Cell Wall Invertase Promotes Fruit Set under Heat Stress by Suppressing ROS-Independent Cell Death

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Reduced cell wall invertase (CWIN) activity has been shown to be associated with poor seed and fruit set under abiotic stress. Here, we examined whether genetically increasing native CWIN activity would sustain fruit set under long-term moderate heat stress (LMHS), an important factor limiting crop production, by using transgenic tomato (Solanum lycopersicum) with its CWIN inhibitor gene silenced and focusing on ovaries and fruits at 2 d before and after pollination, respectively. We found that the increase of CWIN activity suppressed LMHS-induced programmed cell death in fruits. Surprisingly, measurement of the contents of H$_2$O$_2$ and malondialdehyde and the activities of a cohort of antioxidant enzymes revealed that the CWIN-mediated inhibition on programmed cell death is exerted in a reactive oxygen species-independent manner. Elevation of CWIN activity sustained Suc import into fruits and increased activities of hexokinase and fructokinase in the ovaries in a heat shock protein genes Hsp90 and Hsp100 in ovaries and HspII17.6 in fruits under LMHS, which corresponded to a lower transcript level of a negative auxin responsive factor IAA9 but a higher expression of the auxin biosynthesis gene ToFZY6 in fruits at 2 d after pollination. Collectively, the data indicate that CWIN enhances fruit set under LMHS through suppression of programmed cell death in a reactive oxygen species-independent manner that could involve enhanced Suc import and catabolism, HSP expression, and auxin response and biosynthesis.

Suc metabolism plays important roles in fruit and seed development through providing not only energy to power numerous cellular processes, but also substrates for synthesis of biopolymers such as starch and cellulose (Ruan, 2012). In parallel, Suc and hexoses derived from Suc degradation also act as signaling molecules to regulate gene expression in response to developmental and environmental cues (Wang et al., 2014; Wang and Ruan 2016). Physiologically, Suc degradation or utilization facilitates Suc translocation through the phloem from leaves to sinks such as developing fruits and seeds by lowering Suc concentration in the recipient sink cells (Ho, 1988; Ruan et al., 1995). There are two enzymes in plants to degrade Suc: Suc synthase (Sus) and invertase (INV). Sus is a glycosyl transferase and reversibly converts Suc in the presence of UDP into UDP-Glc and Fru. By contrast, INV is a hydrolase that irreversibly hydrolyses Suc into Glc and Fru. Based on its subcellular location, INV is classified into cell wall invertase (CWIN), vacuolar invertase (VIN), and cytoplasmic invertase (CIN; Sturm, 1999).

CWIN activity is essential for fruit and seed development and hence crop yield (Ruan, 2014). For example, mutation of the CWIN gene INCW2 in maize (Zea mays) reduced maize grain weight by 80% (Lowe and Nelson, 1946; Miller and Chourey, 1992). Silencing CWIN expression in tomato (Solanum lycopersicum) resulted in fruit and seed abortion (Jin et al., 2009; Zanor et al., 2009). Conversely, overexpression of CWIN in rice (Oryza sativa) driven by its native promoter enhanced grain filling and yield (Wang et al., 2008). An increase in CWIN transcription or activity is also commonly observed upon pathogen infection in a wide range of plant species (Benhamou et al., 1991; Scharte et al., 2005), indicating a role for CWIN in defense against pathogens, possibly through Glc signaling (Ruan, 2014). For instance, constitutive expression of a yeast (Saccharomyces cerevisiae) INV gene in the apoplasms increased tobacco (Nicotiana tabacum) resistance to potato virus Y...
(Herbers et al., 1996), whereas RNA interference (RNAi)-mediated repression of CWIN in tobacco compromised its response to infection by the oomycete Phytophthora nicotianae (Essmann et al., 2008).

CWIN may also have important roles in plant responses to abiotic stresses, such as heat and drought (Pressman et al., 2006; Boyer and McLaughlin, 2007). Recently, overexpression of a CWIN gene CIN1 from Chenopodium rubrum in tomato reduced floral and fruit abortion under salinity, likely through enhanced sink activity, which is attributed to increased sucrosyl metabolic activity and auxin and cytokinin levels (Albacete et al., 2014a, 2014b). These authors further found that the transgenic tomato lines exhibited higher water use efficiency under drought, in part owing to improved carbohydrate metabolic flux in the leaves (Albacete et al., 2015). With global warming, heat stress is becoming a more frequent event and consequently an important limiting factor for crop production (Fedoroff et al., 2015; Prasch and Sonnewald, 2013). Indeed, 2014 and 2015 were the warmest years since records began in 1880 (National Climatic Data Center 2015). In comparison to vegetative growth, plant reproductive development including floral development and fruit and seed set is particularly vulnerable to abiotic stresses (Ruan, 2014; Fragkostefanakis et al., 2016). For example, heat stress can cause 80% floral abortion in tomato (Ruan et al., 2010) and seed or grain abortion of up to 88% in rapeseed (Brassica napus; Young et al., 2004) and maize (Dupuis and Dumas, 1990). It has been predicted that the average annual temperature will increase 1.5°C to 5.8°C by 2100 (Zinn et al., 2010), and there will be approximately 2.5% to 16% crop yield loss globally for every 1°C increase in temperature (Battisti and Naylor, 2009). Thus, increasing fruit and seed set under heat stress is of critical importance for sustaining crop production in the face of climate change. To this end, CWIN could be a candidate target for exploring its potential to confer heat tolerance in reproductive organs as illustrated below.

In tomato, for example, cultivars with higher CWIN activity in anther and fruit had a lower fruit abortion rate under heat stress compared to cultivars with lower CWIN activity (Firon et al., 2006; Li et al., 2012), and an increase in CWIN activity was observed during ovary-to-fruit transition (Palmer et al., 2015). However, it remains to be seen whether genetically increasing CWIN activity can indeed improve fruit and seed set under heat stress and, if so, what is the underlying physiological and molecular basis.

Research on plant heat stress responses has, thus far, mostly focused on the effects of short-term severe heat stress (SSH), for example, 43°C to 45°C for 2 h in tomato (Frank et al., 2009) and 37°C for 3 h in Arabidopsis (Pecinka et al., 2010). However, compared to SSH, long-term moderate heat stress (LMHS) is a more frequent event under field conditions especially amid gradually increasing temperature associated with climate change (Sato et al., 2006). The response mechanisms of plants to LMHS may be drastically different from those to SSHS (Yeh et al., 2012; Hancock et al., 2014). For example, mitochondrial cochaperone MGE2 is required for the tolerance of Arabidopsis vegetative growth to LMHS (35°C for 9 d), but not for its response to SSHS (44°C for 150 min; Hu et al., 2012; Yeh et al., 2012). Despite its importance in developing strategies to improve crop adaptability to global warming, information on the effects of LMHS on plant development in general and on early reproductive development in particular remains scarce.

There are variations in optimal growth temperature among tomato cultivars (Rudich et al., 1977). The cultivar employed in the current study, XF-2, was originally bred for growth in the high-latitude area of Xinjiang Uyghur Autonomous Region (34°25′-48°10′N) in China. Thus, it prefers a cooler growth temperature. In our preliminary experiment, it was found that the temperature regime of 28/20°C (day/night, with daily average temperature of 24°C) incurred significant fruit abortion, as compared to the control temperature regime of 25/18°C (day/night, with daily average temperature of 21.5°C). These observations are consistent with reports that the optimal daily average temperature for tomato fruit set is at approximately 18 to 22°C (Adams et al., 2001), and a temperature regime at or exceeding 30/20°C (day/night) elicits flower abortion (Rudich et al., 1977; Adams et al., 2001). Based on these considerations, we chose 28/20°C as a moderate heat stress in this study. We aimed to evaluate the roles of CWIN in fruit set under LMHS (28/20°C versus a control temperature of 25/18°C) by using a transgenic tomato in which the endogenous CWIN activity is elevated through silencing its inhibitor (Jin et al., 2009). The analyses uncovered several important new findings, including (1) the increase in CWIN activity improved tomato fruit set under LMHS; (2) the elevation of CWIN activity inhibited LMHS-induced programmed cell death (PCD) in young fruits without affecting cell proliferation probably in a reactive oxygen species (ROS)-independent manner; and (3) the regulation of fruit set shifts from paternal control by pollen under severe heat stress to maternal control by ovary under LMHS.

RESULTS

CWIN-Elevated Transgenic Tomato Was Characterized by Sustained Fruit Set and Suc Import and Increased HXK and FXK Activities under LMHS

We first investigated the possible difference in fruit set and yield between wild-type and CWIN-elevated transgenic tomato. The tomato line used here is a determinate cultivar, which generally produces three trusses. Therefore, the fruit set and yield was investigated in the three trusses, which reflects yield phenotype at the whole plant level. Under optimal conditions, there was no difference in fruit set and fruit biomass, hence yield, between the two genotypes (Fig. 1, A–C). Under LMHS, however, fruit set was significantly reduced, resulting in reduction in whole fruit biomass in the wild-type plants (Fig. 1, A–C). By contrast, both parameters were sustained in the transgenic plants.
These data indicate an enhanced reproductive performance in the transgenic tomato under LMHS.

Previous studies have shown that pollen is more sensitive to heat stress than ovary/fruit, and thus pollen viability represents a bottleneck for fruit set and yield (Peet et al., 1998; Zinn et al., 2010). We thus tested whether this is the case under LMHS and whether the improved fruit set in the transgenic tomato resulted from the enhanced development of the pollen or of the ovary and fruit. It was found that LMHS did not decrease pollen viability in either wild-type or transgenic plants, and no difference was found between the two genotypes under LMHS conditions (Fig. 2A). Furthermore, the harvest of old fruits at 21 d after pollination (dap) in wild-type plants reactivated the growth and development of distal fruits in the truss under LMHS (Fig. 2B), which otherwise appeared to be stunted and thus considered to have aborted. Many tomato cultivars can form parthenocarpic fruits, especially under heat stress (Barringer et al., 1981; Sato et al., 2001). However, these reactivated fruits were not parthenocarpic since they contained normally developing seeds (Fig. 2C). These findings show that pollination and fertilization proceeded normally, and these processes are not the major limiting factors for fruit set under LMHS. This outcome indicates that the

Figure 1. Fruit set and biomass were reduced in the wild type but sustained in CWIN-elevated transgenic (RNAi) tomato under LMHS. A, Images of fruit trusses showing improved fruit set in the RNAi plants under LMHS in comparison with that of the wild type. B and C, Fruit set percentage and dry weight in the first three trusses of wild-type and RNAi tomato plants measured at 21 dap. Each value is the mean ± se of at least three biological replicates. In B and C, values with different letters indicate significant differences (P ≤ 0.05). LMHS (28/20°C, day/night, 14/10 h) was imposed when the first flower bud was 3 mm in length and kept for the rest of the life cycle versus a control temperature of 25/18°C.

Figure 2. LMHS did not affect pollen viability and reactivation of fruit and seed development following the harvest of older fruits. A, Pollen viability expressed as the percentage of viable pollen grains based on fluorescein diacetate (FDA) staining. B, Reactivated fruits in the distal end of the truss after the harvest of older fruits at 21 dap in wild-type plants. Arrows indicate the peduncle sites of removed older fruits. Asterisks indicate the reactivated fruits. C, Transverse sections of the reactivated fruits in B, showing the developing seeds (arrows). Bars = 1.0 and 0.5 cm in B and C, respectively. Each value in A is the mean ± se of four biological replicates. No significant difference was found between the two temperature regimes or between the wild-type and RNAi plants.
improved fruit set in transgenic tomato under LMHS is a function of maternal effect. Based on this conclusion, further experiments were focused on ovary and fruit development.

It has been reported that CWIN activity was nearly doubled in 10 dap fruits of the transgenic tomato in which the CWIN inhibitor \textit{INVINH1} was silenced using an RNAi approach (Jin et al., 2009). Measurement of enzyme activities in ovaries at 2 d before pollination (dbp) and fruits at 2 dap, the key stages determining fruit set and yield potential (Palmer et al., 2015), revealed there was a 35% to 41% increase of CWIN activity in the transgenic 2-db.p ovaries and 2-dap fruits under both control and LMHS conditions compared with that from wild-type plants (Fig. 3A). The data analyses were conducted for the ovary and fruit stages separately to ensure a direct comparison between the wild-type and transgenic plants. There was no equivalent difference between the transgenic and wild type in the activities of VIN, CIN, andSus (Supplemental Fig. S1). These results suggest the enhanced fruit set under LMHS in transgenic tomato is contributed by the increase in CWIN activity rather than other Suc-degrading enzymes. The elevated CWIN activity was largely achieved through suppressing expression of the CWIN inhibitor gene \textit{INVINH1} in the RNAi plants (Supplemental Fig. S2A) without affecting the ovary mRNA levels of \textit{LIN5} and \textit{LIN6} (Supplemental Fig. S2, B and C), two CWIN genes expressed strongly and weakly, respectively, in tomato fruit (Fridman and Zamir, 2003). The only exception is that the transgenic plant exhibited a higher level of \textit{LIN5} mRNA in the 2-dap fruits than that of wild type under LMHS (Supplemental Fig. S3B). These data are overall consistent with our previous report on the transgenic tomato plants in which the elevation of CWIN activity was achieved at the posttranslational level due to reduced CWIN inhibitor mRNA level and consequently its protein abundance, thereby releasing extra endogenous CWIN activity (Jin et al., 2009). CWIN could promote fruit and seed set through increasing sink strength and thus facilitating Suc import (Ruan et al., 2012). The import rate of Suc into 2-dap fruits was then evaluated according to Li et al. (2012). After excising the fruits, the exudate from the pedicel attached to the plant was collected, and Suc in the exudate was quantified spectrophotometrically. It was found that the Suc import rate into 2-dap fruit was sustained in the CWIN-elevated transgenic plants but significantly reduced in the wild-type plants under LMHS (Fig. 3B). However, increased CWIN activity did not affect photosynthesis and total plant biomass (Supplemental Fig. S3), indicating that CWIN promotes fruit set through increasing sink strength without significant impact on source activity.

Surprisingly, although CWIN activity and Suc import were promoted in the transgenic fruits, there were no significant differences between wild-type and transgenic plants in Suc, Glc, and Fru levels under both control and LMHS conditions (Supplemental Fig. S4). One possible explanation for this phenomenon is that sugar metabolic fluxes could be promoted in the transgenic ovary/fruit under LMHS, as recently shown in CWIN-overexpressed tomato leaves under drought (Albacete et al., 2015). Therefore, the activities of hexose kinases (HXKs) were measured. HXK and, to a lesser extent fructokinase (FRK), are central to sugar metabolism and signaling. Interestingly, the activities of both enzymes were similar between the two genotypes in 2-db.p ovaries under control conditions (Fig. 3, C and D). However, their responses to LMHS were different, with the transgenic plants displaying increased HXK and FRK activities in 2-db.p ovaries and increased FRK activity in 2-dap fruits, a response absent from the wild-type plants (Fig. 3, C and D).

Figure 3. Effects of LMHS on CWIN activity, Suc import rate, and activities of HXK and FRK in wild-type (WT) and CWIN-elevated transgenic (RNAi) tomato ovaries and fruits. A, CWIN activity in ovaries of 2 dbp and fruits of 2 dap. B, Suc import rate into 2-dap fruits. C, HXK activities in 2-db.p ovary and 2-dap fruit. D, FRK activities in the same samples in C. Each value is the mean ± se of four biological replicates. Values with different letters indicate significant differences (P ≤ 0.05). The data from 2-db.p ovaries and 2-dap fruits were analyzed separately as indicated by the dash line in A, C, and D.
Elevation of CWIN Activity Suppressed LMHS-Induced PCD in Fruit without Affecting Cell Proliferation

In broad terms, fruit abortion under heat stress could result from the activation of PCD and the repression of cell division and expansion (Ruan et al., 2012; Liu et al., 2013). To test whether LMHS induced PCD, we conducted terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which detects the cleavage of nuclear DNA, a hallmark of PCD or apoptosis (Wang and Ruan, 2016). As a negative control, the terminal deoxynucleotidyl transferase was omitted from the TUNEL reaction mixture. As expected, no PCD signals were detected from the treated pericarp sections of wild-type 2-dap fruits under LMHS (Supplemental Fig. S5B). In contrast, massive fluorescent PCD signals were detected in the pericarp and placenta regions of the positive control, in which the sections were treated with DNase I prior to TUNEL assay (Supplemental Fig. S5E). Further labeling on the same sections with 4',6-diamidino-2-phenylindole (DAPI) revealed the locations of the nuclei (Supplemental Fig. S5, C, F, I, and L). Under high magnification, it is clear that the PCD signals were located in nuclei (Supplemental Fig. S5, G versus H). Having established the TUNEL assay, we then examined possible PCD in 2-dap fruits under both normal and LMHS conditions. No PCD signals were detected in the samples from either wild-type or transgenic plants under the control condition (Supplemental Fig. S6). Under LMHS, however, the fluorescent PCD signal was detected in the inner pericarp in three of the four wild-type fruits examined (Fig. 4B) but with no PCD signal detected in all four CWIN-elevated transgenic fruits (Fig. 4E). Extensive PCD signals were also detected in the fruit placenta and the lower part of the seeds connecting it in three of four wild-type fruits (Fig. 4H). By contrast, only a few sporadic signals were observed in placenta and seed joint regions in one of the four transgenic fruits tested (Fig. 4K). Interestingly, no PCD signal was found in the columella region or outer pericarp under LMHS. The same sections were relabeled with DAPI to show the presence of the nuclei (Fig. 4, C, F, I, and L), which were not overlayed with images of the TUNEL-assay because the strong DAPI signal masked or blurred the PCD signals. Nevertheless, the presence of nuclei is clear and appeared to be colocalized with the TUNEL signals (Supplemental Fig. S5, G versus H). We did not detect any PCD signals in 2-dbp ovaries in either wild-type or transgenic plants under normal or LMHS conditions.

In plants, the transcripts of nuclease and protease genes are often up-regulated during PCD; thus, they are referred to as PCD-related genes (Bozhkov and Jansson, 2007; Aleksandrushkina and Vanyushin, 2009). In tomato, the mRNA levels of the nuclease gene TBN1, the Cys protease gene RD19α, and the Ser protease gene SBT3 were increased during PCD in the abscission zone of flowers (Bar-Dror et al., 2011). Cupin domain protein gene pirin is also a PCD-related gene, which was
up-regulated during camptothecin-induced PCD in tomato suspension cells (Orzaez et al., 2001). The mRNA levels of these four PCD-related genes were thus measured using qRT-PCR. It was found that, in comparison to the wild type, the CWIN-elevated 2-dap transgenic fruits (RNAi) exhibited significantly lower mRNA levels for TBN1 and SBT3 under control conditions and for all the four PCD genes under LMHS (Fig. 5). Moreover, LMHS increased the mRNA levels of SBT3 and RD19a in the wild-type fruits but not in CWIN-elevated transgenic plants (Fig. 5, B and C). It is noteworthy that the transcript levels of the four PCD-related genes were similar in 2-dbp ovaries between wild-type and transgenic plants under LMHS (Fig. 5). Furthermore, LMHS had no effect on the expression of these genes in 2-dbp ovaries.

Changes in CWIN activity may affect cell division as reported in developing seeds of *Vicia faba* (Weber et al., 1996) and the endosperm of maize CWIN mutant (INCW2; Vilhar et al., 2002). Our analyses revealed no difference in cell number and cell size of 2-dbp ovaries or 2-dap fruits between the transgenic and wild-type plants under both control and LMHS conditions, and LMHS had little effect on cell number or size in these organs (Supplemental Fig. S7). Consistent with these observations, no difference was found at the mRNA levels for a set of selected cell cycle genes between the wild-type and transgenic plants under LMHS conditions (Supplemental Fig. S8). These genes include two cyclin-dependent kinase (CDK) genes, *CDKA1* and *CDKB1:1*, and three cyclin genes, *CycA2;1*, *CycB2;1*, and *CycD3;1*, which are relatively highly expressed in the early stage of tomato fruit development (Joubès et al., 1999, 2000, 2001; Kvarnheden et al., 2000).

**Figure 5.** Effect of LMHS on expression levels of PCD-related genes in wild-type and CWIN-elevated transgenic (RNAi) tomato. A, Transcript levels of the nuclease gene *TBN1* in ovary of 2 dbp and fruit of 2 dap. B to D, Transcript levels of the Cys protease gene *RD19a* (B), Ser protease gene *SBT3* (C), and cupin-domain protein gene *pirin* (D) from the same samples as in A. Each value is the mean ± se of four biological replicates. The data from 2-dbp ovaries and 2-dap fruits were analyzed separately. Values with different letters (a–c) indicate significant differences (*P* ≤ 0.05).

**LMHS Did Not Elicit Oxidative Responses, and Elevation of CWIN Activity Had No or Little Effect on Antioxidant Enzyme Activities and ROS Content in Tomato Ovary or Fruit**

Heat stress often leads to excessive accumulation of ROS, causing oxidative damage of DNA, proteins, and lipids, which triggers PCD and consequently the abortion of fruit and seed (Vaccà et al., 2004; Laloi et al., 2004; Foyer and Noctor, 2005). To evaluate the possible involvement of oxidative stress in PCD and tomato fruit abortion under LMHS, some major biochemical markers of the oxidative response were targeted for analyses. These include the activities of three key antioxidant enzymes and the H₂O₂ content (Sunkar et al., 2003, 2006). In plants, enzymatic removal of ROS is mainly executed through the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT; Smirnoff, 1993), and their activities are often induced by severe heat stress (Liu et al., 2013). Surprisingly, LMHS had no effect on the activities of all three enzymes in either wild-type or transgenic 2-dbp ovaries (Fig. 6, A–C). Similar results were found in 2-dap fruits, except for the SOD activity, which was slightly reduced by LMHS in transgenic fruits (Fig. 6A). Significantly, no difference was observed between the wild-type and transgenic plants in the activities of these three enzymes in ovary or fruit under LMHS (Fig. 6, A–C).

ROS in plants comprises H₂O₂, singlet oxygen (¹⁰O₂), superoxide (O₂⁻), and the hydroxyl radical (OH⁻). Among them, H₂O₂ is thought to be relatively stable and abundant (Bienert et al., 2007) and thus the most studied ROS species (Liu et al., 2013). LMHS had no significant effect on H₂O₂ content in wild-type 2-dbp ovaries and 2-dap fruits, nor in 2-dap transgenic fruits.
A mild increase in H$_2$O$_2$ content, however, was observed in the 2-dbp ovaries in the transgenic plants following the LMHS treatment (Fig. 6D). Importantly, no difference was observed in H$_2$O$_2$ content between wild-type and transgenic ovaries or fruits under either control or LMHS conditions (Fig. 6D). Although the transgenic and wild-type plants had a similar H$_2$O$_2$ content in extracts from ovaries/fruits under LMHS, there might be a difference in cellular distribution of H$_2$O$_2$ between the two genotypes. We therefore conducted 3, 3'-diaminobenzidine-based tissue printing on sections from the ovaries and fruits to localize H$_2$O$_2$ as described by Liu et al. (2014). The analyses did not detect any visible differences in H$_2$O$_2$ distribution at the cellular level between wild-type and transgenic 2-dbp ovaries or 2-dap fruits, irrespective of the temperature conditions (Supplemental Fig. S9, A–H). Interestingly, H$_2$O$_2$ was evenly distributed in 2-dbp ovaries and 2-dap fruits (Supplemental Fig. S9, A–H) but became tissue specific in 10-dap fruits in which H$_2$O$_2$ was abundant in the pericarp, seed, and septum, but not in the locale region (Supplemental Fig. S9, K and L), indicating a developmental control on the H$_2$O$_2$ cellular distribution. There also appeared to be a decrease in H$_2$O$_2$ intensity during the ovary-to-fruit transition under both control and LMHS conditions (Supplemental Fig. S9, A–D versus E–H).

Due to the presence of different ROS in plant cells, the profile of H$_2$O$_2$ content and localization may not necessarily reflect the actual redox status in the tissue. Therefore, we measured malondialdehyde (MDA), which is the end-product of lipid peroxidation caused by ROS and hence is an excellent marker for overall oxidative stress and damage (Sunkar et al., 2003; Liu et al., 2013). Again, no difference was observed in the MDA content of 2-dbp ovaries or 2-dap fruits between wild-type and transgenic plants under either control or LMHS conditions, and LMHS did not elicit an increase of MDA content in these organs (Fig. 6E).

The data above indicate that LMHS did not elicit significant oxidative responses in tomato ovaries/fruits. This result differs from scenarios under severe heat stress, which often causes oxidative stress and damage to plant cells (Vacca et al., 2004; Laloi et al., 2004; Foyer and Noctor, 2005; De Pinto et al., 2012). To validate the findings obtained under LMHS (Fig. 6), the H$_2$O$_2$ and MDA contents were measured in 2-dbp ovaries after a 24-h treatment of SSHS (36/30°C). The analyses showed...
significant increases in both the H$_2$O$_2$ and MDA contents of wild-type and transgenic ovaries (Fig. 7). Noticeably, the H$_2$O$_2$ content increased to a lesser extent in transgenic ovaries than in those of wild-type plants. Consistent with activity assays (Fig. 6), the expression of antioxidant enzyme genes SOD2, APX2, and CAT2 was not increased by LMHS in either wild-type or transgenic ovaries/fruits (Fig. 8, A–C). Under SSHS, however, the transcript levels of these three genes were significantly increased in wild-type 2-dbp ovaries (Fig. 8, D–F). In the transgenic ovaries, however, only the expression of CAT2 was increased by SSHS (Fig. 8F). Clearly, SSHS caused a significant antioxidant response in wild-type tomato ovaries, and the elevation of CWIN activities appeared to have alleviated some of these responses. Collectively, the data (Figs. 6–8; Supplemental Fig. S9) show substantial differences in the response of ovaries and fruits to SSHS and LMHS, with the former, but not the latter, inducing an oxidative response at the level of gene expression, enzyme activity, and ROS content.

**Figure 7.** SSHS (36/30°C) elicited significant increases in the content of H$_2$O$_2$ and MDA in 2-dbp ovaries in both wild-type and CWIN-elevated transgenic (RNAi) tomato. A, H$_2$O$_2$ content in 2-dbp ovaries after 24-h treatment of SSHS. B, MDA content in the same samples. Each value is the mean ± se of four biological replicates. Values with different letters indicate significant differences (P ≤ 0.05).

**CWIN-Elevated Transgenic Tomato Exhibited a Sustained or Increased Expression of Genes Encoding Heat Stress Transcription Factors and Heat Shock Proteins under LMHS**

Heat stress transcription factors (Hsfs) and heat shock proteins (Hsps) are central in conferring heat tolerance (Saidi et al., 2011; Hahn et al., 2011). Previous studies in tomato have identified three Hsf genes (HsfA1, HsfA2, and HsfB1) and four Hsp genes (Hsp100, Hsp90, Hsp70, and HspII17.6) that play important roles in heat stress response (Mishra et al., 2002; Chang-Schaminet et al., 2009; Giorno et al., 2010; Hahn et al., 2011; Li et al., 2012). Thus, the transcript levels of these genes were measured to determine if elevation of CWIN affects them under LMHS. Although the mRNA levels of the three Hsf genes were similar between wild-type and transgenic ovaries/fruits under LMHS, their responses to LMHS were different (Fig. 9, A–C). For example, their transcript levels in 2-dbp ovaries were reduced by LMHS in the wild-type 2-dbp ovaries but were unaffected in the transgenic ones (Fig. 9, A–C). Within the 2-dap fruits, the transcript levels of HsfA2 were increased by LMHS in the transgenic plants but remained unchanged in wild-type plants (Fig. 9B). The qRT-PCR analyses also revealed that the transcript levels of Hsp90 and Hsp100 were decreased in 2-dbp ovaries by LMHS in wild-type plants but were unaffected in transgenic plants, which led to their higher levels in transgenic plants under LMHS (Fig. 9, D and E). Within the 2-dap fruits, the mRNA levels of HspII17.6 and Hsp100, but not Hsp90, were increased by LMHS in both wild-type and transgenic plants (Fig. 9, D–F). The CWIN-elevated transgenic plants exhibited a significantly higher transcript level of HspII17.6 in 2-dap fruits relative to the wild-type plants under LMHS, because LMHS increased its mRNA levels more in the transgenic plants (Fig. 9F). The mRNA level of Hsp70, however, was similar between the wild-type and transgenic ovaries/fruits under both conditions (Supplemental Fig. S10).

**Elevation of CWIN Activity Reduced the Expression of Negative Auxin Responsive Gene IAA9 and Sustained the Expression of Auxin Biosynthesis Genes ToFZY1 and ToFZY6**

Auxin plays important roles in fruit set and development, and there is compelling evidence of crosstalk between sugar- and auxin-signaling pathways (LeClere et al., 2010; Wang and Ruan, 2016). Therefore, we finally investigated the transcript levels of genes involved in auxin response and biosynthesis. This analysis includes two auxin-responsive genes (IAA9 and ARF7) and two FZY genes (ToFZY1 and ToFZY6). IAA9 and ARF7 are transcription factors negatively regulating the auxin response and signaling during tomato fruit set (Wang et al., 2005; De Jong et al., 2009), whereas the two tomato FZY genes are YUCCA-like genes encoding flavin monooxygenases, which are rate-limiting enzymes in...
the synthesis of indole-3-acetic acid (IAA; Expósito-Rodríguez et al., 2011). The analyses revealed a significantly lower level of IAA9 mRNA in transgenic tomato than that in the wild type in 2-dbp ovaries and 2-dap fruits under both control and LMHS conditions (Fig. 10A) but with no difference detected for the transcript level of ARF7 between the two genotypes (Fig. 10B). ToFZY1 and ToFZY6 are highly expressed in young tomato fruits (Expósito-Rodríguez et al., 2011). LMHS reduced the transcript levels of ToFZY1 in 2-dbp ovaries and ToFZY6 in 2-dap fruits in the wild type but not in the CWIN-elevated transgenic plants (Fig. 10, C and D).

DISCUSSION

The roles of CWIN in plant responses to biotic stresses have been widely acknowledged largely based on transgenic studies (Benhamou et al., 1991; Herbers et al., 1996; Essmann et al., 2008; Sun et al., 2014). Its potential role in abiotic stress responses, however, remains less certain. Nevertheless, observations on positive correlations between CWIN gene expression or activity and grain and fruit set under abiotic stress (e.g., Boyer and McLaughlin 2007; Li et al., 2012) have led to the suggestion that CWIN may be a major player conferring tolerance of reproductive organs to abiotic stress (Ruan et al., 2012; Ruan, 2014). In tomato, reduced CWIN, CIN, and Sus activities were associated with fruit -yield reduction under salinity (Balibrea et al., 1999). A genotypic comparison indicates that CWIN is important for tomato fruit development under salinity through sustaining sink activity and growth (Balibrea et al., 2003), a view supported by recent findings from ectopic over-expression of a foreign CWIN gene in both tomato fruits and leaves under salinity (Albacete et al., 2014b). Here, we tested whether increase of native CWIN activity may confer tolerance to LMHS by using transgenic tomato plants in which the endogenous CWIN activity is elevated through silencing its inhibitor. This approach avoids the potential complication of misexpression of the transgene in tissues or cells that otherwise do not express the corresponding endogenous gene, a problem often resulting in pleiotropic effects (e.g., Jin et al., 2009; Fragkostefanakis et al., 2016). We found that tomato fruit set was sustained under LMHS in the CWIN-elevated tomato but dramatically reduced in wild-type plants (Fig. 1, A–C), substantiating the view that CWIN exerts positive effects on fruit set and growth under heat stress. Further analyses...
revealed that the positive effect is likely achieved through suppressing PCD and altering the expression of genes involved in heat shock response and auxin signaling and biosynthesis. Strikingly, in contrast to that observed under SSHS, the impact under LMHS appears to be exerted independent of oxidative response and paternal effect. Together, our data provide a new understanding of how CWIN-mediated Suc metabolism could regulate fruit set under LMHS, a major limiting factor for crop yield during climate change.

CWIN Promotes Fruit Set under LMHS by Increasing Sink Strength without Affecting Leaf Photosynthesis

Severe heat stress often dramatically reduces leaf photosynthesis (Camejo et al., 2005). Thus, it is important to determine whether decreased fruit and seed set by heat stress is due to source or sink limitation. Unlike severe heat stress, moderate heat stress generally has no detrimental effect on photosynthesis (Lafta and Lorenzen, 1995; Yeh et al., 2012; Hancock et al., 2014). Consistently, we found that LMHS adopted in this study also had no effect on the leaf photosynthesis and total plant biomass in either wild-type or transgenic tomato (Supplemental Fig. S3), indicating that tomato fruit set under LMHS is likely sink limited. This is reflected by a higher Suc import rate in CWIN-elevated fruits under LMHS (Fig. 3B) and enhanced expression of two YUCCA-like auxin biosynthesis genes and reduced expression of a negative auxin signaling gene IAA9 in the CWIN-elevated ovaries and fruits (Fig. 10). Elevated CWIN Activity Enhances Tomato Fruit Set under LMHS, Possibly through Fueling Heat Stress Response and Enhancing Auxin Signaling and Biosynthesis

It is well established that CWIN-mediated Suc catabolism plays key roles in sink development, in part through facilitating photoassimilate import to sink organs (Cheng et al., 1996; Sturm and Tang, 1999; Ruan et al., 2010). For instance, down-regulation of CWIN expression in carrot (Daucus carota) reduced the partitioning of photoassimilates to tap roots, leading to a small root phenotype (Tang et al., 1999). Compared to a heat-sensitive tomato cultivar, a heat-tolerant genotype had higher CWIN activity in flowers and young fruits, which corresponds to a higher Suc import rate to...
fruitlets (Li et al., 2012). The causality between CWIN activity and heat tolerance, however, has not been established in these previous studies. Here, we show that although Suc import rate into 2-dap fruits was similar between the CWIN-elevated and wild-type plants, it is sustained in the former but reduced in the latter under LMHS (Fig. 3, A and B), indicating that elevation of CWIN activity facilitates Suc partitioning into fruits under LMHS. Along with enhanced Suc import, hexose phosphorylation may also be improved in transgenic tomato under LMHS, as reflected by small but significant increases in HXK and FRK activities in 2-dbp ovaries and FRK activity in 2-dap fruits in transgenic tomato under LMHS, a response not found in the wild-type tomato (Fig. 3, C and D). These data suggest that Glc and Fru phosphorylation for subsequent sugar and energy metabolism may be promoted in the CWIN-elevated transgenic plants under LMHS, which may increase the metabolic carbohydrate fluxes, rendering no increase in bulk hexose content observed in the CWIN-elevated tomato under LMHS (Supplemental Fig. S4). A similar scenario was recently found in tomato leaves in which over-expression of a CWIN gene did not affect leaf hexose content but increased the levels of Glc-6-P and Fru-6-P, leading to a delay in leaf senescence under drought (Albacete et al., 2015). Hsfs and Hsps play important roles in the heat response of plants through preventing the misfolding of protein and regulating the intracellular transport and degradation of proteins (Vierling, 1991; Saidi et al., 2011; Hahn et al., 2011). For example, cosuppression of HsfA1 in tomato led to extreme sensitivity of plants and fruits to elevated temperatures (Mishra et al., 2002). The other two Hsf proteins, HsfA2 and HsfB1, have also been revealed to play positive roles in heat tolerance of tomato through forming a complex with HsfA1 (Chan-Schaminet et al., 2009; Giorno et al., 2010; Hahn et al., 2011). A recent study showed HsfA2 acts as a coactivator of HsfA1 to control the transcription of heat response genes, including some Hsps, thereby conferring pollen thermotolerance (Fragkostefanakis et al., 2016). Synergistically, constitutive overexpression of Hsp17.7 in carrot (Malik et al., 1999) or Hsp101 in Arabidopsis (Arabidopsis thaliana; Queitsch et al., 2000) increased its heat tolerance. Our analyses showed that the transcript levels of HsfA1, HsfA2, HsfB1, and Hsp90 were reduced in 2-dbp wild-type ovaries but were sustained in the CWIN-elevated ovaries (Fig. 9, A–D). Within the 2-dap fruits, HsfA2 exhibited an increase in its mRNA level in response to LMHS in the CWIN-elevated plants, but not in the wild-type, and HspII17.6 displayed a greater degree of increase in its transcript level in the transgenic plants than that in the wild-type under LMHS (Fig. 9, B and F). The sustained or enhanced expression of Hsfs and Hsps by CWIN may reflect the transgenic ovaries or fruits being better adapted to LMHS. Given that the heat stress response, including the synthesis of Hsfs and Hsps, is an energy-expensive metabolic process (Finka et al., 2012; Mittler et al., 2012), it is conceivable that the sustained Suc import and increased HXK and FKX activities (hence Glc and Fru phosphorylation) observed in the CWIN-elevated transgenic plants (Fig. 3) may contribute to heat tolerance through fueling the synthesis of Hsfs and Hsps under LMHS (Fig. 11). Apart from its central role in sugar metabolism, HXK could also act as a sugar sensor to modulate development through crosstalk with other processes, including auxin signaling (Moore et al., 2003; Sheen, 2014 and refs. therein). Suc and Glc have also been shown to promote auxin biosynthesis (Wang and Ruan, 2013).
Thus, the sustained Suc import and enhanced HXK activity in the CWIN-elevated plants under LMHS (Fig. 3) may also promote tomato fruit set through impacting on auxin signaling and synthesis pathways. Indeed, compared to that in the wild type, the transcript level of IAA9, a transcription factor negatively regulating auxin responses in tomato (Wang et al., 2009), was significantly lower in 2-dbp ovaries and 2-dap fruits in the CWIN-elevated plants under both control and LMHS conditions (Fig. 10A). This result suggests an enhanced response to auxin in the transgenic ovaries and fruits. Furthermore, while LMHS decreased the expression of YUCCA-like auxin biosynthesis genes ToFZY1 in 2-dbp ovaries and ToFZY6 in 2-dap fruits in the wild type, their transcript levels were sustained in the CWIN-elevated transgenic plants (Fig. 10, C and D), implying a more steady homeostasis of auxin, a major hormone known to stimulate fruit set directly or indirectly (Dorcey et al., 2009; Ruan et al., 2012). Given the altered mRNA levels of the auxin responsive gene, IAA9 and YUCCA-like auxin synthesis genes ToFZY1 and ToFZY6 as discussed above, and the role of auxin in cell division (Wang and Ruan, 2013), it was somehow surprising that no difference was observed in cell number or size in ovaries and fruits between the two genotypes (Supplemental Fig. S7). One reason for this phenomenon is perhaps because the activity of cell division and expansion in tomato fruit are very low at 2 dbp and 2 dap ( Tanksley, 2004). Whether a change in cell number or size occurs between the transgenic and wild type in a later stage of fruit development remains to be determined.

Elevated CWIN Activity Inhibited PCD in a ROS-Independent Manner under LMHS

Abiotic stress-induced fruit and seed abortion may result from the inhibition of cell division or activation of PCD or a combination of both (Ruan et al., 2012). Association of low CWIN activity with expression of some PCD genes has been previously observed. For example, Suc feeding restored the expression of a CWIN gene, ZmIncw2, and a VIN gene, ZmiVer2, in maize kernel under drought, which correlated with reduced expressions of PCD genes ZmRIP2 and ZmPLD1, encoding ribosome-inactivating protein and phospholipase D, respectively (Boyer and McLaughlin, 2007). Similarly, a heat-tolerant tomato genotype exhibiting high CWIN activity in young fruits also had low expression of SPLDa1, a paralog of ZmPLD1 (Li et al., 2012). We demonstrated that genetic increase in CWIN activity indeed suppressed the expression of a cohort of PCD genes in 2-dap tomato fruits, encoding nuclease TBN1, Ser protease SBT3, Cys protease RD19a, and cupin-domain protein, pirin (Fig. 5), leading to an inhibition

Figure 11. A schematic model illustrating how elevation of CWIN activity could enhance tomato fruit set under LMHS. Elevated CWIN activity by silencing its inhibitor promoted Suc import into and metabolism and signaling within tomato ovaries and fruits, which enhanced fruit set under LMHS. The positive effect may be achieved through suppressing the expressions of PCD-related genes and hence PCD and promoting heat response (i.e. increasing the expression of genes encoding Hsfs and Hsps) and enhancing auxin biosynthesis and signaling. Surprisingly, elevated CWIN activity and LMHS had no effect on H$_2$O$_2$ and MDA content, indicating CWIN inhibited PCD in an ROS-independent manner under LMHS, which is different from the scenario under severe heat stress. It is postulated that increased heat response and auxin signaling might contribute to the reduction of PCD-gene expression. Alternatively, or in parallel, CWIN activity might inhibit PCD under LMHS through promoting NO signaling and/or inhibiting PA signaling or affecting G-protein-mediated sugar signaling.
of PCD measured by TUNEL assay (Fig. 4). Moreover, the increased mRNA levels of SBT3 and RD19a observed in the wild-type fruits under LMHS were blocked in the CWIN-elevated transgenic plants (Fig. 5). These data provide direct evidence on the role of CWIN in suppressing PCD-related genes and the PCD phenotype in reproductive organs under LMHS (Fig. 11).

Related to our finding, the loss-of-function of CWIN in the maize mutant mn1 led to a reduction of cell division and expansion in the mutant endosperm (Vilhar et al., 2002) and induction of PCD in the placental-chalazal region of kernels (Kladnik et al., 2004). In our case, however, LMHS increased tomato fruit abortion in the wild type through triggering PCD (Figs. 1, 4, and 5) but with no or little effect on cell number and size (Supplemental Fig. S7). Noticeably, the inhibition of PCD by elevated CWIN activity under LMHS also took place without affecting cell number and cell size (Fig. 4; Supplemental Fig. S7) or the transcript levels of cell cycle genes encoding cyclins and CDKs (Supplemental Fig. S8). The analyses suggest different models by which CWIN regulates PCD in different species or organs.

It is remarkable that LMHS did not lead to an oxidative response, based on the measurements of the activities of major antioxidant enzymes, the transcript levels of genes encoding these enzymes, and the content of H$_2$O$_2$ and MDA (Figs. 6 and 8). This is in contrast to the finding that severe heat stress-induced PCD is attributed to excessive accumulation of ROS (Vacca et al., 2004; Laloi et al., 2004; Foyer and Noctor, 2005). Moreover, the elevated CWIN activity in the transgenic plants also had no effect on these parameters (Figs. 6 and 8). Thus, the induction of PCD by LMHS and the inhibition of PCD by elevated CWIN activity appear both independent of an oxidative response or ROS accumulation. The above view is complemented by findings from the positive control of SSHS, which significantly increased the content of H$_2$O$_2$ and MDA in both genotypes (Fig. 7). SSHS also increased the expression of SOD2, APX2, and CAT2 in wild type 2-dbp ovaries (Fig. 8). Thus, different from LMHS, SSHS caused significant oxidative stress and antioxidant response in tomato, as reported for other plant systems (Foyer and Noctor, 2005). Importantly, elevation of CWIN activity appears to alleviate the oxidative response to SSHS. For example, in the CWIN-elevated ovaries, the SSHS-induced H$_2$O$_2$ content was much less than that in the wild type (Fig. 7A). Furthermore, only the expression of CAT2, but not those of SOD2 and APX2, was increased by SSHS in the transgenic ovaries, whereas all three genes were up-regulated by SSHS in the wild-type ovaries (Fig. 8). These findings suggest that, different from the scenario under LMHS, the elevated CWIN activity appears to alleviate the oxidative stress and antioxidant response caused by SSHS. Overall, the data indicate that CWIN regulates the responses of tomato fruit set to SSHS and LMHS in an oxidative stress-dependent and-independent manner, respectively.

The regulation of PCD in plants involves a complex network, in which many different components, apart from H$_2$O$_2$, play their respective key roles (Wang et al., 2013). For example, NO could depress H$_2$O$_2$ signaling, and the balance between H$_2$O$_2$ and NO determines the incidence of PCD (Wang et al., 2013). It has also been suggested that polyamines (PAs) and their analogs may directly induce PCD in plants and animals in the absence of H$_2$O$_2$ (Moschou and Roubelakis-Angelakis, 2014). NO signaling operates through a member of the SnRK2 family, a component in the sugar signaling pathway (Besson-Bard et al., 2008). Furthermore, both NO and spermine were involved in the Glc-induced inhibition of seed germination in Lotus japonica (Zhao et al., 2009). Thus, it is possible that NO and PA might have responded to CWIN-mediated Suc catabolism and Glc signaling to alleviate PCD in the transgenic fruits under LMHS (Fig. 11). In addition, the involvement of G-protein signaling in regulation of PCD was also suggested (Urano and Jones, 2014, and refs. therein) and G-protein coupled plasma membrane receptor, RGS, might sense apoplasmic Glc released from CWIN activity (Ruan, 2014). Further studies on NO, PA, and G-protein signaling pathways will help to determine if these components are involved in CWIN-mediated PCD under LMHS.

It is worth noting that LMHS-induced PCD appeared in specific regions of the 2-dap fruits, namely in the joint area between the placenta and seeds and in the inner pericarp (Fig. 4). We showed previously that the mRNA of CWIN was most abundantly localized in the placenta connecting young seeds (Jin et al., 2009). Significantly, while LMHS treatment reduced CWIN activity in 2-dbp ovaries but not in the 2-dap fruits (Fig. 3A), it induced PCD in the fruits but not in the ovaries (Fig. 4). The spatial match between CWIN expression and PCD in the fruit placenta and seed region and the sequential events of the LMHS-induced reduction of CWIN activity and appearance of PCD during ovary-to-fruit transition indicate that reduction of CWIN activity by LMHS causes PCD in this region. It is intriguing why PCD also took place in the inner pericarp (Fig. 4, A and B) where the CWIN gene was not expressed (Jin et al., 2009). One possibility is that a PCD signal (e.g., NO or PA, see above) may be initially elicited from the CWIN-enriched placenta and seed region and diffusive to the nearby inner pericarp to induce local PCD. Regardless, the data show that the regions surrounding the developing seeds appear to be more prone to LMHS-induced PCD than the other areas in young tomato fruit. There might be evolutionary advantages for this PCD heterogeneity within a young fruit. Stress-induced PCD in these areas likely results in seed death, and hence fruit abortion, early in development, which may serve as an effective way to save resources for the survival of a few remaining fruits and seeds. The finding could help design more precise biotechnological approaches to prevent seed and fruit abortion under abiotic stress.
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**Materials and Methods**

**Plant Cultivation and Heat Treatments**

Homozygous T<sub>1</sub> generation of a CWIN-elevated transgenic tomato (*Solanum lycopersicum* XF-2) was used, in which CWIN activity was elevated by silencing its inhibitor using an RNAi approach (Jin et al., 2009). Tomato plants were cultivated in a greenhouse under control conditions (25/18°C, day/night, 14/10 h). When the first flower bud in the first inflorescence was 3 mm in length, one-half of the plants were exposed to LMHS (28/20°C, day/night, 14/10 h). For measurement of sugar content, three ovaries (2 dbp) or young fruits were harvested at 3 dap and cut into halves to examine seed development within the fruit. There were four biological replicates for both the pollen viability and fruit reactivation assays, that is, with samples collected from four individual plants.

**Assay of Enzyme Activities and Sugar Content**

Activities of CWIN, VIN, CIN, Sus, and hexose kinases were assayed spectrophotometrically according to Tomlinson et al. (2004). The activities of antioxidant enzymes, including SOD, APX, and CAT, were assayed based on the method of Lee and Lee (2000). The first three ovaries/fruits in the truss were pooled as one biological sample for enzyme assays with four samples collected from four plants.

For measurement of sugar content, three ovaries (2 dbp) or young fruits were harvested from one plant, pooled as one biological replicate, and ground into a fine powder in liquid nitrogen. The powder was extracted with 600 μL of methanol for 15 min at 70°C. Glc, Fru, and Suc in the supernatant were quantified spectrophotometrically as described by King et al. (1997).

**H<sub>2</sub>O<sub>2</sub> Quantification and Localization**

H<sub>2</sub>O<sub>2</sub> was extracted according to Veljovic-Jovanovic et al. (2003). Thiobarbituric acid reacts with MDA, the end-product of lipid peroxidation, to form a red pigment, which can be measured spectrophotometrically according to Tomlinson et al. (2004). The activities of antioxidant enzymes, including SOD, APX, and CAT, were assayed based on the method of Lee and Lee (2000). The first three ovaries/fruits in the truss were pooled together for H<sub>2</sub>O<sub>2</sub> quantification as one biological replicate.

**Lipid Peroxidation Assay**

Triobarbituric acid was used to detect lipid peroxidation according to Sunkar et al. (2003). Thiobarbituric acid reacts with MDA, the end-product of lipid peroxidation, to form a red pigment, which can be measured spectrophotometrically. Briefly, three 2-dap ovaries or 2-dap fruits were homogenized in 0.7 M 0.1% trichloroacetic acid solution on ice. After centrifugation at 10,000 g for 5 min, the supernatant was collected and used for the MDA assay. The first three ovaries/fruits in the truss were pooled together for lipid peroxidation assay as one biological replicates.

**Measurement of Suc Import Rate into Young Fruits**

Thiobarbituric acid was used to detect lipid peroxidation according to Sunkar et al. (2003). Thiobarbituric acid reacts with MDA, the end-product of lipid peroxidation, to form a red pigment, which can be measured spectrophotometrically. Briefly, three 2-dap ovaries or 2-dap fruits were homogenized in 0.7 M 0.1% trichloroacetic acid solution on ice. After centrifugation at 10,000 g for 5 min, the supernatant was collected and used for the MDA assay. The first three ovaries/fruits in the truss were pooled together for lipid peroxidation assay as one biological replicates.

**TUNEL Assay for PCD**

TUNEL assay was conducted largely based on Wang and Ruan (2016). Four ovaries/fruits were harvested from four different individual plants, representing four biological replicates. Ovaries (2 dbp) and young fruits (2 dap) were fixed in 4% paraformaldehyde solution in PBS (130 mM NaCl, 3 mM Na<sub>2</sub>PO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.0) containing 0.1% Tween 20 and 0.1% Triton X-100. The samples were embedded in paraffin following dehydration in an ethanol series from 30% to 100%. Longitudinal consecutive sections were cut at 7-μm thickness each. TUNEL was conducted using a cell death detection kit from Roche (In Situ Cell Death Detection Kit, Fluorescein) based on the manufacturer’s
Cell Number and Size

Feulgen staining was employed to measure cell number in 2-dbp ovaries and 2-dap fruits (Lindström et al., 2006). This method depends on acid hydrolysis of nuclear DNA to release aldehyde groups, which react with Schiff’s reagent staining the nucleus red (Fox, 1969). Briefly, one ovary or fruit was fixed in ethanol and acetic acid solution (3:1) for 24 h. After washing twice in water, the sample was macerated with 5 mM HCl for 30 min in an ice bath followed by 60 min at 20°C. The macerated sample was then stained with Schiff’s reagent in the dark for 1 h, followed by an enzymatic digestion of cell walls with 1% cellulase (w/v) in 50 mM sodium acetate buffer (pH 5.0) at 40°C for 3 h. After centrifugation at 10,000 g for 5 min, the pellet containing nuclei was resuspended in 1 mL of H2O. The number of nuclei in a fixed volume of suspension was counted with the help of a cell-counting chamber, and the cell number per ovary or fruit was then calculated.

To determine cell size, 2-dbp ovaries and 2-dap fruits were fixed in freshly prepared fixing solution (4% paraformaldehyde, 0.1% glutaraldehyde, 2 mM CaCl2, and 50 mM PIPES, pH 6.8) under vacuum (~20 kPa). After washing in PIPES and dehydration through a graded series of ethanol from 10% to 100% in 10% increments, the samples were infiltrated and embedded in LR White resin. Sections at 1 μm in thickness were cut longitudinally through the middle of the ovaries and fruits using glass knives on a Reichert Ultracut E microtome. Sections were stained with 1% methylene blue in 1% Borax. Cell size was calculated by counting cell number per square millimeter of the visual field. Four ovaries/fruits from four individual plants were used for both cell number and size assays.

RNA Extraction and Quantitative RT-PCR Assay

Total RNA was purified from three ovaries (2 dbp) or young fruits (2 dap) using RNasy Plant Mini Kit (Qiagen), and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Quantitative RT-PCR assay was conducted using SYBR Green detection chemistry and run in duplicate with a Rotor-Gene Q (QIAGEN) quantitative RT-PCR machine. Detailed information of all primer pairs used is presented in Supplemental Table S1. SAND was chosen as the reference gene to normalize data (Expósito-Rodríguez et al., 2008). The first three ovaries/fruits in the truss were pooled together for RNA extraction. In each case, there were four biological replicates (i.e. four pools) from four individual plants.

Net Photosynthesis Measurement

Net photosynthesis of the fully expanded leaves was measured at 20 d following LMHS treatment using a Li-6400 CO2 analyzer (Li-COR Incorporation) according to Li et al. (2012). The instrument was set at a light intensity of 500 μmol m−2 s−1 and a CO2 concentration of 380 ppm.

Statistical Analyses

One-way ANOVA test was carried out for data analyses using IBM SPSS Statistics 20.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers listed in Supplemental Table S1.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Activities of Suc-degrading enzymes in wild-type and CWIN-elevated transgenic (RNAi) tomato under control and LMHS conditions.

Supplemental Figure S2. Effects of LMHS on transcript levels of CWIN inhibitor and CWIN genes in wild-type and CWIN-elevated transgenic (RNAi) tomato.

Supplemental Figure S3. Effects of LMHS on Pn and plant biomass in wild-type and CWIN-elevated transgenic (RNAi) tomato.

Supplemental Figure S4. Effects of LMHS on G6c, Fru, and Suc content in wild-type and CWIN-elevated transgenic (RNAi) tomato.

Supplemental Figure S5. Technical controls for TUNEL assay to detect PCDS in pericarp of 2-dap tomato fruits under LMHS.

Supplemental Figure S6. No PCD was detected in wild-type and CWIN-elevated transgenic (RNAi) fruits under control (optimal) condition.

Supplemental Figure S7. Effects of LMHS on cell number and cell size in wild-type and CWIN-elevated transgenic (RNAi) ovary and fruit.

Supplemental Figure S8. Effect of LMHS on expression levels of CKDs and cyclins in wild-type and CWIN-elevated transgenic (RNAi) tomato.

Supplemental Figure S9. Localization of H2O2 in wild-type and CWIN-elevated transgenic (RNAi) ovary and fruit.

Supplemental Figure S10. Effect of LMHS on transcript levels of Hsp70 in wild-type and CWIN-elevated transgenic (RNAi) tomato.

Supplemental Table S1. Primer sequence information, amplicon lengths, and qPCR efficiency of genes of interest used in qPCR assay.

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LITERATURE CITED


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