Positive regulatory role of strigolactone in plant responses to drought and salt stress

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This report provides direct evidence that strigolactone (SL) positively regulates drought and high salinity responses in *Arabidopsis*. Both SL-deficient and SL-response [*more axillary growth* (*max*)] mutants exhibited hypersensitivity to drought and salt stress, which was associated with shoot- rather than root-related traits. Exogenous SL treatment rescued the drought-sensitive phenotype of the SL-deficient mutants but not of the SL-response mutant, and enhanced drought tolerance of WT plants, confirming the role of SL as a positive regulator in stress response. In agreement with the drought-sensitive phenotype, *max* mutants exhibited increased leaf stomatal density relative to WT and slower abscisic acid (ABA)-induced stomatal closure. Compared with WT, the *max* mutants exhibited increased leaf water loss rate during dehydration and decreased ABA responsiveness during germination and postgermination. Collectively, these results indicate that cross-talk between SL and ABA plays an important role in integrating stress signals to regulate stomatal development and function. Additionally, a comparative microarray analysis of the leaves of the SL-response *max2* mutant and WT plants under normal and dehydrative conditions revealed an SL-mediated network controlling plant responses to stress via many stress- and/or ABA-responsive and cytokinin metabolism-related genes. Our results demonstrate that plants integrate multiple hormone-response pathways for adaptation to environmental stress. Based on our results, genetic modulation of SL content/response could be applied as a potential approach to reduce the negative impact of abiotic stress on crop productivity.

hormonal regulation | plant adaptation | transcriptome analysis

Strigolactones (SLs), a small class of carotenoid-derived compounds, were first characterized more than 45 y ago as seed germination stimulants in root parasitic plants, such as *Striga*, *Orobanche*, and *Phelipanche* species (1, 2). SL was later reported as a root-derived signal that can enhance symbiosis between plants and arbuscular mycorrhizal fungi (AMF), possibly through its ability to induce AMF hyphal branching (3). More recently, SL was reported to play an important role in the suppression of shoot branching by inhibiting the outgrowth of axillary buds (4, 5).

In the nonmycotrophic *Arabidopsis*, *more axillary growth* (*MAX*) genes, namely *MAX1*/*AT2G26170*, *MAX3*/*AT2G44990*, and *MAX4*/*AT4G32810*, have been identified to encode enzymes that are involved in the SL-biosynthetic pathway. Additionally, two genes, *MAX2*/*AT2G42620* and *BRC1*/*AT3G18550* (*branched 1*), have been reported to play a role in SL responses (2). *MAX3* and *MAX4* encode carotenoid cleavage dioxygenase 7 (CCD7) and CCD8, respectively, which catalyze sequential carotenoid cleavage reactions to produce an apocarotenone called carlactone, a proposed SL precursor (6). MAX1 is a cytochrome P450 monooxygenase that is presumably involved in a catalytic step downstream of MAX3 and MAX4 (2). *MAX2* encodes an F-box leucine-rich repeat protein that acts as the substrate-recruiting subunit of an Skp, Cullin, and F-box (SCF)-type ubiquitin E3 ligase complex. Given the structural similarity between MAX2 and the auxin receptor transport inhibitor response 1 (TIR1), MAX2 has been suggested to be involved in SL perception (2). *Arabidopsis max3* and *max4* mutants have a 70–75% reduction in SL content as determined by *Striga* germination assays (4). Unlike *max3* and *max4*, whose branching phenotype is rescued by exogenous application of SL, the branching phenotype of *max2* is not. These data provide further evidence that *max2* is a SL-signaling and not a SL-biosynthetic mutant (4). The *BRC1* is induced by SL and encodes a TCP-type transcription factor (TF) that acts downstream of MAX2 in the regulation of shoot branching (2).

Environmental stresses, such as drought and high salinity, adversely affect plant growth and productivity. Various phytohormones, such as abscisic acid (ABA), brassinosteroid, and cytokinin (CK), have been shown to cooperatively regulate adaptive responses to these stressors (7–9). Currently, the role of SL in plant stress responses, if any, remains unknown. In the

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Significance

Environmental stresses, such as drought and high salinity, adversely affect plant growth and productivity. Although various phytohormones are known to be involved in regulation of plant stress responses, the role of strigolactone (SL) in this important process remains elusive. By using different molecular and physiological approaches, we provide compelling evidence that, in *Arabidopsis*, SL acts as positive regulator of plant responses to drought and salt stress, which was associated with shoot- rather than root-related traits. Comparative transcriptome analysis suggests that plants integrate multiple hormone-response pathways—at least SL, abscisic acid, and cytokinin pathways—for adaptation to environmental stress. Our findings demonstrate that genetic modulation of SL content/response could provide a new approach for development of crops with improved stress tolerance.

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE48949).

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present study, various SL-deficient and SL-signaling mutants were functionally analyzed to determine the involvement of SL in regulating drought and salt stress responses. Results demonstrate that SL acts as a positive regulator of stress signaling networks and diverse ABA signaling pathways by regulating the expression of many stress and/or ABA-responsive genes involved in plant development and abiotic stress response. Furthermore, impaired SL signal transduction also led to the down-regulation of CK oxidase/dehydrogenase (CKX) encoding genes that are required for CK degradation (7). Collectively, these results indicate that coordinated cross-talk between SL, ABA, and CK signaling networks regulates plant adaptation to adverse environmental conditions.

Results

SL-Deficient and SL-Response *max* Mutants Exhibit Hypersensitivity to Drought and Salt Stress. To determine the potential involvement of SL in the response of *Arabidopsis* to abiotic stress, the ability of the *Arabidopsis max* mutant and WT plants to survive drought and high salinity was examined. Two independent lines from each of the SL-biosynthetic *max3* and *max4* mutants, as well as the SL-signaling *max2* mutant, were subjected to a drought tolerance assay in which water was withheld from 3-wk-old plants growing in soil. WT and all *max* mutant plants displayed similar plant size and growth rate without the application of drought stress (Fig. 1 *A* and *C* and Fig. S1 *A* and *C*). A significantly lower number of SL-deficient or SL-response *max* mutant plants, however, survived the drought stress compared with WT plants (Fig. 1 *B* and *D* and Fig. S1 *B* and *D*). For the salt tolerance test, 3-wk-old plants were irrigated with 200 mM NaCl instead of water for 6 d. Plants were subsequently supplied with water for 4 d, and the effect of salt stress on plants was then evaluated. A significantly higher number of SL-deficient and SL-signaling *max* mutant plants died compared with WT plants in response to salinity stress (Fig. S2*A*). A germination assay conducted on germination medium (GM) agar plates amended with 100 mM NaCl also resulted in reduced germination rates for the *max* mutants compared with WT plants (Fig. S2*B*). These data indicate that *max* mutants are hypersensitive to salt stress at the germination and vegetative stages of growth, and further establish that a reduction in endogenous SL content or impairment in SL signaling compromises the plant's ability to tolerate drought and salt stress. Thus, SL plays an important role in the regulation of plant responses to abiotic stress.

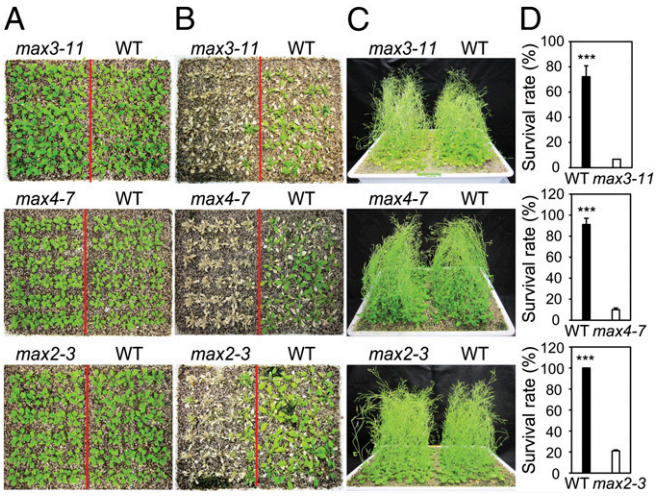

Fig. 1. Hypersensitivity of SL-deficient and SL-signaling *max* mutant plants to drought stress. (*A*) Three-wk-old WT and SL-deficient *max3-11* and *max4-7* and SL-signaling *max2-3* mutant plants before being subjected to a drought stress. (*B*) WT and mutant plants subjected to a drought stress and then rewatered for 3 d. Inflorescences were removed from the surviving plants before photographing. (*C*) Unstressed (control) WT and *max* plants grown in parallel with the drought test. (*D*) Percent survival rates of WT and mutant plants. Data represent the mean and SE from data pooled from three independent experiments ($n = 30$ per genotype per experiment). Asterisks indicate significant differences as determined by a Student *t* test (***$P < 0.001$).

Exogenous Application of SL Rescues the Drought-Sensitive Phenotype of SL-Deficient Mutants and Enhances the Drought Tolerance of WT Plants. To further confirm the role of SL in drought stress, the effect of exogenous SL on the phenotype of the SL-deficient and SL-response *max* mutant and WT plants subjected to drought stress was determined. A comparison of the *max* mutants subjected to drought stress with or without exogenous application of SL revealed that the drought-sensitive phenotype of the SL-deficient *max3* and *max4* mutants could be rescued when sprayed with SL to almost the same level of WT plants (Figs. 1 and 2), whereas no significant effect of SL application was observed on SL-response *max2* plants (Fig. 2). Furthermore, SL-treated WT plants were much more tolerant to drought than the untreated WT plants, as evidenced by their higher survival rate (100% survived among SL-treated plants vs. ~29% survived among water-treated plants; Fig. 2). These data further support the role of SL as a positive regulator of plant response to drought stress.

SL-Deficient and SL-Signaling *max* Mutants Are Less Sensitive to Exogenous ABA Than WT Plants. Plant responses to ABA and abiotic stresses are interrelated. ABA is induced by abiotic stresses and ABA signaling plays a pivotal role in controlling plant adaptation to many types of abiotic stress (9). To determine if ABA is involved in SL-mediated plant responses to stress, we analyzed responsiveness of the *max* mutants to various concentrations of ABA during germination and postgermination developmental stages. We observed that exogenous ABA more severely inhibited germination and postgermination growth of WT seedlings compared with that of the *max* mutants, indicating that the *max* mutants have reduced sensitivity to ABA than WT at the stages examined (Fig. 3 and Fig. S3). These results suggest the existence of cross-talk between SL and ABA signaling pathways in the regulation of plants stress responses.

Root Growth of *max* and WT Plants Under High Salinity and Osmotic Stress. One of the successful strategies exhibited by plants to deal with osmotic stress is to alter root-related traits, particularly root growth (10, 11). To gain insight into mechanisms that render *max* mutant plants more sensitive to abiotic stress, we examined root growth in *max* and WT plants under salt and osmotic stresses. In our experimental design, various concentrations of mannitol were used to induce osmotic stress. Root growth in the *max* mutant and WT plants was inhibited to similar extents by treatments with different concentrations of NaCl and mannitol (Fig. S4), indicating that the stress-sensitive phenotype of *max* plants is not associated with a differential effect on root growth or development, at least to 11 d of growth.

Comparison of Dehydration-Induced Water Loss Rates, ABA-Mediated Stomatal Closure, and Stomatal Density in the *max* Mutant and WT Plants. It was of interest to determine whether an alteration in shoot-related traits was the cause of the stress-sensitive phenotype observed in *max* plants. Leaf water status and water loss rates of WT and *max* mutant plants exposed to dehydration were compared. Seventeen-day-old plants were subjected to dehydration by removing them from their growth medium and placing them on a paper towel. The plants were then periodically weighed to determine leaf relative water content (RWC) and rate of water loss. By using this dehydration assay, it was observed that SL-deficient and SL-signaling *max* mutant plants lost water faster

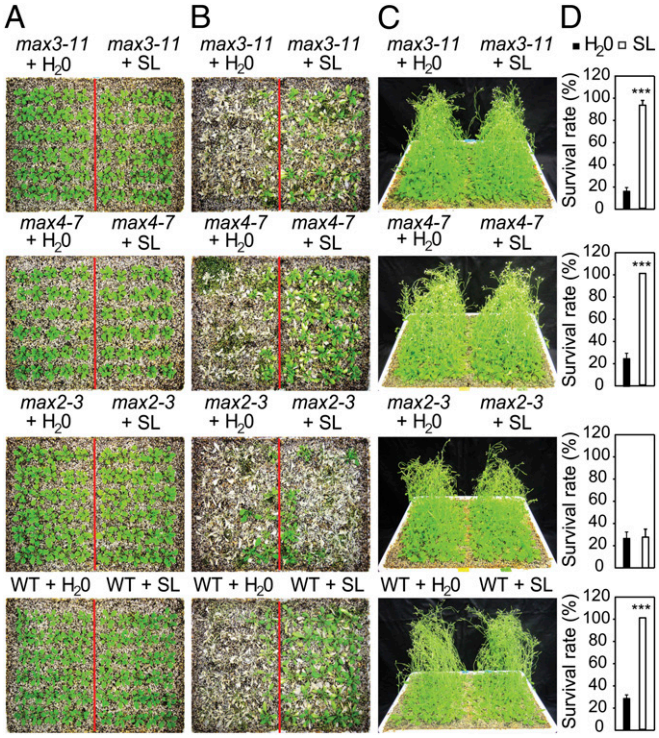

Fig. 2. Effect of SL treatment on survival of SL-deficient and SL-response mutants and WT plants. (*A*) Three-wk-old WT and SL-deficient *max3-11* and *max4-7* and SL-signaling *max2-3* mutant plants before drought stress. (*B*) Three-wk-old plants sprayed with 5 mL of 5 μM SL or water [sprayed once at 4:00 PM (the first day and from the 7th to the 13th days) and twice at 10:00 AM and 4:00 PM (from the second day to the sixth day) during water withholding period] and subjected to a drought stress. Plants were photographed 3 d subsequent to rewatering and after removal of inflorescences from the surviving plants. (*C*) Nonstressed WT and *max* plants sprayed with 5 mL of 5 μM SL or water as shown in *B*. (*D*) Percent survival of mutant and WT plants sprayed with SL or water and subjected to a drought stress as described earlier. Data represent the mean and SE from data pooled from three independent experiments ($n = 30$ plants per genotype per experiment). Asterisks indicate significant differences as determined by a Student *t* test (***$P < 0.001$).

than WT plants (Fig. 4*A*), suggesting that an altered transpiration rate might be responsible for the lower tolerance of *max* plants to water deficit stress.

It is well established that ABA regulates turgor pressure in guard cells of leaves, inducing stomatal closure when water stress is perceived, and that alteration in ABA-mediated stomatal closure and/or density can affect transpiration rates during water stress (12). Because WT and *max* mutant plants displayed different degrees of ABA responsiveness (Fig. 3 and Fig. S3), ABA-mediated stomatal movement in WT and *max* plants was analyzed. As shown in Fig. 4 *B* and *C*, stomatal cells of the SL-deficient and SL-signaling *max* mutants closed more slowly than in WT plants in response to ABA treatment. Additionally, stomatal density was higher in *max* mutant lines than in WT plants (Fig. 4*D*). These data indicate that the slower ABA-mediated stomatal closure and higher stomatal density observed in the *max* mutants contribute to an increased water loss rate, rendering the *max* mutants more sensitive to drought than WT.

Comparative Transcriptome Analysis of Leaves of the SL-Response *max2-3* and WT Plants Under Well-Watered and Dehydrative Conditions. A comparative transcriptome analysis of leaves of WT and SL-signaling *max2-3* plants under normal and dehydrative conditions was conducted by using the *Arabidopsis* 44K DNA oligo microarrays using the experimental design illustrated in Fig. S5 *A* and *B*. This was done to identify genes involved in the downstream pathways affected by SL-mediated responses to abiotic stress. The microarray data can be accessed through Gene Expression Omnibus (accession no. GSE48949). Results of the microarray analyses are summarized in Dataset S1. A comparison of the *max2-3* and WT leaf transcriptomes under nonstress conditions revealed 231 up-regulated and 262 down-regulated genes in *max2-3* with respect to the WT, by using the criteria of fold change at least two and a false discovery rate-corrected *P* value (i.e., *q*-value) <0.05 (*max2-3* well-watered control 0 h vs. WT well-watered control 0 h; Fig. S5*C* and Datasets S2 and S3). Among the 262 down-regulated genes in this comparison, 50 genes are drought-inducible (Fig. S5 *D*, *i*, and Dataset S4), among which nine are also ABA-inducible in at least five independent ABA treatment data sets among the 13 ABA treatment datasets contained in Genevestigator (Dataset S5).

A greater number of stress-inducible and/or ABA-inducible genes that are down-regulated in the *max2* mutant were expected to be identified when plants were exposed to water stress. To verify this assumption, transcriptomes of leaves of *max2* and WT plants exposed to drying for 2 or 4 h were compared. Approximately 1,022 and 2,767 genes were down-regulated (with a ratio ≥2) in *max2* plants dehydrated for 2 and 4 h, respectively, in comparison with similarly treated WT plants (*max2-3* dehydrated vs. WT dehydrated at 2 h and 4 h; Fig. S5*C* and Dataset S3). A Venn diagram constructed from the two down-regulated gene sets (2, 4) identified 491 and 955 genes, respectively, as dehydration-inducible (Fig. S5 *D*, *ii* and *iii* and Dataset S4), many of which are also ABA-inducible (Dataset S5), suggesting that ABA signaling-mediated dehydration-responsive gene expression is affected in *max* mutants. This significant overlap was further corroborated by the high Z-scores obtained by using Genesect at VirtualPlant (version 1.3; Dataset S6). The relatively lower expression of a number of drought- and/or ABA-inducible genes in *max* mutants under nonstress and drought stress conditions may be correlated with the drought-sensitive phenotype of these plants. A significant proportion (43.89%) of the down-regulated genes identified in unstressed *max2* vs. unstressed WT overlapped with down-regulated genes recorded in dehydrated *max2* vs. dehydrated WT comparisons (Fig. S5 *D*, *iv*, and Dataset

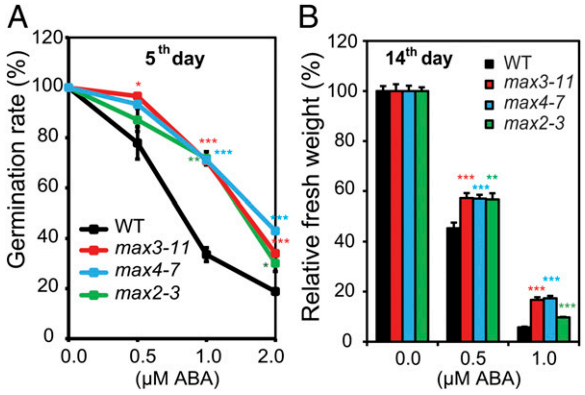

Fig. 3. Response of SL-deficient and SL-signaling *max* mutant plants to exogenous ABA treatment. (*A*) Percent germination of SL-deficient *max3-11* and *max4-7* mutant, SL-signaling *max2-3* mutant, and WT seeds treated with different levels of exogenous ABA. Data represent the mean plus SE of data pooled from three independent experiments ($n = 50$ seeds per genotype per experiment). (*B*) Relative fresh weight of SL-deficient *max3-11* and *max4-7* mutant, SL-signaling *max2-3* mutant, and WT seedlings to application of different concentrations of exogenous ABA. Relative fresh weights of all seedlings were determined after 14 d of incubation at 22 °C. Data represent the mean and SE ($n = 6$, where each replicate is composed of seven pooled plants). Asterisks indicate significant differences as determined by a Student *t* test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

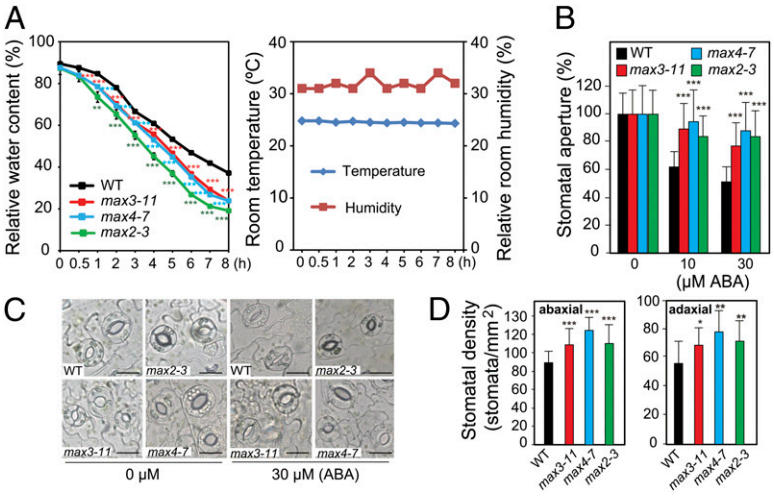

Fig. 4. RWC, relative size of the stomatal aperture and stomatal density of the WT and SL- deficient and SL-signaling *max* mutant plants. (*A*) Time course of RWC of WT and SL-deficient *max3-11* and *max4-7* and SL-signaling *max2-3* plants exposed to dehydration stress. Data represent the mean and SE ($n = 5$, where each replicate represents the weight of six plants). Room temperature and relative room humidity data recorded during the course of the experiment are also presented. (*B*) Average size of the stomatal aperture of rosette leaves from 3-wk-old WT and *max* mutant plants in the presence or absence of ABA presented as a percentage relative to the size of stomatal aperture in WT and mutant plants not exposed to ABA, which was defined as 100%. Epidermal peels were treated with ABA for 1 h after stomatal preopening under light conditions. Data represent the mean and SD ($n = 200$). (*C*) Guard cells of 3-wk-old WT and *max* mutant plants exposed to 30 μM ABA for 1 h or left unexposed. (Scale bars: 20 μm.) (*D*) Average stomatal density on the abaxial and adaxial sides of rosette leaves from 3-wk-old WT and *max* mutant plants. Data represent the mean and SD ($n = 30$). Asterisks indicate significant differences as determined by a Student *t* test (*$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$).

S4). The reliability of our microarray data were validated by examining the expression of several genes by using real-time quantitative PCR (RT-qPCR; Fig. S6).

Several dehydration- and/or ABA-inducible genes down-regulated in *max2* mutant plants encode regulatory and functional proteins, such as AP2-, myeloblastosis (MYB)-, and NAC-type TFs, CIPKs and leucine-rich repeat kinases, heat shock proteins, ATP-binding cassette (ABC) transporters, LEA (late embryogenesis abundant) proteins, α/β-hydrolases, glycosyltransferases, and glycoside hydrolases (Fig. S6 and Dataset S3). A subset of these genes is related to metabolism categories (Figs. S7 and S8), in particular regulatory and functional genes involved in flavonoids biosynthesis, such as production of anthocyanin pigment 1 (*PAP1*)/*MYB75*, *PAP2*/*MYB90*, *CHS*, *FLS1*, *DFR*, and *LDOX* (13, 14) (Dataset S7). Flavonoids are known to protect plants against various environmental stresses (15). The majority of the flavonoid biosynthesis-related genes identified in the present study are inducible by drought in an ABA-independent manner, suggesting that SL also modulates stress response independently of ABA (Dataset S7). Genes involved in CK degradation, namely *CKX1*, *CKX2*, *CKX3*, and *CKX5*, were repressed in *max2* mutant under normal and dehydrative conditions (Dataset S7). These genes have previously been correlated with stress and ABA responses (16–18). Thus, the down-regulation of a number of genes with known links to stress-, ABA-, and CK-signaling pathways is concomitant with reduced drought and salt tolerance of *max* mutant plants, clearly establishing the link of SL with plant stress responses.

A closer look at the up-regulated gene sets identified an interesting phenomenon. A significant number of photosynthesis-related genes, almost all of which were down-regulated by dehydration in an ABA-independent mechanism, were up-regulated in *max2* vs. WT plants, especially under dehydration stress conditions (Fig. S9 and Dataset S8). These data suggest a correlation between misregulation of photosynthesis-related genes, which would result in an enhanced photosynthesis and reduced drought tolerance of the *max* plants. In addition, microarray and RT-qPCR analyses indicated that, among the three known SL-biosynthetic genes, *MAX3* and *MAX4* were significantly induced by dehydration in leaves of WT plants (Fig. S10*A* and Dataset S2), further supporting that modulation of SL synthesis is important for plant adaptation to stresses.

Discussion

Considerable research has been carried out in the past 20 y to elucidate the functional role of various hormones in plant responses to environmental stress and the molecular events involved in hormone-mediated adaptation to abiotic stress (7–9). Little information is available, however, regarding the function of SL in plant abiotic stress responses despite the fact that this hormone was discovered more than 45 y ago. In the present study, a loss-of-function approach was used to explore the regulatory role of SL in plant response and adaptation to abiotic stress. Results demonstrated that all the examined *max* mutants displayed increased sensitivity to drought and salt stress (Fig. 1 and Figs. S1 and S2), thus implicating the involvement of SL as a positive regulator in abiotic stress responses. This premise was further supported by the rescue of the drought-sensitive phenotype of SL-deficient *max3* and *max4* mutants but not of the SL-response *max2* mutant, and the enhancement of drought tolerance of WT plants by exogenous application of SL to the plants (Fig. 2). It is worthy to note that no remarkable change in shoot branching phenotype of the WT plants was observed during exogenous SL treatment, which is in agreement with previous findings (4, 5).

Consistent with the drought-sensitive phenotype, leaves of *max* mutant plants were found to lose water more rapidly and to a greater extent than leaves of WT plants when they were subjected to dehydration (Fig. 4*A*). Importantly, no significant differences in root growth were observed in nutrient medium grown mutant and WT plants subjected to salinity and osmotic stress treatments (Fig. S4). These data indicate that the higher transpiration rate may contribute to the reduced tolerance of the *max* mutants against drought stress. The increased transpiration rate in *max* mutants was associated with increased stomatal density and alterations in ABA-mediated stomatal closure (Fig. 4 *B*–*D*). Additionally, all *max* mutants tested here exhibited a lower sensitivity to various concentrations of ABA compared with WT plants during the germination and young seedling stages of growth (Fig. 3 and Fig. S3). The reduced sensitivity to ABA may be responsible for the slower stomatal closure, leading to greater loss of water and the overall reduced drought tolerance of *max* mutants. Several studies have demonstrated a close relationship between drought tolerance, leaf water status, stomatal density, and rates of stomatal closure. For instance, mutations in the *Arabidopsis* K^+ *uptake transporter 6* (*KUP6*), *KUP8*, and *GORK* (*guard cell outward rectifying* K^+ *channel*) resulted in reduced ABA sensitivity, impaired ABA-mediated stomatal closure, and decreased plant survival under drought stress (19). Conversely, overexpression of *EDT1* (*enhanced drought tolerance1*) in *Arabidopsis* conferred enhanced drought tolerance that was associated with a lower transpiration rate and lower stomatal density (20). In the present study, the ABA-insensitive phenotype and increased stomatal density and water loss noted in SL biosynthesis and SL signaling impaired mutants (Figs. 3 and 4)

all point to SL positively regulating abiotic stress responses, at least in part through ABA signaling.

A comparative transcriptome analysis of the SL-response *max2* mutant and WT plants under well-watered and dehydrative conditions was conducted to gain insight into the molecular mechanisms involved in SL-mediated drought stress responses. The results indicate that the down-regulation of many stress- and/or ABA-inducible genes encoding a wide range of stress-related regulatory and functional proteins (21, 22) may be responsible for the decreased levels of stress tolerance observed in the *max* mutant plants. For instance, dehydration-, high salinity-, and ABA-inducible *AtNAC2*/*AT5G39610* (Fig. S6 and Dataset S3), which was previously reported as a positive regulator of salt stress response (23), was down-regulated in *max2* plants relative to WT under normal and dehydration conditions. Similarly, expression of *CIPK1*/*AT3G17510*, a positive regulator of ABA and osmotic stress responses (24), was repressed in unstressed *max2-3* compared with unstressed WT plants (Dataset S3). More importantly, down-regulation of *ABCG22*/*AT5G06530* and *ABCG40*/*AT1G15520* ABA importer genes in well-watered and dehydrated *max2* leaves, respectively, compared with the respective WT may explain the impaired stomatal function observed in the *max* mutants and thus the drought-sensitive phenotype (Figs. 1 and 4 and Dataset S3). This viewpoint is supported by data indicating that *abcg22* and *abcg40* mutants are drought-sensitive as a result of higher transpirational water loss resulted from diminished stomatal closure compared with WT plants (25, 26). Additionally, recent studies reported that the constitutive overexpression of *CKX* genes, namely *CKX1*, *CKX2*, *CKX3*, and *CKX4*, resulted in higher ABA sensitivity, enhanced ABA response and improved drought and salt tolerance in transgenic *Arabidopsis* and tobacco plants (17, 18). The transcriptome analysis conducted in the present study revealed that, among seven *Arabidopsis CKX* genes, four (*CKX1*, *CKX2*, *CKX3*, and *CKX5*) were down-regulated in the *max2* mutant with and/or without being subjected to dehydration (Dataset S7). The down-regulation of the four *CKX* genes may have contributed to the decreased stress tolerance of *max* plants by reducing their ABA response. Furthermore, many genes involved in flavonoid biosynthesis (13, 14) were significantly repressed by the dehydration stress treatment in *max2-3* plants relative to the level of expression in WT plants (Dataset S7). This suppression may result in a decrease in the biosynthesis of flavonoid compounds, which are known to ameliorate the injury to plant cells induced by various abiotic stresses (15). In addition, the drought-sensitive phenotype of the *max* mutant plants might also be attributed to an enhanced photosynthesis, as suggested by the up-regulation of a subset of photosynthesis-related genes, which are negatively regulated under dehydration conditions, in the stressed *max2* mutant relative to the stressed WT (Fig. S9 and Dataset S8). The enhancement of photosynthesis in the *max* plants was also supported by the increased transpiration rate observed in these plants, which resulted, at least in part, from enhanced stomatal activity observed during dehydration treatment (Fig. 4). An increased rate of the highly energy demanding photosynthesis could make the plant more vulnerable to stressful environments as it consumes resources that are limitedly available during stress. Thus, one of the strategies plants use to adapt to stresses is growth reduction by decreasing photosynthesis, as supported by the observed down-regulation of photosynthesis-related genes in plants in response to stresses (Dataset S8) (27, 28), to reallocate limited energy resources from the developmental programs toward efficient stimulation of defense pathways against harmful stresses (7, 29). Furthermore, a large portion of the identified SL signaling-mediated photosynthesis- (Dataset S8) and flavonoid biosynthesis-related genes (Dataset S7) were down- and up-regulated, respectively, by dehydration in an ABA-independent mechanism, suggesting that SL controls stress response not only in an ABA-dependent (Figs. 3 and 4 and Dataset S5) but also in an ABA-independent manner.

In accordance with a positive regulatory role for SL in drought responses, among the three known biosynthetic *MAX* genes, *MAX3* and *MAX4* were found to be significantly induced by dehydration treatment in leaves of WT plant (Fig. S10*A*). The induced expression of *MAX3* and *MAX4* by stress may trigger an efficient activation of SL biosynthesis, leading to the activation of SL signaling, thereby enabling plants to better adapt to adverse conditions. The observed up-regulation of *MAX3* and *MAX4* by high salinity and ABA treatments in WT plants further supports this mechanism (Fig. S10*B*). The expression of *MAX3* and *MAX4* was strongly induced after 2 h of salt treatment, whereas that of *MAX1* was mostly unchanged. All the SL-biosynthesis *MAX1*, *MAX3*, and *MAX4* genes exhibited a tendency to be induced by longer periods of ABA treatment, especially *MAX3* and *MAX4*, which were induced more than four- and eightfold after 10 h of treatment with ABA (Fig. S10*B*). Furthermore, repression of *MAX3* as well as *MAX1* in the SL-response *max2* mutant by dehydration relative to the dehydrated WT plants further supports the premise that stress-regulated SL homeostasis is essential for plant adaptation to abiotic stress (Dataset S3).

Overall, results of the present study demonstrated that SL positively modulates stress responses in *Arabidopsis* through ABA-dependent and ABA-independent pathways. Biological processes responsible for plant adaptation to adverse environmental conditions are complex, requiring the involvement of multiple hormone regulatory pathways. This complexity is reflected, for instance, in the functions of ABA, CK, and SL in the regulation of stomatal closure and leaf senescence, two traits that are closely related to stress responses and adaptation. Whereas ABA and SL promote the senescence of leaves, CK delays leaf senescence (30, 31). On the other hand, ABA (32) and SL (the present study) act as positive regulators of stomatal closure and thus stress response, whereas CK acts as a negative regulator of the same process (18, 33). Importantly, results of the present study demonstrate the potential for the use of genetic engineering to improve the stress tolerance of crop plants by manipulating SL biosynthesis and/or SL signaling as suggested by the enhanced drought tolerance exhibited by WT plants sprayed with SL (Fig. 2).

Materials and Methods

Plant Materials and ABA and High Salinity Treatments. The *Arabidopsis max2-3* (SALK_092836), *max2-4* (SALK_028336), *max3-11* (SALK_023975), *max3-12* (SALK_015785), *max4-7* (SALK_082552), and *max4-8* (SALK_072750) mutants used in this study are in Columbia genetic background and were obtained as in a previous study (4). For ABA and high salinity treatments, *Arabidopsis* WT plants were grown on GM agar plates for 14 d (22 °C, 16 h light/8 h dark cycle, 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux density) and treated with water (hydroponic control), 100 μM ABA, or 250 mM NaCl for the indicated time periods.

Assessment of Drought, Salt, and Osmotic Stress Tolerance. ***Drought stress tolerance assay.*** The same tray method was used to evaluate drought tolerance as previously described (18). Seedlings were grown in soil by using Dio Propagation Mix no. 2 for Professional soil (Dio Chemicals). When an SL treatment was applied, 30 plants grown in soil trays were sprayed with 5 mL of 5 μM SL (*rac*-GR24; Chiralix) or water control once (on the first day and from the 7th to 13th days) at 4:00 PM and twice at 10:00 AM and 4:00 PM (from the second day to the sixth day). Rewatering was performed when the greatest difference in appearance was discerned between the control and mutant plants. Trays were photographed 3 d subsequent to rewatering and after removal of inflorescences from the survived plants.

Salt stress tolerance assay. WT and mutant plants were grown in soil for 3 wk, as they were in the drought tolerance test. Each tray was then irrigated at the bottom of the tray with a total of 2 L of 200 mM NaCl instead of water for 6 d. The plants were then irrigated with water from the seventh day onward. Plant survival was recorded 4 d later (day 10).

Germination assay. Seeds were sown on GM medium containing 1% sucrose with or without 100 mM NaCl. Those with expanded cotyledons were recorded as germinated seeds.

Root growth assay. Four-day-old plants germinated and grown on vertical GM plates were transferred onto 0.5× Murashige–Skoog plates containing 1.2% (wt/vol) agar with and without the indicated concentration of NaCl or mannitol. Root growth of vertically grown seedlings was examined 7 d after transfer.

Stomatal Closure Assay and Measurement of Stomatal Density. ABA-induced stomatal closure was assessed as previously described (19) with slight modifications. Epidermal peels from leaves of 3-wk-old plants grown on GM plates were incubated for 4 h in a solution containing 10 mM KCl, 0.2 mM $CaCl_2$, and 10 mM Mes·KOH (pH 6.15) under white light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The peeled strips were subsequently incubated in a solution containing the same buffer plus ABA. Guard cells were photographed by using a light microscope equipped with a digital camera. Two hundred stomatal apertures were measured for each mutant and WT plant. Stomatal density was determined by counting the number of stomates on the abaxial and adaxial sides of leaves of 3-wk-old mutant and WT seedlings. Leaves were cleared with chloral hydrate and visualized by the light microscopy. All stomates from 30 images of the central region of the leaves from eight individual plants were counted for each mutant and WT.

Assay for Sensitivity to ABA. Germination and growth inhibition assays were performed on GM medium containing 1% sucrose and various concentrations of ABA as described previously (18).

Expression Analyses. Total RNA was extracted with an RNeasy Plant Mini Kit (Qiagen). Procedures described previously (34) were used for cDNA synthesis and RT-qPCR. Polyubiquitin 10 (*UBQ10*) was used as a reference gene for expression analysis. Primer pairs used to examine the expression levels of specific genes are listed in Dataset S9.

Dehydration Treatment and Microarray Analysis. WT and *max2-3* plants (30 plants each) were grown in soil as described previously (18) and in the drought tolerance assay. Aerial portions of 24-d-old plants were detached and exposed to dehydration by placing them on paper towels on a laboratory bench. At the indicated time points, RWC of treated samples was measured ($n = 5$). Rosette leaves of three independent WT and SL-signaling *max2-3* mutant plants treated for 0, 2, 4, and 6 h were then collected to make three biological replicates for microarray and expression analyses. Microarray analysis by using the *Arabidopsis* Oligo 44K DNA microarray (version 4.0; Agilent Technology) was performed as described previously (35, 36). The microarray data and a detailed protocol were deposited in the Gene Expression Omnibus database (accession no. GSE48949). MapMan (http://mapman.gabipd.org) and VirtualPlant (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/) were used to analyze the data. In some cases, ABA and stress-responsive gene expression was analyzed by using Genevestigator (www.genevestigator.com) or the *Arabidopsis* eFP browser (http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi).

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