# Drought stress-induced irregularities in male organ development cause stage-specific morpho-physiological and transcriptome changes in tomato

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**Abstract**

Drought limits the growth and productivity of plants. Reproductive development is sensitive to drought but the underlying physiological and molecular mechanisms remain unclear in tomato. Here, we investigated drought effect on tomato floral development using morpho-physiological and transcriptome analyses. Drought induced bud and flower abortions, and reduced fruit set/yield, triggered by male sterility due to abnormal anther and pollen development. Under drought stress (DS), anthers at pollen mother cell to meiotic (PMC-MEI) stage survived while anthers at tetrad to uninucleate microspore (TED-VUM) stage aborted. PMC-MEI stage had lower ABA increase, reduced IAA and higher sugar contents under DS relative to well-watered. However, TED-VUM stage had higher ABA increase, higher IAA level and no accumulation of soluble sugars, indicating abnormal carbohydrate and hormone metabolisms. Moreover, RNA-Seq analysis identified altogether ¿15,000 differentially expressed genes that were assigned to multiple pathways, suggesting tomato anthers utilize complicated mechanisms to cope with drought. Major genes involved in tapetum/microspore development and ABA homeostasis were drought-induced while those involved in sugar utilization and IAA metabolism were repressed at PMC-MEI stage. Our results suggest crosstalks between phytohormones and carbohydrate metabolism at different anther stages under DS and provide novel insight into molecular mechanisms of drought tolerance in tomato.

## 1. INTRODUCTION

Water deficit is one of the most important abiotic stress restricting plant production. As a consequence of climate change, drought events are projected to increase in intensity, duration, and frequency (Lesk et al., 2016). Thus, the improvement of drought-resistance of crops represents an urgent need that demands the identification of key regulators and pathways as potential targets for drought-resistance improvement. Reproductive stage drought stress causes more severe damage and yield loss than any other stage of development of crop plants (Dorion et al., 1996; Sheoran and Saini, 1996). Therefore, an effective way to improve drought-resistance of crops is to select for yield and its components during reproductive development under drought stress. Reduction in grain yield due to drought has mainly been attributed to male sterility because the male organ is more drought sensitive than the female organ which remains fertile under stress condition that causes sterility in the male (Bingham, 1966; Saini and Aspinall, 1981). Hence, the male development under drought has attracted greater research attention than the female. Commercially, tomato is the most important vegetable crop. However, it is susceptible to abiotic stresses including drought. An earlier study examined the effects of drought on tomato utilizing vegetative tissues (Seng, 2014). However, for a fruit vegetable crop like tomato, fruit set is the most important trait for evaluating drought tolerance, thus evaluating drought tolerance of the reproductive organs especially the male is of enormous importance.

Anther, the male organ of flowering plants comprises of concentric cell layers including the epidermis, endothecium, middle layers and tapetum, the innermost layer surrounding a central locule that contains sporogenous cells. Sporogenous cells develop into pollen grains, the male gametophytes within the locule. During pollen development, anther wall layers play important roles in nutrition, protection, and pollen release. The tapetum serves as a source of energy for the developing microspores, secrete enzymes e.g. callase that releases the tetrads from the callose wall (Izhar and Frankel, 1971) and precursors for exine wall formation. Initially, tapetum development proceeds normally and at later stage, undergoes program cell death (PCD) and disintegrates (Parish and Li, 2010). The timely tapetum-specific PCD and disintegration are necessary for normal pollen development. Precocious or postponed tapetum degeneration results in male sterility (Bhadula and Sawhney, 1988; Graybosch and Palmer, 1985). The modified epidermal cells, the stomia modulate pollen release process. Failure of stomia cells to degenerate leads to male sterility due to anther indehiscence (Cecchetti et al., 2013). Abnormalities in anther and pollen development leading to induction of male sterility as a result of drought have been extensively investigated in cereal crops (Ji et al., 2010; Saini and Westgate, 1999). However, there are limited or no information on the impacts of drought on anther and pollen development in tomato.

Carbohydrate metabolism and sugar movement from source organs to sink tissues such as anthers are important processes for pollen development. Studies with cereals have shown that disturbances in these processes lead to male sterility associated mainly with abnormal starch accumulation in the pollen (Koonjul et al., 2005; Oliver et al., 2005). Sucrose accumulation, inadequate starch build-up in pollen grains and subsequent abortion of pollen development are attributed to repression of genes involved in sucrose and starch metabolism in drought and cold-stress anthers (Koonjul et al., 2005; Oliver et al., 2005). Additionally, drought-stressed rice anthers accumulate more starch granules in connective tissues than in pollen grains (Jin et al., 2013; Lalonde et al., 1997). However, studies on changes in starch and soluble sugar accumulation and the underlying molecular mechanisms in developing anthers under drought stress are scarce in fruit vegetable crops and for tomato in particular, there are no reports.

Phytohormones are important endogenous chemical messengers that modulate plant growth and development and responses to adverse stress factors. Among the phytohormones, abscisic acid (ABA) is the key plant hormone known to mediate responses to abiotic stresses such as drought and temperature (Zhang et al., 2006). In reproductive tissues, there is increasing evidence of a strong correlation between ABA level increase and pollen sterility during abiotic stresses. Higher pollen abortion and greater reduction in grain set with concomitant higher ABA accumulation in anthers of drought-susceptible than drought-tolerant wheat cultivars have been reported (Dong et al., 2017; Ji et al., 2011). Besides, there is proof of crosstalk between ABA and sugar signaling. The expression of*OSINV4* , a cell wall invertase gene, was repressed by ABA in cold stress rice anthers (Oliver et al., 2007). Recent study implicated auxin (IAA) in the control of plant response to abiotic stress during reproductive development. Reduction in pollen fertility and grain yield was associated with reduced accumulation of endogenous IAA in rice spikelets due to drought-induced repression of YUCCA genes that are involved in IAA biosynthesis (Sharma et al., 2018). Jasmonic acid (JA) has been demonstrated to participate in drought stress responses in vegetative tissues (De Ollas Valverde et al., 2015; Du et al., 2013) but there are no reports on JA mediation of drought responses in reproductive organs. However, induction of stigma exsertion as a result of reduction in endogenous JA in tomato anthers under high temperature has been reported (Pan et al., 2019). Although phytohormones play critical roles in regulating crops responses to drought stress, the dynamics of endogenous hormone metabolisms and the relationships with behavioural patterns of anthers and pollen at different stages of development under drought stress are not well understood.

In this study, we have examined the effects of drought stress on anther morpho-physiological and molecular responses in tomato. The major objectives of the study were to determine: (a) the consequences of subjecting anthers of varied developmental stages to water deficit on flowering phenology, flower development anther morphology and fruit set/yield (b) whether different stages of anther development response differently to drought stress (c) the effect of drought stress on male gametophyte fertility (d) the histological and cytological changes in anthers at different stages under drought stress (e) effects of drought stress on the levels of

endogenous IAA, ABA, and JA; the contents of sucrose, glucose and fructose; and starch accumulation at different stages of anther development (f) the underlying molecular mechanisms determining the physio-morphological changes in the male organ at different stages of development under stress conditions. Findings in this study will contribute to the understanding of the behavioural patterns and defects in the anther, tapetum, and pollen at different stages of development and the associated physiological and molecular mechanisms in response to drought stress in tomato.

## 2. MATERIALS AND METHODS

### 2.1 Plant material and growth conditions

Three-week-old seedlings of tomato (*Solanum lycopersicum*L.*)* cultivar 'Micro-Tom' were planted in pots containing 150 g of soil composing of peat, vermiculite and perlite (4:2:1) and grown in a growth chamber with $24/20^0$C (day and night) temperatures, constant 60% relative humidity, 16 hours photoperiod and light intensity of 150 µmol $m^{-2}$ $s^{-1}$. Plants were watered regularly until reproductive development. About binucleate stage of pollen development of the first flower buds, plants were exposed to two watering regimes: Well-watered (WW), soil moisture 65 ± 5%, and drought stress (DS), soil moisture 6 ± 3% by withholding watering in the DS plants. Soil moisture was monitored using HH2 Soil Moisture Meter (Delta-T Devices, Cambridge, UK) until it decreased to 5%. The soil moisture was then maintained at 6 ± 3% for 4 days, thereafter normal watering resumed. Meanwhile, the WW plants were watered normally. Pollen development stages were estimated by measurement of bud length and staining with 4, 6-diamidino-2-phenylindole (Regan and Moffatt, 1990).

### 2.2 Phenotypic and fruit yield assessments

The first six trusses on each plant were tagged and only buds and flowers on tagged trusses were used to evaluate responses to drought stress. On each truss, the position of each bud was recorded. Observations in respect of anther bud development pattern (aborted or reached anthesis), flower development pattern (aborted or set fruit), flower and stamen morphology were made commencing the day watering was suspended, through the stress period to anthesis of the ultimate bud on each tagged truss after re-watering. Flowering phenology was determined by counting the number of opened flowers per plant each day. Bud abortion, fruit set, and flower abortion were expressed as percentages of number aborted buds, flowers that set fruit and aborted respectively, to the total number of flower buds that occurred on all tagged trusses from the time watering was withheld to the time drought stress was suspended. Flowers were observed at anthesis for any developmental anomalies and stamen length was determined using digital callipers. Fruit yield was determined at maturity stage and weighed using digital balance.

### 2.3 Assessment of male gametophyte fertility

Viability of the male gametophyte was determined by microscopic observation of pollen grains stained with Alexander's stain (Alexander, 1967) using Nikon Eclipse 90i microscope with attached Nikon DS-Ri1 camera for all opened flowers from time of suspension of watering to anthesis of the last bud after rewatering. Pollen stained red were considered viable. Pollen viability was calculated using the formula:

$$Pollen\,viability\,(\%) = \frac{Number\,of\,viable\,pollen}{\text{Total number of pollen}} \times 100$$

The pollen viability of the aborted buds was considered as zero. To confirm the viability of the male gametophyte, artificial pollinations of the stigmata of flowers on WW plants with pollen of flowers from DS plants (WW × DS) and stigmata of flowers on DS plants with pollen of flowers from WW plants (DS × WW) were carried out. Pollination in each case was from the time of suspension of watering to anthesis of the last bud on tagged trusses after rewatering.

### 2.4 Histo-cytological observations

Anthers at six different developmental stages: meiotic (MEI), tetrad (TED), early uninucleate microspore (EUM), vacuolated uninucleate microspore (VUM), binucleate (BIN)) and mature pollen (MP) adapted from Polowick and Sawhney, (1992, 1993a, b) were sampled from WW and DS plants after 4 d of DS and at different days after rewatering (DARW). The samples were fixed and post fixed in 2.5% glutaraldehyde and 1% Osmium tetroxide ($OsO_4$) in phosphate buffer (PBS 0.1M, pH7.0) respectively, dehydrated in a graded series of ethanol and absolute acetone. Samples were infiltrated consecutively with 1:1 mixture of absolute acetone and final spur resin mixture, 1:3 mixture of absolute acetone and final resin mixture, and lastly with final resin. Samples were embedded in spur resin and heated at 70°C for more than nine hours. 2-3µm and 6µm thick sections were cut using LEICA EM UC7 utratome (LKB) and stained with uranyl acetate and potassium iodide solution respectively, observed and photographed using Nikon Eclipse 90i microscope. Thin sections from the same samples were double-stained with uranyl acetate and alkaline lead citrate and viewed using Hitachi Model H-7650 transmission electron microscope (TEM).

### 2.5 Transcriptome, soluble sugar and phytohormones analyses

Anthers were divided into three stages based on the similarity of response to 4 d DS during phenotypic evaluation experiment-stage I, [PMC-MEI]: were anthers at pollen mother cell and younger (PMC) and MEI stages with length [?] 2.8mm; stage 2, [TED-VUM]: were anthers at TED, EUM and VUM stages with lengths [?] 4mm to [?] 6.4mm; stage 3, [BIN-MP]: were anthers at transition to pollen mitosis 1 and early binucleate (BIN) to mature pollen (MP) stages with length >7mm. On 4 d of DS, samples from WW and DS plants were collected for each stage, immediately frozen in liquid nitrogen and stored at -75°C until used.

#### 2.5.1 Preparation of RNA-Seq library and Sequencing

Total RNA was extracted utilizing Trizol reagent (Invitrogen). RNA quantity and quality were determined by NanoDrop 1000 spectrophotometer (Thermo Scientific Inc.) at $OD_{260/280}$, 1% agarose gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Following the protocol described by Zhong et al., (2011), strand-specific RNA-Seq libraries were prepared using 1ng/µl of total RNA sample and sequenced on Illumina HiSeq 2500 system according to the manufacturer's instructions (Illumina).

#### 2.5.2 Analysis of RNA-Seq data

The raw RNA-Seq reads were processed to remove sequences with adapter contamination, low-quality nucleotides in excess of 10% and unknown nucleotides greater than 50 using Trimmomatic (Bolger et al., 2014). Processed sequences with length less than 40 bp were also got rid of. Ribosomal RNA sequences were removed after been discovered by aligning reads to ribosomal RNA database (Quast et al., 2012). The remaining clean reads were mapped to reference tomato genome using HISAT software that allowed up to two mismatches (Kim et al., 2015). The expression level of each gene was determined by counting the number of fragments that mapped to each gene and then normalized to number of fragments per kilobase of transcript sequence per millions (FPKM) base pairs sequenced using HTSeq software. A gene with FPKM value [?] 0.1 was considered expressed. To recognize differentially expressed genes (DEGs), the FPKM values of each gene from WW and DS anthers were analyzed using DESeq software (Anders and Huber, 2010). A rigid cut-off, $\log_2$ fold change >1 and p-adjusted $< 0.05$ was set as thresholds to consider a gene significantly differentially expressed. GOseq (Young et al., 2010) was used to analyze functional enrichment of specific gene ontology (GO) terms for DEGs. KEGG pathways significantly enriched with DEGs were determined using the KOBAS software.

#### 2.5.3 Validation of RNA-Seq data

RNA was extracted using the plant RNA kit (OMEGA) with three replications. cDNA was obtained using PrimeScript RT Reagent Kit following the manufacturer's protocol (TaKaRa, Japan) and then utilized for RT-qPCR reactions performed in Bio-Rad CFX96 (Bio-Rad, USA) using SYBR® Green Realtime PCR Master Mix (TaKaRa, Japan). Gene-specific primers utilized in this study are listed in Table S1. Relative gene expression levels were normalized utilizing *SlUbi3* as a reference gene and data analyzed using $2^{-Ct}$ method (Livak and Schmittgen, 2001).

### 2.5.4 Extraction and quantification of sucrose, fructose and glucose

Anthers were ground into fine powder in liquid nitrogen. 2 ml ethanol (80%) was added to 0.2 g of the ground tissue and incubated at 80 0C for 30 minutes and centrifuged at 12000 rpm for 20 min at room temperature. The precipitate was re-suspended in 80% ethanol and re-extracted. The supernatants were pooled and incubated at 90°C until dryness, then 3 ml of dd$H_2O$ was added to the pellets and filtered using 0.45 µm microporous membrane. High-performance liquid chromatography (HPLC) analysis of sucrose, glucose, and fructose contents was performed from three biological replicates of each sample as described in a recently published paper (Pan et al., 2019).

### 5.5 Phytohormones extraction and quantification

The extraction and purification of auxin (IAA), abscisic acid (ABA) and jasmonic acid (JA) were carried out following previously described method (Fu et al., 2012; Pan et al., 2008) with slight adjustments. The sample was pulverized in liquid nitrogen. 0.1g of powder was homogenized in 1 ml of ethyl-acetate containing 25 µl of internal standards of d2-IAA (Sigma-Aldrich), d5-JA (QCC) and d6-ABA (OlchemIm Ltd, Czechoslovakia), agitated for 12 h at 140 rpm at 4°C, centrifuged for 10 minutes at 12000 rpm at the same temperature and the supernatant collected. The sample was re-extracted once with 1 ml ethyl-acetate. The supernatants were pooled, dried using nitrogen gas, precipitate re-suspended in 0.5 ml of 70% methanol, and centrifuged for 10 min at 12000 rpm at 4°C. Three aliquots of 0.2 ml of sample supernatant were placed in separate snap-cap vials and analyzed using HPLC-mass spectrometry as previously described (Pan et al., 2019). The Agilent Zorbax XDB C 18 column (150 x 2.1 mm, 3.5 µm) was employed for the HPLC analysis as described earlier (Chen et al., 2018).

### 2.6 Data Analysis

Data were analyzed for statistically significant difference using Student's t-test in Microsoft Excel 2019 and presented as means with standard deviations using GraphPad Prism, version 9.0 (GraphPad Software, Inc., La Jolla, CA, USA).Venn diagrams were constructed utilizing the Venny web tool (Oliveros, 2007).

## 3. RESULTS

### 3.1 Effects of drought stress on flower development and fruit set

To assess the effects of drought stress at reproductive stage on flower development and fruit set, tomato plants were drought stressed by withholding watering and allowed to grow at soil moisture 6 ± 3% for 4 d, then normal watering resumed (Figure 1A). On suspending watering, the first emerged buds were at about binucleate stage of pollen development (Figure S1A). At the end of the drought stress (DS), drought-stressed plants were severely wilted and stunted (Figure S1B). The first significant impact of drought stress on reproductive development was on flower production. Under DS, significant reduction in the number of opened flowers/day occurred between 1 d before suspending DS and 7 days after rewatering (DARW) with nearly zero flower production between 1-7 DARW. The second period of flower production occurred between 8 and 19 DARW, peaking on 11 DARW, suggesting that drought stress can postpone flowering (Figure 1B). Further, we found that 25% of flower buds on DS plants became yellowish and distorted in appearance and finally aborted. Strikingly, bud abortion occurred mainly at three specific stages of pollen development: tetrad (TED), early uninucleate microspore (EUM) and vacuolated uninucleate microspore (VUM) (Figure 1C), indicating that these stages are most sensitive to drought stress. In addition, 38.6% of opened flowers aborted resulting altogether in a total of 63.4 % flower (flower buds and opened flowers) abortion in DS plants, which was significantly higher than that in WW plants (Figure 1D). Consistently, fruit set in the DS plants was 36.6% which was markedly lower than 77.5% in WW plants, a reduction of 52.8% (Figure 1D). Subsequently, fruit yield per plant in DS plants was decreased by 57.4%. Together, our data suggest that bud and flower abortions are the main contributors to yield loss under drought condition.

All anthers subjected to preanthesis drought stress for 4 d had significantly shorter stamen at anthesis compared to WW stamen (Figure 1E). They also displayed three types of phenotypes: type-1 appeared very similar to the normal flower; type-2 had anther lobes often reflexed and freed from the style in the neck

region; type-3 had extremely shortened stamen and mostly with stigma extended above the cone. Most of the flowers were type-1, followed by type-3 and then type-2 represented by 65.3%, 27% and 7.7% respectively. Interestingly, type-1, 2 and 3 flowers often but not always occurred on the same truss, with type-3 flower first appearing, followed by type-2 and then type-1 flowers (Figure 1E). Although the lengths of all preanthesis DS anthers were significantly shorter, the decrease was more dramatic for the type-3 anther which stamen was 1.6 mm shorter than the pistil compared to 0.2 mm higher than the pistil in the WW flower (Figure S1D), suggesting that the stigma exsertion is attributed to extreme shortening of the stamen and not elongation of the style. In short, drought stress affected the reproductive development of tomato in diverse ways.

### 3.2 Drought stress reduces the fertility of the male organ

To ascertain whether drought affected the male gametophyte, pollen fertility was determined. On average, the pollen viability of the DS plants was reduced by 31.9% (Figure 2A). Surprisingly, the pollen viability of DS flowers was similar to that of WW flowers after 4 d of DS suggesting starch accumulation might be reduced but not prevented in pollen of maturing anthers. In contrast, type-2 and 3 flowers recorded zero pollen at anthesis after rewatering (Figure 2A).

Because low fruit set coincided with overall low pollen viability of DS plants, it was speculated that male sterility was the main contributor of bud and flower abortions and subsequently the poor fruit yield performance of DS plants. To confirm our speculation, reciprocal cross-pollinations were performed. When the stigmas of flowers on WW plants were pollinated with pollen of flowers from DS plants (WW$\times$ DS), fruit set was 55.5% lower than in WW plants, which was consistent with higher flower abortion rate (Figure 2B), indicating that DS severely affected the male fertility. In contrast, when the stigmas of flowers on DS plants were pollinated with sound pollen of flowers from WW plants (DS $\times$ WW), flower abortion and fruit set were 14.3% and 85.7% which were moderately lower and higher respectively than those in WW plants (Figure 2B), indicating that the female fertility was not significantly affected. These results suggest that drought stress-induced irregularities in male organ are the main cause of the low fruit set/yield of drought-stressed tomato plants.

### 3.3 Histo-cytological analysis of anther development under drought stress and recovery conditions

To understand details of the effects of drought stress on the male development, anthers at six developmental stages as earlier described (Chen et al., 2018; Polowick and Sawhney, 1992, 1993a, b, 1995) were examined under light microscope (LM) and transmission electron microscope (TEM). The six stages included: meiotic (MEI), tetrad (TED), early uninucleate microspore (EUM), vacuolated uninucleate microspore (VUM), binucleate (BIN) and mature pollen (MP).

Pre-meiotic anthers from DS plants exhibited no distinct alterations from WW anthers (Not shown). However, defects were obvious at all of the later stages examined after 4 d drought-stress treatment and rewatering (Figure 3A). At MEI stage, the sporogenous and tapetum tissues were constricted and moved apart especially in the outer tapetum region after 4 d of DS anther (arrow 1) so that the sporogenous tissue no longer filled the locular space; the type-3 anther wall layers and the sporogenous tissue were collapsed and not distinctly demarcated. At other stages, after 4 d of DS, abnormalities included: degenerated and enlarged pollen (arrows 2 and 4 respectively); ectopic callose wall dissolution and precocious released of microspores at TED stage; compressed and compactly parked microspores at EUM stage; dwindled tapetum layer at TED and EUM stages and its enhanced disintegration and dissolution at VUM stage; premature induction of anther dehiscence (arrow 3), partial degeneration of stomia cells (arrow 5) and the large number of pollen grains retained in anther locules due to inefficient dehiscence at MP stage. In the type-2 anther, the tapetum was hypertrophic, persistent at VUM stage and exhibited delayed degeneration at BIN stage; together with type-3 anther, the anthers were collapsed at BIN stage so that two instead of a common locule in each anther lobe were observed (arrow 6) and a total lack of dehiscence due to persistent stomia cells at MP stage (arrow 7). Additionally, in the type-3 anthers, premature disintegration and dissolution of the callose wall, tapetum and sporogenous tissues were observed with conspicuous empty locule (arrow 8) from TED to MP stages,

although debris of degenerated microspores (arrow 9) was sometimes observed in the locule at some stages).

The effects of DS on pollen development were further revealed by TEM observations (Figure 3B, Figure S1E). Microspores were completely dissolved and absent in the locule from TED to MP stages in the type-3 anther indicating that drought during mid-meiotic stage can prevent microsporogenesis. Developing pollen cells at different stages of development in anther of DS (4 d), type-2 and type-3 were highly plasmolyzed with constricted protoplasm separated from the pollen cell wall (arrow 10) and had abnormally numerous small or large vacuoles (Figure 3B). Other abnormities observed in the developing pollen included expansion of the middle lamella after 4 d of DS at MEI stage (Figure S1E); premature microspore wall formation at TED stage after 4 d DS and at MEI stage in type-3 anther (Figure 3B, Figure S1E); in the type-2 anthers at TED stage, failure of tetrad formation and lack of callose wall around individual tetrad of microspores' nuclei were observed indicating delayed cleavage of meiotic mother cell's (MMC) cytoplasm and impaired callose deposition and/or ectopic callose dissolution, although evidence of meiosis was the presence of multiple nucleoli in the cytoplasm of MMC (Figure 3B) suggesting that drought during early meiotic stage can prolong microsporogenesis and delay microspore mother cell cleavage.

In addition, TEM observations revealed disturbances in tapetum development caused by drought stress (Figure 3C). Under well-watered condition, the tapetum degeneration occurred at VUM stage. However, in DS (4 d) anthers, from MEI to EUM stages the tapetum underwent ectopic degeneration with constricted protoplasm at MEI stage (Arrow 11) and completely devoid of protoplasmic contents and cellular structure at TED and EUM stages respectively. After re-watering, in the type-2 anthers, excessive vacuolation of tapetum cells was observed at MEI and TED stages, and the tapetum persisted and maintained cellular structure up to VUM stage and degeneration was delayed until BIN stage. Although persistent and developed under well-watered condition between 10-12 DARW, the tapetum in the type-2 anthers exhibited signs of plasmolysis, with cells contracted and separated from the tapetum cell wall (arrow 11), adjacent tapetum cells (arrow 12) and adjacent middle layer cells (arrow 13). In the type-3 anthers, obvious abnormalities included ectopic degeneration of the tapetum cells beginning at MEI stage and their complete absence from anthers at TED to VUM stages. In short, drought stress spanning the period from early meiotic to binucleate stages affects anther and pollen development through induction of tapetum/pollen degeneration and dissolution, delayed tapetum degeneration and stomia persistence.

### 3.4 Transcriptome changes in anthers at different stages of development under drought stress

To study possible molecular adjustments of tomato anthers under drought, Illumina RNA Sequencing (RNA-Seq) was used to investigate the transcriptome of anthers at 3 different development stages: pollen mother cell to meiotic cell stage (PMC-MEI), tetrad to vacuolated microspore stage (TED-VUM) and binucleate to mature pollen stage (BIN-MP) from well-watered (WW) and drought-stressed (DS) plants. After filtering, a total of 924,197,894 clean reads were generated from 18 libraries with high consistency between replicates (Pearson's r, 0.86-0.99, Table S2). Out of that, 862,078,735 reads, uniquely aligned to specific genomic regions of *S. lycopersicum* reference genome with an average mapping rate of 98.2 % (Table S3) and were used for differential gene expression (DEG) analysis. In total, 15,431 DEGs were identified (Table S4A). Among them, 3427, 2780, and 815 genes were up-regulated at PMC-MEI, TED-VUM and BIN-MP stages, respectively (Figure 4A, Table S4B), whilst 3450, 3560 and 1399 genes were down-regulated at PMC-MEI, TED-VUM and BIN-MP stages, respectively (Figure 4A, Table S4C), indicating that the number of DEGs decreased with age of anther. Greater number of genes was down-regulated at TED-VUM indicative of its high level of vulnerability to drought stress. Confirmation of RNA-Seq results with RT–qPCR analysis using 18 selected drought-responsive genes revealed that the expression patterns from RT–qPCR analysis were consistent with those from RNA-Seq analysis (Figure S2A).

To have an idea of the possible roles of drought-regulated genes, enrichment analyses of GO terms and KEGG pathways were performed. A total of 225 well-defined functional groups were significantly enriched in drought-responsive genes at different development stages (Tables S5 and S6). All biological processes significantly enriched in up-regulated genes occurred only at one stage such as 'regulation of gene expression' (GO: 0010468), 'transmembrane transport' (GO: 0055085) and 'response to water' (GO: 0009415) at PMC-

MEI, TED-VUM and BIN-MP stages, respectively (Table S5A). With regards down-regulated genes, some enriched biological processes were observed at more than one stage, for example, 'carbohydrate metabolic process' (GO: 0005975) at TED-VUM and BIN-MP stages; 'lipid metabolic process' (GO: 0006629) at PMC-MEI and TED-VUM stage; 'amide biosynthetic process' (GO: 0043604) at PMC-MEI and BIN-MP stages and 'metabolic process' (GO:0008152) at all three stages. Other biological processes were enriched only at one stage, example 'photosynthesis' (GO: 0015979) at TED-VUM stage (Table S 6A), suggesting that carbohydrate biosynthesis function was significantly repressed by drought stress at mid to late stages of anther development. Similarly, KEGG analysis showed only one pathway 'Ribosome' was highly significantly enriched in down-regulated DEGs at two stages whereas others were only enriched at TED-VUM stage, such as 'Photosynthesis'and 'Biosynthesis of secondary metabolites' (Figure 4B, Table 7B). Strikingly, only the pathway 'plant hormone signal transduction' was highly enriched in up-regulated DEGs at PMC-MEI stage (Figure 4B, Table S7A). Together, our GO and KEGG data suggest drought stress may primarily affect carbohydrate and secondary metabolic processes including hormone pathways in tomato anthers.

### 3.5 Impact of drought stress on tapetum-specific expressed genes

To determine whether drought affected the development of the tapetum and microspore at transcription level, 52 tapetum- and pollen- related expressed genes were identified (Table S8). RNA-Seq data showed that*Solanum lycopersicum EXCESS MICROSPORES* (*SlEMS* ),*ABORTED MICROSPORES* (*SlAMS* ), *DEFECTIVE TAPETUM DEVELOPMENT and FUNCTION1* (*SlDTF1) DYSFUNCTIONAL TAPETUM1 (SlDYT1)* and *CALLOSE DEFECTIVE MICROSPORE1* (*SlCDM1* ) were markedly down-regulated whereas SlMYB80 was up-regulated under DS condition at PMC-MEI stage. At TED-VUM stage, *SlAMS* , *MALE STERILITY1* (*SlMS1* ) and *ANTHER 7* (*SlAT7)* were significantly drought-repressed (Figure 5). The up-regulation of at most a gene at PMC-MEI stage while all examined genes were down-regulated at TED-VUM stage is in line with the considerable amount of damage to the tapetum and/or microspores caused by drought stress in anthers at TED-VUM stage than at PMC-MEI stage as revealed by histocytological analysis and suggest a dominant and positive role of SlMYB80 in drought tolerance mechanism early in anther development.

### 3.6 Drought stress disturbed sucrose and starch metabolism and soluble sugar transport

Consistent with 'carbohydrate metabolic process' and 'photosynthesis' pathway significantly enriched in drought-repressed genes, 16 DEGs involved in sucrose cleavage, sugar phosphorylation and starch synthesis, and 33 DEGs associated with sugar transport were identified (Table S9). Gene expression analysis showed that the sucrose cleavage genes including *cell wall invertase 3-like (SlCWINV3-like)* and sucrose synthase (*SUS* ) *TOMSSF* were significantly repressed at PMC-MEI stage. Whereas ß-*fructofuranosidase* and *SlSUS6*were significantly up-regulated at TED-VUM stage, *SlCWINV3-like*and *SlSUS6* were significantly repressed at BIN-MP (Figure 6A) stage, suggesting that sucrose hydrolysis is impaired at PMC-MEI and BIN-MP stages but not at TED-VUM stage under drought stress. The sugar transporters, *SlSWEET16* and *SlSTP8* were notably up-regulated at PMC-MEI stage. *SlSWEET16* was markedly induced at both TED-VUM and BIN-MP stages (Figure 6A), suggesting that monosaccharide transport is generally not prevented in tomato anther under drought stress. Further utilization of hexose sugars in metabolic processes requires their phosphorylation, catalyzed by hexokinases that phosphorylate both glucose and fructose, and fructokinases that specifically phosphorylate fructose (Granot et al., 2013). While*Slhxk1* and *Slhxk2* were both significantly repressed at PMC-MEI stage, *Slhxk1* was significantly down-regulated at TED-VUM stage. In addition, the rate-limiting enzymes in starch biosynthesis,*Slagpl3* and *TOMADPGPPs* were both significantly down-regulated at PMC-MEI and TED-VUM stages. However, ß-amylase 8, the starch hydrolyzing gene was significantly up-regulated at BIN-MP stage (Figure 5A). Our data suggest hindered anther sink strength and starch biosynthesis while starch degradation is enhanced during pollen development under DS.

Under DS condition, sucrose, fructose and glucose contents were strikingly higher at PMC-MEI and BIN-MP stages, although glucose was significantly lower at PMC-MEI stage whereas their contents remained similar to those in WW anthers at TED-VUM stage (Figure 6B). In drought-stressed anthers, the pattern of starch accumulation in the developing pollen was comparable to that in WW anthers from MEI to VUM stage.

However, at BIN and MP stages, starch accumulation in pollen grains of DS anthers was attenuated, whereas more starch accumulated in the pollen grain under WW condition, (Figure 6C). After rewatering, type-2 anthers accumulated more starch granules in the connective and endothecium tissues at PMC-MEI stage, whereas starch accumulation in the pollen grains was decreased at BIN–MP stage, though not to the same extent as in anthers DS 4 d (Figure S3), suggesting partial recovery of carbohydrate metabolic pathway after rewatering. In type-3 anthers, starch accumulation in the pollen and anther walls was abolished (Figure S3) across all the examined stages suggesting that carbohydrate metabolic pathway never recovered after rewatering in the type-3 anthers.

### 3.7 Effect of drought on phytohormones metabolism and signaling

The expression profiles of IAA, ABA and JA metabolic and signaling genes based on RNA-Seq data and their corresponding endogenous contents were analyzed. A total of 38 DEGs encoding enzymes involved in auxin metabolism (17) and signaling (21) were at least regulated at one stage (Table S10). The IAA biosynthesis gene, *SlTAR2,* was significantly down-regulated at PMC-MEI stage, whereas *SlTAR1-like* ,*SlTAR* 2, *ToFZY1* and *ToFZY2* were significantly up-regulated at TED-VUM stage (Figure 7C). Consistently, the content of endogenous IAA was significantly reduced at PMC-MEI stage whereas it was moderately increased at TED-VUM under DS condition (Figure 7B).

A total of 19 DEGs associated with ABA metabolism and signaling were identified (Table S10). At PMC-MEI stage, two dehydrogenase/reductase genes, 3-*oxoacyl-CoA reductase* 1 and *SlSDR12-like* involved in ABA biosynthesis were significantly up-regulated, while*Slcyp707a1,* encoding ABA 8'-hydroxylase involved in ABA catabolism, was markedly induced. Correspondingly, the core components of ABA signaling genes including one ABA receptor, *SlPYL9* , two*protein phosphatase 2C* (*PP2C* ) genes (*SlPP2C37* and*SlDIG3),* and two SNF1-RELATED PROTEIN KINASE2 (*SnRK2* ) genes (*SlSRK2C* and *AY222455* ) were significantly up-regulated (Figure 7). Additionally, two known direct downstream transcription factor targets of *SnRK2, SlAREB* , homologue of*AtAREB2* and *SlABF* , homologue of *AtABF3* (Figure S2B) which are regulators of ABA-responsive genes, were exclusively significantly up-regulated at PMC-MEI stage, suggesting that ABA signaling is induced early in anther development under drought stress. At TED-VUM stage, only *SlSDR12-like* and *SlDIG3* were significantly up-regulated and only a few genes were moderately induced at BIN-MP stage such as *Slcyp707a1* , *SlPP2C37* and*SlSRK2C* (Figure 7), suggesting that ABA signaling is impaired under drought condition at TED-VUM and BIN-MP stages. Consistently, endogenous ABA content was significantly increased at PMC-MEI and TED-VUM stages with an increase of 205% and 395%, respectively (Figure 7B). Together, these results suggest that ABA, in conjunction with its catabolic gene might play a significant role in drought tolerance mechanism in the early stages of anther development.

A total of 21 DEGs associated with JA metabolism and signaling were recognized in at least one stage (Table S10). RNA-Seq analysis of four genes involved in JA biosynthesis revealed that at PMC-MEI stage,*SlLOX5* exhibited significant increase. At TED-VUM and BIN-MP stages, all four genes: *SlLOX5* , *SlOPR3* ,*Phospholipase A2* and *SlAOC* were down-regulated under drought stress although none was significant at BIN-MP stage (Figure 7C). Invariably, under DS, endogenous JA contents were significantly decreased at TED-VUM and BIN-MP stages (Figure 7B).

## 4. DISCUSSION

Successful development of the anther in flowering plants is very crucial to ensuring plant fertility and productivity because it produces and delivers the male gamete to the female gametophyte for efficient fertilization (Borg et al., 2009). However, anther development is often perturbed by abiotic stresses such as drought resulting in male sterility and yield reduction (Jin et al., 2013; Nguyen and Sutton, 2009). Nevertheless, the developmental flaws and the underlying physiological and molecular mechanisms remain unclear in tomato. In this study, we examined the effect of drought stress on anther development using morpho-physiological and molecular analyses in tomato. Reproductive development is extremely sensitive to abiotic stresses but during flower ontogeny, some stages are more sensitive (Sato et al., 2002). In our study, drought induced bud abortion specifically at tetrad (TED), early uninucleate (EUM) and vacuolated uninucleate microspore

(VUM) stages (Figure 1 C) whereas more advanced and younger buds in the proximal and distal regions of a truss respectively, were drought tolerant. The advanced buds proceeded to anthesis during or immediately after the stress, before bud abortion set in while the younger buds recovered and developed to anthesis after rewatering. This indicates that the break in flowering is the result of bud abortion and the inherent drought tolerance of the younger buds permitted growth resumption after transient growth arrest, led to the second peak and extended period of flower production. Thus, drought altered flowering phenology (Figure 1B) through induction of irreversible and reversible arrest of anther bud development, and delayed anthesis. Drought-induced reduction in yield is attributed to flower and pod abortions (Fang et al., 2010; Kokubun et al., 2001). However, we, demonstrated that in addition to flower abortion, bud abortion is a crucial factor limiting yield in tomato. Additionally, all preanthesis DS anthers had reduced length but the reduction in stamen length was much more severe in type-3 flower that the stigma extended above the anther cone (Figures 1E, Figure S1D), similar to heat-induced stigma exsertion (Pan et al., 2019). Suggesting that the underlying mechanisms involved in stamen shortening leading to stigma exsertion under both stresses in tomato might be similar.

Male sterility due to poor pollen viability is the most important limiting factor affecting yield under drought stress (Jin et al., 2013). In the present study, drought stress caused marked reduction in pollen viability (Figure 2A) and subsequently low fruit set in accordance with previous reports (Saini and Aspinall, 1981). However, the effect of DS on pollen viability varied with stage of anther development. It was more severe for anthers between meiotic and binucleate stages which produced anthers that lacked pollen or had pollen that were severely malformed (Figures 3A and B). Further, male sterility due to anther indehiscence and stigma exsertion were observed in our study (Figure 1E and Figure 3A) in line with previous reports under low and high temperatures (Kiran et al., 2019; Pan et al., 2019). Our results suggest that reduction in fruit yield is due mainly to the reduction in pollen viability but secondary factors including anther indehiscence and stigma exsertion also play role. Importantly, the drought-tolerant and susceptible anthers identified are of significant importance for identifying major players involved in drought stress tolerance that can facilitate drought tolerance breeding in tomato.

The production of viable pollen grains depends on the normal development and function of the tapetum (Kawanabe et al., 2006). However, abiotic stresses especially extreme temperatures can cause early or delayed tapetum degeneration leading to pollen abortion (Oda et al., 2010). The current study revealed that drought stress induced both precocious and delayed tapetum degeneration (Figure 3A and C) in agreement with previous reports (Jin et al., 2013; Oda et al., 2010 ). The early tapetum degeneration might have led to the early dissolution of the callose wall and subsequently, the ectopic release of microspores and abnormal microspore wall formation at meiotic (type-3) and tetrad stages (Figure 3B, S1), in line with earlier reports under low-temperature stress (Gothandam et al., 2007; Mamun et al., 2006). Moreover, it might constitute the main underlying cause of pollen and flower bud abortions at tetrad and early uninucleate stages. However, at later stages, pollen abortion might not be related to tapetum degeneration since it is already in a degenerate state at these stages even in WW anthers (Figure 3A and C). Therefore, other factors including but not limited to defects in pollen structure such as plasma membrane and organelles malfunction might be involved in bud and pollen abortions at later stages, consistent with the high number of GO terms in the cellular component significantly enriched in down-regulated DEGs (Table S6).

Drought-induced altered expression of genes involved in tapetum/microspore development causes abnormal tapetum development and male sterility (Jin et al., 2013). In our study, except for *SlMYB80* which was markedly induced at PMC-MEI stage, DS repressed the examined tapetum expressed genes at PMC-MEI and TED-VUM stages (Figure 5). The synthesis and deposition of callose provide a temporary wall that separates microsporocytes, meiotic cells and microspores of the tetrad and subsequently degraded by the enzyme callase secreted by the tapetum to release the microspore (Lu et al., 2014; Scott et al., 2004). *SlCDM1* , a C3H zinger finger TF and *SlMYB80* regulate callose metabolism during microsporogenesis (Lu et al., 2014; Zhang et al., 2007). The large number of tapetum and microspore expressed genes down-regulated with conspicuous defects in tapetum, pollen and callose wall in this study, is consistent with (Jin et al., 2013). Additionally, mutants of *MYB80* are male sterile with altered tapetum and pollen development in

many crops (Phan et al., 2012; Xu et al., 2014). Therefore, the up-regulation of *SlMYB80* at PMC-MEI stage is consistent with normal pollen development (Figure 2A) of type-1 anthers. Suggesting a positive and overriding role of *SlMYB80* in early anther development under drought stress. Our results suggest that drought stress between meiosis and early uninucleate microspore stages is detrimental and can induce irreversible arrest of tapetum and pollen development in tomato.

In flowering crops starch forms the principal storage food reserve in mature male gametophyte (Dorion et al., 1996; Jin et al., 2013). Storage of starch in mature pollen relies on uninterrupted sucrose supply to the anther and its unimpeded cleavage principally by cell wall invertases into monosaccharides which are conveyed into cells via monosaccharide transporters and subsequently used in starch biosynthesis (Dorion et al., 1996; Ruan et al., 2010). Our study clearly showed that sucrose, glucose, fructose, and starch accumulated abnormally in developing drought-stressed anthers (Figure 6 and S3), suggesting that drought affected carbon allocation and processes implicated in starch biosynthesis. Sucrose and starch metabolic genes were differentially drought-regulated. The sucrose cleavage genes*SlCWINV3-like,* and *TOMSSF* at PMC-MEI stage, and*SlSUS6* at BIN-MP stage were noticeably down-regulated, resulting in higher sucrose levels in agreement with past reports (Dorion et al., 1996; Nguyen et al., 2010). In contrast,*β-φρυςτοφυρανοσιδασε* and *SlSUS6* were induced which correlated with lower sucrose levels at TED-UM stage.

Committing glucose to participate in starch biosynthesis and other metabolic processes requires its phosphorylation by hexokinase which is specifically expressed in the tapetum and developing pollen (Granot et al., 2013; Suwabe et al., 2008). We observed two hexokinase genes, *Slhxk1* and *Slhxk2* generally repressed at all three stages of development (Figure 6A) resulting increased levels of fructose and glucose, in agreement with Nguyen et (al., 2010). In addition, drought repressed the expression of *ADGPase* gene, the rate limiting enzyme in starch biosynthesis, consistent with Lalonde et al., (1997). Interestingly,*β-αμψλασε 8* , the starch hydrolyzing enzyme was induced at BIN-MP stage. These results suggest that reduction pollen viability due to reduced starch accumulation under drought stress is highly associated with diminished sugar utilization and hydrolysis of de novo and/or existing starch in maturing anthers.

Previous studies provided evidence of IAA involvement in abiotic stress responses in vegetative organ (Min et al., 2014; Sakata et al., 2010). In reproductive organs, drought- reduction of IAA biosynthesis genes expression and contents reduces pollen and spikelet fertility in rice (Sharma et al., 2018). In our study, gene expression analysis showed that the tryptophan *aminotransferase of Arabidopsis -related* (TAR) family member, *SlTAR2*was largely repressed at PMC-MEI stage whereas two TAR genes, including*SlTAR2 and SlTAR1-like genes* , and two YUC genes, ToFZY1 and ToFZY2 were significantly induced at TED-VUM stage (Figure 7C), which were in agreement with endogenous IAA levels (Figure 7B). Intriguingly, the TED-VUM stage, that had IAA biosynthesis genes and content significantly induced and increased respectively under drought stress, exhibited severe pollen abnormalities (Figure 3B, DS (4 d)) and anther buds abortion (Figure 1C). On the contrary, the PMC-MEI stage with conspicuous attenuation in IAA biosynthesis and amount had a good number of its anthers (type-1) producing viable pollen (Figure 2A) and excellent fruit set (Figure S1C), inconsistent with Sharma et al., (2018).

JA plays roles in multiple abiotic stresses including drought stress (de Ollas et al., 2013; De Ollas Valverde et al., 2015). We demonstrated that, with exception of *SlLOX5* which was moderately increased at PMC-MEI stage, drought repressed the expression of JA biosynthesis genes at all stages of anther development with concomitant significant decrease in JA levels in anthers at TED-VUM and BIN-MP stages (Figure 7) similar to Pan et al., (2019) under high-temperature stress. In *Arabidopsis*JA-deficient mutants lines exhibit anther indehiscence (Cecchetti et al., 2013). Interestingly, JA reduction at BIN-MP stage occurred concurrently with extreme reduction in fruit set (Figure S1C) which might be due to male sterility as a result of impaired anther dehiscence since a large number of pollen grains were retained in mature anthers (Figure 3A, DS (4 d). It is speculated that anther indehiscence exhibited by type-2 and type-3 anthers at anthesis (Figure 3A) is correlated with low JA levels.

Drought stress triggers ABA biosynthesis and increases its content in reproductive organs with a lower and higher level increase in ABA concentrations associated with abiotic stress tolerance and susceptibility

respectively (Zhang et al., 2006). In this study, many ABA metabolic and signaling genes were differentially expressed at different stages of anther development (Table S10). Expression analysis revealed that the*dehydrogenase/reductase* (*SDR* ) genes: *3-oxoacyl-CoA reductase* 1and *SlSDR12-like,* involved in ABA biosynthesis were significantly induced at PMC-MEI stage and *SlSDR12-like* was induced at TED-VUM stage while *Slcyp707a,* that inactivates bioactive ABA was highly and moderately induced at PMC-MEI and BIN-MP stages respectively (Figure 7C). It appears that the regulation of ABA homeostasis rests on the coordinated expression of ABA biosynthesis and catabolic genes. At PMC-MEI stage (drought tolerant), higher expression of ABA biosynthesis coincided with higher expression of *Slcyp707a*and lower ABA accumulation. On the contrary, at TED-VUM stage (drought susceptible) ABA overproduction coincided with higher and reduced expression of ABA biosynthesis and catabolic genes respectively (Figure 7B, C), consistent with a study in which distinct drought tolerant and susceptible wheat cultivars were used (Ji et al., 2011). Thus, up-regulation of ABA inactivation pathway appears as a core mechanism for modulating ABA level and conferring drought tolerance in tomato anthers consistent with Ji et al., (2011) but inconsistent with Jin et al., (2013). The inconsistency might be related to differences in crop species, stress level and duration, and technique used to analyze the transcriptomes. Put together, our results suggest that ABA and its catabolic pathway play a decisive role in regulating ABA homeostasis and drought tolerance in tomato anthers. Further, it is speculated that antagonistic interactions between ABA and IAA confers drought tolerance in pre-meiotic anthers, and between ABA and JA, in controlling anther dehiscence in meiotic anthers in tomato.

To sum up, it is explicit that increase bud and flower abortions and subsequent reduction in fruit yield of DS plants were largely attributed to drought-induced male sterility caused by abnormalities in anther, tapetum and pollen development resulting in attenuation of pollen fertility, anther indehiscence and stigma exsertion. Under drought stress, different stages of anther development exhibit differential responses with the sensitivity of anthers to drought stress spanning the period from meiotic mother cell stage to binucleate stage with TED-VUM stage, the most sensitive to drought, whereas PMC-MEI stage the most drought tolerant. The drought tolerance exhibited by the PMC-MEI stage is probably the result of moderate increase in ABA level due to the high-level expression of its catabolic enzymes which maintains a level of ABA optimum to trigger signaling and activating ABA-dependent drought adaptive gene expression and repression of IAA signaling (Figure 8). Our findings provide insight into the behavioural patterns and defects in the anther, tapetum and pollen at different development stages and associated physiological and molecular mechanisms in response to drought stress and give a novel insight into potential drought tolerance mechanism which can be engineered for improvement of drought tolerance in tomato.

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## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

## AUTHORS' CONTRIBUTIONS

ATLS and GL conceived and designed the research. ATLS conducted most of the experiments. MF helped analyzed the data. ATLS and MA wrote the manuscript. MA and GL critically revised the manuscript. All the authors read and approved the manuscript.

## COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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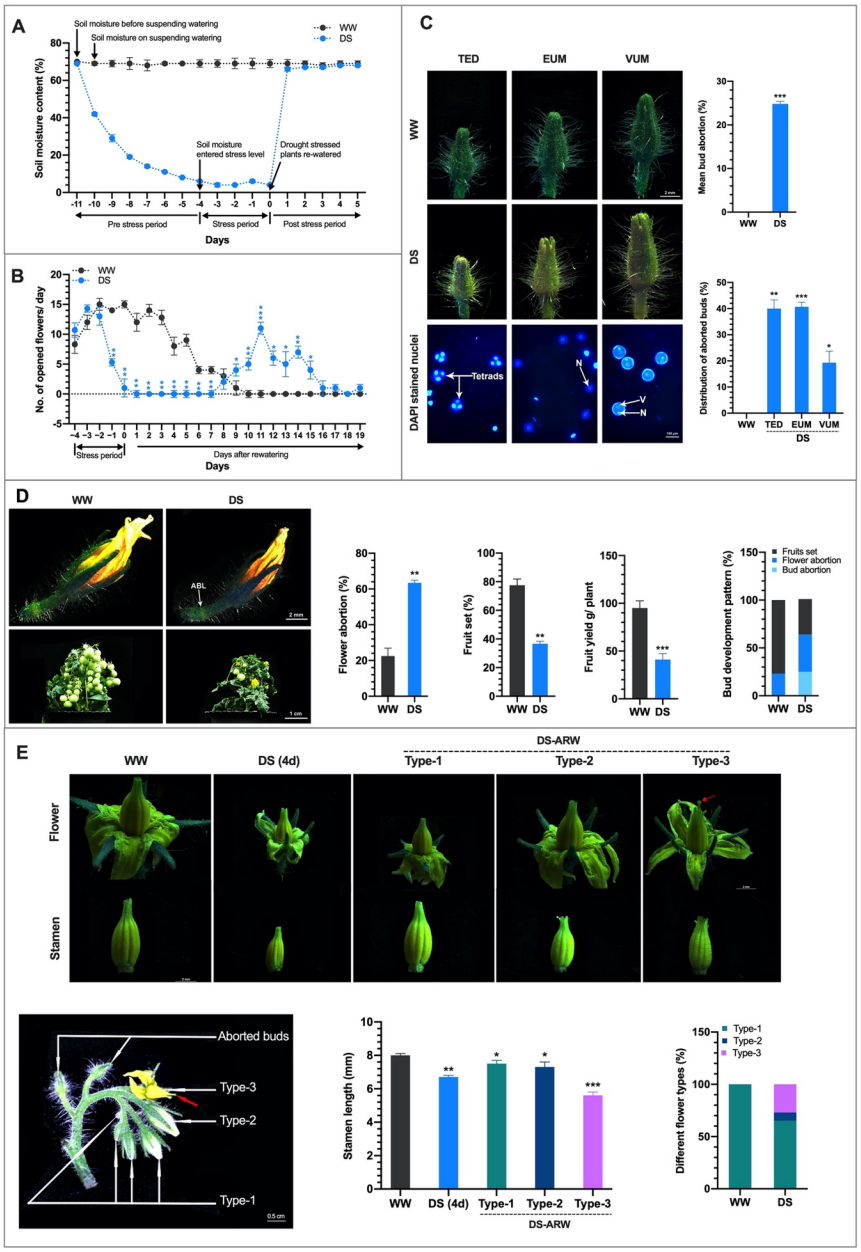


**Figure 1.** Tomato anther development and fruit yield under drought stress. **(A)** Soil moisture content (%) during the period watering was suspended to 5 days after re-watering (DARW). (**B)** Number of opened flowers/day during the stress period to 19 DARW. **(C)** Aborted flower buds at 3 specific stages of anther development from drought-stressed (DS) plants. Bud development stage was determined by staining with DAPI and measurement of bud length. TED, tetrad stage; EUM, early uninucleate microspore stage; VUM, vacuolated uninucleate microspore stage. (**D)** Flower abortion, fruit set and fruit yield of DS plants. Aborted flowers abscised at the abscission layer between the upper and lower pedicel after failure of fertilization. Fruits were harvested at maturity and weighed to determine the fruit yield in gram/plant. ABL, abscission layer.**(E)** Morphology of flowers and stamens 4 d after DS and rewatering (DS-ARW). Flowers were sampled after 4 d of DS (DS 4 d), between 9-11 DARW (type-3, red arrow indicates stigma exsertion); 10-12 DARW

(type-2); 11-19 DARW (type-1). Values presented are means ± SD (n=3 biological replicates, 4 plants each); * $p$ <0.05; **$p$ <0.01; *** $p$ < 0.001 (t-test).

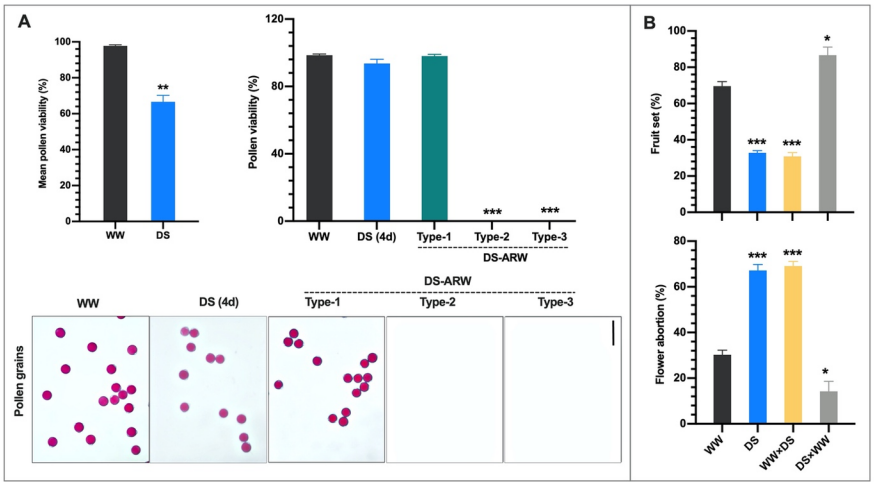


**Figure 2.** Fertility of the male organ under drought stress.**(A)** Mean pollen viability of DS plants and individual flowers from DS plants after 4 d of drought stress and at different days after rewatering (DS-ARW) for type-1, type-2 and type-3. Scale bar: 100 µm.**(B)** Fruit set and flower abortion of DS plants, stigma of flower on WW plants crossed pollinated with pollen of flowers from DS plants (WW× DS) and stigma of flowers on DS plants crossed pollinated with pollen of flowers from WW plants (DS× WW). Values presented are means ±SD (n=3 biological replicates, 4 plants each). * $p$ <0.05; **$p$ <0.01; ***$p$ < 0.001 (t-test).

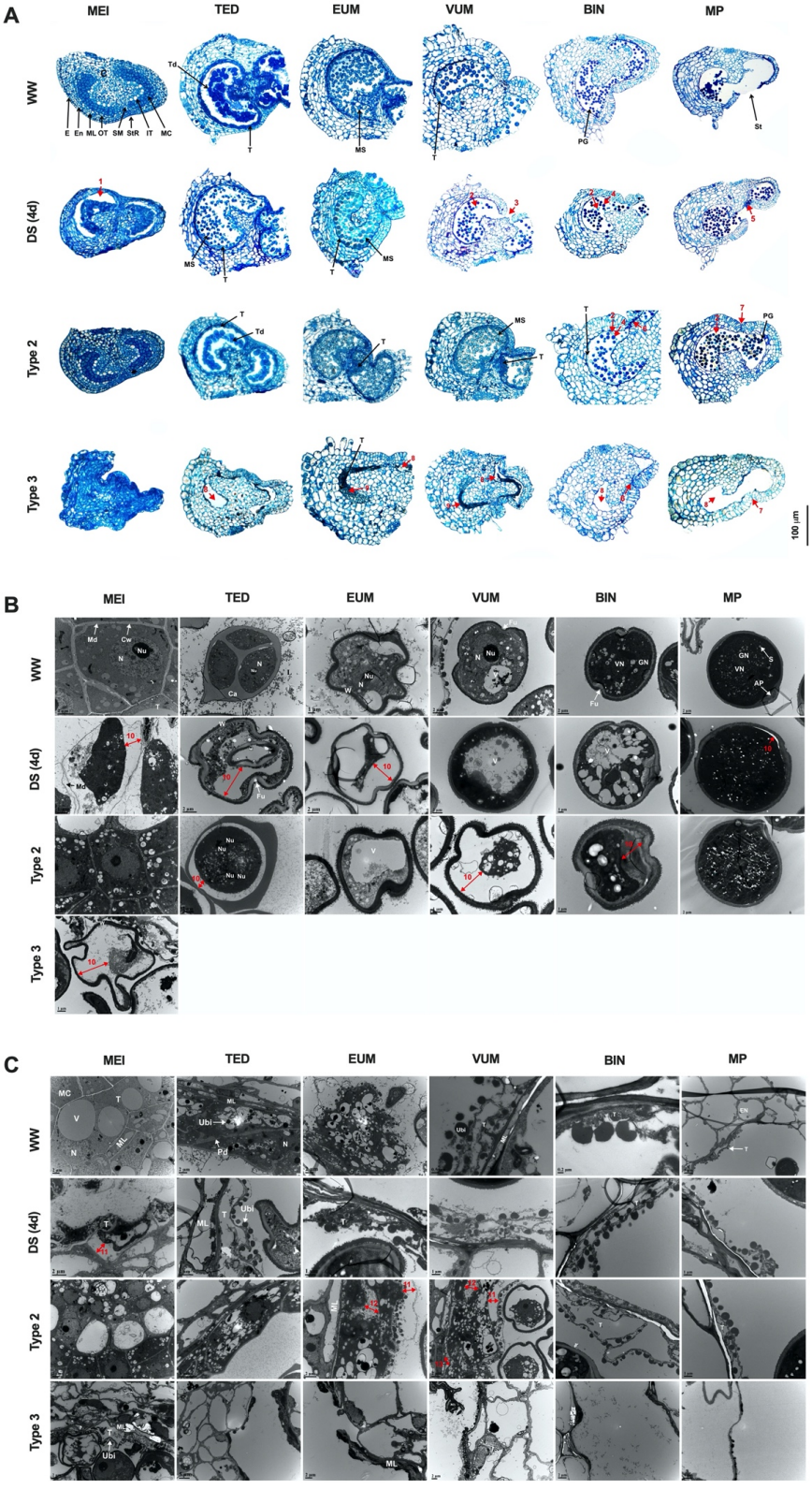

**Figure 3.** Comparison of the anther, tapetum and pollen development under drought stress and recovery conditions. **(A)**Cross-sections of anthers at six different stages of development under DS and recovery conditions. **WW** , anthers from well-watered plants; **DS (4 d),** anthers from drought-stressed plants after 4 d of DS; **type-2 and type-3** , anthers (DS at meiotic stage)from drought-stressed and rewatered plants between 1-11 and 1-12 days after rewatering respectively depending on the stage of development. MEI, meiotic; TED, tetrad; EUM, early uninucleate microspore; VUM, vacuolated uninucleate microspore; BIN, binucleate; MP, mature pollen; E, epidermis; En, endothecium; ML, middle layer; OT, outer tapetum; SM, septum; StR, stomium region; IT, inner tapetum; MC, meiotic cell; C, connective tissue; Td, tetrad; T, tapetum; MS, microspore; PG, pollen grain; St, stomium. **(B)** Transmission electron micrographs (TEMs) of developing pollen. Cw, cell wall; Nu, nucleolus; N, nucleus; Md, middle lamella; Ca, callose; W, pollen wall; V, vacuole: Fu, furrow; VN, vegetative nucleus; GN, generative nucleus; AP, aperture; S, starch granule. **(C)** TEMs of developing tapetum. ML, middle layer; Ubi, ubisch body; Pd, plasmodesma. Arrows: 1, gap between disengaged sporogenous and tapetum tissues; 2, aborted pollen; 3, precocious anther dehiscence; 4, abnormally enlarged pollen; 5, persistent stomium cells adhered to connective tissue; 6, collapsed anther; 7, persistent stomium cells adhered together; 8, empty locules; 9, debris of degenerated microspore; 10, gap between pollen wall and pollen protoplasm; 11, gap between tapetum wall and shrank tapetum cell protoplasm; 12, gap between two adjacent tapetum cells; 13, gap between tapetum and middle layer cells. Spots with the same number depict the same feature.

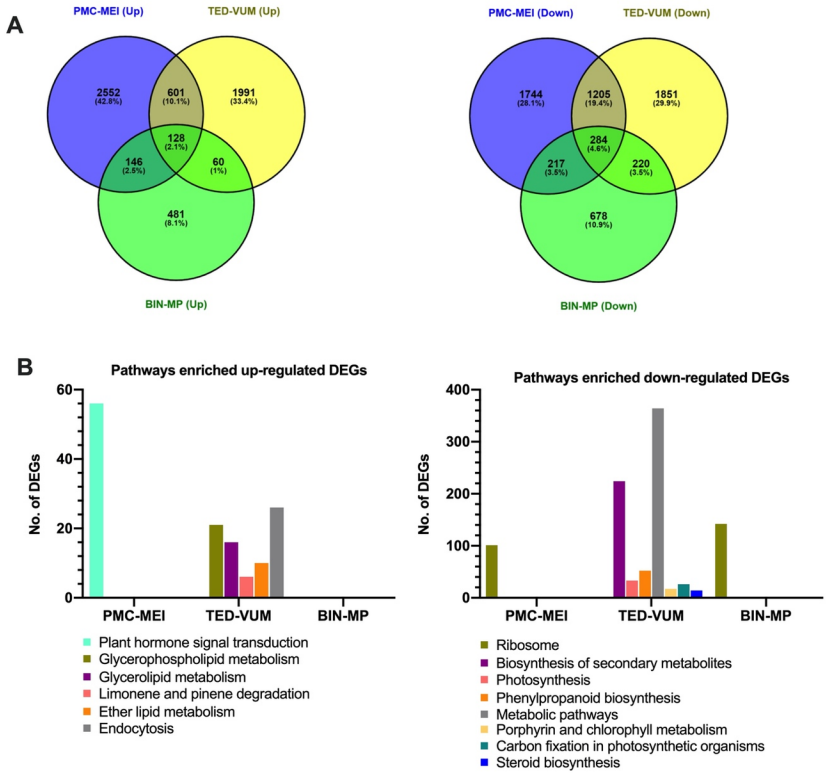


**Figure 4.** Drought-responsive genes in tomato anthers**(A)** Number of DEGs Up- and down-regulated by drought stress in tomato anthers at three stages of development. **(B)** KEGG pathways significantly enriched in DEGs Up- and down-regulated by drought stress in tomato anthers.

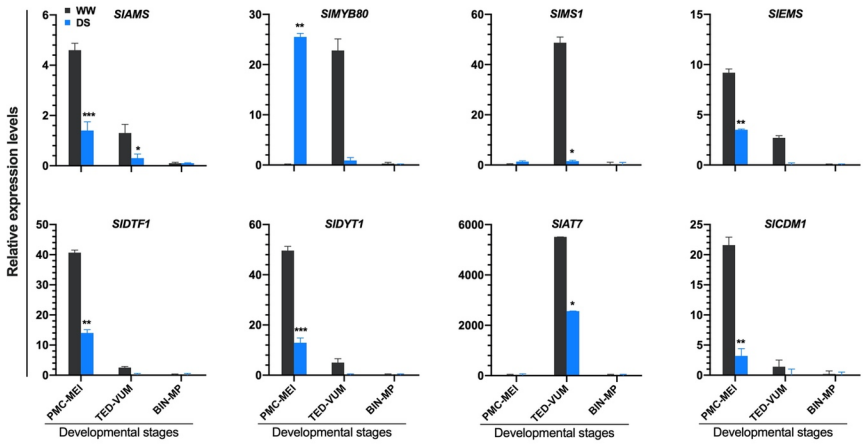


**Figure 5.** Drought induces changes in the expression of tapetum and pollen expressed genes in tomato anthers. Values are means $\pm SD$ ($n = 3$ replications, 24 plants each). * $p$<0.05; ** $p$ <0.01; *** $p < 0.001$ (t-test).

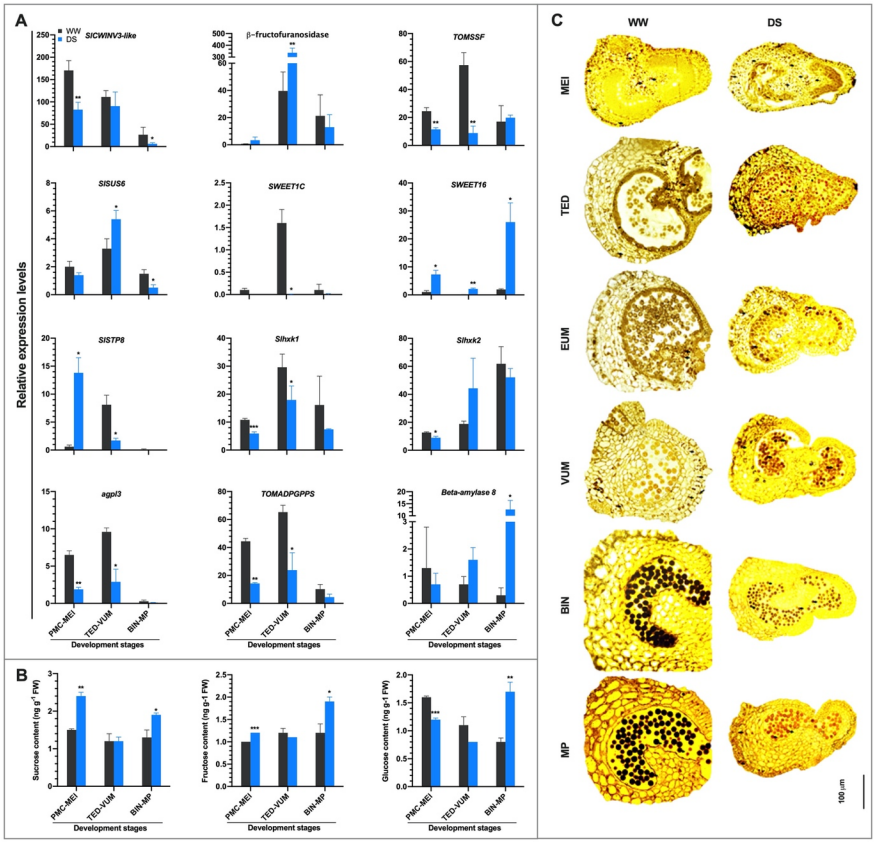


**Figure 6.** Comparison of sucrose and starch metabolism in developing anthers exposed to drought stress. **(A)** Expression analysis of genes involved in sucrose and starch metabolism, and sugar transport based on RNA-Seq data. **(B)** Sucrose, glucose, and fructose contents in anthers of DS plants. Anthers of equivalent

developmental stages were sampled from WW and DS plants 4 d after drought stress. Values are mean ± *SD* ($n$ = 3 replications, 24 plants each). *$p$ <0.05; **$p$ <0.01; ***$p$ < 0.001 (t-test). **(C)** Starch accumulation in tomato anthers determined by staining with Iodine Potassium Iodide (IKI) solution. **WW** , anthers from well-watered plants;**DS** , anthers from drought-stressed plants; **MEI** , anthers at meiotic stage; **TED** , anthers at tetrad stage; **EUM** , anthers at early uninucleate microspore stage; **VUM** , anthers at vacuolated uninucleate microspore stage; **BIN** , anthers binucleate stage; **MP** , anthers at mature pollen stage.

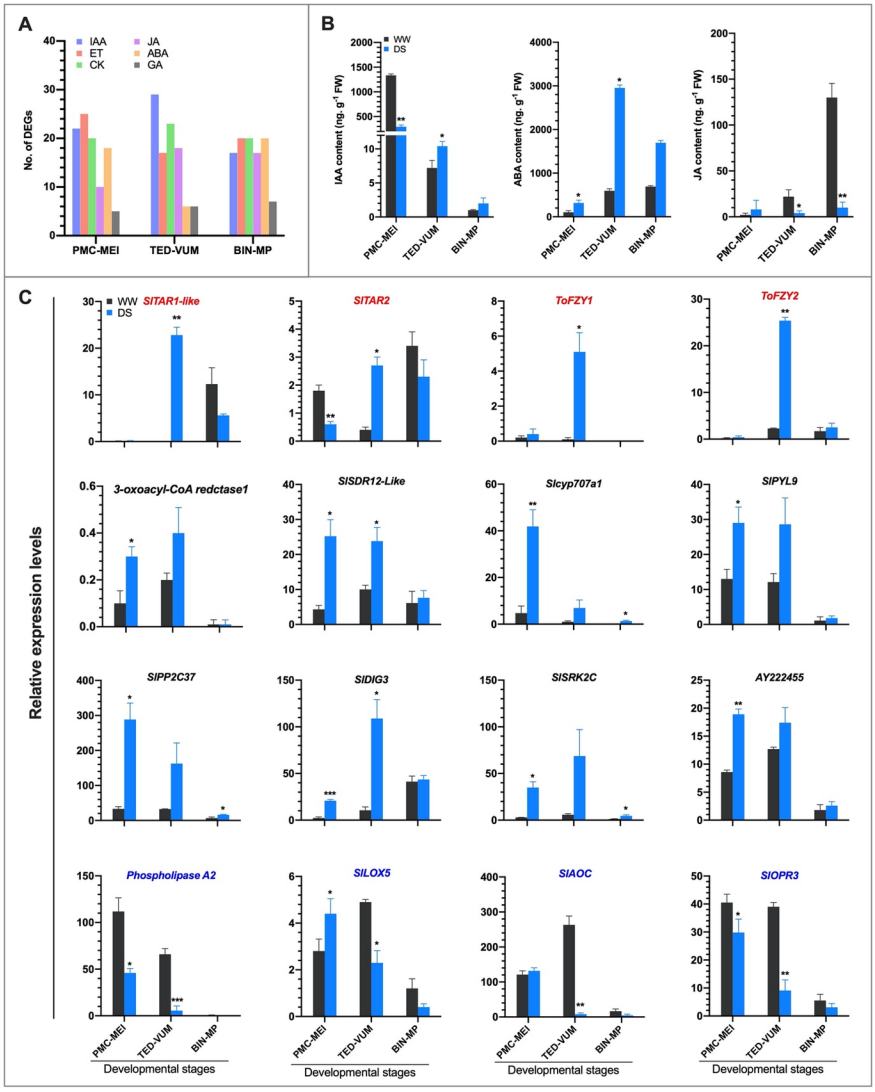


**Figure 7.** Drought stress induces alterations in IAA, ABA and JA metabolism and signaling in tomato anthers. **(A)** Distribution (%) of different phytohormones metabolic and signaling DEGs in tomato anthers. **(B)** Contents of IAA, ABA and JA in anthers 4 d after DS. **(C)** Expression analysis of genes involved in IAA (red titles), ABA (black titles) and JA (blue titles) metabolism and /or signaling in DS anther based on RNA-Seq data. Values are means ±*SD* ($n$ = 3 replications, 24 plants each). * $p$<0.05; **$p$ <0.01; ***$p$ < 0.001 (t-test).

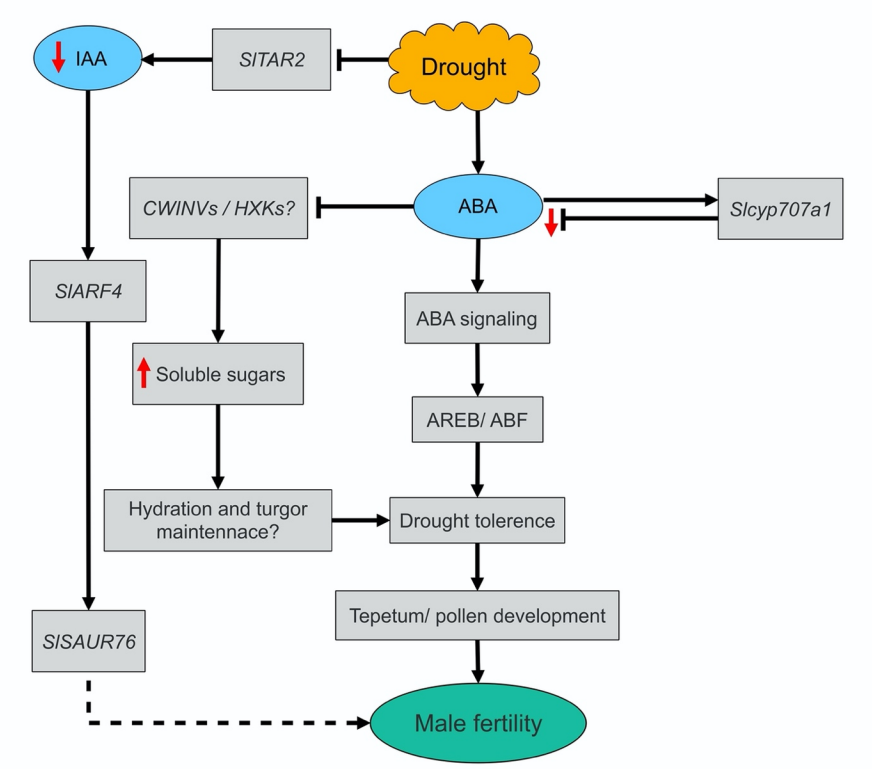


**Figure 8.** Propose model of drought tolerance mechanisms in tomato anthers during early stages of development. ABA: abscisic acid; IAA: indole-3-acetic acid; CWINVs: cell wall invertases; HXKs: hexokinases; AREB: abscisic acid responsive element binding protein; ABF: abscisic acid responsive element binding factor.