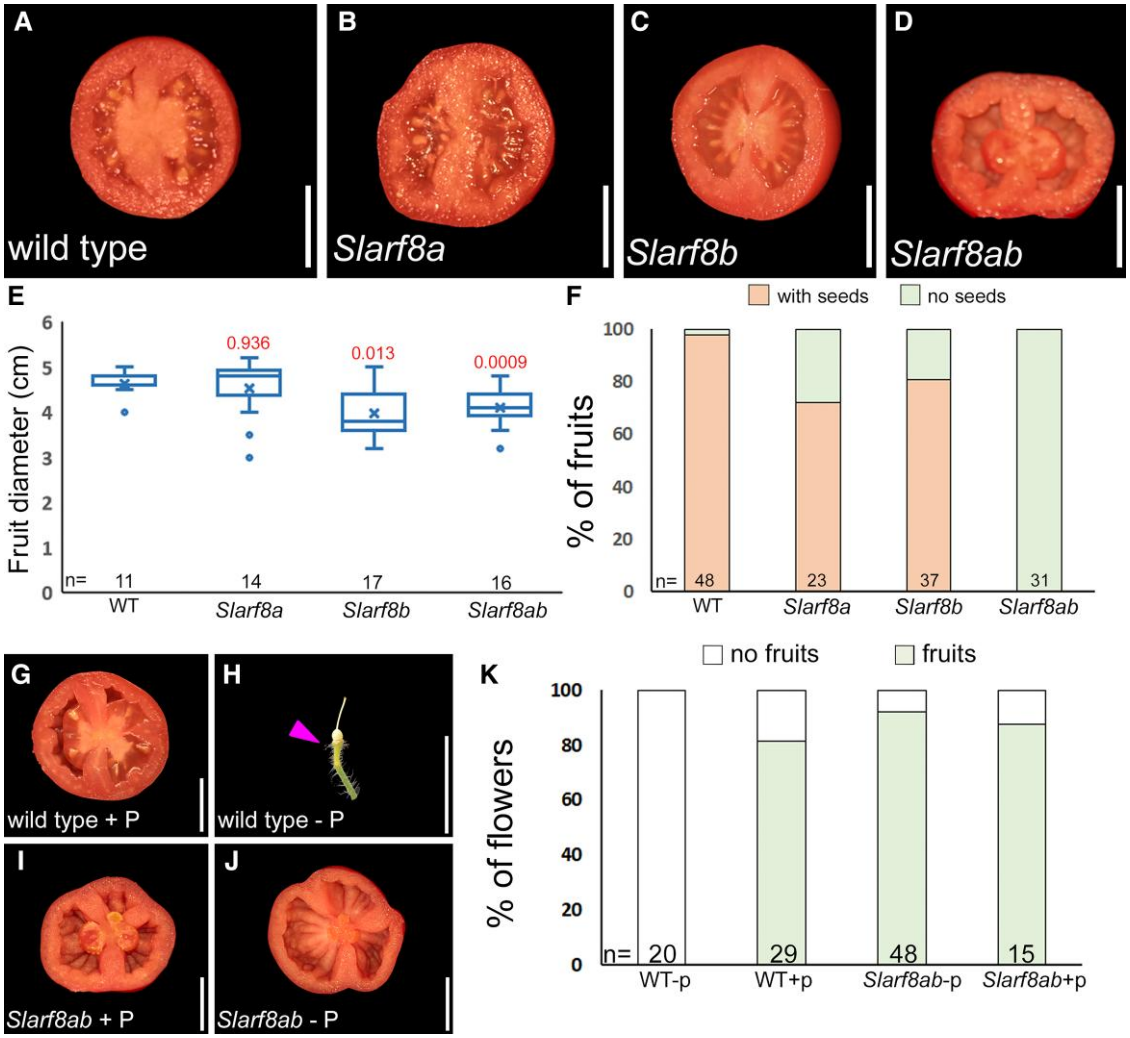

Figure 1. Fruit phenotypes of *Slarf8a Slarfb* mutants. **A to D)** Representative photographs of cut, self-fertilized fruits of the indicated genotypes. *Slarf8ab—Slarf8a Slarf8b*. Individual images were digitally extracted for comparison. Scale bar: 2 cm. **E)** Quantification of the fruit diameter of cut, self-fertilized fruits of the indicated genotypes; *n* = number of fruits quantified. *P*-values represent differences from the wild type, as determined by the Dunnett test. Lower and upper whiskers indicate the minimum and maximum values, respectively; lower, middle, and upper horizontal lines indicate the first quartile, median, and third quartile, respectively; X indicates the mean. **F)** Quantification of the percentage of seed bearing (orange) and seedless (green) fruits from the indicated genotypes; *n* = number of fruits analyzed. **G to J)** Representative photographs of fruits of the indicated genotypes with or without pollination (±*P*, respectively), showing the fertilization-independent fruit set of *Slarf8a Slarf8b (Slarf8ab)*. **H)** The senescent remains of an unpollinated wild-type flower. Individual images were digitally extracted for comparison. Scale bar: 2 cm. **K)** Quantification of the percentage of fruit set in the indicated genotypes and treatments. *Slarf8ab—Slarf8a Slarf8b*. −p, unpollinated; +p, pollinated; *n* = number of flowers analyzed.

stages of fruit development. The underexpressed genes included 2 MADS-BOX genes (Solyc01g092950/SlMADS2 and Solyc01g087990), implicated in the control of fruit set (Joldersma and Liu 2018), the auxin-responsive gene *SlIAA16* (Solyc01g097290), and a pistil-specific extensin-like gene (Solyc02g078100) (Supplemental Fig. S5). Interestingly, several of the genes affected by *Slarf8a Slarf8b*, including *SlGA20ox-1*, Solyc01g087990, and Solyc02g078100, were similarly affected by natural or parthenocarpic fruit set (Tang et al. 2015) (Supplemental Table S1). We validated the effect of *Slarf8a Slarf8b* on the expression of several of the identified genes in 2 stages of gynoecium development, S2 and S3. In most cases, the effect was also apparent in the S3 stage (Supplemental Fig. S5). We also compared the DEG from our data with the DEG obtained from a related published dataset, from *Solanum pimpinellifolium* plants overexpressing *miR167a*, which targets *SpARF6* and *SpARF8* genes (Supplemental Table S1) (Liu et al. 2014). Thirty-eight genes were underexpressed in both *Slarf8a Slarf8b* and *35S:MIR167a* (Supplemental Table S1), including *SlIAA16*. Forty genes were overexpressed in *Slarf8a Slarf8b* and *35S:miR167a* (Supplemental Table S1). These results suggest that class A SlARFs affect several pathways that are central to fruit formation and development, and may help in future

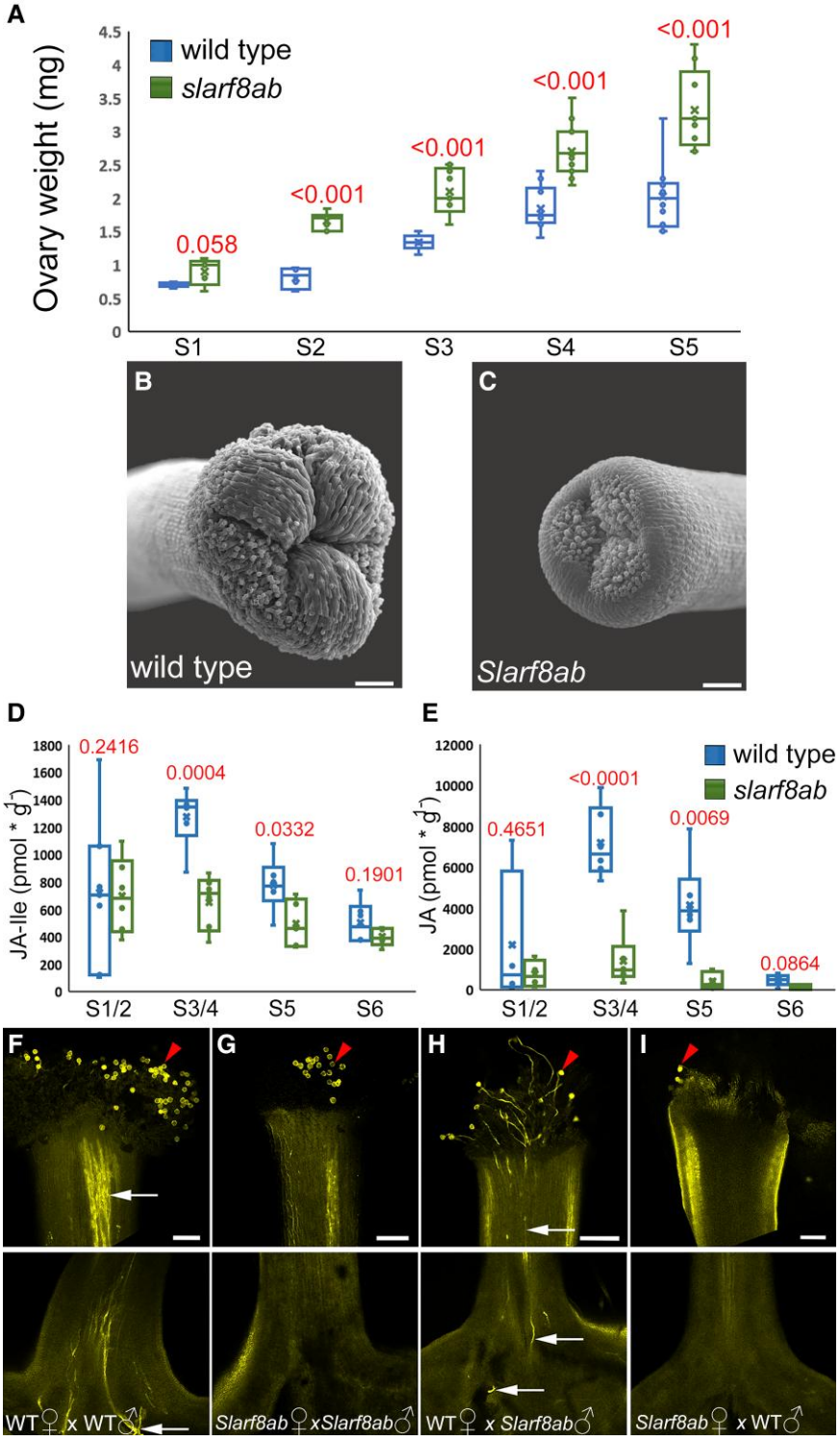

Figure 2. Phenotypes of *Slarf8a Slarf8b*)*Slarf8ab*) flowers. **A)** Ovary weight of the indicated genotypes and stages of ovary development. The flower-bud stages were classified in sequential developmental stages according to bud and gynoecia size, opening of the sepals, color of the petals, and opening of the flowers. Bars represent the SE of at least 3 biological replicates. Statistically significant differences according to the Student *t*-test are indicated. Scanning Electron Microscope image of the stigma from wild type **B)** and *Slarf8a Slarf8b* (*Slarf8ab*, **C)** flowers. Scale bar: 100 μm. **D, E)** Jasmonate levels in developing gynoecia. Gynoecia of the respective stages were extracted, and levels of jasmonic acid (JA) and JA-isoleucine (JA-Ile) were determined. Bars represent the SE of at least 3 biological replicates. Statistically significant differences according to the Student *t*-test are indicated. In **A), D)**, and **E)**, lower and upper whiskers indicate the minimum and maximum values, respectively; lower, middle, and upper horizontal lines indicate the first quartile, median, and third quartile, respectively; X indicates the mean. **F to I)** In vivo pollen tube growth assay. Confocal laser scanning microscope images showing pollen and pollen tubes stained by aniline blue in the gynoecia of the indicated genotypes. Each pollination was repeated with 5 to 6 flowers, all showing the same result. Red arrowheads indicate pollen grains on the stigma, white arrows show staining of callose indicative for pollen tubes within the style. The female parent is listed first in the crosses. Note that pollen tube growth was detectable in WT ovaries only. Scale bar: 100 μm.

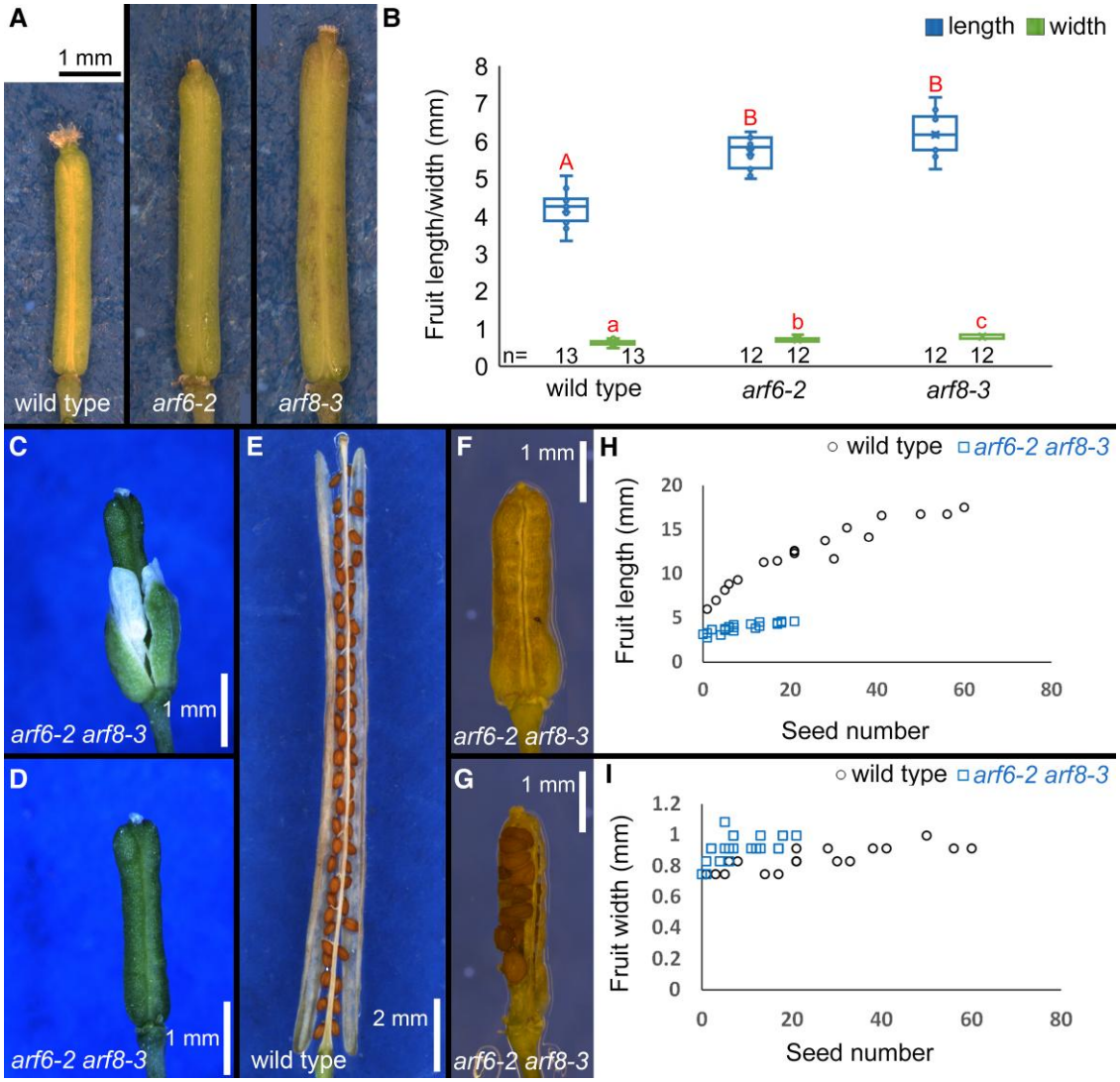

Figure 3. Fruits of Arabidopsis *Atarf6* and *Atarf8* mutant combinations. **A)** Fruit appearance 11 d after emasculation of flowers of indicated genotypes. **B)** Fruit lengths (blue) and widths (green) of the indicated genotypes; *n* = number of plants quantified. Letters above data indicate statistically distinguishable classes by Tukey–Kramer multiple comparison statistical test, $P < 0.05$. Length and width data were analyzed separately, and statistical groups are indicated by uppercase letters for length data and lowercase letters for width data. Supplemental Table S2 shows data from a separate experiment with a larger set of genotypes. Lower and upper whiskers indicate the minimum and maximum values, respectively; lower, middle, and upper horizontal lines indicate the first quartile, median, and third quartile, respectively; X indicates the mean. **C, D)** Unpollinated *arf6-2 arf8-3* fruits without emasculation. The gynoecium was not pollinated because *arf6-2 arf8-3* mutant stamen filaments are short and the anthers are indehiscent. Shown is the same fruit before **C)** and after **D)** manually removing the outer organs of the 14th flower down from the youngest open flower. **E)** Wild-type mature fruit about 2.5 wk after natural self-pollination, opened gently to reveal seeds. **F, G)** *arf6-2 arf8-3* mature fruit 17 d after manual pollination with wild-type pollen. The same fruit is shown before **F)** and after **G)** opening to reveal seeds. Fruit length **H)** or width **I)** versus seed number for wild-type and *arf6-2 arf8-3* mutants. Scale bar: 1 mm **A, C, D, F, G)** or 2 mm **E)**. Additional genotypes and statistical analysis of the data in **H)** and **I)** are presented in Supplemental Fig. S8 and Tables S2, S3.

identification of putative class A SlARF targets that mediate the effect of auxin on fruit set.

SlARF8A and *SlARF8B* are required for jasmonate production and female fertility

To understand why *Slarf8a Slarf8b* plants do not produce seeds, we explored flower function and anatomy. In reciprocal pollinations, *Slarf8a Slarf8b* double mutants produced viable pollen but were female-sterile, similarly to *S. pimpinellifolium p35S:AtMIR167a* plants described previously (Liu et al. 2014). Stereo microscopy and scanning electron microscopy (SEM) revealed that *Slarf8a Slarf8b* mutant styles were shorter than wild-type styles, and that mutant stigmas had very few papillae and were shorter and narrower than wild-type stigmas (Fig. 2, B and C; Supplemental Fig. S6). In vivo pollen-tube germination and growth assays showed that neither wild-type nor *Slarf8a Slarf8b* pollen grains were able to

germinate on the stigma of the *Slarf8a Slarf8b* double mutant (Fig. 2, F to I). In agreement, *Slarf8a Slarf8b* mutants had no seeds, both when allowed to self-pollinate (Fig. 1F) and when pollinated by wild-type pollen. These gynoecium phenotypes resemble those of jasmonate-insensitive tomato mutants (Li et al. 2004; Schubert et al. 2019). Therefore, we measured the levels of JA and its bioactive conjugate JA-Ile in the ovaries of wild type and *Slarf8a Slarf8b* double mutants. In the wild type, JA and JA-Ile levels rose between Stages 1 to 2 and Stages 3 to 4, peaking 3 to 4 d before anthesis (Schubert et al. 2019). The *Slarf8a Slarf8b* double mutants had low JA-Ile and JA levels throughout ovary development (Fig. 2, D and E). Therefore, *SlARF8* genes promote JA production during tomato flower maturation.

AtARF6 and AtARF8 both inhibit and promote Arabidopsis fruit growth

Arabidopsis *Atarf8* mutants also have parthenocarpic fruit growth (Vivian-Smith et al. 2001; Goetz et al. 2006). However, fruits of emasculated *Atarf8* mutant flowers grow substantially less than do fertilized wild-type fruits, suggesting that additional AtARFs might control Arabidopsis fruit growth. Indeed, *AtARF8* acts partially redundantly with *AtARF6* to control hypocotyl and leaf growth, and to promote flower maturation before pollination (Nagpal et al. 2005; Reeves et al. 2012; Reed et al. 2018). We found that after emasculation, both *Atarf6* and *Atarf8* single mutant fruits grew longer and wider than did wild-type fruits (Fig. 3, A and B; Supplemental Table S2). Thus, AtARF6 and AtARF8 each inhibit fruit growth in the absence of seed set, suggesting that they may act redundantly in fruits as they do in other tissues. However, after emasculation, siliques of the *Atarf6 Atarf8* double mutant remained as short as those of wild type, although they did grow wider than wild-type gynoecia (Supplemental Table S2). Thus, the enhanced fruit elongation caused by loss of either AtARF6 or AtARF8 was blocked in the absence of both. Similarly, fruits of emasculated *Atarf6/+ Atarf8* sesquimutant plants were shorter than those of *Atarf8-3* single mutant plants (Supplemental Table S2). This nonlinear gene dosage effect is similar to that seen previously for hypocotyl elongation (Reed et al. 2018). Unfertilized *Atarf6 Atarf8* double mutant gynoecia also remained green and retained their perianth organs (Fig. 3, C and D).

These results suggested that AtARF6 and AtARF8 may promote or inhibit fruit elongation depending on gene dosage and possibly other factors. We therefore examined fruit growth after fertilization in flowers deficient in AtARF6 and/or AtARF8. Although *Atarf6 Atarf8* flowers do not normally form seeds, we obtained partial seed set in some flowers after manual pollination (Crawford and Yanofsky 2011). To silence *AtARF6* and *AtARF8* in a more limited domain, we also drove expression of the *AtMIR167a* miR167 precursor gene behind the *SEEDSTICK* (*STK*) gene promoter, which is expressed in the transmitting tract, ovule funiculi, and seed coat, but not in the style or stigma (Pinyopich et al. 2003; Mizzotti et al. 2014) (Supplemental Fig. S7, A and B). *Atarf6 Atarf8* double mutants and *pSTK:MIR167a* lines formed up to half as many seeds as did wild-type fruits (Fig. 3, E to I; Supplemental Fig. S8, A and C). Incomplete fertilization of *Atarf6 Atarf8* and *pSTK:MIR167a* plants arose from poor pollen tube growth into the lower portion of the gynoecium (Supplemental Fig. S7, C to E). Despite setting seeds, fruits of manually pollinated *Atarf6 Atarf8* and *pSTK:MIR167a* gynoecia were shorter than those of wild-type gynoecia, even when these were pollinated with limiting amounts of pollen to cause partial seed set (Cox and Swain 2006; Ripoll et al. 2019) (Fig. 3, H and I; Supplemental Fig. S8, A, C and Table S3, A, C). Reciprocal crosses between wild type and *pSTK:MIR167a* revealed that the AtARF6/8 promotion of fruit growth after fertilization was controlled maternally (Supplemental Fig. S8D and Table S3D). The *Atarf8-3* single mutation also decreased the (Seed number × Fruit elongation) interaction, indicating a reduced fruit-elongation response to seed number. As also found for emasculated siliques, after fertilization, *Atarf6* and *Atarf8* mutant fruits were each wider than wild-type fruits (Supplemental Fig. S8B and Table S3B).

These results suggest that AtARF6 and AtARF8 inhibit longitudinal and radial fruit growth in the absence of fertilization, but together promote longitudinal growth after fertilization. It is plausible that auxin from developing seeds signals to switch between these activities. In agreement, and consistent with previous studies (Dorcey et al. 2009; Fuentes et al. 2012; Figueiredo et al. 2015; Shinozaki et al. 2020), we found that IAA levels increased in both ovules and whole gynoecia within 24 h after pollination, although only the increase in whole gynoecia was statistically significant (Supplemental Fig. S9). Such an increase in auxin levels upon fertilization could switch AtARF6 and AtARF8 to activate rather than repress genes required for growth.

Overall, these results suggest that AtARF6 and AtARF8 control the switch between repression and activation of fruit elongation following fertilization in Arabidopsis.

Slarf8a and *Slarf8b* mutations increase yield stability under nonoptimal conditions

Extreme temperatures lead to yield loss and reduced fruit set (Charles and Harris 1972; Wahid et al. 2007; Alsamir et al. 2021). The parthenocarpic and seedless phenotype of *Slarf8a Slarf8b* double mutants and the partial seedless phenotypes of single *Slarf8a* and *Slarf8b* mutants (Figs 1 and 2; Supplemental Fig. S3) prompted us to test whether altering SlARF8 activity can increase yield in extreme temperatures. We hypothesized that an intermediate dosage of these paralogous genes might enable at once high-quality fruit and higher yield stability. We therefore grew wild type, *Slarf8a/+Slarf8b/+*, *Slarf8a*, *Slarf8a Slarf8b/+*, and *Slarf8a Slarf8b* in a greenhouse with controlled hot temperatures (with an amplitude of 32 to 38 °C day/28 to 30 °C night) and tested

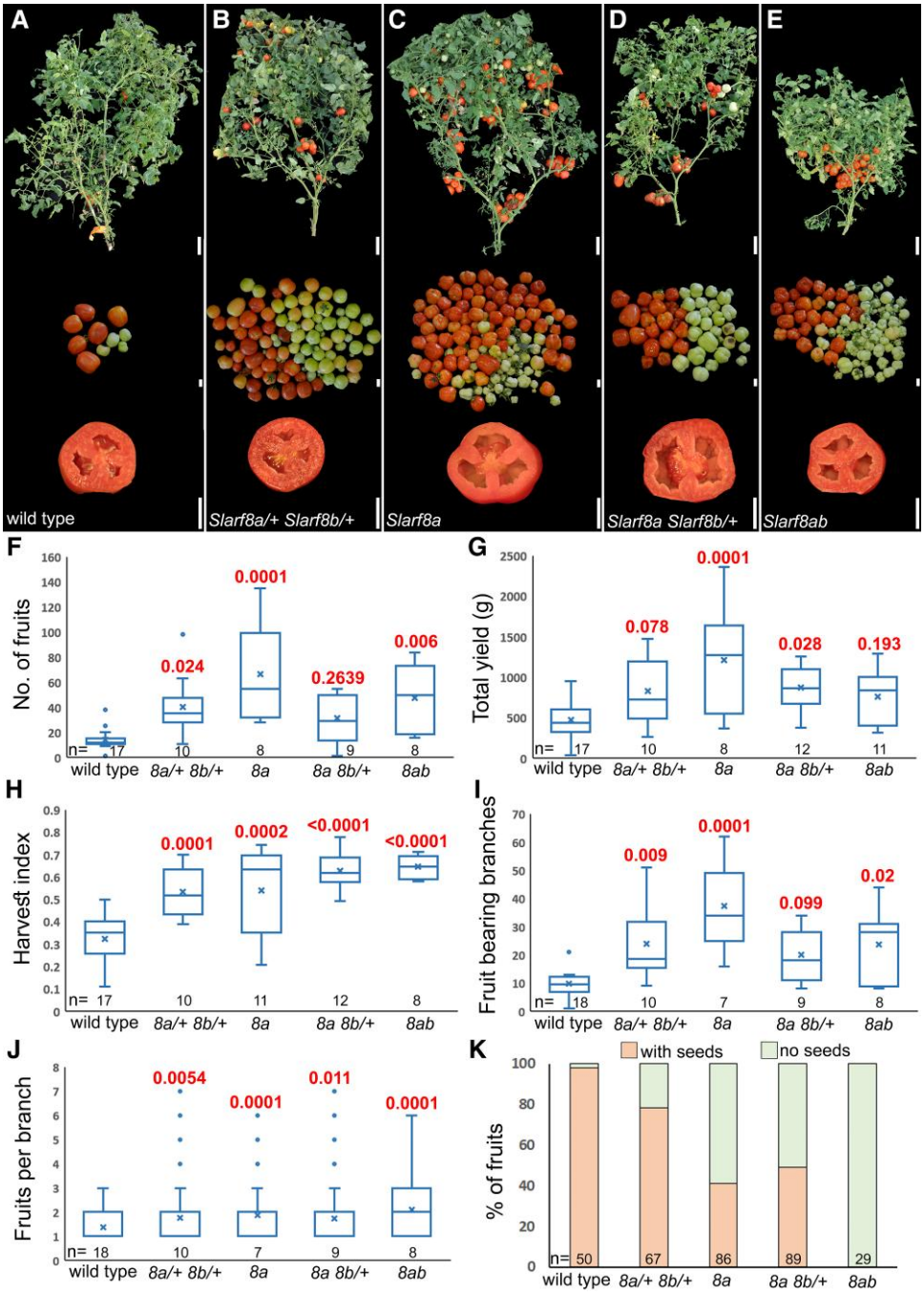

Figure 4. Effect of mutations in *Slarf8* genes on yield of plants grown in controlled hot conditions. Plants were grown in a controlled greenhouse under 34 °C day/28 °C night temperatures. **A to E)** Mature plants at the end of the experiment, fruits of a single representative plant and a representative cut fruit from plants of each of the indicated genotypes. Individual images were digitally extracted for comparison. Scale bar: 10 cm (whole plants), 2 cm (fruits). Quantification of the total number of fruits **F)**, total yield in grams **G)**, harvest index: total yield/plant weight **H)**, number of fruit-bearing branches per plant **I)**, and the number of fruits per fruit-bearing branch **J)** in the indicated genotypes; *n* = number of quantified plants or inflorescences. *P*-values represent differences from the wild type, as determined by the Dunnett test. Lower and upper whiskers indicate the minimum and maximum values, respectively; lower, middle, and upper horizontal lines indicate the first quartile, median, and third quartile, respectively; X indicates the mean. **K)** Quantification of the percentage of natural parthenocarpy of fruits from the indicated genotypes. The orange color represents fruits with seeds and the green color represents parthenocarpic, seedless fruits; *n* = number of fruits analyzed. Genotypes are abbreviated as follows: *8a—Slarf8a; 8a 8b/+—Slarf8a Slarf8b/+; 8ab—Slarf8a Slarf8b*.

their growth and yield performance. The plants were allowed to self-pollinate. Under these conditions, wild-type plants produced a very low number of fruits (Fig. 4, A, F, and G). In contrast, most of the *Slarf8* mutant combinations produced higher fruit number, resulting in higher total yield than the wild type (Fig. 4, A to G). Harvest index, the ratio

of total fruit yield to total plant weight, is an important agronomical trait that indicates the efficiency of fruit production (Kwon et al. 2020). All the *Slarf* mutant combinations had significantly and substantially higher harvest index than the wild type. *Slarf8a Slarf8b* had the highest harvest index, due to the combination of its compact plant habit and high yield (Fig. 4H). While *Slarf8a Slarf8b* fruits did not grow placenta with locular gel, *Slarf8a* and *Slarf8a/+Slarf8b/+* had more locular gel, as compared to the double mutant. Plants with reduced *SlARF8* had slightly smaller fruits (Supplemental Fig. S10) and produced more seedless fruits (Fig. 4K). Therefore, *Slarf8* mutations can increase yield stability under hot conditions by relaxing the control of fruit set. Under our standard, unstressed growth conditions, yield parameters of the different *Slarf8* mutant combinations were similar to those of the wild type, except some combinations that had slightly increased yield (Supplemental Table S4).

Extreme cold temperatures can also decrease yield (Charles and Harris 1972). We therefore tested whether the *Slarf8a* and *Slarf8b* mutant combinations can increase yield also under cold-temperature stress (16 °C day/10 °C night). The plants were allowed to self-pollinate. Most of the *Slarf8a* and *Slarf8b* mutant combinations produced more fruits, which led to a higher yield and higher harvest index than the wild type (Fig. 5, A to G). This indicates that reduced SlARF activity relaxes the control on fruit set also in the cold. In addition to the effect on yield, leaves of *Slarf8a Slarf8b* double mutants remained green and appeared healthy in the cold when wild-type leaves had turned yellow and/or purple, suggesting higher general resistance to the cold (Supplemental Fig. S11). Interestingly, all genotypes including the wild type produced only seedless fruits in the cold (Fig. 5, A to D and J).

The experiments in controlled heat and cold stress conditions suggested that mutations in *SlARF8A* and *SlARF8B* might increase yield stability under extreme temperature stress. We therefore tested the yield performance of the different *Slarf8* mutant combinations under ambient heat stress conditions. An initial field test was performed with several mutant combinations. In this experiment, plants were grown outdoors in the field in Rehovot, Israel between March and August 2019, during which they experienced several heat episodes (Supplemental Table S5). Under these conditions, wild-type plants produced a very low number of fruits, and these fruits were relatively small, while plants carrying mutant alleles of *Slarf8a* and *Slarf8b* had increased fruit number relative to the wild type, resulting in a higher total fruit weight (Supplemental Fig. S12). In agreement with the experiments in controlled conditions, we observed an effect for some heterozygous mutant alleles. We therefore performed a second experiment with several genotypes with a gradually reduced *SlARF8* dose. Plants were grown in a net-house in the soil under field conditions, with no temperature control, in Rehovot, Israel, between May and August 2021, during which they experienced 3 to 5 h of temperature above 40 °C every day for several weeks (Supplemental Table S5). The plants were allowed to self-pollinate. The wild-type plants had a very low number of fruits. In contrast, all of the *Slarf8* mutant combinations had a substantially and significantly higher number of fruits, which resulted in higher total fruit weight and higher harvest index (Fig. 6, A to H; Supplemental Fig. S13). The best-performing genotype was *Slarf8a*, with over 3-fold more fruits and over 4-fold fruit weight relative to the wild type. This suggests that partial reduction of *Slarf8* dose bypasses the effect of temperature on yield. As vegetative growth and fruit appearance are normal in single *Slarf8a* mutants, they can be attractive for breeding purposes.

Changing SlARF8 dose increases yield via several traits

While *Slarf8a Slarf8b* double mutants were parthenocarpic and seedless, other mutant combinations with intermediate SlARF8 dose were not parthenocarpic and were only partly seedless. Therefore, the effect of SlARF8s on fruit set appears to be more complex than just affecting parthenocarpy. We therefore examined the developmental basis of the improved yield of *Slarf8* mutants. Pollen viability assays under normal and controlled heat conditions showed no statistically significant difference between wild type, *Slarf8a* and *Slarf8a Slarf8b* plants (Supplemental Fig. S14). We monitored flowering time, time of initial fruit production, number of fruit-bearing branches, and number of fruits per fruit-bearing branch in the different experiments under controlled or ambient temperature stress. In the second field experiment, *Slarf8a*, *Slarf8a/+ Slarf8b/+*, and *Slarf8a Slarf8b/+* all flowered significantly and substantially earlier than the wild type. Interestingly, *Slarf8a Slarf8b* double mutants flowered at the same time as the wild type, suggesting a complex interaction between SlARF8A and B with respect to flowering time (Fig. 6K). Most of the tested *Slarf8* mutant combinations made substantially and statistically significantly more fruit-bearing branches than the wild type, in all tested temperature-stress conditions, although the extent differed in the different conditions (Figs 4I, 5H, and 6I). Average number of fruits per fruit-bearing branch was significantly higher in all mutant combinations in the controlled heat and cold conditions, but not in the field experiment (Figs 4J, 5I, and 6J). To further understand the basis for more fruits per branch, we counted the number of flowers and the number of fruits on specific inflorescences. While all the tested genotypes produced a similar number of flowers per inflorescence, all tested *Slarf8* mutant combinations produced more fruits per branch in comparison with the wild type (Fig. 7, A and B). This indicates that more flowers set fruit in the *Slarf8* mutants. In the controlled heat and cold conditions, the different *Slarf8* mutants started to produce fruits earlier than the wild type (Fig. 7, C and D; Supplemental Figs S10C and S11C). Therefore, the substantially increased fruit number and fruit weight of the *Slarf8* mutants results from a combination of earlier onset of fruit set, production of more fruit-bearing branches, and more flowers that produce fruit. The relative

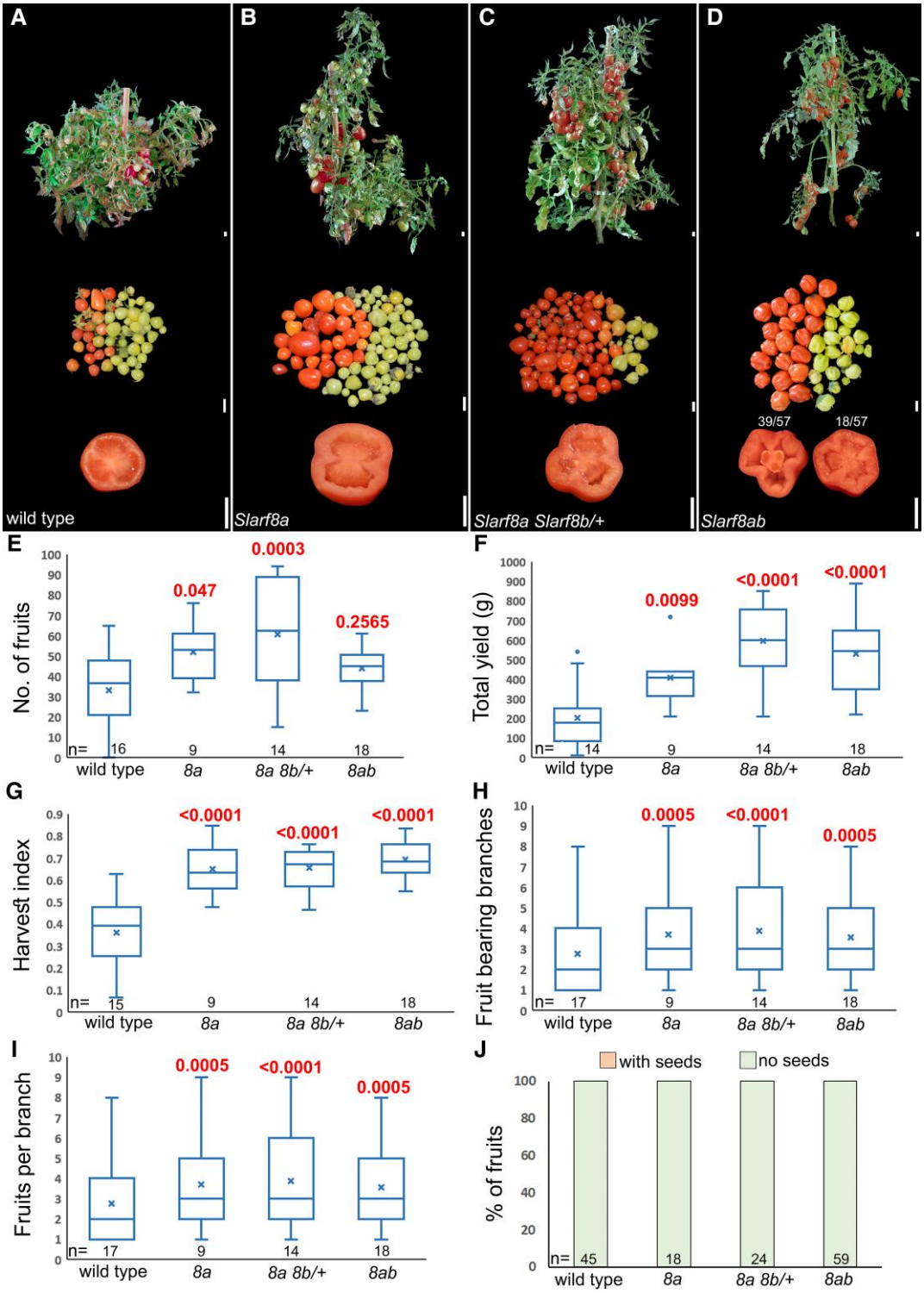

Figure 5. Effect of mutations in *Slarf8* genes on yield of plants grown in controlled cold conditions. Plants were grown in a controlled greenhouse under 16 °C day/10 °C night temperatures. **A to D)** Mature plants at the end of the experiment, fruits of a single representative plant, and a representative cut fruits from the indicated genotypes. Individual images were digitally extracted for comparison. Scale bar: 10 cm (whole plants), 2 cm (fruits). Quantification of the total number of fruits **E)**, total yield in grams **F)**, harvest index: total yield/plant weight **G)**, number of fruit-bearing branches per plant **H)**, and the number of fruits per fruit-bearing branch **I)** in the indicated genotypes; n = number of plants or inflorescences quantified. *P*-values represent differences from the wild type, as determined by the Dunnett test. Lower and upper whiskers indicate the minimum and maximum values, respectively; lower, middle, and upper horizontal lines indicate the first quartile, median, and third quartile, respectively; X indicates the mean. **J)** Quantification of the percentage of natural parthenocarpy in the indicated genotypes. The orange color represents fruits with seeds, and the green color represents parthenocarpic, seedless fruits; n = number of fruits quantified. Genotype abbreviation is as in Fig. 4.

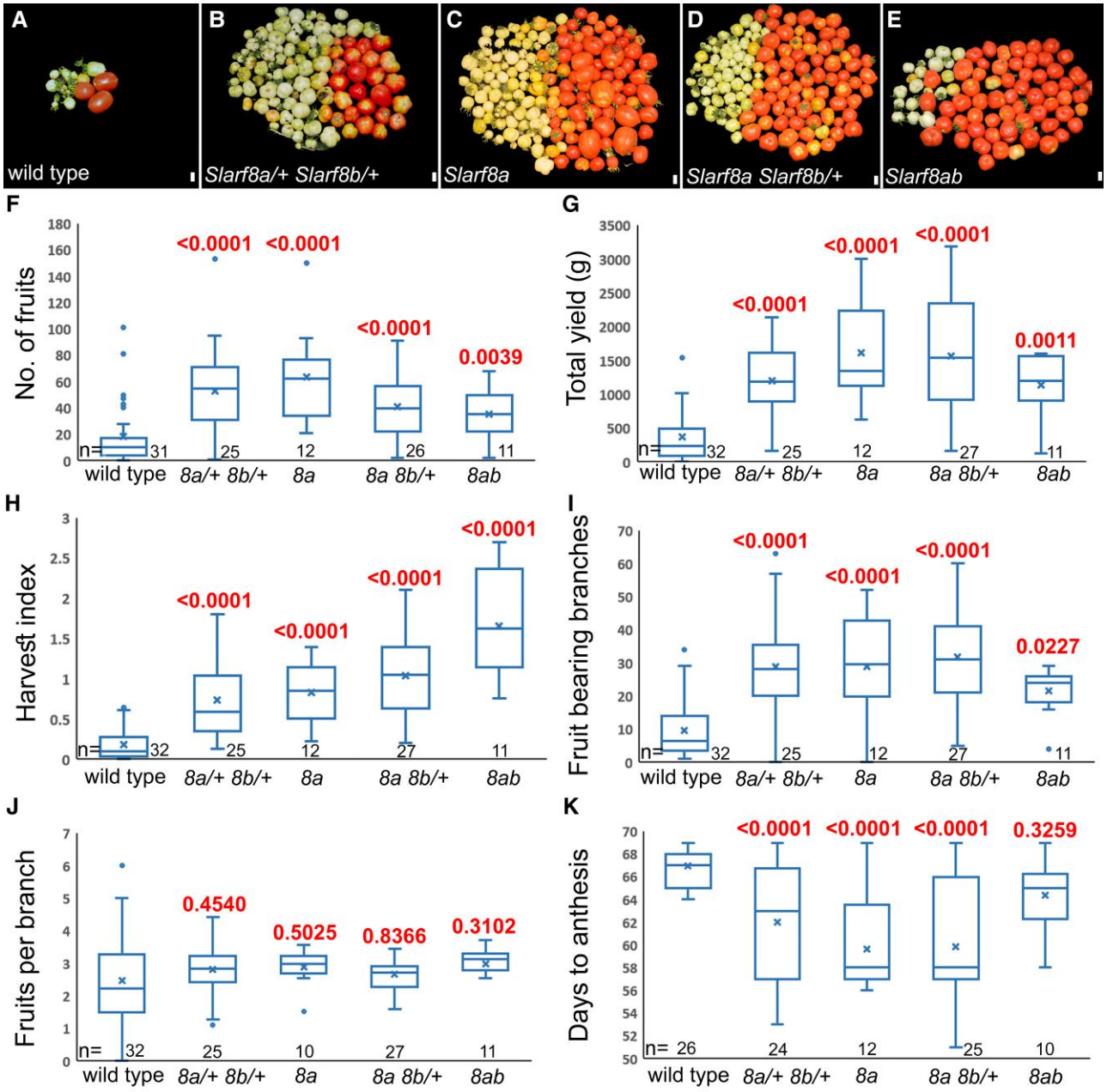

Figure 6. Effect of mutations in *Slarf8* genes on yield of plants grown in ambient heat-stress conditions. Plants were grown in a net-house in the soil in the summer under field conditions, with no temperature control, during which they experienced several hours of temperature above 40 °C every day for several weeks. **A to E)** Fruits of a single representative plant of the indicated genotypes. Individual images were digitally extracted for comparison. Scale bar: 2 cm. Quantification of the total number of fruits **F)**, total yield in grams **G)**, harvest index: total yield/plant weight **H)**, number of fruit-bearing branches per plant **I)**, number of fruits per fruit-bearing branch **J)**, and days to anthesis of the first flower **K)** in the indicated genotypes; *n* = number of plants or inflorescences quantified. *P*-values represent differences from the wild type, as determined by the Dunnett test. Lower and upper whiskers indicate the minimum and maximum values, respectively; lower, middle, and upper horizontal lines indicate the first quartile, median, and third quartile, respectively; X indicates the mean. Genotype abbreviation is as in Fig. 4.

contribution of each component differs depending on the *Slarf8* dosage and the external conditions.

Discussion

Our results show that the Class A ARFs from the ARF6/8 clade are important regulators of fruit formation and development in both tomato and Arabidopsis. In tomato, absence of *SlARF8* activity causes formation of parthenocarpic seedless fruits. Absence of either AtARF6 or AtARF8 in Arabidopsis similarly increases fruit growth before fertilization, and the 2 together promote fruit elongation after seed set. Reducing SlARF8 activity results in reduced sensitivity of tomato fruit set to environmental conditions, leading

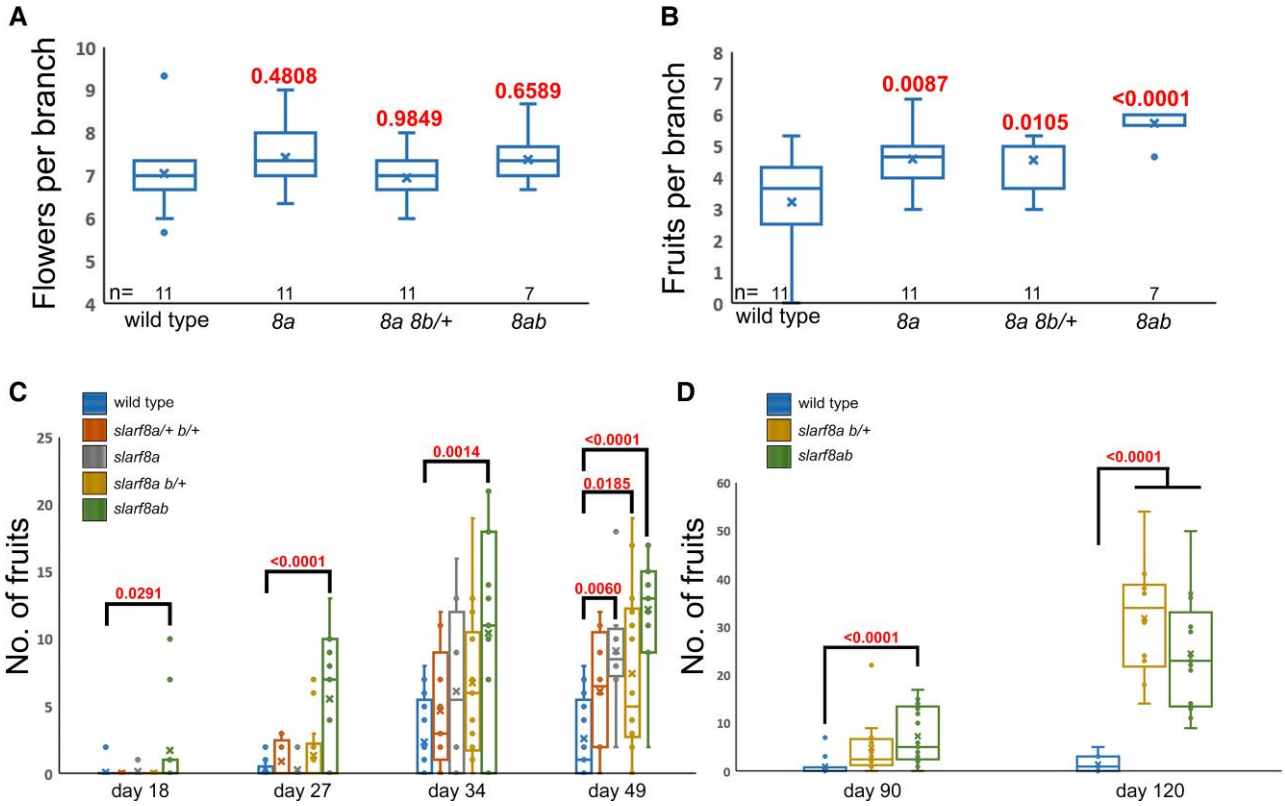

Figure 7. Increased and earlier fruit set in *Slarf8* mutants. Number of flowers per inflorescence **A)** and number of fruits on the same inflorescences **B)** of the indicated genotypes; n = number of inflorescences quantified. *P*-values represent differences from the wild type, as determined by the Dunnett test. Quantification of the total number of fruits per plant in the indicated genotypes and time points under heat conditions in the controlled heat **C)** and controlled cold **D)** experiments. Number of plants for each genotype (n = **C**, **D**): wild type: 17, 7 to 14; *Slarf8a*/+ *Slarf8b*/+: 9; *Slarf8a*: 8, *Slarf8a 8b*/+: 14, 4 to 12; and *Slarf8ab*: 11, 6 to 19. *P*-values indicate differences from the wild type, as determined by the Dunnett test. Lower and upper whiskers indicate the minimum and maximum values, respectively; lower, middle, and upper horizontal lines indicate the first quartile, median, and third quartile, respectively; X indicates the mean.

to increased yield stability. Interestingly, partial reduction of *SlARF8* activity leads to a substantial yield increase in extreme temperatures with minimal pleiotropic effect.

ARF proteins can repress or promote fruit growth

Various combinations of loss of tomato *SlARF8A* or *SlARF8B* caused partial fruit growth without fertilization. A parallel study reached similar conclusions and support the model that a switch in auxin response underlies fruit set (Hu et al., in press). Similarly, the *Sliaa9/e* mutant, in which class A SlARF gene activation activity should be increased (Israeli et al. 2019), has excess fruit growth (Wang et al. 2005, 2009; Hu et al. 2018). These results suggest that ARF-Aux/IAA complexes may repress fruit growth in the absence of fertilization. Similarly, in eggplant both downregulation and upregulation of *SmARF8* led to the formation of parthenocarpic fruits, and in strawberry loss of *FvARF8* increased fruit growth (Du et al. 2016; Zhou et al. 2020). Conversely, in Arabidopsis, AtARF6 and AtARF8 were required for normal fruit elongation even after fertilization and seed formation. Although we have not obtained tomato plants lacking the entire clade of *SlARF6A*, *SlARF8A*, and *SlARF8B*, *S. pimpinellifolium p35S:MIR167a* plants with silencing of these 3 genes had small ovaries that did not grow parthenocarpically (Liu et al. 2014). Promotion of fruit growth by Class A ARFs is in agreement with the *Sliaa9/e* phenotype. Thus, if ARFs regulate genes that promote growth, as is generally found in most shoot tissues, then the results suggest that in wild-type plants Class A ARFs are switched between gene repression and gene activation states. In several plants, including Arabidopsis, strawberry and tomato, fertilization triggers an increase in auxin production in ovules (Mapelli et al. 1978; Dorcey et al. 2009; Fuentes et al. 2012; Figueiredo et al. 2015; Figueroa and Browse 2015; Liao et al. 2018) (Supplemental Fig. S9), and may then trigger fruit set by switching Class A ARFs from repressors to activators of gene expression and growth. Thus, the *arf8* mutations relieve repression of fruit set, thereby bypassing normal regulation by fertilization-induced auxin production.

It is likely that the ARFs regulate fruit growth largely cell-autonomously in response to auxin movement from ovules. *SlARF8A* and *SlARF8B* are expressed in placenta, septum, and pericarp tissues that need to grow substantially as fruits form. *AtARF6* and *AtARF8* are also normally expressed

in the septum and placenta and probably act in this tissue to control Arabidopsis fruit growth or differentiation (Wu et al. 2006; Crawford and Yanofsky 2011). That the valves of unpollinated *Atarf6 Atarf8* fruits are wider than those of unpollinated wild-type fruits suggests that in the double mutant, growth of valves may be released from growth inhibition but still be constrained from elongation by inner tissues. This suggests that growth in different tissues of the fruit may be regulated distinctly. For example, AtARF6 and AtARF8 are needed for septum breakdown (Crawford and Yanofsky 2011), and failure of this process might leave residual extra tissue that may limit elongation but not affect thickening.

It is uncertain how removing an ARF-Aux/IAA repressor complex by mutating particular *ARF* genes would increase activation of growth-promoting genes. Similarly, in several other cases, mutations in Class A *ARF* genes increased rather than decreased molecular auxin response. For example, partially reduced activity of several ARFs led to increased auxin response in Arabidopsis roots (Vanneste et al. 2005), tomato *Slarf5*/*Slmp* mutants had elevated DR5 auxin reporter activity but reduced leaf blade growth (Israeli et al. 2019), and partial loss of ARF activity in Arabidopsis led to increased hypocotyl elongation (Reed et al. 2018). One possible explanation is that in *arf* mutants other transcription factors can access the promoters of growth-promoting genes to activate them. These could be other ARFs with less repressor activity, or proteins of other classes such as PIFs or BZR/BES proteins that can also promote growth (Oh et al. 2014). Binding by alternative regulators under different light conditions has been shown to occur at promoters of some Arabidopsis *SAUR* growth-promoting genes (Dong et al. 2019). Another possibility is that mutating *ARF* genes reduces negative feedbacks on Aux/IAA repressor protein level, such that the equilibrium or timing of the expression of growth genes is changed. Further work will be needed to explore these dynamics, and how additional ARFs contribute to fruit set and fruit growth (Schruff et al. 2004; Ellis et al. 2005; Hao et al. 2015; Breitel et al. 2016).

Effects on flower maturation

ARF6 and ARF8 are angiosperm-specific ARFs, and previous and present results indicate that they play important roles in flower development, maturation and fertility in both Arabidopsis and tomato (Nagpal et al. 2005; Reeves et al. 2012; Finet et al. 2013; Liu et al. 2014; Mutte et al. 2018). Arabidopsis mutants lacking both AtARF6 and AtARF8 are both male- and female-sterile, whereas the corresponding tomato mutants are so far only female-sterile, with the caveat that an *Slarf6a Slarf8a Slarf8b* triple mutant is not yet available. This may be explained in part by the differing roles of JA in the 2 species. In Arabidopsis, JAs are required for stamen filament elongation, anther dehiscence and pollen viability, whereas in tomato they are rather required for female but not male fertility (Li et al. 2004; Browse and Wallis 2019). In Arabidopsis and as suggested here also in tomato, ARF6/8 ARFs promote a burst of expression of JA biosynthesis genes just before flowers open (Reeves et al. 2012). In Arabidopsis flowers, JA might also amplify auxin response in a reinforcing feedback between the 2 hormone signaling pathways, before being turned off after flowers open (Reeves et al. 2012). In tomato, JA rises also before flower opening and a positive feedback pathway involving JA-induced MYB21 family transcription factors amplifies JA production (Schubert et al. 2019). Deficiency in JA signaling results in defects of ovule development and in the formation of seedless fruits, which is accompanied by upregulation of genes encoding other ARFs and AUX/IAA proteins during flower development, suggesting that JA represses auxin response (Schubert et al. 2019). Further work may reveal the extent to which the regulatory circuitries underlying these feedbacks are conserved between Arabidopsis and tomato.

Altering *SlARF* gene dosage to improve fruit-set stability

Our findings suggest that a possible approach to increase yield stability under unstable climate may be to breed plants that bypass the effects of variable weather to produce fruits under all conditions, analogous to breeding for day-neutral flowering time for different latitudes. While further investigation in an array of varieties and large-scale field experiments is required before such mutants can be widely used for breeding, the current research provides a promising starting point for such experiments. In particular, partially reducing the activity of a subset of Class A ARFs increases stable fruit set in an array of different environments. Thus, each genetic combination of *Slarf8a* and *Slarf8b* alleles (0 to 3 functional alleles) has a slightly different effect on flexibility and stability of fruit set. Moreover, in addition to their effects on fruit set, *SlARF8A* and *SlARF8B* genes also have dose-dependent effects on plant stature and on leaf size and shape. We suggest that different *Slarf* mutant combinations can be used to optimize the resource allocation to vegetative and reproductive tissues and may thus contribute to both yield stability and desirable vegetative traits. For example, dwarf stature could be particularly valuable in urban varieties (Kwon et al. 2020), and compact and determinant plants are suitable for processing tomato (Park et al. 2014).

Whereas *Slarf8a Slarf8b* mutant plants produced fruit after emasculation and are therefore truly parthenocarpic, genotypes with a partial reduction in *SlARF8* gene dosage, such as *Slarf8a*, *Slarf8a*/+ *Slarf8b*/+, and *Slarf8a Slarf8b*/+, produced only some seedless fruits and did not produce fruits after emasculation. Nevertheless, fruit set in these genotypes was equally or more robust in extreme temperatures compared to the fully parthenocarpic genotypes. It is therefore possible that in these genotypes the robust fruit set is only partially related to the control of fertilization-dependent fruit set and reflects an additional role of SlARF8A and SlARF8B in the developmental responses to temperature. Indeed, we observed early flowering, more fruit-bearing

branches and more flowers setting fruits in some of the SlARF8-deficient genotypes, and these changes may contribute to overall fruit production. Each of these traits may be less sensitive to extreme temperatures in the mutants.

Materials and methods

Plant material and growth conditions (tomato)

Tomato [*S. lycopersicum* cv M82 (LA3475)] plants were used throughout the study. Seeds were germinated and seedlings grown in a growth room or a growth chamber for 2 to 4 wk. The seedlings were then transferred to a greenhouse with a natural daylight and temperature or controlled temperature in the hot and cold experiments. For field trails, the seedlings were grown in a commercial nursery and planted in the field 30 d after seeding. Unless stated otherwise, plants were allowed to self-pollinate.

The following plant materials were described before: *Slarf5/Slmp*, *Slarf7*, *Slarf19a*, and *Slarf19b* (Israeli et al. 2019), and *Sliaa9/e* (Berger et al. 2009). The *Slarf8a* and *Slarf8b* mutants were generated during this study as described below.

Plant material and growth conditions (Arabidopsis)

All *Arabidopsis thaliana* plants used were in the Columbia ecotype. *Atarf6-2* and *Atarf8-3* insertion mutation alleles in *AtARF6* (At1g30330) and *AtARF8* (At5g37020) were previously described (Nagpal et al. 2005), and were detected by PCR using a T-DNA left border primer (JMLB) and gene-specific primers (ARF6-R7 or ARF8-3081R, Supplemental Table S6), or with primers flanking the insertion mutations to detect the wild-type alleles. Seeds were surface sterilized with 95% ethanol followed by bleach solution (2:1 H_2O: bleach with 2 or 3 drops of Tween-20), plated on Murashige and Skoog salts (Murashige and Skoog 1962) containing 1% (w/v) sucrose and 0.6% (w/v) Phyto-agar (pH 5.7), cold stratified for 1 to 3 d at 4 °C, and then grown at 22 °C under a 16-h-light/8-h-dark photoperiod. For flower and fruit assays, plants were transplanted to a soil mixture of peat:vermiculite (1:1) and grown under long day conditions.

Constructs for transgenic Arabidopsis plants

To drive *GUS* or *MIR167a* behind the Arabidopsis *SEEDSTICK* promoter (*pSTK*), the 1.85-kb *pSTK* promoter was PCR-amplified from wild-type genomic DNA using the primers pSTK-F and pSTK-R (Supplemental Table S6), and cloned into pENTR/D-TOPO vector (Invitrogen) to obtain pENTR-*pSTK*. *MIR167a* was amplified from genomic DNA using primers miR167aF1-Spe and miR167aR2-Sac (Supplemental Table S6) and cloned between *SpeI* and *SacI* sites of pKGW (Karimi et al. 2002) to obtain pKGW-*MIR167a*. pENTR-*pSTK* was recombined by LR clonase (Invitrogen) into pBGWFS7 (Karimi et al. 2002) to obtain *pSTK:GUS*, and into pKGW-*MIR167a* to obtain *pSTK:MIR167a*. Constructs were introduced into Agrobacterium strain GV3101 by electroporation and transformed into plants by the floral dip method (Clough and Bent 1998).

CRISPR/Cas9 construct design and generation of mutant tomato plants

Constructs were designed to generate defined deletions within the coding sequence of each target gene, using 2 sgRNAs alongside the Cas9 endonuclease gene. The primers for plasmid construction are listed in Supplemental Table S6. sgRNAs were designed using the CRISPRp server (http://cbi.hzau.edu.cn/crispr). The constructs were assembled as detailed in Xie et al. (2015). Briefly, the sgRNAs were divided into 2 parts. Each part was amplified using a sgRNA spacer primer and terminal specific primers containing a FokI site. After FokI digestion, the fragment was inserted into the BsaI digested modified pUC57-cloning vector containing a U6 promoter and subsequently subcloned into the binary vector pMR286. *SlARF8A* and *SlARF8B* were targeted by a single construct containing 2 sgRNAs targeting either *SlARF8A* or *SlARF8B*. Several positive T0 plants harboring the Cas9 cassette were recovered and mutant alleles identified in the T1 progeny of these plants (Supplemental Fig. S2). For *Slarf8a*, 2 alleles were obtained, containing a (i) 5-bp deletion 222 bp after the ATG, causing a stop codon after 86 amino acids (AA) (instead of 844 AA, *Slarf8a-1*) and (ii) 1-bp insertion 224 bp after the ATG, causing a stop codon after 88 AA. The *Slarf8a-1* allele was used for further experiments. For *Slarf8b*, several independent T0 plants were recovered harboring an identical deletion between the 2 guides, starting 100 bp after the ATG start codon and causing a stop codon after 46 AA (instead of 842 AA) (see Supplemental Fig. S2).

Controlled pollinations and fruit growth assays (Arabidopsis)

For tracking ovule fertilization, stigmas of late stage 12 flowers (mature but not yet self-pollinated) were dusted with pollen from *pLAT52:GUS* plants (Johnson et al. 2004). At 24 h after pollination, carpel valve walls were removed and gynoecia were stained with X-gluc overnight at 37 °C (Wu et al. 2006). Tissues were cleared in a 70%, 80%, and 95% ethanol series, mounted in chlorohydrate:water (8:3) and viewed on a Nikon E800 microscope (model E800, Nikon, Tokyo, Japan) using differential interference contrast optics and photographed with a Spot cooled color digital camera (Spot v2.1 software, Diagnostic Instruments, Sterling Heights, MI, USA).

To measure parthenocarpic fruit growth, open flowers were removed from primary inflorescence apices of adult plants. Sepals, petals, and stamens were removed from 2 to 4 stage 12 mature flower buds on each apex, and plants were grown for an additional 10 to 12 d before measuring silique length and width. Images of flowers or siliques were obtained using a stereomicroscope (Wild Type 308700, Switzerland), equipped with a Leica DFC420 camera (Leica,

Switzerland). Siliques were either measured directly using a camera lucida attachment on the dissecting microscope, or images were exported from Leica Application Suite 2.8.1 software to ImageJ (Java-based image processing program, National Institutes of Health, USA) for measurements. For limited pollination assays, gynoecia emasculated as above were dusted with small amounts of wild-type pollen and then measured and photographed after fruit growth 10 to 20 d later.

Statistical analyses of Arabidopsis fruit growth

We investigated differences among genotypes in the effect of seed number on fruit length or width by conducting linear regression using the *lm* function from the *stats* package in the statistical software R, version 4.2.0 (R Core Team 2022, https://www.R-project.org/). We fit quadratic curves to each genotype so that the intercept and slope were allowed to vary between genotypes, but the quadratic term was held constant. We modeled fruit length as a function of seed number, seed number squared, genotype, and the interaction between seed number and genotype. For the comparisons among genotypes shown in Supplemental Table S3, A, C and Fig. S8, A, C, respectively, this full model fit significantly better than models that excluded the seed number squared term. The resulting regression model gives an excellent fit to the data and explains 96% of the variance in fruit length for the data in Supplemental Fig. S8A. For the analyses of fruit width data (Supplemental Fig. S8B and Table S3B) and maternal and paternal effects (Supplemental Table S3D and Fig. S8D), we used a reduced model that included only effects of seed number, seed number squared, and genotype. In this case, adding an interaction between seed number and genotype failed to improve the fit to the data.

Tomato transformation and tissue culture

M82 seeds were used to generate transgenic tomato plants according to McCormick (1991) and as described in detail (Israeli et al. 2019). Seeds were sterilized and germinated on Nitsch medium for 7 to 10 d, until seedlings formed cotyledons. Cotyledons were dissected and incubated for 24 to 48 h. The cotyledons were then subcultured with 0.35 to 0.4 O.D. diluted agrobacterium GV3101 containing the transformation construct. The cotyledons were incubated for additional 48 h and then moved to J1 culture media for 1 to 2 wk. Appearing calli or shoots were transferred to J2 culture media for further shoot organogenesis. The culture media was replaced every 2 wk until small plants formed. Plants were removed from the cotyledons and transferred into J3 culture media for further growth. After establishing a vital meristem, plants were transformed to a rooting medium, and following rooting plants were transplanted to soil for further analysis and crosses.

Scanning electron microscopy

For SEM, tissues were fixed in 30% ethanol under vacuum for 10 min, followed by dehydration in an increasing ethanol series up to 100% ethanol. Fixed tissues were critical-point dried, mounted on a copper plate, and coated with gold using a Polaron Gold Sputter Coating unit. Samples were viewed using a JEOL JSM-IT-100 LV microscope. The images were taken with an accelerating voltage of 10 to 20 kV at high vacuum mode and secondary electron image. Images were adjusted uniformly using Adobe Photoshop CS6.

Pollen-tube growth and pollen viability assays

Pollen-tube growth assays were performed according to Muschietti et al. (1994), Higashiyama et al. (1998), and Kikuchi et al. (2007). Briefly, gynoecia were harvested 24 h after hand pollination, cut longitudinally in half, and immediately transferred into an ethanol:acetic acid (6:1) solution. After incubation at 4 °C overnight, samples were washed several times in absolute ethanol followed by 2 washing steps with 70% (v/v) ethanol and water for 30 min each. Gynoecia were cleared by incubation in 8 M NaOH for 30 to 45 min and then washed several times in distilled water to completely remove NaOH. The samples were then stained in a 0.005% (w/v) aniline blue solution (pH 9 to 10) overnight. Gynoecia were placed on a microscope slide, slightly squeezed, and imaged using a LSM780 (Zeiss GmbH, Germany) and 405 excitation wavelength. Fluorescence was recorded at 454 to 576 nm.

For pollen-viability assays, flower buds were collected from plants grown under either normal (28 °C/22 °C, day/night) or heat (34 °C/28 °C, day/night) conditions, 2 d after anthesis. Anthers were dissected to release pollen grains, which were then fixed on a microscope slide using 10 μL Alexander staining solution with minor adaptations as described (Peterson et al. 2010). Viable pollen grains stained magenta-red and nonviable grains stained blue-green. At least 6 images (10× objective) were taken from each slide under a light microscope (DM500, Leica), equipped with a digital camera (ICC50 W, Leica). Pollen grains were counted using the multipoint tool from ImageJ software (Schneider et al. 2012).

IAA quantification

For IAA quantification, wild-type stage 12 mature flowers were pollinated or emasculated (unpollinated) and then at various time points dissected and flash frozen in liquid nitrogen. Three to 5 replicates were used. Silique samples had 15 to 80 pooled siliques weighing between 12 and 36 mg; and ovule samples had ovules pooled from 25 siliques estimated to weigh 2.5 mg. Five hundred picograms of $^{13}C_6$-IAA internal standard (Cambridge Isotope Laboratories, Andover, MA, USA) were added to each sample, and samples were extracted and purified as described in Andersen et al. (2008). IAA was then quantified by combined gas chromatography-selected reaction monitoring–mass spectrometry (MS) as described in Edlund et al. (1995).

Determination of JA and JA-Ile

JA and JA-Ile were quantified using 10 to 50 mg of homogenized gynoecia per sample as described (Schubert et al. 2019).

Briefly, gynoecia were collected in the greenhouse at the different developmental stages and flash frozen in liquid nitrogen. Extraction was performed with methanol supplied with [$^2H_6$] JA and [$^2H_2$] JA-Ile (50 ng each) as internal standards. After solid phase extraction on HR-XC column (Chromabond, Macherey-Nagel), eluates were subjected to ultra-performance liquid chromatography–tandem MS according to Balcke et al. (2012). The contents of JA and JA-Ile were calculated using the ratio of analyte and internal standard peak heights.

Heat and cold experiments

Plants were grown in pots in a growth chamber under normal temperature for 3 to 4 wk, before the first flowers/inflorescence were fully developed, and then transferred to a greenhouse with controlled conditions. For controlled heat-stress experiments, plants were grown under controlled heat conditions, with an amplitude of 32 to 38 °C day/28 to 30 °C night. For cold stress, plants were grown under 16 °C day/10 °C night. Plants were kept under these conditions until harvest, which took place 120 d after germination in the heat and 150 d after germination in the cold, when the wild-type plants ceased making fruits and were dying. For the (first) outdoor field experiment, plants were planted in March, and experienced several heat waves on the warmer days of the Israeli summer during the time of flowering and fruit production (specific temperatures in Supplemental Table S5). Under these conditions, fruit production was severely affected in wild-type plants. The plants were harvested during August. In the second field experiment, 4-wk-old seedlings were transplanted to a brown-red degrading sandy loam soil under a high ceiled insect-proof net-house (50-mesh) in a randomized block design. The plots were drip-irrigated up to a field capacity and covered with a black mat to control weeds. Plants were grown during June to August 2021, and experienced multiple episodes of extreme heat along all growth stages (Supplemental Table S5). Under these conditions, fruit production was severely affected in wild-type plants. Plants were harvested 110 d after germination, when the first fruits began to decompose. For yield-related trait measurements, fruits were harvested and weighed individually from each plant. The number of fruit-bearing inflorescences and the number of fruits in each inflorescence were recorded. Red and green fruits were counted separately, where fruits at the breaker stage were counted in the red category. Yield index was calculated as the ratio of total fruit weight to plant vegetative weight.

RNA extraction and RNAseq analysis (tomato)

RNA was extracted using the Plant/Fungi Total RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions including DNase treatment. To compare gene expression between wild type, *Slarf8a Slarf8b* and *Slarf19a Slarf19b* using RNAseq, gynoecia were collected from flowers 5 d before anthesis and total RNA was extracted. Two biological replicates were used. Sequencing libraries were prepared according to the Illumina TruSeq RNA protocol and sequenced on an Illumina HiSeq2000 platform.

Accession numbers

Sequence data used in this study can be found in the Sol Genomic Network under the following accession numbers: SlARF5/SlMP—Solyc04g081240; SlARF6A—Solyc00g196060/Solyc12g006340; SlARF7—Solyc07g042260; SlARF8A—Solyc03g031970; SlARF8B—Solyc02g037530; SlARF19A—Solyc07g016180; SlARF19B—Solyc05g047460; ENTIRE/SlIAA9—Solyc04g076850; Pistil-specific extensin-like protein—Solyc02g078100; SlGA20ox1—Solyc03g006880; SlCKX2—Solyc10g017990; SlIAA16—Solyc01g097290; MADS-box transcription factor—Solyc01g087990; SlMADS2—Solyc01g092950; AtARF6—At1g30330; AtARF7—At5g20730; AtARF8—At5g37020. STK—At4g09960.

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Author contributions

A.I., B.H., J.W.R., and N.O. conceived and designed the research project. A.I., N.M., R.S., and N.T. preformed the experiments in tomato. J.C.S.Y., M.-F.W., E.E.R., and J.W.R. performed the experiments in Arabidopsis. R.S. and B.H. performed the tomato pollen tube growth assay. K.L. and B.H. measured the IAA levels in Arabidopsis and jasmonate levels in tomato, respectively. M.L. analyzed the RNAseq data in tomato. A.I., B.H., J.W.R., I.E., and N.O. analyzed the data and wrote the manuscript with comments from all authors.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Spatial expression patterns of tomato class A *SlARF* genes in the ovary.

Supplemental Figure S2. CRISPR/Cas9-generated alleles of *SlARF8A* and *SlARF8B*.

Supplemental Figure S3. Fruit phenotypes of single, double, and triple *Slarf* mutants.

Supplemental Figure S4. *SlARF8A* and *SlARF8B* promote plant and leaf growth partially redundantly.

Supplemental Figure S5. Effect of *Slarf8ab* on the expression of selected genes.

Supplemental Figure S6. Ovaries of wild type and *Slarf8a Slarf8b* double mutants.

Supplemental Figure S7. *pSTK:GUS* expression pattern and effects of reduced *AtARF6* and AtARF8 activity on pollination.

Supplemental Figure S8. Effects of seed number on fruit growth after fertilization.

Supplemental Figure S9. IAA levels in Arabidopsis ovules and gynoecia.

Supplemental Figure S10. Plant height, fruit size, and timing of fruit production in *Slarf8a and Slarf8b* mutant combinations under heat stress conditions.

Supplemental Figure S11. Plant height, fruit size, and timing of fruit production in *Slarf8a* and *Slarf8b* mutant combinations under cold stress conditions.

Supplemental Figure S12. Effect of mutations in *Slarf8* genes on yield of plants grown under ambient heat-stress conditions.

Supplemental Figure S13. Effect of *Slarf8* mutations on the number and weight of red and green fruits under ambient heat stress.

Supplemental Figure S14. Pollen viability of *Slarf8* mutants.

Supplemental Table S1. Tomato RNAseq data.

Supplemental Table S2. Arabidopsis fruit lengths and widths 10 d after emasculation.

Supplemental Table S3. Statistical analysis of Arabidopsis fruit growth.

Supplemental Table S4. Yield parameters of tomato *Slarf8* mutants in nonstressed conditions.

Supplemental Table S5. Temperature and humidity data for the field experiments.

Supplemental Table S6. List of primers.

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Conflict of interest statement. NO and AI are inventors in a Provisional patent application No. 63/267,407, INCREASING YIELD STABILITY IN PLANTS that includes data described in this paper.

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