

A shade-hyposensitive tomato line shows altered auxin homeostasis and higher fruit yield under high-density field conditions

Esteban Burbano-Erazo^{1,2*} , Silvana Francesca^{3*} , Miguel Simon-Moya¹, Julia Palau-Rodriguez¹ , Andrea Berdonces¹, Laura Valverde¹ , Jose M. Perez-Beser¹, Matteo Addonizio³, Jaume F. Martinez-Garcia¹ , Maria Manuela Rigano³  and Manuel Rodriguez-Concepcion¹ 

¹Institute for Plant Molecular and Cell Biology (IBMCP), CSIC-Universitat Politècnica de València, 46022, Valencia, Spain; ²Doctorate in Biotechnology, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Avda. Diagonal 643, 08028, Barcelona, Spain; ³Department of Agricultural Sciences, University of Naples Federico II, 80055, Portici (Naples), Italy

Summary

Authors for correspondence:

Maria Manuela Rigano

Email: mrigano@unina.it

Manuel Rodriguez-Concepcion

Email: manuelrc@ibmcp.upv.es

Received: 11 March 2025

Accepted: 23 June 2025

New Phytologist (2025) **247**: 2839–2851
doi: 10.1111/nph.70384

Key words: auxin, field, fruit, shade, tomato, yield.

- Plants detect the presence of nearby vegetation as a reduced ratio of red to far-red light (low R : FR). This proximity shade signal can be simulated in the lab by supplementing white light (W) with FR (W + FR). While shade avoidance strategies are considered undesirable in agricultural crops, FR supplementation enhances plant growth and fruit quality in tomato (*Solanum lycopersicum*).
- Here we compared the response of different tomato genotypes to W + FR in the lab and identified one *Solanum pennellii* introgression line (IL2-2) with a shade-tolerant phenotype at the seedling stage.
- Compared to the shade-avoider parental genotype M82, IL2-2 plants showed reduced elongation upon W + FR exposure and a disrupted expression of auxin-related genes both under W and W + FR. At harvest, W + FR treatment improved M82 fruit quality by increasing °Brix, ascorbic acid, and carotenoids, and these quality traits remained virtually unchanged in IL2-2. Under high density (HD) conditions, fruit quality traits were hardly impacted by planting density or genotype, but IL2-2 showed improved fruit yield.
- Our findings suggest that IL2-2 could serve as a valuable genotype for high-density or intercropping agrosystems.

Introduction

Plants use light not only as an essential source of energy for photosynthesis but also as a signal informing of the presence of nearby vegetation that can potentially become competitors. Leaves and other photosynthetic (i.e. green) tissues absorb red light (R) but not far-red light (FR). As a consequence, light reflected from the leaves of neighboring plants has a much lower R : FR ratio than direct sunlight. This decrease in R : FR is a signal of vegetation proximity that does not require actual shading or reduced light intensity. The low R : FR signal (herein referred to as proximity shade) is perceived by the phytochrome family of photoreceptors, particularly phytochrome B (phyB). After phyB-dependent perception, the signal is transduced by a network of PHYTOCHROME INTERACTING FACTORS (PIFs), a group of transcription factors that function as inducers of the proximity shade response (Casal & Fankhauser, 2023; Martinez-Garcia & Rodriguez-Concepcion, 2023). In the lab, proximity shade can be simulated by enriching white light (W)

with FR, hence reducing the R : FR ratio without reducing total light intensity. This simulated shade (W + FR) treatment triggers elongation growth and degradation of photosynthetic pigments (Chls and carotenoids) in plants that are shade-intolerant, including *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), and most crops (Cagnola *et al.*, 2012; Bou-Torrent *et al.*, 2015; Chitwood *et al.*, 2015; Llorente *et al.*, 2016; Fanwoua *et al.*, 2019; Ji *et al.*, 2019, 2020, 2021; Kim *et al.*, 2019; Ortiz-Alcaide *et al.*, 2019; Sun *et al.*, 2020; Casal & Fankhauser, 2023; Li *et al.*, 2024; Shomali *et al.*, 2024). The response to proximity shade, referred to as shade avoidance syndrome (SAS), is strongly attenuated in shade-tolerant species such as *Cardamine hirsuta*, an edible relative of *Arabidopsis*. Exposure of *Cardamine* plants to W + FR causes no elongation growth, and it only leads to a minor decrease in the levels of Chls and carotenoids compared to W-grown controls (Molina-Contreras *et al.*, 2019; Morrelli *et al.*, 2021).

Domestication has led to increased tolerance to proximity shade as the SAS involves responses that are not desirable from an agronomic perspective, such as excessive elongation growth at the expense of defense against biotic stress. However, the idea that

*These authors contributed equally to this work.

attenuating shade-avoidance responses would benefit crops might be an oversimplification (Gommers *et al.*, 2013; Casal & Fanhauer, 2023). Agricultural practices directly impacted by proximity shade, such as high density planting or intercropping, also involve additional light-independent competence for access to water and nutrients and increased risk of infections associated with crowded environments. Besides, it remains to be demonstrated that the SAS mechanisms deduced from studies using FR supplementation to simulate shade also apply to actual plant proximity shade in the field. In tomato, a number of recent studies have observed positive effects of FR supplementation in plant growth but also in fruit quantity (number and weight) and quality (sugar content) (Fanwoua *et al.*, 2019; Ji *et al.*, 2019, 2020; Kalaitzoglou *et al.*, 2019). However, it is unclear whether these conclusions could also apply to agricultural settings involving proximity shade with no FR supplementation. For example, high density conditions are associated with low R : FR but they also involve reduced light intensity in the lower strata of the crop plants and increased competition for resources.

Here we investigated the variability in the response to simulated shade in tomato during short exposure to W + FR at early stages of development (e.g. in seedlings) and long-term exposure up to the fruit-bearing stage. We further identified a *Solanum pennellii* introgression line showing hyposensitivity to proximity shade in terms of growth and a gene expression profile consistent with a heavily impaired homeostasis of auxin, the main hormone controlling proximity shade-induced elongation. Furthermore, we compared how FR supplementation in the glasshouse and high-density conditions in the field influenced long-term plant growth and fruit traits of this shade-tolerant line and the shade-avoider parental line.

Materials and Methods

Plant materials and treatments

A protocol was set up on plants at the seedling stage to investigate the response to proximity shade in tomato (*Solanum lycopersicum* L.) genotypes Money Maker, San Marzano, M82, and Micro-Tom. Additionally, two populations of tomato landraces and introgression lines (IL) were evaluated. In particular, we used 21 landraces (8 from South America, 4 from United States, 9 from other European and world regions) and 20 *S. pennellii* introgression lines (IL) belonging to a collection available at the University of Naples Federico II, Department of Agricultural Sciences (details hosted in the LabArchive repository, doi: 10.6070/H4TT4NXN). For all the genotypes, seeds were sterilized and plated on solid 1/2-strength Murashige & Skoog (1/2MS) medium containing 1% (w/v) agar without sucrose or added vitamins (Barja *et al.*, 2021). Plates were incubated in the dark for 4 d. Only seeds that germinated at the same time were transferred to soil and kept for 2 d in a walk-in growth chamber referred to as TCH under a photoperiod of 8 h of darkness and 16 h of white light (W) at a photosynthetic photon flux density (PPFD) of $150 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ at $25 \pm 1^\circ\text{C}$ during the day and

$22 \pm 1^\circ\text{C}$ during the dark (night) period. W was obtained using a mix of 5 Philips MAS LEDtube 1500mm HO 23W840 and 4 MAS LEDtube 1500mm HO 23W830 LED tubes, arranged in an alternating pattern. To simulate proximity shade, GreenPower LED module HF far-red (Philips) tubes supplying FR were added between the racks of LED tubes supplying W. As a result, in the same TCH chamber, there was an area illuminated with W (R : FR of 3.14) and another area illuminated with W + FR (simulated shade, R : FR of 0.17) separated by an opaque panel so W-grown plants were not exposed to residual FR. W and W + FR light spectra of this chamber (TCH) are shown in Supporting Information Fig. S1. After the 2-d adaptation period under W, seedlings were either left under W or transferred to W + FR. Eleven days after the treatment, morphological measurements were done using the IMAGEJ software (Schneider *et al.*, 2012). For long-term W + FR exposure, MicroTom plants were grown in plastic pots (0.66 l, 12 cm diameter) in the TCH chamber for 4 months, until the fruit-bearing stage. In the case of IL2-2 plants and the M82 parental, growth beyond the seedling stage was carried out in a glasshouse room (referred to as IGR) equipped with W and W + FR light sources to support the same photoperiod and light intensity (PPDF) and quality (R : FR ratio) values similar to those in the TCH chamber. Light spectra in the IGR box are shown in Fig. S1. Seeds were sown in seed trays, and after germination, the seedlings were transferred to plastic pots (9 l, 21 cm diameter) with commercial substrate Vegetal Radic PP (Tecno Grow, Tercomposti, Italy). Plants were grown in the IGR box with $24 \pm 3^\circ\text{C}$ air temperature during the day and $18 \pm 3^\circ\text{C}$ during the night. Plants were irrigated using the following full nutrient solution: $\text{Ca}(\text{NO}_3)_2$ 1.27 g l^{-1} ; P_2O_5 0.26 g l^{-1} ; K_2O 0.23 g l^{-1} ; MgSO_4 0.31 g l^{-1} . The electrical conductivity (EC) of the NS was $1.8 \pm 0.1 \text{ dS m}^{-1}$. The pH was monitored daily and maintained at 6.0 ± 0.3 . All plants were grown under W (PPDF of $190 \pm 15 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 2 wk. Thereafter, the plants were subjected to two different light treatments using a custom-made lighting system developed by MEG s.r.l. (Milan, Italy). As previously outlined, the light treatment comprised enrichment with FR light, maintaining the same flux density (as the W control). Consequently, only the R : FR was reduced from 9 (W) to 0.13 (W + FR). The analysis of morphological, physiological, and molecular parameters was conducted on plants that had reached the 30-d growth stage and at the end of the reproductive cycle.

Arabidopsis thaliana (L.), Heynh wild-type (Col-0), single *sav3-5*, and triple *pif4-101 pif5-3 pif7-1* (*pif457*) mutants have been described before (Pastor-Andreu *et al.*, 2024). For hypocotyl assays, seeds were surface-sterilized and sown on solid 1/2MS growth medium without sucrose or vitamins at two different densities: low (2 seeds cm^{-2}) or high (18 seeds cm^{-2}). After stratification (3–4 d in the dark at 4°C), plates with seeds were incubated for 7 d in a dedicated growth chamber (model D1200PL; Aralab, Madrid, Spain) at 22°C and continuous W emitted from LED light tubes that provided a PPDF of $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ (R : FR of 3.55). The light spectrum of this *Arabidopsis* chamber (termed ACH) is shown in Fig. S1.

Hypocotyl length was measured as indicated elsewhere (Urdin-Bravo *et al.*, 2024). Three biological replicates were done. Each replicate was done on a different plate and included 25–30 seedlings per genotype and per treatment (density). For the high-density condition, we plated *c.* 150 seeds in a square region of 28 × 28 mm, but only seedlings growing in the center of this area were used for measuring the hypocotyl length, whereas the remaining seedlings were discarded. Hypocotyl measurements of individual seedlings from the three independent replicates were pooled together for data presentation.

Open field experiment

The field trials were carried out in experimental farms (ARCA2010 in Acerra, Italy, and Fundación Cajamar in Paiporta, Spain). At the end of April 2024, 3 wk after sowing, when the third true leaf was fully expanded, tomato plants (genotypes M82 and IL2-2) were transplanted in the open field. Sunlight spectrum in a sunny April day at the Spanish site is shown in Fig. S1. Plants were arranged in a randomized block design with four replications per treatment and eight plants per biological replication. Plant density was kept at 3 plants m⁻² for normal farming practice (low density, LD), or 6 plants m⁻² for high density (HD). The experimental field was irrigated with a timer-controlled drip irrigation system of 4 l h⁻¹ (one emitter per plant). A class A evaporation pan was used to estimate evapotranspiration and adjust irrigation time to ensure that the amount of water supplied to each plant restored the soil to its field water capacity at each irrigation round. The irrigation and nutrition of the plants was done according to the management practices of the area. Border plants were discarded.

Assessment of pigment content

The leaf pigment index and Chl content parameters were determined on fully expanded leaves using a portable meter SPAD-502 Plus (Konica Minolta Optics, Tokyo, Japan). Leaf Chls and carotenoids were also quantified by HPLC-DAD (Barja *et al.*, 2021).

Photosynthetic parameters

Gas exchange and Chl fluorescence measurements were conducted on healthy, young, fully expanded leaves (typically the third leaf counting from the apical meristem) of adult plants. Leaf gas exchanges were measured at 30 d after transplant with a portable photosynthesis system (LCA 4; ADC BioScientific Ltd, Hoddesdon, UK) equipped with a broadleaf chamber (cuvette window area, 6.25 cm²). All the measurements were conducted in an open system at a temperature of 24 ± 0.5°C, a CO₂ concentration of 400 ± 10 µmol m⁻² s⁻¹, and a relative humidity of 70–80%. The measurements were carried out with a transparent top leaf chamber, allowing measurements at ambient light intensity while including or excluding FR light. From gas-exchange measurement, the following instantaneous parameters were measured: sub-stomatal CO₂ (vp_m), transpiration

(*E*; mmol m⁻² s⁻¹), stomatal conductance (*g*; mol m⁻² s⁻¹), and photosynthetic rate (µmol m⁻² s⁻¹).

Fruit traits

Fruits were quantified and weighed after harvest, before collecting pericarp samples (that were snap-frozen in liquid nitrogen for storage) and counting seed numbers. Fruit equatorial area was calculated using the IMAGEJ software from pictures taken from above of harvested fruits. Total solid soluble content (°Brix) was measured with a refractometer (Hanna Instruments, Padova, Italy). Fruit firmness, expressed as the maximum force (kg cm⁻²) needed for penetration of the probe (8 mm diameter) into the fruit through the tomato skin, was measured using a PCE-PTR200 penetrometer (PCE Instruments, Capannori, Italy). Pericarp tissue was used to measure ascorbic acid using a colorimetric method (Francesca *et al.*, 2022) and carotenoids by HPLC-DAD (Barja *et al.*, 2021).

RNA sequencing

Total RNA was extracted from the first two completely expanded leaves of 2-wk-old plants exposed to W + FR (or W as a control) for 24 h. The tissue was collected and immediately frozen in liquid nitrogen for storage at -80°C. RNA extraction was carried out using the RNeasy Mini Kit (Qiagen) according to the method reported by the manufacturer. Three independent replicates of total RNA (1 µg, RIN between 7.1 and 8.6) extracted from leaves of different plants growing under the same conditions were used for RNA sequencing by BGI Tech Solutions (<https://services.bgi.com>). A total of 12 samples were tested using the DNBSEQ platform, with an average yield of 6.68 G data per sample. The average alignment ratio of the sample comparison genome (*S. lycopersicum* 4081.JGI.ITAG2.4.v.2201) was 97.82%. The BGI DrTom pipeline (<https://eu-biosys.bgi.com/help/en/mrna/>) was used to estimate gene expression, identify DEGs, and analyze enrichment according to GO terms or KEGG pathways. Raw data are deposited at NCBI, BioProject PRJNA1231488.

RT-qPCR analyses

Total RNA for RT-qPCR was isolated from frozen leaf samples using the Purelink RNA mini kit (Thermo Fisher Scientific, Waltham MA, USA). Determination of RNA integrity and quantity as well as cDNA synthesis were carried out as described (Ezquerro *et al.*, 2023). Transcript abundance of *YUC9* (Solyc06g083700) and *PIF7b* (Solyc06g069600) was assessed using the following gene-specific primers (5'–3'): *YUC9*_qF, ACCGTTGAACTTGTCACTGG; *YUC9*_qR, GCACCAGCTAGCCCTTTCC; *PIF7b*_qF, GCGCTCCCCTCATTTATCCA; and *PIF7b*_qR, TTTGGGGCTGATGGATTCGG. The tomato gene *Solyc07g025390* was used as an endogenous reference gene for normalization (González-Aguilera *et al.*, 2016). The RT-qPCR was carried out on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) using three independent samples and two technical replicates of each sample.

Statistical analyses

Statistical analyses, including *t*-test and two-way ANOVA with means comparison tests, were carried out using the statistical software R v.4.1.0 (<https://www.R-project.org/>). Two-way ANOVA was applied when analyzing data with two factors, for example, genotype (with more than one level) and treatment (light or density, with two levels). In cases when the analysis focused solely on the treatment with two levels, a *t*-test was used to compare the means of the two groups.

Results

Tomato genotypes show a range of responses to simulated proximity shade (W + FR)

Tomato plants of different accessions (Money Maker, San Marzano, M82 and MicroTom) were germinated in darkness, and evenly grown 4-d-old seedlings were transferred to soil and incubated under W for two additional days. Then, half of them were transferred to W + FR (referred to as simulated shade) and the other half were left under W for 11 d. At the end of the treatment, different parameters associated with the SAS were measured (Fig. 1a). All accessions tested showed longer epicotyls and lower leaf Chl contents when exposed to W + FR (Fig. 1b). Leaf area also increased under simulated shade, even though the difference with W controls was not statistically significant in San Marzano (Fig. 1b). In the case of hypocotyl elongation, by far the most studied SAS phenotype in Arabidopsis (Martinez-Garcia & Rodriguez-Concepcion, 2023), only MicroTom plants failed to significantly respond to the W + FR treatment (Fig. 1b). To check whether MicroTom plants responded to the W + FR treatment at the molecular level, we used marker genes shown to be upregulated (*YUC9*) or downregulated (*PIF7b*) by low R : FR in seedlings of the tomato Ailsa Craig cultivar (Sun *et al.*, 2020). RT-qPCR analysis showed a strong *YUC9* upregulation and *PIF7b* downregulation in leaves of MicroTom seedlings exposed for 24 h to W + FR compared to W controls (Fig. 1c), confirming that MicroTom plants perceived and transduced the low R : FR signal.

To next investigate long-term responses to proximity shade, the MicroTom accession was selected based on its small size, which allowed for growing plants and estimating fruit yield in the growth chamber. In this case, plants were germinated and grown for 4 d as described above, and then they were left under W or transferred to W + FR for 4 months. FR supplementation increased fruit yield, likely due to slightly increased fruit size (area), with no differences detected in plant weight (Fig. 1d). Ripe fruit from plants grown under W + FR showed an improved content of total soluble solid ($^{\circ}$ Brix) compared to W controls, but no significant differences were detected in firmness or in seed number per fruit (Fig. 1d). The enhancement of fruit production and $^{\circ}$ Brix has been previously associated with FR-supplemented conditions in several tomato cultivars (Fanwoua *et al.*, 2019; Ji *et al.*, 2019, 2020; Kalaitzoglou *et al.*, 2019), confirming that most short-term and long-term responses to low

R : FR are conserved in different tomato accessions, including MicroTom.

Screening for shade-tolerant tomato lines

We next used the experimental conditions optimized for seedlings to screen for tomato lines showing attenuated responses to proximity shade. To increase genetic variability, we used collections of introgression lines (ILs) and landraces (Figs 2, S2). In particular, 20 ILs covering the genome of the wild tomato relative *S. pennellii* in the genetic background of the tomato (*S. lycopersicum*) cultivar M82 (Eshed & Zamir, 1995) and 21 landraces from Europe, South America, the United States, and other world regions (Ruggieri *et al.*, 2014) were included in the screening for shade-hyposensitive lines. As a read-out, we used epicotyl elongation under W + FR compared to W, as it was the phenotypic trait showing the strongest response and dynamic range (Fig. 1).

Most ILs and landraces were found to display an exacerbated epicotyl elongation response to low R : FR compared to M82 (Fig. 2a). Among the few lines that showed a reduced response to W + FR, IL2-2, and E109 seedlings showed the strongest elongation phenotype (Fig. 2a) and a similar epicotyl length under W compared to M82 (Fig. S2). To validate the shade-hyposensitive phenotype of these two lines, a second round of experiments was performed using only lines M82, IL2-2, and E109. The results confirmed that IL2-2 seedlings showed a significantly reduced response to simulated shade compared to the M82 parental line, not only in terms of epicotyl elongation but also when measuring the increase in hypocotyl length (Fig. 2b). In the case of the E109 line, however, the elongation response of hypocotyls and epicotyls was similar to that of M82 seedlings (Fig. 2b). We therefore concluded that only the IL2-2 line showed an attenuated elongation response to simulated shade (Fig. 2c).

To test whether other short-term responses to low R : FR were also attenuated in IL2-2, we quantified the levels of photosynthetic pigments (carotenoids and Chls) by HPLC after simulated shade exposure (Fig. 2d). M82 and IL2-2 seedlings germinated and grown in plates were transferred to soil and left under W before exposing them to W + FR (or left under W) for 24 h. Treatment with W + FR caused a reduction in the levels of carotenoids and Chls in M82 seedlings, whereas no statistically significant changes were detected in the IL2-2 line (Fig. 2d).

Auxin-related genes are misregulated and respond less to W + FR treatment in IL2-2 plants

The response of M82 and IL2-2 lines to simulated shade was next compared at the transcriptomic level. Dark-germinated M82 and IL2-2 seedlings were transferred to soil at Day 4 after imbibition and grown under W for 11 d. Then, some of them were left under W and others were exposed to W + FR for 24 h before extracting RNA from leaves. Following RNA sequencing (RNA-seq) and bioinformatic analyses, genes with more than twofold change (FC) in expression ($\log_2\text{FC} > 1$, *P*-value < 0.05) were considered differentially expressed genes (DEGs) (Fig. 3). The reduced morphological and metabolic responses to simulated

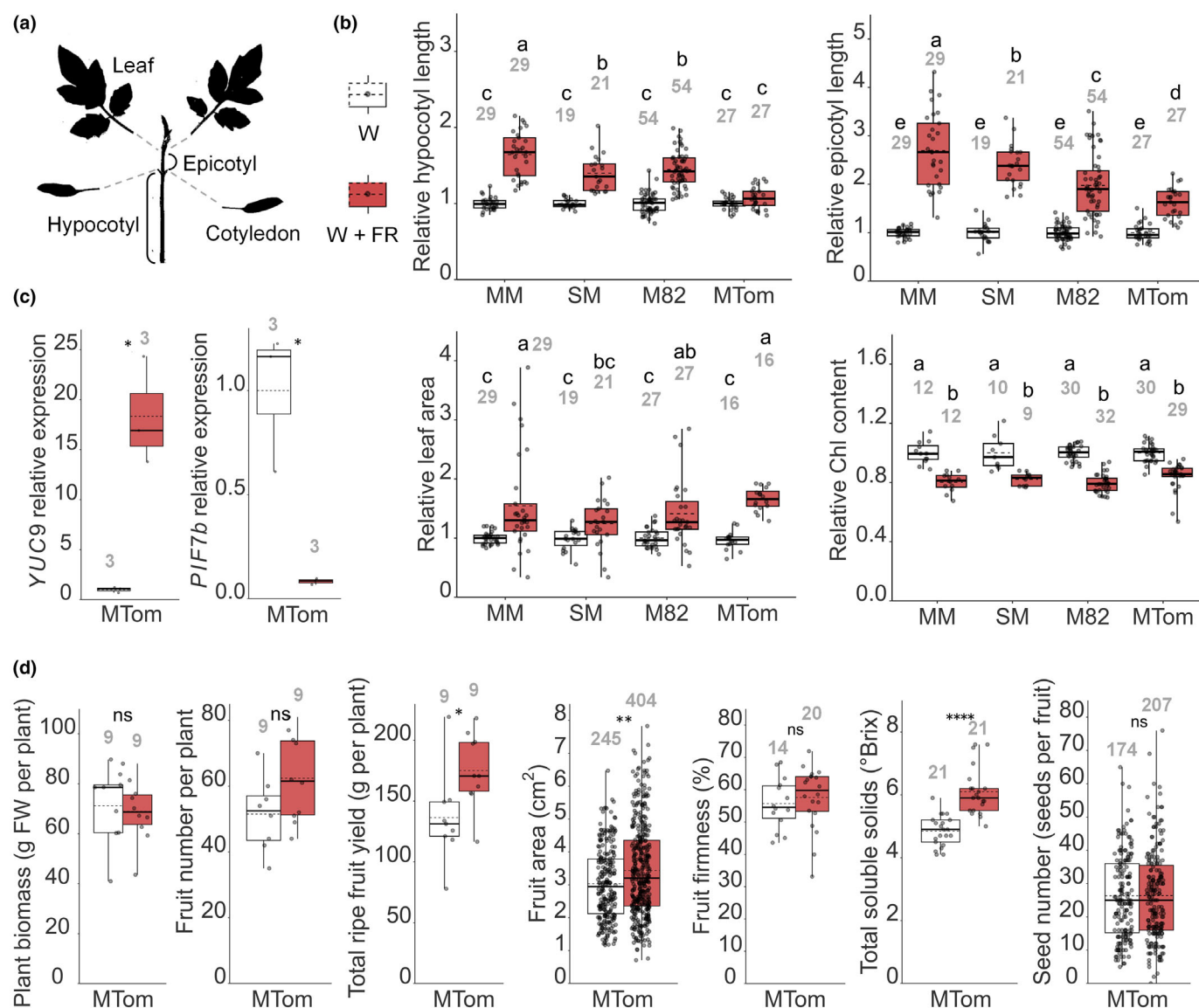


Fig. 1 Tomato accessions show a range of responses to simulated shade. (a) Schematic representation of tomato seedling organs used to quantify the impact of simulated shade. (b) Effect of simulated shade in different organs of tomato seedlings of the MoneyMaker (MM), San Marzano (SM), M82, and MicroTom (MTom) accessions germinated under darkness for 4 d and grown under white light (W) for 2 d and then exposed to far-red-supplemented W (W + FR) or left under W for 11 d. (c) Reverse transcription quantitative polymerase chain reaction analysis of shade marker genes *YUC9* and *PIF7b* in leaves from W-grown 2-wk-old MicroTom seedlings exposed to simulated shade (W + FR) or left under W for 24 h. (d) Quantification of total biomass and fruit-associated features of MicroTom plants grown under W or W + FR for 4 months. In all the plots, dots represent individual data points and numbers in gray indicate sample size (*n*). The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the dotted line within the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (two-way ANOVA followed by Duncan's multiple range test, $P < 0.05$) or asterisks (t -test: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

shade displayed by IL2-2 seedlings (Fig. 2) appeared in contrast with a substantial increase in number of genes differentially expressed in response to the W + FR treatment compared to M82 (Fig. 3a). Gene Ontology Biological Process (GO-P) enrichment analyses showed that the 292 genes responding to simulated shade in M82 but not in IL2-2 seedlings were involved in light-regulated processes related to growth, including auxin signaling, photomorphogenesis, phototropism, and cell wall biogenesis (Fig. 3b). Consistent with the central relevance of auxin

for shade-promoted growth in tomato (Cagnola *et al.*, 2012; Bush *et al.*, 2015; Sun *et al.*, 2020), the highest enrichment *Q*-value was found for 'auxin-activated signaling pathway' genes, which encode proteins required for auxin transport (PIN3, PIN4, BG1LA, and BG1LE) and response (IAA1, IAA7, IAA17, IAA22, and ARF10B) (Fig. 3c). Furthermore, four auxin signaling genes encoding SAUR and ATHB2 homologs were found among the top 20 genes most upregulated by simulated shade in M82 plants (Fig. 3d). Most of these auxin-related genes were

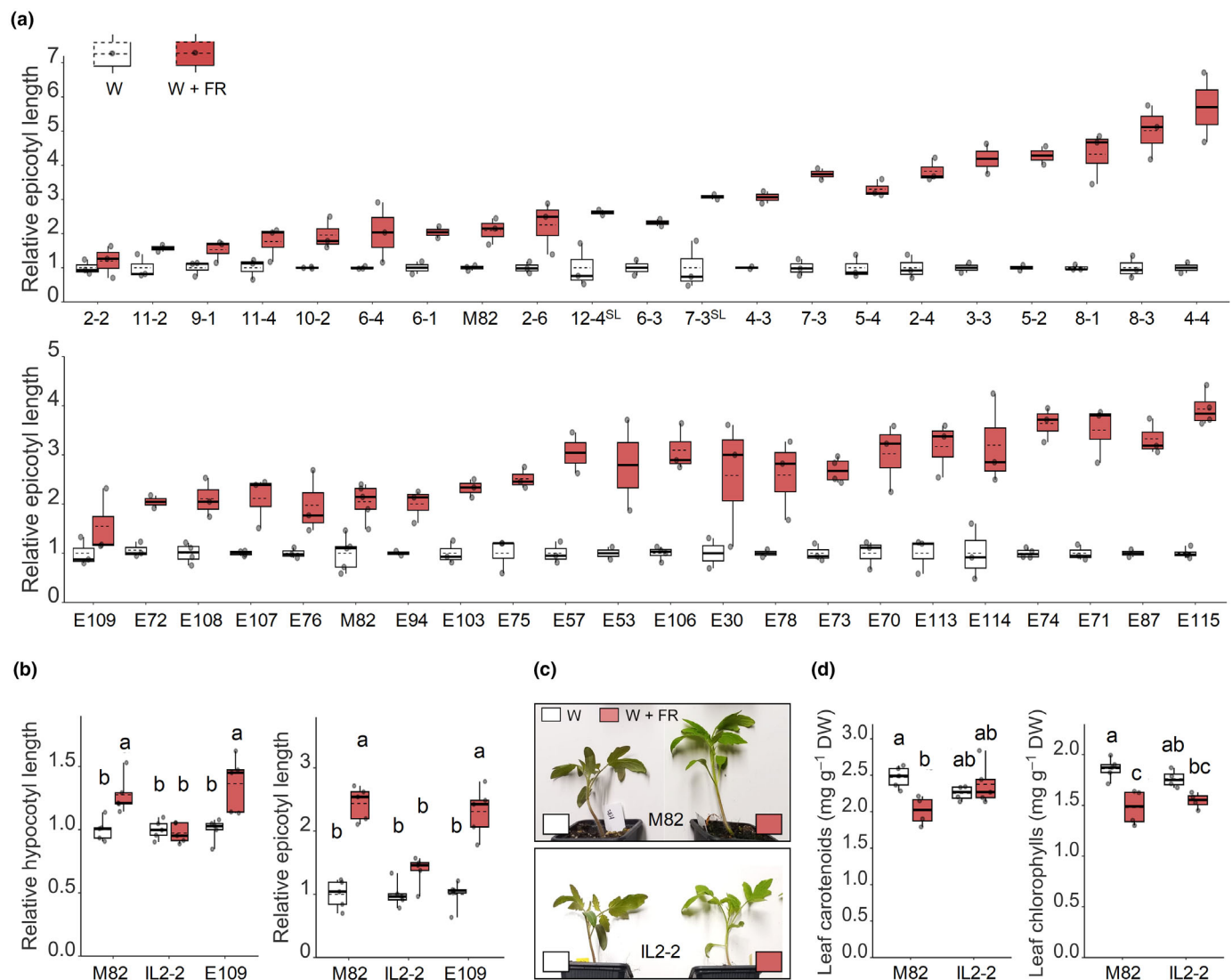


Fig. 2 The tomato introgression line IL2-2 shows an attenuated response to simulated shade. Seeds from different tomato lines were germinated and grown under white light (W) and then exposed to far-red (FR)-supplemented W (W + FR) or left under W for 11 d. (a) Epicotyl elongation of the indicated tomato introgression lines (ILs, upper plot) and landraces (lower plot) compared to their corresponding M82 controls in response to simulated shade. SL, sub-line. Values correspond to three replicates ($n = 3$) and they are shown relative to W controls. (b) Hypocotyl and epicotyl length of the indicated lines ($n = 5$). (c) Representative images of seedlings from the IL2-2 line and its M82 parental. (d) Levels of photosynthetic pigments in the indicated seedlings ($n = 5$). In boxplots, dots represent individual data points. The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the dotted line within the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (two-way ANOVA followed by Duncan's multiple range test, $P < 0.05$).

upregulated in IL2-2 compared to M82 in plants grown under W but they showed similar levels in M82 and IL2-2 plants after the W + FR treatment (Fig. 3c,d). GO-P analysis of the 304 genes that were differentially expressed when comparing M82 and IL2-2 seedlings grown under control (W) conditions showed the second highest Q -value for the same category of auxin-activated signaling pathway genes that was overrepresented in shade-regulated M82 genes. A different enrichment analysis of the same 304 DEGs using KEGG pathways showed the highest Q -value for tryptophan metabolism, a category that included genes for auxin biosynthetic enzymes such as TAA3/TAR2,

YUC5, and YUC9 (Fig. 3e). Again, these genes were misregulated in IL2-2 and reached similar expression levels as the M82 parental following exposure to simulated shade. Also similar to the auxin signaling and transport genes, biosynthetic genes were less responsive or responded differently to W + FR in IL2-2 seedlings, likely contributing to the reduced shade-triggered elongation responses observed in the introgression line (Fig. 2). In the case of genes involved in photosynthetic pigment biosynthesis, M82 and IL2-2 seedlings showed a much more similar profile both under W and in response to the W + FR treatment (Fig. S3).

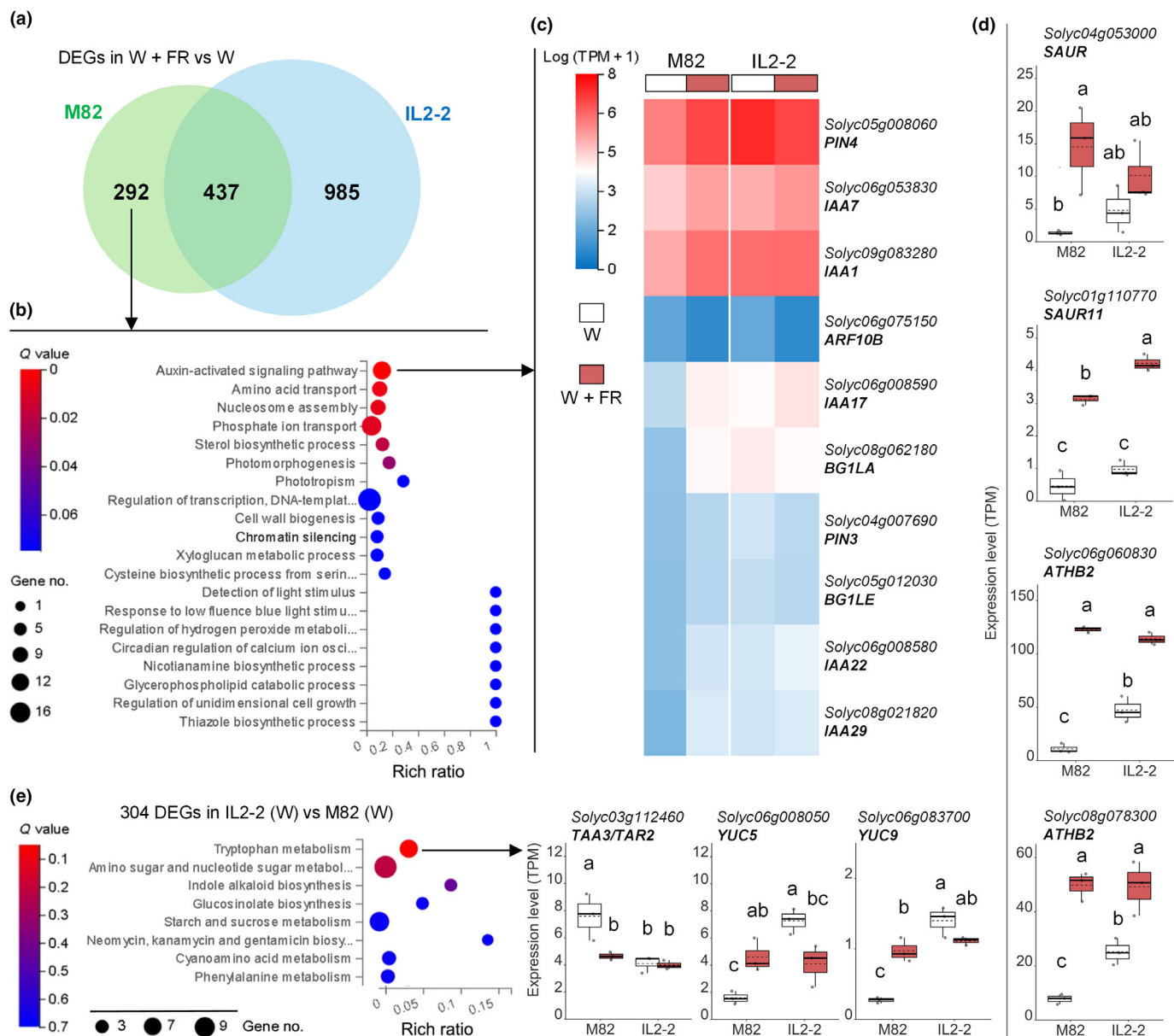


Fig. 3 IL2-2 plants show an altered expression of auxin-related genes. Two-week-old tomato M82 and IL2-2 seedlings were treated with W + FR (or W as a control) for 24 h and then used for RNA sequencing using three independent replicates ($n = 3$). (a) Venn diagram of differentially expressed genes (DEGs) in plants exposed to simulated shade. (b) GO term (Biological Process) enrichment analyses of the 292 DEGs specific of M82. The hyper function in R software was used to calculate the enrichment (Rich) ratio and the Q-value, which represents the significance value of enrichment (Q-values lower than 0.05 correspond to significant enrichment). The size of the bubbles represents the number of DEGs annotated to a particular GO term. (c) Heatmap comparison of transcript abundance (mean log₂ TPM + 1) for the genes included in the GO term 'auxin-activated signaling pathway'. (d) Expression of the four auxin signaling genes found among the top 20 most highly upregulated DEGs in simulated shade-treated M82 plants. Transcripts Per Kilobase Million (TPM) values are represented. (e) KEGG pathway enrichment analyses of the DEGs found between M82 and IL2-2 seedlings grown under control (W) conditions and transcript levels of the genes included in the term 'tryptophan metabolism'. Bubble plot and box plots were made as described in (b, d). In boxplots, dots represent individual data points. The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the dotted line within the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (two-way ANOVA followed by Duncan's multiple range test, $P < 0.05$).

The shade-hyposensitive phenotype of IL2-2 does not alter fruit quality under FR-supplemented light

To analyze long-term responses to simulated shade in IL2-2 plants, they were grown together with the parental line M82 in a

glasshouse room that, similar to the growth chamber used to this point, had separate areas illuminated with W or W + FR. Following germination in seed trays, M82 and IL2-2 seedlings were transferred to 9-l plastic pots. After 2 wk under W in the glasshouse room, half of the pots were left under W, and the other

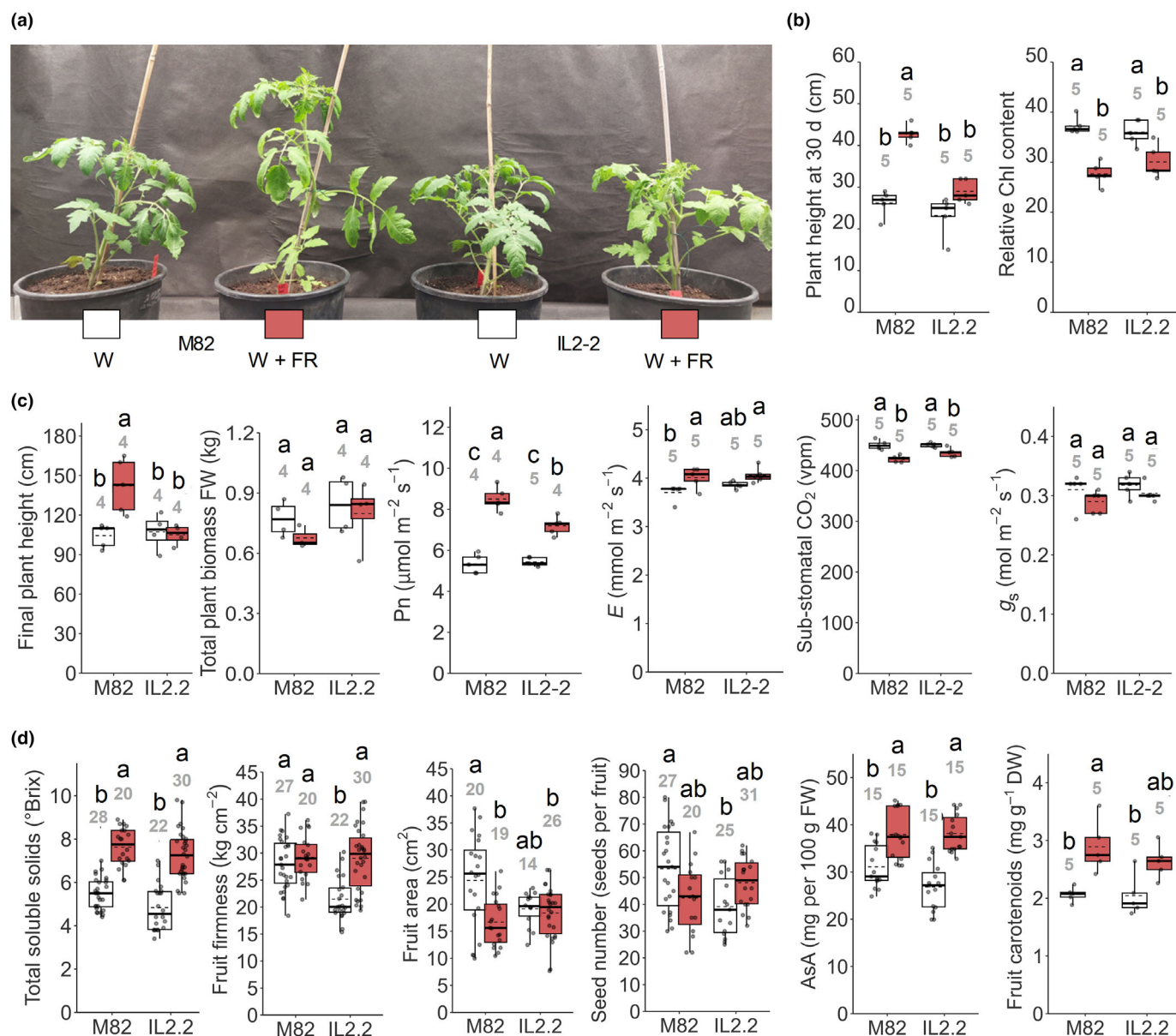


Fig. 4 IL2-2 plants elongate less than M82 but show a similar enhancement of fruit quality under FR-supplemented light. (a) Representative images of tomato M82 and IL2-2 plants germinated and grown under white light (W) for 20 d and then transferred to W + far-red (FR) or left under W for 10 more days. (b) Plant height and Chl content of plants like those shown in (a). (c) Plant height, biomass, and photosynthetic parameters of M82 and IL2-2 plants kept under W or W + FR until fruit production. (d) Quantification of fruit-associated features from the plants described in (c). In boxplots, dots represent individual data points, and numbers in gray indicate sample size (n). The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the dotted line within the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (two-way ANOVA followed by Duncan's multiple range test, $P < 0.05$).

half exposed to W + FR. Similar to that observed in the growth chamber with 15-d-old seedlings that had been exposed for 11 d to either W or W + FR (Fig. 2c), 30-d-old M82 plants growing in the glasshouse room and exposed for 10 d to either W or W + FR were taller and paler under simulated shade (Fig. 4a). This result confirmed that the low R : FR conditions in the glasshouse room were working to activate the response to proximity shade. In the case of IL2-2 plants, their height was virtually unaffected by the FR supplementation of W light (Fig. 4b). By

contrast, the shade-induced Chl drop was similar in IL2-2 plants and M82 controls (Fig. 4b). When plants produced fruit, we compared both the vegetative and the reproductive (fruit) phenotypes under W or W + FR. Continuous growth under W + FR resulted in longer plants in the case of M82, whereas no significant difference in plant height was observed for the IL2-2 line growing under W + FR compared to control W conditions (Fig. 4c). While total plant biomass was unaffected by the treatment or the genotype, W + FR treatment resulted in higher

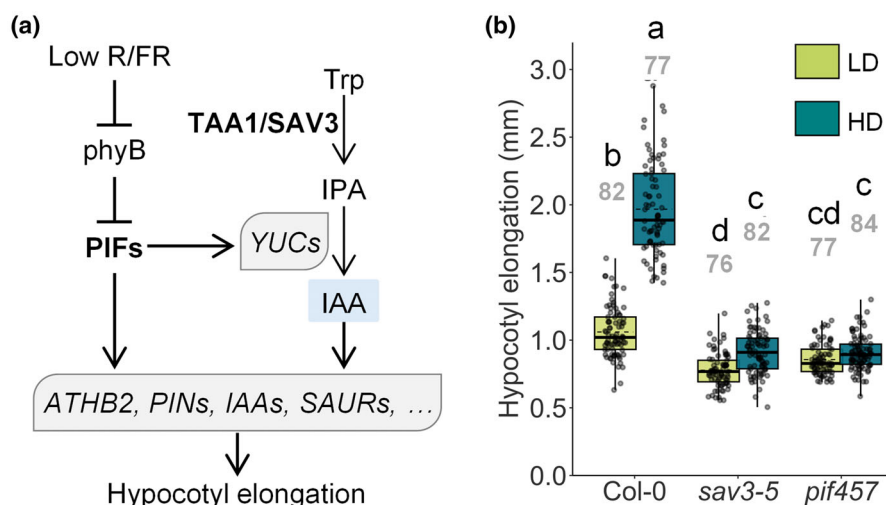


Fig. 5 Auxin is required for hypocotyl elongation under high planting densities. (a) Cartoon depicting the main proximity shade signaling components and their connection with auxin production and hypocotyl growth. (b) Arabidopsis *sav3-5* and *pif457* mutant lines and their wild-type parental (Col-0) were plated either at low-density (LD) or high-density (HD) and grown for 1 wk under continuous W. Dots represent measurements of hypocotyl length of individual seedlings growing in three different plates (replicates) that are pooled together. Numbers in gray indicate sample size (*n*). The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the dotted line within the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (two-way ANOVA followed by Duncan's multiple range test, *P* < 0.05).

photosynthetic (*P_n*) and transpiration (*E*) rates in M82, increases that appeared attenuated in the IL2-2 line (Fig. 4c). In both genotypes, FR supplementation reduced the substomatal CO₂ concentration without significantly changing the stomatal conductance rate (*g_s*) (Fig. 4c). Likely due to the relatively small size of the pots used in this experiment, fruit production was very low in our glasshouse conditions (less than a dozen fruits per plant), making it impossible to reliably calculate yield. As for fruit traits, M82 plants grown under W + FR conditions produced fruit with higher soluble solid content (°Brix) but only marginal differences in firmness compared to W controls (Fig. 4d), mostly paralleling that observed for the MicroTom accession (Fig. 1d). Also similarly, seed number per fruit was unaffected by FR supplementation (Fig. 4d). By contrast, M82 plants growing under simulated shade produced fruit of slightly smaller size (area). A deeper analysis of M82 ripe fruit showed that W + FR treatment resulted in increased accumulation of reduced ascorbate (vitamin C) and carotenoids (pro-vitamin A), hence adding nutritional quality to the tomatoes (Fig. 4d). A very similar fruit and seed phenotype was observed for the IL2-2 line under W + FR, indicating that its shade hyposensitivity mostly impacts plant vegetative growth (height) but does not prevent the positive effects on fruit quality provided by FR supplementation.

IL2-2 plants show an improved fruit yield under high-density conditions in the field

We next aimed to test whether some of the conclusions derived from the results using simulated shade (W + FR) and pot-grown plants in the glasshouse could also apply to soil-grown plants in open field conditions exposed to a natural proximity

shade provided by neighboring plants. As IL2-2 plants showed a strong alteration of auxin-related processes (Fig. 3), we first examined the relevance of this hormone for enhanced growth under higher density conditions (Fig. 5). For this purpose, we employed Arabidopsis plants, in which a direct link between low R : FR perception by phyB and PIF-regulated auxin production has been well established (Casal & Fankhauser, 2023; Martinez-Garcia & Rodriguez-Concepcion, 2023). In this pathway, tryptophan (Trp) is converted to indole-3-pyruvic acid (IPA) by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1), also known as SHADE AVOIDANCE 3 (SAV3), and then flavin monooxygenases of the YUCCA (YUC) subfamily catalyze the oxidative decarboxylation of IPA to indole-3-acetic acid (IAA), the major natural auxin (Fig. 5a). PIF4, PIF5, and PIF7 are the three main PIFs inducing the expression of YUC genes, and they also regulate the expression of other genes involved in auxin homeostasis, transport, and signaling, eventually promoting hypocotyl elongation (Fig. 5a). Mutants deficient in SAV3 (*sav3-5*) or in PIF4, PIF5, and PIF7 (*pif457*) are therefore defective in IAA synthesis and hypocotyl elongation in response to W + FR (Pastor-Andreu *et al.*, 2024). To check whether this auxin-dependent pathway was also important to regulate growth in response to plant density conditions, *sav3-5* and *pif457* lines together with a wild-type (Col-0) control were germinated and grown under W for 1 wk on plates at two different densities: low density (LD) and high density (HD). While WT plants were significantly longer in the HD setting, the length of *sav3-5* or *pif457* hypocotyls was similar under LD and HD (Fig. 5), confirming that auxins are required for enhanced growth under high planting densities.

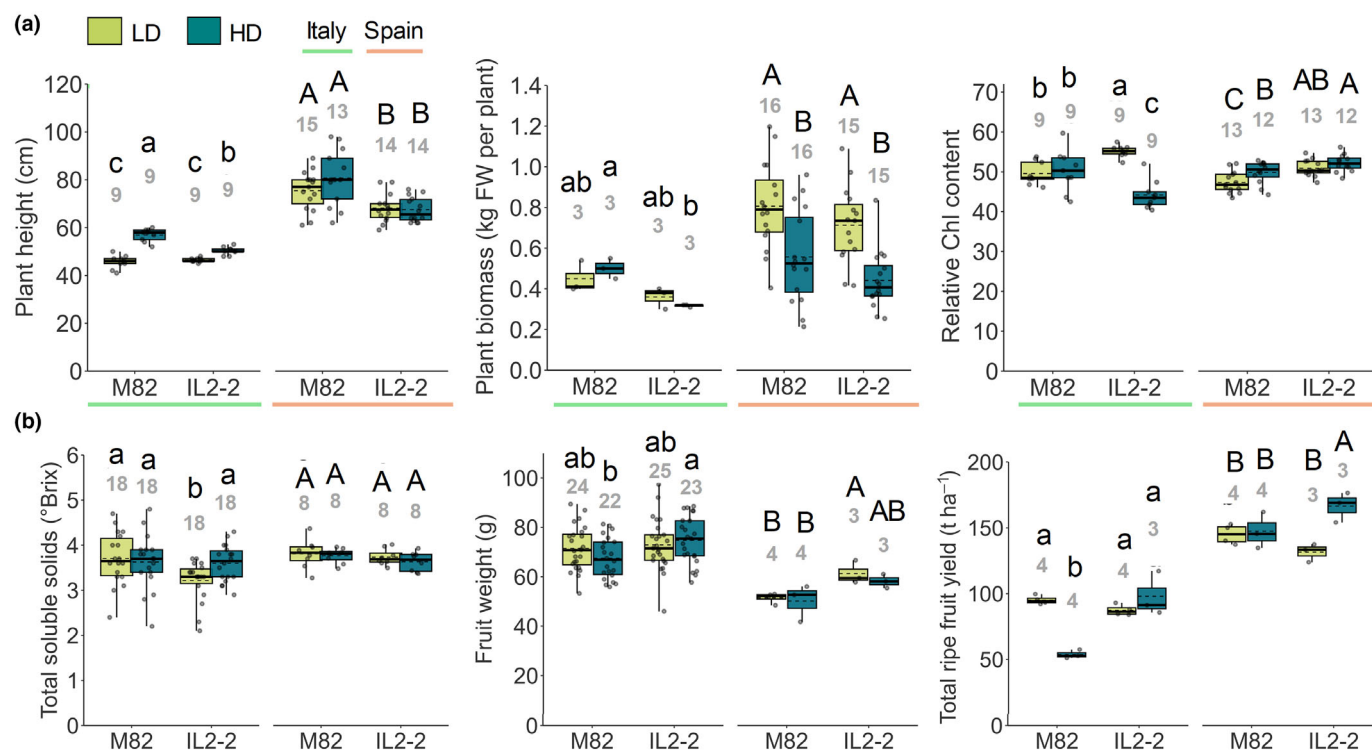


Fig. 6 IL2-2 plants perform better than M82 under high planting densities. Tomato M82 and IL2-2 plants were grown in open fields located in Italy and Spain either at low density (LD) or high density (HD) conditions. (a) Plant height, biomass, and leaf Chl levels at the time of harvest. (b) Ripe fruit features and total yield. Dots represent individual data points, and numbers in gray indicate sample size (n). The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the dotted line within the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (two-way ANOVA followed by Duncan's multiple range test, $P < 0.05$).

Open field trials with tomato were then carried out using the same pool of M82 and IL2-2 seeds in two different locations: Acerra (Campania, Italy) and Paiporta (Valencia, Spain). Plants were germinated in the glasshouse and transferred to the field after 3 wk, at the end of April 2024. They were grown in rows separated by 1 m, at two different densities: 3 plants m^{-2} (referred to as low density, LD) or 6 plants m^{-2} (high density, HD). The same row had two alternating groups of plants of each genotype. About 3 months after planting, plants were analyzed, and fruits were harvested and used for analysis (Fig. 6). The timeline of temperature and relative humidity records at both sites is shown in Fig. S4. At the time of harvest, the plants from the Italian location were smaller and had produced less fruit compared to those in Spain (Fig. 6). Besides particularities in soil composition and farming practices of the two locations, such differences might derive from wider temperature oscillations in the Italian field (Fig. S4). In both locations (Italy and Spain), M82 plants tended to be taller (but not heavier) under HD conditions, a phenotype that was attenuated in IL2-2 plants (Fig. 6a). This effect of the HD conditions in the field paralleled that of W + FR irradiation in the glasshouse (Fig. 4c). In the case of Chl levels, however, the drop triggered by W + FR treatment (Fig. 4b) was only observed when comparing IL2-2 plants grown under HD and LD in the Italian field (Fig. 6a). Plant density conditions or genotype did not substantially influence fruit soluble content (°Brix),

which was only statistically reduced when the IL2-2 line was grown under LD in Italy. Similarly, fruit weight was hardly affected by the plant genotype or the density of the culture (Fig. 6b). Most strikingly, fruit yield per hectare was similar in M82 and IL2-2 under LD conditions in both locations, but it was higher in the introgression line under HD (Fig. 6b).

Discussion

Tomato is a shade avoider plant that exhibits a pronounced multilevel response to proximity shade (low R : FR) as shown here and elsewhere (Cagnola *et al.*, 2012; Chitwood *et al.*, 2012, 2015; Bush *et al.*, 2015; Fanwoua *et al.*, 2019; Ji *et al.*, 2019, 2020, 2021; Kim *et al.*, 2019; Sun *et al.*, 2020; Li *et al.*, 2024; Shomali *et al.*, 2024). In this work, we compared the most conspicuous phenotypic responses, that is enhanced growth and decreased accumulation of photosynthetic pigments, in different tomato accessions grown under the same experimental conditions (Fig. 1). SAS in domesticated tomatoes is quite variable and attenuated compared to the strong shade avoidance displayed by wild tomato species (Chitwood *et al.*, 2012; Bush *et al.*, 2015). We confirmed that the domesticated accessions that we used for this work show similar leaf expansion and Chl loss responses to low R : FR but a remarkable variation in stem elongation responses, being strongest in MoneyMaker and weakest in MicroTom

(Fig. 1b). In particular, the epicotyl elongation response was the most reproducible and it was evident even in MicroTom, an accession that showed no significant hypocotyl elongation upon W + FR exposure in our experimental conditions (Fig. 1b). Based on these results, we carried out a screening for tomato lines with an attenuated epicotyl growth upon exposure to W + FR that led us to the identification of the *S. pennellii* introgression line IL2-2 (Figs 2, S2). While this line showed a clear attenuation of shade-induced stem elongation in both seedlings and adult plants, the response to proximity shade in terms of Chl loss was quite variable (Figs 2, 4, 6). Consistently, RNAseq data confirmed that IL2-2 plants show an impaired shade-triggered regulation of genes involved in growth (i.e. auxin-related) but not of those involved in photosynthetic pigment production (i.e. MEP, carotenoid, and Chl biosynthetic pathways) (Figs 3, S3). These results together confirm that the molecular pathways regulating elongation and photosynthetic pigment accumulation in response to proximity shade (low R : FR) in tomato are different and can be genetically separated, as previously shown in Arabidopsis (Toledo-Ortiz *et al.*, 2010; Bou-Torrent *et al.*, 2015; Morelli *et al.*, 2021).

The perception and transduction of the low R : FR signal to regulate SAS responses is best understood in Arabidopsis (Martinez-Garcia & Rodriguez-Concepcion, 2023). The light signal is sensed by phytochromes, with phyB playing the most relevant role in proximity shade. Under high R : FR, active phyB represses SAS responses by interacting with PIFs to repress their activity (Leivar & Monte, 2014). Members of the so-called PIF quartet (PIFQ: PIF1, PIF3, PIF4, and PIF5) and PIF7 directly contribute to the SAS (Martinez-Garcia & Rodriguez-Concepcion, 2023). While both *pif4* and *pif7* mutants show a decreased elongation in response to W + FR, the *pif7* mutant shows a WT phenotype in terms of simulated shade-triggered photosynthetic pigment loss (Toledo-Ortiz *et al.*, 2010; Bou-Torrent *et al.*, 2015; Morelli *et al.*, 2021). Because PIF1 and PIF3 are more associated with the repression of photosynthetic development whereas PIF4 and PIF5 are more related to the promotion of growth, it is possible that the molecular mechanism responsible for the shade tolerance phenotype of IL2-2 (mostly affecting elongation growth) utilizes signal transduction pathways involving PIF4, PIF5, or/and PIF7. These particular PIFs promote shade-triggered growth by preferentially regulating genes involved in the biosynthesis and transport of hormones, with a prominent role of the auxin IAA (Iglesias *et al.*, 2018; Casal & Fankhauser, 2023; Martinez-Garcia & Rodriguez-Concepcion, 2023). IAA synthesized in shade-exposed cotyledons through the TAA/YUC pathway (Fig. 5a) is transported to hypocotyls, where it stimulates cell elongation. Consistently, low R : FR regulates the expression of genes encoding PIN-FORMED (PIN) polar-auxin-transport efflux carriers. Upon arrival in hypocotyls, auxin binding to TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFBs) receptors leads to the degradation of the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins, which regulate the expression of genes for AUXIN RESPONSE FACTORS (ARFs). Other auxin-related genes rapidly induced by low R : FR include members of the *AUX/IAA* and the *SMALL AUXIN UP RNA (SAUR)* gene families, which are often upregulated in

both cotyledons and hypocotyl tissues. Direct control of the expression of genes involved in both auxin-biosynthesis (e.g. *YUCs*), transport (e.g. *PINs*), and response (e.g. *IAAs*, *SAURs*) by Arabidopsis PIF4, PIF5, and PIF7 (Iglesias *et al.*, 2018; Casal & Fankhauser, 2023) rapidly links the perception of low R : FR with activated plant growth.

In tomato, transcriptomic analyses have also found a central role for auxins in the response to low R : FR (Cagnola *et al.*, 2012; Bush *et al.*, 2015; Sun *et al.*, 2020). We observed that genes related to auxin synthesis (*TAR1/TAA3*, *YUC5*, and *YUC9*), transport (*PIN3* and *PIN4*), and signaling (*ARF10B*, *IAA1*, *IAA7*, *IAA17*, *IAA22*, *IAA29*, and *SAUR*) show an altered expression in IL2-2 plants compared to the M82 parental in the absence of the low R : FR signal (Fig. 3). W-grown IL2-2 plants also show increased expression of *BGILA* and *BGILE*, tomato homologs of the rice *BIG GRAIN 1 (BG1)* gene whose overexpression increases auxin basipetal transport, auxin sensitivity, and grain size (Liu *et al.*, 2015). Furthermore, two tomato homologs of the transcription factor ATHB2, a key early transducer of the low R : FR signal that reduces auxin responses (Iglesias *et al.*, 2018; He *et al.*, 2020), are also upregulated in W-grown IL2-2 (Fig. 3d). Interestingly, most of these genes show a reduced response to W + FR in the IL2-2 line, reaching transcript levels similar to those in shade-treated M82 plants (Fig. 3). These observations suggest that IL2-2 plants have a strongly altered auxin homeostasis that hardly impairs growth or fruit development under high R : FR conditions but prevents a normal response to low R : FR (Figs 2, 4, 6). Further work should establish the molecular basis of this characteristic phenotype.

Despite most of the work on the participation of auxin in SAS responses having been done in lab conditions using W + FR to simulate proximity shade, our results using Arabidopsis *pif457* and *sav3* mutants grown at different densities confirmed that this hormone-dependent SAS pathway is also key to enhancing growth under HD conditions (Fig. 5). In agreement, the system-level rearrangement of auxin homeostasis deduced in IL2-2 plants (Fig. 3) not only prevents normal elongation growth in response to FR supplementation in the glasshouse (Figs 2, 4) but also under HD conditions in the field (Fig. 6). From the agronomic point of view, the IL2-2 line has several advantages. First, it is very similar to the M82 parental in terms of growth, biomass, photosynthesis, and fruit quality under high R : FR conditions, that is when grown under W (Fig. 4) and LD (Fig. 6). The only significant changes were detected in fruit firmness and seed number in the glasshouse, which were slightly decreased in IL2-2 (Fig. 4d) and in leaf Chl content and fruit weight in the field, which were increased (Fig. 6). These differential phenotypes arguably derive from the genomic introgression present in the IL2-2 line, and at least some of them are compatible with the altered auxin homeostasis phenotype observed in these plants. Second, it does not interfere with the beneficial effects that FR supplementation provides for fruit quality, that is increased content of soluble solids (°Brix), ascorbic acid (vitamin C) and carotenoids (pro-vitamin A) in ripe fruit (Fig. 4d). And third, under HD conditions in the field, it produced more fruit per area of cultivated land compared to the M82 parental (Fig. 6b).

The work reported here demonstrates that the identification of shade-tolerant tomato lines can be beneficial for agronomic practices involving high planting density. Our screening for shade-tolerant tomato lines used W + FR to simulate shade and epicotyl elongation as an output of the response (Figs 2, S2). Implementing new screenings using HD instead of FR supplementation might identify new lines with improved performance in agricultural settings involving proximity shade. Another option would be to measure shade-triggered Chl loss instead of epicotyl elongation as an output. Several methods exist for the high-throughput analysis of Chl levels indoors or in open fields (Tayade *et al.*, 2022), allowing us to design large-scale screenings. An added advantage of a Chl-based screening is that the molecular mechanism responsible for the shade tolerance phenotype will likely be different from those responsible for the elongation-based phenotypes. Therefore, stronger lines could be created by combining the two types of mechanisms (i.e. the two molecular pathways) causing the reduced response to low R : FR. We speculate that these lines could also perform better when intercropped with taller plants. Intercropping is a conservation agriculture practice that involves growing two or more crops in close proximity to one another. Intercropping promotes biodiversity and enhances crop resilience to extreme environmental changes, but it also increases crop yields in both low-input (traditional) and high-input (intensive) agrosystems (Tilman, 2020; Li *et al.*, 2023). However, the development of intercropping is currently limited by the reduced toolbox of crop varieties amenable to this farming practice. Screenings for shade-tolerant lines or targeted manipulation of specific components of the low R : FR signal perception and transduction should provide a solution for this challenge.

Acknowledgements

We thank the staff at the IBMCP Metabolomics Platform for technical support. This study was part of the PRIMA project UT0PIQ funded by the Italian Ministero dell'Istruzione e del Merito (MIUR) to MMR (reference E79J21005760001) and the Spanish Agencia Estatal de Investigación (AEI, MCIN/AEI/10.13039/501100011033) and European Commission NextGeneration EU/PRTR to MR-C (reference PCI2021-121941). Additional funding for MMR and MR-C came from the EU/COST-funded ReCrop network (Reproductive Enhancement of CROP resilience to extreme climate, CA22157). We also acknowledge the support of MICIN/AEI grants PID2020-115810GB-I00, RED2022-134577-T and PID2023-149584NB-I00 to MR-C and PID2020-115782GB-I00, PLEC2022-009323, and PID2023-149395NB-I00 to JFM-G and Generalitat Valenciana grants AGROALNEXT/2022/067 to MR-C and PROMETEU/2021/056 to JFM-G. EB-E received a predoctoral fellowship from Colombia's Colciencias Doctorado Exterior program (MINCIENCIAS885/2020). JP-R is supported by a predoctoral fellowship from AEI (PRE2021-099195).

Competing interests

None declared.

Author contributions

EB-E, SF, MS-M, JFM-G, MMR and MR-C conceived the project and designed the experiments; EB-E, SF, MS-M, JP-R, AB, LV, JMP-B and MA performed the experiments; EB-E, SF, JFM-G, MMR and MR-C analyzed and discussed the data; EB-E, SF, JFM-G, MMR and MR-C wrote the paper; all authors reviewed and approved the manuscript. EB-E and SF contributed equally to this work.

ORCID

Esteban Burbano-Erazo  <https://orcid.org/0000-0001-5056-9893>
 Silvana Francesca  <https://orcid.org/0000-0003-2981-0413>
 Jaume F. Martinez-Garcia  <https://orcid.org/0000-0003-1516-0341>
 Julia Palau-Rodriguez  <https://orcid.org/0009-0009-2480-7152>
 Maria Manuela Rigano  <https://orcid.org/0000-0001-7826-9067>
 Manuel Rodriguez-Concepcion  <https://orcid.org/0000-0002-1280-2305>
 Laura Valverde  <https://orcid.org/0000-0002-0131-8817>

Data availability

The data that support the findings of this study are included as part of the article or as Supporting Information (Figs S1–S4). Raw RNAseq data are deposited at NCBI, BioProject PRJNA1231488.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Light spectra of growth chambers.

Fig. S2 Differential responses of tomato accessions to simulated shade.

Fig. S3 Heatmap comparison of the expression of genes involved in photosynthetic pigment biosynthesis.

Fig. S4 Timeline of temperature and humidity records at the experimental fields in Acerra (Italy) and Paiporta (Spain).

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