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A novel strigolactone-miR156 module controls stomatal behaviour during drought recovery

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v) Abstract

miR156 is a conserved microRNA whose role and induction mechanisms under stress are poorly known. Strigolactones are phytohormones needed in shoots for drought acclimation. They promote stomatal closure ABA-dependently and independently; however, downstream effectors for the former have not been identified. Linkage between miR156 and strigolactones under stress has not been reported.

We compared ABA accumulation and sensitivity as well as performances of wt and miR156-overexpressing (miR156-oe) tomato plants during drought. We also quantified miR156 levels in wt, strigolactone-depleted and strigolactone-treated plants, exposed to drought stress.

Under irrigated conditions, miR156 overexpression and strigolactone treatment led to lower stomatal conductance and higher ABA sensitivity. Exogenous strigolactones were sufficient for miR156 accumulation in leaves, while endogenous strigolactones were required for miR156 induction by drought. The "after-effect" of drought, by which stomata do not completely re-open after rewatering, was enhanced by both strigolactones and miR156. The transcript profiles of several miR156 targets were altered in strigolactone-depleted plants.

Our results show that strigolactones act as a molecular link between drought and miR156 in tomato, and identify miR156 as a mediator of ABA-dependent effect of strigolactones on the after-effect of drought on stomata. Thus, we provide insights into both strigolactone and miR156 action on stomata.

vi) Key words

Abscisic acid (ABA), After-effect of drought, Hormone signalling, Osmotic stress, *Solanum lycopersicum*, Stomata, Stress-responsive microRNA

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INTRODUCTION

It has been estimated that more than 90% of water uptake in plants is lost through transpiration. Stomata represent a fundamental checkpoint balancing the entry of carbon dioxide and the exit of water, and their regulation is at the core of the main acclimation strategy to water scarcity (Matthews, Vialet-Chabrand, & Lawson, 2017). Upon drought, tolerance mechanisms operate at

different spatial and temporal scales with rapid stomatal closure as the basis for preventing shoot water loss (Tardieu, Simonneau, & Muller, 2018). MicroRNAs (miRNAs) and phytohormones have been associated to the regulation of guard cell development and movement (Curaba, Singh, & Bhalla, 2014; Ding, Tao, & Zhu, 2013).

miRNAs are a widespread class of endogenous, small RNA molecules (19-24 nt in length) that negatively regulate gene expression at the transcriptional, post-transcriptional, and translational levels (Nozawa, Miura, & Nei, 2012). In animals, under certain conditions, miRNAs are being looked at as hormones, because of their cell-to-cell and also long-distance movement coupled to signalling activity (Bayraktar, Van Roosbroeck, & Calin, 2017). While some plant miRNAs are species-specific in terms of expression patterns and targets, others, including miR156, are very conserved. The modulation of miR156 is crucial throughout development for correct leaf formation, tillering/branching, plastochron, panicle/tassel architecture, and timing of age-dependent reproductive transition along with fruit ripening and fertility (Wang & Wang, 2015). In spite of a few differences among species, the pattern of mature miR156 accumulation in response to environmental stimuli is also rather conserved (Khraiwesh, Zhu, & Zhu, 2012). Indeed, miR156 is consistently induced by a variety of abiotic stresses such as drought, osmotic stress, heat, cold, salinity, and macro-nutrient deficiency (Cui, Shan, Shi, Gao, & Lin, 2014; Ding, Fromm, & Avramova, 2012; Hsieh et al., 2009; H. H. Liu, Tian, Li, Wu, & Zheng, 2008; Stief et al., 2014). As for its role under stress, it was initially postulated that miR156 increase is merely needed to stall flowering until stress is over. This effect would be exerted via miR156-mediated inhibition of the age-dependent pathway to flowering. In support of this view, miR156-overexpressing plants (miR156-oe hereafter) are late-flowering. Under long-day, inductive conditions the flowering time in Arabidopsis (*Arabidopsis thaliana*) tends to inversely correlate with miR156 levels in a range of environmental conditions (Cui et al., 2014; May et al., 2013). However, it is also becoming clear that this miRNA may have more direct functions in stress acclimation. For instance, it was shown that miR156-oe plants outperform wt controls both in *Medicago sativa* under drought (Arshad, Feyissa, Amyot, Aung, & Hannoufa, 2017) and in Arabidopsis under osmotic/high salinity stress (Cui et al., 2014) or recurring heat (Stief et al., 2014). These functions may be exerted by miR156 through post-transcriptional repression of one or more members of the wide family of SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) transcription factors (Arshad et al., 2017; Cui et al., 2014; Stief et al., 2014), the best characterised among miR156 targets. However, these studies

145 do not clarify which cascade of molecular events induces miR156 under stress, and whether
146 miR156 can affect stomatal regulation.
147 Abscisic acid (ABA) is the best-characterised phytohormone among several affecting stomatal
148 functioning. Its direct application, as well as endogenous synthesis in phloem companion,
149 mesophyll and guard cells, trigger stomatal closure (Bauer et al., 2013; Kuromori, Sugimoto, &
150 Shinozaki, 2014; S. A. M. McAdam & Brodribb, 2018; Merilo et al., 2018). ABA signalling is known
151 to affect miRNA production (Lian et al., 2018; Speth, Willing, Rausch, Schneeberger, & Laubinger,
152 2013; Yan et al., 2017), but to our knowledge, no miRNAs have been demonstrated to affect ABA
153 sensitivity.
154 Strigolactones, the most recently discovered class of phytohormones, also play a role in this
155 scenario. ABA and strigolactones share a carotenoid precursor, from which strigolactone synthesis
156 proceeds through a partially known series of enzymes including DWARF27 (D27), CAROTENOID
157 CLEAVAGE DIOXYGENASE7 (CCD7) and 8 (CCD8), and MORE AXILLARY GROWTH1 (MAX1) to
158 produce bioactive strigolactones (Waters, Gutjahr, Bennett, & Nelson, 2017). Strigolactones
159 modulate several aspects of plant development and interactions with rhizosphere organisms
160 (Andreo-Jimenez, Ruyter-Spira, Bouwmeester, & Lopez-Raez, 2015; Cardinale, Korwin Krukowski,
161 Schubert, & Visentin, 2018; Lanfranco, Fiorilli, Venice, & Bonfante, 2018; Lopez-Raez, 2016).
162 Furthermore, plants with defective strigolactone synthesis or signalling in several dicot plants are
163 hypersensitive to drought, salt and osmotic stress (Cardinale et al., 2018). In Arabidopsis, *Lotus*
164 *japonicus* and tomato, strigolactones positively control stomatal movements as components of a
165 systemic stress signal (Tardieu, 2016). Indeed, mutants in strigolactone biosynthesis exhibited
166 reduced stomatal closure (Ha et al., 2014; J. Liu et al., 2015; Visentin et al., 2016). Conversely,
167 enhanced stomatal closure and drought tolerance was observed in plants treated with exogenous
168 strigolactones (Lv et al., 2018; Visentin et al., 2016; Y. Zhang, Lv, & Wang, 2018) or in which shoot
169 strigolactone biosynthesis is increased by grafting onto a low-strigolactone rootstock (Visentin et
170 al., 2016). The effect of strigolactones on stomatal closure depends, at least in part, on ABA
171 synthesis, transport and/or sensitivity. Accordingly, strigolactone depletion decreases sensitivity to
172 exogenous ABA in several species (Bu et al., 2014; Ha et al., 2014; J. Liu et al., 2015; Lv et al., 2018;
173 Visentin et al., 2016) and sensitivity to endogenous ABA in stressed tomato (Visentin et al., 2016).
174 On the other hand, treatment with the synthetic strigolactone analogue racemic GR24 (*rac*-GR24)
175 increases sensitivity to ABA in tomato (Visentin et al., 2016). It deserves attention that the above-
176 described model may be confined to dicot plants, as it does not apply to rice – most strigolactone-

biosynthetic mutants of which produce more ABA than the wild-type (wt) and thus are more resistant to drought (Haider et al., 2018).

In this varied landscape, the possibility of a functional connection between miR156 and strigolactones in the regulation of stomatal movements and drought avoidance has never been investigated. miR156g was *in silico* predicted to directly target the transcripts of the strigolactone biosynthetic gene *MAX1* in Arabidopsis (Marzec & Muszynska, 2015), but experimental proof of this is currently lacking. Additionally, a functional link in the context of shoot development, via stabilisation of specific SPL proteins by strigolactones, is already known in gramineous plants (J. Liu, Cheng, Liu, & Sun, 2017; Song et al., 2017). The fact that the transcripts of several SPL factors are targeted by miR156 offers a potential integration point between hormone- and miRNA-mediated signalling (Kerr & Beveridge, 2017; M. Liu et al., 2017; Song et al., 2017). Nevertheless, it is still unknown whether this or a similar mechanism is operational under stress.

In this work, we used different approaches to clarify the link between miR156 and strigolactones with regards to stomatal regulation in tomato. We investigated the effect of miR156 overexpression on stomatal function and ABA metabolism/sensitivity as well as the transcript stability of strigolactone biosynthetic genes. Moreover, the effect of strigolactones on miR156 levels were assessed by i) treatment with exogenous strigolactones coupled to the use of a strigolactone-depleted transgenic line, and ii) the application of drought stress to increase both strigolactone and miR156 levels in shoots. The results identified strigolactones as a molecular component linking drought to miR156 accumulation. Furthermore, they allowed us to integrate miR156 in a model that describes the connections between strigolactones and ABA in tomato (Cardinale et al., 2018), thus offering insights into both strigolactone and miR156 action on stomata.

MATERIALS AND METHODS

Plant materials and growth conditions

The tomato *CCD7*-silenced line 6936 (kind gift by Dr H. J. Klee, University of Florida) (Vogel et al., 2010) and its wt M82 were grown in a growth room set at the following conditions: 16/8 h day/night cycle, 25°C, 65% humidity, and 200 µmol s^{-1} m^{-2} of photosynthetic photon flux density (PPFD). Seeds were sterilized in 4% (v/v) sodium hypochlorite containing 0.02% (v/v) Tween 20, rinsed thoroughly with sterile water, and plated on MS medium with 0.8% w:v agar, pH 5.9. Ten days after, seedlings were transferred to an inert substrate and pots were watered with Hoagland

solution twice per week. Drought-stress kinetics were performed by uprooting plants of both wt and *CCD7*-silenced genotypes and transferring them in vermiculite hydrated with only 20 ml of water (stressed group), or transferred in wet vermiculite (well-watered controls). A sub-group of wt plants were leaf-sprayed with $GR24^{5DS}$ (see dedicated paragraph below) 24 h before the beginning of stress, while all other plants were mock-treated with a solution of 0.01% v/v acetone in water. Throughout the experiment, each condition and genotype was represented by 5 plants.

The tomato miR156-oe line overexpressing the *AtMIR156b* primary transcript and its wt (MicroTom) were kindly provided by Dr. G. Silva (University of Sao Paulo, Brazil) (Ferreira e Silva et al., 2014). Plants were grown in 1-liter pots filled with a commercial substrate (Terra Nature, NPK 12:14:24 imported by Raw Materials Europe, NL) composed of sandy-loam soil/expanded clay/peat mixture (2:1:1 by weight) and maintained under greenhouse conditions. Two drought time-courses were performed on 4-week-old plants by withholding water for 15 days - starting from day zero - on 5 plants per line and treatment; an irrigated control group was kept for each genotype. After 10 days, once severe water stress levels were reached, the plants were watered to allow for recovery. Stomatal conductance was measured throughout the experiment, while leaf samples were collected for water potential measurements at day zero, ten and fifteen (see below for physiological analytical methods).

Treatment with exogenous strigolactones

A 5 µM solution of $GR24^{5DS}$ (StrigoLab Srl, Turin, Italy) in 0.01% v/v acetone in water was sprayed on leaves of unstressed plants until runoff, while control plants were sprayed with a corresponding solution of acetone only. This pure enantiomeric form of the synthetic strigolactone analogue GR24 was preferred over commercial *rac*-GR24 due to the possibly confounding bioactivity by the other enantiomer ($GR24^{ent\text{-}5DS}$) contained in the racemic mixture (Scaffidi et al., 2014). Stomatal conductance (see below) was measured 2 and 24 h after treatment, while samples for the quantification of mature miR156 were collected 2, 6 and 24 h after treatment (in the absence of stress), deep frozen and stored at -80°C until analysis. When $GR24^{5DS}$ treatment was imposed on wt plants to be subsequently stressed, harvesting times were i) 24 h after treatment (well-watered samples; with stress beginning immediately after harvest); ii) after 3 h of stress (water-stressed samples; with re-watering and the beginning of recovery happening immediately after harvest); iii) 24 h after the beginning of stress, i.e. 21 h into recovery (recovered samples).

241

242 **Gene-transcript quantification and miR156 target-site detection**

243 For transcript quantification from axillary buds, at least 30 stem sections were excised at the level
244 of leaf insertion from 10-week-old MicroTom plants (eight plants each, for wt and miR156-oe
245 plants) to obtain two lots of 50 mg (fw) per genotype, and freeze-dried until analysis. For
246 quantification in shoot tissues, the same procedure was applied on 200 mg (fw) of tomato leaves.
247 Total RNA was extracted by using Spectrum™ Plant Total RNA Kit (SIGMA), and treated with DNase
248 I (ThermoScientific) at 37°C for 30 min to remove residual genomic DNA. First-strand cDNA was
249 synthesized from 1 µg of purified total RNA using the High-Capacity cDNA Reverse Transcription
250 Kit (Applied Biosystems, Monza, Italy) according to the manufacturer's instructions.
251 For targeted miR156 cDNA synthesis, a modified protocol with a stem-loop primer (Pagliarani et
252 al., 2017) was followed in samples of wt M82 and *CCD7*-silenced plants obtained from the
253 drought-stress experiments described above. For transcript quantification of candidate genes by
254 quantitative reverse-transcription PCR (qRT-PCR), random primers were used to reverse transcribe
255 total RNA. qRT-PCR analysis was carried out in a StepOnePlus system (Applied Biosystems) using
256 the SYBR Green (Applied Biosystems) method on 10 ng of cDNA (50 ng for *SlCCD7* transcripts). For
257 loci and primers (which were used at 400 nM), see Table S1. Transcript concentrations were
258 normalised on the geometric mean of *SlsnRU6* and *SlEF-1*α transcript concentrations used as
259 endogenous controls. Three independent biological replicates were analysed as a minimum, and
260 each qRT-PCR reaction was run in technical triplicates. Transcript amounts were quantified by the
261 $2^{-\Delta\Delta Ct}$ method. Putative target genes of miR156 were predicted *in silico* using the psRNATarget
262 algorithm with default setting parameters (http://plantgrn.noble.org/psRNATarget/) (Dai & Zhao,
263 2011).
264

265 **Stomatal conductance and aperture measurements**

266 During the drought-stress time-course on miR156-oe plants and their MicroTom wt, stomatal
267 conductance was measured daily between 10:00 and 12:00 am on two randomly selected, fully
268 developed apical leaves for each plant with a portable system (SC-1 Leaf Porometer for Stomatal
269 Conductance Measurements, Decagon Device, WA, USA). Leaf water potential was measured
270 using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA,
271 USA) (Scholander, Bradstreet, Hemmingsen, & Hammel, 1965) on one leaf per plant, immediately
272 after gas exchange quantification. During the drought stress time-course on *CCD7*-silenced plants

and their M82 wt, stomatal conductance was measured on two randomly selected, fully developed apical leaves for each plant with the same portable system as above. The selected time-points for full measurements were: i) immediately before stress start, between 8:00 and 10:00 am; ii) 3 h later, when stomatal conductance values were about 20% of the irrigated controls (after which, the plants were watered to start recovering); iii) 6 and 24 h after the beginning of stress, i.e. 3 and 21 h into the recovery period. In order to quantify guard-cell reactivity to exogenous ABA treatments, leaves of 4-week-old wt (MicroTom) and miR156-oe plants were sprayed with varying concentrations of ABA in water or with water alone until drip-off, then let dry for 1 h before quantifying stomatal conductance as above. For the quantification of stomatal pore areas in the two genotypes, mature excised leaves were pre-incubated in a MES-KCl buffer (10 mM MES-KOH/50 mM KCl, pH 6.15) under light for 2 h to promote stomatal opening before treatments. Leaves of three plants per line (wt and miR156-oe) were then incubated for 3 h in MES-KCl buffer containing 10 µM ABA. Subsequently, the abaxial epidermis of ABA-treated and untreated leaves of both genotypes was peeled off following a published procedure (Hopper, Ghan, & Cramer, 2014), and stomatal apertures were measured with the ImageJ software on images captured using a calibrated light microscope. The aperture areas of a minimum of 30 stomata from three independent leaves per treatment were quantified.

Extraction and quantification of ABA

Plant tissues (each sample approximately 20 mg fw) were ground using 3-mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany) with a MM 301 vibration mill at a frequency of 27.0 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany). One ml ice-cold methanol/water/acetic acid (10/89/1, v/v) and internal standard mixtures [containing 20 pmol of each of (-)-7′,7′,7′-2H3-phaseic acid; (-)-7′,7′,7′-$^{2}H_3$-dihydrophaseic acid; (-)-8′,8′,8′-$^{2}H_3$-neophaseic acid; (+)-4,5,8′,8′,8′-$^{2}H_5$-ABA-GE; (-)-5,8′,8′,8′-$^{2}H_4$-7′-OH-ABA; (+)-3′,5′,5′,7′,7′,7′-$^{2}H_6$-ABA] were added to each sample. After 1 h shaking in the dark at 4°C, the homogenates were centrifuged (20 000 *g*, 10 min, 4°C), and the pellets were then re-extracted in 0.5 ml extraction solvent for 30 min. The combined extracts were purified by solid-phase extraction on Oasis® HLB cartridges (60 mg, 3 ml, Waters, Milford, MA, USA), then evaporated to dryness in a Speed-Vac (UniEquip) and finally analysed by UPLC-ESI(-/+)-MS/MS (Turečková, Novák, & Strnad, 2009).

Statistical analysis

Significant differences among treatments were statistically analysed by applying a one-way ANOVA test, and Tukey's *HSD* post-hoc test was used for mean separation when ANOVA results were significant ($P < 0.05$). Significant differences of pairwise comparisons were assessed by the Student's *t*-test. The SPSS statistical software package (SPSS Inc., Cary, NC, USA, v.22) was used.

RESULTS

miR156-oe plants have lower stomatal conductance and acclimate better to drought

To test the hypothesis that miR156 induction under drought is relevant to the plant water balance, the physiological responses of wt (MicroTom) and miR156-oe plants were analysed during a drought and recovery time-course. Well-watered miR156-oe plants displayed a significantly lower stomatal conductance than wt (day zero in Fig. 1A), while water potential in the same leaves did not significantly differ between the two genotypes (well-watered samples in Fig. 1B). This pattern of higher stomatal conductance in the wt was maintained throughout the experiment for the irrigated controls. In stressed plants, miR156 overexpression correlated with lower stomatal conductance in the initial phases of the dehydration kinetics (day two, Fig 1A). At the time of the most severe drought stress level the situation reversed, as stomatal conductance of miR156-oe plants was slightly but significantly higher than wt (day seven and ten, Fig. 1A). At this stage, leaf water potential was significantly lower in wt than miR156-oe plants (water-stressed samples in Fig 1B, corresponding to day ten in Fig. 1A). At day fifteen, i.e. five days after re-irrigation, leaf water potential of both wt and miR156-oe plants had recovered to levels comparable to those preceding the drought spell (recovered samples in Fig. 1B). However, unlike wt plants, stomatal conductance of miR156-oe tomato plants had completely failed to recover at this time (day fifteen, Fig. 1A). These data suggest that: i) miR156 overexpression can lead to better acclimation to drought; ii) the guard cells of miR156-oe plants respond more slowly to changes in water availability than those of wt; and iii) under irrigated conditions, stomatal conductance is most likely controlled by a non-hydraulic, miR156-related signal in tomato.

The low stomatal conductance of miR156-oe plants is not due to increased free ABA

Considering the pivotal role of ABA in stomatal closure, we reasoned that the physiological effects of miR156 overexpression on stomatal opening observed in the above experiments may be due to increased ABA levels. Hence, we set to quantify ABA and its main catabolites in the leaves of wt and miR156-oe plants under well-watered conditions (day zero in Figure 1). Unexpectedly given

their low-transpiration phenotype, free ABA was significantly less concentrated in the leaves of miR156-oe plants than in wt (Fig. 2A). Accordingly, transcripts of the ABA biosynthetic gene *SlNCED1* were somewhat lower in miR156-oe plants, albeit not significantly (Fig. 2B). While glycosylated ABA levels were similar in wt and miR156-oe plants, the ABA catabolite phaseic acid showed a non-significant trend towards higher concentrations in miR156-oe plants than in the wt (Fig. 2A). Consistently, transcript amounts of the ABA-hydroxylating genes *SlCYP707A1* and *SlCYP707A2*, which catalyse a key step in phaseic acid production from ABA, were significantly higher in miR156-oe than wt leaves (Fig. 2B). Other related metabolites, such as dihydro-phaseic acid, neo-phaseic acid and 7'-hydroxy-ABA, were below the limit of detection in both genotypes (n.d. in Fig. 2A). Thus, miR156 overexpression seems to decrease endogenous ABA levels, at least partly by inducing degradation. These data therefore exclude that the low stomatal conductance rates observed in miR156-oe plants under well-watered conditions are due to increased ABA content in total leaf tissues.

miR156 promotes stomatal closure in response to ABA

Since leaves of the miR156-oe genotype contained less free ABA than those of the wt and yet they had more closed stomata, we hypothesized that miR156 may increase guard cell sensitivity to ABA. To further address this point, we quantified stomatal conductance in wt and miR156-oe plants 1 h after treatment with increasing concentrations of ABA, in comparison with the corresponding mock treatment with water. The results clearly showed that unlike the wt, guard cells of miR156-oe plants are oversensitive to ABA. In fact, in miR156-oe plants the decrease in average conductance values was already significant at 1 µM ABA concentration. Also at higher ABA concentrations the decrease of stomatal conductance relative to the mock-treated controls was more marked in miR156-oe plants than in the wt (Fig. 3). To confirm physiological data at the morphological level, we as well assessed stomatal closure by determining stomatal pore areas after 1 h exposure to 10 µM exogenous ABA. In the absence of ABA in the floating medium, stomatal pore areas were significantly wider in wt than miR156-oe leaves (Fig. S1A), confirming the stomatal conductance differences occurring between the two genotypes under unstressed and untreated conditions (Fig. 1 and 3). Upon ABA treatment, average stomatal pore areas in miR156-oe plants decreased by about 60% with respect to non-treated stomata in the same genotype. By contrast, mean stomatal aperture in wt plants decreased by about 30% only, compared to the untreated control (Fig. S1B and C). These results indicate that guard cells have higher sensitivity to

exogenous ABA in miR156-oe than wt plants. This in turn supports the notion that the lower stomatal conductance in well-watered miR156-oe vs wt plants might be due, at least partly, to higher sensitivity to endogenous ABA, which would more than compensate for the lower ABA content.

Strigolactones are needed and sufficient for miR156 induction in tomato, both under normal conditions and under drought

As outlined in the introduction, both the miR156 and strigolactone biosynthetic pathways are induced by drought in leaves; both are needed for full resistance to abiotic stress; and both enhance guard cell sensitivity to ABA in tomato. This was shown above for miR156 and in a previous work for strigolactones (Visentin et al., 2016). Thus, we set to explore if not only a correlation but also a causal link exists between strigolactones and miR156 that may be relevant to stomatal functioning.

As a first approach, we compared the levels of mature miR156 in leaf tissues 2, 6 and 24 h after treatment with the strigolactone analogue $GR24^{5DS}$ with mock-treated controls. Results from this experiment indicate that exogenous strigolactones are sufficient to increase the concentration of the mature miRNA starting a few hours after treatment and up to 24 h later (Fig. 4A).

We next tested whether endogenous strigolactones are required for miR156 induction under stress, by quantifying mature miR156 in leaves of wt (M82) and *CCD7*-silenced tomato plants under irrigated and stress conditions. Severe stress levels are achieved quickly (within a few hours) in this experimental set-up, by transferring the plants to dry or wet vermiculite substrate for the stressed and control group, respectively. The two genotypes showed a very similar, low concentration of miR156 under irrigated conditions, while wt leaves treated 24 h earlier with $GR24^{5DS}$ had higher concentrations than mock-treated leaves (Fig. 4B), consistently with the results in Fig. 4A. At the most severe stress point, the concentration of mature miR156 increased in wt leaves, as expected. Conversely, in plants impaired in strigolactone biosynthesis, no induction could be observed (water-stressed samples in Fig. 4B). Additionally, the results in Fig. 4B clearly show a powerful synergic effect of $GR24^{5DS}$ pre-treatment and drought on mature miR156 levels. This is obvious both in stressed samples, and especially after re-watering (for 24 h in the recovered samples in Fig. 4B), when miR156 amounts decrease towards pre-stress levels in the leaves of mock-treated wt plants, , while they steadily increase up to 450 folds in the $GR24^{5DS}$-pre-treated leaves.

Strigolactones promote sustained stomatal closure ("after effect") during recovery from drought

Based on the above data, miR156 appears to promote the after-effect of drought, and its inducibility by stress seems to completely depend on strigolactones in tomato. Therefore, we reasoned that if the strigolactone-miR156 module is operational in guard cells, then strigolactones should positively affect the extent of the after-effect of drought as well. To elucidate this point, stomatal conductance data (Fig. 4C) were collected during the same experiment reported in Fig. 4B. Data showed clearly that $GR24^{5DS}$-treated plants displayed an enhanced after-effect of drought, thus mimicking the physiological response of miR156-oe plants. Consistently, *CCD7*-silenced plants did the opposite and recovered their stomatal conductance faster than wt plants. Overall, these data attested that strigolactones promote sustained stomatal closure during recovery from drought (the so-called "after-effect") in tomato, as miR156 does.

Finally, we tested whether exogenous strigolactones could directly induce stomatal closure. In Arabidopsis, treatment with *rac*-GR24 induces a fast stomatal closure that has been demonstrated to be ABA-independent (Lv et al., 2018). Thus, we measured stomatal conductance in unstressed, wt tomato leaves treated with $GR24^{5DS}$ compared with the mock-treated controls. Our results clearly showed that stomatal conductance levels of wt tomato decreased significantly within 2 h of $GR24^{5DS}$ treatment, while they they started recovering at 24 h (Fig. 4D).

Strigolactones affect the transcript accumulation profiles of putative SPL factors

The above results, together with previous observations on the activation of the strigolactone biosynthetic pathway in leaves under drought (Visentin et al., 2016), suggested that a strigolactone-miR156 module does exist in tomato. This module may be fully operational under and after drought, thanks to an initial stress-induced increase of leaf strigolactones. If so, then we should be able to identify transcripts of putative miR156 targets that are dysregulated, in a stress- and strigolactone-dependent fashion. Among all loci encoding putative SPL factors in tomato (Salinas, Xing, Hohmann, Berndtgen, & Huijser, 2012), four (*Solyc03g114850*, *Solyc07g062980*, *Solyc10g009080* and Solyc04g045560) have been reported as candidate targets of miR156 under drought (M. Liu et al., 2017). Accordingly, in our experiments, the transcripts of all of them followed a decreasing trend in droughted wt tomatoes. However, there was no difference between wt and *CCD7*-silenced leaves at this time-point, suggesting that neither strigolactones nor miR156-mediated post-transcriptional gene silencing are needed for this decrease. Conversely

during recovery, accumulation patterns of *Solyc07g062980*, *Solyc10g009080* and *Solyc04g045560* in wt and *CCD7*-silenced plants diverged from those of miR156. This finding is consistent with the hypothesis that after drought - i.e. when the stomatal after-effect becomes apparent - these transcripts are destabilised by miR156, which in turn is induced by strigolactones (Fig. 5). On the contrary, the transcript profiles of *COLORLESS NON-RIPENING* (*CNR*, *Solyc02g077920*) and of the orthologue of *SPL9*/*15* in Arabidopsis (*Solyc10g078700*) (Silva et al., 2019) showed no significant changes during drought and they both increased during recovery in the two genotypes, mirroring the related miRNA expression trend (Fig. 5). A significant increase of steady-state transcript concentrations of both *CNR* and *SPL9/15* was instead observed in *CCD7*-silenced plants under pre-stress conditions. It should be noted here that transcripts from all of these genes are validated targets of miR156 in unstressed wt plants, and they are almost undetectable in miR156-oe plant tissues (Silva et al., 2019; X. Zhang et al., 2011).

miR156 does not target the transcripts of strigolactone-biosynthetic genes for degradation in tomato

The transcripts of the strigolactone-biosynthetic gene *MAX1* have been predicted *in silico* to be direct targets of miR156 in Arabidopsis (Marzec & Muszynska, 2015). This would in principle lead to transcript degradation and thus lower strigolactone production in miR156-oe plants. Indeed, in axillary buds of miR156-oe potatoes, the strigolactone content was lower than in wt (Bhogale et al., 2014), but the molecular underpinnings were not investigated at that time. To search for all possible links between miR156 and strigolactones, and given that strigolactones are known to feedback regulate their own synthesis at the transcriptional level, we also checked whether miR156 might affect the stability of strigolactone biosynthetic genes in tomato. We addressed this issue *in silico* first, by searching for miR156 target sequences on transcripts of *D27*, *CCD7*, *CCD8* and *MAX1* in Arabidopsis and tomato. However, no acceptable predictions satisfying the requirements for identification of miRNA targets in plants were obtained (Axtell & Meyers, 2018; Dai & Zhao, 2011; Jones-Rhoades & Bartel, 2004). This contrasts with available information for *MAX1* and miR156g in Arabidopsis (Marzec & Muszynska, 2015).

Additionally, we quantified transcripts of strigolactone-biosynthetic genes in roots, where they are mostly expressed, and axillary buds of miR156-oe and wt plants. However, no evidence of miR156-driven transcript destabilisation was found, and even higher transcript concentrations in the miR156-oe plants was observed for some genes in either tissue (Fig. 6A and B). Therefore,

negative regulation by miR156 on strigolactone biosynthetic genes at the transcript stability level was not observed in tomato. Of course, indirect effects at the protein or metabolite level cannot be excluded at this stage. Nonetheless, the latter information would not change the answer to the question being asked here, i.e. if miR156 directly destabilizes the transcripts of strigolactone-biosynthetic genes, as suggested by Marzec and Muszynska (2015).

DISCUSSION

miR156 induction by drought requires strigolactones in tomato

Almost nothing is known about the molecular cues modulating miR156 levels under any conditions. Even though specific changes in the epigenetic landscape at the *MIR156a* and *c* loci are reported to be important for correct miR156 expression during Arabidopsis development (Xu, Zhang, & Wu, 2018), no information are available about the signalling path connecting it to stress. Partly filling this gap, we found that miR156 induction by drought was completely dependent on the efficient synthesis of endogenous strigolactones. We also observed that exogenous strigolactones increased mature miR156 levels in tomato leaves, both in the absence and especially in combination with drought stress. These findings make strigolactones the first identified molecular component in the drought-triggered pathway leading to miR156 induction, and to its stress-related effects (Cui et al., 2014; Stief et al., 2014).

The role of the strigolactone-miR156 module in stomata

Given the demonstrated effects of strigolactones on stomata in several plant species (Bu et al., 2014; Ha et al., 2014; J. Liu et al., 2015; Lv et al., 2018; Visentin et al., 2016), and the above described functional connection between strigolactones and miR156, we sought to investigate whether miR156 may act at the stomatal level specifically. When we compared the physiological performances of miR156-oe with those of wt plants, stomatal conductance of the former was indeed lower under irrigated conditions, suggesting a positive control of miR156 on stomatal closure. This trait of miR156-oe plants is opposite to strigolactone-related mutants, and reminiscent of tomato plants whose shoots experience high strigolactones. This latter situation can be achieved either by treating with exogenous strigolactones (which induce miR156, as stress does), or grafting a wt scion onto a strigolactone-depleted rootstock (which induces higher strigolactone biosynthesis in the shoot, higher ABA sensitivity in guard cells and lower stomatal conductance) (Ha et al., 2014; J. Liu et al., 2015; Visentin et al., 2016). It is noteworthy that in

miRNA156-oe plants subjected to severe stress, stomatal conductivity was higher than in the wt (as observed in miR156-oe *M. sativa* by Arshad et al., 2017), reversing the pattern observed upon irrigation. This could be explained by non-ABA-dependent signal(s) acting downstream of hydraulic signals generated by severe drought. Consequently, miR156-oe plants lost less water under severe drought. This was attested by their water potential dropping less than wt, and by the fact that their better water status overweighed higher ABA sensitivity in terms of stomatal closure.

The effects of the strigolactone-miR156 module under recovery might be executed via the post-transcriptional repression of SPL genes such as *Solyc10g009080*, which most convincingly showed a divergent transcript profile with miR156 in this phase. Their ultimate function under stress is worthy of further experimental investigation. It should be noted here that, in drought stress conditions, the transcripts of all four SPL genes suggested to be miR156 targets decreased in *CCD7*-silenced leaves as much as in the wt. As in these transgenic tissues miR156 levels did not rise as a consequence of drought, we must conclude that their regulation during stress is more likely to occur at the transcriptional rather than post-transcriptional level, contrarily to previous suggestions (M. Liu et al., 2017).

miR156 is a possible mediator of ABA-dependent effects of strigolactones on stomata

Strigolactone effects on stomata are known to be both ABA-dependent and independent. Indeed, treatment with exogenous strigolactones induces fast, ABA-independent closure of stomata but also higher sensitivity to ABA (Brun et al., 2019; Li et al., 2017; J. Liu et al., 2015; Lv et al., 2018; Visentin et al., 2016; Y. Zhang et al., 2018), while strigolactone-related mutants have impaired ABA-dependent responses to osmotic stress (reviewed in Cardinale et al., 2018).

We found several indications rather pointing to miR156 affecting the ABA-dependent subset of strigolactone effects on stomata, under irrigated conditions. Firstly, stomatal closure in response to exogenous strigolactone treatment (ABA-independent in Arabidopsis) (Lv et al., 2018) was achieved within 2 h in tomato, when miR156 induction was not significant yet; and started decreasing by 24 h, when miR156 levels were highest (Fig. 4A *vs* Fig. 4D). Secondly, under irrigated conditions, miR156-oe plants had lower stomatal conductance but also significantly less free ABA in leaves, possibly due to weaker biosynthesis and especially accelerated ABA conversion to phaseic acid. Accordingly, the activation of ABA catabolism is a reported effect of increased strigolactone synthesis in the shoot, and of exogenous strigolactone treatment in different tissues (Ferrero et al., 2018; Lechat et al., 2012; Toh et al., 2012; Visentin et al., 2016). Thirdly, stomatal

closure by exogenous ABA was more complete in miR156-oe plants than in wt, which may be explained by higher sensitivity to, or more effective transport of ABA. Higher ABA sensitivity would more than compensate lower ABA levels and justify reduced stomatal conductance in miR156-oe plants. As in other priming phenomena, the strigolactone-dependent increase in ABA sensitivity may be exerted at or downstream the perception and signalling level. Consistently, several ABA-responsive genes are less induced by drought in the *max2* signalling mutant of Arabidopsis than in the wt (Ha et al., 2014). Finally, but not less importantly, stomatal conductance of miR156-oe plants recovered much less than wt following drought stress. This response was opposite to *CCD7*-silenced plants, while resembling that of plants treated with exogenous strigolactones prior to stress imposition. This set of features converges on the idea that the strigolactone-miR156 pathway promotes the "after-effect" of drought, which is thought to be part of a wider stress-memory mechanism (Lämke & Bäurle, 2017). After rewatering in fact, stomatal conductance never quite reaches the levels of unstressed plants even though water potential has, and this response could depend on non-hydraulic signals such as ABA (Ding et al., 2012; Galmes, Medrano, & Flexas, 2007; Lovisolo, Perrone, Hartung, & Schubert, 2008). Thus, genetic and pharmacological evidence indicates that both strigolactones and miR156 increase sensitivity to ABA in a number of species, including tomato. This reinforces the possibility of a strigolactone-miR156 module setting basal stomata sensitivity to ABA in accordance with the water-balance history of the plant. Of course, our data do not exclude that part of miR156 effects may also be exerted ABA-independently.

In conclusion, the results of the present work provided insight into the induction of miR156 under drought. They also demonstrated a cause-effect link between miR156 accumulation and the regulation of water relations and stomatal functioning. Particularly, we suggest that miR156 might not directly mediate the short-term, transient and likely ABA-independent stomatal closure triggered upon $GR24^{5DS}$ treatment. Rather, the strigolactone-miR156 module seems to set basal sensitivity of guard cells to ABA. This is most obvious after a drought period has occurred, when the module has been activated but hydraulic signals are no longer active. We propose that the module is also relevant for the establishment of specific drought responses, since its activation might promote acclimation to (recurring) water-limiting conditions by increasing sensitivity to ABA and drought avoidance. Tomato plants conditionally depleted of miR156, or overexpressing it, will allow discriminating between the stress-related, strigolactone-dependent effects of miR156 on stomata, and the long-term developmental consequences of its constitutive dysregulation.

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FIGURE LEGENDS

Figure 1. Effects of miR156 overexpression on tomato physiological performances during a drought and recovery time-course. **(A)** Stomatal conductance (g_s) of wt (MicroTom, empty symbols) and miR156-oe plants (black symbols) under well-watered conditions (ww, squares) and water stress followed by recovery (ws/rec, circles). Water withdrawal started at day zero, while recovery started by irrigation ten days later. Differences in g_s for well-watered plants were statistically significantly different at all points except day seven, but were not reported on the graph for better clarity. **(B)** Leaf water potential (Ψ_{leaf}) of wt and miR156-oe plants under different water conditions (well-watered = day zero, water-stressed = day ten and recovered plants = day fifteen of time-course in A). Data represent the mean ± SE of *n* = 5 biological replicates from two independent experiments. In (A) and (B), * indicates significant differences between genotypes for the same time-point and condition as determined by the Student's *t*-test, while in (B), lower case letters mark significant differences between different conditions in wt or miR156-oe plants separately, as determined by a one-way ANOVA test (P <0.05).

Figure 2. Effect of miR156 overexpression on ABA metabolism in unstressed wt (MicroTom) and miR156-oe plants (day zero in Fig. 1). The concentrations of **(A)** free ABA, abscisic-β-D-glucosyl ester (ABA-GE), phaseic acid (PA), dihydro-phaseic acid (DPA), neo-phaseic acid (neo-PA), 7'-hydroxy-ABA (7'OH-ABA) and of **(B)** transcripts of the ABA catabolic genes *SlCYP707A1* and *SlCYP707A2* and of the ABA biosynthetic gene *SlNCED1* were quantified. Gene transcript abundance was normalised to endogenous *SlEF-1*α and *SlsnRU6*, and presented as fold-change value over mean values of wt plants, which were set to 1. Data represent the mean ± SE of *n* = 5 biological replicates. * indicates significant differences between genotypes, as determined by a Student's *t*-test (P <0.05). n.d. = not detectable.

Figure 3. Effects of miR156 overexpression on stomatal responses to ABA treatment. Induced stomatal closure was quantified by measuring stomatal conductance 1 h after treatment with increasing concentrations of ABA in wt (MicroTom) and miR156-oe leaves, and expressed as a percentage of mean values for mock-treated leaves in the same genotype, which were set to 100. Data are the means ± SE of *n* = 5 biological replicates. Different letters indicate significant differences between different treatments in the same genotype as determined by a one-way ANOVA test, while * indicates significant differences between genotypes for the same condition, as attested by Student's *t*-test (P <0.05).

Figure 4. Strigolactones affect miR156 production and promote the after-effect of drought. **(A)** Effect of $GR24^{5DS}$ (5 µM) compared to mock treatment on the concentration of mature miR156 in leaf tissues during a short-term time-course in unstressed wt plants (0, 2, 6 and 24 h after treatment, *n* = 3 each sample being a pool of 3 leaflets). **(B)** Mature miR156 levels in leaves of wt (treated with exogenous strigolactones or mock-treated), and mock-treated *CCD7*-silenced plants during a quick drought time-course. Stress was imposed by uprooting plants at time zero (well-watered samples) and transferring them into dry vermiculite, with irrigated controls being transferred into wet vermiculite. Water-stressed and recovered samples were harvested 3 or 24 h (respectively) after the beginning of stress, having been re-watered right after stress peaking. For strigolactone and mock treatment, leaves were sprayed as in (A) 24 h before time zero (the beginning of stress). Data represent the mean ± SE of *n* = 3 biological replicates from three independent experiments. Target RNA abundance was normalised to endogenous *SlEF-1α* and *SlsnRU6* transcripts and presented as fold-change value over mock-treated wt tissues, which were set to 1. Different letters indicate significant differences as determined by a one-way ANOVA test (*P* <0.05). **(C)** Stomatal conductance values for the experiment in (B). One-way ANOVA test (*P* <0.05) was applied to detect differences among genotypes within a given time point. **(D)** Normalised stomatal conductance of wt (M82) plants upon treatment with $GR24^{5DS}$. The aerial parts of plants were sprayed with a 5 µM $GR24^{5DS}$ solution, and stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) was measured 2 and 24 h after the treatment. Data are presented as percentage of stomatal conductance over average g_s values of mock-treated plants, which were set to 100%. Data represent the mean ± SE of *n* = 4 biological replicates from three independent experiments. Different letters indicate statistically significant differences among treatments as determined by a one-way ANOVA test (*P* <0.05).

Figure 5. Effects of drought on the transcript concentration of six miR156 targets in leaves of wt (M82) and *CCD7*-silenced (SL-) tomato plants. *SlCCD7* transcripts are also displayed as a control for stress and transgene effect on this key strigolactone-biosynthetic gene. Gene transcripts were normalised to endogenous *SlEF-1*α and *SlsnRU6* in stressed and recovered plants from the drought time-course of Fig. 4B. Stress was imposed by uprooting plants at time zero (well-watered samples) and transferring them into dry vermiculite. Water-stressed and recovered samples were harvested 3 or 24 h (respectively) after the beginning of stress, having been re-watered right after stress peaking. Data are presented as fold change over the mean values for well-watered plants (time zero) of the same genotype, which were set to 1. Data represent the means ± SE of *n* = 5

(Solyc07g062980, Solyc10g009080 and Solyc10g078700) or n = 3 (Solyc03g114850, Solyc04g045560, Solyc01g090660 and Soly02g077920) biological replicates. Different letters indicate significant differences between conditions and genotypes within a given bar cluster as determined by a one-way ANOVA test (P <0.05).

Figure 6. Effects of miR156 overexpression on transcripts of strigolactone-biosynthetic genes. Transcript amounts of the putative (*SlD27*) or confirmed (*SlMAX1*, *SlCCD7* and *SlCCD8*) strigolactone-biosynthetic genes were quantified in **(A)** roots or **(B)** axillary buds of wt (MicroTom) and miR156-oe plants. Gene transcripts were normalised to endogenous *SlEF-1*α and *SlsnRU6* and presented as fold change over mean wt values, which were set to 1. In (A) data represent the means ± SE of n = 4 biological replicates from two independent experiments; in (B), each of two replicates was the pool of at least 30 axillary buds from 8 plants. *SlCCD7* transcript was not detectable in samples represented in (B). * indicates significant differences between genotypes as determined by a Student's t-test (P <0.05).

SUPPLEMENTARY DATA

The following supplementary data are available:

Supplementary Figure S1. Effects of miR156 overexpression on stomatal reactivity to exogenous ABA expressed as pore areas.

Supplementary Table S1. List of primer pairs used in this work, with target gene names and/or *Solyc* codes.

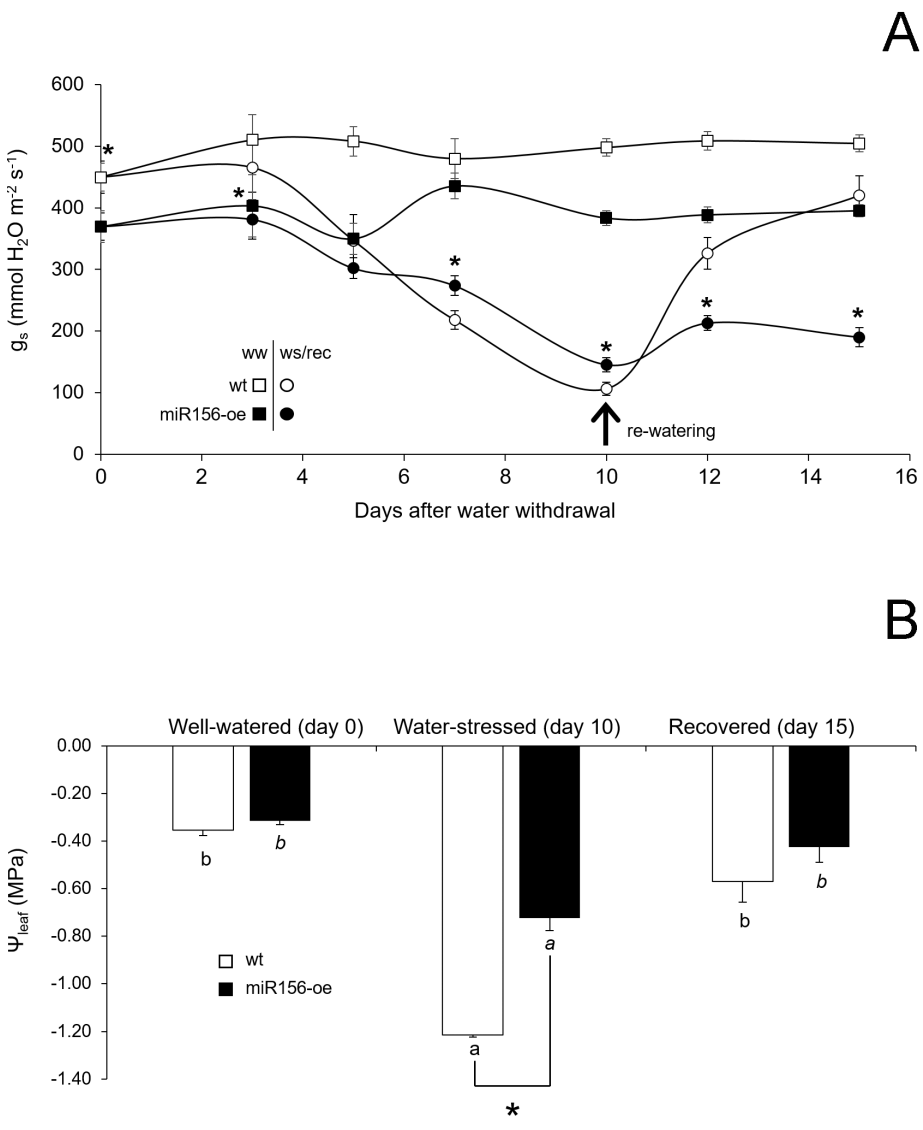
A
600
500
400
300
200
100
0
g_s (mmol H_2O m^{-2} s^{-1})
ww | ws/rec
wt □ ○
miR156-oe ■ ●
re-watering
0 2 4 6 8 10 12 14 16
Days after water withdrawal
B
Well-watered (day 0)
Water-stressed (day 10)
Recovered (day 15)
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
-1.20
-1.40
Ψ_{leaf} (MPa)
wt
miR156-oe
b
b
a
a
b
b
*

Figure 1

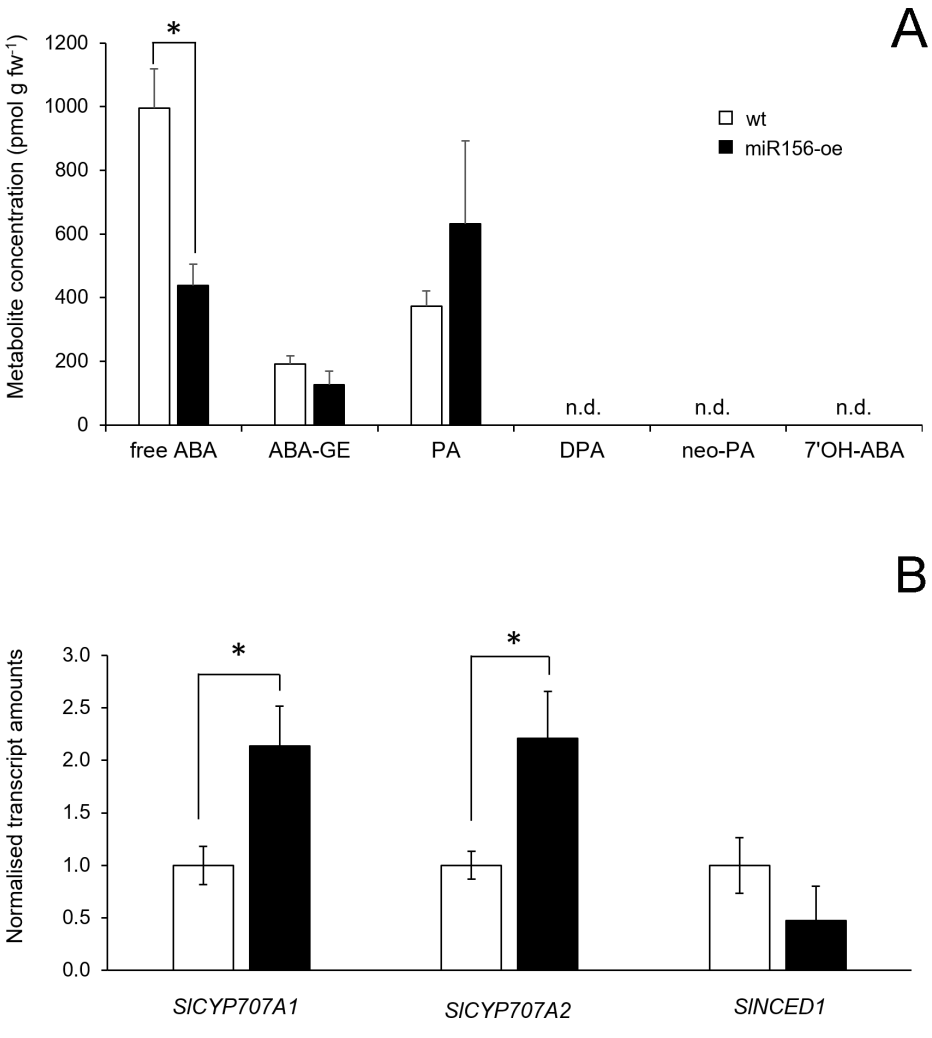
A
Metabolite concentration (pmol g fw-1)
1200
1000
800
600
400
200
0
*
wt
miR156-oe
n.d.
n.d.
n.d.
free ABA
ABA-GE
PA
DPA
neo-PA
7'OH-ABA
B
Normalised transcript amounts
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
SlCYP707A1
SlCYP707A2
SlNCED1

Figure 2

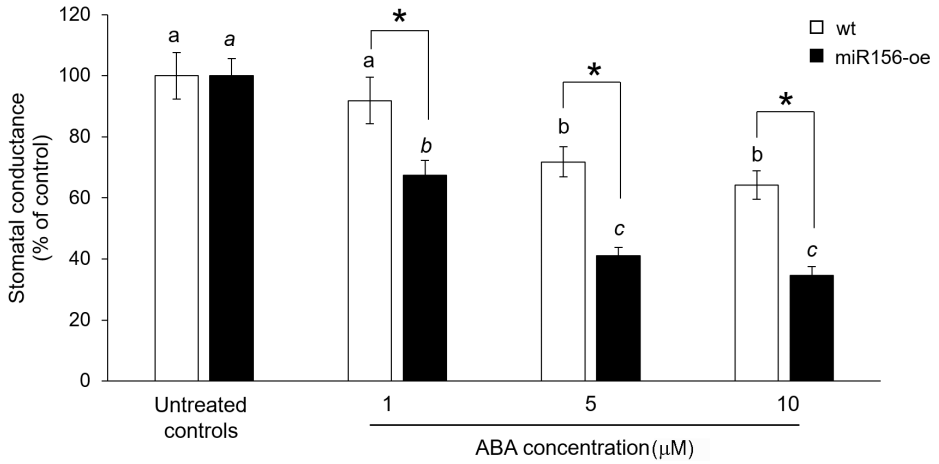
Stomatal conductance
(% of control)
120
100
80
60
40
20
0
a
a
a
b
b
c
b
c
*
*
*
wt
miR156-oe
Untreated
controls
1
5
10
ABA concentration(μM)

Figure 3

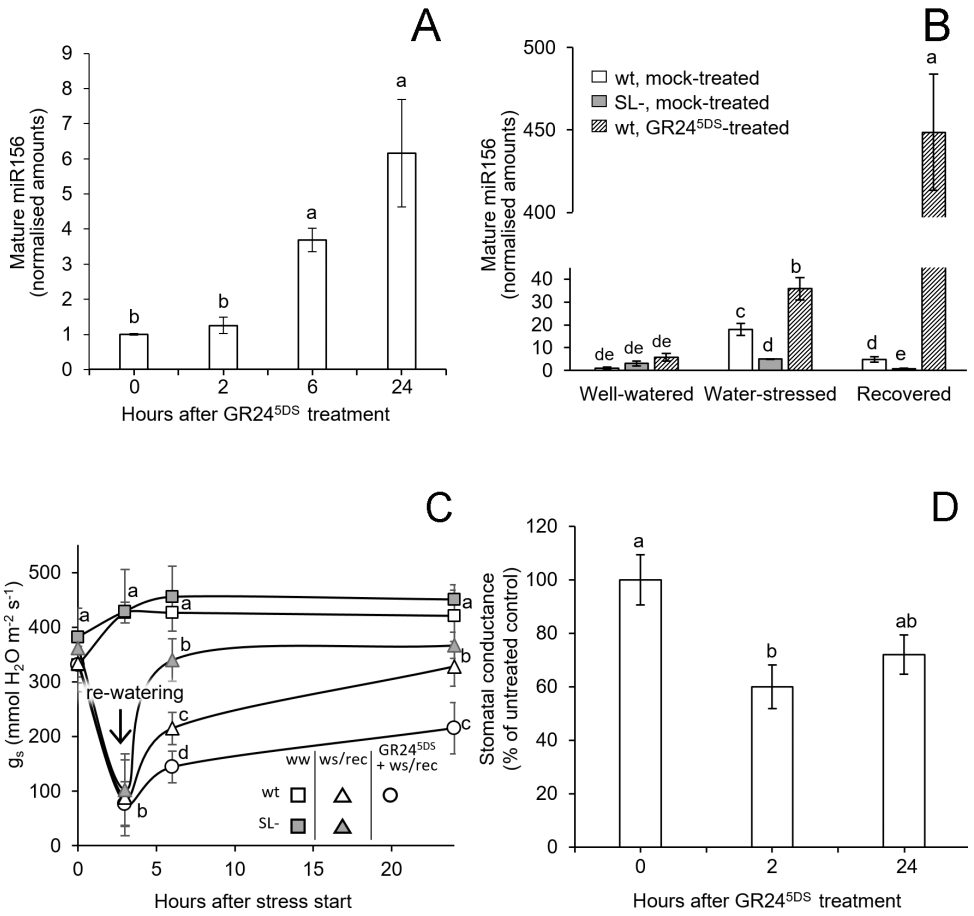
A
Mature miR156 (normalised amounts)
Hours after GR24^{5DS} treatment
B
wt, mock-treated
SL-, mock-treated
wt, GR24^{5DS}-treated
Mature miR156 (normalised amounts)
Well-watered
Water-stressed
Recovered
C
g_s (mmol H_2O m^{-2} s^{-1})
re-watering
ww
ws/rec
GR24^{5DS} + ws/rec
wt
SL-
Hours after stress start
D
Stomatal conductance (% of untreated control)
Hours after GR24^{5DS} treatment

Figure 4

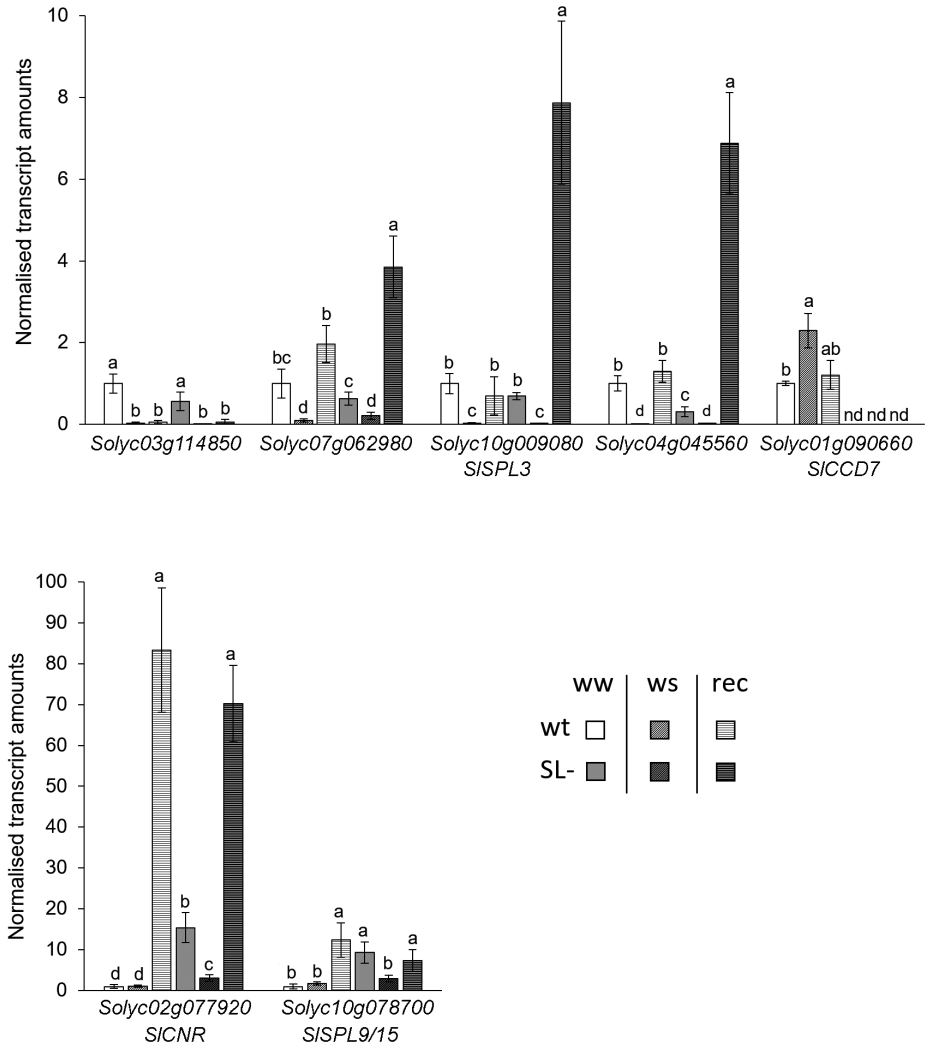
Normalised transcript amounts
Solyc03g114850
Solyc07g062980
Solyc10g009080
SlSPL3
Solyc04g045560
Solyc01g090660
SlCCD7
nd nd nd
Solyc02g077920
SlCNR
Solyc10g078700
SlSPL9/15
ww
ws
rec
wt
SL-

Figure 5

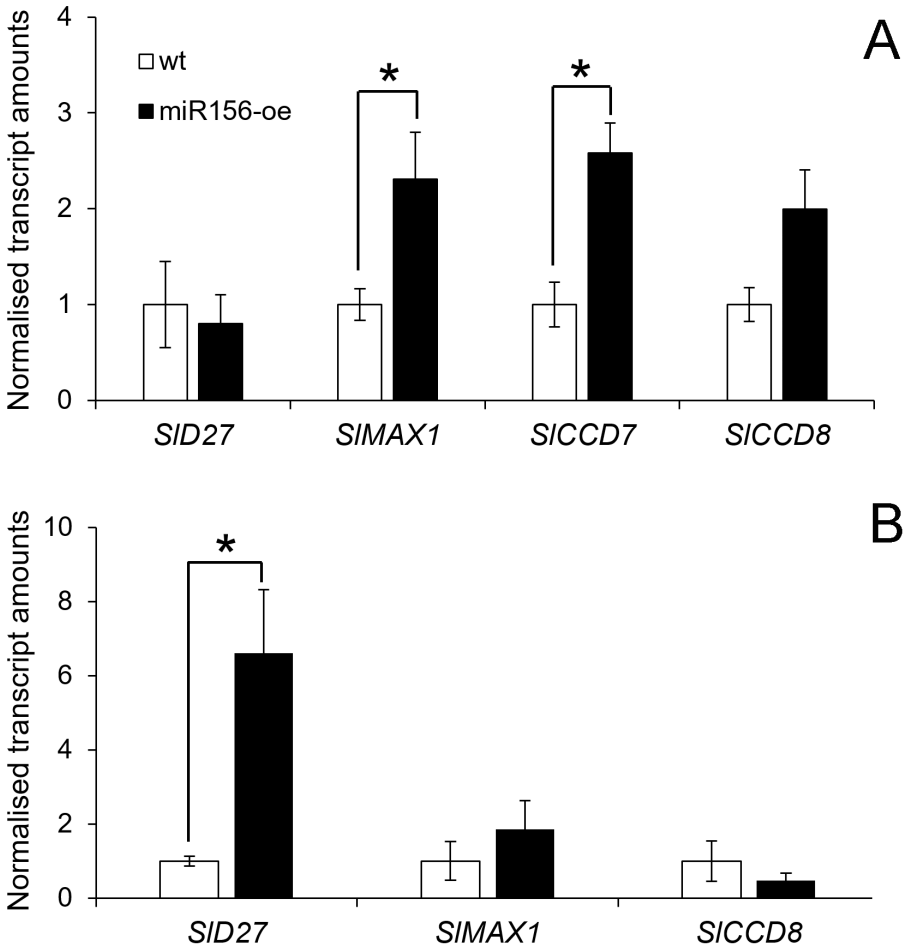
A
Normalised transcript amounts
4
3
2
1
0
wt
miR156-oe
*
*
SlD27
SlMAX1
SlCCD7
SlCCD8
B
Normalised transcript amounts
10
8
6
4
2
0
*
SlD27
SlMAX1
SlCCD8

Figure 6

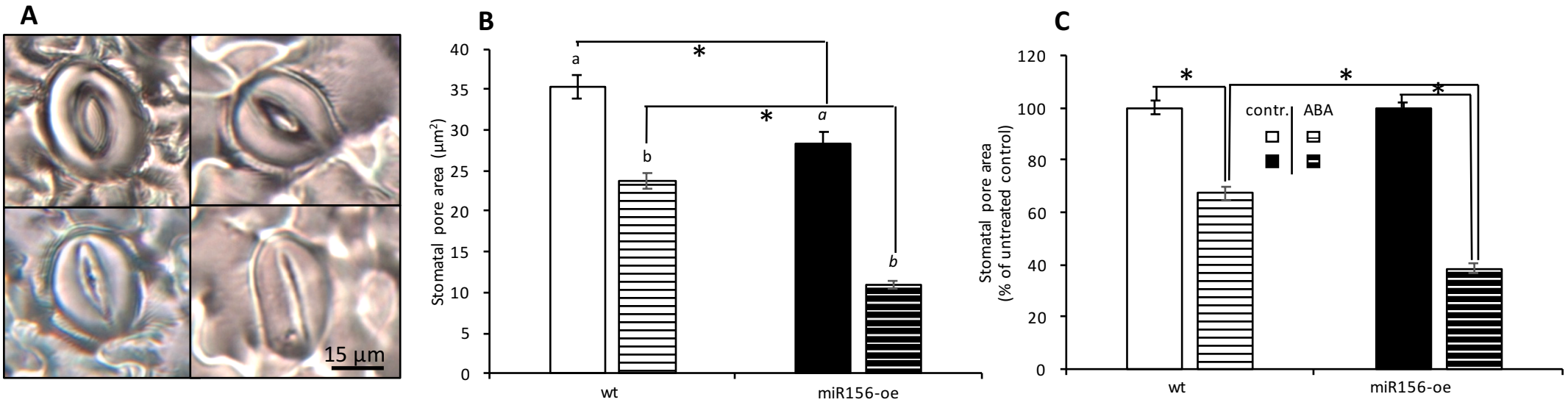

Figure S1. Effects of miR156 overexpression on stomatal reactivity to exogenous ABA. **(A)** Representative images of stomata from wt (MicroTom, left-hand panels) and miR156-oe plants (right-hand panels), before (upper panels) or after treatment (lower panels) with ABA 10 µM for 1 h. **(B)** Raw and **(C)** normalised stomatal aperture areas measured on abaxial epidermal peels of the two genotypes, before and after ABA treatment. Data represent the mean and ± SE of $n > 30$ biological replicates from two independent experiments. * indicates significant differences between genotypes under the same conditions, while different letters mark significant differences between treated and untreated leaves of the same genotype as determined by a Student's *t*-test ($P < 0.05$).

Visentin *et al*
Figure S1

Table S1. List of primers used in this work, with target gene names and/or *Solyc* codes.

primer/target name and/or *Solyc* code	sequence	reference
stem-loop miR156	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC-3'	Chen *et al.* (2005)
Mature miR156 MIMAT0009138 (from miRBase)	5'-GTCGTATCCAGTGCAGGGT-3' 5'-TTGACAGAAGATAGAGAGCACG-3'	this work
SlNCED1 Solyc08g016720.1	5'-AGGCAACAGTGAAACTTCCATCAAG-3' 5'-TCCATTAAAGAGGATATTACCGGGGAC-3'	Sun *et al.* (2012)
SlCYP707A1 Solyc04g078900.3	5'-CCCAGAGTTCTTTCCTGATCCACAA-3' 5'-GAATGCCACTACCAGATCCTACCAC-3'	Sun *et al.* (2012)
SlCYP707A2 Solyc08g075320.3	5'-TCGAAAAAGGATACAATTCGATGCC-3' 5'-CTGCAATTTGTTCGTCAGTGAGTCC-3'	Sun *et al.* (2012)
SlCNR Solyc02g077920.2	5'-GGCAGCCAAATAACCTACTTTC-3' 5'-CAATTTGCTTAGAAATCCGGG-3'	this work
SlSPL15 Solyc10g078700.1	5'-TCAGCTACCAGGACCAGTTATCAT-3' 5'-CGTCGATTCCTTGATCCCC-3'	this work
putative miR156 target 1 Solyc03g114850.2	5'-CATTCAGGGGCTATCAGGGA-3' 5'-TGGACATTCTACCTGCCGAA-3'	this work
putative miR156 target 2 Solyc07g062980.2	5'-CAAGACACCACTGCCCATTG-3' 5'-AAAGGTCCAACAACGATGCC-3'	this work
putative miR156 target 3 Solyc10g009080.2	5'-TCCAGGAGAAGGGTCAAGTT-3' 5'-TCGAAGTACCAACAGACAAAAGT-3'	this work
putative miR156 target 4 Solyc04g045560.2	5'- TAACTCCACAAGCACGAGGT-3' 5'- AGTGGTCCCTGAAAGCTTGA-3'	this work
putative *SlD27* Solyc09g065750.3	5'-TGTTCTTCTTCATGCAGGCAAAT-3' 5'-GCTGTTGCAATCTGCTTGGT-3'	this work
SlCCD7 Solyc01g090660.3	5'-GTTGCTCTTACCAATGGTTCAATTT-3' 5'-TACATTCATCATGGAAGGATCAAAGTT-3'	Kohlen *et al.* (2012)
SlCCD8 Solyc08g066650.3	5'-CCAATTGCCTGTAATAGTTCC-3' 5'-GCCTTCAACGACGAGTTCTC-3'	Kohlen *et al.* (2012)
SlMAX1 Solyc08g062950.3	5'-CGCCCTTAGTTGCCAGAGAA-3' 5'-GCCAACCAAACCCATGTTCC-3'	this work
SlsnRU6	5'-GGGAACGATACAGAGAAGATTAGC-3' 5'-ACCATTTCTCGATTTGTGCGT-3'	this work
SlEF-1α *Solyc06g005060.3*	5'-CTCCATTGGGTCG TTTTGCT-3' 5'-GGTCACCTTGGC ACCAGTTG-3'	Digilio *et al.* (2010)

References not listed in the main bibliographic section are:

Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. 2005. Real-time quantification of microRNAs by stem–loop RT–PCR. Nucleic Acids Research **33**(20), e179.

Digilio MC, Corrado G, Sasso R, Coppola V, Iodice L, Pasquariello M, Bossi S, Maffei ME, Coppola M, Pennacchio F, Rao R, Guerrieri E. 2010. Molecular and chemical mechanisms involved in aphid resistance in cultivated tomato. New Phytologist **187**(4), 1089-1101.

Sun L, Sun Y, Zhang M, Wang L, Ren J, Cui M, Wang Y, Ji K, Li P, Li Q, Chen P, Dai S, Duan C, Wu Y, Leng P. 2012. Suppression of *9*-cis-*Epoxycarotenoid dioxygenase*, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. Plant Physiology **158**(1), 283–298.