The interaction of strigolactones with abscisic acid during the drought response in rice

Imran Haider1,2,*, Beatriz Andreo-Jimenez1,*, Mark Bruno3, Andrea Bimbo1, Kristýna Floková1,4, Haneen Abuauf2, Valentine Otang Ntui2,†, Xiujie Guo2, Tatsiana Charnikhova1, Salim Al-Babili2,3, Harro J. Bouwmeester1,4, and Carolien Ruyter-Spira3

1 Laboratory of Plant Physiology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands
2 Bioactives Lab, Biological and Environmental Sciences and Engineering Division, King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Kingdom of Saudi Arabia
3 Faculty of Biology, University of Freiburg, D-79104 Freiburg, Germany
4 Plant hormone biology group, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands

*These authors contributed equally to this work.
† Present address: International Institute of Tropical Agriculture, Nairobi, Kenya
‡ Correspondence: h.j.bouwmeester@uva.nl

Received 3 November 2017; Editorial decision 27 February 2018; Accepted 8 March 2018

Editor: Richard Napier, University of Warwick, UK

Abstract

Both strigolactones (SLs) and abscisic acid (ABA) biosynthetically originate from carotenoids. Considering their common origin, the interaction of these two hormones at the biosynthetic and/or regulatory level may be anticipated. Here we show that, in rice, drought simultaneously induces SL production in the root, and ABA production and the expression of SL biosynthetic genes in the shoot. Under control conditions, the ABA concentration was higher in shoots of the SL biosynthetic rice mutants dwarf10 (d10) and d17 than in wild-type plants, while a similar trend was observed for the SL perception mutant d3. These differences were enhanced under drought. However, drought did not result in an increase in leaf ABA content in the rice mutant line d27, carrying a mutation in the gene encoding the first committed enzyme in SL biosynthesis, to the same extent as in the other SL mutants and the wild type. Accordingly, d10, d17, and d3 lines were more drought tolerant than wild-type plants, whereas d27 displayed decreased tolerance. Finally, overexpression of OsD27 in rice resulted in increased levels of ABA when compared with wild-type plants. We conclude that the SL and ABA pathways are connected with each other through D27, which plays a crucial role in determining ABA and SL content in rice.

Keywords: Abscisic acid, biosynthesis, drought, DWARF27, rice, strigolactones.

Introduction

Due to their sessile nature, plants must adjust their growth and development to continuously changing environmental conditions (Wolters and Jürgens, 2009). Plant hormones, such as abscisic acid (ABA), auxin, cytokinin, gibberellin, ethylene, brassinosteroids, jasmonic acid, salicylic acid, and strigolactones (SLs), play a central role in regulating these adaptive
responses (Santner and Estelle, 2009). However, plant hormones do not act alone but are interconnected and modulate each other’s activity at the level of biosynthesis, degradation, and signalling by crosstalk mechanisms (Zhang et al., 2013).

ABA is a well-studied phytohormone that not only regulates several developmental processes such as seed maturation and dormancy, but is also intricately involved in the adaptation of plants to abiotic stresses (i.e. drought, salinity, etc.) (Nambara and Marion-Poll, 2005; Finkeinstein, 2013). It is generally accepted that under drought, the ABA level accumulates in the root and is then transported to the shoot via the xylem, which results in stomatal closure that limits water loss and the accumulation of reactive oxygen species (ROS) (Ko and Helariutta, 2017). However, it has been shown that ABA is also directly generated in the shoot in response to drought (Borghi et al., 2015). In addition, ABA stimulates the formation of DEHYDRINS and LATE EMBRYOGENESIS ABUNDANT (LEA) proteins that are involved in osmotic adjustment and other protection mechanisms (Shinozaki and Yamaguchi-Shinozaki, 2007). In plants, ABA is derived from C9-carotenoid precursors synthesized in the plastids (Moise et al., 2014; Nisar et al., 2015). The first committed step in ABA biosynthesis is the oxidative cleavage of the epoxy-carotenoids 9-cis-violaxanthin or 9-cis-neoxanthin to xanthoxin (Schwartz et al., 1997), catalysed by 9-cis-epoxycarotenoid dioxygenases (NCEDs) and considered to be the rate-limiting step in ABA biosynthesis (Tan et al., 1997; Burbidge et al., 1999; Schwartz et al., 2003). Xanthoxin is further converted, in the cytosol, by a short-chain dehydrogenase/reductase (SDR) to abscisic aldehyde that is oxidized to form ABA by abscisic aldehyde oxidase (AAO3) (Seo et al., 2000; Cheng et al., 2002) (Supplementary Fig. S1 at JXB online).

SLs were initially isolated from root exudates as seed germination stimulants of root parasitic plants of the Orobanchaceae (Striga, Phelipanche, and Orobanche spp.) (Cook et al., 1966; Bouwmeester et al., 2003; Xie et al., 2010) and later identified as hyphal branching stimulants of arbuscular mycorrhizal fungi (AMF) (Akiyama et al., 2005). Some years later, SLs were also recognized as hormones involved in the inhibition of shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008) and a range of other biological processes, including root development, hypocotyl growth, responses to environmental conditions, photo-morphogenesis, and secondary growth in vascular plants, as well as protonema branching and colony extension in the moss Physcomitrella patens (Ruyter-Spira et al., 2013; Zhang et al., 2013; Al-Babili and Bouwmeester, 2015). When growing under low phosphate levels, plants display increased biosynthesis and exudation of SLs (Yoneyama et al., 2007), which stimulates the hyphal branching of symbiotic AMF that acquire phosphate from the soil and provide it to the host in return for photosimilates (Bouwmeester et al., 2007; López-Ráez et al., 2008).

Based on studies with mutants, so far eight genes have been identified in several plant species, with roles in SL biosynthesis and signalling (Waters et al., 2017; Jia et al., 2018). Among them, five have been shown to be required for SL biosynthesis. In rice and Arabidopsis, DWARF27 (D27) (Lin et al., 2009; Waters et al., 2012) encodes a β-carotene isomerase converting all-trans-β-carotene into 9-cis-β-carotene (Alder et al., 2012). MORE AXILLARY GROWTH 3 (MAX3)/DWARF17 (D17) (Booker et al., 2004; Zou et al., 2006) and MORE AXILLARY GROWTH 4 (MAX4)/DWARF10 (D10) (Sorefan et al., 2003; Arite et al., 2007) encode the carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD8, respectively. MORE AXILLARY GROWTH 1 (MAX1) encodes a cytochrome P450 monoxygenase (Booker et al., 2005). In vitro studies with D27, CCD7, and CCD8 showed that these enzymes are sufficient to convert all-trans-β-carotene into carlactone (CL), an intermediate with obvious structural similarities to canonical SLs (Alder et al., 2012; Seto et al., 2014). The formation of CL starts with the D27-catalysed isomerization of all-trans-β-carotene (Bruno and Al-Babili, 2016), followed by the CCD7-catalysed stereospecific cleavage (Bruno et al., 2014) that leads to the intermediate 9-cis-β-apo-10’-carotenal that is then converted by CCD8 into CL (Alder et al., 2012; Bruno et al., 2017). Recently, we demonstrated that a rice MAX1 homologue (carlactone oxidase) catalyses the conversion of CL into the SL parent molecule, 4-deoxyorobanchol (ent-2’-epi-5-deoxystrigol), by forming the B-C lactone ring, and that a second rice MAX1 homologue (orobanchol synthase) forms orobanchol by introducing a hydroxyl group into 4-deoxyorobanchol (Zhang et al., 2014). Arabidopsis MAX1 has been shown to oxidize CL to a different SL-like molecule, carlactonic acid, which is methylated into methyl-carlactonoate (McCLA)—that can bind to the receptor D14 (see below)—by an as yet unidentified enzyme (Abe et al., 2014). More recently, Brewer et al. (2016) revealed that in Arabidopsis LATERAL BRANCHING OXIDOREDUCTASE (LBO), an oxidoreductase-like enzyme of the 2-oxoglutarate and Fe(II)-dependent dioxygenase (2OGD) superfamily, converts McCLA into an unidentified SL-like compound (Brewer et al., 2016). SL mutants have a high-tillering/branching phenotype, which in the case of biosynthetic mutants can be rescued by exogenous application of GR24 (Wang and Li, 2011), but not in the SL perception and downstream signalling mutants (Umehara et al., 2008). Up to now, there are three genes known to be involved in the latter process: MORE AXILLARY GROWTH 2 (MAX2)/DWARF3 (D3) encodes an F-box protein (Stirnberg et al., 2007); DWARF14 (D14), an α/β-fold hydrolase (Hamiaux et al., 2012, Nakamura et al., 2013; de Saint Germain et al., 2016; Yao et al., 2016); and SUPPRESSOR OF MORE AXILLARY GROWTH2-LIKE 6,7,8 (SMXL6,7,8)/DWARF53 (D53) that encode repressors of SL signalling (Jiang et al., 2013; Zhou et al., 2013; Wang et al., 2015; Soundappan et al., 2015).

Considering their common biosynthetic origin from carotenoids (Matusova et al., 2005), a relationship between ABA and SL production may be anticipated. Indeed, ABA-deficient tomato mutants impaired in different steps of ABA biosynthesis exhibit reduced SL levels (López-Ráez et al., 2008, 2010), suggesting that ABA affects SL accumulation. Conversely, Torres-Vera et al. (2014) reported that the SL-deficient tomato mutant slcedb had reduced levels of ABA. Similarly, an SL-deficient CCD7RNAi lotus line showed reduced ABA levels when exposed to a combination...
of phosphate deficiency and osmotic stress, compared with the corresponding wild type (Liu et al., 2015).

Because in the above-mentioned studies SL deficiency was shown to reduce ABA levels, it can be expected that SL-deficient plants are compromised in their response to drought. Interestingly, two independent studies investigated drought tolerance in Arabidopsis SL mutants (Bu et al., 2014; Ha et al., 2014). Ha et al. (2014) reported that SLs act as a positive regulator in the plant’s response to drought and salt stress. The exogenous application of GR24 rescued the drought-sensitive phenotype of SL-deficient mutants (max3 and max4) but not of the insensitive mutant (max2). In addition to this, the max mutants showed reduced sensitivity to exogenous ABA compared with wild-type plants. In the study by Bu et al. (2014), the drought sensitivity phenotype was confined to max2. However, ABA levels in the wild type and max2 were similar under control and drought conditions.

To obtain a better understanding of the relationship between SLs and ABA during drought, in the present study we first studied whether SLs are affected by drought. Hereto, we quantified SL content and analysed the expression level of SL biosynthesis genes in rice exposed to drought conditions. Furthermore, we investigated the consequences of mutations in SL biosynthesis and signalling and D27 overexpression on ABA content. Our results show that SL biosynthesis/perception interferes with ABA formation as observed in d10, d17, and d3 mutants, and suggest that the SL biosynthetic enzyme D27 is unique in its ability to regulate the levels of both plant hormones.

Materials and methods

Plant materials, growth conditions, and drought treatments

Rice (Oryza sativa L. cv. Shiokari) was used in this study. Rice seeds were first surface sterilized with 70% ethanol for 30 s and with 2% sodium hypochlorite (v/v) for 30 min. The seeds were then rinsed five times in sterile double-distilled water and immersed in water in the dark for 2 d at 28 °C to induce germination. Finally, germinated seeds were transferred to the climate room in pots filled with a mixture of sand and soil (1:1) (for the drought tolerance assay) or silver sand (for others). The conditions in the climate room were: temperature, 28 °C day/25 °C night; photoperiod, 10 h light/14 h dark; 75% relative humidity, and light intensity of 570 μM m−2 s−1. The rice plants were watered twice a week using modified half-strength Hoagland nutrient solution (López-Ráez et al., 2008). To study the effect of long-term drought conditions in rice, four seedlings of japonica variety Shiokari were grown together in three litre volume pots. The pots were filled with silver sand equally (the same soil volume in all the pots) and watered at saturated levels until plants were 4 weeks old. After this stage, drought was imposed by water withholding until a field capacity (FC) of 45% was reached. This condition was maintained for 9 d. The drought was maintained by weighing the pots every day and watering with nutrient solution until the desired water level. After the drought period, plant material was collected for further biomass and hormone quantification. To study the effect of strong drought conditions, stress by water withholding was imposed on 2-week-old seedlings (five-leaf stage) during 10 d.

To test the drought tolerance of rice SL mutants (d10, d17, d27, and d3), seeds of mutants and the wild type were germinated on half-strength Murashige and Skoog (MS) medium. The rice seedlings (16 plants per pot and five pots for each line) were grown in a climate room in three litre pots (diameter 19 cm, depth 14.5 cm) filled with a mixture of sand and soil (1:1). To minimize experimental errors, each pot was filled with the same weight of soil and supplied with the same volume of water. The drought treatment and water loss rate experiment under dehydration conditions were performed according to Zhang et al. (2012) with minor modifications. When plants reached the five-leaf stage, watering was withheld for 12 d. Watering was then resumed for 3 d to allow plants to recover, after which the survival rates were calculated. To rule out the possible effect of differences in plant architecture between the rice lines studied for the drought resistant/sensitive phenotypes, a pooled survival experiment was also performed. In this experiment, eight plants from the d27 line (drought sensitive) and eight plants from the d10 line (drought resistant) were sown in the same pot with a total of eight replicates. The rest of the procedure was similar to the drought survival assay performed for the individual lines.

To detect the water loss rate under dehydration conditions, flag leaves were detached from plants (n=9) and weighed at 0, 0.5, 1, 2, 3, 4, 5, and 6 h after their removal from the plant (at 24 °C). Water loss rates were calculated as the percentage of initial fresh weight.

Generation of OsD27 overexpression lines in rice

Full-length cDNA of OsD27 was PCR amplified using primers D27-OE-F and D27-OE-R (Supplementary Table S1), digested by BamHI and SpeI, and then inserted into the corresponding sites downstream of the ubiquitin promoter in the binary vector pTCK303 (Wang et al., 2004). The resulting plasmid was designated as pPubi::D27 and introduced into rice variety Shiokari using Agrobacterium tumefaciens strain EHA105 as previously described (Hiei et al., 1994).

Strigolactone and abscisic acid analysis

For SL analysis in rice, plants were grown, and root extracts were collected and extracted according to Jamil et al. (2011b). ABA was analysed as previously described (López-Ráez et al., 2010) with minor modifications. For SL quantification from root extracts, 0.5 g of fresh root tissue was manually ground in liquid nitrogen. Samples were taken in 10 ml cold glass tubes and 2 ml of ethyl acetate containing 0.01 nmol ml−1 ethyl acetate solution containing GR24 as internal standard (IS) was added and mixed by vortexing. After 15 min sonication (in a Branson 3510 ultrasonic bath), each sample was centrifuged for 10 min at 2000 rpm. The organic phase was transferred to 4 ml glass vials. The samples were re-extracted with another 2 ml of ethyl acetate (without IS) and combined in the same 4 ml glass vials. The collected samples were dried in a speed vacuum (Thermo Scientific SPD121P SpeedVac). The residue was dissolved in 50 μl of ethyl acetate and diluted with 4 ml of 100% hexane. This solution was loaded in a pre-conditioned Silica gel GracePrep SPE (200 mg/3 ml column). After washing with 2 ml of 100% hexane, SL was eluted with 2 ml solvent mixtures of hexane:ethyl acetate (10:90). ABA was extracted using 0.2 g of leaf material in a 4 ml glass vial containing 1 ml of ethyl acetate and 0.01 D6-ABA (IS) and combined in the same 4 ml glass vials. The collected samples were dried in a speed vacuum (Thermo Scientific SPD121P SpeedVac). The residue was dissolved in 50 μl of ethyl acetate and diluted with 4 ml of 100% hexane. This solution was loaded in a pre-conditioned C18 GracePrep column (100 mg/1 ml). ABA was eluted with 1 ml of 100% acetone. The solvent mixtures were evaporated under speed vacuum and the residue was dissolved in 200 μl of acetonitrile-water (25:75, v/v). The samples were filtered through Minisart SRP4 0.45 μm filters (Sartorius, Germany) for ultra-performance liquid chromatography–tandem spectrometry (UPLC-MS/MS), as previously described (López-Ráez et al., 2010; Jamil et al., 2011b). Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters). Calculations of ABA and SL 4-DO concentration were based on a
Analysis of transcript levels
Total RNA was extracted from rice roots and shoots using a combination of three protocols: TRIZol reagent (Invitrogen), the RNasey Mini Kit (Qiagen), and the DNase-I Kit (Qiagen), as previously described (http://www.untergasser.de/lab/protocols/rna_prep_comb_trizol_v1_0.htm). RNA concentration, quality, and integrity were checked using a NanoDrop ND-1000 UV-Vis spectrophotometer and standard gel electrophoresis. Reverse transcription reaction was performed with the Bio-Rad iScript cDNA Synthesis Kit using 1 μg of total extracted RNA following the manufacturer’s instructions. Primers used for real-time quantitative reverse transcription–PCR (qRT-PCR) analysis are listed in (Supplementary Table S1). qRT-PCR was performed with the Bio-Rad iQ5 instrument using Bio-Rad SYBR Green Supermix to monitor dsDNA synthesis following the manufacturer’s instructions. Three independent biological replicates were used, and each PCR was done in triplicate. Relative expression levels of genes were determined using the comparative Ct method as previously described (Livak and Schmittgen, 2001), and the rice Ubiquitin (ubi) gene (Supplementary Table S1) was used as the internal control to normalize target gene expression (Lin et al., 2009).

Stomatal density
Leaf stomatal density was determined by using the silicon rubber impression technique (Smith et al., 1989). The method and impression material are described by Giday et al. (2013). Imprints of the leaf surface from the abaxial surfaces of the more developed leaf were taken from 6-week-old plants (three biological replicates). Imprints were later smeared with nail polish in the mid-area between the central vein and the leaf edge, and after drying the polish was peeled off from the imprint and mounted for microscope count. Numbers of stomata from three randomly selected non-overlapping rectangular fields of view (×20 magnification, 0.68 mm²) per biological replicate were counted under a light microscope (Zeiss Imager D2, Germany) and Axiocam camera (Zeiss, Germany).

Carotenoid extraction from rice seedlings for quantitative HPLC analysis
The extraction was performed under dim light by adding 2 ml of acetone to 10 mg of finely ground powder of plant material, followed by sonication (duty-cycle 30, output 2) and centrifugation (5 min at 3000 g). This procedure was repeated three times, after which 2 ml of PE:DE (2:1, v/v) was added to the collected supernatants. Water was added up to 14 ml followed by centrifugation for 5 min at 3000 g. The organic phase was collected and the extraction was repeated once more. The combined organic phases were evaporated to dryness and dissolved in exactly 100 μl of CHCl₃—from which 10 μl was injected for HPLC analysis.

Substrates were quantified spectrophotometrically at their individual λmax using molar absorption coefficients given in the literature (Britton et al., 1995). Protein concentration was determined using the Bio-Rad protein assay kit. For HPLC analysis, a Waters system equipped with a photodiode array detector (model 996) was used. Separation was performed using YMC-Pack C₃0 reversed phase columns from YMC Europe (150 mm length×3 mm internal diameter, 5 μm particles, system 1; and 250 mm length×4.6 mm internal diameter, 5 μm particles, system 2). The column temperature was set at 35 °C. The solvent system consisted of solvent A, methanol/tert-butyl methyl ether (1:1, v/v); and B, methanol/tert-butyl methyl ether/water (5:1:1, v/v/v). In plant tissue analysis, the column was developed at a flow rate of 1 ml min⁻¹ with 100% B followed by a gradient to 18% B within 65 min. Then, the flow was subsequently increased to 1.5 ml min⁻¹ to 0% B within 2 min and maintained for another 11 min. Finally, the column was re-equilibrated to starting conditions for 10 min.

Results
SL production is induced under drought
To study the effect of drought on SL production, five-leaf stage rice plants were grown under a continuous, mild water deficit. Mild drought conditions resulted in a reduction in plant biomass, particularly of the shoot (Supplementary Fig. S2A), showing that this level of water deficit is indeed limiting plant growth. ABA increased in the shoot of mildly drought-stressed plants, but not in the roots (Supplementary Fig. S2B). SL levels strongly increased in the roots when plants were exposed to mild drought (Fig. 1). Roots of plants grown under drought contained a higher level of 4-deoxyorobanchol and a higher sum of the three putative methoxy-5-deoxystrigol isomers 1–3 (2-fold), identified before in rice (Jamil et al., 2011a; Cardoso et al., 2014), when compared with plants grown under control conditions (Fig. 1).
These results were supported by the increased expression of some of the SL biosynthetic genes (Fig. 2). In the shoot under drought, expression of D27 and the MAX1 homologs Os01g0700900, Os01g0701400, Os02g0221900, and Os06g0565100 was up-regulated. In the root, expression of D10 and Os01g0700900 was up-regulated. Of the genes tested, only D27 was expressed more highly in the shoot than in the root.

**Effect of drought on the survival rate of rice SL mutants**

The induction of SL biosynthesis under drought could imply that SLs are involved in the adaptation to drought. To explore their contribution to drought tolerance, we analysed how drought affects rice SL mutants. For this purpose, we performed a drought survival assay. The SL-deficient d10 and d17 and SL-insensitive d3 showed significantly higher survival rates (~85, 75, and 45%, respectively), compared with the wild type (20%). In contrast, none of the d27 plants survived (Fig. 3A, B).

To rule out the possibility that the difference in drought tolerance is due to differences in plant architecture and/or biomass between the mutants, which may cause differences in pot water availability, d27 and d10 were analysed in a pooled experiment, in which these two genotypes were grown in the same container. Also under these conditions, the d27 mutant is more drought sensitive than d10 (Supplementary Fig. S3).

As water loss by transpiration is one of the most important parameters contributing to drought sensitivity, we measured water loss rates of detached wild-type and SL mutant leaves. The detached leaves of d10, d17, and d3 lost water more slowly than wild-type leaves, but d27 leaves evaporated water significantly faster (Fig. 3C). In addition to this, we examined the stomatal density in one of the drought-resistant d-mutants (d10) and the drought-sensitive d27. Stomatal density was significantly lower in d10 when compared with the wild type, while stomata size of d10 showed a tendency to be larger; however, this was not significant. Also, no differences were observed for d27 (Fig. 4).

**ABA content in rice SL mutants**

To study if the above-described differences in drought survival, and transpiration rate, between the rice genotypes are linked with differences in ABA levels in the leaf, we quantified ABA in rice SL mutants and the wild type under control conditions and at several time points after water withholding to create different levels of drought.

Under control conditions, ABA leaf content was significantly higher in d10 and d17 (1.5-fold) when compared with wild-type plants, but did not differ significantly between d27 and the wild type (Fig. 5A). A tendency for increased ABA levels was observed for d3, although this was not significant. The trend for root ABA in control conditions was the same (data not shown). ABA content was also measured in leaf material that was harvested 2, 6, and 10 d after water withholding. After 2 d, we observed a higher increase in ABA levels in d10, d17, and d3 (1.2- to 1.3-fold) than in

---

**Fig. 2.** The effect of mild drought on the expression level of SL biosynthetic genes in rice. The expression level of rice SL biosynthetic pathway genes was measured in root (light grey) and shoot (dark grey). Long-term mild drought (35–45% field capacity) was applied during 9 d (Experiment 1). The D17 gene was not expressed at detectable levels. Significant differences between control ‘C’ and drought ‘D’ treatments in the different tissues, as determined by Student’s t-test, are indicated by asterisks (*P<0.05, **P<0.001). Bars represent the mean of five biological replicates (each replicate consisting of a pool of four plants ±SE.
After 6 d of water withholding, ABA content was no longer significantly different between the lines but still showed a similar trend to after 2 d of water withholding. After 10 d of drought, ABA content was again higher in d10, d17, and d3 (1.1-fold); however, in d27 ABA levels were significantly lower (0.90-fold), when compared with the wild type (Fig. 5B). To study whether these subtle changes in ABA levels under drought correlate with gene expression, we measured the expression of two ABA biosynthetic genes, NCED3 and NCED4. After 6 d of drought, the expression of

The wild type. After 6 d of water withholding, ABA content was no longer significantly different between the lines but still showed a similar trend to after 2 d of water withholding. After 10 d of drought, ABA content was again higher in d10, d17, and d3 (1.1-fold); however, in d27 ABA levels were significantly lower (0.90-fold), when compared with the wild type (Fig. 5B). To study whether these subtle changes in ABA levels under drought correlate with gene expression, we measured the expression of two ABA biosynthetic genes, NCED3 and NCED4. After 6 d of drought, the expression of

Fig. 3. Plant survival and detached leaf water loss rates of SL-deficient and -insensitive rice mutants and their wild type. (A) The phenotype of five-leaf stage plants before stress and after 12 d of water withholding (strong drought) followed by re-watering and 3 d of recovery (Experiment 2). (B) Survival rate after recovery (five independent biological replicates, each pot containing 16 plants) (Experiment 2). (C) Water loss rate of detached flag leaves (n=9). Bars represent the mean ±SE. Significant differences between mutants and wild-type plants as determined by Student’s t-test are indicated by asterisks (*P<0.05, **P<0.01).

Fig. 4. Stomatal density and size in wild-type, d10, and d27 plants. Barplots show the stomatal size and density from 6-week-old plants in the leaf abaxial side. Bars represent the mean ±SE. An asterisk represents a significant difference compared with the wild type after a pairwise Student t-test (*P<0.05). Microscopy pictures taken for the measurements can be seen at the bottom.
both biosynthetic genes was lower in d3 and d17, while it was higher in d27, compared with the wild type (Supplementary Fig. S4). This might be the result of negative feedback on gene expression by the higher ABA levels in d3 and d17, and positive feedback in d27 by the lower ABA levels (Fig. 5A, B). However, expression of NCED3 and NCED4 in d10 remained similar to that in the wild type (Supplementary Fig. S4). We also tested the expression of two ABA catabolic genes, CYP707A-2 and CYP707A-3, but we did not find any differences from the wild type (data not shown).

Taken together, the results suggest that SL deficiency in rice might stimulate ABA accumulation in the shoot, and that D27 is involved in this, especially under drought conditions. However, the underlying mechanism cannot be explained by changes in the expression level of the above-described genes.

Constitutive overexpression of OsD27 in rice results in increased levels of ABA

Because the lower ABA level in d27 is in contrast to the higher ABA level observed in the other SL biosynthetic/signalling mutants, we addressed the possible function of OsD27 in ABA formation. For this purpose, we analysed shoot ABA levels in two independent OsD27 overexpression lines (D27-OE1 and D27-OE2), under both control and drought conditions. The lines did not show morphological changes when compared with their wild type (Supplementary Fig. S5A). D27 overexpression in these lines was confirmed by qRT-PCR and was between 1000- and 2300-fold higher than for the corresponding wild type (Supplementary Fig. S5B).

Under control conditions, shoot ABA content was significantly increased in both OsD27 overexpression lines D27-OE1 and D27-OE2 (∼96% and 32%, respectively) compared with wild-type plants (Fig. 6A), while ABA content in d27 did not differ from that of the wild type. After 10 d of water withholding, only one of the OsD27 overexpression lines had a higher ABA level than the wild type, whereas the ABA level in d27 was significantly lower (Fig. 6B). We also analysed OsD27 expression in d10, d17, and d3 mutants, which contain elevated ABA levels under control conditions, and observed a significantly higher expression in these SL mutants (Fig. 6C). These results show that in these mutant lines ABA content is positively correlated with D27 gene expression levels. This correlation is also observed when wild-type plants are exposed to drought, during which D27 expression is induced. All this may be due to a positive feedback of SL deficiency on OsD27 expression in the SL mutants, which may contribute to their increased ABA levels and hence their increased drought tolerance.

Carotenoid content in the d27 mutant and OsD27 overexpression lines

In a further attempt to elucidate how D27 affects ABA biosynthesis, we examined the carotenoid composition and content in 2-week-old wild-type plants, the d27 mutant, and the two OsD27 overexpression lines, grown under normal conditions. Although all investigated carotenoids (all-trans- and 9-cis-β-carotene, neoxanthin/violasxanthin, and lutein) showed the tendency to be decreased in the d27 mutant compared with its wild type, this decrease was only significant for neo-/violaxanthin, lutein, and trans-β-carotene. Interestingly, the carotenoid levels were increased (2-fold; Fig. 7) in the D27-OE2 overexpressing line that only showed a minor increase in ABA content (Fig. 6A).

Discussion

Rice is known to be a large water consumer. Water scarcity is the major limitation for high yield in rain-fed rice and, even under drought conditions, rice yield is severely compromised (Kumar et al., 2008). Exploring ways to produce more rice with less water is essential for food security. This starts with understanding how rice adapts to drought. In the present study, the involvement of SLs in the drought response was assessed in japonica rice cv. Shiokari, together with its interaction with the abiotic stress hormone ABA.

The results show that exposure to drought increases the expression of some of the SL biosynthetic genes in root and shoot and that this results in increased SL accumulation in roots. The increase in SL production was more pronounced during a period of stable mild drought with a 10-fold increase...
in 38-day-old plants (Fig. 1A, B) compared with a 2-fold increase in 25-day-old plants (Supplementary Fig. 7A, B) than under an increasing drought stress. In tomato, Ruiz-Lozano et al. (2016) also reported an increase in SLs during drought, but only when plants are colonized with AMF. Perhaps, AMF colonization improved the plant’s water uptake and alleviated the stress imposed by water shortage, transforming it from a severe into a mild form of drought stress. In another study using tomato, the expression of the SL biosynthetic gene \textit{Slccd8} was also found to be increased during drought in shoot material from the wild type (WT) and \textit{d10}, \textit{d17}, and \textit{d3} mutants grown under control conditions. Bars represent the mean ±SE from six (A, B) and three (C) biological replicates. Significant differences between mutants, overexpression lines, and the wild type, as determined by a pairwise comparison Student's t-test, are indicated by asterisks (*\(P<0.05\), **\(P<0.01\), ***\(P<0.001\)).

It is well known that different types of drought provoke a different repertoire of plant responses inherent to the different strategies that plants use for their survival during these conditions (Pinheiro and Chaves, 2011). During longer periods of mild drought, stomata remain open, allowing photosynthesis, and plants have the possibility to adapt their architecture. For rice it has been reported that upon drought, plants reduce their leaf production and leaf area, and suppress tillering (Bouman and Tuong, 2001). The latter response is a typical response attributed to the SLs and is in line with our observation of increased root SL levels upon exposure to drought. Besides this, SLs may also be involved in root architectural changes that occur during drought. At the same
time, drought also induced the expression of SL biosynthetic genes, particularly D27, in the shoot (Fig. 2), suggesting that SL production is also increased in above-ground tissues. However, it is unclear which role shoot-based SLs play under these conditions.

During the more severe drought conditions that follow mild spells of drought, stomata need to be closed in order to prevent further water losses. Photosynthesis will be compromised, and the plant should no longer invest in (root) growth. For the latter response, a decrease in SL production and an increase in ABA production are expected. Indeed, in the present study, it is observed that while mild drought resulted in a 10-fold induction of SL production, exposure to more severe drought conditions only led to a 2- to 3-fold increase in SL production (Supplementary Fig. S7). It could be that even longer periods of severe drought will shut down root-based SL biosynthesis completely. This situation would be similar to what is observed in tomato (Visentin et al., 2016) where the expression of SL biosynthesis genes in the root was shut down during the drought, which was suggested to be a prerequisite to allow rapid accumulation of ABA in the shoot.

The effect that SL deficiency/insensitivity had on ABA levels in the SL mutants was reflected in their drought survival rates in dry down assays, where the higher ABA-producing lines d10, d17, and d3 were more drought tolerant and the low ABA-producing line d27 was more drought susceptible when compared with wild-type plants (Fig. 3). These differences correlated with their water loss rates as observed in detached leaf assays. Also, other studies showed that water loss is regulated by stomatal aperture through ABA levels (Supplementary Fig. S7). It could be that even longer periods of severe drought will shut down root-based SL biosynthesis completely. This situation would be similar to what is observed in tomato (Visentin et al., 2016) where the expression of SL biosynthesis genes in the root was shut down during the drought, which was suggested to be a prerequisite to allow rapid accumulation of ABA in the shoot.

The underlying mechanism by which D27 controls ABA biosynthesis and signalling genes shows that D27 is highly conserved across the entire green lineage, whereas CCD7, CCD8, and SL signalling genes are far less conserved, or not even detected in the Chlorophytes (Delaux et al., 2012; Ruyter-Spira and Bouwmeester, 2012). Considering that ABA is already produced in the Chlorophytes (Hartung, 2010), and SLs seem to be a later addition to the repertoire of plant hormones, it can be hypothesized that D27 was a more ancient role, perhaps in regulating ABA biosynthesis.

The underlying mechanism by which D27 controls ABA levels in rice is currently not known. In vitro experiments demonstrated that D27 is not directly involved in the formation of the ABA precursors 9-cis violaxanthin or 9-cis-neoxanthin from their all-trans precursors (Bruno and Al-Babili, 2016). However, D27 was found to isomerize α-carotene and β,β-cryptoxanthin (Bruno and Al-Babili, 2016). Perhaps, the absence of undefined end-products resulting from these side branches of the carotenoid pathway may somehow impact on ABA biosynthesis. Another possibility is that, considering that D27 acts on multiple substrates from the same pathway, D27 overexpression or D27 inactivity will have a large impact on the flux of the carotenoid pathway, ultimately resulting in altered levels of its final products such as ABA.

Although ABA was increased in both D27 overexpression lines, ABA levels in line D27-OE1 were higher than in D27-OE2. This correlated with a higher expression level of NCED3 in D27-OE1 (Supplementary Fig. S6). However, in its turn, line D27-OE2 contained higher
carotenoid levels (neo-/violaxanthin) than *D27-OE1* (Fig. 7). The latter was also observed when an ABA biosynthesis gene, β-carotene hydroxylase (*DSM2* or *BCH*), was overexpressed in rice (*Du et al.*, 2010), showing that overexpression of ABA biosynthesis genes does not always lead to increased levels of ABA but may also lead to accumulation of pathway intermediates through an as yet unknown mechanism. Lutein was also found to be increased in line *D27-OE2* when compared with the wild type. Interestingly, the concentration of lutein and neo-/violaxanthin was found to be decreased in leaves of *d27*. Considering that lutein is the end-product of the β,ε branch of carotenoids and ABA the end-product of the β,β branch, it is striking that besides β-carotene, α-carotene has also recently been identified as a substrate for *D27* (*Bruno and Al-Babili*, 2016).

Our findings show that SLs play an active role in drought physiology. Moreover, our results suggest a connection between the SL and ABA pathways, and a crucial role for *D27* in determining ABA and SL content in rice, indicating that this gene may be a promising target for drought improvement in crops. Further studies on how *D27* fine-tunes the level of ABA and SLs in different stress responses, as well as in developmental processes, should increase our understanding of the role of *D27* in regulating SL and ABA homeostasis and hence in determining drought tolerance in plants.

**Supplementary data**

Supplementary data are available at *JXB* online.

Fig. S1. Shared biosynthesis pathway of strigolactone (SL) and abscisic acid (ABA).

Fig. S2. Biomass (A) and ABA content (B) from rice plants under drought and control conditions (Experiment 1).

Fig. S3. Pooled drought survival assay of the *d27* and *d10* SL biosynthetic mutant lines (Experiment 5).

Fig. S4. ABA biosynthetic *OsoNCED3* and *OsoNCED4* gene expression.

Fig. S5. Phenotypic and transcriptional comparison of *D27* overexpressor and *d27* mutant plants, together with their wild type.

Fig. S6. *NCED3* expression levels in shoots of *D27*-overexpressing plants and their wild type (Experiment 4).

Fig. 7. Effect of strong drought on SL content in rice root extracts.

Table S1. Primer sequences used for gene expression analysis and overexpression lines.

**Acknowledgements**

We thank Professors J. Li and Y. Wang for kindly providing the rice *OsoD27* overexpression lines. We thank Francel Verstappen for his technical support at WUR in the Netherlands. This work was supported by the Higher Education Commission (HEC) of Pakistan to IH, Netherlands Organization for Scientific Research (NWO; Equipment grant, 834.08.001) to HJB, and King Abdullah University of Science and Technology (KAUST) and the EU (METAPRO: FP7 KBBE-2009-3-1-01). We acknowledge a private donor who supported BAJ’s work via the Wageningen University Fund.

**References**


