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Constitutive expression of *Arabidopsis* bZIP transcription factor *AREB1* activates cross-signaling responses in soybean under drought and flooding stresses

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ABSTRACT

Abiotic stress, such as drought and flooding, are responsible for considerable losses in grain production worldwide. Soybean, the main cultivated oilseed in the world, is sensitive to both stresses. Plant molecular mechanisms answer via crosstalk of several signaling pathways, in which particular genes can respond to different stresses. Previous studies confirmed that overexpression of transcription factor *AtAREB1* confers drought tolerance in soybean. However, plants containing this gene have not yet been tested under flooding. Thus, the objective of this study was to characterize genetically modified (GM) soybean plants overexpressing *AtAREB1* under drought and flooding conditions in comparison to its genetic background. Physiological and biochemical measurements were performed. In addition, the expression level of genes commonly activated under both stresses was evaluated. The results supported the role of the *AtAREB1* gene in conferring tolerance to water deficit in soybeans. Furthermore, under flooding, the GM line was efficient in maintaining a higher photosynthetic rate, intrinsic efficiency in water use, and instantaneous carboxylation efficiency, resulting in higher grain yield under stress. The GM line also presented higher protein content, lower concentration of hydrogen peroxide, and lower expression levels of genes related to fermentative metabolism and alanine biosynthesis. These results indicate that in addition to drought stress, plants overexpressing *AtAREB1* exhibited better performance under flooding when compared to the non-GM line, suggesting a cross-signaling response to both abiotic factors.

1. Introduction

Grain-producing crops, such as soybean, are exposed to harsh environments that can limit their growth and development. In some regions, severe drought periods may occur, while in others, the frequency of intense rainfall may lead to waterlogging of the soil or even

Abbreviations: ATP, adenosine triphosphate; CAT, catalase; GM, genetically modified; ROS, reactive oxygen species; RH, relative humidity; SOD, superoxide dismutase; TF, transcription factor; WD, water deficit.

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flooding (Fisher et al., 2017). Since these environmental conditions have been occurring naturally for centuries, it drove plant evolution to modulate adaptive responses through signaling pathways that can overlap and integrate into several levels (Bailey-Serres and Voesenek, 2010). The routes of abiotic stress responses that appear to be independent signaling pathways may interact through cross-signaling (Knight and Knight, 2001). Thus, cross-signaling is of significant interest to select candidate genes that increase tolerance to multiple stresses.

The soybean crop is sensitive to drought, and when affected, it can lead to losses of up to 40 % in production (Farias et al., 2001; Liang, 2016). In contrast, among the four major crops, namely soybean, wheat, maize, and rice, only rice plants are adapted to waterlogging of the soil, but all are sensitive to total submersion (Bailey-Serres et al., 2012). The main consequence of flooding is the reduction in oxygen availability to the roots, leading to adenosine triphosphate (ATP) deficiency due to Krebs cycle inhibition and, consequently, the electron transport chain (Rocha et al., 2010).

The development of tolerant genotypes is among the strategies to mitigate the effect of abiotic stress on soybean crops. Abiotic stress tolerance has been successfully achieved, mainly via the overexpression of transcription factors (TFs). Some examples include the Dehydration-Responsive Element-Binding protein 1 and 2 (DREB1 and DREB2) in *Arabidopsis thaliana* (Liu et al., 1998) and the Abscisic Acid-Responsive Element-Binding protein 1 (AREB1) in *Arabidopsis* (Kim et al., 2004; Fujita et al., 2005), soybean (Barbosa et al., 2012; Leite et al., 2014), rice (Oh et al., 2005), and *Arachis hypogaea* (Li et al., 2013). These studies showed higher performance of different plants overexpressing TFs under drought stress.

Conversely, studies about flooding-responsive genes are mostly focused on rice crops. Genes encoding the Ethylene Response Factors (ERF) family, such as SUBMERGENCE-1 (SUB1A) (Xu et al., 2006) and SNORKEL (SNK1 e SNK2) (Hattori et al., 2009), are linked to submersion tolerance. However, their orthologues (SUB1 and SNK) have not been identified in *Arabidopsis* and soybean. In contrast, studies with flooding-related genes in soybean are far from conclusive, lacking enough information to understand the tolerance mechanisms in this species.

In general, TFs can act in response to multiple stresses due to their activity, promoting or repressing the expression of genes involved in the protection of cellular structures and/or in the modulation of enzymes related to cellular metabolism. In a recent study, approximately 75 % of the bZIP TF family identified in soybeans was shown to be differentially expressed under drought and waterlogging (Zhang et al., 2018).

The bZIP family AREB/ABF genes are amongst the TFs identified in *Arabidopsis* that confer abiotic stress tolerance in plants (Choi et al., 2000; Uno et al., 2000; Yoshida et al., 2010). Three members of this family, AREB1/ABF2, AREB2/ABF4, and ABF3 were found to be involved in responses related to drought (Choi et al., 2000; Uno et al., 2000; Kang et al., 2002; Kim et al., 2004; Fujita et al., 2005; Furihata et al., 2006) and osmotic stress (Fujita et al., 2013; Yoshida et al., 2010).

Soybeans overexpressing the *Arabidopsis* AREB gene (*AtAREB1*) exhibited superior physiological responses under drought in greenhouse and field conditions (Barbosa et al., 2012; Marinho et al., 2015; Fuganti-Pagliarini et al., 2017). However, the characterization of these plants under flooding conditions has not yet been done. Considering that some genes exhibit cross-signaling and activate response mechanisms in response to multiple stresses, this study aimed to characterize GM soybeans containing the *35S-AtAREB1* gene construct under both drought and flooding stress.

2. Materials and methods

2.1. Plant material and experimental conditions

Two soybean lines were used for the experiments: line 'GM 1Ea2939' containing the *35S-AtAREB1* gene construct; and cultivar 'BR16', which is the genetic background of the GM 1Ea2939. Line GM 1Ea2939 was previously obtained via *Agrobacterium tumefaciens* transformation and characterized under drought conditions (Marinho et al., 2015). It was the chosen line for this experiment due to its better performance and higher expression of drought-inducible genes under water deficit when compared to other lines transformed with the same gene (Marinho et al., 2015; Fuganti-Pagliarini et al., 2017).

Seeds were pre-germinated for 96 h on Germitest® paper, moistened with distilled water, and incubated in a germination chamber at 25 ± 1 °C and 100 % relative humidity (RH). After germination, seedlings were transferred to individual pots (15 cm external diameter x10 cm base x11 cm height) filled with 1.1 kg of substrate, composed of soil: sand mixture (1:1). Initially, two seedlings were transferred to each pot and then inoculant (Nitragin Cell Tech HC®, Novozymes, Franklinton, North Carolina, EUA) was added. Pots were then kept in the greenhouse, with the temperature set to 28 ± 2 °C. After plantlets reached complete emergence and establishment, one plantlet was removed to keep one plantlet per pot.

The experiments were kept at ideal irrigation maintenance (near field capacity) until plantlets reached the V3 stage (Fehr and Caviness, 1977), when water deficit and flooding stress were applied. Under the water deficit treatment, irrigation suspension followed the methodology proposed by Marinho et al. (2015). Under flooding treatment, the pots were placed inside larger ones, which were then flooded to 5 cm above the soil surface. Under the control condition, the plants were irrigated daily. A randomized complete block design was used, with a 2 × 3 factorial design (two genotypes and three water conditions: control – C, water deficit – WD, and flooded – F), with six repetitions. After drought and flooding stress were applied, stomatal conductance (*gs*) was monitored (LI-6400XT, LI-COR) until it reached 0.02 mol H_2O m^{-2} s^{-1}, pre-established as a value for a stress indicator (Flexas et al., 2006). When this value was achieved, 7 days after initial stress, physiological analysis were made, as well as sample collection of roots and leaf tissue for further biochemical and molecular analysis.

2.2. Physiological analysis

Measurements of the photosynthetic rate (*A*), intercellular CO_2 concentration (*Ci*), stomatal conductance (*gs*), and transpiration rate (*E*) were carried out in the central leaflet of the third fully expanded trifoliate leaf (apex-base direction) using a portable infrared gas analyzer (LI-6400XT model, LI-COR) with a 90 % red + 10 % blue light source and 2 cm^2 chamber. All measurements were carried out in the greenhouse during the morning period (between 9:00 and 11:00 a.m.) and under good luminosity conditions, considering the following parameters: photosynthetically active radiation (PAR) 1000 μmol m^{-2} s^{-1}; reference of CO_2 400 μmol mol^{-1}; reference of water ranging from 18 to 20 mmol mol^{-1}; and CO_2 flux400 μmol s^{-1}.

Chlorophyll (SPAD Index) was measured in one lateral leaflet from the same aforementioned trifoliate leaf using a portable chlorophyll meter (SPAD-502, Minolta).

After gas exchange measurements, the instantaneous and intrinsic water use efficiency (WUE and WUE*i*, respectively) were calculated, as well as instantaneous carboxylation efficiency (iCE).

2.3. Biochemical analysis

Leaf and root tissues were collected from three biological replicates, each with two plants. Two hundred (200) mg of each tissue were ground in liquid nitrogen and resuspended in phosphate-buffered saline (PBS, 100 mM, pH 7.5; adapted from Gratão et al., 2014) to quantify the total soluble proteins and determine superoxide dismutase (SOD; EC 1.15.1.1) and catalase enzyme (CAT; EC 1.11.1.6) activities. Separately, one hundred (100) mg of ground tissue was resuspended in trichloroacetic acid to determine the hydrogen peroxide content (H_2O_2), according to Alexieva et al. (2001).

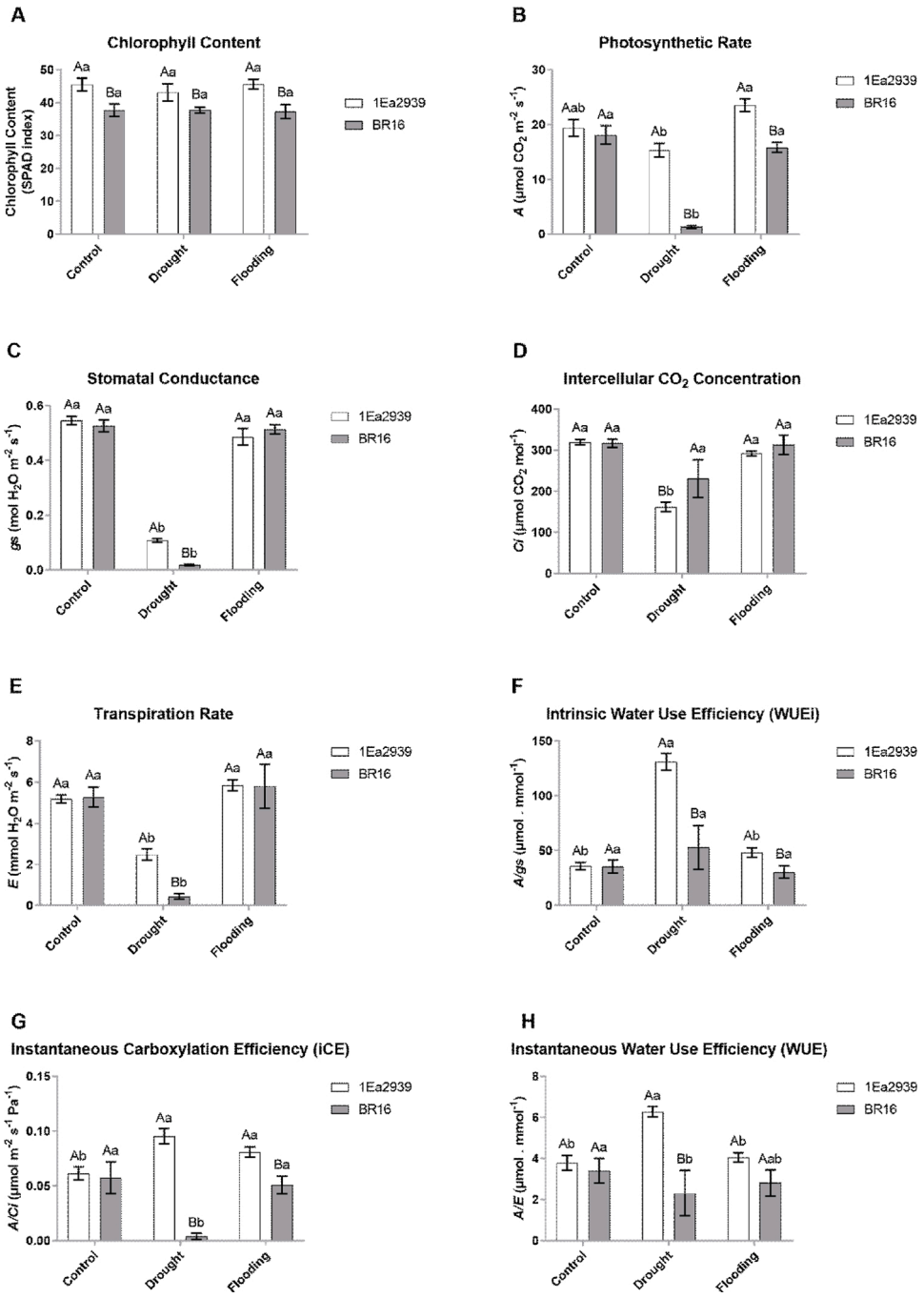

Fig. 1. Physiological changes of GM 1Ea2939 (*35S-AtAREB1*) and the conventional cultivar BR16 under drought and flooding stress. A) chlorophyll content (SPAD index); B) photosynthetic rate (*A*); C) stomatal conductance (*gs*); D) intercellular CO_2 concentration (*Ci*); E) transpiration rate (*E*); F) intrinsic water use efficiency (WUEi); G) instantaneous carboxylation efficiency (iCE); H) instantaneous water use efficiency (WUE). Values represent mean ± standard error. Letters represent statistical differences by Tukey's test ($p \leq 0.05$). Capital letters compare genotypes within the same treatment and small letters compare treatments within the same genotype.

Total soluble protein concentration was determined by the Bradford method (1976), using 0.1 mL of root extract and 0.05 mL of leaf extract in triplicate. The protein concentration was calculated according to the standard protein curve of bovine serum albumin (BSA). Absorbance was measured in a spectrophotometer at a wavelength of 595 nm. The total soluble protein concentration was expressed as μg protein/g.

SOD activity was determined according to Broetto (2014) with minor modifications, using 0.1 mL of root and leaf extracts in triplicate. Absorbance was measured in a spectrophotometer at a wavelength of 560 nm. One unit of SOD (U SOD) was defined as the amount of enzyme needed to inhibit the photoreduction of nitro tetrazolium blue chloride (NBT) by 50 %. The activity of this enzyme was expressed as U SOD/mg protein^{-1}.

CAT activity was determined according to Azevedo et al. (1998) using 0.15 mL of plant extracts in triplicate. Catalase activity was determined by following the rate of decomposition of H_2O_2 by the decrease in an absorbance at 240 nm within 1 min (Beutler, 1975). Enzyme activity was calculated using the molar extinction coefficient of 36 M cm^{-1} (Anderson et al., 1995) and expressed as μmol H_2O_2 min^{-1} mg protein^{-1}.

The H_2O_2 concentration was determined according to Alexieva et al. (2001), using 0.2 mL of plant extracts in triplicate. Absorbance was measured in a spectrophotometer at the wavelength of 390 nm, and a standard curve was used to calculate the concentration. H_2O_2 concentration was expressed as μmol H_2O_2/g.

2.4. Gene expression analysis through RT-qPCR

Total RNA was isolated from the tissues of three biological replicates (two plants per replicate) using the TRIzol reagent (Invitrogen,

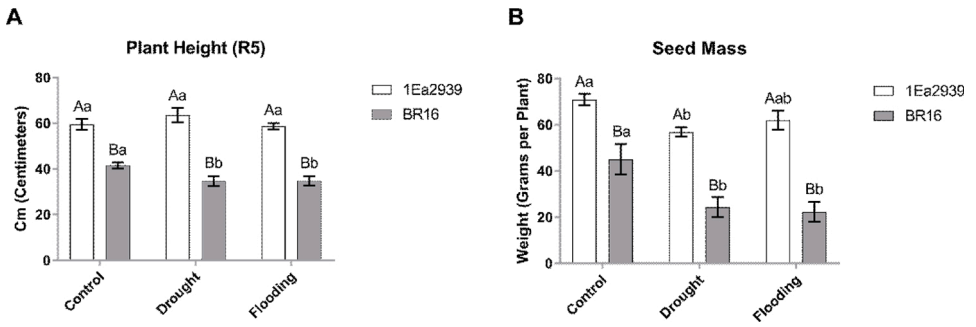

Fig. 2. Phenotypic effect on the GM 1Ea2939 line (*35S-AtAREB1*) and conventional cultivar BR16 under drought and flooding stresses. (A) Plant height (R5 Stage). (B) Seed mass. Values represent mean ± standard error. Letters represent statistical differences by Tukey's test ($p \leq 0.05$). Capital letters compare genotypes within the same treatment and small letters compare treatments within the same genotype.

Carlsbad, Califórnia, EUA) according to the manufacturer's instructions. Samples were treated with DNAse I (Invitrogen, Carlsbad, Califórnia, EUA), and the cDNA was synthesized from isolated RNA by reverse transcriptase using the SuperScript III First Strand Synthesis (Thermo Fisher Scientific, Waltham, Massachusetts, EUA). RT-qPCR was performed using the SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, EUA) in a 7300 RT-qPCR Thermocycler (Applied Biosystems/Life Technologies, Grand Island, NY, USA). Standard curves were produced from serial dilutions of a cDNA pool to estimate the efficiency of the PCR amplification with each pair of primers. The primer concentrations were adjusted to achieve efficiency rates higher than 90 %.

Apart from the transgene *AtAREB1*, expression of the other stress-inducible soybean genes were assayed: drought-responsive genes, such as Dehydrin-like (Glyma.09G185500), Heat Shock Protein (*HSP70*, Glyma.17G072400), and Late Embryogenesis Abundant (*LEA18*, Glyma.17G164200) selected from Fuganti-Pagliarini et al. (2017); and flooding-responsive genes encoding Alanine aminotransferase 1 (*GmAlaAT1*, Glyma.07G045900), Alanine aminotransferase 2 (*GmAlaAT2*, Glyma.01 G026700), Alcohol dehydrogenase (*ADH*, Glyma.04G240800), and Sucrose synthase (*SuSy*, Glyma.13 G114000) selected from Nakayama et al. (2017). In addition, expression of the gene encoding 9-cis-epoxycarotenoid dioxygenase (*NCED3*, Glyma.15 G250100) was also surveyed, selected from Rodrigues et al. (2015). The *β-actin* (Glyma.15 G050200) and *FYVE* zinc finger (Glyma.13 G114700), previously identified as stable reference genes under flooding (Nakayama et al., 2014) and drought stress (Marcolino-Gomes et al., 2015), respectively, were used as an endogenous control for RT-qPCR analyses. Primers used in the experiments are listed in Table S1. For *AtAREB1*, normalized data were presented. For the other target genes, calibration of samples under stress was done using the control treatment after data normalization. The data was analyzed using Rest2009 software (Pfaffl et al., 2002).

2.5. Growth and yield analyses

A second experiment was installed following the same materials and conditions as described above. A randomized complete block design was used, using a 2 × 3 × 9 factorial design (two genotypes, three water conditions, and nine replicates). Plants were kept at near field capacity until it reached the vegetative stage V3 (Fehr and Caviness, 1977), when the drought and flooding stresses were applied for 7 days. Plant height was measured at the R5 stage. When the plants were fully developed, seeds were collected from each plant individually and weighed on precision scale to quantify the seed's mass per plant.

2.6. Statistical analysis

The software RStudio (RStudio Team, 2015) was used to verify if the residues exhibited normality in distribution through the Shapiro-Wilk test. Data were then submitted to ANOVA and means compared by Tukey's test ($p \leq 0.05$).

3. Results

3.1. Physiological changes and the phenotypic effect observed on both drought-tolerant and conventional genotypes

The GM 1Ea2939 line showed higher chlorophyll content compared to cultivar BR16 (Fig. 1A) under both stresses and the control treatment. In both stress treatments, GM 1Ea2939 also exhibited superior performance when analyzing the photosynthetic rate (*A*) (Fig. 1B). Stomatal conductance (*gs*) was drastically reduced during WD in both genotypes, but GM 1Ea2939 still maintained superior performance (Fig. 1C). Intercellular CO_2 concentration (*Ci*) was reduced during WD but BR16 exhibited higher values in comparison to GM 1Ea2939 (Fig. 1D). Results for the transpiration rate (*E*) showed a similar pattern to what was observed for *gs* (Fig. 1E). With regards to the intrinsic water use efficiency (WUEi) and instantaneous carboxylation efficiency (iCE), GM 1Ea2939 showed better results (Fig. 1F and G, respectively). Under WD, the GM line also exhibited better efficiency of instantaneous water use (WUE) than the conventional cultivar (Fig. 1H).

Phenotypically, the plant height of GM 1Ea2939 showed better performance in all conditions tested (Fig. 2A). In contrast to BR16, the GM line did not show differences between the control and stressed conditions. Physiological changes of GM 1Ea2939 reflected in superior grain yield when compared to BR16 in all conditions (Fig. 2B). In addition, the GM line showed higher yield stability under drought and flooding.

3.2. Effect of the overexpression of AtAREB1 on protein concentration and ROS levels

Protein concentration was higher in GM 1Ea2939 compared to BR16 in both tissues (Fig. 3A–B). With regards to the experimental conditions, proteins accumulated more under flooding stress in roots (Fig. 3B). In leaves, H_2O_2 accumulated more in BR16 in the control and under WD, whereas in GM 1Ea2939, it remained stable in all conditions (Fig. 3C). In roots under flooding, BR16 exhibited higher H_2O_2 concentration in comparison to GM 1Ea2939 (Fig. 3D). Lower accumulation of this ROS under flooding was observed when comparing the control and drought conditions (Fig. 3C–D). Antioxidant activity was observed in leaves, where SOD activity was detected to be higher in BR16 than in GM 1Ea2939 (Fig. 3E). Under control condition, SOD content was higher in BR16 compared to GM 1Ea2939 in the roots (Fig. 3F). In the same cultivar, CAT activity was also higher than in the GM line under WD and flooding in roots (Fig. 3H), however, there was no difference in leaf tissue (Fig. 3G).

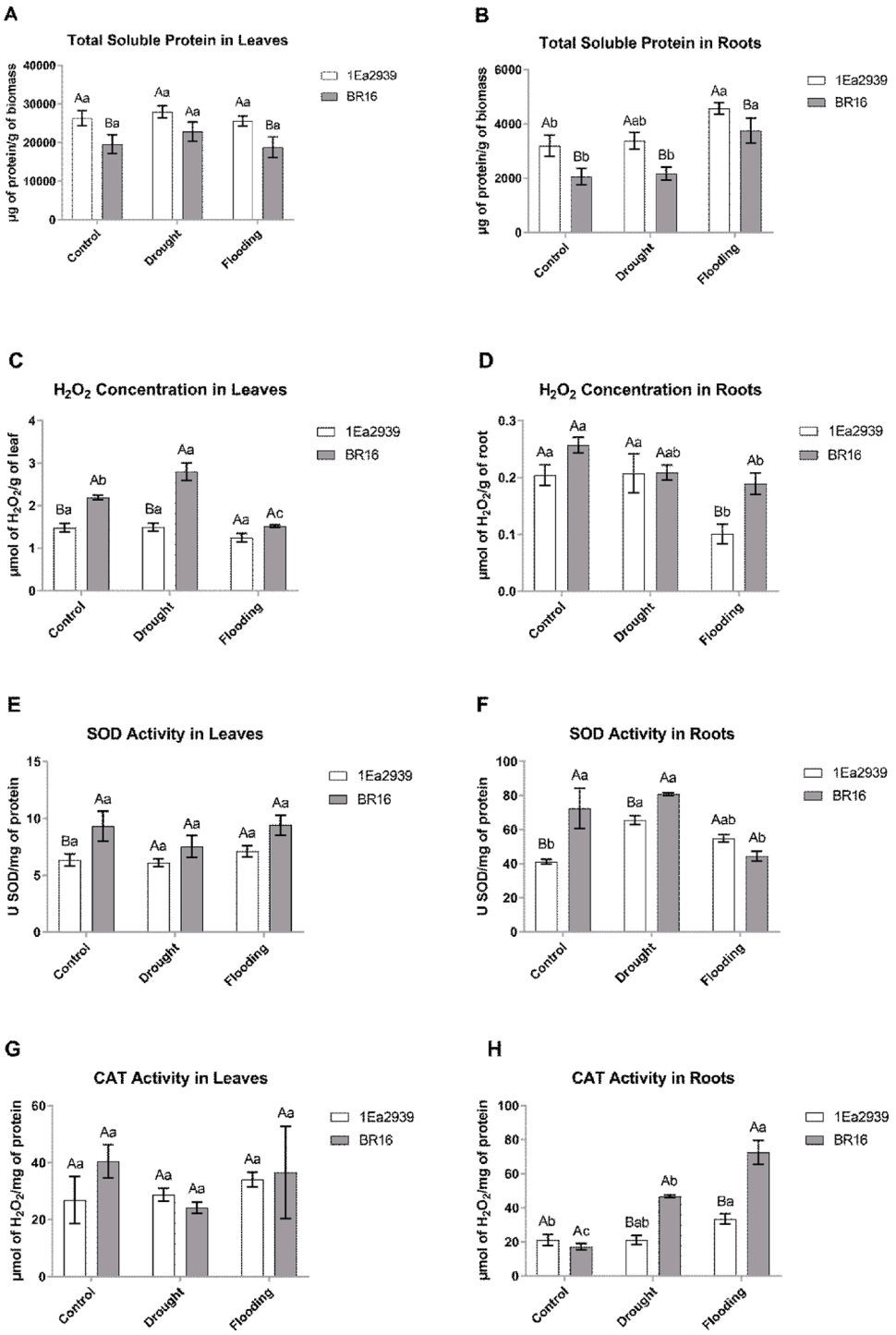

Fig. 3. Total soluble protein, H_2O_2, SOD, and CAT analysis of the GM 1Ea2939 line (*35S-AtAREB1*) and conventional cultivar BR16 under water deficit and flooding stress. A) Total soluble protein genotype effect in leaves, B) in roots and C) H_2O_2 concentration in leaves; D) H_2O_2 concentration in roots E) SOD activity in leaves F) SOD activity in roots; G) CAT activity in leaves; H) CAT activity in roots. Values represent mean ± standard error. Letters represent statistical differences by Tukey's test ($p \leq 0.05$). Capital letters compare genotypes within the same treatment and small letters compare treatments within the same genotype.

3.3. Changes in the expression of stress-inducible genes

Expression of the transgene *AtAREB1* was confirmed in the GM line (Fig. 4). Since *AtAREB1* is under the control of constitutive promoter 35S, it was expressed in all conditions (C, WD, and F) and tissues tested, but not on BR16 due to its absence in the conventional cultivar.

Other stress-responsive genes were also evaluated. *NCED3* expression in GM 1Ea2939 was observed in both tissues and water conditions (Fig. 5A). Drought-responsive *LEA18* was only induced under drought, with higher expression detected in BR16 roots (129.6-fold increase) when compared to the same tissue in GM 1Ea2939 (105.2-fold increase; Fig. 5B). Dehydrin expression was significantly increased (up-regulated) in leaves and roots of BR16, with a 1426.6-fold and 1049.8-fold increase, respectively (Fig. 5C). Similar to *LEA18*, dehydrin was more expressed in BR16 than in GM 1Ea2939. *HSP70* gene expression increased in leaves (11.3-fold increase) and in roots (8.1-fold increase) of BR16 under drought but decreased in the roots (0.6-fold decrease in GM 1Ea2939 and 0.4-fold decrease in BR16) of both cultivars under flooding (Fig. 5D).

The expression of flooding-responsive gene *ADH* was higher under flooding in BR16 roots (18.3-fold increase) when compared to the same tissue in GM 1Ea2939 (5.2-fold increase; Fig. 5E). Similarly, *GmAlaAT1* had increased expression under flooding in BR16 roots (4.3-fold increase) when compared to the same tissue in GM 1Ea2939 (2-fold

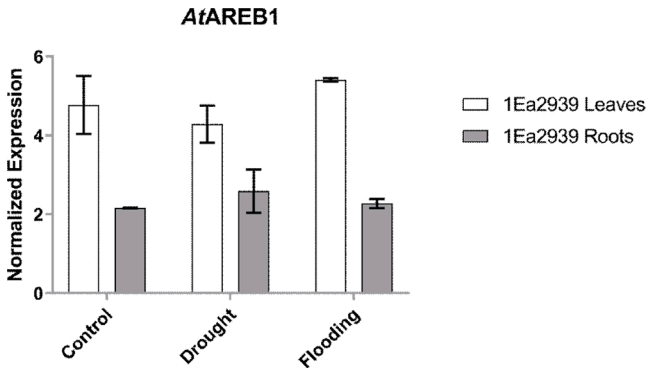

Fig. 4. Normalized expression of the *AtAREB1* gene in the GM 1Ea2939 line (*35S-AtAREB1*) under water deficit and flooding stress, normalized by endogenous genes *β-actin* and *FYVE*. Values represent mean ± standard error.

increase; Fig. 5F). In contrast, *GmAlaAT2* expression was not significant under flooding in either genotype or tissue type (Fig. 5G). In fact, it was downregulated under drought in BR16 leaves. The *SuSy* gene was upregulated in BR16 roots under both stresses, whereas in GM 1Ea2939, it was not altered (Fig. 5H).

4. Discussion

Under abiotic stressees such as drought and flooding, plants activate a network of inner adaptive responses that ultimately translate into a phenotypical answer to cope with the negative environmental stimuli. A series of results in this paper explored these plant responses and the impact of drought and flooding stress on a GM line overexpressing the transcription factor *AtAREB1* (GM 1Ea2939) in comparison to its non-transgenic background (BR16).

Physiologic responses, such as chlorophyll content, in GM 1Ea2939 were observed to be superior to the chlorophyll found in BR16, regardless of the water condition (Fig. 1A). This result supports the photosynthetic rate found in the GM line, which was also higher in both conditions (Fig. 1B). Previous work showed that photosynthesis was reduced in plants under both drought (Pinheiro and Chaves, 2011) and flooding (Caudle and Maricle, 2012). In this study, GM 1Ea2939 plants stood out for maintaining gas exchange even under drought stress, supporting previous work where the same line also exhibited higher transpiration and stomatal conductance under a shortage of water (Marinho et al., 2015).

The decrease in photosynthesis under flooding stress has also been demonstrated in other studies (Caudle and Maricle, 2012; Liu et al., 2014). In plants, flooding tolerance results from an ability to maintain photosynthesis during stress (Caudle and Maricle, 2012), either by returning to normal levels or achieving a stable rate of photosynthetic activity. Photosynthesis is associated with stomatal conductance. Interestingly, in contrast to what was observed in the water deficit experiments, plants tested with flooding did not present differences in stomatal conductance. Furthermore, photosynthesis can also be associated with non-stomatal limitations, such as an increase or decrease of the RuBisCO enzyme (Hu et al., 2013). Thus, since the soluble protein content was observed to be higher in GM 1Ea2939 (Fig. 3A–B), we inferred that this contributed to its higher photosynthetic rate under flooding.

Other physiological changes were also induced under flooding. The GM line showed better instantaneous water use efficiency and instantaneous carboxylation efficiency, both of which are associated with the plants' capacity to use water and carbon to realize photosynthesis. The higher physiological values observed in GM 1Ea2939 indicated that one of the factors contributing to flooding tolerance might be its higher efficiency in maintaining photosynthetic activity even under stress. We showed that these responses are variable between different genotypes. In addition, these physiological differences also had an impact on plant growth and grain yield. The GM plants were more productive in all conditions tested and maintained higher stability when under drought and flooding stress (Fig. 2).

The physiological results suggested that the transformation resulted in profile changes that affected the plants even in a non-stress situation, as seen by the increased chlorophyll content (Fig. 1A), plant height, seed mass (Fig. 2), soluble protein content (Fig. 3A–B), and SOD activity (Fig. 3E–G). Similar results were observed in other crops, as an increased chlorophyll content was directly related to photosynthetic activity, which promoted the grain filling process of stay-green genotypes observed in species, such as wheat (Peingao, 2013), sorghum (Borrel et al., 2014), and maize (Zhang et al., 2019). Indeed, Fuganti-Pagliarini et al. (2017) demonstrated that line 1Ea2939 exhibited a longer cycle (150 days) than the wild-type (130 days). However, a senescence pattern in leaves and stems should also be evaluated to correlate it with the photosynthetic ability of stay-green genotypes. As discussed by Marinho et al. (2015), analysis of the yield components of the 1Ea2939 line showed that despite that the AREB gene is being driven by the constitutive promoter 35S, which is often associated with negative growth effects, it did not impair its agronomic performance under greenhouse conditions. It was later shown that even under field conditions, this GM line exhibited better performance when compared to the wild-type and other GM lines (Fuganti-Pagliarini et al., 2017).

Biochemically, the GM line accumulated more soluble proteins in both analyzed tissues. Fuganti-Pagliarini et al. (2017) showed that plants overexpressing *AtAREB1* had increased grain protein in field experiments for two consecutive crops. Proteins are distributed across all plant cells and are essential in the structuring and maintenance of the cells (Bray et al., 2000). Approximately 40 % of soluble proteins located within photosynthetic tissues are RuBisCO (Feller et al., 2008), which acts on carbon fixation by the carboxylation of ribulose 1,5-biphosphate (RuBP). Thus, it is possible to infer that the higher protein content present in the GM line was correlated with the increased chlorophyll concentration and the higher photosynthetic activity observed in these plants.

Higher protein concentration was also observed in roots under flooding in both genotypes. Being the tissue that is submerged during flooding, roots are severely affected under these conditions (Sakazono et al., 2014). In water, oxygen diffusion is approximately 10,000 times slower than in air (Pepper and Gentry, 2014). For this reason, when roots are exposed to water for longer periods, they suffer from anaerobic stress that can alter protein content and patterns (Sachs et al., 1980).

Reactive oxygen species (ROS) are also a by-product of stress conditions. In low concentrations, ROS can act as stress signaling molecules. However, in higher doses, they become toxic for plants, leading to cellular death (Gechev and Hille, 2005). Hydrogen peroxide, for instance, is capable of diffusing across membranes and is a central signaling compound in cross-tolerance mechanisms (Blokhina and Fagerstedt, 2010; Foyer and Noctor, 2003). In leaves, BR16 exhibited higher levels of H_2O_2 in control and drought conditions (Fig. 3C). Similarly, Silva (2017) observed increased levels of H_2O_2 in soybean leaves after drought followed by rehydration. One of the first responses against critical levels of ROS is SOD enzyme activity, which detoxifies the superoxide anion (O_2^-), leading to H_2O_2 formation (Alscher et al., 2002). Lower SOD levels in leaves were observed in the GM line compared to BR16 (Fig. 3E–F). Combined with the higher levels of H_2O_2 found in BR16, these results indicated that the O_2- conversion into H_2O_2 happened on a larger scale, suggesting more severe stress in this cultivar.

In roots, the GM line exhibited lower H_2O_2 concentration in all conditions and lower SOD activity under control and drought conditions. In addition, CAT activity was higher in BR16 under drought and flooding (Fig. 3H). According to Damanik et al. (2016), CAT levels were increased in flooding-sensitive genotypes, similar to that observed in BR16. Higher CAT activity was also observed in roots under drought stress of other species, such as chickpea (Mafakheri et al., 2011), canola

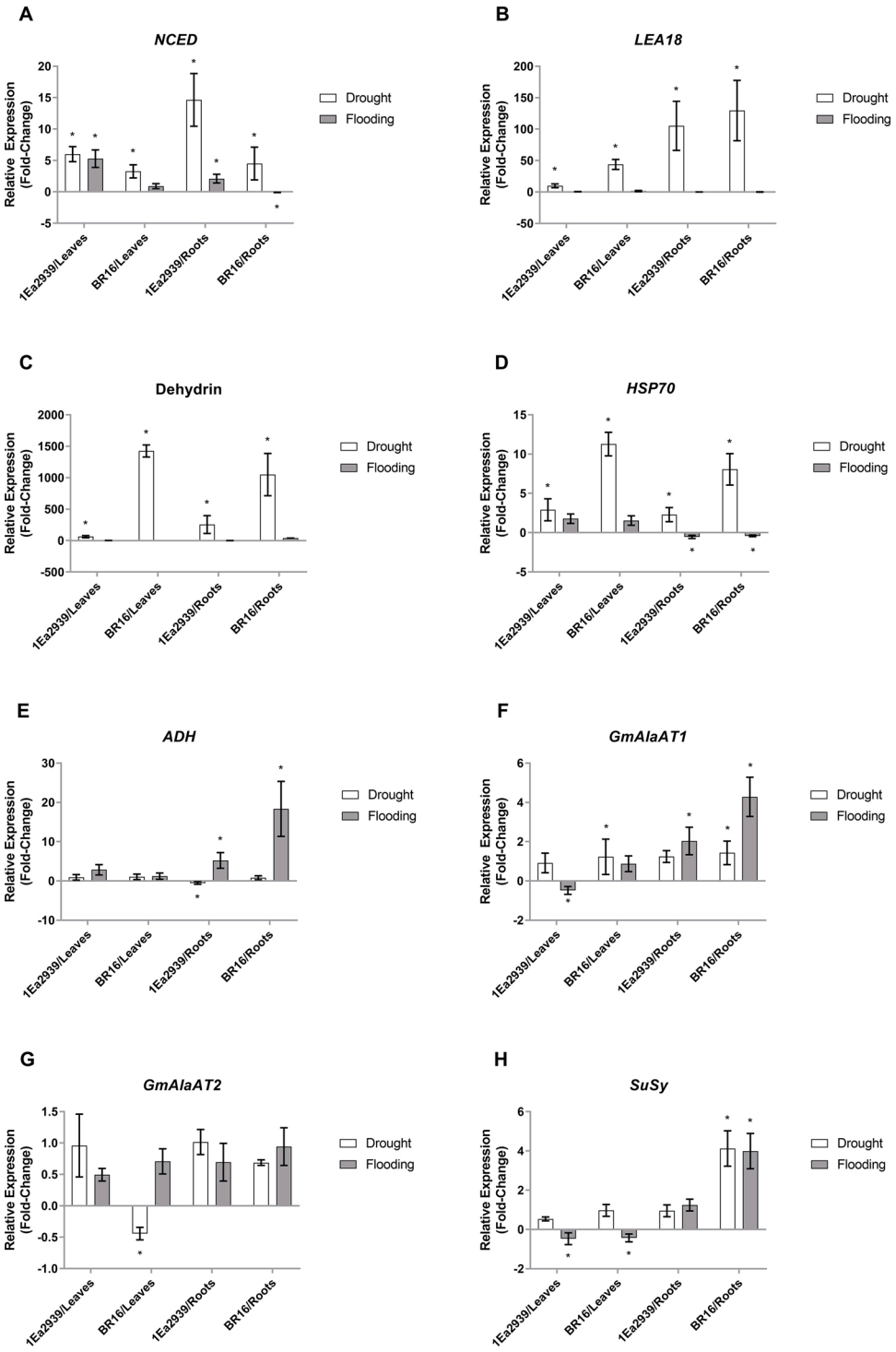

Fig. 5. Relative expression of drought- and flooding-responsive genes in leaves and roots of GM line *35S-AtAREB1* (1Ea2939) and conventional cultivar BR16. A) *NCED3* (Glyma15G40070) B) *LEA18* (Glyma.17G164200); c) Dehydrin (Glyma.09G185500); D) *HSP70* (Glyma.17G072400); E) *ADH* (Glyma.04G240800); F) *GmAlaAT1* (Glyma.07G045900); G) *GmAlaAT2* (Glyma.01G026700); H) *SuSy* (Glyma.13G114000). Relative expression was calibrated by the control condition and normalized by endogenous genes β-actin and FYVE. Values represent mean ± standard error. *Significant results $p < 0.05$ (Rest2009).

(Mirzaee et al., 2013), wheat (Naderi et al., 2014), and soybean (Masoumi et al., 2010). These results support the hypothesis that the conventional cultivar suffered more from the effects of the abiotic stress compared to the GM line. Thus, even if enzyme production was higher in BR16, the antioxidant system was not balanced, resulting in a high concentration of remaining H_2O_2.

The *AtAREB1* gene, present within the GM 1Ea2939 line, was successfully expressed in all conditions (Fig. 4). Since *AtAREB1* requires the presence of ABA to be activated (Uno et al., 2000; Furihata et al., 2006; Yoshida et al., 2010), we also evaluated the expression of *NCED3*, a key precursor of ABA biosynthesis. We detected *NCED3* expression under drought in both tissue types and genotypes, supporting previous evidence (Iuchi et al., 2001; Frey et al., 2012; Rodrigues et al., 2015). In our experiments, *NCED3* was upregulated under flooding on both tissues but only of the GM line, suggesting that *AtAREB1* was also expressed in this condition. Taken together with the *AtAREB1* expression data and both the physiological and biochemical results, we demonstrated the TF role under flooding stress.

Drought-responsive genes, such as *LEA18*, dehydrin, and *HSP70,* and flooding-responsive genes, such as *ADH*, *AlaAT1,* and *SuSy,* were all activated under their respective conditions. These genes were more modulated in BR16, supporting biochemical results related to oxidative

stress, indicating that GM 1Ea2939 sensed the effects less than the conventional cultivar. More specifically, *LEA18* was highly expressed under drought (Fig. 5B), as also demonstrated in other studies (Fuganti-Pagliarini et al., 2017). The expression of this gene occurred in both genotypes and tissue types, but it was significantly activated in BR16. Dehydrin, another drought-related gene, is known for being usually expressed under drought and cold stress (Puhakainen et al., 2004). Expectedly, due to its higher susceptibility to the lack of water, a higher quantity of dehydrins were found in BR16 under drought conditions. The *HSP70* gene acts as a molecular chaperone, involved in the folding, translocation, and degradation of proteins under stressed conditions, as well as on several cellular processes (Park and Seo, 2015). In our experiments, *HSP70* was expressed only under drought (Fig. 5D) and similar to the other drought-responsive genes, it was more activated in BR16 when compared to GM 1Ea2939.

During flooding, hypoxia stress occurs due to low oxygen concentrations, which limits energy production through aerobic pathways. Therefore, to obtain energy, three main anaerobic pathways are activated during stress: ethanol pathway, lactic acid pathway, and a pathway that is plant-specific, which produces alanine, involving the enzyme *AlaAT*. In plants under normal oxygen conditions, these pathways are absent or have very low activity but are rapidly induced at low concentrations of O_2 (Sousa and Sodek, 2002). *ADH* is an enzyme that catalyzes the reduction of toxic acetaldehyde to ethanol and is activated under hypoxia and anoxia stresses (Preiszner et al., 2001). *ADH* expression was identified only in roots in our experiments, being the tissue that is submerged under flooding (Fig. 5E). This gene was strongly induced in the conventional cultivar, suggesting that it needed more glycolysis activation to produce energy and, consequently, maintained glycolysis and cytosolic pH via activation of the fermentative pathway (Good and Crosby, 1989). Rizal and Karki (2011) also observed higher *ADH* expression in flooding-sensitive plants when compared to flooding-tolerant ones.

Genes that code for the *AlaAT* enzymes were also observed, namely subclass I *GmAlaAT1* and subclass II *GmAlaAT2* (Fig. 5F–G). The latter gene did not show significant differences under flooding (Fig. 5G), supporting results observed by Rocha et al. (2010), whom demonstrated that *AlaAT2* was less expressed than *AlaAT1*. This is due to the *AlaAT2* gene being expressed only in mitochondria, which are not intensively affected during stress. In contrast, *AlaAT1* is expressed in the cytosol, so it is significantly affected in hypoxia situations. This gene was induced in roots under flooding in both genotypes, exhibiting more intense activation of stress-responsive pathways in BR16 when compared to the GM line.

The sucrose synthase (*SuSy*) enzyme is associated with sucrose metabolism, acting as either a synthetase or invertase (Koch, 2004). *SuSy* was significantly expressed in BR16 roots under both stress conditions (Fig. 5H). This gene was particularly induced in BR16 roots under flooding, showing that it activated energy-producing pathways. Consequently, BR16 needed more NAD^+ regeneration within the fermentative pathway, which, in turn, induced the *ADH* observed in this genotype.

AtAreb1 is a transcription factor that regulates a large number of genes. Thus, it may be activating other routes that contribute to the response to anoxia, such as promoting amino acid biosynthesis or other compounds, or even resulting in morphological changes, such as aerenchyma development. Additional studies are needed to identify these mechanisms.

The results of this study indicate superior performance of plants overexpressing the *AtAREB1* gene under drought and provide new insights regarding its behavior in response to water excess. Under flooding, the GM line stood out by exhibiting better physiological performance in comparison to the conventional cultivar, also reflected in the higher grain production. They also accumulated more proteins, less H_2O_2, and had less activation of the antioxidant enzymes CAT and SOD, suggesting more efficient control of ROS. Additionally, stress-responsive genes related to fermentative metabolism and alanine biosynthesis were less induced due to the overexpression of *AtAREB1*. Collectively, these data indicated that the defensive mechanisms activated by *AtAREB1*, in addition to being associated with drought-tolerance, also promote better performance of soybean under flooding.

5. Contributions

MBFA performed the experiments and wrote the manuscript. LMMH, CFR, ACBO, and ALN conceived the project and revised the manuscript. KYS, NK, and KN contributed with gene constructs and revised the manuscript. EGGB revised the manuscript. LACM performed the biochemical analysis. PB performed statistical analysis and revised the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2020.153338.

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