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Cryptochrome 1a of tomato mediates long-distance signaling of soil water deficit

Victor D'Amico-Damião[a], Ian C. Dodd[b], Reginaldo Oliveira[a], José C.B. Lúcio[a], Davi R. Rossatto[a] and Rogério F. Carvalho [a,*]

1. Department of Biology Applied to Agriculture, São Paulo State University (UNESP), 14884-900 Jaboticabal, Brazil
2. The Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK

* Corresponding author at: Department of Biology Applied to Agriculture, São Paulo State University (UNESP), 14884-900 Jaboticabal, Brazil
E-mail address: R.F. Carvalho (rogerio.f.carvalho@unesp.br)

ORCID IDs: Victor D'Amico-Damião (0000-0001-9497-7063), Ian C. Dodd (0000-0003-2725-859X), Reginaldo Oliveira (0000-0002-0974-0537), José C. B. Lúcio (0000-0003-4297-6071), Davi R. Rossatto (0000-0001-9510-8345), Rogério F. Carvalho (0000-0003-1270-7372)

Abbreviations: *A*, photosynthesis; ABA, abscisic acid; C_i, internal CO_2 concentration; crys, cryptochromes; DAS, days after sowing; DW, dry weight; *E*, leaf transpiration; FW, fresh weight; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; ROS, reactive oxygen species; RWC, relative water content; TW, turgid weight; WUE, water use efficiency

ABSTRACT

Although the blue light photoreceptors cryptochromes mediate the expression of genes related to reactive oxygen species, whether cryptochrome 1a (cry1a) regulates local and long-distance signaling of water deficit in tomato (*Solanum lycopersicum* L.) is unknown. Thus the *cry1a* tomato mutant and its wild-type (WT) were reciprocally grafted (WT/WT; *cry1a*/*cry1a*; WT/*cry1a*; *cry1a*/WT; as scion/rootstock) or grown on their own roots (WT and *cry1a*) under irrigated and water deficit conditions. Plant growth, pigmentation, oxidative stress, water relations, stomatal characteristics and leaf gas exchange were measured. WT and *cry1a* plants grew similarly under irrigated conditions, whereas *cry1a* plants had less root biomass and length and higher tissue malondialdehyde concentrations under water deficit. Despite greater oxidative stress, *cry1a* maintained chlorophyll and carotenoid concentrations in drying soil. Lower stomatal density of *cry1a* likely increased its leaf relative water content (RWC). In grafted plants, scion genotype largely determined shoot and root biomass accumulation irrespective of water deficit. In chimeric plants grown in drying soil, *cry1a* rootstocks increased RWC while WT rootstocks maintained photosynthesis of *cry1a* scions. Manipulating tomato *CRY1a* may enhance plant drought tolerance by altering leaf pigmentation and gas exchange during soil drying via local and long-distance effects.

Keywords: abiotic stress; *cry1a* mutant; drought; root-shoot signaling; *Solanum lycopersicum* L.; water deficit

1. Introduction

Soil water deficit decreases crop yields by restricting plant growth and development [1] and changing the expression of thousands of genes [2]. Mild soil drying can induce partial stomatal closure to maintain leaf water status without affecting photosynthesis (*A*), thereby increasing instantaneous water use efficiency (WUE), the ratio of *A* to transpiration (*E*). However, prolonged soil drying usually decreases leaf water potential, thereby limiting *A* via both stomatal and non-stomatal mechanisms [3]. Reactive oxygen species (ROS) generated during photosynthetic processes can damage cellular membranes, stimulating the upregulation of antioxidant system components [4]. Furthermore, drought affects the biosynthesis and degradation of photosynthetic

pigments, although enhanced carotenoid content can act as an antioxidant in photosynthetic membrane lipids, augmenting plant drought tolerance [5]. Thus soil drying affects plant physiological, biochemical and morphological responses. However, many environmental factors also alter plant responses to drought stress.

Incident light is utilized in the photosynthetic process to split water molecules at photosystem II, which undergoes severe changes during drought stress [6]. For example, high light stress could aggravate the effects of water deficit, increasing ROS formation if electron transport rate is compromised [5]. Furthermore, light quality can affect tolerance to water deficit, as adding green (530 nm) light to a control treatment of red (660 nm) and blue (450 nm) LEDs improved tomato (*Solanum lycopersicum* L.) drought tolerance by decreasing stomatal conductance without limiting photosynthesis, thereby increasing instantaneous water use efficiency [7]. In addition, using LED lights with peaks in the blue and red spectral regions enhanced chlorophyll a/b ratio in pepper (*Capsicum annuum* L.) seedlings compared to growing plants with compact fluorescent lamps (peaks at green and red spectral regions), thereby increasing electron transport rate while decreasing non-photochemical quenching during water deficit [8]. While light quality can enhance drought tolerance by affecting photosynthesis, it is also important to consider its photomorphogenetic role.

Photomorphogenesis is the process by which light modulates plant growth and development from seed germination to senescence. Higher plants encode photoreceptor proteins sensitive to changes in the light quality, quantity, duration and direction. Currently, five plant photoreceptor families are described: UV-B resistance locus 8 (ultraviolet-B light photoreceptor); cryptochromes (crys), phototropins and zeitlupes (ultraviolet-A/blue light photoreceptors); and phytochromes (red/far-red light photoreceptor) [9]. Of these, crys photoreceptors (and other) regulate a wide range of physiological and developmental processes, from seed germination to fruiting. In *Arabidopsis thaliana*, three crys were identified (cry1, cry2, and cry3) and characterized (cry1 and cry2) as mediators of seedling de-etiolation, anthocyanin accumulation and cotyledon expansion as well as circadian rhythm and photoperiod-dependent flowering [10,11]. In tomato, one of the most important vegetables in the world, four cry genes within a multigene family were identified: *CRY1a*, *CRY1b*, *CRY2* and *CRY3*. The nuclear proteins cry1a and cry2 photoreceptors (responsive to both a low- and high-blue light fluence rates) are active in the photomorphogenic responses, while it is not clear if

the *CRY1b* gene encodes a functional photoreceptor in tomato. Expression of *CRY3* encodes a DASH protein located in semi-autonomous organelles, that acts in DNA repair mechanisms. Furthermore, cry1a regulates photomorphogenic responses related to early root growth, hypocotyl and stem elongation, pigments biosynthesis and fruit yield [12,13,14]. Several papers have started to investigate whether these cry-mediated effects alter plant drought tolerance.

Changes in gene expression, biochemical responses and stomatal opening might be involved in crys mediating plant drought stress responses [15]. For example, crys up-regulate (*eg RD29A*, *ADH1* and *ABA2* in Arabidopsis) and down-regulate (*eg LEA*, *RAB18*, *NIA1*, *APX1* and *NAC* in *Brassica napus*) the expression of ABA/stress-responsive genes [16,17]. Moreover, in tomato grown under optimal conditions (available water and white light), *cry1a* mutant leaves had 20% higher ABA concentrations than wild type plants [18], which may enhance drought tolerance by improving the regulation of water status via stomatal closure, lessening the risk of plant water deficit [19,20]. However, the physiological responses of *cry1a* plants to soil water deficit has not been investigated.

Grafting techniques have been widely used to understand local and long-distance regulation of plant drought stress responses [21,22]. For example, soil water deficit causes the plant hormone ABA to accumulate throughout the plant, and reciprocal grafting experiments using wild-type and ABA-deficient mutants can resolve the relative importance of roots and shoots in regulating plant responses [23]. Since cry1 positively affects Arabidopsis root growth by decreasing *cry1* mutant root extension growth under blue light [24], it might alter plant drought responses by affecting water uptake. Furthermore, since the *cry1a* mutant enhances foliar ABA accumulation of tomato [18] which might affect stomatal regulation of plant water status, we hypothesized that cry1a is involved in long-distance signaling of plant drought stress responses. In other species, cryptochrome-mediated water deficit responses have investigated cry1, but tomato has not been investigated. Since cry2 has been associated with flowering and fruiting in tomato but *CRY2* is unstable under high blue light fluences in Arabidopsis, whereas tomato cry1a controls several physiological and developmental process, our efforts focused on the tomato *cry1a* mutant. For the first time, we explored the role of the tomato *CRY1a* gene on plant water stress responses using the photomorphogenetic mutant *cry1a* and grafting to understand the role of this

photoreceptor in long-distance signaling [21,22]. Thus we grew own-rooted and reciprocally grafted *cry1a* and WT tomato plants under well-watered conditions and in drying soil, to evaluate biomass accumulation, plant water relations, stomatal anatomy and biochemical responses.

2. Materials and methods

2.1. Plant material, growth condition and grafting technique

Sterilized seeds (5% NaClO solution for 10 mins followed by thorough washing with water) of the tomato (*Solanum lycopersicum* L.) *cry1a* mutant [12] and its wild type (WT; cv. Moneymaker) were used. Seeds were placed in trays containing a substrate (1:1 mixture of pine bark base:expanded vermiculite) supplemented with 1g L^{-1} of NPK fertilizer 10:10:10 and 4 g L^{-1} of lime. The plants were grown in a naturally lit greenhouse under an average temperature of 27ºC (SD ± 2.37ºC) and a relative humidity of 60% (SD ± 13%), under a 12 h/12 h (light/dark) photoperiod at photosynthetic photon flux density (PPFD) of 450–700 µmol m^{-2} s^{-1}. Fifteen days after sowing (DAS) on the same substrate, the plants were transferred to 200 mL pots, and grafting performed.

Fifteen-day old plants were grafted by the splice method with the aid of a scalpel blade and grafting clips, to obtain the following graft combinations: WT/WT, *cry1a*/*cry1a*, WT/*cry1a*, *cry1a*/WT (scion/rootstock) (Supplementary Fig. S1). Immediately after grafting, the basal quarter of the pots was submerged in water (in a floating moist chamber at 25ºC ± 2ºC and a high relative humidity: 88% ± 10%) under a 12 h/12 h (light/dark) photoperiod at 45 µmol m^{-2} s^{-1} supplied by white light LEDs, until the graft union had completely healed (30 DAS). During this period, own-rooted plants of *cry1a* and WT genotypes remained in the same conditions as grafted plants. After the graft union had healed, own-rooted and grafted plants were transferred to 2.8 L pots containing the same substrate, where they were irrigated daily until the beginning of the water deficit (40 DAS).

2.2. Water deficit treatment

Preliminary tests with the same pot, substrate and growth conditions aimed to determine soil water holding capacity and plant responses to soil drying. Pots were uniformly filled with the equivalent of 500 g of substrate. After irrigating the substrate to the drip point, the pots were allowed to drain overnight to determine the drained capacity (1.74 g g^{-1}). Furthermore, plant evapotranspiration was monitored daily (gravimetrically) to estimate soil water availability. After suspending irrigation for ten days, soil moisture had declined to 30% of field capacity (0.53 g g^{-1}) while the irrigated treatment (receiving 500 mL daily, split between early morning and late afternoon) maintained values close field capacity. At the end of the experiment, the substrate was oven dried (105°C for 24 h) to determine the soil moisture. At 50 DAS, the own-rooted and grafted plants were harvested for analysis described below. For further biochemical analyzes, plants were immediately freeze-dried in liquid nitrogen and stored at -80ºC.

2.3. Growth analysis

Plant height and maximum root length were determined using a graduated ruler. Shoots and roots were separated, the roots washed to remove the substrate and oven dried at 60°C for 72 h to measure their dry weights using an analytical scale (Model AA-200, Denver Instrument Company, New York, USA).

2.4. Leaf pigments content

Chlorophyll a+b and carotenoids pigments were extracted (acetone 80%) from the fifth fully expanded leaf (25 mg) and determined by spectrophotometry (DU-640, Beckman Coulter, Fullerton, USA) at 663 nm (chlorophyll *a*), 647 nm (chlorophyll *b*) and 470 nm (carotenoids) [25].

2.5. Lipid peroxidation and H_2O_2 content

Lipid peroxidation was evaluated from the fifth fully expanded leaf (500 mg) and roots (700 mg) according to the content of thiobarbituric acid-reactive substances present in the malondialdehyde (MDA) form and the content of hydrogen peroxide (H_2O_2), as determined by reaction with potassium iodide. MDA content was estimated

using an extinction coefficient of 1.55×10^{-5} mol^{-1} cm^{-1}. H_2O_2 content was determined using a known concentration curve as a standard [25].

2.6. Leaf relative water content

The relative water content (RWC) was determined with discs (5 mm diameter) of fifth fully expanded leaf, approximately 200 mg of fresh weight, according to the following equation: (FW-DW)/(TW-DW)×100 (FW, fresh weight; DW, dry weight; TW, turgid weight). The discs were immediately weighed to determine the fresh weight (FW) and subsequently the discs were placed in petri dishes with deionized water for 6 h to determine the turgid weight (TW). Then, the discs were dried at 60°C for 48 h to obtain the dry weight (DW).

2.7. Stomatal measurements

Stomatal density was obtained from the fifth fully expanded leaf using "super glue" to obtain impressions of paradermal sections of the abaxial epidermis using a glass slides microscope and were counted using an optical microscope with micrometric ruler [25]. Stomatal pore area was obtained from the same glass slides microscope impressions, and digitized using an optical microscope and a video camera coupled to a microcomputer (IM50, Leica, Copenhagen, Denmark). After images digitization, the stomatal pore area was measured using a Photoshop image processing software (CS5 Extended, Adobe Systems, San Jose, USA).

2.8. Leaf gas exchange

Photosynthesis (*A*) and leaf transpiration (*E*) of the fifth fully expanded leaf was measured between 9 and 11 am using a gas exchange system (LCpro, Analytical Development Co., Hoddeston, UK) illuminated with a blue-red LED light source supplying 1,200 μmol m^{-2} s^{-1} PPFD. The water use efficiency (WUE) was calculated by dividing *A* by *E*.

2.9. Data analyses

The experimental design used was completely randomized with five biological replicates (plants) per treatment, except for leaf gas exchange measurements which were with three biological replicates per treatment. Except for the growth analysis, all measurements comprised 3 technical replicates per leaf. Furthermore, stomatal measurements were performed with 10 technical replicates (randomly selected fields of view) per leaf. Each experiment was repeated at least three times, with data from a representative experiment reported. Own-rooted plants were compared in a 2 × 2 factorial scheme, consisting of two genotypes (WT and *cry1a*) and two conditions (irrigated and water deficit), using two-way analysis of variance (ANOVA) to determine the main effects of genotype, watering treatment and their interaction (Experiment 1). The grafted plants (Experiment 2) were compared in a 4 × 2 factorial scheme, comprising four graft combinations (WT/WT, *cry1a*/*cry1a*, WT/*cry1a* and *cry1a*/WT) and two conditions (irrigated and water deficit) using two-way ANOVA. Furthermore, a three-way ANOVA determined the main effects of rootstock, scion and water treatment. Mean values were compared using Tukey's HSD test (significance at $p \leq 0.05$) via AgroEstat software (www.agroestat.com). Linear regressions determined significant ($P < 0.05$) relationships between variables (r^2 and *P*-values shown; Supplementary Table S1) combining data from both own-rooted and grafted plants.

3. Results

3.1. Response of own-rooted tomato plants to water deficit

The *cry1a* mutant was 20% taller than the WT in both irrigated and water deficit conditions (Fig. 1A). Water deficit decreased plant height by 10%, averaged across both genotypes (Fig. 1E). Although taller, the *cry1a* mutant had 13% lower shoot biomass in both irrigated and water deficit conditions (Fig. 1C). Water deficit decreased shoot biomass by 24%, averaged across both genotypes. The *cry1a* mutant also had less root biomass and length (22 and 15% respectively) than WT plants (Fig. 1B and D). Although water deficit decreased root biomass by 11%, maximum root length increased by 12% (averaged across genotypes). The decrease in root biomass seemed accentuated in *cry1a* while the increase in root length seemed greater in WT plants (Fig. 1B and D).

Thus *cry1a* plants prioritized shoot elongation over shoot biomass accumulation, and had a diminished root system, but otherwise responded similarly to water deficit as WT plants (Supplementary Fig. S2C).

In irrigated conditions, *cry1a* leaves had 29% lower pigment (chlorophyll a+b and carotenoid) contents than WT plants (Fig. 2A and B). The two genotypes responded differently to water deficit (as indicated by significant genotype x water interactions), as *cry1a* maintained pigment content but WT plants decreased both chlorophyll a+b and carotenoid contents by 44% (Fig. 2A and B). Thus water deficit decreased pigment content of WT plants, but *cry1a* plants maintained pigment content.

Shoot and root malondialdehyde (MDA) content of the *cry1a* mutant was higher (63% and 20% respectively) than WT plants in both irrigated and water deficit conditions (Fig. 2C and E). Although there were no significant genotypic effects on shoot hydrogen peroxide (H_2O_2) content (Fig. 2D), *cry1a* mutant exhibited 15% lower root H_2O_2 content compared to WT plants (averaged across water availabilities; Fig. 2F). Soil water deficit significantly increased whole plant MDA and H_2O_2 contents to a similar extent in both genotypes (no significant genotype x water interactions). Thus MDA accumulation of *cry1a* plants was greater than in WT plants, with similar shoot and root H_2O_2 contents of both genotypes.

Leaf relative water content (RWC) of *cry1a* was higher than the WT irrespective of soil moisture (Fig. 3A). Both genotypes responded similarly to water deficit (as indicated by no significant genotype x water interaction), which decreased leaf RWC by 5%. Stomatal density of *cry1a* mutant was 31% lower than the WT in both irrigated and water deficit conditions (Fig. 3B). Water deficit increased stomatal density to a similar extent (by 21%) in both genotypes (no significant genotype x water interaction). Stomatal pore area of both genotypes was similar, with soil water deficit decreasing it by 75% respectively (averaged across genotypes; Fig. 3C and D). Thus the greater leaf RWC of *cry1a* plants was associated with decreased stomatal density, but otherwise the two genotypes responded similarly to water deficit.

Under well-watered conditions, photosynthesis (A) and transpiration (E) of *cry1a* mutant was 14% and 21% less respectively than WT plants (Fig. 4A and B). Soil water deficit decreased A similarly in both genotypes by 13%, but had less effect on E of *cry1a* (significant genotype x water interaction), since E of well-watered *cry1a* plants was lower (Fig. 4B). Water use efficiency (WUE = ratio of A/E) was higher in WT

plants, and increased similarly under water deficit by 34% (averaged across genotypes; Fig. 4A and C). Both genotypes showed similar photosynthetic and WUE responses to water deficit.

3.2. Responses of reciprocally grafted plants to water deficit

Plant height was determined by scion, but not rootstock, genotype (Fig. 5A and E), with *cry1a* scions 14% taller (averaged over both rootstocks). Nevertheless, a WT rootstock enhanced height of a *cry1a* scion (by 12% compared to *cry1a* self-grafts) while a *cry1a* rootstock enhanced height of a WT scion (by 23% compared to WT self-grafts), as indicated by a significant scion x rootstock interaction. Soil drying decreased plant height by 17% (averaged over all graft combinations) independently of scion or rootstock. WT scions were 21% heavier than *cry1a* scions (independent of the rootstock). Soil drying decreased shoot dry weight by 21% (averaged over all graft combinations) independently of scion or rootstock (Fig. 5C). Thus scion stem elongation of reciprocally grafted plants was increased by a chimeric rootstock, but otherwise rootstock genotype did not affect scion biomass accumulation or response to soil drying.

Scion and rootstock had no significant effect on root length (Fig. 5B), but soil drying stimulated root length by 11% (averaged across all graft combinations). Scion determined root dry weight, with WT scions resulting in 46% heavier roots independent of rootstock (Fig. 5D). Root systems of all graft combinations responded similarly to soil drying.

While soil water deficit decreased pigment content of WT scions by 16% independent of rootstock, pigment content of *cry1a* scions depended on the rootstock (Fig. 6A and B**)**. Soil drying decreased pigment content of *cry1a*/WT plants (as indicated by significant scion x rootstock x water interactions). Thus the rootstock affected pigment responses of *cry1a* scions to water deficit, with a WT rootstock phenotypically reverting the response of *cry1a* self-grafts (*cry1a*/*cry1a* maintained pigment content).

Under well-watered conditions, shoot hydrogen peroxide (H_2O_2) content was similar in all graft combinations, but soil drying elicited scion-dependent responses (Fig. 6D**)**. Thus soil drying increased shoot H_2O_2 content of WT scions by 30%

independent of rootstock, while there was no change in *cry1a* self-grafts and *cry1a*/WT plants. These changes in ROS generation were only partially reflected in shoot malondialdehyde (MDA) accumulation. Plants grown on a *cry1a* rootstock generally had 11% lower shoot MDA concentrations (Fig. 6C**)**. Soil drying increased MDA concentrations of WT scions by 43% (averaged over both rootstocks), but had no effect in *cry1a* scions. Thus scion affected shoot H_2O_2 content while rootstock affected shoot H_2O_2 and MDA accumulation, with scion determining the response of shoot H_2O_2 and MDA content to soil drying.

WT rootstocks had 63% higher root H_2O_2 content than *cry1a* rootstocks independent of soil water availability, but scion genotype also had a significant effect on *cry1a* rootstocks (Fig. 6F**)**. Thus a WT scion approximately halved root H_2O_2 content of *cry1a* rootstocks independent of soil water availability. Soil drying increased root H_2O_2 content of all graft combinations similarly. Under well-watered conditions, root MDA concentration was similar in all graft combinations, but soil drying increased root MDA accumulation by 20% (averaged across all graft combinations; Fig. 6E). Soil water deficit had variable effects on root MDA content in the different graft combinations (as indicated by significant scion x rootstock x water interactions). Root MDA content of WT self-grafted plants increased by 41% under soil drying, but did not change in *cry1a*/WT plants. Thus *cry1a* rootstocks had lower root H_2O_2 contents in drying soil, but this did not affect MDA accumulation.

Leaf relative water content (RWC) was primarily rootstock-dependent (Fig. 7A), with scions on *cry1a* rootstocks showing 4% higher RWC (averaged over both soil water availabilities). Nevertheless, the response of RWC to soil drying was scion-dependent, with soil drying decreasing RWC of WT scions by 3% while *cry1a* scions maintained RWC, as indicated by a significant scion x soil drying interaction. Maintenance of leaf RWC in *cry1a* scions was associated with their 18% lower stomatal density independent of soil water availability or rootstock (Fig. 7B). Stomatal pore area was determined by scion, but not the rootstock (Fig. 7C and D), and was 16% lower in *cry1a* scions than WT scions (averaged over both rootstocks). A *cry1a* rootstock decreased stomatal pore area of a WT scion by 26%, independent of soil water availability. Soil drying decreased stomatal pore area of all graft combinations, but to a greater extent in WT scions than *cry1a* scions (significant scion x soil water availability

interaction). Thus maintenance of leaf water status in *cry1a* scions was rootstock-independent and attributed to their decreased stomatal density.

Under well-watered conditions, photosynthesis (*A*) and transpiration (*E*) of WT/*cry1a* plants was 36% and 21% more than WT self-grafts (Fig. 8A and B). Soil water deficit restricted leaf gas exchange, with effects of both scion and rootstock. WT self-grafts showed the greatest restriction, with a limited response in *cry1a*/WT plants. In drying soil, a *cry1a* rootstock enhanced *A* and *E* of a WT scion, while a WT rootstock enhanced *A* and *E* of a *cry1a* scion. Soil drying increased WUE by 7% (averaged across all graft combinations; Fig. 8C), with the most prominent response in self-grafted *cry1a* plants. Rootstock did not affect WUE response to soil drying, but plants with a *cry1a* rootstock generally had a higher WUE.

3.3. Correlations between plant variables

Since shoot dry weight was correlated with photosynthesis (*A*) across both experiments (Supplementary Table S1), correlation analysis attempted to explain variation in *A*. *A* declined linearly with transpiration (*E*) as the soil dried (Fig. 9B), suggesting a stomatal limitation. However, *A* was not correlated with leaf relative water content (RWC; Fig. 9A), suggesting non-hydraulic mediation of stomatal conductance. Although *A* was not correlated with chlorophyll content (Fig. 9D) as mutant scions maintained *A* even with reduced foliar pigment content, it declined linearly with hydrogen peroxide (H_2O_2) content (Fig. 9C), suggesting a further non-stomatal limitation.

4. Discussion

While previous work characterized the effects of tomato *CRY1a* under optimal conditions [13,18], for the first time we demonstrate this gene is also involved in regulating leaf pigmentation, root lipid peroxidation and leaf gas exchange responses to water deficit. Whereas water deficit decreases pigment (chlorophyll and carotenoid) concentrations of WT plants, the *cry1a* mutant maintains pigment levels. Moreover, the *CRY1a* photoreceptor modulates long-distance signaling of water deficit, with scion x rootstock x water availability interactions affecting leaf pigments and leaf transpiration

(*E)*, and also root malondialdehyde (MDA) content of grafted plants. While further work is needed to understand the mechanistic basis of this regulation, *CRY1a* mediation of root-shoot communication of soil water deficit does not seem hydraulically regulated, since relative water content (RWC) was not correlated with any other variable, such as leaf gas exchange measurements (Fig. 9A). Physiological responses of own-rooted and reciprocally grafted plants are discussed in turn.

4.1. cry1a photoreceptor positively regulates tomato growth

As expected, soil drying decreased stem elongation, shoot and root biomass accumulation but enhanced primary root length (Fig. 1), with an attenuated root growth response in *cry1a*. Altered source-sink partitioning [13] might explain this response, with *cry1a* showing greater shoot growth than root growth compared to WT plants independent of soil water availability (Supplementary Fig. S2A). However, these morphological responses were generally independent of soil drying (no genotype x soil water availability interactions), indicating that *CRY1a* positively regulates root extension and biomass accumulation in tomato. Repeated periods of soil drying and re-watering seem necessary to accentuate root biomass accumulation of tomato in response to soil water deficit [26]. Although *cry1a* plants grew taller independent of soil moisture, they accumulated less shoot biomass and leaf area (Supplementary Fig. S2B). However, such growth inhibition did not seem to be hydraulically-mediated, as *cry1a* plants had a higher leaf RWC (Fig. 3A), especially in drying soil. Decreased *E* of *cry1a* plants (Fig. 4B), especially when well-watered, was caused by their lower stomatal density (Fig. 3B) as stomatal pore area was similar to WT plants independent of soil moisture (Fig. 3C and D). Similarly, lower stomatal density of the Arabidopsis *atdtm1* mutant was associated with increased relative water content [27]. While the lower stomatal density (and leaf *E*) of *cry1a* under well-watered conditions might delay soil moisture depletion, ultimately both genotypes showed similar morphological (Fig. 1) and physiological (Fig. 4) responses to soil drying.

Blue light activates crys to positively regulate downstream expression of the *HY5* (*LONG HYPOCOTYL 5*) gene, encoding the well-characterized light signaling transcription factor; HY5 a photomorphogenesis promotor [28]. Under optimal soil moisture and light conditions, tomato lines overexpressing *OFPs* (*OVATE FAMILY*

429 *PROTEINS*) upregulated foliar *HY5* and *CAT2* (*CATALASE 2*) expression, thereby
430 increasing total chlorophyll while decreasing hydrogen peroxide (H_2O_2) and MDA
431 contents [29]. When grown under blue light in well-watered conditions, *cry1a* mutant
432 and *SlCRY1a* overexpressing lines had lower and higher *SlHY5* transcript levels than
433 WT plants, respectively [30]. Taken together, these results are consistent with the
434 biochemical (leaf pigments and shoot MDA concentrations) responses of well-watered
435 own-rooted *cry1a* plants grown in a naturally lit greenhouse (Fig. 2). In contrast, since
436 drought gradually increases *HY5* expression [31], *SlHY5* overexpressing lines decreased
437 H_2O_2 and MDA accumulation compared to WT, as their enzymatic antioxidant defense
438 systems (eg SUPEROXIDE DISMUTASE, SOD; and CAT) were enhanced [32]. Thus
439 the functional *SlCRY1a* in the WT likely positively regulates the expression of *SlHY5*,
440 thereby promoting the activity of antioxidant enzymes thus decreasing membrane lipid
441 peroxidation during soil drying.

442 Nevertheless, leaf pigment (carotenoid and chlorophyll) responses of the two
443 genotypes to soil water deficit greatly differed (Fig. 2A and B). Under well-watered
444 conditions, the *cry1a* mutant had lower pigment concentrations than WT plants, but
445 higher concentrations under soil water deficit. Soil drying significantly decreased
446 pigment concentrations of WT plants but maintained (or tended to increase)
447 concentrations in *cry1a*. Drought concentrated the leaf pigments in thinner *cry1a* leaves
448 [13,18], by reducing chloroplast ultrastructure volume in fully expanded tomato leaves
449 [33]. In other words, genotypic differences in leaf structure accentuated soil drying
450 effects, resulting in divergent pigment responses to drought in WT (decreased) and
451 *cry1a* (maintained) plants.

452 Although greater light absorption suggested by higher chlorophyll concentrations
453 of *cry1a* plants growing in drying soil did not enhance shoot H_2O_2 (a representative
454 ROS) content relative to WT plants (Fig. 2D), greater oxidative damage (measured as
455 MDA accumulation) occurred (Fig. 2C) perhaps because other reactive molecules were
456 generated. Indeed, crys modulate ROS homeostasis in a blue light dependent manner,
457 which implies the expression of genes whose products are related to ROS scavenging,
458 H_2O_2 signaling and ROS formation [15,34]. Nevertheless, high light exposure of low-
459 light adapted *cry1a* plants resulted in less photoinhibition than the WT [35], indicating
460 complex relationships between ROS generation and oxidative damage. However, these
461 molecular responses may not be specifically light-mediated, as root H_2O_2 content of

462 *cry1a* and WT plants was similar in drying soil, yet *cry1a* roots accumulated greater
463 MDA concentrations in drying soil. Nevertheless, following cry activation, ROS
464 signaling in the apex of the primary root of transgenic Arabidopsis that overexpressed
465 *CRY1* was approximately 2.5-fold greater than *cry1cry2* double mutant roots [36].
466 Irrespective of the sources of oxidative stress (excess light absorption or soil drying),
467 greater oxidative damage of *cry1a* plants growing in drying soil (Fig. 2C and E)
468 occurred even at higher leaf water status (Fig. 3A), indicating *CRY1a* is involved in
469 mediating tolerance to oxidative stress.

470 Despite greater oxidative damage of *cry1a* plants growing in drying soil (Fig. 2C),
471 leaf photosynthesis (*A*) was comparable to WT plants (Fig. 4A). Although *A* of *cry1a*
472 plants was 13% lower than WT plants (averaged across soil moisture treatments), both
473 genotypes decreased *A* similarly in response to soil drying (no genotype x soil moisture
474 interaction) as transpiration decreased. Thus diminished *A* of *cry1a* could be explained
475 by decreased stomatal density limiting internal CO_2 concentration (C_i) even in well-
476 watered plants [20], with drought-induced stomatal closure (Fig. 3C) imposing an
477 additional limitation. While further *A*-C_i analysis is required to distinguish the
478 physiological importance of stomatal and non-stomatal limitation of *A* in these
479 genotypes, they maintained similar leaf water use efficiency (WUE) independent of soil
480 water availability.

481

482 *4.2. Importance of tomato cry1a in regulating long-distance-signaling of water deficit*

483

484 Differential shoot and root growth of own-rooted *cry1a* and WT plants (Fig. 1)
485 [13,18] seemed entirely scion-mediated, as reciprocal grafting experiments revealed no
486 rootstock effects (Fig. 5). However, when WT *Nicotiana attenuata* scions were grafted
487 onto photoreceptor-silenced rootstocks, only phyB1 and B2 (but not cry2) delayed shoot
488 growth both in field and glasshouse growth conditions [37], indicating that
489 photoreceptor levels in the roots can exert systemic effects on shoot growth. Greater
490 biomass of WT scions increased root biomass independent of rootstock, with similar
491 assimilate partitioning across all graft combinations (Supplementary Fig. S2F). Despite
492 these differences in root biomass, root extension of all graft combinations was similar
493 within a soil moisture treatment (Fig. 5B), indicating limited shoot-to-root signaling
494 mediated by cry1a. Increased, or maintenance of, root biomass allocation with soil

drying in tomato [26] results from greater shoot (than root) sensitivity to water deficit, and seemed independent of location (root or shoot) of the *CRY1a* gene.

However, cry1a mediated root-shoot communication determining leaf pigment and root malondialdehyde (MDA) concentrations as the soil dried, since there were significant scion, rootstock and water interactions (Fig. 6A, B and E). Grafting *per se* (independent of rootstock genotype) prevented *cry1a* scions upregulating shoot MDA and hydrogen peroxide (H_2O_2) concentrations in response to water deficit (cf. Fig. 2C and D; Fig. 6C and D), consistent with transcriptome profiles of tomato under biotic stress strongly differing (6% of all genes) between own-rooted and self-grafted plants [38]. In addition, self-grafts from two different grapevine genotypes result in the differential expression of genes involved in oxidative stress (eg *GLUTATHIONE S-TRANSFERASES*, *ASCORBATE OXIDASE*, *POLYPHENOL OXIDASE*, and *PEROXIDASE* genes) compared to heterografts [39], indicating complex oxidative responses may account for inconsistent results between own-rooted and grafted plants.

Nevertheless, a *cry1a* rootstock enhanced leaf pigment concentrations of WT scions, but only in well-watered plants (Fig. 6A and B). However, this extra light-absorbing capacity did not enhance foliar oxidative stress (Fig. 6C and D). Greater root MDA accumulation of own-rooted *cry1a* plants under water deficit (Fig. 2E) did not occur in grafted plants, independent of the scion. However, a *cry1a* scion decreased drought-induced root MDA accumulation of WT rootstocks (Fig. 6E) despite similar rootstock H_2O_2 levels (Fig. 6F). Furthermore, a WT scion decreased root H_2O_2 levels of *cry1a* rootstock independent of soil moisture. While drought-induced H_2O_2 generation can cause lipid peroxidation [40], it is also involved in long-distance signaling (via second messengers) of essential physiological processes that regulate tomato drought stress responses [41], e.g. root growth [42] and stomatal closure [43]. HY5 is a candidate molecule to regulate H_2O_2 root-shoot communication by interacting with CRY1a activity in tomato [30,32], since local activity (shoot or root) of HY5 transcription factor (protein) mediates downstream mobile signals from shoots to roots in Arabidopsis regulating plant growth [44], and is likely important in *CRY1a* mediated root-shoot communication in tomato (Fig. 10).

Decreased stomatal density of *cry1a* (Fig. 3B) was not rescued by grafting onto a WT rootstock (Fig. 7B), yet *cry1a*/WT plants had a lower relative water content (RWC) than self-grafted *cry1a* plants (across both soil water availabilities). This was likely

528 because *cry1a*/WT plants transpired more, which increased photosynthesis (*A*; Fig. 8A
529 and B) irrespective of soil moisture. For instance, lower RWC of the tomato *not*
530 (*notabilis*; ABA-deficient) mutant under water deficit was related to its enhanced
531 transpiration rate [45]. Whether a WT rootstock can enhance whole plant hydraulic
532 conductance of *cry1a* requires further investigation. In contrast, neither rootstock or soil
533 drying altered stomatal density of WT scions, nor did rootstock affect the responses of
534 stomatal pore area to drying soil. Indeed, the stomatal density of reciprocally grafted
535 seedlings of a drought-tolerant and a drought-sensitive tomato genotypes was primarily
536 scion (but not rootstock) determined across both soil water availability (watered or
537 drought) conditions [46]. Nevertheless, WT scions on a *cry1a* rootstock showed an
538 attenuated RWC response to soil drying, despite similar stomatal closure to WT self-
539 grafts. Again, rootstock-mediated changes in whole plant hydraulic conductance seem
540 necessary to explain the regulation of leaf gas exchange in these graft combinations
541 [47].
542
543 **5. Conclusions**
544
545 In conclusion, although own-rooted plants were not directly compared with
546 grafted plants, *CRY1a* appears to have both local and long-distance roles in regulating
547 leaf pigmentation and gas exchange during water deficit responses. Thus own-rooted
548 *cry1a* plants maintained foliar pigment concentrations in drying soil, possibly related to
549 maintenance of leaf RWC due to lower stomatal density (a local effect). Furthermore, a
550 WT rootstock maintained *cry1a* transpiration under water deficit, independently of
551 changes in stomatal density or pore area or leaf water status, which enhanced
552 photosynthesis (a long-distance effect). While soil drying causes stomatal limitation of
553 photosynthesis that seems independent of leaf water status, further work is needed to
554 understand how *CRY1a* downregulates photosynthesis, especially since greater lipid
555 peroxidation occurred in own-rooted *cry1a* plants and photosynthesis declined linearly
556 with shoot H_2O_2 concentration.
557
558 **Author contributions**
559

V.D.D., I.C.D. and R.F.C. designed, interpreted, wrote and approved the manuscript for publication. V.D.D., R.O. and J.C.B.L. performed the experiments and collected the data. V.D.D. and D.R.R. performed the leaf gas exchange experiments and collected the data. V.D.D., I.C.D. and R.F.C. analyzed the results and constructed the figures.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version.

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Figures

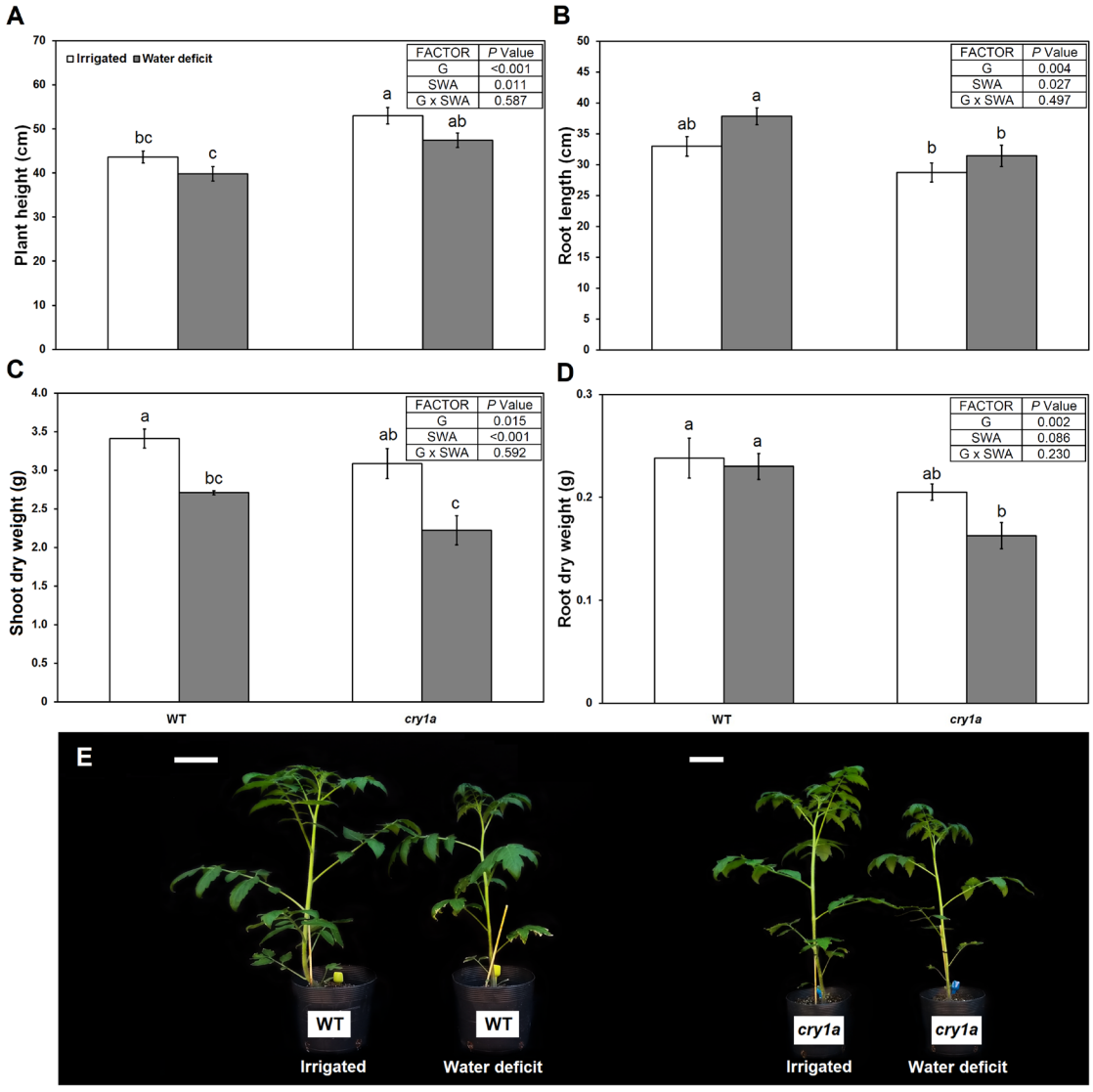
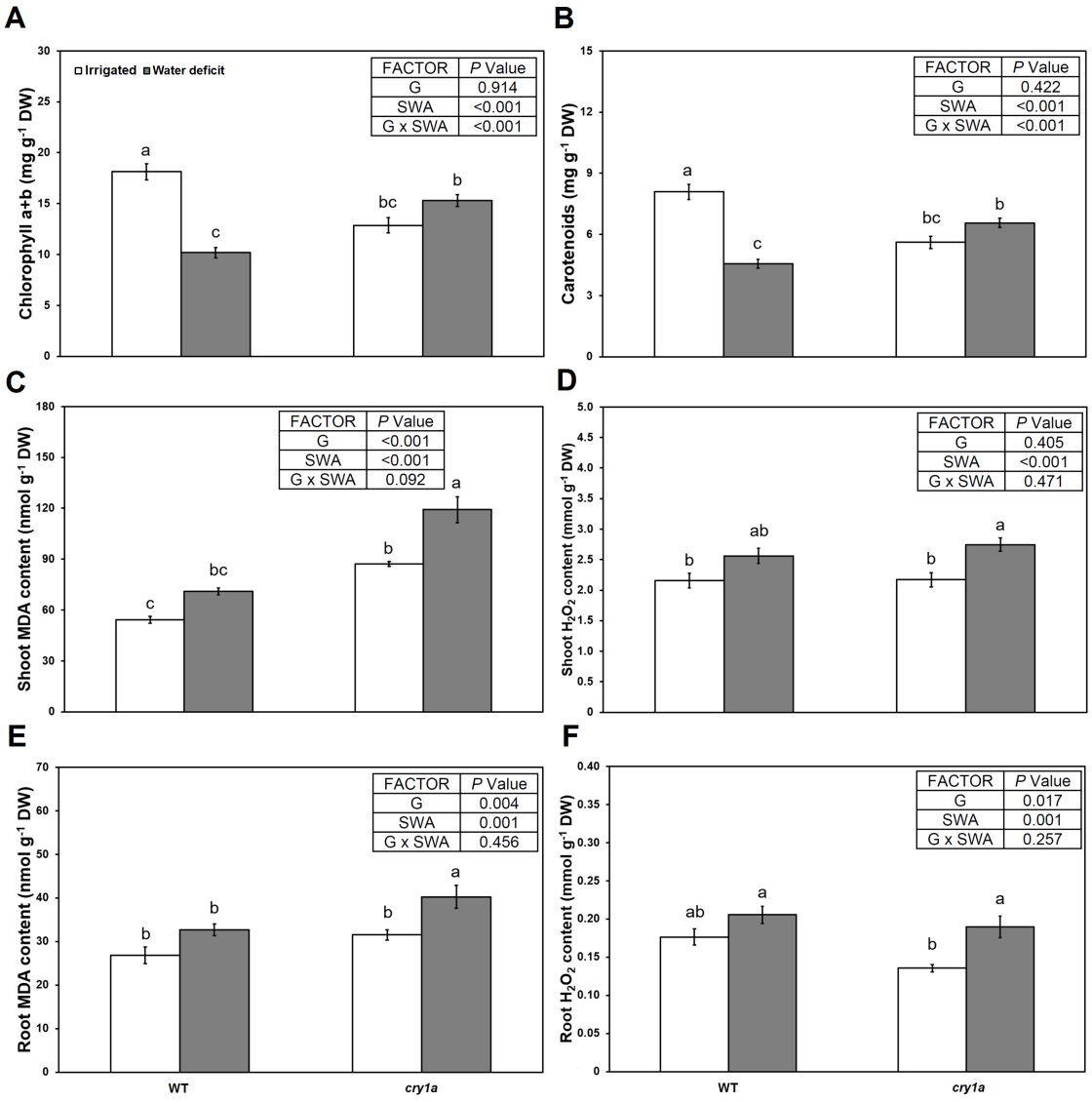


Fig. 1. Growth analysis of tomato *cry1a* mutant and wild-type (WT) grown under irrigated or water deficit conditions.

(A) plant height; (B) root length; (C) shoot dry weight; (D) root dry weight. The data are shown as the mean values of 5 plants of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the

766 treatments. The mean values were compared using the Tukey's HSD test (significance
767 at $p \leq 0.05$). The tables summarize the significance (*P*-values) of genotype (G), soil
768 water availability (SWA), and their interactions (G x SWA) after ANOVA. (E) Tomato
769 cv. Moneymaker phenotypes of own-rooted plants of *cry1a* mutant and wild-type (WT)
770 submitted to water deficit treatment or daily irrigated treatment. Pictures were
771 digitalized and exhibited followed by correspondent scale bar (10 cm).
772



773

774 **Fig. 2.** Biochemical analysis of tomato *cry1a* mutant and wild-type (WT) grown under
775 irrigated or water deficit conditions.

776 (A) chlorophyll a+b; (B) carotenoids; (C) shoot malondialdehyde content; (D) shoot
777 hydrogen peroxide content; (E) root malondialdehyde content; (F) root hydrogen
778 peroxide content. The data are shown as the mean values of 5 plants (3 technical
779 replicates) of each treatment and the bars represent ± SEs. The distinct letters above the
780 bars indicate significant differences between the treatments. The mean values were
781 compared using the Tukey's HSD test (significance at $p \leq 0.05$). The tables summarize

the significance (*P*-values) of genotype (G), soil water availability (SWA), and their interactions (G x SWA) after ANOVA.

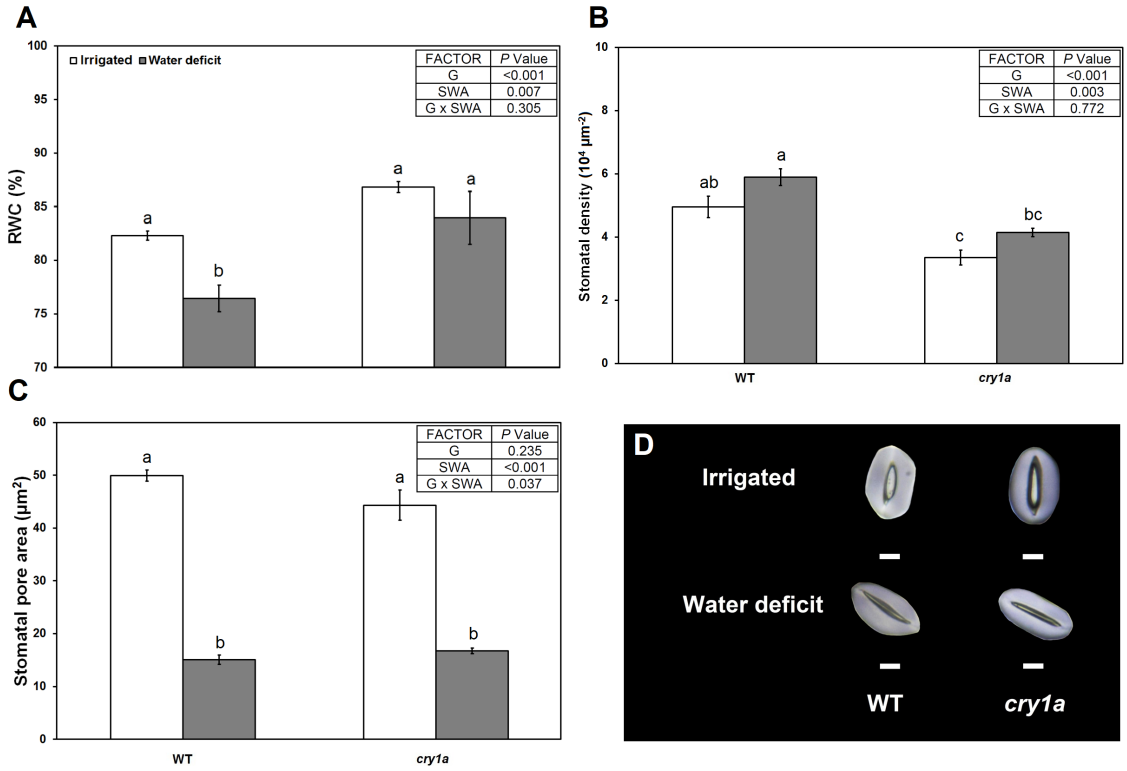


Fig. 3. Water relations and stomatal measurements of tomato *cry1a* mutant and wild-type (WT) grown under irrigated or water deficit conditions.

(A) relative water content (RWC); (B) stomatal density; (C) stomatal pore area. The data are shown as the mean values of 5 plants (10 technical replicates) of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the treatments. The mean values were compared using the Tukey's HSD test (significance at $p \leq 0.05$). The tables summarize the significance (*P*-values) of genotype (G), soil water availability (SWA), and their interactions (G x SWA) after ANOVA. (D) Representative images of leaf abaxial epidermis stomata from *cry1a* and WT plants grown under irrigated condition or subjected to water deficit condition. Scale bars: 10 µm.

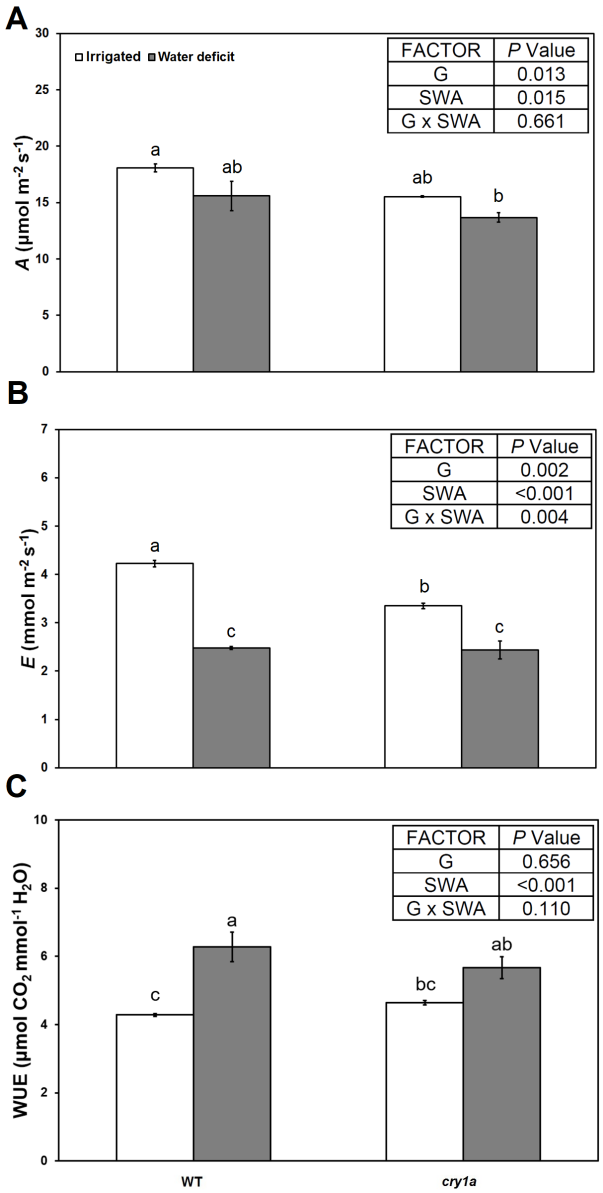


Fig. 4. Gas exchange of the fifth fully expanded leaf of tomato *cry1a* mutant and wild-type (WT) grown under irrigated or water deficit conditions.

(A) photosynthesis (*A*); (B) leaf transpiration (*E*); (C) water use efficiency (WUE). The data are shown as the mean values of 3 plants (3 technical replicates) of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the treatments. The mean values were compared using the Tukey's HSD test (significance at $p \leq 0.05$). The tables summarize the significance (*P*-values) of genotype (G), soil water availability (SWA), and their interactions (G x SWA) after ANOVA.

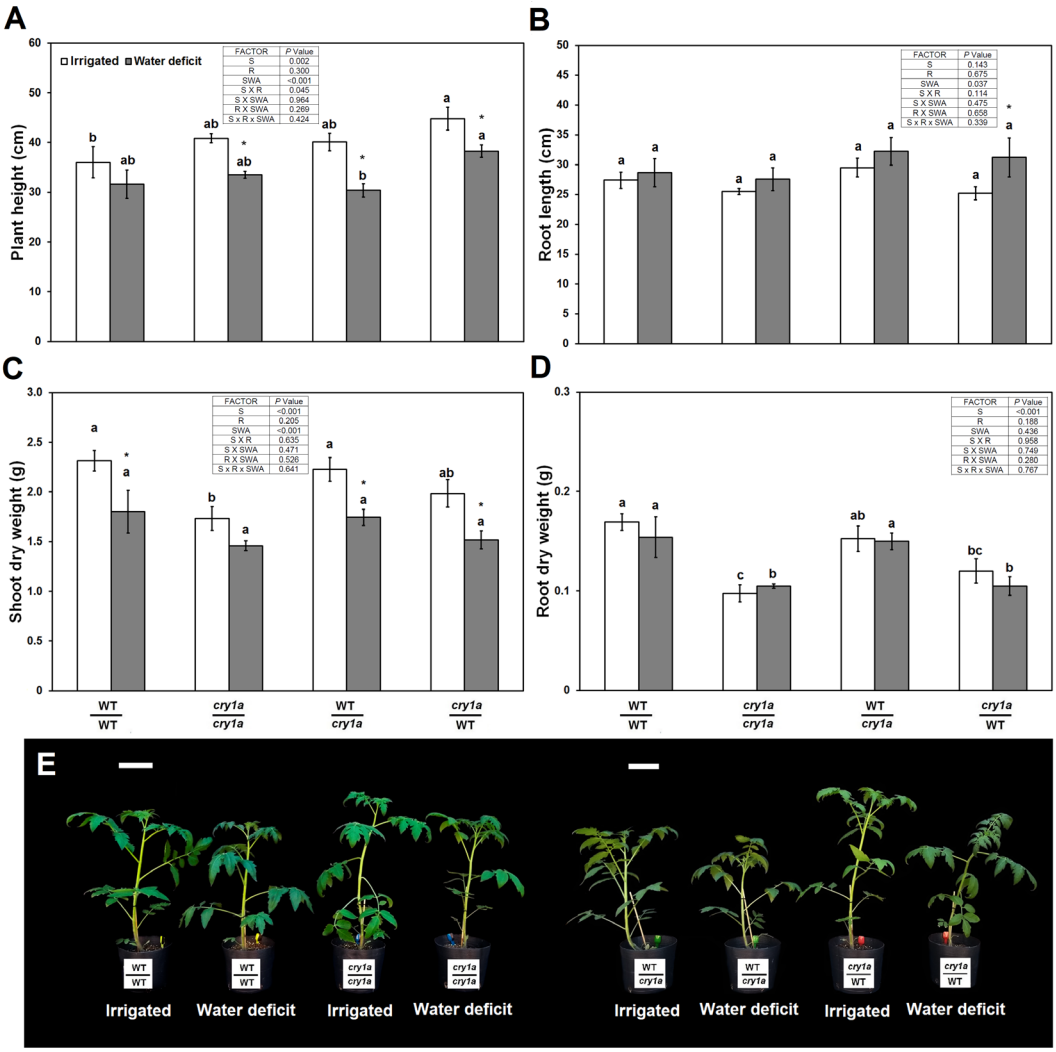


Fig. 5. Growth analysis of self-grafts and heterografts of tomato *cry1a* mutant and wild-type (WT) grown under irrigated or water deficit conditions.

(A) plant height; (B) root length; (C) shoot dry weight; (D) root dry weight. The data are shown as the mean values of 5 plants of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the graft combinations within the same growth condition, and the asterisks represent significant differences between the growth conditions in the same graft combination. The mean values were compared using the Tukey's test (significance at $p \leq 0.05$). The tables summarize the significance (*P*-values) of scion (S), rootstock (R), soil water availability (SWA), and their interactions (S x R; S x SWA; R x SWA; S x R x SWA) after ANOVA. (E) Tomato cv. Moneymaker phenotypes of reciprocal grafted plants of *cry1a* mutant and wild-type (WT) submitted to water deficit treatment or daily irrigated treatment. The genotype below represents the rootstock and the genotype above represents the scion. Pictures were digitalized and exhibited followed by correspondent scale bars (10 cm).

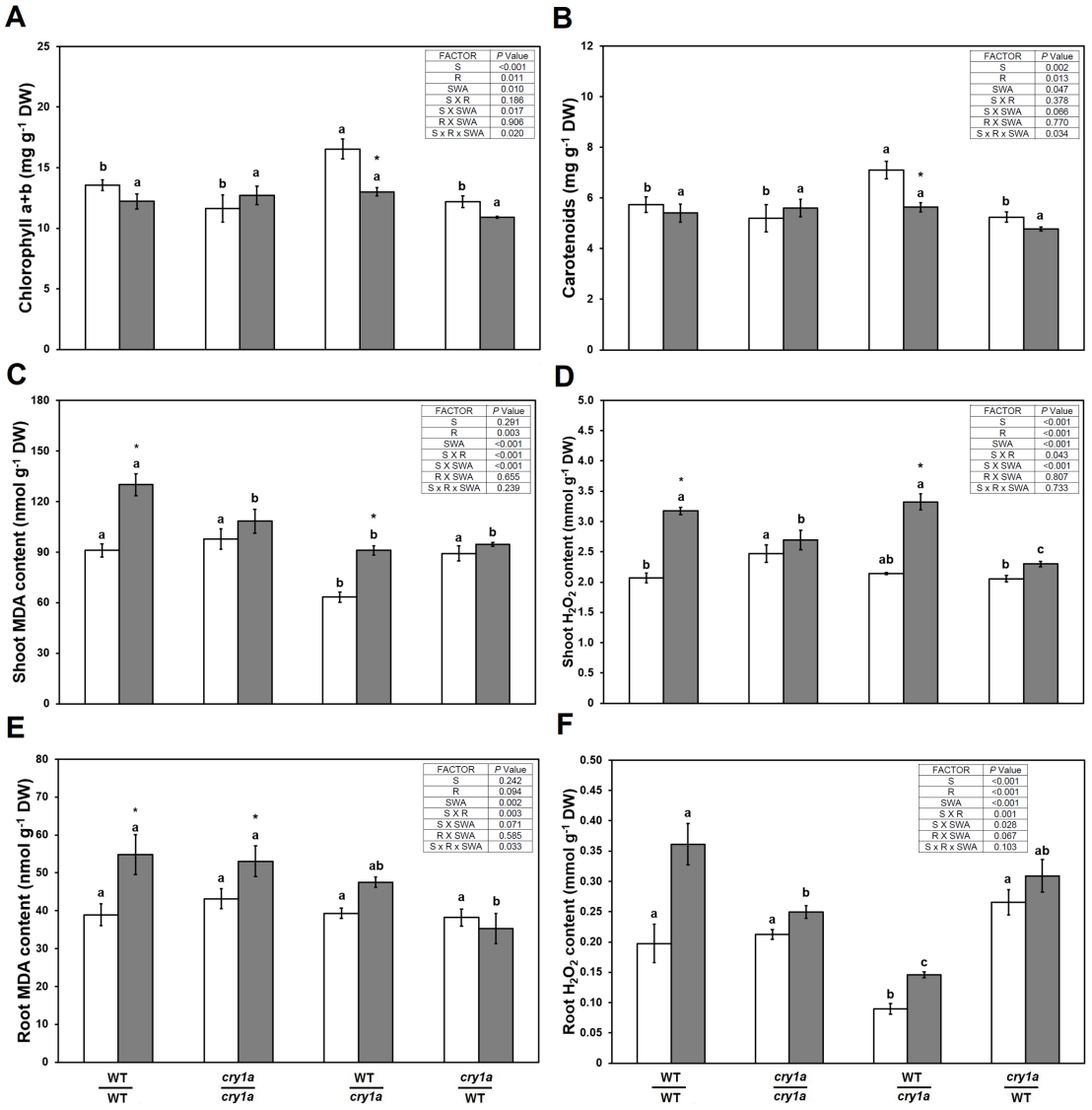


Fig. 6. Biochemical analysis of self-grafts and heterografts of tomato *cry1a* mutant and wild-type (WT) grown under irrigated or water deficit conditions.

(A) chlorophyll a+b; (B) carotenoids; (C) shoot malondialdehyde content; (D) shoot hydrogen peroxide content; (E) root malondialdehyde content; (F) root hydrogen peroxide content. The data are shown as the mean values of 5 plants (3 technical replicates) of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the graft combinations within the same growth condition, and the asterisks represent significant differences between the growth conditions in the same graft combination. The mean values were compared using the Tukey's test (significance at $p \leq 0.05$). The tables summarize the significance (*P*-values) of scion (S), rootstock (R), soil water availability (SWA), and their interactions (S x R; S x SWA; R x SWA; S x R x SWA) after ANOVA.

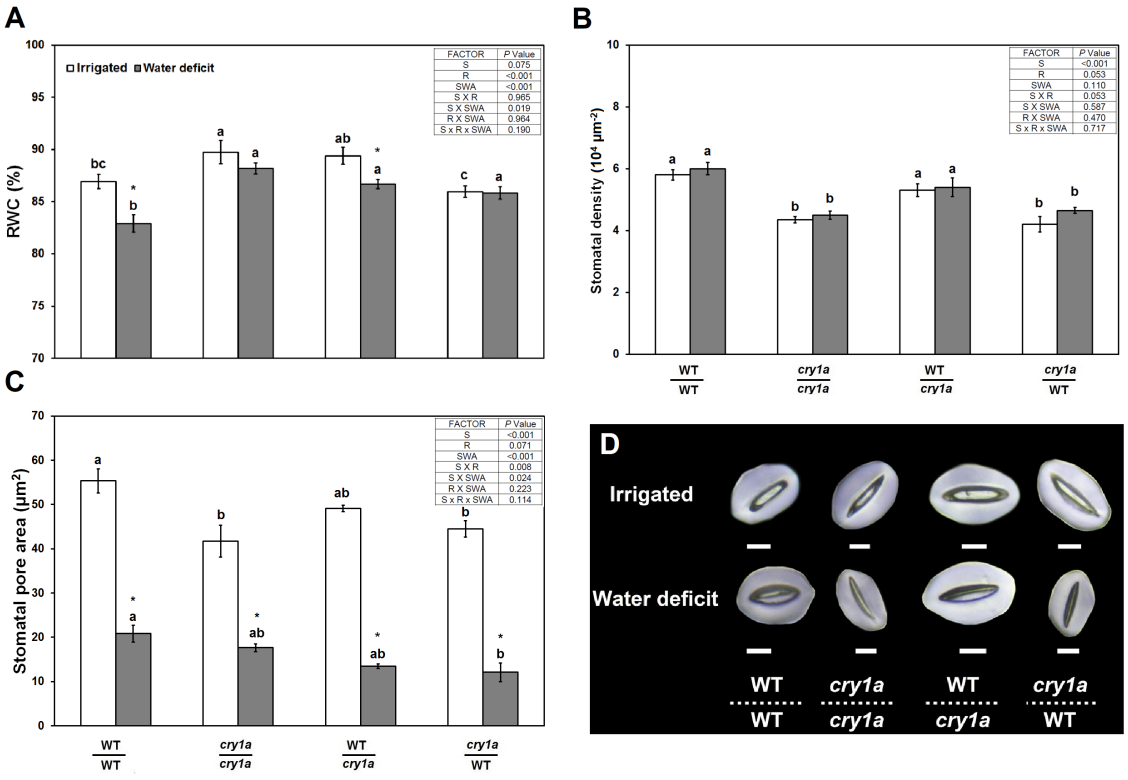


Fig. 7. Water relations and stomatal measurements of self-grafts and heterografts of tomato *cry1a* mutant and wild-type (WT) grown under irrigated or water deficit conditions.

(A) relative water content (RWC); (B) stomatal density; (C) stomatal pore area. The data are shown as the mean values of 5 plants (10 technical replicates) of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the graft combinations within the same growth condition, and the asterisks represent significant differences between the growth conditions in the same graft combination. The mean values were compared using the Tukey's test (significance at $p \leq 0.05$). The tables summarize the significance (*P*-values) of scion (S), rootstock (R), soil water availability (SWA), and their interactions (S x R; S x SWA; R x SWA; S x R x SWA) after ANOVA. (D) Representative images of leaf abaxial epidermis stomata from self-grafts and heterografts of *cry1a* and WT plants grown under irrigated condition or subjected to water deficit condition. Scale bars: 10 µm.

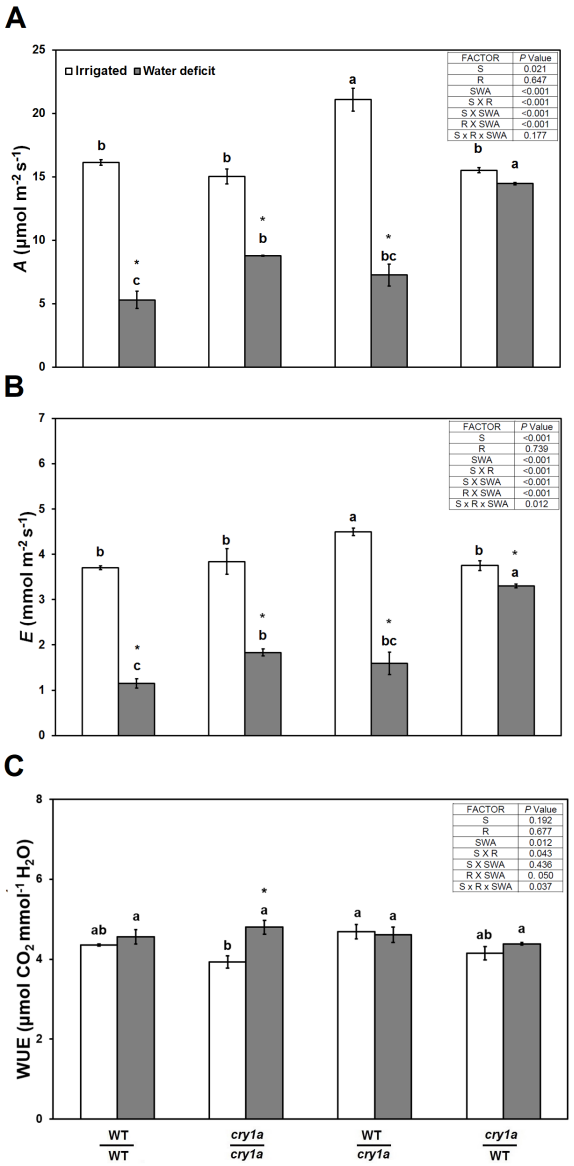


Fig. 8. Gas exchange of the fifth fully expanded leaf of self-grafts and heterografts of tomato *cry1a* mutant and wild-type (WT) grown under irrigated or water deficit conditions.

(A) photosynthesis (*A*); (B) leaf transpiration (*E*); (C) water use efficiency (WUE). The data are shown as the mean values of 3 plants (3 technical replicates) of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the graft combinations within the same growth condition, and the asterisks represent significant differences between the growth conditions in the same graft combination. The mean values were compared using the Tukey's test (significance at $p \leq 0.05$). The tables summarize the significance (*P*-values) of scion (S), rootstock (R), soil water availability (SWA), and their interactions (S x R; S x SWA; R x SWA; S x R x SWA) after ANOVA.

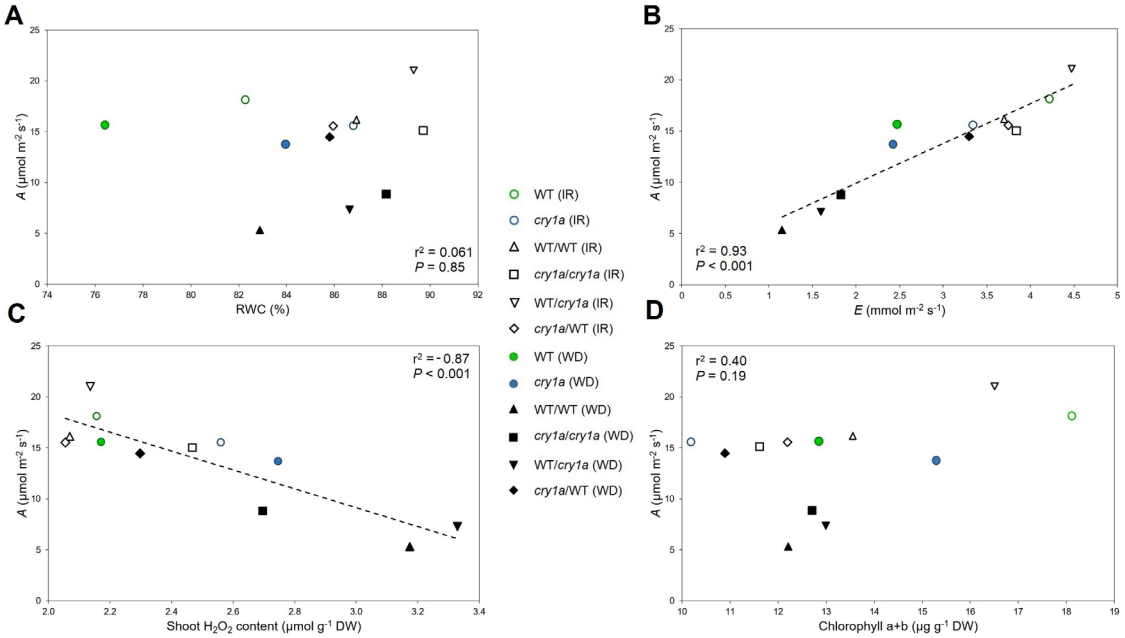


Fig. 9. Relationships between leaf photosynthesis (*A*) and (A) relative water content (RWC); (B) transpiration (*E*); (C) hydrogen peroxide (H_2O_2) content; (D) chlorophyll a+b in own-rooted, self-grafts and heterografts (indicated as scion/rootstock) of tomato *cry1a* mutant and wild-type (WT) grown under irrigated (IR) or water deficit (WD) conditions.

Each point represents a soil water availability treatment x genotype or graft combination. Linear correlations (r^2 and *P*-values) were fitted by dashed lines when $P < 0.05$.

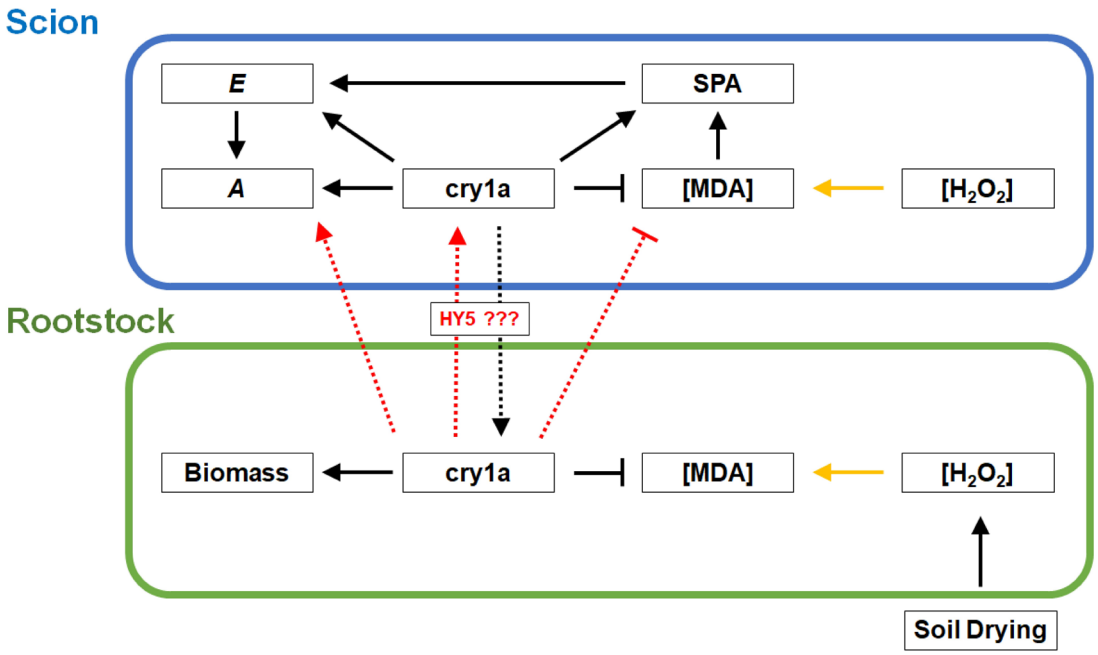


Fig. 10. Schematic figure of tomato cry1a effects on stomatal (stomatal pore area = SPA), oxidative stress (H_2O_2 and MDA concentrations), leaf gas exchange (*A*, *E*) and root biomass responses of grafted plants.

Positive effects are represented by lines ending in an arrow, and negative effects are represented by lines ending in a bar. Root-shoot communication (as scion x rootstock x soil water availability interactions) is represented by dashed lines (red, root-to-shoot; black, shoot-to-root). Relationships within oxidative stress pathways are indicated by a yellow line, with the putative role of the HY5 transcription factor mediating unknown (???) downstream mobile signals indicated.

Supplementary figures

Fig. S1

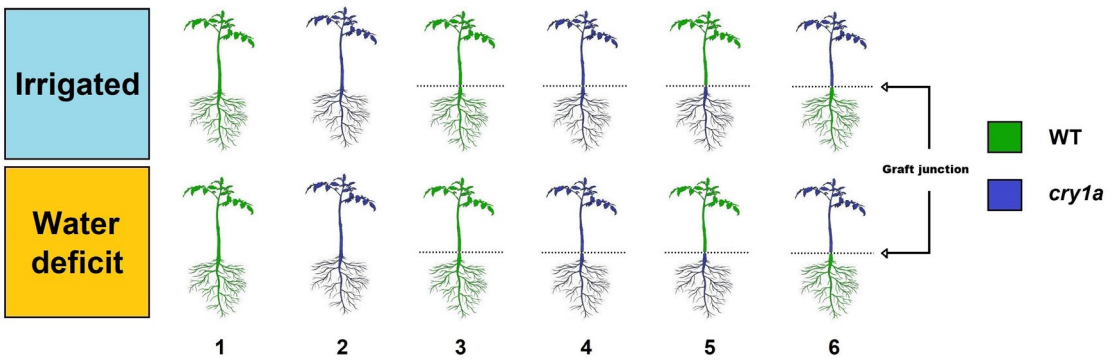


Fig. S1. Reciprocal grafting of tomato (*Solanum lycopersicum* L.) *cry1a* mutant and wild-type (WT) scion/rootstock combinations under different growth conditions to study cryptochrome 1a signaling during water deficit

(1) WT, (2) *cry1a*, (3) WT/WT, (4) *cry1a*/*cry1a*, (5) WT/*cry1a* and (6) *cry1a*/WT.

Fig. S2

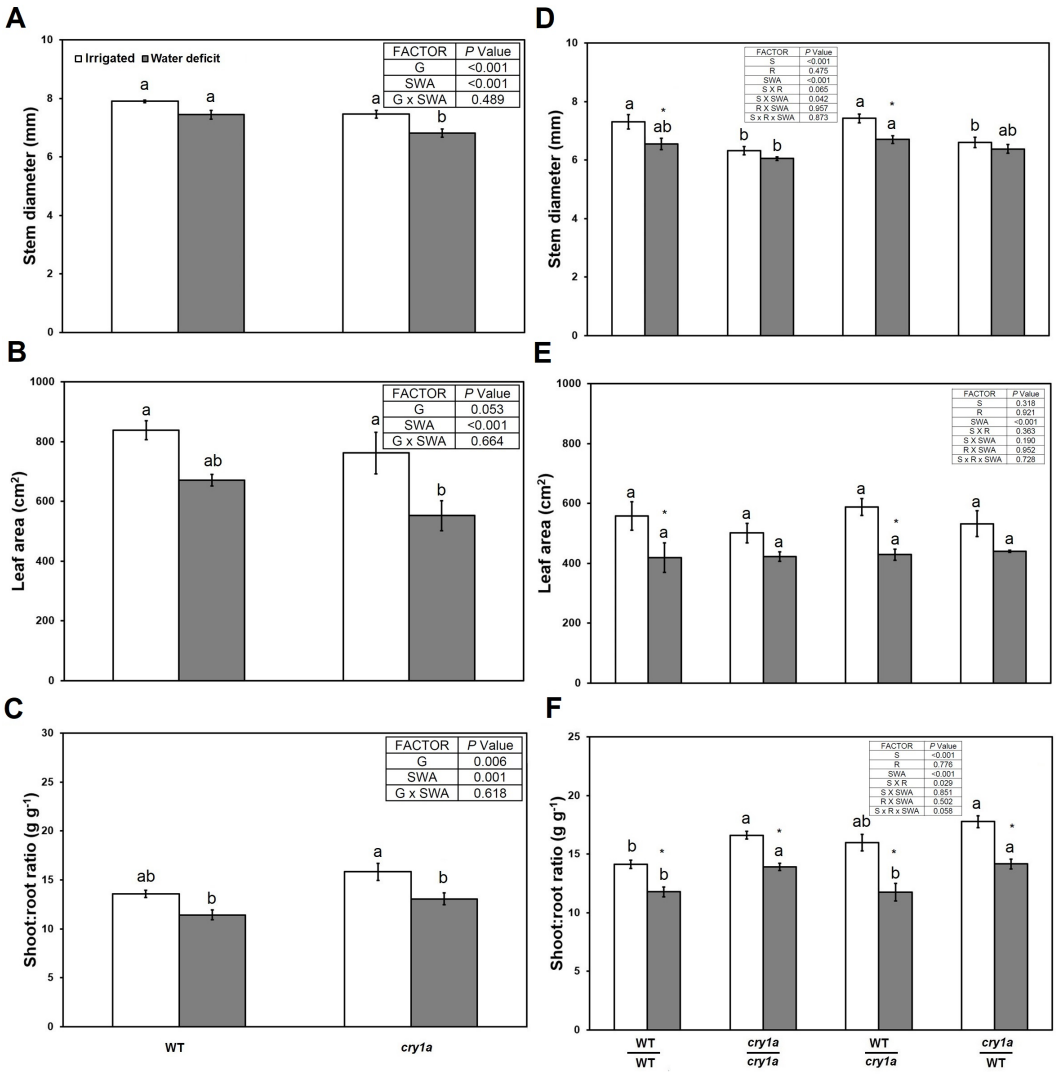


Fig. S2. Supplementary growth analysis of tomato *cry1a* mutant and wild-type (WT) (A-C) and reciprocal grafts combination (D-F)

(A) stem diameter; (B) leaf area; (C) shoot:root dry weight ratio; (D) stem diameter; (E) leaf area; (F) shoot:root dry weight ratio, grown under irrigated or water deficit conditions. The data are shown as the mean values of 5 plants of each treatment and the bars represent ± SEs. The distinct letters above the bars indicate significant differences between the plants (genotypes or graft combinations) within the same growth condition, and the asterisks represent significant differences between the growth conditions in the same plant (genotypes or graft combinations). The mean values were compared using the Tukey's test (significance at $p \leq 0.05$). The tables summarize the significance (*P*-values) of genotype (G), soil water availability (SWA), and their interactions (G x SWA) after ANOVA or scion (S), rootstock (R), soil water availability (SWA), and their interactions (S x R; S x SWA; R x SWA; S x R x SWA) after ANOVA.

949 **Supplementary table**

950 **Table S1.** Linear correlation coefficient between parameters with r^2 and asterisks for *P* values.

	Chlorophyll a+b	Shoot MDA	Root MDA	Shoot H_2O_2	Root H_2O_2	Stomatal density	Stomatal pore area	RWC	*A*	*E*	Shoot DW
Shoot MDA	-0.39										
Root MDA	-0.27	**0.74****									
Shoot H_2O_2	-0.23	**0.58***	**0.71***								
Root H_2O_2	-0.53	**0.62***	0.41	0.26							
Stomatal density	0.28	0.13	0.22	0.076	-0.086						
Stomatal pore area	0.30	**-0.61***	-0.39	**-0.59***	-0.27	-0.14					
RWC	-0.14	-0.6	0.36	0.065	0.0087	-0.40	0.36				
A	0.40	**-0.75****	**-0.79****	**-0.87*****	-0.55	-0.20	**0.66***	0.061			
E	0.33	**-0.75****	**-0.69***	**-0.83*****	-0.38	-0.27	**0.79****	0.29	**0.93*****		
Shoot DW	0.43	**-0.70***	**-0.80****	-0.40	-0.49	-0.16	0.47	-0.42	**0.58***	0.45	
Root DW	0.43	**0.64***	**-0.74****	-0.28	**-0.62***	-0.030	0.12	-0.56	0.44	0.22	**0.90*****

951 Linear correlation coefficient between parameters with r^2 and asterisks for *P* values in own-rooted, reciprocal and self-grafted tomato plants grown under
952 irrigated or water deficit condition. Text in bold highlights significant correlations plotted in figures 1–8. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.