Chapter 3-2

Drought-tolerant soybean development: evaluation of GM lines under greenhouse and field conditions

Renata Fuganti-Pagliarini[1,2], Silvana Regina Rockenbach Marin[1,3], Mayla Daiane Côrrea Molinari[3], Daniel de Amorim Barbosa[3], Hugo Molinari[4], Liliane Márcia Mertz-Henning[1], Norman Neumaier[1], José Renato Bouças Farias[1], Kazuo Nakashima[5], Kazuko Yamaguchi-Shinozaki[6,7], Alexandre Lima Nepomuceno[1]

[1] Embrapa Soja. Rodovia Carlos João Strass. Acesso Orlando Amaral. Warta. PO. Box 231 86001-970, Londrina, PR, Brazil

[2] National Council for Scientific and Technological Development (CNPq), Brazil

[3] Londrina State University – Biological Sciences Center. P.O. 10.011, 86057-970, Londrina, PR, Brazil

[4] Embrapa Agroenergia. Parque Estação Biológica. s/nº, Brasília, Distrito Federal 70297-400, Brazil

[5] Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki 305-8686, Japan

[6] Laboratory of Plant Molecular Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

[7] Research Institute for Agricultural and Life Sciences, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

* Corresponding author e-mail*: alexandre.nepomuceno@embrapa.br*

Abstract

Drought is one of the greatest sources of environmental stress that has resulted in both economic and yield losses in many soybean-producing regions. The use of biotechnological tools aimed to produce plants with increased drought tolerance and productivity is the result of major investments in scientific and technological research. During the last decades, transcription factors (TFs) and key genes of important drought-responsive pathways have been used to develop genetically modified (GM) plants with increased tolerance to abiotic stress. To develop soybean lines with improved drought tolerance, genes encoding dehydration-responsive element binding protein (DREB) TFs and ABA-responsive element-binding proteins (AREB) as well as the *GolS* and *NCED* genes that encode the enzyme galactinol synthase (GolS, EC 2.4.1.123) of the raffinose family of oligosaccharides (RFOs) and the key enzyme in abscisic acid (ABA) biosynthesis 9-*cis*-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51), respectively, were successfully introduced in soybean plants. After transformation, it was imperative to characterize the resultant drought tolerance of the GM lines.

Thus, the soybean GM lines containing genetic constructions to overexpress *AtDREB1A*, *GmDREB1A*, *AtDREB2A*, *AtAREB1*, *AtGolS2*, and *AtNCED3* were phenotyped based on molecular and agronomical traits, growth parameters, and survival rates under water deficit conditions in experiments carried out under greenhouse and field conditions for more than one crop season. All obtained results were compiled and are presented in this report. Overall, the GM lines are promising and show improved drought tolerance as diverse defense mechanisms aimed at surviving periods of water scarcity were targeted while retaining the productivity of the crop. These outcomes highlight soybean drought-tolerance pathways and indicate that the use of biotechnological tools in agricultural research can help producers minimize both yield and financial losses during drought-stricken crop seasons.

Keywords: *Glycine max*, transcription factor, DREB, AREB, ABA, galactinol synthase, 9-*cis*-epoxycarotenoid dioxygenase, water deficit, yield

Introduction

Drought is one of the most stressful environmental factors currently affecting crops of economic importance. Therefore, yield reductions are likely to remain ongoing and financial/economic losses are inevitable in drought-prone environments. According to a new report from the Food and Agriculture Organization (FAO) of the United Nations, natural disasters have cost the agricultural sectors of developing countries a staggering 96 billion USD in either damaged or lost crops and livestock between 2005 and 2015. Drought—which has recently battered farmers across the globe—was one of the leading culprits. Eighty-three percent of all economic losses due to drought that were documented by FAO study were absorbed by agriculture, with a price tag of 29 billion USD (FAO 2018).

As an important global commodity, soybeans are not exempt from water deficit problems and neither is Brazil, which is the second highest soybean producer worldwide and one of the few countries that could considerably increase its production over the next decades. Losses due to drought during 37 Brazilian harvests from the 1976/77 and 2013/14 crop seasons were estimated to have cost 79,62 billion USD (Ferreira 2016). To illustrate the importance of soybeans to the Brazilian economy, according to the Brazilian Institute of Geography and Statistics (IBGE), in 2015, agriculture was the only economic sector that did not reduce its contribution to the Gross Domestic Product (GDP) and instead increased its contribution 1.8% from that of the previous year, which was primarily due to the influence of soybeans and corn (FAO 2016).

Despite these positive numbers, drought periods have generated recurrent and significant losses in soybean yields. This scenario is not likely to change in the upcoming decades; instead, the climate predictions of the IPCC (Intergovernmental Panel on Climate Change) indicate that changes in both the frequency and

intensity of extreme climate events must be expected. For the coming years, it is very likely that daytime maximum and minimum temperatures will increase, accompanied by an increased frequency of hot days. It is also very likely that heat waves will become more frequent and that the number of cold waves and frost days (in applicable regions) will decline. Increases in the number of high-intensity precipitation events are also likely in many locations. In addition, the frequency of summer droughts is likely to increase in many interior continental locations, and droughts—as well as floods—associated with El Niño events are also likely to intensify. Furthermore, tropical cyclone mean and peak wind intensities and peak precipitation intensity are likely to increase. This expectancy indicates that sustainable crop production critically depends on the development of cultivars that are more tolerant to abiotic stress in general and to drought stress in particular, which may be accomplished through available genetic engineering techniques. Therefore, both the identification of genes that confer water deficit tolerance and the development of GM plants that express key responsive genes with current biotechnological tools have become a priority for agricultural research given the current global climate change conditions (Ramiro et al. 2016).

In response to environmental changes, plants, which are sessile organisms, have evolved a set of morphological, physiological, biochemical, cellular, and molecular mechanisms to metabolically cope during water deficit periods (Fang and Xiong 2015). The products of these stress-inducible genes may be classified into two groups. The first group includes proteins that are mostly associated with abiotic stress tolerance and include molecules, such as chaperones, late embryogenesis abundant (LEA) proteins, osmotins, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases. The second group is comprised of regulatory proteins, which are protein factors involved in the subsequent regulation of signal transduction and stress-responsive gene expression, and include various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules like calmodulin-binding protein (Hasegawa et al. 2000, Shinozaki and Yamaguchi-Shinozaki 2007). Nonetheless, drought induces the expression of abscisic acid (ABA)-dependent and ABA-independent genes (Shinozaki and Yamaguchi-Shinozaki 2000, Yamaguchi-Shinozaki and Shinozaki 2005), which points to the existence of a complex regulatory mechanism involved in the perception of abiotic stress signals (Shinozaki and Yamaguchi-Shinozaki 2000, Zhu 2001).

Among the drought stress-tolerant genes currently used, transcription factors (TFs) and key genes in drought-responsive pathways show great potential for the development of plants more resistant to water deficits. Specifically, transcription factors have shown great potential as they are able to recognize and bind to specific DNA sequences in the regulatory regions of target genes, activating and regulating the expression of the downstream genes responsible for cellular protection processes under conditions of dehydration (Shinozaki and Yamaguchi-Shinozaki 2007). The dehydration-responsive element binding protein (DREB)

TF is part of an ABA-independent drought-response pathway. By interacting with the *cis*-C-repeat/dehydration response element (CRT/DRE) [consensus sequence (A/G)CCGAC] by the AP2 DNA-binding domain, which is present in the promoter region of target genes, these TFs mediate downstream gene expression in response to environmental stress. The insertion of the TF *AtDREB1A*, which is under the control of the stress-inducible rd29A promoter, has been found to successfully improve drought tolerance responses in *Arabidopsis thaliana* (Liu et al. 1998, Jaglo-Ottosen et al. 1998, Gilmour et al. 1998), tobacco (Kasuga et al., 2004), rice (Dubouzet et al. 2003, Oh et al. 2005, Ito et al. 2006), maize (Qin et al. 2004, 2007), wheat (Pellegrineschi et al. 2004, Gao et al., 2009), and peanut plants (Bhatnagar-Mathur et al. 2004, 2007, Devi et al. 2011, Vadez et al. 2013). Also, *AtDREB1A* has been successfully introduced into soybeans with promising results for the improvement of drought tolerance (Polizel et al. 2011, Rolla et al. 2013, Fuganti-Pagliarini et al. 2017).

The DREB2A protein, which belongs to the DREB family, has been used to develop genetically modified drought-tolerant plants. In *Arabidopsis*, the overexpression of a constitutively active (CA) DREB2A form was found to result in significantly improved tolerance to drought and heat stress (Sakuma et al. 2006a, 2006b). *AtDREB2A* homologous genes have been studied in maize (Qin et al. 2007), rice (Dubouzet et al. 2003), sunflowers (Almogueva et al. 2009), wheat (Terashima and Takumi 2009), and chrysanthemums (Liu et al. 2008). In addition, *AtDREB2A* was successfully introduced into soybeans (Engels et al. 2013). Recently, Mizoi et al. (2013) identified a soybean DREB2 gene, *GmDREB2A;2,* and showed that its heterologous expression in *Arabidopsis* induced stress-inducible genes, such as *RD29A*, *RD29B*, *HsfA3*, and *HSP70*, and improved stress tolerance. Marinho et al. (2020, submitted) successfully introduced the *GmDREB2A;2* TF in soybean plants.

When considering the ABA-dependent TFs, the ABA-responsive element-binding protein (AREB) family has shown interesting results with regard to conferring drought tolerance. In *Arabidopsis*, AREB acts as the major TF family under conditions of abiotic stress (Yamaguchi-Shinozaki and Shinozaki 2005, Kobayashi et al. 2008, Lee et al. 2010, Yoshida et al. 2015) and has been reported to regulate environmental stress responses and ABA signaling during the vegetative stage (Jakoby et al. 2002, Fujita et al. 2005, Yoshida et al. 2010). These TFs target the expression of water deficit-responsive genes by binding to conserved *cis*-elements, called ABA-responsive elements (ABRE; PyACGTGG/TC) present in the promoter regions of target-genes (Barbosa et al. 2012). In *A. thaliana*, the overexpression of *AREB1* has been found to result in ABA hypersensitivity, the induction of drought-responsive genes like *RD29B*, and improved water deficit tolerance (Fujita et al. 2005). In *Glycine max*, plants overexpressing *AtAREB1* TF showed interesting results that supported the potential of *AtAREB1* TF for improving drought tolerance (Barbosa et al. 2012, Leite et al. 2014, Marinho et al. 2016, Fuganti-Pagliarini et al. 2017).

Transcription factors have not been the only focus of research to improve drought tolerance in plants,

genes have also been associated with key response mechanisms, such as *GolS* and *NCED*, which encode the enzyme galactinol synthase (EC 2.4.1.123) from the raffinose family of oligosaccharides (RFOs) and the key enzyme in ABA biosynthesis 9-*cis*-epoxycarotenoid dioxygenase (EC 1.13.11.51), respectively. Raffinose family oligosaccharides (RFOs), such as raffinose, stachyose, and verbascose, act as osmoprotectants and are known to be involved in responses to adverse environmental conditions. In drought tolerance responses, RFOs are able to regulate osmotic potential and protect both enzymes and membranes from different sources environmental stress (Panikulangara et al. 2004, Pattanagul and Madore 1999). The *GolS* genes have been reported to be upregulated by abiotic stress in many plant species, such as rice (*Oryza sativa*; Takahashi et al. 1994), grapes (*Vitis vinifera;* Pillet et al. 2012), tobacco (*Medicago falcata*; Zhuo et al. 2013), and *Salvia miltiorrhiza* (Wang et. 2012). In particular, drought tolerance has been reported for plants expressing *GolS* genes like *A. thaliana* (Taji et al. 2002), tomatoes (*Solanum lycopersicum* Mill. cv Moneymaker; Downie et al. 2003), coffee [*Coffea arabica* (Santos et al. 2011) and *Coffea canephora* (Santos et al. 2015)], *Populus tricocarpa* (Zhou et al. 2014), and soybeans (Marcolino-Gomes et al. 2014, Rodrigues et al. 2015).

As ABA is an important hormone that triggers the responses of plants to adverse environmental conditions (Barbosa et al. 2012, Cao et al. 2013, Takeuchi et al. 2014, Park et al. 2015), the genes related to its metabolism are also targets for genetic manipulation. Accordingly, several studies have reported a improved performance of plants under water deficit conditions due to the overexpression of genes that encode enzymes of the ABA biosynthetic pathway (Iuchi et al. 2001, Endo et al. 2008), specifically, the *NCED3* gene, which encodes 9-*cis*-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51), a key enzyme in ABA biosynthesis (Bhaskara et al. 2012, Behnam et al. 2013). In *Arabidopsis*, the overexpression of *AtNCED3* has been found to increase endogenous ABA levels, promote the transcription of drought- and ABA-inducible genes, and improve drought tolerance in GM plants (Iuchi et al. 2001). The *NCED3* gene has also been reported to be strongly induced under simulated drought or greenhouse conditions as well as in several economically important crops like tomatoes (Burbidge et al. 1999), common beans (Qin and Zeevaart 1999), cowpeas (Iuchi et al. 2000), avocados (Chernys and Zeevaart 2000), peanuts (Wan and Li 2006), turmeric (Ahrazem et al. 2012), citrus (Rodrigo et al. 2006, Neves et al. 2013, Pedrosa et al. 2015), and soybeans (Molinari et al. 2020).

Here, we evaluated the drought tolerance of soybean lines genetically modified for the *AtDREB1A*, *AtDREB2A*, *GmDREB2A*, and *AtAREB1* TFs and the *AtGolS2* and *AtNCED3* genes under both greenhouse and field conditions. The plants were phenotyped by drought tolerance in water-deficit and control treatments based on molecular, physiological, growth, agronomical, and survival parameters. It is important to highlight that the data obtained under greenhouse conditions indicated the potential of the DREB and AREB TFs and *GolS* and *NCED* genes to develop genetically modified drought-tolerant soybean lines. These data were generated under controlled conditions in which light, temperature, water, weed, insect, and disease levels

were monitored. According to Passioura (2012), results obtained in greenhouses may not be representative of the way in which plants behave throughout an entire season under actual field conditions. As such, this study presents a comparison of the results obtained in the field, where researchers can accurately gauge whether a technology is successful, with those obtained under greenhouse conditions. Similarly to that of other countries, field tests constitute a legal requirement of the Brazilian National Technical Biosafety Commission that must be both fulfilled and approved prior to the authorization of a commercial product.

Furthermore, the combined knowledge obtained from both greenhouse and field tests may provide new insights into the mechanisms of drought tolerance in soybean plants that may help breeders to choose the best performing lines for their breeding programs and develop cultivars that may be released to producers, which continue to face production challenges associated with drought conditions.

Soybeans genetically modified for drought tolerance

Brief description of the obtention of the soybean GM lines

Several soybean conventional cultivar backgrounds were used to obtain the GM lines containing the different genetic constructions: *rd29A:AtDREB1A*, *rd29A:AtDREB2A*, *35S:AtAREB1*, *35S:GolS2*, *35S:AtNCED3*, *35S:GmDREB2AFL*, and *35S:GmDREB2ACA*. As a standard protocol, these constructions were introduced via electroporation into the *Agrobacterium tumefaciens* strain EHA 105 (Hood et al. 1993) as described by Casali and Preston (2003). Both *rd29A:AtDREB1A* and *rd29A:AtDREB2A* were under the control of the inducible promoter *rd29A*. The other five genetic constructions were under the control of the constitutive promoter CaMV *35S* (Cauliflower mosaic virus). All vectors contained the *NOS* terminator (*A. tumefaciens* nopaline synthase) and two marker genes in the cassette structure: the *bar* gene (phosphinothricin acetyl transferase), which confers resistance to the herbicide ammonium glufosinate and was used as a selective agent; and the *NPTII* gene (Neomycin phosphotransferase), which confers resistance to the antibiotic kanamycin and was used to select the colonies containing the inserted transgene.

The genetic backgrounds (soybean conventional cultivars) were transformed using the *A. tumefaciens* method described by Paz et al. (2006). A modification was introduced that aimed to improve injury from infection; thus, each cotyledon was scratched 10 to 12 times using a stainless-steel micro brush. Seedlings developed during the selection process were transferred to a substrate/sand (1:1) mixture with the substrate containing soil/sand/organic compounds (3:2:2). Seedlings were maintained in a growing chamber and acclimated for at least 1 week. After which, the seedlings were transferred to a greenhouse and were molecularly evaluated to identify the presence of the transgenes of interest.

The confirmation of possible positive events was performed through conventional PCR assays using specific primers to identify the inserts. Thus, genomic DNA was extracted from leaf tissues (Doyle and Doyle

1987). The PCR reaction was performed in a final volume of 25 μL composed of 5 μM of each forward and reverse primer, 0.4 mM dNTPs, 2 mM magnesium chloride, 1 U Taq DNA polymerase, and 50 ng μL^{-1} DNA. Amplifications were performed in a Veritti® (Applied Biosystems, Foster City, USA) thermocycler using the following cycling parameters: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72° C for 30 s, with a final elongation cycle of 72 °C for 5 min. The PCR products were analyzed using 1% agarose gel electrophoresis (1x SB) stained with ethidium bromide.

Plants positive for the transgenes were grown in a greenhouse for event selection with 3:1 Mendelian segregation in the T_1 generation, and the generation was allowed to progress to obtain homozygous seeds. Homozygous plants were used in the subsequent experiments to evaluate growth, survival, molecular, physiological, and agronomical parameters under greenhouse (GH) and field conditions in the water-deficit and control treatments.

Brief description of water deficit experiments

Greenhouse (GH) conditions

To phenotype the GM lines under GH conditions, several experiments were carried out. As a standard procedure, seeds from all GM events and from conventional soybean cultivars (WT plants) were treated with Vitavax® Thiram 200 SC (200 g L^{-1}; ADAPAR) for health quality purposes and then allowed to germinate on Germitest® paper for 96 h at 25 ± 1 °C and 100% relative humidity (RH). All plants used in these GH experiments had been previously identified as being positive for the target gene of interest.

In one of the experiments carried out under GH conditions with *AtDREB1A* lines, after germination, the plants were cultivated in pots containing sand and soil with 15% gravimetric humidity (GravH) for 31 days post-sowing until reaching the reproductive stage R1 (Fehr et al. 1971). Immediately after reaching R1, irrigation was withheld in the drought treatment pots until the GravH values decreased to 5% (moderate water deficit, MoWD). Twenty-nine days later, irrigation was further reduced to 2.5% GravH (severe water deficit, SWD) for approximately 30 days until harvest. The control plants were kept at 15% GravH throughout the experiment. To keep the pots at the desired GravH, they were weighed twice a day and water was added as needed (Casagrande et al. 2001). Photosynthesis (*A*), stomatal conductance (*gs*), the transpiration rate (*E*), and chlorophyll content were measured for each treatment (i.e., BR 16-treated, BR 16-control, *AtDREB1A* line P58-treated, and P58-control) under moderate and severe water deficits using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, USA) and a SPAD-502 chlorophyll meter (Sakai, Japan). Plant height was measured in each treatment. An analysis of variance (ANOVA) and Tukey post-hoc tests were performed using SAS software (Cary, USA). Anatomical analyses were also performed for samples at the R2 development stage with a MoWD (5% GravH) as well as at R4 stage with a SWD (2.5% GravH).

In another experiment conducted under GH conditions, after germination, the seedlings were

transferred to 1-L pots filled with a substrate mixture of soil:sand:organic compounds (3:2:2). Each pot contained only one seedling. All seedlings were maintained in a greenhouse at 28 ± 2 ºC, with temperature and relative humidity (RH) recorded every 5 min with a Hobo U14-002 thermistor (Onset®, Bourne, USA). The experiment was set in a complete randomized block design with a factorial arrangement of treatments, two water conditions (water deficit, WD; control, C), two plant types (GM and non-GM plants), and nine replications. Pots containing the GM lines were maintained at a 100% soil field capacity (FC) through daily irrigation with a fixed water volume sufficient to saturate the substrate until plants reached the phenological stage V4. At this stage, one day before WD induction, all pots were saturated with water at the end of the afternoon to allow the excess water to be drained overnight. The following morning, the pots were wrapped in polyethylene bags, and the central region of each pot was covered with cotton around the stem base in order to prevent water loss by evaporation. Then, the pots with control plants were maintained at 100% soil FC, while irrigation was withheld in the WD group, which was monitored daily in relation to stomatal conductance (*gs*). As a standard protocol to confirm the induction of the WD, when plants showed *gs* values less than 200 mmol H2O m^{-2} s^{-1} (Flexas et al. 2004, Salinet 2009), the gas exchange parameters (*A*, sub-stomatal CO_2 (*Ci*), *E*, and gs) were measured on the central leaflet of the third fully-expanded trifoliate leaf (apex-base direction) with an LC pro-SD portable infrared gas analyzer (ADC BioScientific, Hoddesdon, UK) in three plants. Measurements were performed inside the greenhouse at 9:00 a.m. (Brazilian daylight savings time) with 1000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). The intrinsic water use efficiency was obtained through the ratio *A/gs.* Thereafter, the same trifoliate leaf was sampled, wrapped in aluminum foil, immersed in liquid nitrogen, and stored at -80 ºC for the analysis of gene expression by RT-qPCR (Real-Time Quantitative Polymerase Chain Reaction).

After seven days of withheld irrigation, the number of nodes (NN) was counted in a sample of five plants, and the leaves were collected. Total leaf area was measured in this 5-plant sample using a LI-3100C leaf area meter (Li-Cor, Lincoln, USA). Then, the leaf blades, stems, petioles, and roots were dried with a forced aeration oven at 60 °C to constant weight so that shoot (leaf blades + stems + petioles) and root dry matter (per plant) could be weighed. Plant height was measured at the start (H1) and end (H2) of the WD period. The mean length of the internodes corresponded to the ratio between H2 and the number of nodes. The relative growth rate in height (RGRH) was calculated according to Eq (1):

$$RGRH\ (\%) = \frac{(H2-H1)}{H1} \times 100 \qquad \text{Eq. (1).}$$

Following the evaluation of the growth parameters and the sampling of the trifoliate leaves for the gene expression analysis, the plants were transferred to 8-L pots filled with a substrate composed of soil:sand:organic compounds (3:2:2) and maintained under continuous irrigation conditions until the end of the cycle, when the agronomical traits of the number of seeds, number of pods presenting seeds, total number of seeds, and yield were evaluated per plant.

Survival experiments were also carried out in the GH GM lines. The experimental design was completely randomized and included 10 plants. Prior to the WD initiation, all pots were saturated with water, drained overnight, and covered with plastic bags until the following morning, after which, irrigation was withheld. The monitoring of the water deprivation symptoms was performed visually and daily. When plants of the drought-sensitive cultivar BR 16 were almost 100% dry, the plants were watered to avoid death. After the plants that remained alive had recovered leaf turgor following rehydration, the number of plants that survived the water deficit was determined.

A hydroponic experiment to simulate the WD in the roots was conducted with lines containing *AtDREB2A* FL according to the protocol of Martins et al. (2008). At the V3 vegetative developmental stage, dehydration treatments lasting 0 (control, without stress), 30, 60, and 90 min under WD conditions were applied in triplicate, with each plant being considered a biological replicate. Triplicate measurements of the photosynthetic rate, stomatal conductance, transpiration, and leaf-air temperature differences were obtained during the treatments using an LI-6400 portable photosynthesis system (Li-Cor, Lincoln, USA). The data were analyzed statistically using the Duncan test at 5% significance ($p < 0.05$) in SAS software (Cary, USA).

Likewise, a molecular characterization was performed of the plants submitted to experiments under GH conditions. In addition to the expression of the transgenes introduced into the soybean genotypes, many other drought response-related genes were analyzed. In the *AtDREB1A* GM lines, the genes assayed were *GmLEA14* (late embryogenesis abundant; Glyma.18G238700), which encodes a contributor to osmotic stress protection in both embryonic and vegetative tissues; *GmGR-RBP*, which encodes a glycine-rich RNA-binding protein (Glyma.12G043000); *GmPI-PLC*, which encodes a phospholipase C (Glyma.14G059200); and *GmSTP*, which encodes a sorbitol transporter protein (Glyma.11G119500; Polizel et al. 2011).

In the *AtAREB1* GM soybean lines, the expression level of the 2C-type protein phosphatase *PP2C* (Glyma.14G195200), G*mSRK2* kinase (Glyma.02G135500), and *GmRAB18* (Glyma.09G185500) genes was assessed based on the results of the cDNA microarrays of *A. thaliana* under water deficit conditions (Fujita et al. 2009, Yoshida et al. 2010).

The GM lines for the *AtGolS2* gene were assayed to determine the expression level of the genes *GmRS1* (Glyma.03G137900) and *GmRS3* (Glyma.19G004400), which encode raffinose 1 and raffinose 3 and the late embryogenesis abundant proteins *LEA2* (Glyma.09G185500) and *LEA6* (Glyma.17G164200), respectively, (Honna et al. 2016). The *AtNCED3* lines were assessed to determine the expression levels of ABA-dependent genes, such as *GmAREB1* (Glyma.04G039300, Glyma.07G213100, and Glyma.02G131700), *GmPP2C* (Glyma.14G195200), *GmSnRK2* (Glyma.02G135500), and *GmAAO3* (Glyma.14G045100; Molinari et al. 2020). For the GM lines containing *AtDREB2A*, a molecular characterization was carried out for samples collected under field conditions (Fuganti-Pagliarini et al. 2017).

Field conditions

Before installing the experiment, all of the necessary documentation to test GM lines under field conditions were submitted and approved by the National Technical Biosafety Commission (CTNBio). The permissions to carry out the experiments were published in the Brazilian Official Journal.

The first field experiment was conducted during the 2011/2012 crop season as a "pilot screening." Subsequent experiments were carried out in the field area located at the National Soybean Research Center (23°11' S, 51°11' W, 630 m altitude; Embrapa Soja, Londrina, PR, Brazil), which is a branch of the Brazilian Agricultural Research Corporation, during the crop seasons of 2013/2014 to 2017/2018. A split-plot design was used in a complete randomized block design with four blocks. The plots corresponded to irrigated (IRR, water from precipitation + irrigation when needed) and non-irrigated (NIRR, water from only precipitation) water conditions and artificially drought simulated (DS) conditions at the vegetative (DSV) and reproductive (DSR) stages. To mimic drought conditions, the plants were sheltered from the rain by rainout shelters programmed to automatically close when rainfall was detected and to open as soon as the rain ceased.

The subplots corresponded to the conventional Brazilian soybean cultivars and GM lines for the *AtDREB1A*, *AtDREB2A*, and *AtAREB1* TFs and the *AtGolS2* and *AtNCED* genes. The area of each subplot was 220 m^2 for the IRR and NIRR treatments. The seeds were sown with 0.5-m spacing between rows and 16 plants m^{-1}. Plants of the soybean cultivar BRS 295RR were used as a 10-m wide isolation border around the experiment, following the stipulations of Brazilian legislation. The air temperature and relative humidity were monitored daily with a weather station located adjacent to the experimental area.

Soil chemical corrections and cultivations were performed according to recommendations for the crop (Embrapa 2013). During the experiment, physiological and agronomic evaluations were performed. The net CO_2 assimilation rate (*A*), transpiration rate (*E*), and stomatal conductance (*gs*) were measured from the central leaflet of the third fully-expanded trifoliate leaf (apex-to-base direction) of one plant located in the middle portion of each subplot with a LCpro-SD portable infrared gas analyzer (ADC BioScientific) calibrated for 1000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) under sunny sky conditions between 9 and 11 a.m. (Brazilian daylight savings time). After gas exchange measurements were taken, both the instantaneous (*A*/*E*) and intrinsic (*A*/*gs*) water use efficiency (WUE) were calculated. The chlorophyll index (SPAD) was measured in one lateral leaflet from the same aforementioned trifoliate leaf using a SPAD-502 portable chlorophyll meter (Minolta). Plant height was calculated as the mean distance between the cotyledonary node and the stem apex from five plants per subplot. The mean length of the internodes corresponded to the ratio between the height per plant and number of nodes per plant. The leaf area index (LAI) corresponded to the ratio between the total leaf area, which was obtained with an LI-3100C area meter (LI-COR), and the soil area occupied by the plants. The total dry matter of the pods and seeds per plant and grain yield per plant were evaluated (10 plants per subplot) at harvest. These measurements were obtained

for all four experimental blocks at the reproductive developmental stage. The percentage of protein and oil content in the soybean grain samples at harvest was determined from whole seeds and grains using the near infrared (NIR) reflectance technique according to the methodology of Heil (2010). For lines containing DREB1A and DREB2A TFs, these parameters were assayed during the crop seasons of 2013/2104 and 2014/2015. For lines containing the *AtAREB1A* FT or the *GolS2* and *NCED3* genes, oil and protein content was sampled in four crop seasons from 2014/2015 to 2017/2018.

All residuals presented normal distributions and met ANOVA assumptions. Thus, the data were analyzed via an ANOVA, and the means were compared by a Tukey post-hoc test ($p \leq 0.05$).

Molecular analyses were performed to evaluate transgene expression under field conditions. Thus, three samples from three different blocks were collected individually based on physiological results. Samples were immediately placed into liquid nitrogen and stored in a freezer at -80 °C until RNA extraction. Total RNA was extracted from the leaf samples using Trizol® reagent. Following RNA extraction, the samples were treated with DNAse I. To verify the presence of any remaining genomic DNA, a conventional PCR was performed. cDNA synthesis was carried out using the Super Script III First Strand kit (Thermofisher Scientific, Waltham, USA) according to the instructions of the manufacturer. The expression levels of the transgenes *AtDREB1A*, *AtDREB2A CA*, and *AtAREB1 FL* were assessed by qPCR. Also, based on a search of the available literature, some genes related to drought responses were selected. These analyses were carried out only for the GM soybean event 1Ab58 (*AtDREB1A*), 1Bb2193 (*AtDREB2A CA*), 1Ea2939 (*AtAREB1)* lines and the conventional cultivar BR 16. Soybean lines containing *AtGolS2*, *AtNCED3*, *GmDREB2A;2 FL*, and *GmDREB2A2; CA* were not included in this evaluation. Thus, the expression level of the chosen genes was quantified under IRR and NIRR conditions. Genes related to drought response (i.e., stomata overture/closure and osmotic adjustment), photosynthesis, and metabolic and hormone pathways (i.e., nitrogen assimilation), drought proteins (e.g., dehydrins, DHNs), heat shock proteins, and water channels were chosen. Therefore, the selected genes were phosphatase *GmPP2C* (Glyma.14G195200), alanine aminotransferase *GmAlaAT* (Glyma.01G026700 and Glyma.07G045900), Δ-1-pyrroline-5-carboxylate synthetase (P5CS; Glyma.18G034300), galactinol/GolS (Glyma.10G145300), late embryogenesis abundant/LEA18 (Glyma.17G164200), DHN (Glyma.09G185500), heat shock protein (Glyma.17G072400), putative soybean aquaporin pip1/UDP galactose transporter (Glyma.12G066800), putative soybean aquaporin pip2/aquaporin transporter/glycerol uptake facilitator (Glyma.12G172500), ribulose-1,5-bisphosphate carboxylase/ oxygenase (small chain; Glyma.13G046200), and chlorophyll a/b binding protein (Cab21; Glyma.16G165800; Fuganti-Pagliarini et al. 2017).

Using the gene sequences obtained from Phytozome, sets of primers for each gene were designed using the Primer3Plus platform available online (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). To verify homo- and heterodimers and hairpin formations, multiple primer analyzer software was used

(http://www.thermoscientificbio.com/webtools/multipleprimer/). Quantitative PCR reactions were carried out in biological and technical triplicate using the Platinum® SYBR Green® qPCRSuperMix-UDG kit with ROX (Thermofisher Scientific, Waltham, USA) according to the instructions from the manufacturer in a 7900HT Fast Real-Time PCR System with a 384-well block (Thermofisher Scientific, Waltham, USA). The β-actin gene (No. Access: GMU60500) was used as the reference gene (Stolf-Moreira et al. 2011).

The efficiency of the amplification reaction was estimated using five serial dilutions of cDNA (1×, 5×, 25×, 125×, and 625×). To compute the efficiency of the reaction (E), the relationship presented in Eq (2) was used:

$$E = \left[10^{\frac{-1}{slope}}\right]^{-1} \quad \text{Eq. (2)}$$

where the =SLOPE (Average Ct value range, log quantity range). Only primers with > 90% efficiency were used. The cycling parameters for the reactions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 1 min. To evaluate the specificity of the amplified products, a dissociation curve was generated at the end of each reaction. The relative expression was determined by normalization to the reference gene β-actin. Expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Bustin 2002).

Results from greenhouse and field characterization

Soybean lines containing DREB transcription factors

Different soybean lines containing the *AtDREB1A* TF were phenotyped under greenhouse conditions. One of the first studies was carried out with the P58 line (Polizel et al. 2011). In this line, *AtDREB1A* gene expression was higher in the genetically modified P58 plants under water deficit conditions, demonstrating transgene stability in the T_2 generations and the induction of the *rd29A* promoter. Drought-responsive genes, such as *GmPI-PLC*, *GmSTP, GmGRP* and *GmLEA14,* were highly expressed in plants submitted to the severe WD treatment (gravimetric humidity at 2.5%). Genetically modified plants showed higher stomatal conductance and consequently higher photosynthetic and transpiration rates. In addition, they had more chlorophyll. The overexpression of *AtDREB1A* may have contributed to a decrease in leaf thickness; however, a thicker abaxial epidermis was observed (Polizel et al. 2011).

A subsequent experiment was carried out with additional *AtDREB1A* lines. Rolla et al. (2013) evaluated GH plants from the P58 and P1142 lines in the T_8 and T_5 generations, respectively. A growth analysis of the plants under well-watered (C, control) and WD conditions showed that the *AtDREB1A* plants exhibited lower heights (C/WD), the same number of nodes (C/WD), a slightly higher number of leaves (WD), and a greater leaf area (C/WD) than that of the BR 16 plants. However, a statistical analysis of these data showed that none of these differences were statistically significant, indicating that the transformation of

soybean plants with the *AtDREB1A* gene under the control of the *rd29A* promoter did not lead to any retardation of the growth of the transformed plants. Although there were no statistically significant differences in the relative growth ratio and the percentage of growth reduction under water deficit conditions when compared to that of the control plants, the *AtDREB1A* lines exhibited a more conservative growth pattern under control conditions and slightly increased their growth rates under WD conditions compared to that of the control plants. Furthermore, although not statistically significant, *AtDREB1A* plants seemed to have a higher number of leaves and a greater leaf area than the BR 16 plants, at least in the latter stages of development (Rolla et al. 2013).

As has already been reported, previous studies under GH conditions showed that the *AtDREB1A* P58 line had a slow-wilting phenotype and was able to maintain a higher rate of photosynthesis and higher photosynthetic efficiency under water-deficit conditions than that of the control plants (Polizel et al. 2011). In this second study, GH data suggested that the higher survival rates of the *AtDREB1A* plants (70%, 60%, and 40% for GM lines P58, P1142, and the conventional cultivar BR16, respectively, after a severe water deficit) were due to lower water use resulting from lower transpiration rates under well-watered conditions in the GH experiments (**Fig. 1A**; Rolla et al. 2013).

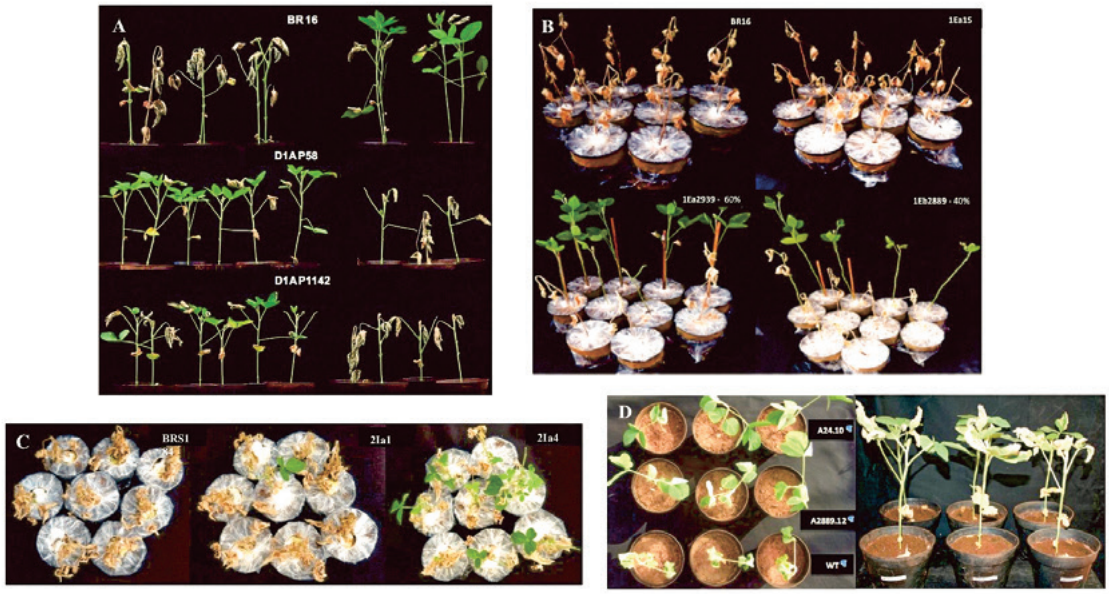

Fig. 1. Survival rate of soybean plants genetically modified with *AtDREB1A* (A), *AtAREB1A* (B and D), and *AtGolS2* (C) and their respective parental cultivar.
In A, DREB1A plants P58 and P1142 show 60% and 70% overall survival rates, respectively, while that of BR 16 is 40%. These lines were submitted to a 6-day water deficit followed by three days of recovery. In B and D, the *AtAREB1A* lines 1Ea15, 1Ea2939, A24, and A2889, and the WT cultivar, BRS 184, are shown. These lines were submitted to 17 days of withhold irrigation followed by 7 days of re-watering. In C, the survival rate of the *AtGols2* lines 2Ia1 and 2Ia4 submitted to withheld irrigation for 20 days followed by nine days of rehydration are shown. The *AtDREB1A* lines were under the control of the stress-inducible rd29A promoter, while the *AtAREB1A* and *AtGolS2* lines were under the control of the constitutive promoter 35S. Adapted from Rolla et al. 2013, Barbosa et al. 2012, and Marinho et al. 2016.

In addition to DREB1A from *Arabidopsis thaliana*, the GM soybean plants were obtained using *GmDREB2A;2 FL* and *GmDREB2A;2 CA* genes, which were under the control of the constitutive promoter CaMV *35S*. These lines were characterized for molecular, physiological, phytometric, and agronomic responses at three developmental periods (i.e., germinative, vegetative, and reproductive; Marinho et al., 2020, submitted). Therefore, three different and independent lines were submitted to WD experiments conducted under GH conditions. Molecular data showed that transgene expression was significantly higher for all three GM events under control and water deficit conditions when compared to that of the conventional background cultivar BRS 283. Drought-responsive genes were also induced in the *GmDREB2A;2 CA* and *GmDREB2A;2 FL* lines. Under the imposed water deficit, in both the vegetative and reproductive developmental stages, the expression level of *LEA6* (Glyma.17G164200), *LEA2* (Glyma.09G185500), and heat shock protein *HSP70* (Glyma.17G072400) genes was higher for all GM events when compared to that of the conventional cultivar BRS 283 (Marinho et al. 2020, submitted). LEA2 and LEA6 are Dehydrins (DHNs), which typically accumulate at the seed maturation stages and in plant tissues in response to drought, high salinity, low temperatures, or treatment with ABA (Battaglia et al. 2008). The contribution of DHNs to the abiotic stress tolerance of plants occurs mainly due to their protective effects on lipid membranes (Bao et al. 2017). Koag et al. (2009) have identified that the interaction of such proteins with lipids in the membrane or with partially denatured proteins helps to protect cells against damage caused by low water potential. Yet, DHNs play critical roles in determining desiccation tolerance by capturing water and stabilizing and protecting the structure and function of proteins and membranes in addition to acting as molecular chaperons (as heat shock proteins) and hydrophilic solutes to protect cells from damage due to water shortages (Hand et al. 2011).

Phytometric data showed a reduction in the growth rate for total seedling length and root length under osmotic conditions (-0.2 MPa of polyethylene glycol, PEG-8000); however, seedlings from the *GmDREB2A;2 FL* line presented a lower reduction in growth during germination. The physiological parameters evaluated indicated that the WD imposition resulted in a sharp reduction in all gas exchange parameters (*gs*, *Ci*, *A*, and *E*). For the *gs* and *Ci* parameters, the results indicated a lower performance of the conventional cultivar BRS 283 when compared to that of the GM events in the WD treatments; however, the interaction between genotypes and water conditions was not significant. Similarly, the *gs* of the cultivar BRS 283 also showed a lower trend compared to that of the GM lines. In contrast, the CO_2 intercellular concentration was higher in the conventional cultivars when compared to that of the GM events (Marinho et al. 2020, submitted).

The physiological responses of the GM plants, especially *A*, indicated improved physiological performance under conditions of water restriction. Drought conditions lead to a deficit in *A* caused by stomata and non-stomata limitations. Therefore, the low values of *A* found in the conventional cultivar BRS 283

might have contributed to its lower tolerance compared to that of the GM plants, which was reflected in a lower green leaf area (Marinho et al. 2020, submitted).

Under GH conditions, when WD was imposed in the vegetative and reproductive developmental stages, the GM *GmDREB2A;2 FL* line presented the highest yield after drought imposition during the reproductive period when compared to that of other materials. These data suggest that it is possible to obtain soybean GM lines with the *GmDREB2A;2* TF with minor damage to final yields, which is extremely desirable when considering a grain crop, such as soybeans. It was proposed that the *GmDREB2A;2 FL* line presented superior performance due to the high expression levels of the transgenes and drought-induced genes. These studies also indicated that the *GmDREB2A;2* TF participates in important responses to water deficits during different periods of soybean development (Marinho et al. 2020, submitted).

For the *AtDREB2A* TF, laboratory and GH studies were performed with the GM soybean lines P1397 and P2193. In a biological oxygen demand (BOD) incubator, leaflets from the T_0 and T_1 generations were dehydrated for 30 and 90 min by subjecting the detached leaves to a temperature of 30 °C and 60% humidity. Leaflets of the non-transformed conventional soybean cultivar BR 16 were used as negative controls. Results of the molecular characterization indicated a greater stability of transgene *rd29A:AtDREB2A CA* expression in the P2193 line compared to that of the P1397 line, which only exhibited notable expression in the control plants from the T_1 generation. Transgene instability in the P1397 line was further demonstrated by the possible repression of gene expression after a 90-min exposure to treatment (Engels et al. 2013).

Under GH conditions, a hydroponic experiment was carried out to simulate drought in the roots of the *AtDREB2A CA* lines. Molecular data obtained from the roots and leaves showed that both GM lines exhibited high expression of the transgene, with the roots of P2193 showing the highest expression levels during water deficit conditions. The physiological parameters examined during WD conditions confirmed the induction of stress. The *A, Ci*, *E*, and leaf-air temperature values of the *AtDREB2A CA* transgenic plants differed from those of the control cultivar BR 16 for each treatment (Engels et al. 2013). These findings confirmed the presence of stress in the hydroponic system, with a tendency to reduce the rates of stomatal conductance, photosynthesis, and transpiration (Jaleel et al. 2009).

The first experiment carried out under field conditions as an initial screening was conducted during the 2011/2012 crop season with the *AtDREB1A* lines P58 and P1142 and the genotype 09D-0077, which resulted from a cross between the P58 line and its isoline, the BR 16 cultivar. The field performance of the P58 and 09D-0077 plants was evaluated under four different water regimes: irrigated (IRR), natural rainfall (NIRR, non-irrigated), and drought simulated (DS), in which plants were sheltered from the rain in the vegetative (DSV) or reproductive (DSR) stages. The drought treatments affected plant productivity as well as their growth and yield components. Under conditions of water deficits in both the vegetative and reproductive stages, the main effects of the *AtDREB1A* gene were observed in the changes in plant height

due to a shortening of the internode, at least at the initial stages of crop growth in the field. However, there were no significant differences in the growth components between P58 and 09D-0077 plants under field conditions. No significant differences were observed in yield components among genotypes, except for the number of nodes, which was higher for the P58 line in the non-irrigated treatment and the 09D-0077 cross under a water deficit during the vegetative stage compared to that of the control plants. Although the DREB plants did not outperform their isoline (cultivar BR 16) in terms of yield, there was a clear tendency towards the superiority of the DREB line with regard to some yield components, such as the number of seeds and the total number of pods when WD was applied during the vegetative stage (Rolla et al. 2013).

The soybean *AtDREB1A* line P58 and *AtDREB2A CA* line P2193 were assayed under field conditions during the crop seasons of 2013/2014 (**Fig. 2**) and 2014/2015 for physiological and agronomical parameters (Fuganti-Pagliarini et al. 2017). The results from the crop season of 2013/2014 for instantaneous (*A*/*E*) and intrinsic (*A*/*gs*) water use efficiency and the LAI did not show any significant interactions between water conditions and plant materials. In each water condition, there were no differences between plant materials with regard to *A*/*E*. The lines 1Ab58 and 1Bb2193 showed similar behaviors to that of the wild type (WT-BR 16) plants with regard to chlorophyll content regardless of the water conditions. With regard to plant height and the mean length of internodes, the lines 1Ab58 and 1Bb2193 showed similar values relative to those of the WT genotype in the IRR and NIRR treatments. With regard to the total dry matter of pods and seeds per plant, the line 1Bb2193 showed lower values in the NIRR treatment for both agronomical traits compared to that of the control plants (Fuganti-Pagliarini et al. 2017).

Fig. 2. Field experiment during the crop season of 2013/2014.
A split-plot design was used in a complete block randomized design with four blocks. The plots corresponded to irrigated (IRR, water from precipitation + irrigation when needed) and non-irrigated (NIRR, water from only precipitation) water conditions and artificially drought simulated (DS) conditions at the vegetative (DSV) and reproductive (DSR) stages. To mimic drought conditions, the plants were sheltered from the rain by rainout shelters programmed to automatically close when rainfall was detected and to open as soon as the rain ceased. Subplots corresponded to the conventional Brazilian soybean cultivar BR 16 (genetic background of GM lines) and the GM lines for the *AtDREB1A*, *AtDREB2A* and *AtAREB1* TFs.

Yield data collected during the crop season of 2013/2014 showed that the WD caused significant losses to both DREB lines. For P58 and P2193, when a WD was applied during the reproductive stage, the yield value reached 805 and 765 kg ha^{-1}, respectively, and was more harmful to the crop compared to that when the WD was imposed during the vegetative stage (yield of 1.509 and 1.632 kg ha^{-1} for the P58 and P2193 lines, respectively). Under irrigated conditions, the values registered were 2.929 and 2.665 kg ha^{-1} for the *AtDREB1A* and *AtDREB2A CA* lines, respectively. It is important to highlight that intense water deficit periods were recorded during important developmental stages, such as flowering and pod filling, that resulted in a decrease in final yield, although the total rainfall of the crop season of 2013/2014 fell within the recommendations for the soybean crop (Fuganti-Pagliarini et al. 2017).

No differences were identified for physiological and agronomic parameters in the field experiment performed during the crop season of 2014/2015, which was probably due to the optimum rainfall volume and homogeneous distribution observed during the whole cycle, which thus resulted in a small water deficit in the plants. According to the data collected by the weather station located at the experiment site, a total rainfall of 790.8 mm was registered. The water recommendations for soybean crops vary between 450 and 800 mm/cycle, depending on the weather conditions, crop management, and the cycle duration (Embrapa 2013).

Although a short water deficit period occurred in October/2014, the experiment was sown on November 6th. Thus, no significant water deficit period occurred during the cycle, and no differences were present between GM lines and the BR 16 cultivar. As no differences were identified, no molecular analyses were performed (Fuganti-Pagliarini et al. 2017).

The gene expression analysis performed on samples collected under field conditions during the crop season of 2013/2014 showed that the expression of the transgenes *AtDREB1A* and *AtDREB2CA* was induced in the NIRR treatment for each respective transgenic line. Among these TFs, higher expression was identified for the *AtDREB1A* gene (line 1Ab58). No expression was identified for the BR 16 soybean conventional cultivar. In the P58 and P2193 lines, higher levels of Cab21 (Glyma16g165800), phosphatase *GmPP2C* (Glyma.14G195200), and putative soybean aquaporin pip1/UDP galactose transporter (Glyma.12G066800) were identified when compared to the background of BR 16, illustrating that WD response mechanisms were activated under field conditions (Fuganti-Pagliarini et al. 2017).

Neither the oil nor protein content in soybean seeds were affected by insertion of the *AtDREB1A* and *AtDREB2A CA* TFs. As no significant difference was present between WD treatments, the data were analyzed combined. During the crop season of 2013/2014, the protein content in the P58 and P2193 lines was 37.8% and 37.7%, respectively. The oil content in the seeds ranged from 20.9% (P58) to 21.4% (P2193). During the crop season of 2014/2015, the overall protein content values were higher (40.1% and 39.2% for the P58 and P2193 lines, respectively) while the oil content values were lower (19.6% for both GM lines) when compared to those of the crop season of 2013/2014, which fell within the parameters accepted by the consumer market, which are protein levels between 40-45% and lipid levels between 18-20% (Embrapa 2015).

Soybean lines containing the AREB transcription factor

Two different genetic constructions with the *AtAREB1* TF were introduced into soybean plants: *35S:AtAREB1 FL* (full-length) and *35S:AtAREB1ΔQT*, a constitutive active form of AREB1 that presents a conserved transcriptional activator P domain plus the native bZIP DNA binding domain (Fujita et al. 2005). The soybean GM lines were obtained for both strategies (Barbosa et al. 2012, Leite et al. 2014).

A GH experiment with soybean lines overexpressing *AtAREB1 FL* was performed to assay the physiological parameters under water deficit and control conditions. The GM lines, A2889.12 and A24.10, showed the ability to survive for a period of 5 days without water and exhibited no leaf damage (**Fig. 1B**). In addition, these lines remained able to grow under WD conditions, which was verified by their higher relative rates of shoot length (RRSL) compared to that of the WT plants (conventional cultivar BR 16; Barbosa et al. 2012).

Furthermore, these lines also displayed better growth and physiological performance under water deficit conditions (i.e., higher RRSL, *gs*, and *A*) when compared to that of the wild type plants, which may

have been related to the responses triggered by the transgene. Particularly, line 1Ea2939 showed a higher total number of pods and seeds and increased dry seed matter compared to WT plants. The best performance of line 1Ea2939 relative to that of the BR 16 plants might have been related to the mechanisms of drought prevention, such as reduced stomatal conductance or leaf transpiration under control conditions with no water restriction (Barbosa et al. 2012).

The other lines, 1Eb2889 and 1Ea15, were obtained with the *AtAREB1 FL* gene and additional experiments were carried out under GH conditions. *AtAREB1* expression was observed in the transgenic lines 1Ea2939 and 1Eb2889 but not in the 1Ea15 line. The phenotypic analyses of the growth parameters indicated that in the early stages of seedling development under well-watered conditions, there were no differences in plant growth among the transgenic lines 1Ea15, 1Ea2939, and 1Eb2889 when compared to that of the conventional soybean cultivar BR 16. These results allowed the authors to infer that the transformation of soybean plants with *AtAREB1*, under the control of the constitutive promoter CaMV *35S*, did not alter the growth characteristics of the transformed plants. Considering the number of nodes (NN), there was a significant interaction between genotype/transgenic lines and water conditions. Thereby, the GM lines 1Ea2939 and 1Eb2889 presented a similar NN under both water conditions, whereas the BR 16 and 1Ea15 plants presented lower values under WD conditions (Marinho et al. 2016).

Physiologically, transpiration data collected throughout the water deficit period revealed that in the first days after withholding irrigation and WD imposition (2–3 days), the transpiration rates of BR 16 and 1Ea15 plants were higher relative to that of the other two GM lines (1Ea2939 and 1Eb2889). This result was probably due to the higher *gs* of the BR 16 and 1Ea15 plants grown under well-watered conditions (C). The differences in the transpiration rates among the genotype/GM lines at the beginning of the WD period resulted in a lower water status in the substrates used to grow the BR 16 and 1Ea15 plants, and as a result, these plants presented lower transpiration rates compared to that of the plants of the GM lines 1Ea2939 and 1Eb2889 (Marinho et al. 2016).

Furthermore, under well-watered conditions, the GM lines 1Ea2939 and 1Eb2889 presented lower stomatal conductance relative to that of the cultivar BR 16, confirming that the lower transpiration rates presented by these plants were due to lower *gs* and not to lower leaf areas. However, under WD conditions, the highest values of *gs* were found for the event 1Ea2939, followed by the event 1Eb2889, when compared to that of the BR 16 and 1Ea15 plants. The differences in *gs* values among genotype/GM lines under WD conditions were likely due to differences in the manner in which the plants depleted the water from the soil throughout the experimental period. Plants that had higher *gs* (BR 16 and 1Ea15) during the initial period after withholding irrigation presented a rapid depletion of water from the soil due to their high transpiration rates. However, the opposite was observed for 1Ea2939 and 1Eb2889 plants (i.e., such plants showed slow water depletion from the substrate) that led to water conservation and the maintenance of higher gas exchange

rates when compared to that of the event 1Ea15 or the cultivar BR 16 (Marinho et al. 2016).

The decreased *gs* values in the GM lines 1Ea2939 and 1Eb2889 resulted in lower intercellular CO_2 concentrations under control conditions. However, this reduction in *gs* was not large enough to promote changes in the photosynthetic rate relative to that of the BR 16 and 1Ea15 plants (Marinho et al. 2016). A decrease in stomatal conductance is one of the first responses to water deficits and usually results in a decreased photosynthetic rate (Anjum et al. 2011), which was not observed in this report.

The expression of the transgene *AtAREB1* was observed in the GM lines 1Ea2939 and 1Eb2889 but was not detected in the line 1Ea15. These lines were obtained independently. The expression level of a transgene depends on its insertion site in the genome, the number of copy/insertions, and the occurrence of gene silencing (Li et al. 2002, Svitashev et al. 2002, Altpeter et al. 2005). The *GmRAB18* gene, which is drought-responsive, was strongly induced in BR 16 and 1Ea15 plants under WD conditions; however, in the GM lines 1Ea2939 and 1Eb2889, the expression of this gene was relatively low, which supports the observed findings of daily transpiration and stomatal conductance, indicating that subjecting the GM lines 1Ea2939 and Eb2889 to WD conditions resulted in lower stress levels compared to those of BR 16 and 1Ea15 plants (Marinho et al. 2016).

The differences in gene expression of the *AtAREB1* transgene and the physiological behavior of the GM lines 1Ea2939 and 1Eb2889 reflected the survival rates and yield components. After 17 days of withholding irrigation followed by 7 days of watering, the cultivar BR 16 and the line 1Ea15 showed 100% mortality, whereas plants of the GM lines 1Ea2939 and 1Eb2889 showed 60 and 40% survival, respectively (**Fig. 1D**). The analysis of yield components showed that the transformation of soybean plants with the *AtAREB1* gene under the control of the constitutive promoter *35S*, which has often been associated with growth abnormalities, did not impair the agronomic performance of the transformed plants. The GM line 1Ea2939 presented a larger total number of pods, higher total dry pod matter, larger number of viable seeds, higher dry matter of viable seeds, higher total dry matter of seeds, and larger total number of seeds per plant (Marinho et al. 2016).

The results obtained under GH conditions indicated that the line 1Ea2939 was the most promising with regard to improved drought tolerance. Thus, this GM line was evaluated in subsequent field experiments. During the crop season of 2013/2014 (**Fig. 2**), line 1Ea2939 showed higher intrinsic water use (*A/gs*) than that of the other plant materials in the NIRR treatment as well as a higher leaf area index (LAI). Among the different materials tested, higher plant height values were registered with line 1Ea2939, which were impaired due to severe water lodging that occurred after a plentiful rain (341.4 mm in 40 days). The results showed lower productivity for the 1Ea2939 plants in the IRR treatment (2.021 kg ha^{-1}) when compared to that of the NIRR treatment (2.153 kg ha^{-1}). This difference of approximately 140 kg ha^{-1} was due to the water lodging that occurred in the IRR treatment. Although productivity and the final potential yield numbers decreased,

data from before the abundant rainfall event showed that line 1Ea2939 exhibited a higher number of nodes (21 nodes/plant in 1Ea2939 plants, while other GM lines and WT plants presented an average ranging from 14 to 15 nodes/plant) and a higher number of pods per plant compared with those of the other plants, indicating great yield potential (Fuganti-Pagliarini et al. 2017). A positive relationship between pods, nodes, and yield or nodes, pods, and seeds has been reported (Kahlon et al. 2011, Egli 2013).

Molecular data showed higher levels of drought-responsive genes in line 1Ea2939, such as phosphatase *GmPP2C* (Glyma.14G195200), alanine aminotransferase *GmAlaAT* (Glyma.01G026700), Cab21 (Glyma.16G165800), and putative soybean aquaporin pip2/aquaporin transporter/ glycerol uptake facilitator (Glyma.12G172500), when compared to that of its genetic background, the conventional cultivar BR 16. These records suggest that the stomata closure exhibited by *AtAREB1 FL* line 1Ea2939 was probably triggered by the combination of different physiological and molecular mechanisms given that Glyma.14G195200 was up-regulated and that *GmPP2C* is closely related to this drought-responsive mechanism. The expression of the light-harvesting chlorophyll a/b-binding (LHCB) proteins (Glyma.16G165800) supported this hypothesis, as these proteins have been found to positively regulate plant drought tolerance by positively controlling stomatal movement through guard cell signaling in response to ABA in *Arabidopsis*, which was also observed for soybean plants (Xu et al. 2012). In summary, considering the molecular and physiological data obtained from the field experiment, it was suggested that the GM line 1Ea2939 targeted more than one mechanism to cope with water deficit periods and presented a combined modulation of the gene expression profile and physiological responses to conserve water and protect its cells from water starvation (Fuganti-Pagliarini et al. 2017).

The oil and protein content from *AtAREB1* lines were determined from the crop seasons of 2013/2014 until 2017/2018. The oil content values ranged from 17.9% during the crop season of 2014/2015 to 21.9% during the crop season of 2017/2018. The protein percentage reached its highest value of 41.8% during the 2014/2015 season and its lowest value during the 2017/2018 season (36.6%). Therefore, the overexpression of the transcription factor *AtAREB1* led to an improved capacity of the soybean crop to cope with drought stress without yield or nutritional losses (Barbosa et al. 2012, Marinho et al. 2016).

Soybean lines containing galactinol synthase gene (AtGolS2)

Soybean lines overexpressing the *35S:AtGolS2* construction were obtained via *Agrobacterium tumefaciens*-mediated transformation. Among these, two lines, 2Ia1 and 2Ia4, were analyzed in drought-simulated and control treatments in both GH and field conditions.

The results from the GH experiments showed that the overexpression of *AtGolS2* in GM plants led to an increase in galactinol and RFO biosynthesis transcripts, such as raffinose 1 (*GmRS1*, Glyma.03G137900) and raffinose 3 (*GmRS3*, Glyma.19G004400) genes (Honna et al., 2016). Such carbohydrate accumulation

could represent an adaptive mechanism to adverse water conditions since increased water retention in the cell can delay senescence and death (Quick et al., 1989). Therefore, the accumulation of galactinol and raffinose transcripts observed in the GM line 2Ia4 could lead to the development of plants that are more tolerant to drought due to RFO accumulation, which may act as osmoprotectors under water deficit conditions by increasing the tolerance to changes resulting from the osmotic adjustment process (Turner et al. 2001). The importance of galactinol and raffinose transcript accumulation due to changes in carbohydrate metabolism under abiotic stress conditions has been previously described by Taji et al. (2002) in *Arabidopsis*. Peters et al. (2007) also observed the accumulation of carbohydrates under adverse abiotic conditions in *Xerophyta viscosa* leaves.

In addition to the role that RFOs play in drought tolerance, it is possible that the interaction between the LEA proteins and RFOs, as suggested by Liu et al. (2010) and Wolkers et al. (2001), increased the capacity of the 2Ia4 plants to survive and recover after a period of severe drought. Both *LEA2* (Glyma.09G185500) and *LEA6* (Glyma.17G164200) presented higher expression levels under WD conditions in the GM line. According to Wolkers et al. (2001), soluble carbohydrates, such as sucrose and trehalose, and LEA proteins act jointly to form glassy structures that bind via hydrogen bonds to minimize the damage caused by abiotic stress. This network formed by carbohydrates and LEA proteins allows for a greater stability of cellular structures, while acting as an anchor for the molecular network, providing stability to macromolecular and cellular structures under extreme environmental conditions (Wolkers et al. 2001).

With regard to the gas exchange parameters assayed in the GH experiment, lower values were observed in all plants under WD conditions, regardless of the plant material evaluated (i.e., GM or conventional background). With regard to soil gravimetric moisture (GraM) content, the *AtGolS2* line 2Ia4 plants showed higher values under WD conditions compared to that of the other plant materials. The water accumulation in the substrate-sand mixture and the increase in galactinol and raffinose transcripts observed in the 2Ia4 plants probably influenced the gas exchange parameter response, thus supporting the possibility that these carbohydrates acted as osmoprotectors during osmotic adjustments under WD conditions (Honna et al. 2016).

In the GH survival experiment, the *AtGolS2* GM lines showed higher survival rates after 21 days of withholding irrigation followed by nine days of rehydration when compared to that of the genetic background (**Fig. 1C**). The conventional cultivar BRS 184 plants showed 100% mortality, while the soybean plants from the GM line 2Ia4 also showed 100% recovery after rehydration, which agrees with the data reported by Taji et al. (2002). The authors of that study demonstrated that *Arabidopsis* plants that overexpressed the gene *35S:AtGolS2* showed complete recovery after 14 days of WD followed by five days of rehydration due to a reduction in leaf transpiration, higher water accumulation in the substrate, and the accumulation of raffinose and galactinol in tissues, suggesting once again that the increased levels of these carbohydrates may have

allowed them to act as osmoprotectors.

In the experiment conducted under field conditions, a higher number of pods with seeds, number of seeds, 100-seed weight, and yield were identified in GM 2Ia4 plants in the IRR and NIRR treatments when compared to that of the conventional background. This result may have been due to the increased synthesis of RFOs, even under well-watered conditions, since a constitutive promoter (*35S*) was used. The oil and protein content in the *AtGolS2* lines was registered from the crop seasons of 2014/2015 to 2017/2018. The percentage values obtained were lower for protein content (ranging between 36-37%) and higher for oil content (ranging between 22-23%) compared to that of the standard pattern determined by the crushing market. However, through a soybean-breeding program, this may be improved as the 2Ia4 plants may be useful for the development of drought-tolerant plants (Honna et al. 2016).

Soybean lines containing the 9-cis-epoxycarotenoid dioxygenase (NCED) gene (AtNCED3)

Cotyledons from the soybean conventional cultivar BRS 184 were transformed through the *A. tumefaciens* method with the construct *35S:AtNCED3*. Two positive events were identified in the T_0 generation, 2Ha11 and 2Ha13. These lines were submitted to molecular, physiological, and agronomical characterization in WD and control treatments under both GH and field conditions (Molinari et al. 2020).

Higher expression levels of the *AtNCED3* gene and the endogenous genes *GmAREB1* (Glyma.04G039300; Glyma.07G213100; Glyma.02G131700), *GmPP2C* (Glyma.14G195200), *GmSnRK2* (Glyma.02G135500), and *GmAAO3* (Glyma.14G045100) were identified in the GM lines when compared to that of the WT plants under WD conditions (Molinari et al., 2020).

A higher expression of the genes from the ABA biosynthesis pathway suggests that the *AtNCED3* gene is involved in the drought response of soybeans. Furthermore, the ABA synthesis pathway was triggered in response to WD conditions, as observed by the higher ABA levels detected under WD conditions in the GM plants. Plants from the GM line 2Ha11 showed an ABA concentration of 166.34 pmol mL^{-1}, while the background cultivar BRS 184 plants under WD conditions and both plant materials under control conditions showed ABA concentrations under 4 pmol mL^{-1}, which is the minimum detection limit of the kit employed (Molinari et al. 2020). The increase in ABA levels and ABA biosynthesis-related genes was previously described in *Arabidopsis* (Iuchi et al. 2001). In peanut plants (*Arachis hypogaea* L.), the constitutive expression of the *AhNCED1* gene in WT *Arabidopsis* plants resulted in higher ABA accumulation in the GM plants in response to drought compared to that of the control plants (Hwang et al. 2010). Similarly, *Caragana korshinskii*, a deciduous perennial shrub of sandy grasslands and deserts, showed ABA accumulation followed by a large increase in *CkNCED1* mRNA levels in detached leaves and stems after dehydration for 4 h at room temperature (Wang et al. 2009). In addition, in *Stylosanthes guianensis*, an important forage legume and cover crop, the dehydration of leaves and roots induced the strong and rapid expression of

SgNCED1, while ABA accumulation was induced by an increase in *SgNCED1* mRNA levels under stress (Yang and Guo 2007). In soybeans, the diurnal oscillation of the *GmNCED3*, *GmNCED4*, and *GmNCED5* genes has been reported, and such oscillation appeared to be limited by light under stressful conditions, the period in which stomatal closure is needed to avoid water loss by evapotranspiration (Rodrigues et al. 2015). All these reports support a strong and direct relationship among the expression of *NCED* genes, increased ABA levels, and the activation of drought responses, such as stomatal closure, to reduce water loss under WD conditions (Molinari et al. 2020).

Likewise, as reported by Molinari et al. (2020), the expression of drought-responsive endogenous genes, such as *GmAREB1*, *GmPP2C*, *GmSnRK2*, and *GmAAO3* in soybeans, has also been described in *Arabidopsis* (Iuchi et al. 2001). Considering the genes from the ABA biosynthesis pathway, an increase in aldehyde oxidase genes (*AAO3*) under WD conditions was also found identified in peanuts (Yang et al. 2011) and peas (Zdunek-Zastocka and Sobczak, 2013). The high expression of *GmPP2C* and *GmSnRK2* identified in soybeans reflects the refined control of ABA synthesis, which is negatively regulated by the inhibition of the NCED enzyme when ABA levels exceeded cell maintenance levels (non-stressed conditions), preventing high hormone levels from indicating a metabolic disturbance (Liu et al. 2016).

Under GH conditions, the gas exchange measurements (*gs*, *Ci*, *A*, *E*) under WD conditions decreased in the GM line 2Ha11. Furthermore, GM plants showed 80% higher intrinsic water use efficiency (*A/gs*) when compared to that of the WT plants under WD conditions. The decrease in the gas exchange parameters observed in the GM event 2Ha11 has also been reported in *Arabidopsis* plants overexpressing *AtNCED3* (Iuchi et al. 2001). A reduction in transpiration was also noted in *Arabidopsis* plants overexpressing *OsNCED3* from rice (Hwang et al. 2010). Furthermore, GM tobacco lines overexpressing *SgNCED1* showed a decrease in transpiration rates and lower photosynthetic rates, which resulted from lower stomatal conductance (Zhang et al. 2008). Detached leaves from tobacco (*Phaseolous vulgaris*) that overexpressed *PvNCED1* also showed lower water loss due to transpiration compared to that of the control plants (Qin and Zeevaart 2002). Enhanced stomatal closure was also observed in *Vicia fava* lines expressing the *AtNCED3* gene (Melhorn et al. 2008). This might also have occurred with the GM event 2Ha11 since reduced gas exchange parameter values were observed under WD conditions, which was probably the result of stomatal closure triggered by an increased in ABA levels (Molinari et al. 2020).

The soybean *AtNCED3* lines were evaluated during the crop seasons of 2015/2016 and 2016/2017. In these experiments, no significant interaction between plant materials and water conditions was observed, probably due to the great amount of rainfall recorded during both crop seasons. According to data collected by the weather station located at the experiment site, a total rainfall of 1,521.4 mm and 1,147.2 mm was registered for the crop seasons of 2015/16 and 2016/17, respectively. The recommendations for soybean crop with regard to water requirements range from 450 to 800 mm/cycle, depending on weather conditions, crop

management, and cycle duration (Embrapa 2013).

Results from field assays comprised of average values from both water conditions (irrigated and non-irrigated treatments) for each cultivar/GM line. In the crop seasons of 2015/2016 and 2016/2017, the GM line 2Ha11 showed increased yield when compared to its genetic background, which was probably a result of higher 100-seed weights and the total number of pods. As observed for the GM line 2Ha11, an increase in the yield components was reported for the GM lines of creeping bent grass overexpressing *VuNCED1*, which showed an increase in plant body biomass and an increased number of tillers under WD conditions (Aswath et al. 2005).

The oil and protein content from the *AtNCED3* lines showed that when considering all crop seasons samples (from 2014/2015 to 2017/2018) overall, line 2Ha10 presented values within the range acceptable by the consumer market for oil and protein percentages in the grain (20% and 42%, respectively), indicating that the overexpression of the *AtNCED3* gene in this line did not imply in changes in the specific compositional characteristics.

Final highlights on the development of drought-tolerant soybean lines

Experiments under GH conditions give the "concept proof" of the genetic strategy; however, these tests under controlled light, temperature, water, weed, insect, and disease levels may not be representative of the way in which plants behave over the entire season in an actual field (Passioura 2012). Yet, in GH environments, plants are not able to express their full potential, as limitations due to pot size, controlled water amounts, temperature fluctuations, diseases, and pests do not challenge the organism as a whole but limit the simulation of actual environmental conditions. As such, reports containing data from field experiments are important given that it is only in the field that researchers can accurately gauge whether the technology has been successful or not in improving the characteristic of interest. In addition, when the objective is the release of a commercial variety, field tests are a legal requirement of regulatory governmental commissions.

We summarized data from soybean GM lines that were characterized in both GH and field conditions. These lines present promising indications for the improvement of soybean drought tolerance. Based on the reported results, the strategy to improve drought tolerance by inserting TFs that regulate the expression of several drought-responsive genes has proven to be an interesting approach to obtain lines that trigger mechanisms to cope with water deficits without compromising either yield or oil and protein content. In soybeans, such approaches may constitute the insertion of either ABA-independent genes, such as *DREB1* and *DREB2*, or ABA-dependent genes, such as *AREB1*, in addition to GM soybean lines overexpressing the *GolS* and *NCED* genes, which are responsible for important drought-defense pathways.

Overall, the soybean GM lines obtained in this study presented protein and oil content values that should be accepted by the crushing industry and that meet quality standards and commercial specifications

(Embrapa 2015). The maintenance of these parameters is essential when developing GM lines as it adds value to the grain and ensures the competitiveness of soy in the world market, enabling possible cultivars obtained from these lines to enter into the feed market for consumption by humans, poultry, pork, cattle, or other farm animals and pets.

Finally, these results have highlighted soybean drought-tolerance pathways and have shown that the use of biotechnological tools in agricultural science can help producers to minimize yield and financial losses during drought-stricken crop seasons.

Acknowledgments

These studies were supported by the Japan International Research Center for Agricultural Sciences (JIRCAS) and Embrapa Soybean. We are grateful to the Science and Technology Research Partnership for Sustainable Development (SATREPS) of the Japan Science and Technology Agency/Japan International Cooperation Agency (JST/JICA), which supported our study. We are also grateful to the Coordination for the Improvement of Higher Education Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq) for granting scholarships to the Master, Ph.D., and postdoctoral students involved in this project. The authors are likewise grateful to the staff and students of the Agrometeorology, Ecophysiology, and Plant Biotechnology laboratories of Embrapa Soybean for their support with all evaluations. We would like to thank Editage (www.editage.com) for English language editing.

Conflict of Interest: The authors declare that they have no conflict of interest.

References

Ahrazem O, Rubio-Moraga A, Trapero A et al. (2012) Developmental and stress regulation of gene expression for a 9-cis-epoxycarotenoid dioxygenase, *CstNCED*, isolated from *Crocus sativus* stigmas. *J. Exp. Bot.* **63**:681-694. doi:10.1093/jxb/err293

Almogueva C, Prieto-Dapena P, Díaz-Martín J, Espinosa JM, Carranco R, Jordano R (2009) The *HaDREB2* transcription factor enhances basal thermotolerance and longevity of seeds through functional interaction with HaHSFA9. *BMC Plant Biol.* **9**:1-12.

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. *Mol. Breed*. **15**:305–327. doi:10.1007/s11032-004-8001-y

Anjum SA, Xie XY, Wang LC, Saleem MF, Man C, Lei W (2011) Morphological, physiological and biochemical responses of plants to drought stress. *Afr. J. Agric. Res.* **6**:2026–2032. doi:10.5897/AJAR10.027

Aswath C, Kim S, Mo S et al. (2005) Transgenic plants of creeping bent grass harboring the stress inducible gene, 9-cis-epoxycarotenoid dioxygenase, are highly tolerant to drought and NaCl stress. *Plant Growth Regul.* **47**:129-139. doi: 10.1007/s10725-005-3380-6

Bao F, Du D, An Y, Yang W, Wang J, Cheng T, Zhang Q (2017) Overexpression of *Prunus mume* dehydrin genes in tobacco enhances tolerance to cold and drought, *Front. Plant Sci.* **8**:151.

Barbosa EGG, Leite JP, Marin SRR, Marinho JP, Carvalho JFC, Fuganti-Pagliarini R, Farias JRB, Neumaier N, Marcelino-Guimarães FC, Oliveira MCN, Yamaguchi-Shinozaki K, Nakashima K, Maruyama K,

Kanamori N, Fujita Y, Yoshida T, Nepomuceno AL (2012) Overexpression of the ABA-dependent AREB1 transcription factor from *Arabidopsis thaliana* improves soybean tolerance to water deficit. *Plant Mol. Biol. Rep.* **31**:719–730. doi: 10.1007/s11105-012-0541-4

Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F, Covarrubias AA (2008) The enigmatic lea proteins and other hydrophilins. *Plant Physiol.* **148**:6-24.

Behnam S, Iuchi M, Fujita Y, Fujita Y, Takasaki H, Osakabe Y, Yamaguchi-Shinozaki K, Kobayashi M, Shinozaki K (2013) Characterization of the promoter region of an *Arabidopsis* gene for 9-cis-epoxycarotenoid dioxygenase involved in dehydration-inducible transcription. *DNA Res* **20**:315-324. doi: 10.1093/dnares/dst012

Bhaskara GB, Nguyen TT, Verslues PE (2012) Unique drought resistance functions of the highly ABA-induced clade A protein phosphatase 2Cs. *Plant Physiol.* **160**:379-395. doi: 10.1104/pp.112.202408

Bhatnagar-Mathur P, Devi MJ, Reddy DS, Lavanya M, Vadez V, Serraj R, Yamaguchi-Shinozaki K, Sharma KK (2007) Stress-inducible expression of AtDREB1A in transgenic peanut (*Arachis hypogaea* L.) increases transpiration efficiency under water-limiting conditions. *Plant Cell Rep.* **26**:2071–2082.

Bhatnagar-Mathur P, Devi MJ, Serraj R, Yamaguchi-Shinozaki, K, Vadez V, Sharma KK (2004) Evaluation of transgenic groundnut lines under water limited conditions. *Intl. Arachis Newslett.* **24**:33–34.

Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB (1999) Characterization of the ABA-deficient tomato mutant notabilis and its relationship with maize Vp14. *Plant J.* **17**:427-431. doi:10.1046/j.1365-313X.1999.00386.x

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* **29**:23–39.

Cao X, Liu X, Zhang Y, Xue X, Zhou XE, Melcher K, Gao P, Wang F, Zeng L, Zhao Y, Zhao Y, Deng P, Zhong D, Zhu JK, Xu HE, Xu Y (2013) An ABA-mimicking ligand that reduces water loss and promotes drought resistance in plants. *Cell Res.* **23**:1043-1054. doi:10.1038/cr.2013.95

Casagrande EC, Farias JRB, Neumaier N, Oya T, Pedroso J, Martins PK, Breson MC, Nepomuceno AL (2001). Expressão gênica diferencial durante déficit hídrico em soja. *Rev. Bras. Fisiol. Veg.* **13**:168-184.

Casali N, Preston A. (2003) *E. coli* Plasmid vectors. *Plasmid* **235**:55–69. doi:10.1385/1592594093

Chernys JT, Zeevaart JAD (2000) Characterization of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. *Plant Physiol.* **124**:343-353. doi: http://dx.doi.org/10.1104/pp.124.1.343

Devi JM, Bhatnagar-Mathur P, Sharma KK, Serraj R, Anwar SY, Vadez V (2011) Relationships between transpiration efficiency (TE) and its surrogate traits in the *rd29A:DREB1A* transgenic groundnut. *J. Agronomy Crop Sci.* **197**:272-283. DOI: 10.1111/j.1439-037X.2011.00464.x

Downie B, Gurusinghe S, Dahal P, Thacker RR, Snyder JC, Nonogaki H, Yim K, Fukanaga K, Alvarado V, Bradford KJ (2003) Expression of a GALACTINOL SYNTHASE gene in tomato seeds is up-regulated before maturation desiccation and again after imbibition whenever radicle protrusion is prevented. *Plant Physiol.* **131**:1347–1359.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bull* **19**:11-15.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet ED, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *Os-DREB* genes in rice, *Oryza sativa* L., encoded transcription activators that function in drought, high-salt and cold-responsive gene expression. *Plant J.* **33**:751-763.

Egli DB (2013) The relationship between the number of nodes and pods in soybean communities. *Crop Sci.* **53**:1668–1676. doi:10.2135/ cropsci2012.11.0663

Embrapa — Empresa Brasileira de Pesquisa Agropecuária (2013) Available at http://www.cnpso.embrapa.br

Embrapa (2015) Comunicado Técnico 86. Teores de óleo e proteina em soja: fatores envolvidos e qualidade para a indústria. ISSN 2176-2889.

Endo Y, Sawada H, Takahashi M, Okamoto M, Ikegami K, Koiwai H, Seo M, Toyomasu T, Mitsuhashi W, Shinozaki K, Nakazono M, Kamiya Y, Koshiba T, Nambara E (2008) Drought induction of *Arabidopsis* 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant Physiol.* 147:1984-1993. doi: 10.1104/pp.108.116632

Engels C, Fuganti-Pagliarini R, Marin SRR, Marcelino-Guimarães FC, Oliveira MCN, Kanamori N, Mizoi J, Nakashima K, Yamaguchi-Shinozaki K, Nepomuceno AL (2013) Introduction of the *rd29A:AtDREB2A CA* gene into soybean (*Glycine max* L. Merril) and its molecular characterization in leaves and roots during dehydration. *Gen. Mol. Biol.* **36**(4):556-565.

Fang Y, Xiong L (2015) General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. Life Sci.* **72**:673–689. DOI 10.1007/s00018-014-1767-0

FAO - Food and Agriculture Organization of the United Nations (2018) Disasters causing billions in agricultural losses, with drought leading the way. http://www.fao.org/news/story/en/item/1106977/icode/ March, 2018.

FAO - Food and Agriculture Organization of the United Nations (2016) http://www.agricultura.gov.br/comunicacao/noticias/2016/03/pib-da-agropecuaria-tem-alta-de-1porcento-em-2015

Fehr WR, Caviness CE, Burmood DT, Pennington JS (1971) Stage of development description for soybeans, *Glycine max* (L.) Merril. *Crop Sci.* **11**:929-931. doi:10.2135/cropsci1971.0011183X 001100060051x

Ferreira RC (2016) Quantificação das perdas por seca na cultura da soja no Brasil. Tese de Doutorado-Universidade Estadual de Londrina. Centro De Ciências Agrárias. Programa de Pós-Graduação em Agronomia. Londrina.

Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant Biol.* **6**:269-279. doi: 10.1055/s-2004-820867

Fuganti-Pagliarini R, Ferreira LC, Rodrigues FA, Molinari HBC, Marin SRR, Molinari MDC, Marcolino-Gomes J, Mertz-Henning LM, Farias JRB, de Oliveira MCN, Neumaier N, Kanamori N, Fujita Y, Mizoi J, Nakashima K, Yamaguchi-Shinozaki K, Nepomuceno AL. (2017) Characterization of Soybean Genetically Modified for Drought Tolerance in Field Conditions. *Front. Plant Sci.* **8**:448, 2017. doi: 10.3389/fpls.2017.00448

Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K (2005) AREB1 is a transcription activator of novel ABRE dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* **17**:3470–3488.

Fujita Y, Nakashima K, Yoshida T, Katagiri T, Kidokoro S, Kanamori N, Umezawa T, Fujita M, Maruyama K, Ishiyama K, Kobayashi M, Nakasone S, Yamada K, Ito T, Shinozaki K, Yamaguchi-Shinozaki K (2009) Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in *Arabidopsis*. *Plant Cell Physiol.* **50**:2123–2132. doi:10.1093/pcp/ pcp147

Gao SQ, Chen M, Xia LQ, Xiu HJ, Xu ZS, Li LC, Zhao CP, Cheng XG. Ma ZY (2009) A cotton (*Gossypium hirsutum*) DRE binding transcription factor gene, *GhDREB*, confers enhanced tolerance to drought, high salt, and freezing stresses in transgenic wheat. *Plant Cell Rep*. **28**:301–311.

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J.* **16**:433–443.

Hand SC, Menze MA, Toner M, Boswell L, Moore D (2011) LEA proteins during water stress: not just for plants anymore. *Annu. Rev. Physiol.* **73**:115–134. doi: 10.1146/annurev-physiol-012110-142203.

Hasegawa PM, Bressan RA, Zhu JK, and Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **51**:463–499.

Heil C (2010) Rapid, Multi-Component Analysis of Soybeans by FT-NIR Spectroscopy. Madison: Thermo Fisher Scientific.

Honna PT, Fuganti-Pagliarini R, Ferreira LC, Molinari MDC, Marin SRR, de Oliveira MCN, Farias JRB, Neumaier N, Mertz-Henning LM, Kanamori N, Nakashima K, Takasaki H, Urano K, Shinozaki K, Yamaguchi-Shinozaki K, Desidério JA, Nepomuceno AL (2016) Molecular, physiological and agronomical characterization, in greenhouse and in field conditions, of soybean plants genetically modified with *AtGolS2* gene for drought tolerance. *Mol. Breed.* DOI: 10.1007/s11032-016-0570-z

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New agrobacterium helper plasmids for gene transfer to plants. *Transgenic Res.* **2**:208–218. doi:10.1007/BF01977351

Hwang SG, Chen HC, Huang WY (2010) Ectopic expression of rice *OsNCED3* in *Arabidopsis* increases ABA level and alters leaf morphology. *Plant Sci.* **178**:12-22. doi: 10.1016/j.plantsci.2009.09.014

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol.* **47**:141–153.

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *Plant J.* **27**:325-333. doi:10.1046/j.1365-313x.2001.01096.x

Iuchi S, Kobayashi M, Yamaguchi-Shinozaki K (2000) A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerance cowpea. *Plant Physiol.* **123**:553-562. doi: http://dx.doi.org/10.1104/pp.123.2.553

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis* CBF1 overexpression induces cor genes and enhances freezing tolerance. *Science* **280**:104–106.

Jakoby M, Weisshaar B, Dröge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F (2002) bZIP transcription factors in *Arabidopsis*. *Trends Plant Sci*. **7**:106–111.

Jaleel CA, Manivannan P, Wahid A, Farooq M, Al-Juburi HJ, Somasundaram R and Panneerselvam R (2009) Drought stress in plants: A review on morphological characteristics and pigments composition. *Int. J. Agric. Biol.* **11**:100-105.

Kahlon CS, Board JE, Kang MS (2011) An analysis of yield component changes for new and old cultivars. *Agron. J.* **103**:13–22. doi:10.2134/agronj2010.0300

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004) A Combination of the *Arabidopsis DREB1A* gene and stress-inducible rd29A promoter improved drought and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.* **45**:346–350.

Koag MC, Wilkens S, Fenton RD, Resnik J, Vo E, Close TJ (2009) The K-Segment of maize DHN1 mediates binding to anionic phospholipid vesicles and concomitant structural changes. *Plant Physiol.* **150**:1503-1514.

Kobayashi F, Maeta E, Terashima A, Takumi SP (2008) Positive role of a wheat *HvABI5* ortholog in abiotic stress response of seedlings. *Physiol. Plant.* **134**(1):74–86.

Lee JS, Kang JY, Park HJ, Kim MD, Bae MS, Choi HI, Kim SY (2010) DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity. *Plant Physiol.* **153**:716–727.

Leite JP, Barbosa EGG, Marin SRR, Marinho JP, Carvalho JFC, Fuganti-Pagliarini R, Cruz AS, Oliveira MCN, Farias JRB, Neumaier N, Guimarães FCM, Yoshida T, Kanamori N, Fujita Y, Nakashima K, Yamaguchi-Shinozaki K, Desidério JA, Nepomuceno AL (2014) Overexpression of the activated form of the AtAREB1 gene (*AtAREB1ΔQT*) improves soybean responses to water deficit. *Gen. Mol. Res.* **13** (3): 6272-6286. DOI http://dx.doi.org/10.4238/2014.August.15.10

Li X-G, Chen S-B, Lu Z-X, Chang T-J, Zeng QC, Zu Z (2002) Impact of copy number on transgene expression in tobacco. *Acta Bot. Sin.* **44**:120–123.

Liu L, Zhu K, Yang Y, Wu J, Chen F, Yu D (2008) Molecular cloning, expression profiling and trans-activation property studies of a DREB2-like gene from chrysanthemum (*Dendranthema vestitum*). J. *Plant Res.* **121**:215-226.

Liu Q, Sakuma Y, Abe H, Kasuga M, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain, separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* **10**:1491–1406.

Liu T, Longhurst AD, Talavera-Rauh F, Hokin SA, Barton MK (2016) The *Arabidopsis* transcription factor ABIG1 relays ABA signaled growth inhibition and drought induced senescence. Amasino R, ed. *eLife* **5**:e13768. doi:10.7554/eLife.13768.

Liu Y, Zheng Y, Zhang Y, Wang W, Li R (2010) Soybean PM2 protein (LEA3) confers the tolerance of *Escherichia coli* and stabilization of enzyme activity under diverse stresses. *Curr. Microbiol.* **60**:373-378. doi: 10.1007/s00284-009-9552-2

Marcolino-Gomes J, Rodrigues FA, Fuganti-Pagliarini R, Bendix C, Nakayama TJ, Celaya B, Molinari HBC, Oliveira MCN, Harmon FG, Nepomuceno A (2014) Diurnal Oscillations of Soybean Circadian Clock and Drought Responsive Genes. *PLOS ONE* **9**:e86402. doi:10.1371 /journal .pone.0086402

Marinho JP, Kanamori N, Ferreira LC, Fuganti-Pagliarini R, Carvalho JFC, Freitas RS, Marin SRR, Rodrigues FA, Mertz-Henning LM, Farias JRB, Neumaier N, Oliveira MCN, Marcelino-Guimarães FC, Yoshida T, Fujita Y, Yamaguchi-Shinozaki K, Nakashima K, Nepomuceno AL (2016) Characterization of Molecular and Physiological Responses Under Water Deficit of Genetically Modified Soybean Plants Overexpressing the *AtAREB1* Transcription Factor. *Plant Mol. Biol. Rep*. **34**:410–426. DOI 10.1007/s11105-015-0928-0

Marinho, JP, Marcolino-Gomes J, Mertz-Henning LM, Caranhato ALH, Fuganti-Pagliarini R, Marin SRR, Neves MC de O, Foloni JSS, Melo CLP de, Farias JRB, Neumaier N, Kidokoro S, Mizoi J, Kanamori N, Yamaguchi-Shinozaki K, Nakashima K, Nepomuceno AL (2020) Overexpression of *GmDREB2A;2 FL* and *GmDREB2A;2 CA* transcription factors enhances drought tolerance during germinative, vegetative and reproductive developmental periods in soybean. *Gen. Mol. Biol.* (Submitted).

Martins PK, Jordão BQ, Yamanaka N, Farias JRB, Beneventi MA, Binneck E, Fuganti R, Stolf R and Nepomuceno AL (2008) Differential gene expression and mitotic cell analysis of the drought tolerant soybean (*Glycine max* L. Merrill Fabales, Fabaceae) cultivar MG/BR46 (Conquista) under two water

deficit induction systems. *Genet. Mol. Biol.* **31**:512-521.

Melhorn V, Matsumi K, Koiwai H, Ikegami K, Okamoto M, Nambara E, Bittner F, Koshiba T (2008) Transient expression of *AtNCED3* and *AAO3* genes in guard cells causes stomatal closure in *Vicia faba*. *J. Plant Res.* **121**:125-131. doi: 10.1007/s10265-007-0127-7

Mizoi J, Ohori T, Moriwaki T, Kidokoro S, Todaka D, Maruyama K, Kusakabe K, Osakabe Y, Shinozaki K, Yamaguchi- Shinozaki K (2013) *GmDREB2A;2,* a canonical dehydration-responsive element binding protein2-type transcription factor in soybean, is post-translationally regulated and mediates dehydration-responsive element-dependent gene expression. *Plant Physiol.* **161**:346-361.

Molinari MDC, Fuganti-Pagliarini R, Marin SRR, Ferreira LC, Barbosa DA, Marcolino-Gomes J, Oliveira MCN, Mertz-Henning LM, Kanamori N, Takasaki H, Urano K, Shinozaki K, Nakashima K, Yamaguchi-Shinozaki K, Nepomuceno AL (2020) Overexpression of *AtNCED3* gene improved drought tolerance in soybean in greenhouse and field conditions. *Gen. Mol. Biol.* **43**(3) https://doi.org/10.1590/1678-4685-gmb-2019-0292

Neves DM, Coelho Filho MA, Bellete BS (2013) Comparative study of putative 9-cis-epoxycarotenoid dioxygenase and abscisic acid accumulation in the responses of Sunki mandarin and Rangpur lime to water deficit. *Mol. Biol. Rep.* **40**:5339-5349. doi:10.1007/s11033-013-2634-z.

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH, Kim JK (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol.* **138**:341–351.

Panikulangara TJ, Eggers-Schumacher G, Wunderlich M, Stransky H, Schöffl F (2004) Galactinol synthase1. A novel heat shock factor target gene responsible for heat-induced synthesis of raffinose family oligosaccharides in Arabidopsis. *Plant Physiol.* **136**(10):3148–3158.

Pattanagul W, Madore M (1999) Water deficit effects on raffinose family oligosaccharide metabolism in Coleus. *Plant Physiol.* **121**(3):987–993.

Park FC, Peterson A, Mosquna J, Yao J, Volkman BF, Cutler SR (2015) Agrochemical control of plant water use using engineered abscisic acid receptors. *Nature* **520**:545-548. doi: 10.1038/nature14123

Passioura JB (2012) Phenotyping for drought tolerance in grain crops: when is it useful to breeders? *Funct Plant Biol.* **39**:851-859. doi:10.1071/FP12079

Paz MM, Martinez JC, Kalvig AB, Fonger TM, Wang K (2006) Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. *Plant Cell Rep.* **25**:206-213. doi: 10.1007/s00299-005-0048-7

Pedrosa AM, Martins CDPS, Gonçalves LP, Costa MGC (2015) Late embryogenesis abundant (LEA) constitutes a large and diverse family of proteins involved in development and abiotic stress responses in Sweet Orange (*Citrus sinensis* L. Osb.). *PLoS One* **10**:1-17. doi: http://dx.doi.org/10.1371/journal.pone.0145785

Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K, Hoisington D (2004) Stress induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. *Genome* **47**:493–500.

Peters S, Mundree SG, Thomson JA, Farrant JM, Keller F (2007) Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): Both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit. *J. Exp. Bot.* **58**:1947-1956. doi: 10.1093/jxb/erm056

Pillet J, Egert A, Pieri P, Lecourieux F, Kappel C, Charon J, Gomès E, Keller F, Delrot S, Lecourieux D (2012) *VvGOLS1* and *VvHsfA2* are involved in the heat stress responses in grapevine berries. *Plant Cell Physiol.* **53**:1776–1792

Polizel A, Medri ME, Nakashima K, Yamanaka N, Farias JR, de Oliveira MC, Marin SRR, Abdelnoor RV, Marcelino-Guimarães FC, Fuganti R, Rodrigues FA, Stolf-Moreira R, Beneventi MA, Rolla AA, Neumaier N, Yamaguchi-Shinozaki K, Carvalho JF, Nepomuceno AL (2011) Molecular, anatomical and physiological properties of a genetically modified soybean line transformed with *rd29A:AtDREB1A* for the improvement of drought tolerance. *Genet. Mol. Res.* **10**(4):3641–3656.

Qin F, Kakimoto M, Maruyama K, Osakabe Y, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K (2007) Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L. *Plant J.* **50**:54-69.

Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K, Yamaguchi- Shinozaki K (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold responsive gene expression in *Zea mays* L. *Plant Cell Physiol.* **45**:1042–1052.

Qin X, Zeevaart JA (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proc. Natl. Acad. Sci. USA* **96**:15354-15361.

doi:10.1073/pnas.96.26.15354
Qin X, Zeevaart JAD. (2002) Overexpression of a 9-cis-Epoxycarotenoid Dioxygenase Gene in *Nicotiana plumbaginifolia* Increases Abscisic Acid and Phaseic Acid Levels and Enhances Drought Tolerance. *Plant Physiol.***128**(2):544-551. doi:10.1104/pp.010663.
Quick P, Siegl G, Neuhaus E, Feil R, Stitt M (1989) Short-term water stress leads to a stimulation of sucrose synthesis by activating sucrose-phosphate synthase. *Planta* **177**:535-546. doi: 10.1007/BF00392622
Ramiro, Melotto-Passarin DA, Barbosa DM (2016) Expression of *Arabidopsis* Bax Inhibitor-1 in transgenic sugarcane confers drought tolerance. *Plant Biotechnol. J.* **14**:1826-1837. doi:10.1111/pbi.12540
Rodrigo MJ, Alquezar B, Zacarias L (2006) Cloning and characterization of two 9-cis-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *J. Exp. Bot.* **57**:633-643. doi: 10.1093/jxb/erj048
Rodrigues FA, Fuganti-Pagliarini R, Marcolino-Gomes J, Nakayama TJ, Molinari HB, Lobo FP, Harmon FG, Nepomuceno AL (2015) Daytime soybean transcriptome fluctuations during water deficit stress. *BMC Genomics* **16**:505. doi: 10.1186/s12864-015-1731-x
Rolla AA, Carvalho JFC, Fuganti-Pagliarini R, Engels C, Rio A, Marin SRR, Oliveira MCN, Beneventi MA, Marcelino-Guimaraes MC, Farias JRB, Neumaier N, Nakashima K, Yamaguchi-Shinozaki K, Nepomuceno AL (2013) Phenotyping soybean plants transformed with *rd29A:AtDREB1A* for drought tolerance in the greenhouse and field. *Transgenic Res* **23**(1):75-87. DOI 10.1007/s11248-013-9723-6
Sakuma Y, Maruyama K, Qin F, Osakabe Y, Shinozaki K, Yamaguchi-Shinozaki K (2006a) Dual function of an *Arabidopsis* transcription factor DREB2A in water-stress-responsive and heat-stress- responsive gene expression. *Proc. Natl, Acad. Sci. USA*. **103**(49):18822-7. doi: 10.1073/pnas.0605639103
Sakuma Y, Maruyama K, Qin F, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006b) Functional Analysis of an *Arabidopsis* transcription factor DREB2A, involved in Drought-Responsive gene expression. *Plant Cell* **18**:1292-1309 doi: http://dx.doi.org/10.1105/tpc.105.035881
Salinet LH (2009) Avaliação fisiológica e agronômica de soja geneticamente modificada para maior tolerância à seca. Universidade de São Paulo Tese de Doutorado. Escola Superior de Agricultura Luiz de Queiroz.
Santos TB, Budzinski IG, Marur CJ, Petkowicz CL, Pereira LF, Vieira LG (2011) Expression of three galactinol dos synthase isoforms in *Coffea arabica* L. and accumulation of raffinose and stachyose in response to abiotic stresses. *Plant Physiol. Biochem.* **49**:441–448.
Santos TBD, de Lima RB, Nagashima GT, Petkowicz CL, Carpentieri-Pípolo V, Pereira LF, Domingues DS, Vieira LG (2015) Galactinol synthase transcriptional profile in two genotypes of *Coffea canephora* with contrasting tolerance to drought. *Genet. Mol. Biol.* **38**(2):182-190. doi:10.1590/S1415-475738220140171.
Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **58** (2): 221–227, 2007. doi:10.1093/jxb/erl164
Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr. Opin. Plant Biol.* **3**: 217–223.
Stolf-Moreira R, Lemos EGM, Abdelnoor RV, Beneventi MA, Rolla AAP, Pereira SS, Oliveira MCN, Nepomuceno AL, Marcelino-Guimarães FC (2011) Identification of reference genes for expression analysis by real-time quantitative PCR in drought-stressed soybean. *Pesq Agrop Brasileira* **46**: 58–65. doi:10.1590/S0100-204X2011000100008
Svitashev SK, Pawlowski WP, Makarevitch I, Plank DW, Somers DA (2002) Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement. *Plant J.* **32**: 433–445. doi:10.1046/j.1365-313X.2002.01433.x
Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J.* **29**: 417-426. doi: 10.1046/j.0960-7412.2001.01227.x
Takahashi R, Joshee N, Kitagawa Y (1994) Induction of chilling resistance by water stress, and cDNA sequence analysis and expression of water stress-regulated genes in rice. *Plant Mol. Biol.* **26**: 339–352
Takeuchi M, Okamoto T, Akiyama T (2014) Designed abscisic acid analogs as antagonists of PYL-PP2C receptor interactions. *Nat. Chem. Biol.* **10**:477-482. doi: 10.1038/nchembio.1524
Terashima A, Takumi S (2009) Allopolyploidization reduces alternative splicing efficiency for transcripts of the wheat DREB2 homolog, *WDREB2*. *Genome* **52**:100-105.
Turner N, Wright G, Siddique K (2001) Adaptation of grain legumes (pulses) to water-limited environments. *Adv, Agron.* **71**: 123-231. doi: 10.1016/S0065-2113(01)71015-2
Vadez V, Rao JS, Bhatnagar-Mathur P, Sharma KK (2013) DREB1A promotes root development in deep soil layers and increases water extraction under water stress in groundnut. *Plant Biol.* **15**:45–52. DOI:

10.1111/j.1438-8677.2012.00588.x.

Wan XR, Li L (2006) Regulation of ABA level and water-stress tolerance of *Arabidopsis* by ectopic expression of a peanut 9-*cis*-epoxycarotenoid dioxygenase gene. *Bioch And Bioph. Res Comm*. **347**:1030-1038. https://doi.org/10.1016/j.bbrc.2006.07.026

Wang X, Wang Z, Dong J (2009) Cloning of a 9-*cis*-epoxycarotenoid dioxygenase gene and the responses of *Caragana korshinskii* to a variety of abiotic stresses. *Genes Genet. Syst.* **84**:397-405. doi:10.1266/ggs.84.397

Wang D, Yao W, Song Y, Liu W, Wang Z (2012) Molecular characterization and expression of three galactinol synthase genes that confer stress tolerance in *Salvia miltiorrhiza*. *J. Plant Physiol.* **169**: 18, https://doi.org/10.1016/j.jplph.2012.07.015

Wolkers WF, McCready S, Brandt WF, Lindsey GG, Hoekstra FA (2001) Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses *in vitro*. *Biochim Biophys Acta* **1544**:196-206. doi: 10.1016/S0167-4838(00)00220-X

Xu YH, Liu R, Yan J, Liu ZQ, Jiang SC, Shen YY, Wang XF, Zhang DP (2012) Light harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in *Arabidopsis*. *J. Exp. Bot.* **63**: 1095–1106. doi:10.1093/jxb/err315

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Tre. Plant Sci.* **10**:88–94.

Yang J, Guo Z (2007) Cloning of a 9-cis-epoxycarotenoid dioxygenase gene (*SgNCED1*) from *Stylosanthes guianensis* and its expression in response to abiotic stresses. *Plant Cell Rep.* **26**:1383-1390. doi:10.1007/s00299-007-0325-8

Yang L, Liang J, Zhou W, Su L, Zhang B, Li L (2011) Isolation and characterization of the aldehyde oxidase2 gene from *Arachis hypogaea* L. *Plant Mol. Biol. Rep.* **29**:544. doi:10.1007/s11105-010-0259-0

Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *Plant J*. **61**:672–685.

Yoshida T, Mogami J, Yamaguchi-Shinozaki K (2015) Omics Approaches Toward Defining the Comprehensive Abscisic Acid Signaling Network in Plants. *Plant Cell Physiol.* **56**: 10.1093/pcp/pcv060.

Zdunek-Zastocka E, Sobczak M (2013) Expression of *Pisum sativum PsAO3* gene, which encodes an aldehyde oxidase utilizing abscisic aldehyde, is induced under progressively but not rapidly imposed drought stress. *Plant Physiol. Bioch.* **71**:57-66. doi: 10.1016/j.plaphy.2013.06.027

Zhang Y, Yang J, Lu S, Cai J, Guo Z (2008) Overexpressing *SgNCED1* in tobacco increases aba level, antioxidant enzyme activities, and stress tolerance. *Plant Growth Regul.* **27**:151. doi:10.1007/s00344-008-9041-z

Zhou J, Yang Y, Yu J. Wang L, Yu X, Ohtani M, Kusano M, Saito K, Demura T, Zhuge Q (2014) Responses of *Populus trichocarpa* galactinol synthase genes to abiotic stresses. *J Plant Res* **127**: 347–358. https://doi.org/10.1007/s10265-013-0597-8

Zhu JK (2001) Cell signaling under salt, water and cold stresses. *Curr. Opin. Plant Biol.* **53**: 247-73.

Zhuo C, Wang T, Lu S, Zhao Y, Li X, Guo Z (2013) A cold responsive galactinol synthase gene from *Medicago falcata* (MfGolS1) is induced by myo‐inositol and confers multiple tolerances to abiotic stresses. *Physiol. Plant.***149**: 67-78. doi:10.1111/ppl.12019