Down-regulation of CBP80 gene expression as a strategy to engineer a drought-tolerant potato

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Summary
Developing new strategies for crop plants to respond to drought is crucial for their innovative breeding. The down-regulation of nuclear cap-binding proteins in Arabidopsis renders plants drought tolerant. The CBP80 gene in the potato cultivar Desiree was silenced using artificial microRNAs. Transgenic plants displayed a higher tolerance to drought, ABA-hypersensitive stomatal closing, an increase in leaf stomata and trichome density, and compact cuticle structures with a lower number of microchannels. These findings were correlated with a higher tolerance to water stress. The level of miR159 was decreased, and the levels of its target mRNAs MYB33 and MYB101 increased in the transgenic plants subjected to drought. Similar trends were observed in an Arabidopsis cbp80 mutant. The evolutionary conservation of CBP80, a gene that plays a role in the response to drought, suggests that it is a candidate for genetic manipulations that aim to obtain improved water-deficit tolerance of crop plants.

Introduction
Innovative plant breeding requires knowledge of the molecular strategies developed by plants to adapt to and resist water deficiency. Recent studies have revealed that the proteins involved in RNA processing affect the ABA (abscisic acid) signal transduction occurring in drought-stressed plants (Fedoroff, 2002; Hugouvieux et al., 2001; Razem et al., 2006). Among these proteins, the cap-binding protein 80 (CBP80, also known as Abscисic Acid Hypersensitive 1, ABH1) gene in Arabidopsis thaliana has been shown to be an important player in the regulation of the ABA transduction pathway and in drought tolerance. Interestingly, its inactivation in A. thaliana leads to an ABA-hypersensitive stomatal closing and reduced wilting during drought (Hugouvieux et al., 2001, 2002; Kmiecik et al., 2002). In addition, Papp et al. (2004) have shown that the loss of function of the Arabidopsis cap-binding protein 20 (CBP20) also confers a hypersensitivity to ABA during germination and increased water-deficit tolerance during drought stress. The CBP80 protein forms a dimer with the CBP20 protein (Kierzkowski et al., 2009), producing the CBC (Cap-Binding Complex), a complex that recognizes and binds to the cap structure of RNA Pol II transcripts in the nucleus.

The cultivated potato (Solanum tuberosum, ssp. tuberosum) is widely known to be very sensitive to water deficits in soil (MacKerron and Jefferies, 1988). To study the genetic factors that improve the resistance of the potato plant to drought, we silenced the CBP80 gene in the tetraploid potato cultivar Desiree. We designed artificial microRNAs (amiRNAs, also known as amiRs) that target the potato CBP80 mRNA and obtained Desiree transgenic lines that contain a silenced CBP80 gene. Our results show that this approach is successful in inactivating gene expression in polyploid plants. Moreover, potato plants with a silenced CBP80 gene display morphological and physiological changes that are essential for their improvements in drought tolerance. These results also support an evolutionary conservation of CBP80 function in the response of Arabidopsis and potato plants to water deficit and suggest that the CBP80 gene may be a useful target for mutagenesis when designing plants that have an improved tolerance to drought.

Results
The insertion of Arabidopsis T-DNA into the CBC genes produced a reduced wilting phenotype during drought stress (Hugouvieux et al., 2001; Papp et al., 2004). We performed an RNAi-mediated silencing of the Arabidopsis CBP20 or CBP80 genes, and these post-transcriptionally silenced cbc mutants showed a significant increase in their tolerance to water deficits (Figure S1). These
results encouraged us to use a similar approach for the gene silencing of CBP80 in potato crops.

**Artificial miRNAs silence the expression of the CBP80 gene in potato**

Because cultivated potato species are autotetraploid and highly heterozygous, we sequenced multiple colonies containing the CBP80 cDNA clones from the cultivar Desiree; we identified three CBP80 allele variants (Figures S2–S4) (Simko et al., 2006).

To design amiRNAs that target the *S. tuberosum* CBP80 mRNA, we identified the cDNA fragments in the Desiree cultivar that did not show polymorphisms. Using a Web MicroRNA Designer (Ossowski et al., 2008), two amiRs were selected. Both amiRs targeted the same fragment of the CBP80 mRNA. Four transgenic lines carrying the T-DNA that contains the amiR80.1 genes and four lines carrying the T-DNA that contains the amiR80.2 genes were obtained. The selected lines were tested for CBP80 mRNA expression silencing efficiency using qRT-PCR (Figure 1a).

Potato plants with a silenced CBP80 gene show improved tolerance to drought

After three seasons of tuber reproduction, all of the plants from the amiR80.2-14 line maintained a stable silencing of CBP80 gene expression, but several plants from the amiR80.1-8 line recovered from silencing (Figure 5). The Desiree and amiR80.2-14 potato plants were subjected to a 25-day water-deficit stress period. The wilting phenotype was visible for all potato plants tested, and the Desiree plants displayed a more pronounced phenotype (Figure 2a). The transgenic plants recovered much more efficiently from water-deficit stress than the Desiree plants (Figure 2a, bottom-right panel). After 7, 11 and 25 days of drought, the transpirational water loss in the transgenic plants was significantly reduced compared with that in the Desiree plants (Figure 2b). The higher level of relative water content (RWC) in the amiR80.2-14 plants indicates their enhanced drought tolerance, and the differences were also observed after reirrigation. The RWC values showed that the amiR80.2-14 line recovered water better than the Desiree plants (Figure 2b).

An accumulation of RAB18 (responsive to ABA 18, AT5G66400) mRNA occurs in *Arabidopsis* plants exposed to low temperature, water stress or exogenous ABA (Lang & Palva, 1992). Therefore, it is possible to identify the induction of the *Arabidopsis* drought stress response by tracking changes in the levels of the RAB18 mRNA (Jeannette et al., 1999). The expression of RAB18 is widely used as a molecular marker in the response of other plants to dehydration and drought (Hong et al., 2008). We used the same approach for the detection of the RAB18 mRNAs in the Desiree and amiR80.2-14 transgenic plants subjected to water deficit, and we found that the accumulation of the RAB18 mRNA

![Figure 1](image-url) Artificial miRNAs silence the expression of the CBP80 gene in potato. Individual plants from two potato transgenic lines, amiR80.1 and amiR80.2, were tested for their ability to silence CBP80 gene expression. (a) Real-time measurements of the CBP80 mRNA level in selected transgenic lines (calculated as a percentage of CBP80 mRNA expression in Desiree plants). Circles indicate selected transgenic lines that were further analysed. Values are shown as the mean ± SD (n = 3) from three independent experiments. (b) Detection of the artificial miRNAs amiR80.1 and amiR80.2 in the Desiree plants and selected transgenic lines using Northern hybridization. (c) CBP80 protein detection in the Desiree plants and amiR80.1-8 and amiR80.2-14 transgenic lines using Western blots.
starts on day 7 of drought treatment. The RAB18 mRNA accumulated to a much lesser extent in the amiR80.2-14 plants than in the Desiree plants (Figure 2c).

Trichome density, stomata density and closure and cuticle structure in the Desiree and transgenic amiR80.2-14 potato plants

Trichomes have been implicated in conferring resistance to drought in some plants (Huttunen et al., 2010). The number of trichomes per surface area unit on the adaxial epidermis of amiR80.2-14 and cbp80 A. thaliana plants underwent a 1.3- and 1.4-fold increase, respectively, compared with the number in the Desiree and Arabidopsis wild-type plants (Figure 3a,b). The abaxial leaf surface of potatoes was significantly different; a 1.6-fold increase in the number of trichomes covering the veins on the abaxial leaf surface of transgenic potato plants (Figure 3c) was found. However, we observed the same trichome density on the abaxial leaf blades when only the areas between the leaf veins were measured (Figure 3d). The trichome density on the abaxial leaf surface was generally very low in both the wild-type and mutant Arabidopsis plants (data not shown). Our data suggest that the increased trichome production on the adaxial leaf blade in the Arabidopsis and potato cbp80 mutant plants may be a factor that improves plant protection against drought stress.

Stomatal density is regulated by environmental factors, such as drought (Yoo et al., 2010). We measured the stomatal density on the abaxial leaf surface of Arabidopsis and cbp80 mutant plants and both leaf surfaces in the Desiree and amiR80.2-14 plants. The number of stomata on the Arabidopsis cbp80 mutants showed a 1.2-fold increase over that of the wild-type plants when the abaxial leaf surfaces were inspected (Figure 4a). The stomata density on the abaxial surface of the leaf is twofold higher in the potato mutants than in the Desiree plants (Figure 4b). We also calculated the stomata density at the adaxial leaf surfaces in Arabidopsis, the cbp80 mutant, Desiree and amiRNA80.2-14 mutant plants. The number of stomata was decreased by a factor of 0.75 in the Arabidopsis cbp80 mutant and 0.8 in the amiRNA80.2-14 mutant plants (Figure 4a,b). Our findings suggest that the increased stomatal density on the abaxial leaf surface and decreased density on the adaxial surface may...
enhance the adaptation of transgenic Arabidopsis and potato plants to drought.

A previous study showed that the disruption of the Arabidopsis CBP80 gene is responsible for the repetitive, ABA-induced elevations of cytosolic Ca^{2+} levels in guard cells, resulting in an enhanced stomatal closure (Hugouvieux et al., 2001). We examined the responsiveness of stomatal closure to ABA in the Desiree and transgenic amiR80.2-14 potato plants. After a saturating humidity treatment, the fully opened stomatal apertures were larger (30%) in the transgenic plants than in the Desiree plants. In the presence of 0.1 μM ABA, the size of the amiR80.2-14 stomatal apertures decreased, but at 1.0 μM ABA, stomatal closure was significantly enhanced compared with the Desiree plants (Figure 4c). The difference in stomatal aperture was still visible at a 5.0 μM ABA treatment but was no longer noticeable at 10 μM ABA. These experiments demonstrate that the guard cell response in potato transgenic plants shows an enhanced stomatal closure when exposed to increased ABA concentrations and is probably one of the major factors responsible for improved water stress tolerance in these plants.

The cuticle is thought to play a critical role in plant drought tolerance because of its ability to slow down the plant cell stress response in instances of drought (Kosma et al., 2009). We examined the thickness and ultrastructure of the cuticles in the adaxial epidermis. The overall thickness of the cuticles in the Desiree plants was slightly larger than that in the mutant potato plants. However, in the Desiree plants, the cuticle was not as compact and contained a higher number of microchannels than the cuticles in the amiRNA80.2-14 plants. Moreover, the border between the cell wall (cw) and cuticle was typically linear in the mutant plants but more labyrinth like in the Desiree plants (Figure 5).

CBP80 protein regulates the level of miR159, MYB33 and MYB101 in ABA-mediated drought response

The induction of miR159 via ABA controls transcript levels of the transcription factors MYB33 and MYB101 during Arabidopsis seed germination (Reyes and Chua, 2007). We investigated whether similar factors are involved in the plant response to drought in wild-type and cbp80 Arabidopsis and potato mutants. In the A. thaliana cbp80 mutant, the level of miR159 was lower (30%) compared with the wild-type plants in control conditions. After drought treatment, however, the level of miR159 was approximately 65% lower in the cbp80 mutant than in the wild-type plants (Figure 6a, upper panel). We found in the Arabidopsis cbp80 mutant relative to wild-type plant subjected to drought conditions the increase in known target mRNAs of miR159, MYB101 and MYB33 (0.9- and
Figure 4  Stomatal density and aperture length on the surface of leaves from wt Arabidopsis thaliana, cbp80 mutants, and Desiree and amiR80.2-14 transgenic plants. (a, b, left upper panels) Tables showing the comparisons of the stomata density on the abaxial leaf surfaces of the A. thaliana wt, cbp80 mutant, Desiree and amiR80.2-14 plants. (a, b, right upper panels) Fluorescent micrographs prepared using the auto adjust option in PhotoPaint showing multiple stomata on the abaxial leaf surfaces of the A. thaliana wt, cbp80 mutant, Desiree and amiR80.2-14 plants. Scale bar, 50 μm. (a, b, left lower panels) Tables showing the comparisons of stomata density on the adaxial leaf surfaces of the A. thaliana wt, cbp80 mutant, Desiree and amiR80.2-14 plants. (a, b, right lower panels) Light micrographs of the stomata on the adaxial leaf surfaces using nail polish immersions in the A. thaliana wt, cbp80 mutant, Desiree and amiR80.2-14 plants. Scale bar, 50 μm. The bars represent the standard deviation. Values are shown as the mean ± SD (n = 7) from seven independent experiments. Asterisk – P < 0.015, Mann–Whitney U-test. (c) Stomatal closing is ABA hypersensitive in transgenic plants. The stomatal aperture of wild-type plants not treated with ABA was set at 100%. Light micrographs of the stomata in the Desiree and amiRNA80.2-14 transgenic plants (left panel) were prepared using the auto adjust option in PhotoPaint. The Desiree and amiRNA80.2-14 mutants were treated with an increased concentration of ABA. (right panel) A graph showing the stomatal aperture size. Data are shown as the mean ± SD of n = 6 independent experiments with 30 stomata per data point. Scale bar, 20 μm. Asterisk – P < 0.00001, Mann–Whitney U-test.
1.7-fold, respectively) (Figure 6a, lower panel). In the amiR80.2-14 plants, the level of miR159 was slightly lower compared with the Desiree plants (10%). After drought treatment, however, the level of miR159 was approximately 20% lower in the transgenic lines than in the Desiree plants (Xie et al., 2011) (Figure 6b, upper panel). Upon the onset of drought, the levels of MYB101 and MYB33 mRNAs were increased in the amiR80.2-14 plants by approximately 3.4- and 3.7-fold, respectively, relative to the Desiree plants growing in the same conditions (Figure 6b, lower panel). We postulate that miR159 and its targets (MYB33 and MYB101 mRNAs) are involved in the ABA-mediated regulation of the plant response to drought as has been shown in Arabidopsis seed germination. Moreover, the CBP80 protein seems to play identical role in the regulation of MYB33 and MYB101 mRNA levels in both processes. We postulate that the up-regulation of both MYB transcription factors improves plant drought tolerance (Figure 6c).

**Discussion**

Gene silencing has been successfully used for the improvement in crop plants. An antisense strategy was used to down-regulate the expression of a gene encoding the enzyme polygalacturonase in tomato plants from the Solanaceae family and silence the gene coding for the granule-bound starch synthase, a key enzyme in the synthesis of amylose in potatoes of the Amflora cultivar (Frizzi and Huang, 2010; Bagwan et al., 2010). Post-transcriptional gene silencing using artificial microRNAs has been shown to very efficiently knock down gene activity in Arabidopsis (Schwab et al., 2006) and Solanaceae tomato and tobacco plants (Qu et al., 2007; Zhang et al., 2011). In our study, artificial miRNAs were used to silence gene activity in potatoes. The cultivated potato is an autotetraploid species, and due to the high heterozygosity of potatoes, polymorphisms in all alleles must be noted during the selection of a particular mRNA fragment to which an amiRNA should hybridize (Simko et al., 2006). Therefore, post-transcriptional silencing may be the only solution for an effective down-regulation of the expression of particular gene alleles.

Two CBP80 mRNA down-regulated potato lines – amiR80.1-8 and amiR80.2-14 – were obtained. Plants from the amiR80.1-8 line that recovered from silencing were found in the tuber progeny. As reported by Kung et al. (2012), recovery from amiRNA-directed silencing can be a result of multiple copy transgene integration and co-suppression. However, it is intriguing that amiR80.1 was not detected in any of the transgenic lines tested. The low expression level of amiR80.1 may be a result of the secondary structure of pre-amiR80.1 and reflect the efficiency of amiRNA maturation. The CBP20 and CBP80 proteins are involved in microRNA biogenesis (Laubinger et al., 2008; Szarzynska et al., 2009). The artificial microRNAs used in our studies were introduced into the MIR319a gene, and we found that Arabidopsis pri-miR319a accumulation in the double mutant cbp20cbp80 (cbc) was approximately 40-fold higher than in the wild-type plants (Figure S6). Therefore, it is very likely that the silencing of the CBP80 potato gene may down-regulate mature amiR production. The amiR80.2-14 plants maintained a stable silencing of the CBP80 gene at both the mRNA and protein levels. As a result, the expression level of the mature amiR80.2 is sufficiently high to maintain a stable silencing of the CBP80 gene in the amiR80.2-14 line.

The amiR80.2-14 transgenic plants subjected to drought stress displayed an improved water tolerance in comparison with the Desiree plants, and the RWC measurements revealed a larger reduction in water loss. Because similar results were observed in the Arabidopsis cbp80 mutant plants, we assume that a similar physiological response to drought takes place in both species. Upon the onset of drought conditions, the potato RAB18 mRNA accumulated less in the transgenic potato plants that had a post-transcriptionally silenced CBP80 gene. In the Arabidopsis plants, a massive increase in ABA levels in drought plants was observed and correlated with the induction of RAB18 expression (Jeanette et al., 1999; Lang & Palva, 1992). The weaker induction of RAB18 expression in transgenic potato plants suggests potential disturbances in the ABA signal transduction pathway.

In Arabidopsis plants, the CBP80 protein functions as a negative regulator in guard cell ABA signalling (Hugouvieux et al., 2002). ABA-hypersensitive stomatal closing is probably also responsible for the reduced wilting of the transgenic amiR80.2-14 potato plants during drought and can be explained by a restricted transpiration in plants (Hugouvieux et al., 2001; Jager et al., 2011). In addition, in the Arabidopsis cbp80 mutant and amiR80.2-14 potato plants, we found that the stomatal density on the abaxial leaf surface and number of trichomes on the adaxial leaf surface were both significantly increased compared with the wild-type Arabidopsis and Desiree plants. It has been shown that similar factors are involved in the resistance of the Arabidopsis cbp20 mutant plant to water deficit (Jager et al., 2011).
Because CBP20 and CBP80 interact to form the CBC, the elevated numbers of stomata and trichomes per surface unit are probably connected, directly or indirectly, to the function of the CBC. Kierzkowski et al. (2009) reported a lack of CBP20 proteins in Arabidopsis cbp80 mutant plants, suggesting that the smaller subunit of AtCBC is only stable in a complex with AtCBP80.

We found a decrease in stomata density on the adaxial leaf surfaces in Arabidopsis and potato transgenic plants. Sunflower and sorghum abaxial stomata face away from light source and close more rapidly than adaxial stomata that face light source when a water deficit is imposed (Turner and Singh, 1984). Consequently, the decrease in stomata frequency on the adaxial leaf blade may be a means to reduce water loss. Surprisingly, the leaf veins were more highly coated with trichomes in potato transgenic plants than in Desiree plants. We conclude that the high levels of trichomes on the leaf veins of potato transgenic plants may also improve water-deficit tolerance.

The differences in thickness of the adaxial cuticle in the Desiree and transgenic potato plants were not significant, but this observation was in contrast to the results shown by the Arabidopsis cebp20 mutant (Jager et al., 2011). However, we observed differences in the cuticle ultrastructure; the amiRNA80.2-14 cuticle was more compact, it contained almost no microchannels, and the border of the cell wall was linear. We suggest that the range of cuticular transpiration in the mutant plants was lower due to the different architecture of the cuticles and the smaller area of exchange for water between the cell wall and cuticle. This conclusion is supported by an experiment that showed that the cuticular transpiration intensity does not depend on cuticle thickness (Riederer and Schreiber, 2001) but on architecture – notably the number of water pores and content of the vaxes (Kerstiens, 2006; Pallardy, 2008).

Arabidopsis 1-day-old seedlings have been shown to accumulate miR159 in response to ABA (Reyes and Chua, 2007). We observed a drought-induced accumulation of miR159 in both the potato amiR80.2-14 and Arabidopsis cebp80 mutant plants. However, upon the onset of drought in the mutated potato and Arabidopsis plants, the level of miR159 increased less than that in wild-type plants. The Arabidopsis MYB33 and MYB101 mRNA transcription factors were the miR159 targets (Reyes and Chua, 2007). Accordingly, we observed an up-regulation relative to the wild-type plants of MYB33 and MYB101 TFs, leading to improved soil drought tolerance (right pathway).
different affinity of miR159 to its targets in the potato and Arabidopsis or different absolute expression levels of MYB101 in the two species tested. Our results showed that miR159, MYB33 and MYB101 were involved in the ABA-mediated regulation of the plant response to drought, as previously demonstrated for the Arabidopsis seedlings treated with ABA (Reyes and Chua, 2007) (Figure 6). Shin et al. (2011) showed that the transcription factor SIMYB1-1 regulates plant tolerance to drought in the potato. The level of STMYB1R-1 mRNA was elevated upon drought stress in our amiR80.2-14 mutant, but it was down-regulated in the Desiree plants (Figure S7). These results suggest that STMYB1R-1 expression is also linked to the CBP80-mediated response to drought.

The majority of morphological, physiological and molecular changes involved in the improvement in plant water-deficit tolerance were similar in both the Arabidopsis cbp80 and potato amiR80.2-14 mutants. These pleiotropic effects are probably a result of microRNA biogenesis and pre-mRNA splicing impairments that affect ABA-dependent plant signalling (Kim et al., 2008; Laubinger et al., 2008; Raczyńska et al., 2010; Szarzynska et al., 2009). Surprisingly, despite the involvement of the nuclear cap-binding complex in many RNA processing events, the CBP80 gene silencing does not lead to any detectable side effects that may negatively influence plant life. Microarray experiments carried out by the Schroeder group (Kuhn et al., 2008) show that in non-ABA-treated plants, mRNA expression levels of only a few genes are affected in the Arabidopsis cbp80 mutant compared with wild-type plants (Hugouvieux et al., 2001). This result was confirmed by similar studies performed in our laboratory (unpublished data). However, this picture changes dramatically when ABA-treated plants are compared (Kuhn et al., 2008). These data indicate that ABA-induced mRNA levels are critically linked to the proper functioning of the CBP80 protein. Thus, the Arabidopsis cbp80 and potato amiR80.2-14 mutants need to be tested for other abiotic and biotic stresses.

The findings of the study presented in this article inspired us to identify genes which expression is influenced by the CBP80 gene activity under drought conditions. As shown in our experiments, products of MIR159, MYB33 and/or MYB101 genes act downstream of CBP80 and could be future targets of genetic manipulations to obtain drought-tolerant potato plants. Evaluating the agronomic and tuber quality of the transgenic potato lines described in this study will be a subject of future interest. Several quantitative trait loci (QTL) are involved in drought tolerance in the potato (Anithakumari et al., 2011; Mir et al., 2012), but post-transcriptional silencing of the CBP80 expression and/or genetic manipulation of its molecular targets can simplify the genetic architecture of this complex trait in potato and other crop plants. The evolutionary conservation of the involvement of CBP80 as well as miR159, MYB33 and MYB101 in the plant response to drought suggests they can be suitable candidates for genetic manipulations that aim to obtain water-tolerant varieties of crop plants.

Experimental procedures

Plant material and growth conditions

The Arabidopsis thaliana ecotype Columbia-0 wild-type plants, homozygous T-DNA insertion cbp20 mutant (Papp et al., 2004) and homozygous cbp80 mutant (Hugouvieux et al., 2001) were grown in soil (Jiffy-7 42 mm; Jiffy Products International AS, Stange, Norway) in a growth chamber with a 16-h day length (150–200 μmol/m²/s), a constant temperature of 22 °C and 70% humidity. Fourteen single-eye plugs from tubers of the potato cultivars Desiree and transgenic lines amiR80.1 and amiR80.2 generated from Desiree plants were grown in plastic pots (15 × 15 × 15 cm) in the greenhouse. Seven 6-week-old plants per cultivar and transgenic lines were evaluated for drought tolerance under greenhouse conditions during the summer season. The drought phenotypes were assessed 3, 4 and 8 days after withholding irrigation. The second group of 5-week-old plants was transferred from the greenhouse to a growth chamber with a controlled environment (16-h light at 23 °C, 8-h dark at 15 °C, 100 μmol/m²/s) 1 week before the drought experiments, and the plants were regularly irrigated. Seven of ten plants from each genotype showing a similar phenotype were selected. Plants were subjected to a 25-day water-deficit stress period.

Construction of Arabidopsis cbp20 and cbp80 RNAi mutants

Two A. thaliana transgenic lines that carry an expression cassette containing 624 bp or 613 bp of inverted repeats interrupted by loops and representing fragments of the CBP20 and CBP80 mRNAs, respectively, were constructed. Both the sense and antisense CBP20 mRNA sequences were amplified using sense and antisense primers (Table S1). The amplified region of the CBP20 gene represented the cDNA fragment between nt 3 and 627 of the coding region, while the CBP80 gene fragment was amplified between nt 928 and 1541 in the appropriate reading frame. The DNA fragments carrying the 624 or 613 bp inverted repeats were cloned into the pHannibal vector. Both of the DNA insertions were transcribed under a strong CaMV 35S promoter and terminated at an OCS terminator. The constructs were transferred to a pART vector, and the A. thaliana plants were transformed using an Agrobacterium-mediated technique (Chen et al., 1994). We obtained 3 independent transgenic lines in the A. thaliana plants with a silenced CBP20 and 11 with a silenced CBP80 gene.

Desiree mutants obtained using artificial microRNAs targeting potato CBP80 mRNA

We designed primers according to the EST 706129 sequence deposited in the NCBI database. The DNA fragments were cloned and sequenced. Figure S2 shows the electrophoretic separation of the PCR products of the CBP80 gene cDNAs. The CBP80 cDNA is 2593 bp long. The CBP80 mRNA fragments that did not show allelic polymorphisms were selected and analysed to find the most effective target sites for RISC slicing using the Web MicroRNA Designer (Ossowski et al., 2008) at www.wmd3.weigelworld.org (Ossowski S, Fitz J, Schwab R, Riester M, Weigel D, pers. commun.). The mRNA target site is marked within the CBP80 cDNA nucleotide sequence presented in Figure S3. Using the same platform, two amiRs with the following nucleotide sequences were selected: amiRNA80.1 – 5′-TAACGTTACAGGCAGGCAGCAGAC – 3′ and amiRNA80.2 – 5′-TAACGTTACAGGCAGGCAGCAGCAGCAGAC – 3′. Both sequences differ at two positions: nucleotides 16 (G to C) and 18 (C to G) from the 5′ end. The post-transcriptional silencing using artificial miRNA was performed as described previously by Schwab et al. (2006) and Ossowski et al. (2008). Briefly, we performed a set of overlapping PCRs, and using the appropriate primers in the two constructs (Table S1), we exchanged miR319a and miR319a* for amiR80.1 amiR80.1* and amiR80.2 amiR80.2*, respectively. The DNA fragments carrying aMIR80.1 and aMIR80.2 genes were cloned into the pHannibal vector as described above and transformed using an Agrobacterium-mediated technique (Chen et al.,
temperature and again for 30 min at 50 °C. The membrane was then exposed to X-ray film for 24 h at 22 °C.

The relative water content (RWC) was calculated according to the following formula: 
\[
\text{RWC} (%) = \frac{[FW - DW] / (SW - DW)}{100}
\] 
where FW, DW and SW are the fresh, dry and saturated weights of the leaf tissues, respectively. The saturated leaf weight was determined after keeping the leaf in distilled water under light at 22 °C until it reached a constant weight and was fully turgid (typically after 4 h). The leaf dry weight was measured after keeping the turgid leaf at 80 °C in an oven for 16 h. The RWC was tested at 0, 7, 11 and 25 days after drought onset and at 9 h and 3 days after rewatering. The RWC was measured from 1 to 2 g samples of three leaves comparable in size to those described by Boguszewska et al. and collected from the third level at the top of the plant (Boguszewska et al., 2010). Six replicates were kept for each treatment.

Analysis of stomatal density

The experiments were conducted using both the fully developed apical leaflets from the compound leaves of 3-week-old potato plants and the fully developed rosette leaves of 35-day-old Arabidopsis plants. The cleared epidermal peels from the potato abaxial and adaxial and Arabidopsis abaxial leaf surfaces were prepared according to Pei et al. (1997) and examined with a light microscope equipped with a Nikon Eclipse Ti camera, DS-Fi1c-U2 optics, and Plan Fluor 10 × DIC L N1. The counts were made on 7 discs and then averaged.

Determination of trichome density

The surfaces of the leaves were examined using a binocular microscope (SterEO Lunar.V12 Zeiss, Oberkochen, Germany) at 20 and 30 × magnification. Trichomes were counted on three potato and five Arabidopsis leaves from five young plants. The plants were 4 weeks old and cultivated under standard conditions. Micrographs were taken using a PowerShot G5 camera (Canon, Tokyo, Japan) or with the use of the Zeiss Lunar.V12 binocular microscope. Each experiment was repeated five times with similar results.

Stomatal movement

The measurements of stomatal aperture were taken as previously described (Hugouvieux et al., 2002; Pei et al., 1997; Savvides et al., 2012). The specimens were flattened in a buffer composed of 10 mM KCl, 0.1 mM EGTa and 10 mM MES-KOH at pH 6.15. Epidermal tissue was stained for 5 min with Evans blue dye to differentiate between living and dead cells. Only living stomata surrounded by living cells in isolated epidermal strips were considered for further analyses. Measurements and stomatal movements were analysed using a light microscope, Eclipse Ti camera, DS-Fi1c-U2 optics and Plan Fluor 20 × DIC L N1 (Nikon, Tokyo, Japan). The ratio of the length and width of the pore between the guard cells was calculated. For every genotype and ABA concentration, 30 measurements were taken using the NIS-Element program.

Leaf cross-sections

Four fully developed leaves collected from the 5th and 6th whorl of the Desiree and amiR80-2-14 plants were subjected to a leaf cross-section study. Measurements of two biological replicates were taken from the section of the leaf that was centrally located and distant from the leaf veins. The procedure for cuticle detection and measurements in transmission electron microscopy were conducted according to Krzeslowska and Wozny (1996). The concentration of the fixative solution was the only modification of the procedure; here, 2% glutaraldehyde and 4% paraformaldehyde in cacodylic buffer were used to fix the leaves. Ultrastructural observations and measurements were conducted on a transmission electron microscope (JEM-1200 EXII JEOL, Tokyo, Japan).

Statistics

Statistical tests were performed using MS-Excel 2007 and the Statistica program. Student’s t-test and the Mann–Whitney U-test were performed. P-values were placed under the individual figures.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers: CBP80 cDNA sequences from the Desiree cultivars – FJ664251, FJ664252 and FJ664253.

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References


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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 A comparison of the Arabidopsis thaliana T-DNA insertion and post-transcriptionally silenced cbp20 and cbp80 mutants.

Figure S2 The amplification of the potato CBP80 full-length cDNA from the Desiree cultivar.

Figure S3 A comparison of the cDNA sequences of the CBP80 gene alleles from the potato cultivar Desiree.

Figure S4 An analysis of the CBP80 amino acid (aa) sequence from the Desiree plants.

Figure S5 The level of silencing of CBP80 gene expression using artificial miRNAs in selected potato plants.

Figure S6 Quantitative real-time PCR of the Arabidopsis pri-mir319a in wild-type and cbp80 mutant plants.

Figure S7 Real-time measurements of the levels of the StMYB1R-1 mRNA using the ΔΔCt method in the Desiree and amiRNA80.2-14 plants after 14 days without watering.

Table S1 The oligonucleotide primers and probes used.