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BBX21 reduces abscisic acid sensitivity, mesophyll conductance and chloroplast electron transport capacity to increase photosynthesis and water use efficiency in potato plants cultivated under moderated drought

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SUMMARY

The B-box (BBX) proteins are zinc-finger transcription factors with a key role in growth and developmental regulatory networks mediated by light. AtBBX21 overexpressing (BBX21-OE) potato (*Solanum tuberosum*) plants, cultivated in optimal water conditions, have a higher photosynthesis rate and stomatal conductance without penalty in water use efficiency (WUE) and with a higher tuber yield. In this work, we cultivated potato plants in two water regimes: 100 and 35% field capacity of water restriction that imposed leaf water potentials between −0.3 and −1.2 MPa for vegetative and tuber growth during 14 or 28 days, respectively. We found that 42-day-old plants of BBX21-OE were more tolerant to water restriction with higher levels of chlorophylls and tuber yield than wild-type spunta (WT) plants. In addition, the BBX21-OE lines showed higher photosynthesis rates and WUE under water restriction during the morning. Mechanistically, we found that BBX21-OE lines were more tolerant to moderated drought by enhancing mesophyll conductance (g_m) and maximum capacity of electron transport (J_{max}), and by reducing abscisic acid (ABA) sensitivity in plant tissues. By RNA-seq analysis, we found 204 genes whose expression decreased by drought in WT plants and expressed independently of the water condition in BBX21-OE lines as *SAP12, MYB73, EGYP1, TIP2-1* and *DREB2A*, and expressions were confirmed by quantitative polymerase chain reaction. These results suggest that BBX21 interplays with the ABA and growth signaling networks, improving the photosynthetic behavior in suboptimal water conditions with an increase in potato tuber yield.

Keywords: BBX21, abscisic acid, photosynthesis, water use efficiency, *Solanum tuberosum*, drought, yield.

INTRODUCTION

Drought stress can induce a wide range of physiological and biochemical responses regulated by genes encoding functional and/or regulatory proteins, to maintain a normal homeostasis and avoid wilting death (Bhargava and Sawant, 2013). During drought stress, abscisic acid (ABA) biosynthesis (Radin and Ackerson, 1981; Robertson et al., 1985) or ABA sensitivity increase (Saez et al., 2006), and initiate a series of events that induce or enhance plant tolerance to the stress. ABA-dependent and ABA-independent signaling pathways have been established (Soma et al., 2021; Zhu et al., 2002). When plants are under stress, the ABA synthesis increases in the tissues and promotes the activity of transcription factors, such as MYBs, DREBs and bZIPs that facilitate the expression of other genes (Soma et al., 2021). Over time, the induction of ABA-responsive control circuits leads to the engagement of downstream genes and proteins, among them heat shock proteins, antioxidant proteins and osmolytes (Zhu, 2016).

Transcription factors are central players that regulate the transcriptional machinery, allowing the correct integration of endogenous and environmental signals. In Arabidopsis and rice, almost 15% of the transcription factors are zinc finger-type proteins, including B-Box proteins (BBXs). BBX proteins are encoded by a family of highly conserved members, varying the number of BBX between species as documented in Arabidopsis (32), rice (29), tomato (30), potato (30), pear (25) and apple (64) (Crocco and Botto, 2013; Huang et al., 2012; Liu et al., 2018; Talar et al., 2017). BBX proteins are involved in seedling photomorphogenesis, anthocyanin synthesis, shade avoidance, flowering, biotic and abiotic stresses (Gangappa and Botto, 2014; Song et al., 2020; Yadav et al., 2020). In Arabidopsis, BBX21 sticks to the HY5 promoter through the B-Box II domain and inhibits the hypocotyl growth during seedling photomorphogenesis (Datta et al., 2007; Holtan et al., 2011). In peels of *Pyrus pyrifolia* fruits exposed to continuous blue light, PpBBX21(AthBBX24-like) negatively regulates anthocyanin biosynthesis by interfering the formation of heterodimer with PpHY5-PpBBX18 (AthBBX21-like) that activates the transcription of PpMYB10 (Bai et al., 2019). These results are compatible with those suggesting that BBX21 and BBX24 are essential transcriptional cofactors of HY5 to regulate photomorphogenesis and anthocyanin biosynthesis in Arabidopsis seedlings (Bursch et al. 2020; Job et al., 2018). Moreover, BBX21 acts as a repressor of shade avoidance, suppressing exaggerated growth downstream COP1 signaling (Crocco et al., 2010). Interestingly, Arabidopsis *bbx21* mutant seeds geminate worse than WT in a media with ABA, suggesting that BBX21 increases stress tolerance by the interference with the interaction between ABI5 and HY5 (Xu et al., 2014). More recently, Kang et al. (2018) showed that BBX21 directly bounds the ABI5 promoter and recruits the chromatin-remodeling factor PICKLE that increases H3K27me3 marks and downregulates ABI5 expression in the presence of ABA. However, there is scarce and fragmented information about the physiological and molecular mechanisms of BBX21 action in other species. Our research group demonstrated that the overexpression of AtBBX21 (BBX21-OE) in potato plants cultivated under non-stressed conditions promotes stronger plants with increased tuber yield that correlates with higher photosynthetic rates at high irradiances (Crocco et al., 2018). We also found that BBX21-OE lines increase the production of phenols and anthocyanins that act as photoprotective compounds to reduce photoinhibition without penalties on the water use efficiency (WUE; Crocco et al., 2018). Additional physiological studies are required to have a better comprehension of BBX21 action in plants cultivated under suboptimal growing conditions.

Potato, the third largest global food crop after wheat and rice, is cultivated for its underground storage stems or tubers, which are rich in starch and other nutrients. Due to their shallow root system, which limits water extraction from the soil, potato plants are sensitive to drought (Obidiegwu, et al., 2015). Water shortage during the tuber bulking period decreases yield to a larger extent than drought during other growth stages. Leaf size, photosynthesis rate, tuber number and yield are all traits severely limited in potato plants grown under water limitations (Evers et al., 2010; Vasquez-Robinet et al., 2008). In addition, genetic diversity in drought tolerance has been documented in potato (Coleman, 2008; Schafleitner et al., 2007). *Solanum tuberosum* subsp. *andigena* plants, with short day requirements and originated from harsh environments, show higher drought tolerance than the commonly consumed potato *S. tuberosum* subsp. *tuberosum* Hawkes (hereafter, referred as *tuberosum*) better adapted to long-day conditions and highly susceptible to drought (Weisz et al., 1994). The higher water tolerance of *andigena* potatoes has been attributed to the induction of heat shock proteins and antioxidant genes encoding proteins in the chloroplast, as well as genes for anthocyanin synthesis and transport (Vasquez-Robinet et al., 2008). Regarding the role of anthocyanins and flavonoids mitigating photoinhibition and drought stresses in potato plants (Chalker-Scott, 1999; Crocco et al., 2018), we hypothesize that the overexpression of BBX21 improves the photosynthetic rates under suboptimal water conditions. Here, we demonstrated that the overexpression of BBX21 induces higher photosynthesis rates during the early hours in the morning with higher WUE and bigger tubers. The better photosynthetic behavior of BBX21-OE lines under moderated drought is associated with enhancing mesophyll conductance (g_m) and maximum capacity of electron transport (J_{max}). In addition, BBX21-OE plants are less sensitive to ABA than wild-type spunta (hereafter referred as WT). In our RNA-seq, we found 204 growth- and stress-related genes, such as *SAP12, MYB73, EGYP1, TIP2-1* and *DREB2A*, whose transcripts decreased by drought in WT plants, but they were expressed independently of the water condition in BBX21-OE lines. The present study demonstrated that BBX21 transcription factor interplays between ABA and growth signaling networks to adjust photosynthesis rate under moderated drought.

RESULTS

BBX21-OE lines are more tolerant under moderated drought

We know that BBX21-OE potato plants are more robust and produce more photosynthesis at saturated irradiances associated with a lower photoinhibition that increases tuber yield in optimal water conditions (Crocco et al., 2018). Here, we studied the performance of BBX21-OE lines in suboptimal soil water potentials, simulating field conditions of potato crops. We cultivated potato plants at field capacity for 28 days after emergence, and then reduced the irrigation to maintain 35% of water at field capacity for 14 days. The field capacity control (well-watered) treatment established −0.4 MPa, while the moderated drought condition reduced the water potential in plant tissues at

−1.2 MPa (Figure 1a). We found that 42-day-old WT plants reduced the stem height from 9 to 2 cm, with the growth inhibition almost imperceptible in BBX21-OE lines (Figure 1b). The drought affected significantly the final height of WT plants (41.07 ± 1.26 cm versus 34.63 ± 0.94 cm), but not of BBX21-OE plants (34.86 ± 1.13 versus 31.72 ± 0.65). In addition, WT plants showed water stress symptoms in basal leaves (Figure S1). The anthocyanin levels increased under drought in WT, and maintained constitutively high independent of the water condition in BBX21-OE lines (Figure 1b). The moderated drought reduced the chlorophyll content in the WT leaves, and it was constitutively higher in BBX21-OE leaves (Figure 1b). These results suggest that BBX21 contributes to reduce the negative effects of drought during the vegetative growth of potato plants.

BBX21-OE lines maintain higher tuber weight under moderated drought

To evaluate the effects of BBX21 overexpression in the yield of potato plants cultivated under moderated drought, we designed a new experiment. We cultivated the plants for 42 days at field capacity and then reduced the water in the pots at 35% field capacity for an additional 28 days. At day 70, the pots with plants were irrigated at field capacity until the end of the experiment at day 96 when we measured yield parameters (Figure 2a). In well-watered pots, the BBX21 overexpression increased the plant dry weight in CH_2, but not in CH_{13} plants, and significantly promoted the number and weight of tubers (Figure 2b). Under moderated drought, BBX21-OE lines showed a significantly higher weight of tubers than WT (70 ± 0.98% versus 59.42 ± 1.16% with respect to WT in well-watered conditions, respectively; Figure 2b). We did not find differences between genotypes in plant dry weight and number of tubers for plants cultivated under moderated drought (Figure 2b). These results suggest that BBX21 improves the yield of potato plants cultivated under moderated drought.

BBX21-OE lines have higher photosynthesis and WUE under moderated drought

We also evaluated photosynthesis and transpiration rate, stomatal conductance (g_s), CO_2 concentration of stomatal cavity (C_i) and WUE in 42-day-old plants cultivated in well-watered or moderated drought (Figures 3 and S2). We found that BBX21-OE lines had higher photosynthesis rates than WT plants between 400 μmol m^{-2} sec^{-1} and 1500 μmol m^{-2} sec^{-1} photosynthetic proton flux density (PPFD), independently of the water condition (Figure 3). However, g_s and transpiration rate were higher in BBX21-OE than WT plants in well-watered conditions; the genotypes differences for both parameters were lost under moderated drought (Figures 3 and S2). In addition, the

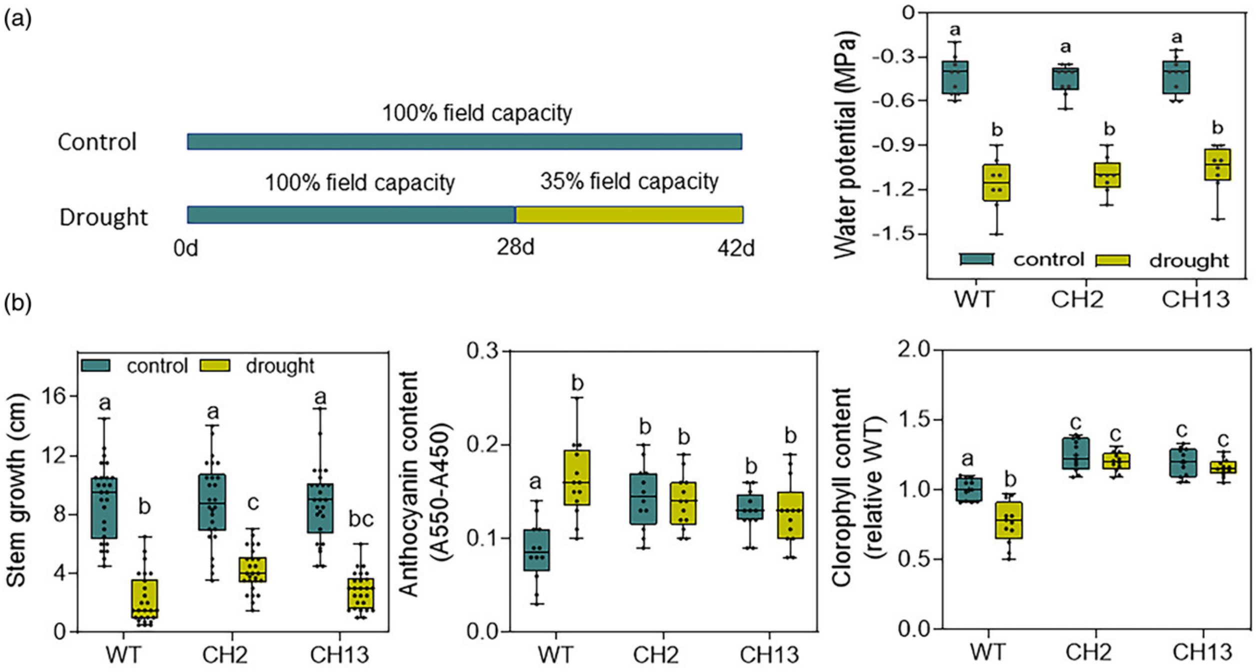

Figure 1. BBX21-OE lines are less sensitive in drought.

(a) Diagram of the experimental design. Wild-type (WT) and BBX21-OE (CH_2 and CH_{13}) plants were cultivated for 28 days at field capacity and then exposed to moderated drought for an additional 14 days (well-watered = 100% and drought = 35% water availability). Leaf water potential in 42-day-old plants at 10:00 h on a sunny day ($n = 9$).

(b) Stem growth ($n = 26$), anthocyanin content ($n = 12$) and chlorophyll levels ($n = 12$) in 42-day-old plants cultivated under control (well-watered) and moderated drought. Data are means ± SE. Different letters denote significant differences among means ($P < 0.05$).

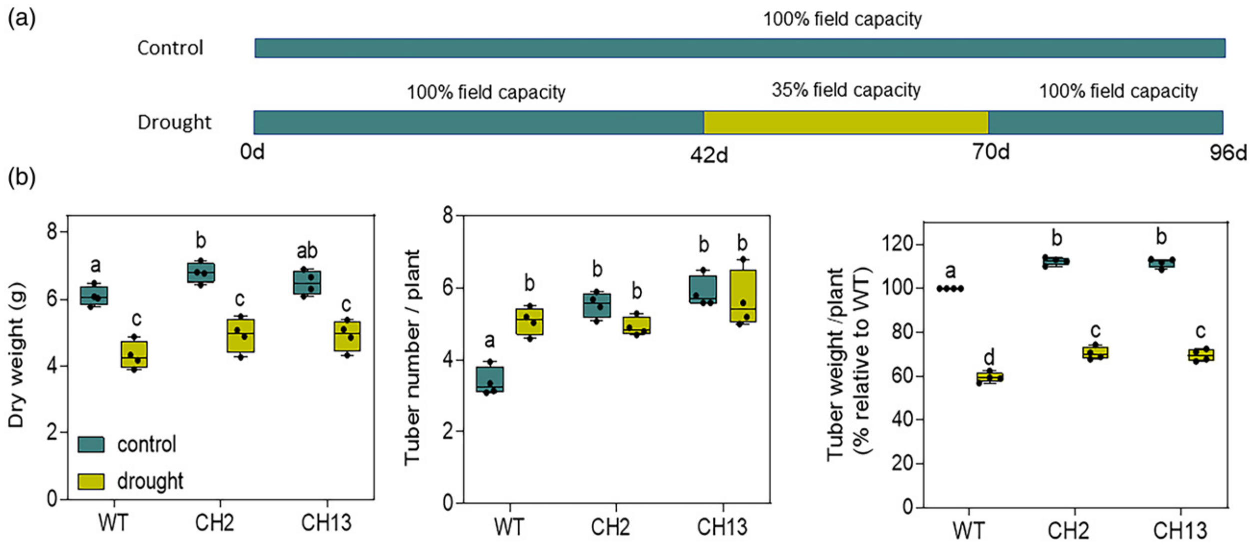

Figure 2. BBX21-OE lines have higher tuber weight and maintain stable the number of tubers in drought.
(a) Diagram of the experimental design using to evaluate yield traits in 96-day-old plants. Plants were cultivated for 42 days at field capacity, then exposed to moderated drought for 28 days (well-watered = 100% and moderated drought = 35% water availability), and then 26 days at field capacity for recovery.
(b) Dry weight, tuber number and tuber weight in 96-old-day plants (WT, CH_2, CH_{13}) cultivated in control (well-watered) and moderated drought. Data are means ± SE of four independent experiments ($n = 4$). Different letters denote significant differences among means ($P < 0.05$).

WUE was higher in BBX21-OE than in WT plants grown under moderated drought (Figure 3). These results suggest that BBX21 overexpression induces a higher biomass for water unity used, maintaining a higher photosynthesis rate at moderated water restriction.

BBX21-OE lines are less sensitive early in the morning under moderated drought

We designed a time-course experiment to study the diurnal pattern of photosynthesis responses under well-watered and moderated drought conditions. We measured the photosynthesis rate at 07:00, 09:00, 14:00 and 19:30 h. The photosynthesis rate increased until 9 h and then decreased until 19:30 h, with the photosynthesis pattern being similar between genotypes, but slightly higher for OE lines cultivated in well-watered conditions. However, under moderated drought, the photosynthesis rate began to decrease earlier in WT than BBX21-OE lines, with the values being double for the transgenic genotype at 9 h (Figure 4). After midday, the photosynthesis rate was almost null for plants cultivated under drought independently of the genotype (Figure 4). The higher photosynthesis rate for BBX21-OE lines in the morning was correlated with higher values of g_s (~0.20 versus ~0.13 mol m^{-2} sec^{-1}), mesophyll conductance, g_m (~0.80 versus ~0.40 mol m^{-2} sec^{-1}) and WUE (~0.13 versus ~0.08 μmol $mmol^{-1}$) under moderated drought (Figure 4). To have a better understanding of the process, we estimated the photosynthetic efficiency per unit of available CO_2 concentration in the chloroplast under saturating irradiances (Phot/C_c ratio) and the C_c/C_i ratio (Figure 5). We found a positive lineal association between photosynthesis and C_c and C_c/C_i ratio ($r^2 = 0.79$ and 0.75, respectively; Figure 5a). We also found significantly higher ratios of Phot/C_c and C_c/C_i in the BBX21-OE lines than WT under moderated drought ($P = 0.0484$ and $P < 0.001$, respectively), these differences between genotypes were lost for plants grown under well-watered conditions (Figure 5b). To have a better understanding of the mechanisms involved in the higher Phot/C_c ratios observed in BBX21-OE lines under drought, we performed net photosynthesis functions for increasing C_c values by manipulating CO_2 concentrations. Surprisingly, the higher photosynthetic efficiency (phot/C_c) of BBX21-OE lines was associated with a higher maximum rate of photosynthetic electron transport (J_{max}) instead of genotype differences in the maximum rate of Rubisco carboxylase activity (V_{cmax}) under moderated water stress (Figure 6). In order to strengthen the analyses of J_{max} estimated through the photosynthesis model developed by Farquhar et al. (1980), we also estimated this parameter by using empirical J_{max} measurements performed by the Li-6400 analyzer by setting the reference cell (CO_2R) at 1500 μmol mol^{-1} and the same pattern was observed (Figure S4c). As expected, we found significant differences in the g_m values between genotypes estimated under normal CO_2 concentrations of 400 ppm in the reference cell (Figure 6b), confirming the results of the time-course experiment at 9 h (Figure 4). In addition, we did not find any association between the net photosynthesis rate and the C_i (Figure S3), suggesting that this parameter does not explain the differences observed in the photosynthesis rate between genotypes under moderated drought. Together, these results suggest that BBX21-OE

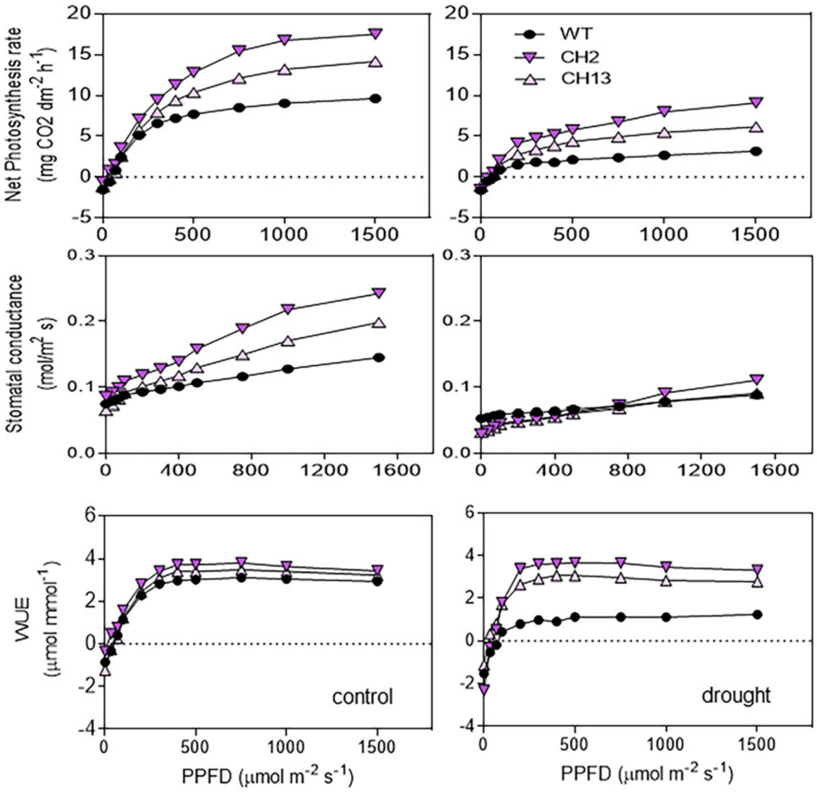

Figure 3. BBX21-OE lines produce higher photosynthesis and water use efficiency (WUE), and similar stomatal conductance in drought.

Net photosynthesis, stomatal conductance (g_s) and WUE as function of PPFD in 42-day-old plants (WT, CH_2 and CH_{13}) cultivated under control (well-watered) and moderated drought (n = 4). Measurements were done between 10:00 h and 15:30 h.

lines cultivated under moderated drought have a higher photosynthesis rate due to two reasons. First, the higher g_m in the morning (Figure 4) is a consequence of a higher CO_2 diffusion to the chloroplast (e.g. C_c/C_i ratio; Figure 5b). Second, the higher J_{max} (Figure 6) contributes to a higher photosynthetic efficiency at the same C_c (e.g. Phot/C_c ratio; Figure 5b).

BBX21 increases water tolerance through reduced ABA sensitivity under moderated drought

In Arabidopsis, it has been documented that BBX21 positively regulates stomatal aperture and *bbx21* mutant seedlings show hypersensitivity to ABA (Xu et al., 2014). Here, we hypothesized that a lower concentration and/or sensitivity to ABA improve the photosynthesis rate of BBX21-OE lines under moderated drought. Firstly, we evaluated the ABA content in the leaves of 42-day-old plants cultivated in well-watered and drought conditions. We did not find any differences in ABA content between WT and OE lines in any water condition (Figure 7a), suggesting that the ABA levels do not explain the differences between genotypes under moderated drought. To evaluate the ABA sensitivity of stomatal opening, we designed a bioassay with detached leaves from 21-day-old plants placed for 1 h in a solution with 0, 1 and 10 µM ABA. We did not find any differences in the stomatal aperture between genotypes at 0 and 10 µM ABA. However, BBX21-OE leaves showed a higher stomatal aperture than WT at 1 µM ABA (Figure 7b). These results suggest that BBX21 overexpression reduces ABA sensitivity in plant tissues. To confirm these results in a more realistic condition, we designed a new experiment spraying the leaves of 42-day-old well-watered plants with 2, 5 and 100 µmol ABA, maintaining a control spraying with a water solution (Figure 8). We found significant and higher differences in photosynthesis rate, g_s and C_c for BBX21-OE lines, compared with WT, at 2.5 µmol but not at 100 µmol ABA (Figure 8). Together these results suggest that BBX21-OE lines are more tolerant under moderated drought because they have a lower sensitivity to ABA.

BBX21 regulates the expression of transcription factors, aquaporins and photosynthesis- and stress-regulated genes under moderated drought

To gain insight into the molecular mechanisms regulated by BBX21 under moderated drought, we studied the transcriptome of 42-day-old potato plants cultivated under well-watered and moderated drought conditions following the same experimental protocol used previously (Figure 1a). Firstly, we compared the genes expressed differentially by drought in WT plants. We found 1494 genes differentially expressed between well-watered and drought in WT. From these genes, we selected 491 upregulated and 1003 downregulated by drought in WT (Tables S1 and S2, respectively). To identify specific target genes of BBX21, we compared the gene expression between WT and BBX21-OE lines cultivated under moderated drought

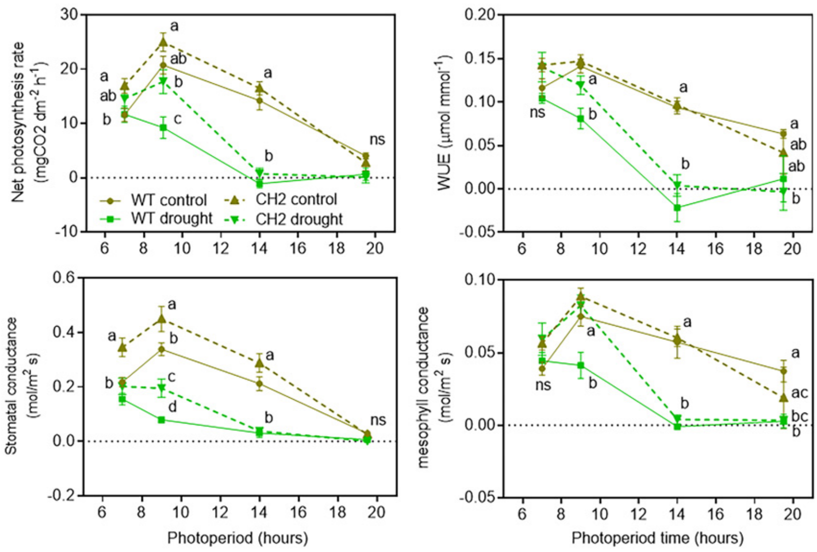

Figure 4. BBX21-OE lines are less sensitive to photoinhibition during the early morning in drought.

Daily march of the net photosynthesis rate, water use efficiency (WUE) stomatal conductance and mesophyll conductance (g_m) in 42-day-old plants (WT and CH_2) cultivated under control (well-watered) and moderated drought ($n = 6$). Data are means ± SE. Different letters denote significant differences among means ($P < 0.05$).

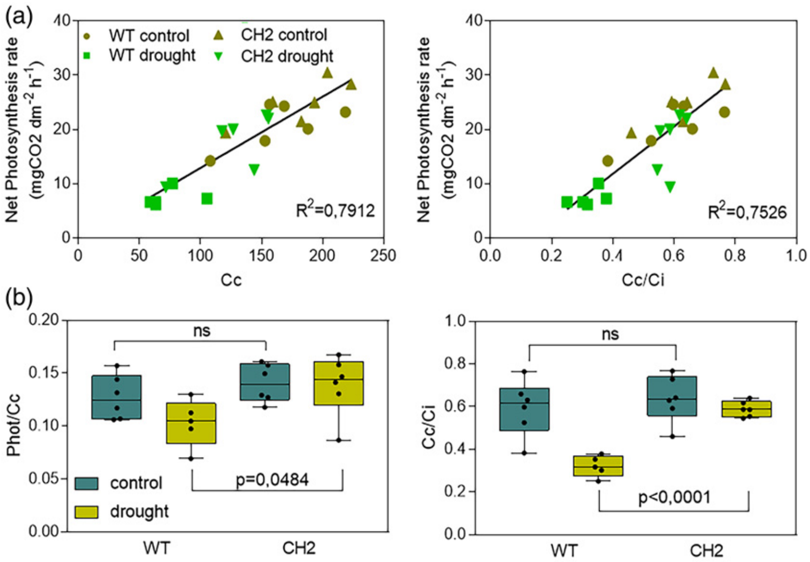

Figure 5. BBX21-OE lines have higher CO_2 diffusion to the chloroplast (C_c/C_i ratio) and biochemical affinity for photosynthesis at the same C_c (Phot/C_c ratio) in drought.

(a) Net photosynthesis rate plotted with C_c (CO_2 concentration in the chloroplast) or with the ratio of C_c/C_i (CO_2 concentration in the chloroplast/CO_2 concentration in the stomatal cavity). The R^2 values of the linear regression analysis are indicated.

(b) Net photosynthesis per C_c unit (Phot/C_c) and C_c/C_i ratio between WT and BBX21-OE lines cultivated in control (well-watered) and moderated drought. Phot/C_c ANOVA was performed with a covariance analysis, using C_c as covariable. Data are means ± SE from those values measured at 09:00 h (Figure 4). Significant differences and P-values between two means are indicated by Student's t-test. ns, not significant.

conditions. We found 841 genes expressed differentially between WT and BBX21-OE genotypes in drought, with 318 genes upregulated and 523 genes downregulated. Then, we proceeded to perform a D-CHIP analysis to classify the BBX21-regulated genes by drought (Figure 9a). We selected two groups of genes (Figure 9a, clusters 1 and 2). Cluster 1 grouped 189 downregulated genes and 21 upregulated genes in WT by drought, and constitutively downregulated in BBX21-OE lines independently of the water condition (Figure 9b; Table S3). Cluster 2 grouped 15 downregulated genes in WT by drought and constitutively stable or high in BBX21-OE lines (Figure 9b; Table S3). For further analysis, we selected some genes downregulated by drought in WT belonging to clusters 1 and 2 (Table S3). Searching for homologous between *S. tuberosum* and *Arabidopsis thaliana,* we found *MYB73, SLAH3, SAUR76, TCP4, DREB2A, SRNK2-5, ERF1, ACS6, EGY1, SAP12* and several heat shock proteins (Table S1). We evaluated the gene expression of *MYB73, EGY1, DREB2A, RCA, SLAH3, TIP2-1* and *SAP12* by quantitative polymerase chain reaction (qPCR; Figure 9c). However, *RUBISCO ACTIVASE* (*RCA*) is absent in the BBX21-regulated transcriptome in drought, we included it because it is required for the light activation of rubisco directly associated with the biochemical photosynthesis (Foyer and Harbinson, 2012). We confirmed that BBX21 regulates the expression of transcription factors (*MYB73*), photosynthesis (*TIP2-1* and *EGY1*) and stress-regulated (*DREB2A* and *SAP12*) genes in plants cultivated under moderated drought. *MYB72* and *TIP2-1* maintain their expression constitutively upregulated, while *EGY1, DREB2A* and *SAP2* are constitutively downregulated (Figure 9c). We could not find any

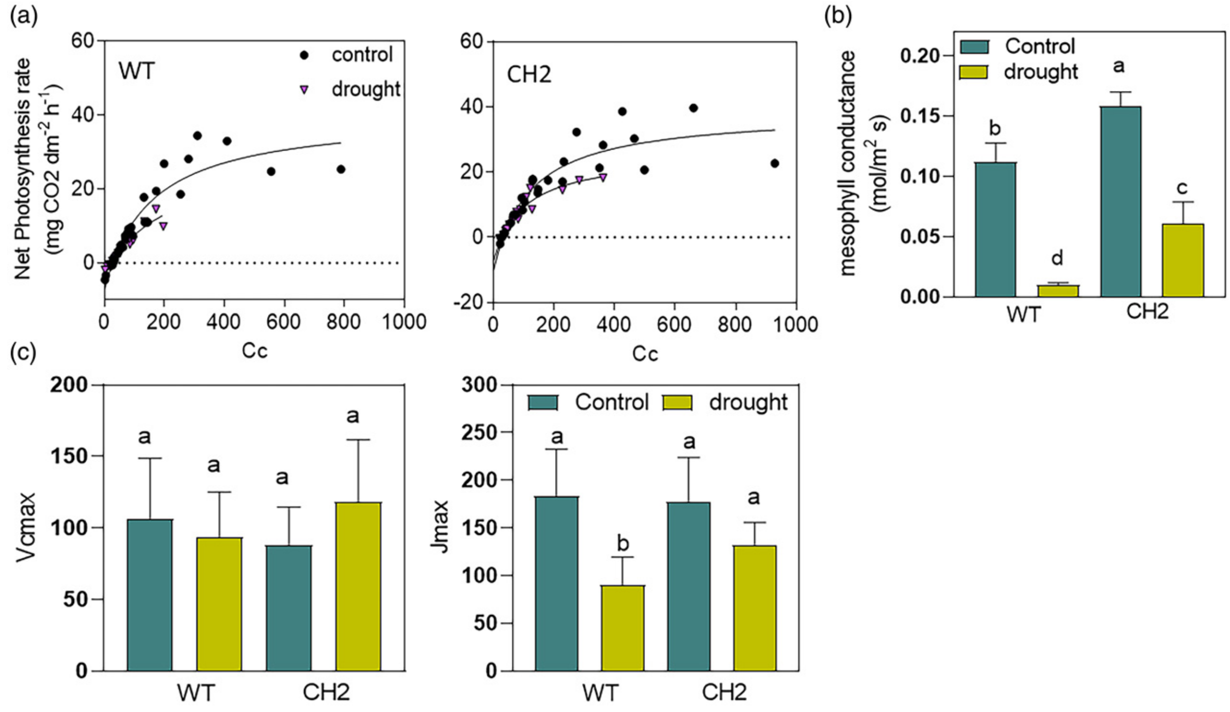

Figure 6. BBX21-OE lines have higher mesophyll conductance (g_m) and maximum capacity of electron capacity (J_{max}) in drought.
(a) Net photosynthesis as a function of the CO_2 concentration in the chloroplast (C_c) in 42-day-old plants of WT and BBX21-OE lines cultivated under control (well-watered) and moderated drought ($n = 4$).
(b) Estimation of mesophyll conductance (g_m) for the measurements performed under normal CO_2 concentrations (400 ppm in the reference cell).
(c) Estimation of J_{max} and maximum rate of Rubisco carboxylase activity (V_{cmax}). Data are means ± SE. Different letters denote significant differences among means ($P < 0.05$).

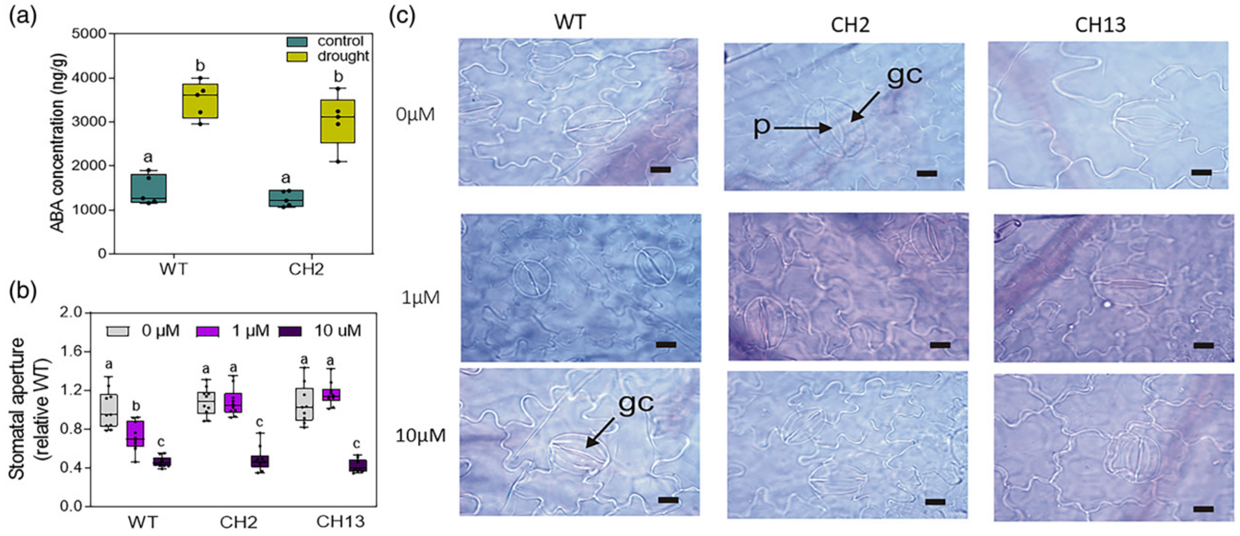

Figure 7. BBX21-OE lines are less sensitive to abscisic acid (ABA) for stomata opening in detached leaves.
(a) ABA content in 42-day-old plants (WT and CH_2) cultivated in control (well-watered) and moderated drought ($n = 5$).
(b) Stomatal aperture in 21-day-old detached leaves of WT and BBX21-OE lines (CH_2 and CH_{13}) exposed to different levels of ABA ($n = 10$). Data are means ± SE relative to WT in well-watered condition. Different letters denote significant differences among means ($P < 0.05$).
(c) Representative photographs show stomata opening in WT and BBX21-OE leaves (CH_2 and CH3) imbibed with 0, 1 and 10 µmol of ABA. gc, guard cell; p, pore. The horizontal lines represent 10 µm.

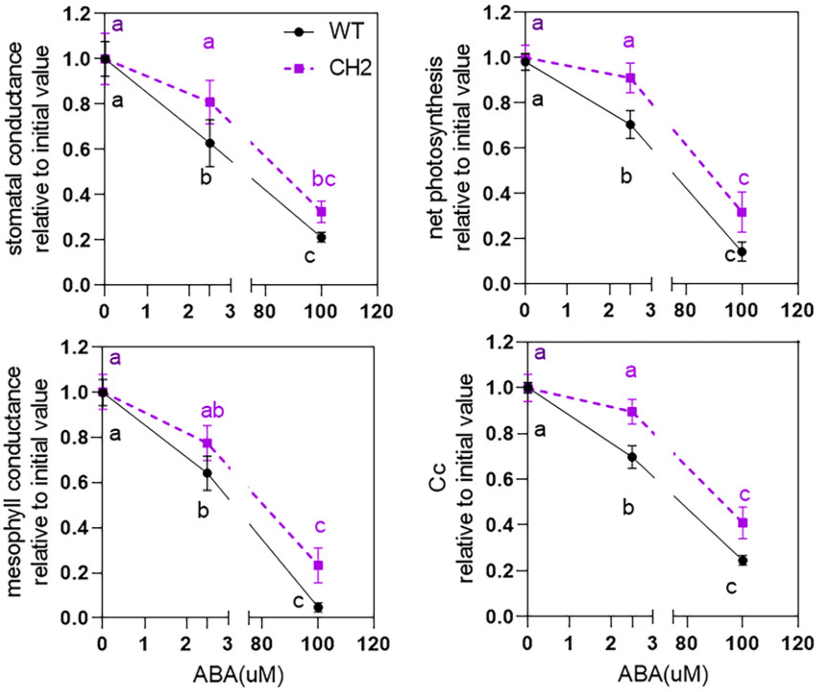

Figure 8. BBX21-OE lines are less sensitive to abscisic acid (ABA) for stomatal conductance in entire 21-day-old plants cultivated in greenhouse conditions.

Stomatal conductance (g_s), net photosynthesis, mesophyll conductance (g_m) and CO_2 concentration in the chloroplast (C_c) were measured in 21-day-old well-watered plants (WT and CH_2) sprayed with water or 2.5 and 100 μmol of ABA in WT and CH_2. Data are means ± SE relative to WT ($n = 10$). Different letters denote significant differences among means ($P < 0.05$).

statistical differences in *RCA* and *SLAH3* genes between genotypes and water conditions (Figure 9c). These results suggest that BBX21 controls the expression of transcription factors, aquaporins, and photosynthesis- and stress-regulated genes to adjust plant growth under moderated drought.

DISCUSSION

BBX21 improves photosynthesis rate under moderated drought

In well-watered conditions, we found that the higher photosynthetic rates of the BBX21-OE lines are strongly associated with a higher g_s and transpiration rate, and an increase of anthocyanins that reduce the photoinhibition without penalty in WUE compared with WT (Figures 1, 3 and S2; Crocco et al., 2018). In contrast, under moderated drought, we showed that BBX21-OE lines have similar anthocyanin levels to WT plants, and maintain higher photosynthesis rates and WUE without differences in transpiration rate and g_s (Figures 1, 3 and S2). However, g_s and g_m emerge as crucial keys for its variation along the photoperiod. In fact, the patterns of g_s and g_m are significantly higher in BBX21-OE than WT plants under moderated drought in the morning (Figure 4). The null photosynthesis values after mid-day are a consequence of the extremely low g_s and the increase of the ABA content under moderated drought independently of the genotypes (Figure 7a). These results strongly suggest that different physiological mechanisms are involved to increase the photosynthesis rates in BBX21-OE compared with WT plants under well-watered and moderated drought.

The advantage of the BBX21 overexpression plants under moderated drought is mainly supported by non-stomatal enhancements for different reasons. First, C_i is lower in BBX21-OE than WT plants, despite that they have similar g_s and transpiration rate (Figures 3 and S2). Second, BBX21-OE lines have higher g_m than WT plants, and consequently a higher C_c/C_i ratio in the chloroplast (Figure 5a). Third, a higher photosynthesis rate in BBX21-OE lines at the same C_c (Phot/C_c ratio) under moderated drought (Figure 5b) is determined by higher J_{max} values, without differences in V_{cmax}, compared with WT plants (Figure 6). Here, we estimated Photo/C_c responses (Figure 6) to avoid the underestimation of the velocity of Rubisco carboxylation (V_{cmax}) through Photo/C_i responses calculated under abiotic stresses (Figure S4a,b; Ethier and Livingston, 2004). The higher J_{max} values of BBX21-OE plants under moderated drought can be explained for the higher chlorophyll content compared with WT plants, and the drastic drop of chlorophylls at the expense of higher levels of anthocyanins in WT (Figure 1b). We confirmed the relevance of J_{max}, together with g_m, as the main factors responsible for the enhancement of photosynthesis in BBX21-OE plants under water stress using the Li-6400 analyzer that calculates J_{max} with empirical measurements (Figure S4c). Finally, the weak association between the photosynthesis rate and C_i (Figure S3) clearly suggests that g_s is not the main cause that explains the higher photosynthesis rates of BBX21-OE lines under moderated drought.

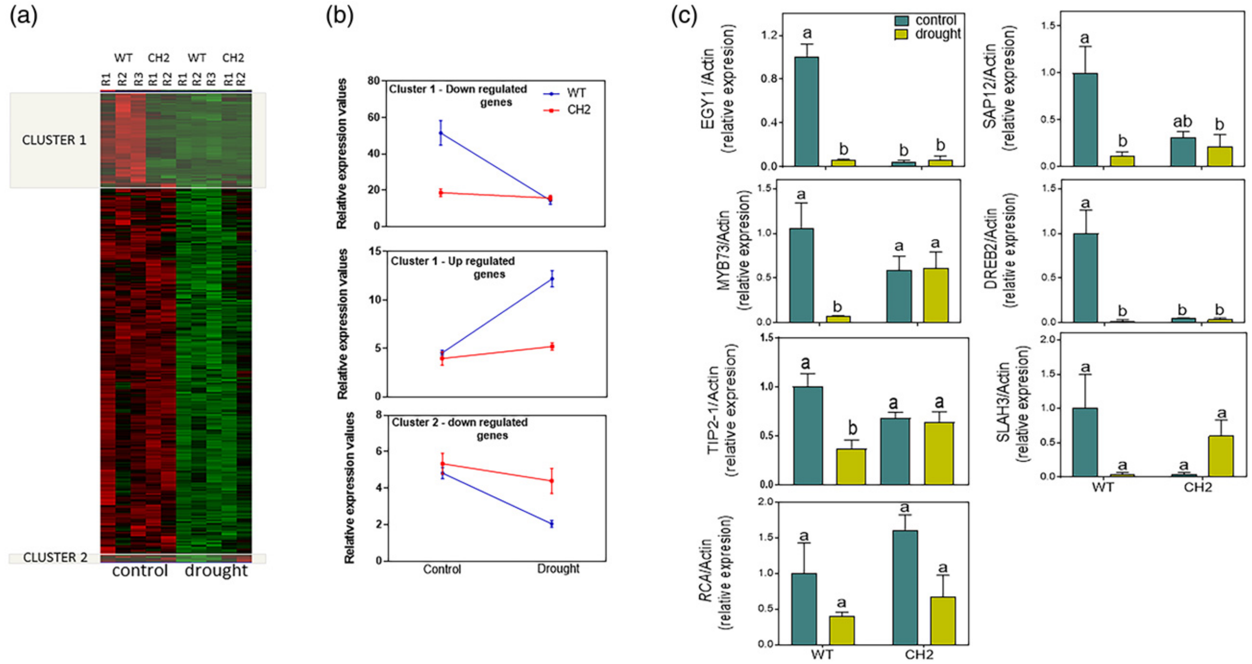

Figure 9. Transcriptome of BBX21-regulated genes in drought and gene expression validation.
(a) Heat map shows 841 genes differentially expressed in WT and BBX21-OE lines in drought. Clusters 1 and 2 are highlighted.
(b) Average expression of genes belonging to clusters 1 and 2 for WT and BBX21-OE lines in well-watered and drought conditions.
(c) Quantitative polymerase chain reaction (qPCR) expression of genes downregulated in WT and constitutively downregulated in BBX21-OE lines. Gene expression of *MYB73, EGY1, DREB2A, RCA, SLAH3, TIP2-1* and *SAP12* was evaluated in 42-day-old plants (WT and CH$_2$) cultivated in control (well-watered) and moderated drought. Data are means ± SE (n = 3). Different letters denote significant differences among means ($P < 0.05$).

According with the main role of the mesophyll conductance found in our work, previous reports found that g_m is associated with the reduction of photosynthesis rate under drought, and it is well correlated with the expression of carbonic anhydrase and aquaporin genes (Flexas et al., 2006; Han et al., 2016; Perez-Martin et al., 2014). We found that *TIP2.1/AQP1* (at3g16240) aquaporin in Arabidopsis shares 92% similarity ($P < 1.1$ e^{-109}) with PGSC00003DMT4000042613 potato gene downregulated by drought in WT in our transcriptome (Cluster 1, Table S3). Using reverse transcriptase (RT)-qPCR, we confirmed that *TIP2.1/AQP1* is significantly reduced by drought in WT but not in BBX21-OE lines (Figure 9c), which probably contributes to enhance a higher photosynthesis rate under water limitations. According with our results, transgenic tobacco plants overexpressing NtAQP1 have a higher g_m than WT by affecting CO$_2$ permeability of biological membranes (Flexas et al., 2006).

BBX21 reduces ABA sensitivity to improve photosynthesis under moderated drought

The ABA content in the leaves of plants cultivated in well-watered conditions is lower than those exposed to moderated drought without significant differences between genotypes (Figure 7a). In contrast, we found that WT and BBX21-OE lines have different ABA sensitivities in a bioassay with detached leaves (Figure 7b). The imbibition of leaves with 1 μmol ABA reduces the stomatal aperture of WT leaves, but it maintains opening the stomata of BBX21-OE leaves. Genotypic differences in the stomatal response disappear with 10 μmol ABA (Figure 7b,c). We confirmed these results in a more realistic condition of entire plants spraying with different doses of ABA to evaluate photosynthesis and related parameters. The overexpression of BBX21 improves the photosynthesis rate, g_s and C_c at 2.5 μmol ABA, but not at higher levels of ABA (Figure 8). These results strongly suggest that BBX21-OE lines are more tolerant to ABA. In addition, these results demonstrate that BBX21 is involved in the ABA signaling pathway controlling photosynthesis responses. This is according with previous reports showing that BBX21 integrates ABA and light signaling. Xu et al. (2014) found that BBX21 positively regulates stomatal aperture and *bbx21* mutant seedlings are hyposensitive to ABA. In another study with Arabidopsis plants, Kang et al. (2018) found that BBX21 directly bounds the ABI5 promoter and recruits the chromatin-remodeling factor PICKLE to modulate the chromatin structure at the ABI5 locus downregulating ABI5 expression to sustain the stomatal aperture in detached leaves exposed to a short pulse of blue or red light. In addition, recent works demonstrated that other BBXs can also be involved in response to drought (Liu et al., 2019; Mbambalala et al., 2020; Xu et al., 2020). While the overexpression of *AtBBX29* enhances the antioxidant and osmolyte capacity in sugarcane plants (Mbambalala et al., 2020), the overexpression of *BBX19* in chrysanthemum reduced drought tolerance through CmBBX19 interferences with CmABF3-dependent transactivation of downstream ABA signaling genes (Xu et al., 2020).

BBX21 regulates the expression of stress- and growth-related genes under moderated drought

Using a RNA-seq comparing 42-day-old WT and BBX21-OE lines cultivated for 14 days under moderated drought, we identified 1494 regulated by drought in WT (Tables S1 and S2) and 844 genes were BBX21-dependent regulated (Figure 9a; Table S3). From those BBX21-regulated genes by drought, we identified 189 and 15 genes downregulated in WT but not in BBX21-OE belonging to clusters 1 and 2, respectively (Figure 9a,b; Table S3). Between them, BBX21 regulates the expression of growth and hormone signaling (*MYB73, TCP14, SUR76, ERF1*), response to stress (several *HSP, DREB2A, SAP12, SNRK2-5*), photosynthesis response (*EGY1, RCA, TIP2.1*) and stomata function (SLAH3; Figure 9b; Table S3). These results confirm that BBX21 is a key regulatory piece that controls the gene expression of different signaling pathways (Gangappa and Botto, 2014; Song et al., 2020; Yadav et al., 2020). In this work, we demonstrated that *EGY1* is regulated by BBX21 in potato plants cultivated under moderated drought (Figure 9c; Table S3). In Arabidopsis, EGY1 is involved in the turnover of photosystem II reaction center under high light stress (Chen et al., 2005; Qi et al., 2020). It has also been documented that EGY1 is a retrograde signal that integrates ABA signaling to maintain chloroplast functionality under ammonium stress in Arabidopsis (Li et al., 2012). On the other hand, BBX21 also controls the expression of structural proteins required for drought tolerance such as *SAP12* and *DREB2A* transcripts (Figure 9c; Table S3). Stress-associated protein (*SAP*) genes encode proteins to protect the cells from the effects of water stress (Giri et al., 2013). In Arabidopsis, SAP12 acts as an early stress sensor, transmitting redox information to other components of the cell through protein interactions (Ströher et al., 2009). In addition, DREB2A is a key transcription factor activated by different stresses such as drought, salt stress and thermotolerance that induces many drought and heat stress-inducible genes via cis-acting dehydration-responsive element, DRE, sequence by an ABA-independent regulatory signaling pathway (Sakuma et al., 2006; Soma et al., 2021), suggesting that BBX21 can operate as integrator of ABA-dependent and -independent signaling pathways.

In nature, plants frequently grow in suboptimal environments. Variation in resources that help plants cope with their growth are common, and plants evolve to compete for limited resources such as water availability. In the context of the global climatic change, it is probable that plants will be more frequently exposed to limitations in water resources. Our work suggests that BBX21 can be modulated to adjust plant growth under moderated stressful environment to increase the production of tubers in potato. The overexpression of BBX21 in potato plants increased the tuber's yield per plant by ~17% with respect to WT under moderated drought (Figure 2b). Future research is needed to have a broader view about the BBX21 mechanisms to improve water stress tolerance at the field trials. Understanding stress signaling and responses will increase our ability to manage water stress tolerance in crops for sustainable agriculture.

EXPERIMENTAL PROCEDURES

Plant material

Potato (*S. tuberosum*) var. Spunta (hereafter referred as WT) and two *AtBBX21* transgenic overexpressing lines (hereafter referred as BBX21-OE, CH_2 and CH_{13}) were used in the experiments. These lines were described previously (Crocco et al., 2018).

Growth conditions to evaluate vegetative growth under moderated drought

The experiments were performed in a greenhouse located at the Faculty of Agronomy, University of Buenos Aires (34°35′S, 58°29′W), with natural radiation (PAR = 500 μmol m^{-2} sec^{-1}), and temperatures ranged between 18 and 24°C. Four independent experiments were carried out between March and November with a photoperiod of about 13.5 h. For each experiment, 16 sprouted tubers of similar size were sown in pots of 10 cm diameter × 15 cm height (1000 cm^3). The soil mix consisted of vermiculite (1/3), peat (1/3) and perlite (1/3). The pots were watered daily at field capacity and fertilized during the experiment with red HAKAPHOS®. The plants were watered daily at field capacity for 28 days. After day 28, the watering was restricted to 35% of the field capacity for 14 days. To evaluate the level of water restriction, the pots were weighed every day during the experiment, and watered when necessary.

Growth conditions to evaluate tuber yield under moderated drought

Four independent experiments were carried out between March and November with a photoperiod of about 13.5 h. For each experiment, 16 sprouted tubers of similar size were sown in pots of 16 cm diameter × 22 cm height (4000 cm^3). The soil mix consisted of vermiculite (1/6), peat (1/6), perlite (1/6), and soil (3/6). The pots were watered daily at field capacity and fertilized during the experiment with red HAKAPHOS®. The plants grew to 96 days old and then were harvested. For the water stress treatment, the plants were watered daily at field capacity for 42 days. After day 42, the watering was restricted to 35% of the field capacity for 28 days, and then the plants were watered again to field capacity until day 96. To evaluate the level of water restriction, the pots were weighed every day during the experiment, and watered when necessary.

Anatomical and morphological determinations

Samples from the central part of the leaf were diaphanized by using Dizeo de Strittmatter's (1973) technique and stained with safranin. In addition, transverse sections of leaves were embedded in paraffin and serially cut at 10–12 μm with Minot-type rotary microtome, and sections were stained with safranin-fast green (Johansen, 1940). Sections were photographed with a Zeiss Axioplan optical microscope and analyzed with the Zeiss AxioCam ERc 5s software (Carl Zeiss Microscopy GmbH, Jena, Germany). The measurements were performed in three biological samples with 10 technical replicates for each genotype. Photos were taken with polarized light. Plant height, and dry and fresh weight of plants were assessed. The height of the principal stem was estimated before water stress (28-day-old plants) and after water stress (42-day-old plants) using a ruler. The aerial part of 42-day-old plants was harvested and weighed with a digital balance. The material was enveloped into a paper and incubated at 60°C during 72 h, and then weighed again to obtain the dry weight.

Chlorophyll and anthocyanin determinations

Chlorophyll determinations were performed in the basal leaves of 42-day-old plants using a SPAD 502 (Minolta) and calculating chlorophyll levels according to the method described by Moran (1982). Leaf discs (6 mm diameter) of the fourth completely expanded leaf of 42-day-old plants were collected for anthocyanin quantifications by spectrophotometry. Each biological sample was the average of two independent leaf discs. Anthocyanin quantification was performed as described by Deikman and Hammer (1995).

Photosynthesis determinations

Leaf water potential (Ψw) was recorded on the fourth expanded leaves in 42-day-old plants, after water stress treatment, using a Scholander-type pressure chamber (Bio-Control, Buenos Aires, Argentina). Net photosynthesis rate, stomatal conductance (g_s), transpiration rate and CO_2 concentration of stomatal cavity (C_i) were measured in 42-day-old plants using an open infrared gas analysis system (Li-Cor 6400). Light functions were measured at 0, 30, 50, 100, 200, 300, 400, 500, 750, 1000 and 1500 μmol m^{-2} sec^{-1}. PPFD was performed in fully expanded leaves using the 6400-02B LED light source chamber. Air flow and CO_2 concentration in the reference cell (CO_2R) were automatically controlled by the equipment at 300 μmol sec^{-1} and 400 μmol mol^{-1} (ppm), respectively. The instantaneous WUE was estimated as the ratio of carbon assimilation and transpiration (net photosynthesis rate/transpiration rate). In a new experiment, net photosynthesis and electron transport rate (J) responses to CO_2 levels were also determined with the following sequence of CO_2 concentrations at the reference cell (CO_2R): 400, 300, 200, 100, 50, 400, 700, 1000 and 1500 μmol mol^{-1}, setting PPFD at 1500 μmol m^{-2} sec^{-1} and air flow at 300 μmol sec^{-1}. C_i and CO_2 concentration in the chloroplast (C_c) functions were fitted to the biochemically based photosynthesis model of Farquhar et al. (1980). Thus, the maximum rate of Rubisco carboxylase activity (V_{cmax}) and the maximum rate of photosynthetic electron transport (J_{max}) were estimated for each treatment and genotype. Temperature corrections for V_{max} and J_{max} were made through the equations reported by Bernacchi et al. (2001) and McMurtrie and Wang (1993), respectively, and R_d was fitted as well. J_{max} was also estimated by using empirical J measurements performed by the Li-6400 analyzer by setting the reference cell (CO_2R) at 1500 μmol mol^{-1}.

Mesophyll conductance and non-stomatal parameters

Mesophyll conductance (g_m) was estimated in both experiments by using the variable J method (Bongi and Loreto, 1989; Harley et al., 1992) through the following equation:

$$g_m = \text{Net photosynthesis}/\{C_i - (\Gamma*[[J + 8(\text{Net photosynthesis} + R_d)]]/[[J - 4(\text{Net photosynthesis} + R_d)]]\}g_m = \text{Net photosynthesis}/\{C_i - (\Gamma*[[J + 8(\text{Net photosynthesis} + R_d)]]/[[J - 4(\text{Net photosynthesis} + R_d))]]\}$$

where R_d is the respiration occurring during the day, not related to photorespiration, and Γ^* is the CO_2 compensation point in the absence of R_d. Net photosynthesis, J and C_i were measured with the Li-6400 System and measurements above described, Γ^* was obtained from the literature at the measuring temperature (25°C; Bernacchi et al., 2002), and R_d was indirectly estimated for each treatment with the method proposed by Yin et al. (2011), based on combined photosynthesis and fluorescence measurements across a range of low irradiances. CO_2 concentration in the chloroplast (C_c) was also estimated by the following equation (Sáez et al., 2018):

$$C_c = [C_i - (\text{Net photosynthesis}/g_m)]$$

J measurements were simultaneously performed with the photosynthesis ones described above. In order to evaluate other non-stomatal limitations related with biochemical affinity of photosynthesis per available CO_2 unit, we used the ratio between net photosynthesis and C_c (Phot/C_c).

ABA bioassays in detached leaves and entire plants

For ABA bioassays with detached leaves, we sampled the stomatal aperture by the determination of the size of guard cells in the third–fifth expanded leaves from 21-day-old plants grown in a white light chamber (PAR = 200 μmol m^{-2} sec^{-1}) with a photoperiod of 16 h light/8 h dark at 22°C. Detached leaves were suspended in stomatal opening solution (15 mM KCl, 10 mM $CaCl_2$ and 10 mM MES-KOH, pH 6.15) in the light for 3 h, and then exposed to fresh stomatal opening solution containing 1 μM ABA and 10 μM ABA for 1 h, maintaining a control without ABA. A total of 15 leaves from five WT and AtBBX21-OE lines were collected, and more than 100 stomata from each line were randomly selected and measured to calculate stomatal aperture (the ratio of width to length) for statistical analysis.

For ABA bioassays with 21-day-old well-watered plants cultivated in a greenhouse under natural radiation, we sprayed leaves with different solutions of ABA at the beginning of the photoperiod. The solution with ABA (99% purity; Sigma-Aldrich, St Louis, MO, USA) was dissolved in a small volume of 96% ethanol to prepare solutions of 0, 2, 5 and 100 μM ABA, containing 0.01% Silwet®. Photosynthesis rate, g_s, g_m and C_c were measured 90 min after ABA application.

ABA determinations by LC–ESI–MS–MS (Villar et al., 2020)

A pool of six of the last totally expanded matured leaves of 42-day-old plants were harvested at 14:30 h. Phytohormones analysis was carried out on tissue collected in nitrogen liquid. Then, lyophilized conservation was performed at −20°C. Samples were ground to powder with a mortar and pestle, and weighed (100–200 mg per sample). The extraction was performed with 2 ml of water deionized (pH 2.8) at 4°C for 30 min. After centrifugation (15 min, maximum speed), the buffer was collected, and the pellet was then re-extracted with 2 ml of fresh buffer (pH 2.8) for an additional. Aliquots of 50 ng deuterated ABA (provided for Olchelmn, Czech Republic) were added as internal standards. Extracts were transferred to other tubes, and mixed with ethyl acetate and partitioned. Then the organic phase was extracted and evaporated at 37°C in Speed-Vac. Dried extracts were dissolved in 50 μl methanol (100%) and placed in vials.

Liquid chromatography analyses were performed using an Alliance 2695 (Separation Module; Waters, Milford MA, USA) quaternary pump equipped with auto-sampler. A Restek C18 (Restek, Bellefonte, PA, USA) column (2.1 × 100 mm, 5 μm) was used at 28°C, with injected volume 10 μl. The binary solvent system used for elution gradient consisted of 0.2% acetic acid in H_2O (solvent B) and MeOH (solvent A), at a constant flow-rate of 200 μl min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied [*t* (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration.

The MS/MS measurements were performed on a Micromass Quatro UltimaTM PT double quadrupole mass spectrometer (Micromass, Manchester City, UK). All analyses were performed using turbo ion spray source (ESI) in negative ion mode with the following settings: capillary voltage −3250 V, energy cone 35 V, RF Lens1 (20), RF Lens2 (0.3), source temp. 100°C, desolvation temp. 350°C, gas cone 100 L h^{-1}, gas desolvation 700 L h^{-1}, collision cell potential of 15 V and multiplier (650). MS/MS parameters were optimized in infusion experiments using individual standard solutions of each hormone. MS/MS product ions were produced by collision-activated dissociation of selected precursor ions in the collision cell of the double quadrupole mass spectrometer, and mass was analyzed using the second analyzer of the instrument. In negative mode, the spectrum for each hormone gave a deprotonated molecule [M–H]. Quantitation was performed by injection of samples in multiple reaction monitoring (MRM) modes, as many compounds could present the same nominal molecular mass. The combination of parent mass and unique fragment ions was used to selectively monitor hormones. MRM acquisition was performed by monitoring the 263/153 and 269/159 for ABA and (2H6)-ABA, 174/130 data were acquired and analyzed using MassLynxTM 4.1 and QuanLynxTM 4.1 (Micromass, Manchester, UK) software. For quantification, values were obtained from a calibration curve previously constructed using known amounts of each hormone and their pure standard (Sigma)/deuterated internal standard ratio. ABA determinations were performed in the Plant Physiology Laboratory, Univ. Nacional de Rio Cuarto, Cordoba, Argentina.

Stranded mRNA-seq library preparation and sequencing

We performed RNA-seq to identify the BBX21-regulated transcriptome associated with moderated drought in potato plants. The plants were irrigated daily at field capacity for 28 days and then the water was restricted to 35% of the field capacity for an additional 14 days. We harvested 42-old-day plant tissues. Samples comprising pooled leaves from at least six independent plants were harvested at midday, isolating total RNA from independent triplicated biological samples. Total RNA was extracted with the Spectrum Plant Total RNA Kit (Sigma-Aldrich). Libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit v2 (ref. RS-122-2101/2) according to the manufacturer's protocol. Briefly, 1 μg of total RNA was used for poly(A)-mRNA selection using Oligo (dT) magnetic beads, and was subsequently fragmented to approximately 300 bp. cDNA was synthesized using reverse transcriptase (SuperScript II, ref. 18064-014; Invitrogen, Waltham, MA, USA) and random primers. The second strand of the cDNA incorporated dUTP in place of dTTP. Double-stranded DNA was further used for library preparation. dsDNA was subjected to A-tailing and ligation of the barcoded Truseq adapters. All purification steps were performed using AMPure XP Beads (ref. A63881). Library amplification was performed with PCR using the primer cocktail supplied in the kit. Final libraries were analyzed using Fragment Analyzer (DNF-473) to estimate the quantity and check size distribution, and were then quantified by qPCR using the KAPA Library Quantification Kit (ref. KK4835; KapaBiosystems, Wilmington, MA, USA) prior to amplification with Illumina's cBot. Libraries were sequenced 1 × 50 on Illumina's HiSeq 2500. The RNA-seq were performed by the Center for Genomic Regulation (CGR), Barcelona, Spain.

RNA-seq analysis and qRT-PCR expression

For RNA-seq data analysis, firstly, we identified genes that are statistically up- and downregulated by water stress in the WT transcriptomes (fold-changes > 2). Then, we contrasted their expression levels against BBX21-OE transgenic transcriptomes to identify BBX21-regulated genes under moderated drought by D-CHIP analysis, we selected those transcripts whose expression levels decreased by water restriction in WT, but they were constitutively downregulated in BBX21-OE lines independently of the water condition. From this group of genes, we searched for homologous between *S. tuberosum* and *A. thaliana* to confirm their expression by qRT-PCR following the methodology cited in Crocco et al. (2018).

For the qRT-PCR, leaf samples of 42-day-old plants were collected and ground in liquid nitrogen. Total RNA was extracted from the frozen samples using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1.5 mg DNA-free RNA template using an oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). RT-qPCR analysis was performed on an optical 96-well plate using SYBR Green PCR master mix (Applied Biosystems, Waltham, MA, USA) and an ABI PRISM 7500 real-time PCR system (Applied Biosystems). Gene-specific primer pairs were designed using Beacon Designer 7.0 (Premier Biosoft, Palo Alto, CA, USA). The primers and the potato code for genes used in this work are listed in Tables S4 and S5, respectively. The expression of each gene was normalized to ACT, and each treatment was standardized to WT expression.

Experimental design and statistical analysis

A completely randomized block design was used for the experiments. The experiments were replicated four times between 2018 and 2019 ($n = 4$). Each block contained WT, CH_2 and CH_{13} lines with 16 plants per genotype and treatment. Statistical analysis was performed with ANOVA followed by comparisons between means with Tukey's *post hoc* test ($P < 0.05$), unless otherwise indicated in the text. Analyses were carried out using Prism (GraphPad Software, San Diego, CA, USA).

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AUTHOR CONTRIBUTIONS

GGO, CDC, ELP and JFB conceived; GGO performed physiological experiments; and GGO and CDC performed molecular experiments; AM prepared the samples and took photographs for anatomical measures; ELP provided technical assistance in photosynthesis experiments; JFB supervised the experiments and wrote the article with the collaboration of GGO and ELP. All the authors read the manuscript.

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest associated with this work.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Representative photograph of 42-day-old WT and BBX21-OE potato plants (CH_2) in well-watered and moderated drought.

Figure S2. Transpiration rate and internal concentration of CO_2 in the stomatal cavity (C_i) as a function of PPFD for 42-day-old plants cultivated under two water conditions ($n = 4$). Data correspond to the experiment in Figure 2.

Figure S3. Net photosynthesis as function of the CO_2 concentration (C_i) in 42-day-old plants (WT, CH_2) cultivated under control (well-watered) and moderated drought ($n = 4$).

Figure S4. (a) Net photosynthesis as function of the CO_2 concentration of stomatal cavity (C_i), (b) V_{cmax} and J_{max} estimated as function of the CO_2 concentration of stomatal cavity (C_i) for each treatment and genotype, and (c) J_{max} estimation by using empirical measurements.

Table S1. Up- and downregulated genes in drought differentially expressed in WT plants.

Table S2. Downregulated genes in drought differentially expressed in WT plants.

Table S3. Genes belonging to clusters 1 and 2 differentially expressed in drought between WT and BBX21-OE lines.

Table S4. Primers used for RT-PCR quantification of genes in potato plants.

Table S5. Potato code for genes used in this study.

REFERENCES

Bai, S., Tao, R., Yin, L., Ni, J., Yang, Q., Yan, X. *et al.* (2019) Two B-box proteins, PpBBX18 and PpBBX21, antagonistically regulate anthocyanin biosynthesis via competitive association with *Pyrus pyrifolia* ELONGATED HYPOCOTYL 5 in the peel of pear fruit. *The Plant Journal*, **100**, 1208–1223.

Bernacchi, C.J., Portis, A.R., Nakano, H., Von Caemmerer, S. & Long, S.P. (2002) Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. *Plant Physiology*, **130**, 1992–1998.

Bernacchi, C.J., Singsaas, E.L., Pimentel, C., Portis, A.R. Jr & Long, S.P. (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and Environment*, **24**, 253–259.

Bhargava, S. & Sawant, K. (2013) Drought stress adaptation: metabolic adjustment and regulation of gene expression. *Plant Breeding*, **132**, 21–32.

Bongi, G. & Loreto, F. (1989) Gas-exchange properties of salt-stressed olive (*Olea europa* L.) leaves. *Plant Physiology*, **90**, 1408–1416.

Bursch, K., Toledo-Ortiz, G., Pireyre, M., Lohr, M., Braatz, C. & Johansson, H. (2020) Identification of BBX proteins as rate-limiting cofactors of HY5. *Nature Plant*, **6**(8), 921–928. https://doi.org/10.1038/s41477-020-0725-0

Chalker-Scott, L. (1999) Environmental significance of anthocyanins in plant stress responses. *Photochemisty and Photobiology*, **70**, 1–9.

Chen, G., Bi, Y.R. & Li, N. (2005) EGY1 encodes a membraneassociated and ATP-independent metalloprotease that is required for chloroplast development. *The Plant Journal*, **41**, 364–375.

Coleman, W.K. (2008) Evaluation of wild *Solanum* species for drought resistance: 1. *Solanum gandarillasii* Cardenas. *Environmental and Experimental Botany*, **62**, 221–230.

Crocco, C.D. & Botto, J.F. (2013) BBX proteins in green plants: Insights into their evolution, structure, feature and functional diversification. *Gene*, **531**, 44–52.

Crocco, C.D., Holm, M., Yanovsky, M.J. & Botto, J.F. (2010) AtBBX21 and COP1 genetically interact in the regulation of shade avoidance. *The Plant Journal*, **64**, 551–562.

Crocco, C.D., Ocampo, G.G., Ploschuk, E.L., Mantese, A. & Botto, J.F. (2018) Heterologous expression of AtBBX21 enhances the rate of photosynthesis and alleviates photoinhibition in *Solanum tuberosum*. *Plant Physiology*, **177**, 369–380.

Datta, S., Hettiarachchi, C., Johansson, H. & Holm, M. (2007) SALT TOLERANCE HOMOLOG2, a B-box protein in Arabidopsis that activates transcription and positively regulates light-mediated development. *The Plant Cell*, **19**, 3242–3255.

Deikman, J. & Hammer, P.E. (1995) Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. *Plant Physiology*, **108**, 47–57.

Dizeo de Strittmatter, C.G. (1973) Nueva técnica de diafanización. *Boletín Sociedad Argentina Botanica*, **15**, 126–129.

Ethier, G.J. & Livingston, N.J. (2004) On the need to incorporate sensitivity to CO2 transfer conductance into the Farquhar–von Caemmerer-Berry leaf photosynthesis model. *Plant, Cell and Environment*, **27**, 137–153.

Evers, D., Lefèvre, I., Legay, S., Lamoureux, D., Hausman, J.-F., Rosales, R.O.G. *et al.* (2010) Identification of drought-responsive compounds in potato through a combined transcriptomic and targeted metabolite approach. *Journal of Experimental Botany*, **61**, 2327–2343.

Farquhar, G., Caemmerer, S.V. & Berry, J. (1980) A biochemical model of photosynthetic CO. *Planta*, **149**, 78–90.

Flexas, J., Ribas-Carbó, M., Hanson, D.T., Bota, J., Otto, B., Cifre, J. *et al.* (2006) Tobacco aquaporin NtAQP1 Is involved in mesophyll conductance to CO_2 in vivo. *The Plant Journal*, **48**, 427–439.

Foyer, C.H. & Harbinson, J. (2012) Photosynthetic regulation. In: Loreto, F., Medrano, H. & Flexas, J. (Eds.) *Terrestrial photosynthesis in a changing environment: a molecular, physiological, and ecological approach*. Cambridge: Cambridge University Press, pp. 20–40.

Gangappa, S.N. & Botto, J.F. (2014) The BBX family of plant transcription factors. *Trends Plant Science*, **19**, 460–470.

Giri, J., Dansana, P.K., Kothari, K.S., Sharma, G., Vij, S. & Tyagi, A.K. (2013) SAPs as novel regulators of abiotic stress response in plants. *BioEssays*, **35**, 639–648.

Han, J.M., Meng, H.F., Wang, S.Y., Jiang, C.D., Liu, F., Zhang, W.F. *et al.* (2016) Variability of mesophyll conductance and its relationship with water use efficiency in cotton leaves under drought pretreatment. *Journal of Plant Physiology*, **194**, 61–71.

Harley, P.C., Loreto, F., Marco, G.D. & Sharkey, T.D. (1992) Theoretical considerations when estimating the mesophyll conductance to CO2 flux by analysis of the response of photosynthesis to CO2. *Plant Physiology*, **98**, 1429–1436.

Holtan, H.E., Bandong, S., Marion, C.M., Adam, L., Tiwari, S. & Shen, Y. *et al.* (2011) BBX32, an Arabidopsis B-Box protein, functions in light signaling by suppressing HY5-regulated gene expression and interacting with STH2/BBX21. *Plant Physiology*, **156**, 2109–2123.

Huang, J., Zhao, X., Weng, X., Wang, L. & Xie, W. (2012) The rice B-box zinc finger gene family: genomic identification, characterization, expression profiling and diurnal analysis. *PLoS One*, **7**, e48242.

Job, N., Yadukrishnan, P., Bursch, K., Datta, S. & Johansson, H. (2018) Two B-box proteins regulate photomorphogenesis by oppositely modulating HY5 through their diverse C-terminal domains. *Plant Physiology*, **176**(4), 2963–2976. https://doi.org/10.1104/pp.17.00856

Johansen, D.A. (1940) *Plant microtechnique*. New York: McGraw-Hill.

Kang, X., Xu, G., Lee, B., Chen, C., Zhang, H., Kuang, R. *et al.* (2018) HRB2 and BBX21 interaction modulates Arabidopsis ABI5 locus and stomatal aperture. *Plant Cell Environment*, **41**, 1912–1925.

Li, B., Li, Q., Xiong, L., Kronzucker, H.J., Krämer, U. & Shi, W. (2012) Arabidopsis plastid AMOS1/EGY1 integrates abscisic acid signaling to regulate global gene expression response to ammonium stress. *Plant Physiology*, **160**, 2040–2051.

Liu, X., Li, R., Dai, Y., Chen, X. & Wang, X. (2018) Genome-wide identification and expression analysis of the B-box gene family in the Apple (*Malus domestica* Borkh.) genome. *Molecular Genetics and Genomics*, **293**, 303–315.

Liu, X., Li, R., Dai, Y., Yuan, L., Sun, Q., Zhang, S. *et al.* (2019) A B-box zinc finger protein, MdBBX10, enhanced salt and drought stresses tolerance in Arabidopsis. *Plant Molecular Biology*, **99**, 437–447.

Mbambalala, N., Panda, S.K. & van der Vyver, C. (2020) Overexpression of AtBBX29 improves drought tolerance by maintaining photosynthesis and enhancing the antioxidant and osmolyte capacity of sugarcane plants. *Plant Molecular Biology Reporter*, **39**(2), 419–433. https://doi.org/10.1007/s11105-020-01261-8

McMurtrie, R.E. & Wang, Y.P. (1993) Mathematical models of the photosynthetic response of tree stands to rising CO2 concentrations and temperatures. *Plant, Cell and Environment*, **16**, 1–13.

Moran, R. (1982) Formulae for determination of chlorophyllous pigments extracted with n, n-dimethylformamide. *Plant Physiology*, **69**, 1376–1381.

Obidiegwu, J., Bryan, G., Jones, G. & Prashar, A. (2015) Coping with drought: stress and adaptive responses in potato and perspectives for improvement. *Frontiers in Plant Science*, **6**, 1–23.

Perez-Martin, A., Michelazzo, C., Torres-Ruiz, J.M., Flexas, J., Fernández, J.E., Sebastiani, L. *et al.* (2014) Regulation of photosynthesis and stomatal and mesophyll conductance under water stress and recovery in olive trees: correlation with gene expression of carbonic anhydrase and aquaporins. *Journal of Experimental Botany*, **65**, 3143–3156.

Qi, Y., Wang, X., Lei, P., Li, H., Yan, L., Zhao, J. *et al.* (2020) The chloroplast metalloproteases VAR2 and EGY1 act synergistically to regulate chloroplast development in Arabidopsis. *Journal of Biological Chemistry*, **295**, 1036–1046.

Radin, J.W. & Ackerson, R.C. (1981) Water relations of cotton plants under nitrogen deficiency. III. Stomatal conductance, photosynthesis, and abscisic acid accumulation during drought. *Plant Physiology*, **67**, 115–119.

Robertson, J.M., Pharis, R.P., Huang, Y.Y., Reid, D.M. & Yeung, E.C. (1985) Drought induced increases in abscisic acid levels in the root apex of sunflower. *Plant Physiology*, **79**, 1086–1089.

Saez, A., Robert, N., Maktabi, M.H., Schroeder, J.I., Serrano, R. & Rodriguez, P.L. (2006) Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatase type 2C ABI1 and HAB1. *Plant Physiology*, **141**, 1389–1399.

Sáez, P.L., Galmés, J., Ramírez, C.F., Poblete, L., Rivera, B.K., Cavieres, L.A. *et al.* (2018) Mesophyll conductance to CO_2 is the most significant limitation to photosynthesis at different temperatures and water availabilities in Antarctic vascular species. *Environmental and Experimental Botany*, **156**, 279–287.

Sakuma, Y., Maruyama, K., Qin, F., Osakabe, Y., Shinozaki, K. & Yamaguchi, S.K. (2006) Dual function of an Arabidopsis transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. *Proceedings of National Academy of Science of USA*, **103**, 18822–18827.

Schafleitner, R., Gutierrez, R., Espino, A.G. & Perez, J. (2007) Field screening for variation of drought tolerance in *Solanum tuberosum* L. by agronomical, physiological, and genetic analysis. *Potato Research*, **50**, 71–85.

Soma, F., Takahashi, F., Yamaguchi-Shinozaki, K. & Shinozaki, K. (2021). Cellular phosphorylation signaling and gene expression in drought stress responses: ABA-dependent and ABA-independent regulatory systems. *Plants* **10**, 756. https://doi.org/10.3390/plants10040756

Song, Z., Bian, Y., Liu, J., Sun, Y. & Xu, D. (2020) B-box proteins: pivotal players in light-mediated development in plants. *Journal of Integrative Plant Biology*, **62**(9), 1293–1309. https://doi.org/10.1111/jipb.12935

Ströher, E., Wang, X.-J., Roloff, N., Klein, P., Husemann, A. & Dietz, K.-J. (2009) Redox-dependent regulation of the stress-induced zinc-finger protein SAP12 in *Arabidopsis thaliana*. *Molecular Plant*, **2**, 357–367.

Talar, U., Kiełbowicz-Matuk, A., Czarnecka, J. & Rorat, T. (2017) Genome-wide survey of B-box proteins in potato (*Solanum tuberosum*)-Identification, *characterization and expression patterns during diurnal cycle, etiolation and de-etiolation. PLoS One*, **12**(5), e0177471.

Vasquez-Robinet, C., Mane, S.P., Ulanov, A.V., Watkinson, J.I., Stromberg, V.K., De Koeyer, D. *et al.* (2008) Physiological and molecular adaptations to drought in Andean potato genotypes. *Journal of Experimental Botany*, **59**, 2109–2123.

Villar, L., Lienqueo, I., Llanes, A., Rojas, P., Perez, J., Correa, F. *et al.* (2020) Comparative transcriptomic analysis reveals novel roles of transcription factors and hormones during the flowering induction and floral bud differentiation in sweet cherry trees (*Prunus avium* L. cv. Bing). *PLoS ONE*, **15**(3), e0230110. https://doi.org/10.1371/journal.pone.0230110

Weisz, R., Kaminski, J. & Smilowitz, Z. (1994) Water-deficit effects on potato leaf growth and transpiration: utilizing fraction extractable soil-water for comparison with other crops. *American Potato Journal*, **71**, 829–840.

Xu, D., Li, J., Gangappa, S.N., Hettiarachchi, C., Lin, F., Andersson, M.X. *et al.* (2014) Convergence of light and ABA signaling on the ABI5 promoter. *PLoS Genetic*, **10**, e1004197.

Xu, Y., Zhao, X., Aiwaili, P., Mu, X., Zhao, M., Zhao, J. *et al.* (2020) A zinc finger protein BBX19 interacts with ABF3 to negatively affect drought tolerance in Chrysanthemum. *The Plant Journal*, **103**, 1783–1795.

Yadav, A., Ravindran, N., Singh, D., Rahul, P.V. & Datta, S. (2020) Role of Arabidopsis BBX proteins in light signaling. *Journal of Plant Biochemistry and Biotechnology*, **29**(4), 623–635. https://doi.org/10.1007/s13562-020-00597-2

Yin, X., Sun, Z., Struik, P.C. & Gu, J. (2011) Evaluating a new method to estimate the rate of leaf respiration in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements. *Journal of Experimental Botany*, **62**, 3489–3499.

Zhu, J.K. (2016) Abiotic stress signaling and responses in plants. *Cell*, **167**, 313–324.

Zhu, J., Gong, Z., Zhang, C., Song, C.-P., Damsz, B., Inan, G. *et al.* (2002) OSM1/ SPY61: a syntaxin protein in Arabidopsis controls abscisic acid–mediated and non-abscisic acid–mediated responses to abiotic stress. *The Plant Cell*, **14**, 3009–3028.