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Original Article

The *Arabidopsis GIBBERELLIN METHYL TRANSFERASE 1* suppresses gibberellin activity, reduces whole-plant transpiration and promotes drought tolerance in transgenic tomato

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ABSTRACT

Previous studies have shown that reduced gibberellin (GA) level or signal promotes plant tolerance to environmental stresses, including drought, but the underlying mechanism is not yet clear. Here we studied the effects of reduced levels of active GAs on tomato (*Solanum lycopersicum*) plant tolerance to drought as well as the mechanism responsible for these effects. To reduce the levels of active GAs, we generated transgenic tomato overexpressing the *Arabidopsis thaliana GA METHYL TRANSFERASE 1* (*AtGAMT1*) gene. *AtGAMT1* encodes an enzyme that catalyses the methylation of active GAs to generate inactive GA methyl esters. Tomato plants overexpressing *AtGAMT1* exhibited typical GA-deficiency phenotypes and increased tolerance to drought stress. GA application to the transgenic plants restored normal growth and sensitivity to drought. The transgenic plants maintained high leaf water status under drought conditions, because of reduced whole-plant transpiration. The reduced transpiration can be attributed to reduced stomatal conductance. *GAMT1* overexpression inhibited the expansion of leaf-epidermal cells, leading to the formation of smaller stomata with reduced stomatal pores. It is possible that under drought conditions, plants with reduced GA activity and therefore, reduced transpiration, will suffer less from leaf desiccation, thereby maintaining higher capabilities and recovery rates.

Key-words: drought stress; *GAMT1*; stomata; transpiration.

INTRODUCTION

Drought and salinity have a major impact on agriculture and food supply, and are responsible for major losses in crop productivity. Water deficit reduces leaf cell turgor, leading to suppression of cell expansion and consequently, inhibition of leaf growth and canopy development, thereby negatively affecting flowering and fruit development (Chaves, Maroco & Pereira 2003). Furthermore, it suppresses photosynthesis and primary carbon metabolism both directly and indirectly (Zhu 2002; Munns & Tester 2008). Plants have adopted various strategies to protect themselves from drought and salinity. Acquired tolerance to osmotic stresses involves changes in growth and development. Drought conditions change the root-to-shoot ratio due to rapid inhibition of shoot growth and maintained growth of roots (Sharp 2002). This integrated growth plasticity involves long-distance communication among different organs, with hormones playing a major role (Munns 2005; Sachs 2005). Growth regulation by drought and salinity is mediated primarily by the stress-related hormone abscisic acid (ABA; Munns 2002; Munns 2005). However, growth-promoting hormones, such as gibberellins (GAs) and cytokinin (CK), are also involved (Magome *et al.* 2004, 2008; Achard *et al.* 2006; Rivero *et al.* 2007; Albacete *et al.* 2008; Ha *et al.* 2012). The balance between these hormones affects plant performance under stress conditions and therefore, their tolerance.

The general effect of GA on plant growth and elongation opposes those of osmotic and water stresses. GAs promote various developmental processes throughout the life cycle of the plant, from seed germination, through leaf expansion, stem elongation, flower induction and development, to fruit set and seed development (Sun & Gubler 2004), all of which are suppressed by osmotic stresses (Hu *et al.* 2007). GA activity is regulated at the levels of biosynthesis, catabolism and signalling (Lange & Lange 2006; Ueguchi-Tanaka *et al.* 2007). The major regulators of GA responses are the DELLA proteins. This group of nuclear regulators functions as suppressors of GA responses. GA binding to its receptor GA Insensitive Dwarf 1 (GID1) leads to the degradation of DELLAs by the 26S proteosome and the stimulation of GA responses (Ueguchi-Tanaka *et al.* 2007; Harberd, Belfield & Yasumura 2009).

Several studies have shown that GA level or signal affects plant response to drought, salinity and other environmental stresses (Magome *et al.* 2004; Achard *et al.* 2006; Shan *et al.* 2007; Li *et al.* 2012). Achard *et al.* (2006) showed that GA-deficient and insensitive *Arabidopsis* mutants exhibit higher tolerance to salt stress. They provided evidence for the contribution of DELLA protein activity to the acquisition of tolerance to osmotic stress. Similar results were obtained by Magome *et al.* (2004), who showed that the GA-deficient

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Arabidopsis mutant *ga1-3* is relatively tolerant to salt stress. They also showed that DDF1, an AP2-like transcription factor induces the transcription of the GA-deactivating gene, *GA2oxidase*, thus reducing the level of endogenous GAs and promoting tolerance to high-salinity stress (Magome *et al.* 2004, 2008). Similarly, overexpression of the AP2-like gene, dehydration-responsive element-binding proteins (*SlDREB*) in tomato, suppresses GA biosynthesis and promotes drought resistance (Li *et al.* 2012). Furthermore, ectopic expression of the gain-of-function mutant version of the GA-signalling suppressor *MhGAI1* (the tea crabapple *DELLA* gene) in tomato promotes drought tolerance (Wang *et al.* 2012). GA treatments to cotton seedlings suppressed the expression of various stress-related genes, including the drought-induced *DREB* (Shan *et al.* 2007), and the same treatments to maize plants reduced the activity of superoxide dismutase (SOD), peroxidase and polyphenol oxidase, all of which contribute to tolerance to osmotic stress (Tuna *et al.* 2008). Achard *et al.* (2008) showed that GA deficiency or inhibition of GA signalling in *Arabidopsis* suppresses ROS accumulation following salt stress. They also showed that salt stress promotes the accumulation of DELLA proteins, which in turn induce the expression of genes encoding ROS-detoxification enzymes. They suggested that DELLA proteins reduce ROS level and thus delay cell death and promote tolerance to osmotic stresses.

In this work, we investigated the effect of GA deficiency on tomato plant tolerance to drought. We generated transgenic tomato overexpressing the *Arabidopsis GA METHYL TRANSFERASE 1* (*AtGAMT1*) gene (Varbanova *et al.* 2007). *AtGAMT1* encodes an enzyme that catalyses the methylation of active GAs to generate inactive GA methyl esters. Siliques of *gamt1 gamt2* double mutant accumulate high levels of active GAs. On the other hand, overexpression of *GAMT1* in *Arabidopsis* reduces the level of the major bioactive GA, GA_4. Furthermore, overexpression of *AtGAMT1* in *Arabidopsis*, tobacco and petunia results in typical GA-deficiency phenotypes (Varbanova *et al.* 2007). Tomato plants overexpressing *AtGAMT1* were semi-dwarf and exhibited increased tolerance to drought. The mechanism by which GA deficiency promotes water-deficit tolerance was investigated.

MATERIALS AND METHODS

Plant material

Tomato (*Solanum lycopersicum*) seedlings and plants were in the M82 background (*SP*$^{-}$). The plants were grown in a semi-controlled greenhouse (unless otherwise stated) under natural day length and a light intensity of ca. 500 μmol m^{-2} s^{-1}.

Plant transformation

The *AtGAMT1* open reading frame downstream of the 35S promoter and upstream of the OCS terminator in pPzp212 (Varbanova *et al.* 2007) was used for transformation. The construct was transferred via *Agrobacterium tumefaciens* to *S. lycopersicum* variety M82 cotyledons, using the transformation and regeneration methods described by McCormick (1991). Kanamycin-resistant T0 plants were grown in the greenhouse and three independent transgenic lines were selected and self-pollinated to generate homozygous transgenic lines.

RNA extraction and cDNA synthesis

Total RNA was isolated from tomato seedlings or leaves of mature plants. Frozen tissues were ground, resuspended in guanidine HCl and then phenol/chloroform was added. Samples were mixed by vortexing for 30 s and after 30 min were centrifuged at 4 °C for 45 min. Ethanol (100%) and 1 M acetic acid were added, and the samples were mixed and stored overnight at –80 °C. NaAc (3 M) was added and samples were washed with cold 70% ethanol. For the synthesis of cDNA we used the Verso cDNA kit (ABgene, Epsom, UK) and 3 μg of total RNA, according to the manufacturer's instructions.

qRT-PCR analyses

qRT-PCR analysis was performed using the SYBR Premix Ex Taq II (RR081Q) kit (Takara Bio Inc., Shiga, Japan). Reactions were performed using a Rotor-Gene 6000 cycler (Corbett Research, Sydney, Australia). A standard curve was obtained for each gene using dilutions of the cDNA sample. Each gene was quantified using the Corbett Research Rotor-Gene software. At least three independent technical repeats were performed for each cDNA sample. Relative expression of each sample was calculated by dividing the expression level of the analysed gene by that of *TUBULIN*. Gene-to-*TUBULIN* ratios were then averaged. For the analyses of GA-stimulated transcript 1 (*GAST1*) we used the forward primer 5′-GTAGCATGACACAGGGCCACA-3′ and reverse primer 5′-TAGCTCTCATATCGGGCAGTAC AA-3′. For the analyses of *AtGAMT1*, we used the forward primer 5′-CGACAGCCATCAACTCCATA-3′ and reverse primer 5′-TCTCATCCAACGACCGAAAC-3′. The forward primer for *TUBULIN* was 5′-CACATTGGTCAGGCCGG TAT-3′ and the reverse primer was 5-ATCTGGCCATC AGGCT-GAAT-3′.

Measurements of stomatal aperture and density

Stomatal aperture and density were determined using the rapid imprinting technique (Geisler, Nadeau & Sack 2000). This approach allowed us to reliably score hundreds of stomata from each experiment simultaneously. Briefly, light-bodied vinylpolysiloxane dental resin (eliteHD+, Zhermack Clinical, Badia Polesine, Italy) was attached to the abaxial side of the leaf and then removed as soon as it dried (~1 min). The resin epidermal imprints were covered with transparent nail polish, which was removed once it dried and served as a mirror image of the resin imprint. The nail-polish imprints were put on glass cover slips and photographed under a bright-field inverted microscope (1M7100; Zeiss, Jena,

Germany) on which a Hitachi HV-D30 CCD camera (Hitachi Kokusai Electric Inc. Tokyo, Japan) was mounted. Stomatal images were later analysed to determine aperture size using the ImageJ software (http://rsb.info.nih.gov/ij/) fit-ellipse tool. A microscopic ruler (Olympus, Tokyo, Japan) was used for size calibration.

Whole-plant transpiration measurements

Whole-plant transpiration rates and relative daily transpiration (RDT) were determined using an array of lysimeters placed in the greenhouse, as described in detail in Sade *et al*. (2009). Briefly, control M82 and transgenic plants were planted in 3.9-l pots and grown under controlled conditions (30/18 °C day/night under natural day length and light intensity of ca. 500 μmol m^{-2} s^{-1}). Each pot was placed on a temperature-compensated load cell with digital output (Vishay Tedea-Huntleigh, Netanya, Israel) and was sealed to prevent evaporation from the surface of the growth medium. A wet vertical wick made up of 0.14 m^2 cotton fibres partially submerged in a 1 L water tank was placed on a similar load cell and used as a reference for the temporal variations in potential transpiration rate. The weight output of the load cells was monitored every 10 s and the average readings over 3 min were logged in a datalogger (Campbell Scientific CR1000 Data Logger, Logan, UT, USA) for further analysis. The plant's daily transpiration (weight loss between predawn and 18:00 h) was normalized to plant weight (at the last well-irrigated predawn point after drainage) and to the neighbouring submerged wick's daily evaporation and was averaged for a given line over all plants (amount taken up by the wick daily = 100%).

Measurements of soil relative volumetric water content (VWC)

VWC was measured using the EC-5 soil moisture sensor combined with the 'ProCheck' interface reader (Decagon Devices, Pullman, WA, USA).

Leaf gas exchange and relative water content (RWC)

Gas exchange in fully expanded leaves, including transpiration, stomatal conductance and net CO_2 assimilation, was determined using a LI-6400 Portable Photosynthesis System (LiCor Inc., Lincoln, NE, USA). Gas exchange was measured at a photosynthetically active radiation (PAR) level of 1200 μmol m^{-2} s^{-1}. Water-use efficiency (WUE) was calculated as net CO_2 assimilation rate divided by transpiration rate. Leaf RWC was measured in control and transgenic plants as follows: fresh weight (FW) was measured immediately after leaf detachment and then leaves were soaked for 8 h in 5 mM $CaCl_2$ at room temperature in the dark, and the turgid weight (TW) was recorded. Total dry weight (DW) was recorded after drying these leaves at 70 °C to a constant weight. RWC was calculated as $(FW - DW)/(TW - DW) \times 100$ (Sade *et al*. 2009).

Measurements of leaf area

The plant's total leaf area was measured with a Li 3100 leaf area meter (Li-Cor area meter, model Li 3100).

Chlorophyll extraction and measurements

Chlorophyll was extracted from fresh leaves and measured spectrophotometrically at 645 and 663 nm (Arnon 1949). Chlorophyll concentration was calculated using the formula: $(20.2 \times A_{645} + 8.02 \times A_{663})/cm^2$.

RESULTS

Tomato plants overexpressing *AtGAMT1* exhibit typical GA-deficiency phenotypes

To examine the effect of GA deficiency on the response of tomato plants to drought, we firstly generated transgenic plants overexpressing the *Arabidopsis AtGAMT1* gene to reduce the levels of active GAs (Varbanova *et al*. 2007). Tomato M82 was transformed with *AtGAMT1* cDNA driven by the 35S promoter (*35S:AtGAMT1*) and transgenic lines were selected on kanamycin. Three resistant lines with mild, medium and strong phenotypes, *GAMT1#14*, *GAMT1#2*, and *GAMT1#17*, respectively, were selected for this study. The plants were self-pollinated and homozygous lines were generated.

The transgenic plants had typical GA-deficiency phenotypes. Leaflets of all lines were smaller than those of M82 and they were a darker green (Fig. 1a,b). The transgene also affected shoot elongation: while the effect was mild in *GAMT#14*, *GAMT#2* plants were semi-dwarf and *GAMT#17* plants were dwarf and bushy (Fig. 1a). qRT-PCR analysis confirmed the expression of *AtGAMT1* in all transgenic lines and the level of expression correlated well with the severity of the phenotype (Fig. 1c). Pigment analysis revealed that chlorophyll content per leaf area was higher in the transgenic leaves (Fig. 2a). We also analysed the expression of the GA-induced gene *GAST1* (Shi *et al*. 1992) in seedlings of control M82 and all three transgenic lines. Its expression was barely affected in *GAMT#14*, but was significantly reduced in *GAMT#2* and *GAMT#17*, with lower expression in the latter (Fig. 2b). GA_3 application to the transgenic plants restored normal growth and development (see later). These results suggest that *AtGAMT1* overexpression reduced the levels of active GAs in the transgenic plants.

Despite the severe effect of the transgene on shoot development, we did not find any clear effect on root development (Supporting Information Fig. S1a). We also grafted control M82 shoots on *GAMT1#17* rootstock and found no effect of the transgenic rootstock on the phenotype of the M82 scion (Supporting Information Fig. S1b).

Overexpression of *AtGAMT1* in tomato promotes tolerance to drought stress

We next tested the performance of the transgenic plants under drought stress using *GAMT1#2* plants. Control (M82)

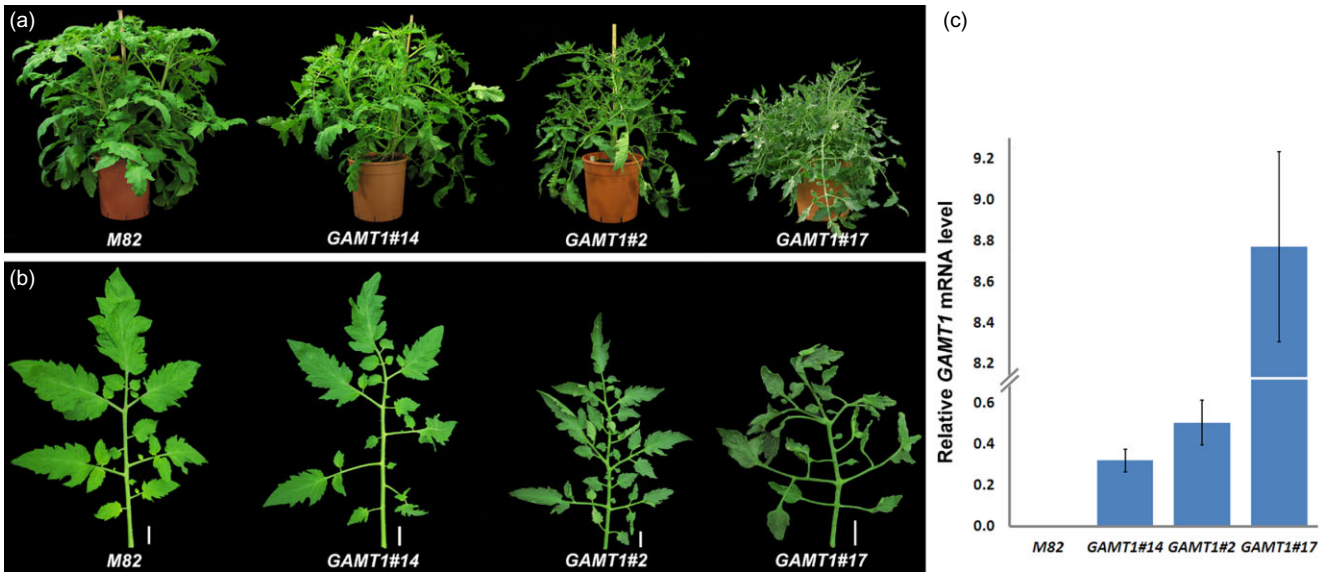

Figure 1. Transgenic tomato plants overexpressing *AtGAMT1*. (a) Representative control M82 and transgenic *GAMT1*#14, *GAMT1*#2 and *GAMT1*#17 plants. (b) Leaf no. 8 of the different lines. (c) Expression levels (qRT-PCR) of the transgene *AtGAMT1* in the different lines. Values (gene-to-*TUBULIN* ratios) in c are means of three biological replicates ± standard error (SE).

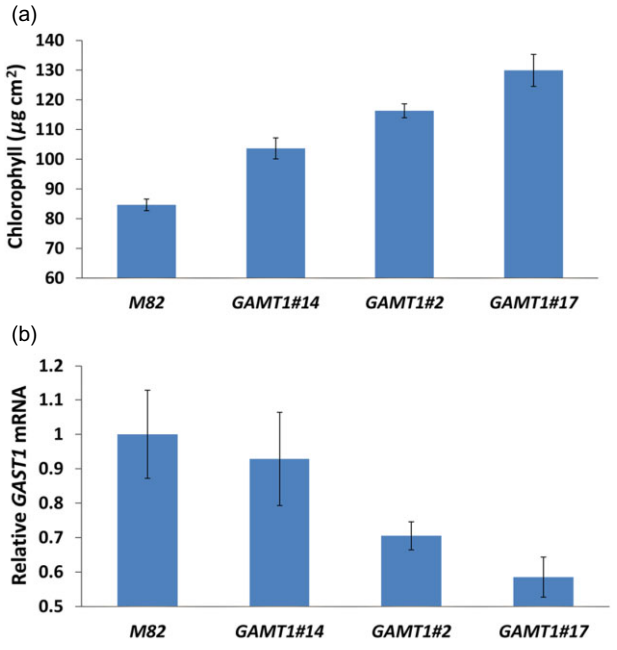

Figure 2. Transgenic tomato plants overexpressing *AtGAMT1* exhibit typical gibberellin (GA)-deficiency phenotypes. (a) Chlorophyll concentration in leaves (expanded leaf no. 6 from the apex) of control M82 and the three transgenic lines. Values are means of five repeats (taken from three different plants) ± standard error (SE). (b) qRT-PCR analyses of *GAST1* expression in control M82 and the three transgenic lines. RNA was extracted from 2-week-old seedlings. Values (gene-to-*TUBULIN* ratio, presented as proportion of the control M82, which was set to a value of 1) are means of three biological replicates (three different plants) ± SE.

and transgenic plants were grown until they produced 10 expanded leaves and then irrigation was stopped for dehydration. After 7 days, non-irrigated control plants started to wilt, but the non-irrigated transgenic plants were still turgid (Fig. 3a). The transgenic plants started to show wilting symptoms 3 days later. Two weeks after the beginning of the drought treatment, we started to irrigate the plants again. Both control M82 and *GAMT1#2* plants recovered, but while M82 leaves had many necrotic lesions, no damage was found in the transgenic leaves (Fig. 3b).

We measured leaf RWC in all of these plants 7 days into the drought treatment, when the non-irrigated control M82 plants exhibited clear signs of wilting. RWC in the non-irrigated control leaves was 50% lower than that in the irrigated control plants (Fig. 3c). At this time point, we did not find any difference in the RWCs of the non-irrigated versus irrigated transgenic leaves.

To confirm that the increased tolerance of the transgenic plants to drought was caused by the reduced levels of active GA, we treated the transgenic plants with exogenous GA. Control M82 and *GAMT1#2* transgenic seedlings with two true leaves were sprayed once a week, for 4 weeks, with 100 μM GA_3. As a control, plants were sprayed with similar amount of water. GA_3 treatments to *GAMT1#2* transgenic plants rescued normal growth and development (Fig. 4a). Four weeks after the beginning of the GA-treatment, we stopped the irrigation for dehydration and after 10 days of drought, control, control plants treated with GA_3, and *GAMT1#2* transgenic plants treated with GA_3 started to wilt. At this time the non-irrigated, mock-treated *GAMT1#2* transgenic plants were still turgid (Fig. 4a,b). The latter started to show wilting symptoms 4 days later (data not shown).

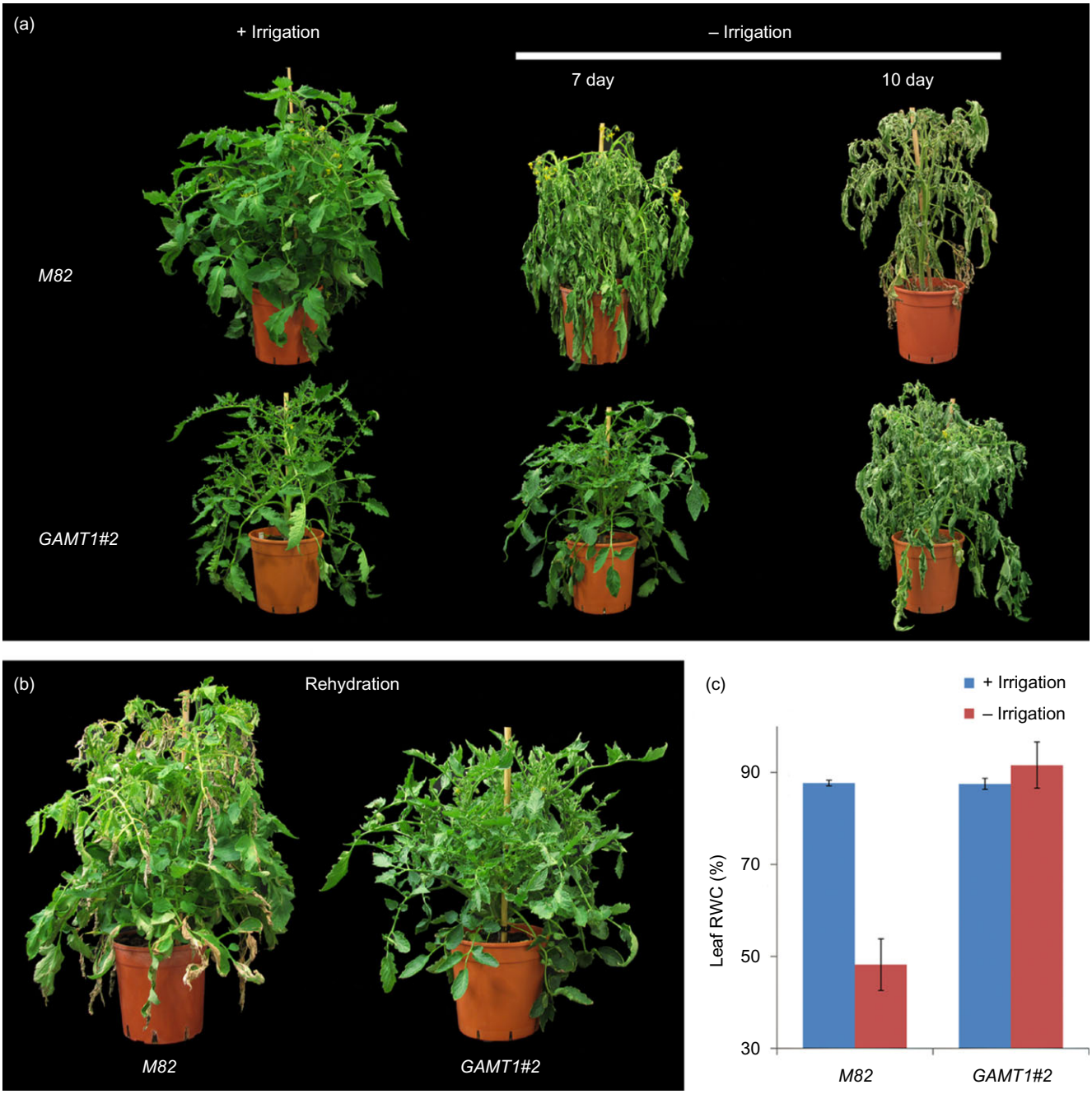

Figure 3. Overexpression of *AtGAMT1* in tomato promotes tolerance to temporary drought stress. (a) Control M82 and transgenic *GAMT1*#2 plants were grown until they produced 10 leaves and then irrigation was stopped for dehydration. Representative plants exposed to 7 or 10 days of drought (–irrigation) are shown. (b) After 14 days of drought stress, plants were rehydrated and recovery was recorded. (c) Average relative water content (% RWC) of control M82 and transgenic *GAMT1*#2 plants grown with (+) irrigation or exposed to 7 days of drought (–irrigation). Values are means of three replicates (three different plants) ± standard error (SE).

We also measured leaf RWC in the different plants when the non-irrigated control M82 plants exhibited clear signs of wilting (10 days into the drought treatment). RWC in the non-irrigated M82, treated or non-treated with GA_3 and in the GA_3-treated transgenic leaves was ca. 20% lower than that found in the irrigated control and transgenic leaves (Fig. 4b). At this time point, we did not find any reduction in the RWCs of the non-irrigated mock-treated *GAMT1#2* transgenic leaves. Similar results were found for all other transgenic lines, that is their phenotype was rescued by GA_3 treatments and they lost resistance to drought (Supporting Information Fig. S2b,c). These results suggest that the increased tolerance of the transgenic plants to transient drought stress is due to the reduced GA levels.

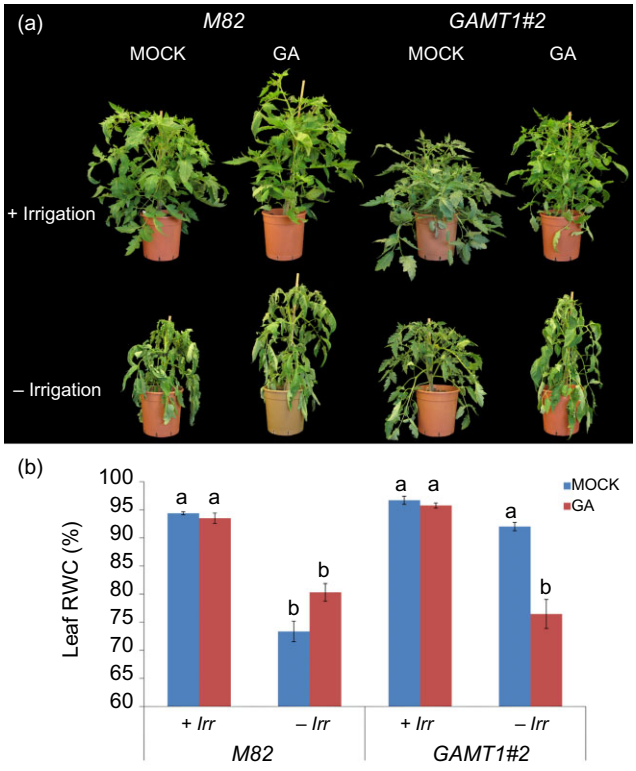

Figure 4. Application of gibberellin (GA) to *GAMT1* overexpressing plants restored normal growth and sensitivity to drought. Control M82 and *GAMT1#2* transgenic seedlings with two true leaves were treated once a week, for 4 weeks, with 100 μM GA_3. Four weeks after the beginning of the GA-treatment, irrigation was stopped for dehydration. (a) Representative GA-treated (GA) and mock-treated (Mock) plants, irrigated (+ irrigation) or exposed to 10 days of drought (– irrigation) are shown. (b) Average relative water content (%RWC) of control M82 and transgenic *GAMT1*#2 leaves taken from GA-treated (GA) or mock-treated (Mock) plants grown with irrigation (+ *Irr*) or exposed to 10 days of drought (–*Irr*). Values are means of four replicates (four different plants) ± standard error (SE). Different letters above the columns represent significant differences between treatments [Tukey–Kramer honestly significant difference (HSD), $P < 0.01$].

AtGAMT1 overexpression reduces whole-plant transpiration under irrigation and during drought stress

To measure whole-plant transpiration in the different lines, we used an array of load cells (lysimeters, see Materials and Methods) placed in the greenhouse and simultaneously followed the daily weight loss of each plant during the well-irrigated regime (Fig. 5a) and through several days of continuous drought (Fig. 5c). All of the transgenic plants showed reduced daily transpiration rate compared to the M82 plants.

The mean RDT of the M82 line was 157 ± 12.6% [mean ± standard error (SE), $n = 5$], which was significantly higher than that of *GAMT1*#2 (120 ± 7%) and *GAMT1*#17 (89 ± 7%; mean ± SE, $n = 6$ and $n = 5$, respectively, Fig. 5b). We did not find significant differences in transpiration during the dark period. Consequently in the non-irrigation regime, M82 plants were the first (at 3 days) to reach minimal soil relative VWC (11.32% ± 1.85, $n = 5$), followed by *GAMT1*#2 and *GAMT1*#14, which reached similar VWCs (13.3% ± 2.6 and 15.4% ± 4.64, $n = 6$ and $n = 6$, respectively) after 4 days of drought. *GAMT1*#17 lines maintained their low and stable transpiration rate throughout the drought treatment and as a result, maintained a significantly higher VWC (26.3% ± 7.66, $n = 5$), even after 6 days of drought (Fig. 5d). These results suggest that the reduced transpiration in the transgenic plants allows them to utilize the available water in the soil more slowly and thus, for longer time than control plants.

To determine if the reduced transpiration, and thus the increased water availability in the soil, is the only cause for the increased tolerance to drought in *GAMT1* overexpressing plants, we exposed the plants to drought stress, but kept similar soil relative VWC. Control M82 and *GAMT1#2* transgenic plants were grown for 4 weeks and then irrigation was stopped for dehydration. Soil relative VWC was measured constantly, using EC-5 soil moisture sensor and the required quantity of water was added to the control M82 plants to ensure equal water availability (similar VWC) for all non-irrigated plants (control and transgenic). Under these growth conditions, M82 and *GAMT1*#2 transgenic plants exhibited similar sensitivity to drought: both started to wilt at 12% VWC and wilting symptoms were similarly increased when VWC reached 6% (Supporting Information Fig. S3). These results suggest that the reduced transpiration is the only cause for the increased drought tolerance of the transgenic plants.

AtGAMT1 overexpression reduces stomatal conductance

Reduced leaf area (Fig. 1) might be the major cause for the lower whole-plant transpiration in the transgenic plants. Although *AtGAMT1* overexpression had no effect on leaf number, it reduced leaflet lamina growth, and thus, whole-plant leaf area in all transgenic lines was smaller than that of control plants (Fig. 6a). The reduced leaf area correlated well with the severity of the phenotype and the reduction in transpiration. Because almost all transpiration occurs via stomata (Hetherington & Woodward 2003), we analysed microscopically the abaxial leaf epidermal tissues. This analysis showed stomatal density in all transgenic lines to be higher than that in control leaves (Figs 6b,7a). We calculated the number of stomata per leaflet and found that leaflets of control and all transgenic lines contain similar number of stomata (Fig. 6d). Because the number of leaves and leaflets was not affected by the transgene (not shown), per plant, the number of stomata was similar in control and transgenic plants. Thus, although we cannot exclude completely the possibility that leaf size had an effect on transpiration, it is probably not a major factor.

We tested whether the differences in whole-plant transpiration between control and transgenic plants were caused by differences in stomatal conductance. We firstly analysed stomatal aperture in the control and three transgenic lines at

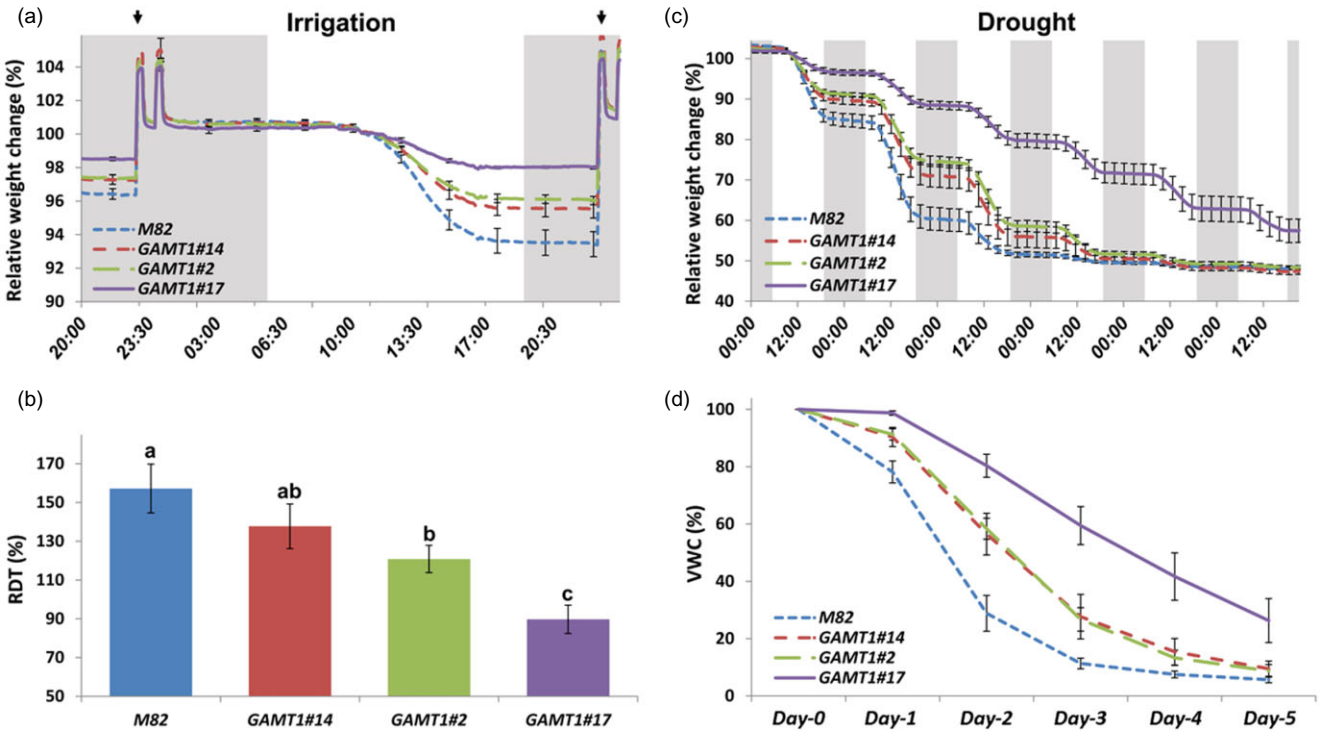

Figure 5. *AtGAMT1* overexpression reduces whole-plant transpiration under irrigation and during drought stress. (a) Average [± standard error(SE)] variations in pot weight of all tested lines (n = at least 5) during a 24 h cycle (white/dark backgrounds indicate day/night, respectively). The cycle consisted of double pulse irrigations (black arrow) followed by drainage and the absence of any weight loss during the night; this was followed by weight loss during the day and the second irrigation pulses. (b) Normalized relative daily transpiration (% RDT; see Materials and Methods) of the different lines on the same day (as in a). Different letters above the columns represent significant differences between lines [Tukey–Kramer honestly significant difference (HSD), $P < 0.05$]. (c) Average (± SE) pot weight variation for all lines during 6 days without irrigation. Pot weight was measured every 10 s and the average readings over 3 min were calculated. For the clarity of the figure, SE bars are shown for values at 2 h intervals. White/dark backgrounds indicate day/night, respectively. (d) Average (± SE) soil relative volumetric water content (% VWC; see Materials and Methods) for all lines during 6 days without irrigation.

different times of the day. Microscopic analyses revealed smaller stomata, as well as all epidermal cells, in the transgenic plants (Fig. 7a). In all tested plants, stomata exhibited maximum opening in the morning, with aperture decreasing thereafter. However, in the morning as well as at noon, stomatal aperture in control plants was larger than that in the transgenic plants (Fig. 7b). The stomatal pore area in the transgenic lines correlated well with the severity of their phenotype (Fig. 1) and with the rate of whole-plant transpiration (Fig. 5). These results suggest that the reduced transpiration in the transgenic plants is due to reduced stomatal conductance.

We also measured gas exchange in control and three transgenic lines using a Li-6400 portable photosynthetic system. The results show that water loss and CO_2 uptake per leaf area increase in the transgenic plants (Supporting Information Fig. S4a,b). However, while water loss per leaf area in *GAMT1#2* plants was ca. 30% higher than that in M82 (0.3 versus 0.2 mol m^{-2} s^{-1}), the number of stomata per leaf area in *GAMT1#2* plants was ca. 80% higher (25.42 versus 14.03 stomata per 0.1 mm^{-2}, Fig. 6b). Thus, per stomata, water loss in *GAMT1#2* plants was much lower than that in M82 plants. The relative increase in CO_2 uptake per leaflet area in the transgenic plants was larger than the relative increase in water loss and thus, the calculated WUE (CO_2 uptake versus water loss) was increased in the transgenic plants (Supporting Information Fig. S4c).

To examine the effect of the transgene on fruit yield, we grew control and transgenic *GAMT1#2* plants in the soil in the greenhouse, under irrigation (daily irrigation) or under low soil moisture conditions (one irrigation per week). Small, but not significant reduction in fruit yield (total fruit weight per plant) was found in the transgenic plants compare to control M82 plants under normal irrigation (Supporting Information Fig. S5). However, when plants were grown under mild drought stress, fruit yield was reduced significantly in control M82 but not in *GAMT1#2* plants.

DISCUSSION

GAMT1 methylates the carboxyl group of various GAs to form the corresponding GA methyl esters (Varbanova *et al.* 2007), which are not biologically active (Weiss *et al.* 1995; Cowling *et al.* 1998), because they cannot bind to the GA receptor GID1 (Ueguchi-Tanaka *et al.* 2005; Nakajima *et al.* 2006). Tomato plants overexpressing *AtGAMT1* exhibit typical GA-deficiency phenotypes, including dwarfism, high chlorophyll levels and reduced expression of the

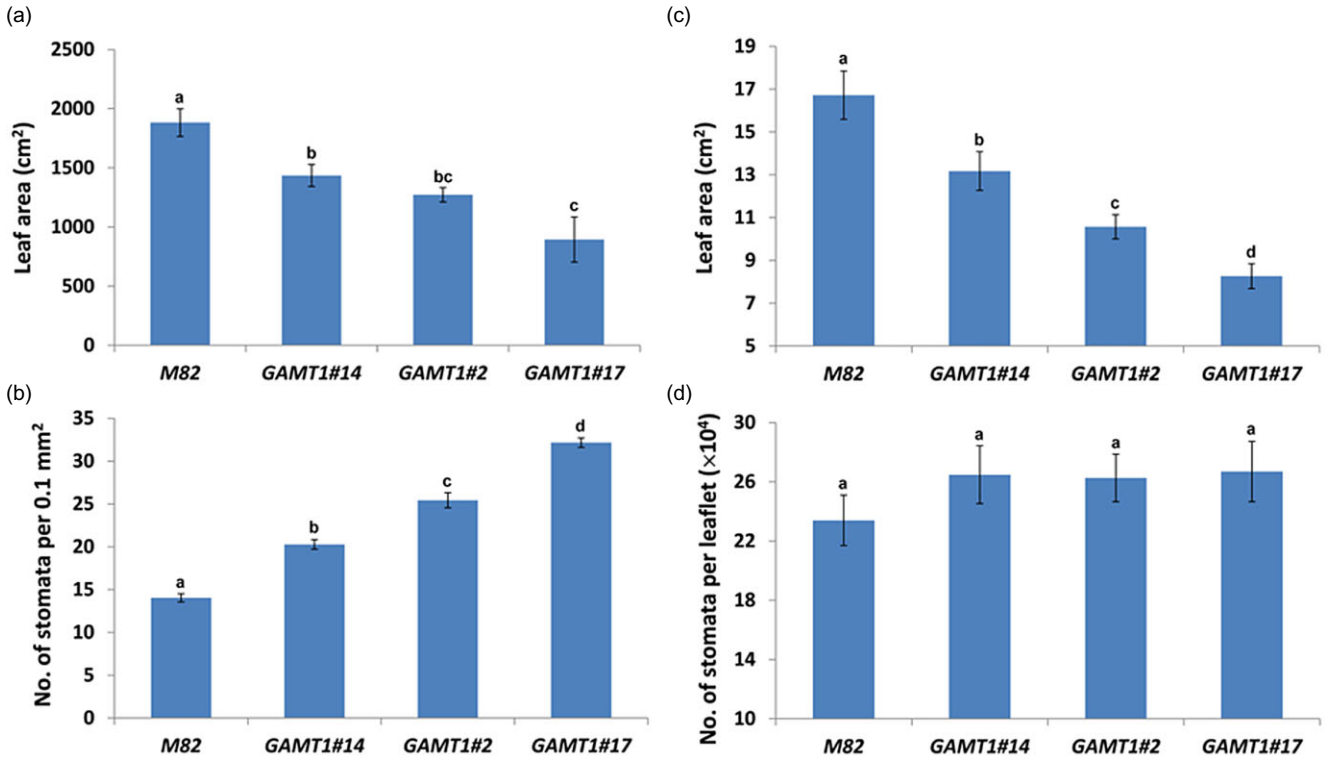

Figure 6. *AtGAMT1* overexpression reduces leaf size and increases stomatal density. (a) Total leaf area of control M82 and the three transgenic lines. Values are means of 5 plants ± standard error (SE). Different letters above the columns represent significant differences between treatments [Tukey–Kramer honestly significant difference (HSD), $P < 0.05$]. (b) Stomatal density (number of stomata per leaf area) in leaf no. 6 from the apex. Values are means of nine repeats taken from three different plants ± SE. Different letters above the columns represent significant differences between treatments (Tukey–Kramer HSD, $P < 0.01$). (c) The area of leaflets that were analysed for stomata density, was measured. Values are means of three leaflets taken from three different plants ± SE. Different letters above the columns represent significant differences between treatments (Tukey–Kramer HSD, $P < 0.05$). (d) The number of stomata per leaflet was calculated using the data presented in b and c ± SE. Different letters above the columns represent significant differences between treatments (Tukey–Kramer HSD, $P < 0.01$).

GA-induced gene *GAST1* (Figs 1,2). The increased chlorophyll concentration (per leaf area) could result from a combination of reduced leaf-cell size with normal level of chlorophyll synthesis (Wolf & Haber, 1960). In tomatoes, but not in *Arabidopsis*, petunia or tobacco (Varbanova *et al.* 2007), *AtGAMT1* overexpression affected leaf morphology. Tomato leaf morphology has been shown to be affected by different factors, including GA, probably due to the enhanced morphogenetic activity (Shani *et al.* 2010; Fleishon *et al.* 2011). GA_3 application to the transgenic plants restored normal growth, leaf shape and colour (Fig. 4). Taken together, the results suggest that *AtGAMT1* overexpression in tomatoes reduces the levels of active GAs, as it does in *Arabidopsis*, petunia and tobacco (Varbanova *et al.* 2007).

The reduced GA levels promoted water-deficit tolerance. The transgenic plants were able to maintain high leaf water status for a longer time than control plants under conditions of low soil water (Figs 3,5). Previous studies have suggested that inhibition of GA levels/activity promotes tolerance to drought stress indirectly, via suppression of growth (Magome *et al.* 2004; Achard *et al.* 2006; Shan *et al.* 2007; Wang *et al.* 2012). Several mechanisms were proposed: redirection of energy resources to support processes involved in drought tolerance, and reduced transpiration by decreasing leaf area (Magome *et al.* 2004; Achard *et al.* 2006). Although leaves of transgenic plants overexpressing *AtGAMT1* were smaller than those of control M82 plants, they contained similar number of stomata, that is stomatal density increased (Fig. 6). Because almost all transpiration occurs via the stomata (Hetherington & Woodward 2003), leaf size in itself is probably not the direct cause of the lower transpiration. The lower GA activity reduced stomatal conductance and this directly affected transpiration rate. The reduced transpiration rate allowed the transgenic plants to utilize the available water in the soil more slowly and thus, for longer time than control plants. This was the only cause for the increased drought tolerance in the transgenic plants: when we kept equal levels of available water during the drought treatment by adding water to the control plants, the different lines (control and transgenic lines) exhibited similar sensitivity to drought (Supporting Information Fig. S3).

Conductance through stomata is modulated by their aperture and by their density (Hetherington & Woodward 2003; Yoo *et al.* 2009; Casson & Hetherington 2010). Yoo *et al.* (2010) have shown that reduced stomatal density decreases conductance and transpiration. Overexpression of

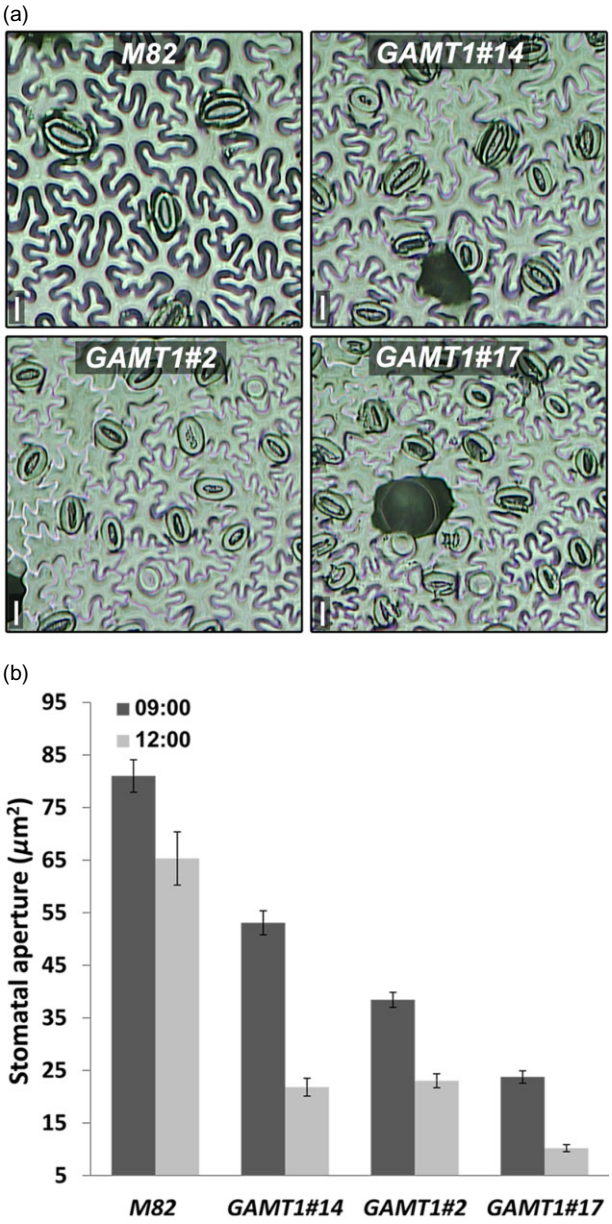

Figure 7. *AtGAMT1* overexpression reduces stomatal conductance. (a) Abaxial leaf epidermal tissues of control M82 and the three transgenic lines. Bars = 20 μM. (b) Stomatal aperture in leaf no. 6 from the apex, measured at 09:00 (dark-grey shading) and 12:00 (light-grey shading) h in control and transgenic leaves. Values are means of ca. 300 measurements (stomata) ± standard error (SE).

the transcription factor *SlDREB* in tomato, suppresses GA biosynthesis and leaf expansion, reduced stomatal density and promotes drought resistance (Li *et al*. 2012). Whether the effect of *SlDREB* on stomatal density is via GA or through other DREB-target genes, is not yet clear. Our results show that reduced GA activity in *AtGAMT1*-overexpressing plants increased stomatal density. However, despite the increased density, whole-plant transpiration was reduced. The reduced transpiration in the *AtGAMT1*-transgenic plants can be attributed to reduced stomatal conductance. *GAMT1* overexpression inhibited the expansion of leaf epidermal cells, leading to the formation of smaller guard cells; the result is smaller stomata with reduced stomatal pores (Fig. 7) and therefore, reduced stomatal conductance.

A reduction in stomatal conductance should lead to reduced CO_2 uptake (Bussis *et al*. 2006). The transgenic plants showed, however, higher CO_2 uptake and water loss per leaflet area (Supporting Information Fig. S4). This is probably due to the increased stomatal density in the transgenic plants, which compensated for the reduced stomatal conductance. Rough calculation shows that per stomata, water loss in the transgenic plants was much lower. WUE (CO_2 uptake versus water loss) was slightly higher in the transgenic plants. This can be attributed to the increased chlorophyll levels in the transgenic leaves, which could affect CO_2 fixation. The higher WUE may contribute to the performance of the plants under restrained soil water conditions.

Drought resistance is an important agricultural trait; however, growth suppression and reduced whole-plant transpiration can affect yield. Preliminary examination showed that fruit yield in the transgenic plants (line *GAMT1#2*) was slightly lower than in control M82 plants under irrigation conditions. However, mild drought stress decreased yield in control, but not in the transgenic plants. This might be due to the ability of the transgenic plants to maintain higher leaf water status and also due to their higher WUE. Thus, it is possible that under severe drought, plants with a lower level of active GAs will perform better.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. *AtGAMT* overexpression had no effect on root development and the transgenic roots had no effect on shoot phenotype. (a) Root system of seven weeks old control and transgenic plants. (b) Grafted plants, from left to right: M82 scion on M82 rootstock, *GAMT1*#17 scion on *GAMT1*#17 rootstock, M82 scion on *GAMT1*#17 rootstock, and *GAMT1*#17 scion on M82 rootstock.

Figure S2. Application of GA to *GAMT1* overexpressing plants restored normal growth and sensitivity to drought. Control M82 and *GAMT1#14* and *GAMT1#17* transgenic seedlings with two true leaves were treated once a week, for 4 weeks, with 100 μM GA_3. (a) Representative GA-treated (GA) and mock-treated (Mock) plants are shown. (b and c) Four weeks after the beginning of the GA-treatment, irrigation was stopped for dehydration. Average relative water content (% RWC) of control M82 and transgenic *GAMT1*#14 (b) and *GAMT1#17* (c) leaves taken from GA-treated (GA) or mock-treated (Mock) plants and grown with irrigation (+ *Irr*) or exposed to 10 days of drought (– *Irr*). Values are means of four replicates (four different plants) ± SE. Different letters above the columns represent significant differences between treatments (Tukey–Kramer HSD, $P < 0.01$).

Figure S3. The reduced transpiration in the transgenic *GAMT1* overexpressing plants is the only cause for their increased drought tolerance. Control M82 and *GAMT1*#2 transgenic plants were grown in the greenhouse for 4 weeks and then irrigation was stopped for dehydration. Similar VWC was kept for all non-irrigated plants (transgenic and control) by adding water to M82 plants. VWC was measured constantly, using the EC-5 soil moisture sensor. Representative irrigated (40% VWC) and non-irrigated plants after different period of drought (8 days = 12% VWC and 10 days = 6% VWC) are shown.

Figure S4. Leaf photosynthetic characteristics including (a) leaf net photosynthesis (A_N) calculated as μmol CO_2 uptake per leaf area per second, (b) stomatal conductance (Gs) calculated as mol of evaporated H_2O per leaf area per second and (c) instantaneous water-use efficiency (iWUE) as determined under saturated light (1200 μmol m^{-2} s^{-1}) at approximately 25 °C, with 390 mmol CO_2. Bars represent means ± SE of four plants. All measurements were conducted with a Li-6400 portable apparatus on young, fully expanded leaves.

Figure S5. Overexpression of *AtGAMT1* reduces fruit yield under irrigation but not under drought conditions. Fruit yield (total fruit weight) of control M82 and transgenic (GAMT1#2) plants grown under normal irrigation (daily irrigation; +) or mild drought stress (one irrigation per week; –) conditions. Fruits were collected when all were red-ripe. Values are means of seven plants ± SE.