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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.

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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 Abscisic 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; Kmieciak *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). Using Northern hybridizations, we were able to detect only amiR80.2 in three of the four potato lines tested (Figure 1b, lanes 5, 13, and 14). Based on a preliminary drought-tolerance test (data not shown), the *amiR80.1-8* and *amiR80.2-14* lines were chosen for Western blotting. The potato CBP80 protein was undetectable in the selected transgenic plants (Figure 1c) (Kierzkowski *et al.*, 2009). Because the *amiR80.1-14* potato line that was transformed with the construct carrying the *aMIR80.1* transgene showed a post-transcriptional gene silencing of the *CBP80* gene at the mRNA and protein levels, we assumed that this line expresses amiR80.1. However, the expression level was below the detection sensitivity of the Northern hybridization.

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 S5). 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 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

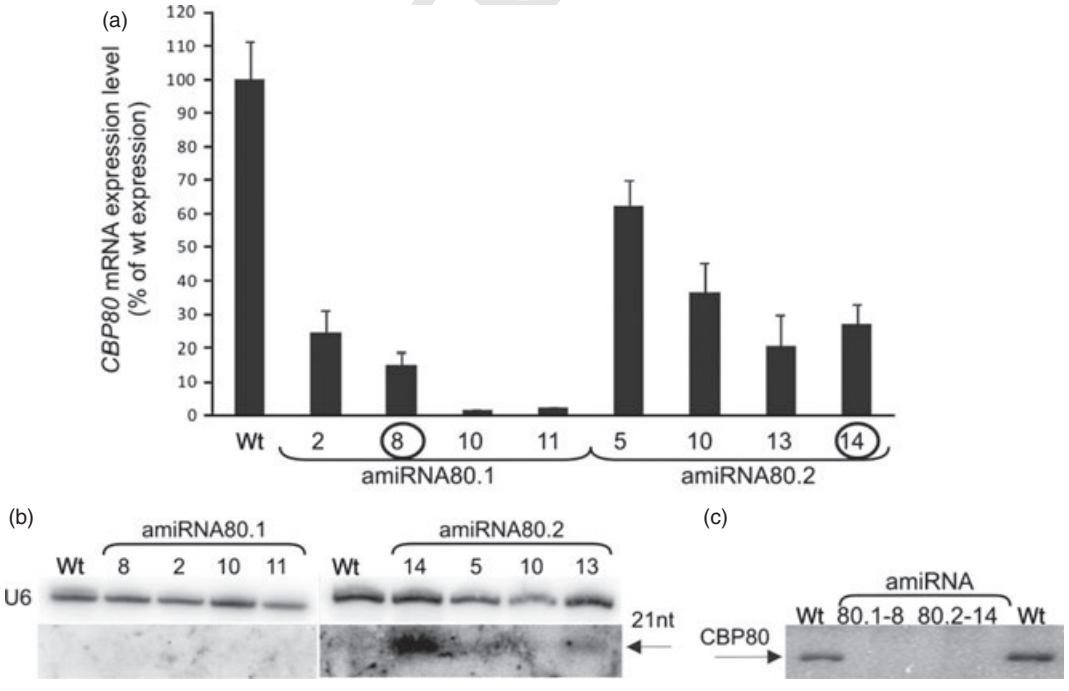

Figure 1 Artificial miRNAs silence the expression of the *CBP80* gene in potato. Individual plants from two potato transgenic lines, amiR80.1 and amiR80.2, were tested for their ability to silence *CBP80* gene expression. (a) Real-time measurements of the *CBP80* mRNA level in selected transgenic lines (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.

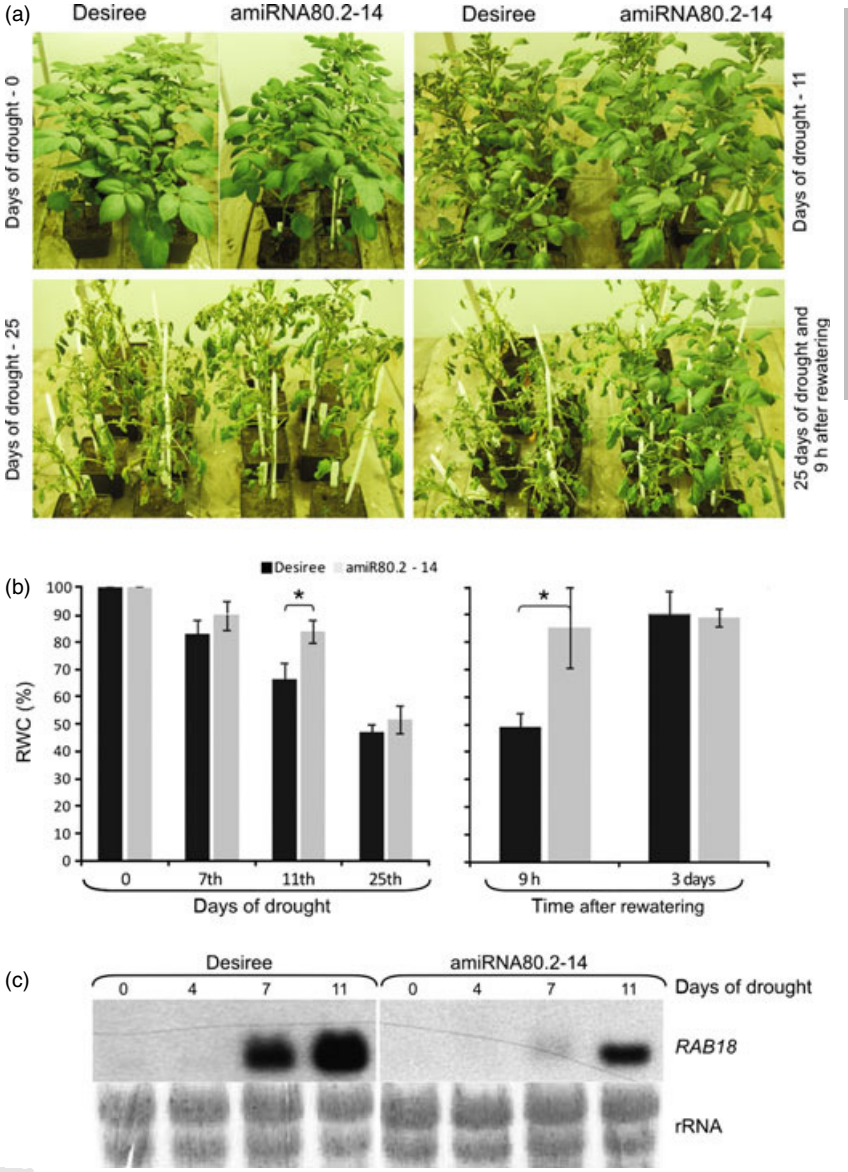

Figure 2 Potatoes with the silenced *CBP80* gene showed an improved tolerance to drought. (a) Six-week-old, well-watered plants were subjected to water stress. Desiree and *amiRNA80.2-14* plants before the application of water stress (upper-left panel) and after 11 and 25 days without watering are shown. Desiree and *amiRNA80.2-14* plants 9 h after rewatering (lower-right panel) are shown. (b) The RWC in leaves from the Desiree and transgenic *amiRNA80.2-14* potato plants after drought stress. The RWC was measured at 0, 7, 11 and 25 days after the introduction of drought stress (left panel). The measurements taken at various time points of drought duration are presented as a percentage of the RWC of the plant at day 0. The right panel shows the RWC upon restarting irrigation after 9 h and 3 days. The leaves were detached and weighed for the initial FW (fresh weight), SW (saturated weight) and DW (dry weight) values. Calculations were conducted as described in the Materials and Methods section, and values are shown as the mean ± SD ($n = 6$) from six independent experiments. Asterisk – $P < 0.001$, Student's *t*-tests. (c) A Northern blot hybridization of the *Arabidopsis RAB18* cDNA probes bound to potato RNAs isolated from the Desiree and *amiR80.2-14* plants after 0, 4, 7 and 11 days without watering.

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

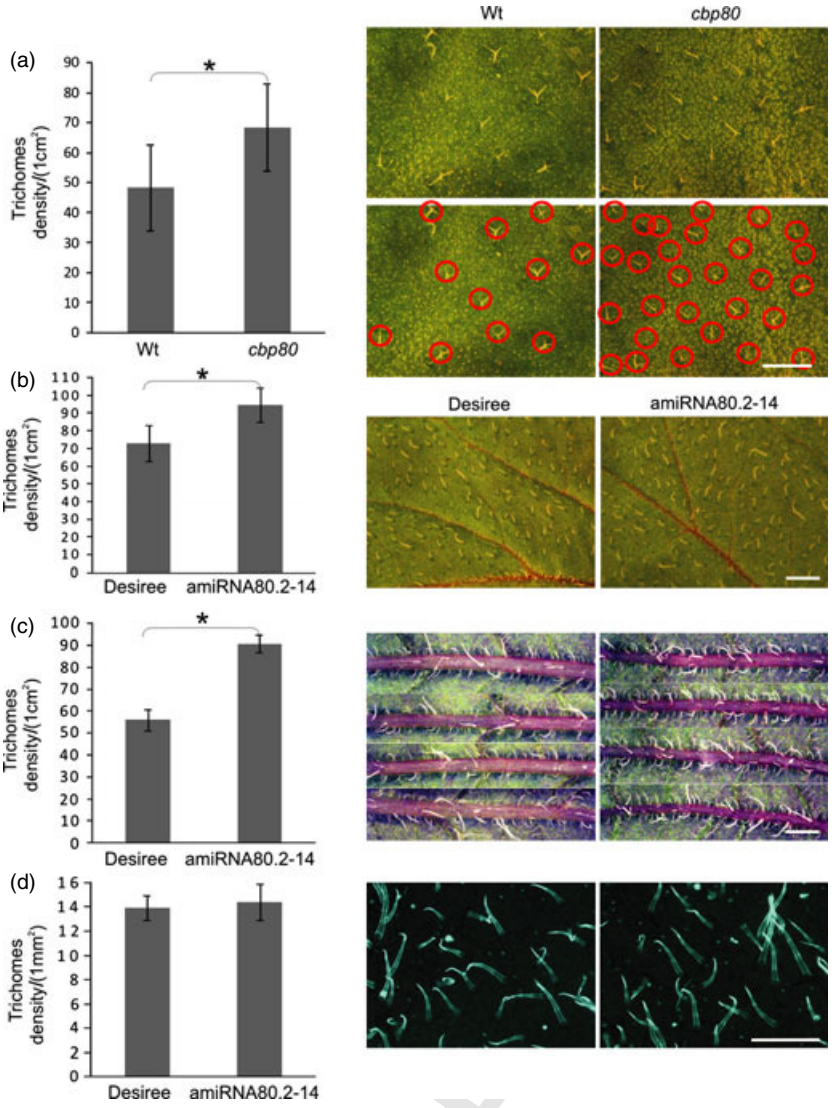

Figure 3 Trichome density on the surfaces of leafs from Desiree and *Arabidopsis thaliana* wild-type and mutant plants. (a, left panel) A table showing a comparison of the adaxial leaf trichomes in *Arabidopsis thaliana* wild-type and *cbp80* mutant plants. (a, right panel) Corresponding binocular light micrographs. The upper and lower panels represent the same micrograph, but in the lower panel, each trichome is circled in red to assist in a comparison of trichome density. Scale bar, 1 mm. (b, left panel) A table showing a comparison of the adaxial leaf trichomes in the Desiree and *amiRNA80.2-14* potato transgenic plants. (b, right panel) Corresponding binocular light micrographs. Scale bar, 2 mm. (c, left panel) A table showing a comparison of the vascular trichomes on the abaxial leaf surface in the Desiree and *amiRNA80.2-14* transgenic potato plants. (c, right panel) Corresponding binocular light micrographs prepared using the auto adjust option in PhotoPaint. Scale bar, 2 mm. (d, left panel) A table showing a comparison of the abaxial leaf surface trichomes in the Desiree and *amiRNA80.2-14* transgenic potato plants. Only the blade areas between the leaf veins were analysed. (d, right panel) Corresponding fluorescent micrograph. Bars represent standard deviation. Values are shown as the mean ± SD (n = 7) from seven independent experiments. Scale bar, 500 μm. Asterisk – $P < 0.025$, Mann–Whitney U-test.

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 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

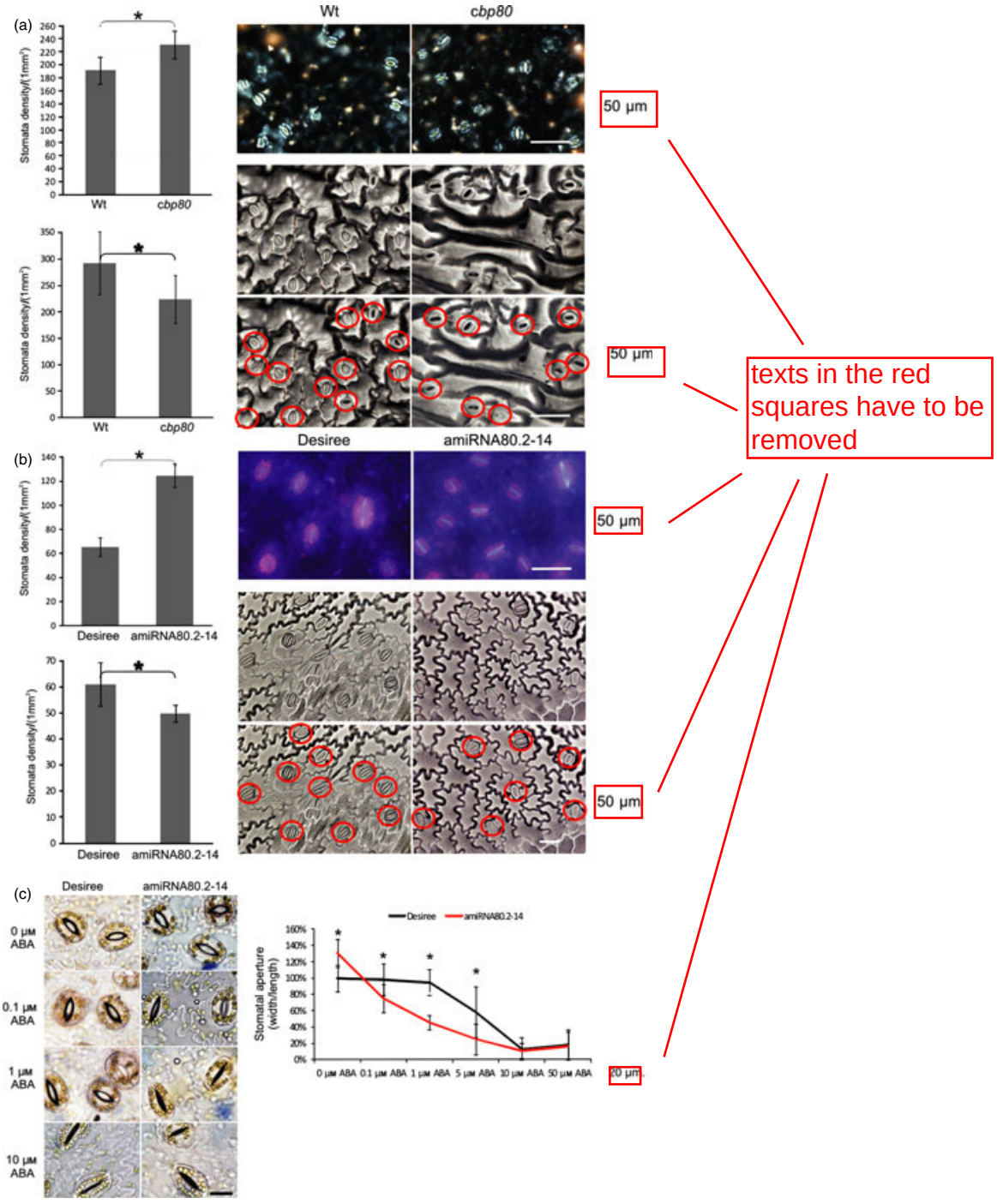

Figure 4 Stomatal density and aperture length on the surface of leaves from wt *Arabidopsis thaliana*, *cbp80* mutants, and Desiree and *amiR80.2-14* transgenic plants. (a, b, left upper panels) Tables showing the comparisons of the stomata density on the abaxial leaf surfaces of the *A. thaliana* wt, *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.

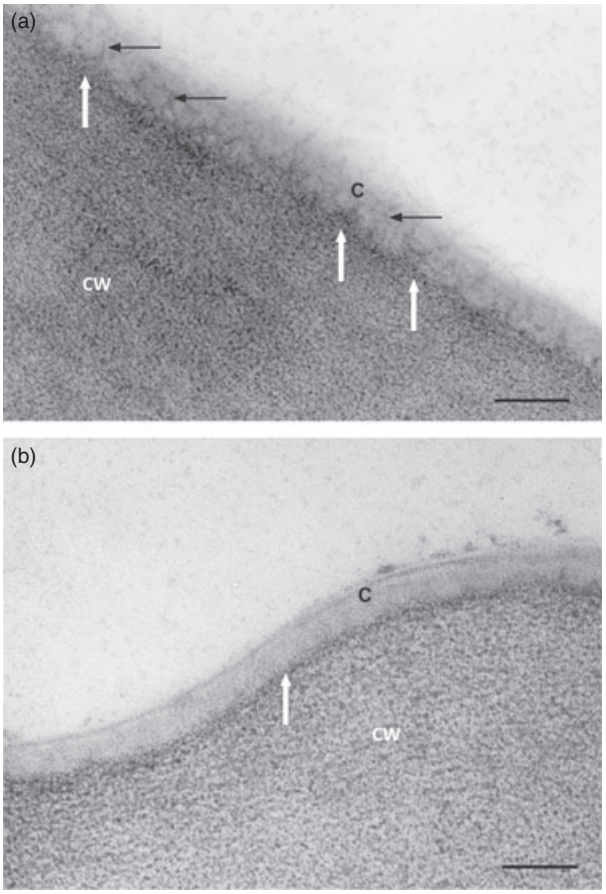

Figure 5 Adaxial cuticle (c) ultrastructure. (a) Desiree with labyrinth borders and a cell wall (cw) – white arrow; Desiree showing microchannels – black arrows. (b) *amiRNA80.2-14* mutant linear border with cw – white arrow. A compact structure of the cuticle, and no microchannels are visible. Scale bar, 100 nm.

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; ~~Jameer~~ *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 (~~Jing~~ *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 *cbp20*x*cbp80* (*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 (Jeannette *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

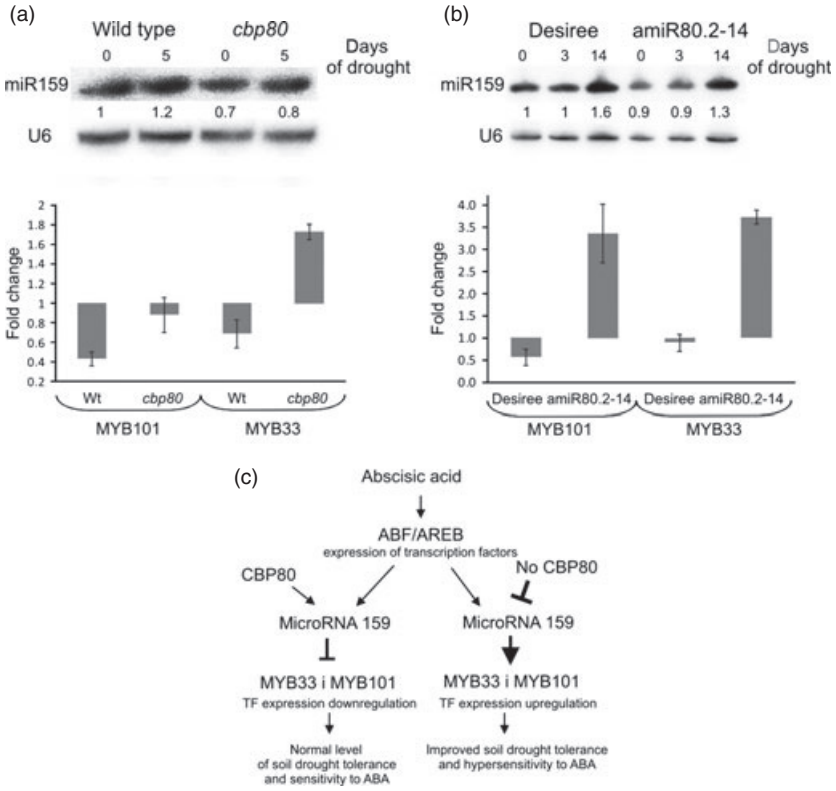

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Figure 6 Northern hybridization and qRT-PCR of the microRNA159 and mRNA of transcription factors *MYB33* and *MYB101* in the Desiree and *amiR80.2-14* plants, respectively. (a, upper panel) A Northern hybridization of the mature miR159 in the *A. thaliana* and the *cbp80* mutant grown under control conditions at days 0 and 5 of drought. (a, lower panel) Real-time measurements of *MYB33* and *MYB101* mRNA levels using the $\Delta\Delta C_T$ method in the *A. thaliana* and the *cbp80* mutant, respectively, after 5 days without watering. The mRNA level is presented as a percentage of the *MYB33* or *MYB101* mRNA level in the control plants at day 0 of drought. (b, upper panel) A Northern hybridization of the mature miR159 in the Desiree and *amiRNA80.2-14* transgenic potatoes grown under control conditions at days 0, 3 and 14 of drought. (b, lower panel) Real-time measurements of *MYB33* and *MYB101* mRNA levels using the $\Delta\Delta C_T$ method in the Desiree and *amiRNA80.2-14* plants, respectively, after 14 days without watering. The mRNA level is presented as a percentage of the *MYB33* or *MYB101* mRNA level in the control plants at day 0 of drought. (c) A schematic diagram showing a model of the evolutionary conservation of the ABA-dependent plant response to drought (left pathway). The silencing of the *CBP80* gene is responsible for the down-regulation of miR159 and subsequent up-regulation of MYB33 and MYB101 TFs, leading to improved soil drought tolerance (right pathway).

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 cbp20* 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 cbp80* 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* mRNA levels in the potato *amiR80.2-14* and *Arabidopsis cbp80* mutants subjected to *drought*. The level of *MYB101* mRNA was significantly more affected in the potato plants than in the *Arabidopsis*. This can be caused by either 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 StMYB1R-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; Raczynska *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 *MIR*159, *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, Norway) in a growth chamber with a 16-h day length (150–200 μmol/m/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/m/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. 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′-TAACGGTACAGGCAGGCCGAC – 3′ and amiRNA80.2 – 5′-TAACGGTACAGGCAGCCGGAC-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.*, 1994). Potato plants were regenerated as described by Mac *et al.* (2004). Polyclonal antibodies raised against the *A. thaliana* CBP80 protein were used to detect homologous proteins in potato by Western blots conducted as described previously (Kierzkowski *et al.*, 2009).

DNA and RNA isolation, cDNA synthesis, and PCR, qPCR and RT-PCR amplification

Total genomic DNA and RNA were isolated from plant leaves using the DNeasy Plant Mini Kit and RNeasy Plant Mini kit (Qiagen, Valencia, CA). Total RNA was used for the reverse transcription reaction performed with Superscript III RT (Invitrogen, Grand Island, NY) and oligo-dT as a primer. The cDNA of the *CBP80* gene from the potato cultivar Desiree was amplified in

PCRs using primers designed according to the EST 706129 sequence deposited at NCBI. All of the primer sequences are shown in Table S1. PCR was performed as previously described by Szarzynska *et al.* (2009). For real-time PCRs, C_T values for all of the *CBP80* transcripts were normalized to the 18S rRNA or cyclophilin cDNA C_T value (Nicot *et al.*, 2005).

RNA gel blot of mature amiRNAs

Northern blots were conducted as described previously by Szarzynska *et al.* (2009).

RAB18 Northern blots

The samples (1 g) of leaves were collected and immediately frozen at the indicated time points. Total RNA was extracted from the frozen leaves using the TRIzol method according to the manufacturer's protocol (Invitrogen, www.invitrogen.com). For Northern blots, 20 μg of total glyoxylated RNA was separated on a 1% (w/v) agarose gel in 15 mM sodium phosphate, pH 6.5 and transferred to Hybond N (Amersham, Buckinghamshire, UK) filters. Hybridization was performed according to Church and Gilbert (1984), with a randomly radiolabeled probe composed of nt 139–618 of the coding sequence of the *A. thaliana RAB18* gene (At5g66400). After hybridization, the membrane was washed twice in 2× SSC with 0.1 (w/v)% SDS at room temperature and again for 30 min at 50 °C. The membrane was then exposed to X-ray film for 24 h at −70 °C.

Relative water content measurements

The relative water content (RWC) was calculated according to the following formula: RWC (%) = [(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 Lumar.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 mMEGTA 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

Anithakumari, A.M., Dolstra, O., Vosman, B., Visser, R.G.F. and van der Linden, C.G. (2011) In vitro screening and QTL analysis for drought tolerance in diploid potato. *Euphytica*, **181**, 357–369.

Bagwan, J.D., Patil, S.J., Mane, A.S., Kadam, V.V. and Vichare, S. (2010) Genetically modified crops: food of the future. *Int. J. Adv. Biot. Res.* **1**, 21–30.
Boguszewska, D., Grudkowska, M. and Zagdanska, B. (2010) Drought-responsive antioxidant enzymes in potato (*Solanum tuberosum* L.). *Potato Res.* **53**, 373–382.
Chen, H., Nelson, R.S. and Sherwood, J.L. (1994) Enhanced recovery of transformants of Agrobacterium tumefaciens after freeze-thaw transformation and drug selection. *Biotechniques*, **16**, 664–668.
Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci.-Biol.* **81**, 1991–1995.
Fedoroff, N.V. (2002) RNA-binding proteins in plants: the tip of an iceberg? *Curr. Opin. Plant Biol.* **5**, 452–459.
Frizzi, A. and Huang, S.S. (2010) Tapping RNA silencing pathways for plant biotechnology. *Plant Biotechnol. J.* **8**, 655–677.
Hong, Y., Zheng, S. and Wang, X. (2008) Dual functions of phospholipase Dalpha1 in plant response to drought. *Mol. Plant*, **1**, 262–269.
Hugouvieux, V., Kwak, J.M. and Schroeder, J.I. (2001) An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis. *Cell*, **106**, 477–487.
Hugouvieux, V., Murata, Y., Young, J.J., Kwak, J.M., Mackesy, D.Z. and Schroeder, J.I. (2002) Localization, ion channel regulation, and genetic interactions during abscisic acid signaling of the nuclear mRNA cap-binding protein, ABH1. *Plant Physiol.* **130**, 1276–1287.
Huttunen, P., Karkkainen, K., Loe, G., Rautio, P. and Agren, J. (2010) Leaf trichome production and repsonses to defoliation and drought in Arabidopsis lyrata (Brassicaceae). *Ann. Bot. Fennici.* **47**, 199–207.
Jager, K., Fabian, A., Tompa, G., Deak, C., Hohn, M., Olmedilla, A., Barnabas, B. and Papp, I. (2011) New phenotypes of the drought-tolerant cbp20 *Arabidopsis thaliana* mutant have changed epidermal morphology. *Plant Biol. (Stuttg)* **13**, 78–84.
Jeannette, E., Rona, J.P., Bardat, F., Cornel, D., Sotta, B. and Miginiac, E. (1999) Induction of RAB18 gene expression and activation of K+ outward rectifying channels depend on an extracellular perception of ABA in *Arabidopsis thaliana* suspension cells. *Plant J.* **18**, 13–22.
Kerstiens, G. (2006) Water transport in plant cuticles: an update. *J. Exp. Bot.* **57**, 2493–2499.
Kierzkowski, D., Kmieciak, M., Piontek, P., Wojtaszek, P., Szweykowska-Kulinska, Z. and Jarmolowski, A. (2009) The Arabidopsis CBP20 targets the cap-binding complex to the nucleus, and is stabilized by CBP80. *Plant J.* **59**, 813–823.
Kim, S., Yang, J.Y., Xu, J., Jang, I.C., Prigge, M.J. and Chua, N.H. (2008) Two cap-binding proteins CBP20 and CBP80 are Involved in processing primary MicroRNAs. *Plant Cell Physiol.* **49**, 1634–1644.
Kmieciak, M., Simpson, C.G., Lewandowska, D., Brown, J.W.S. and Jarmolowski, A. (2002) Cloning and characterization of two subunits of *Arabidopsis thaliana* nuclear cap-binding complex. *Gene*, **283**, 171–183.
Kosma, D.K., Bourdenx, B., Bernard, A., Parsons, E.P., Lu, S., Joubes, J. and Jenks, M.A. (2009) The impact of water deficiency on leaf cuticle lipids of *Arabidopsis*. *Plant Physiol.* **151**, 1918–1929.
Krzeslowska, M. and Wozny, A. (1996) Lead uptake, localization and changes in cell ultrastructure of Funaria hygrometrica protonemata. *Biol. Plant.* **38**, 253–259.
Kuhn, J.M., Hugouvieux, V. and Schroeder, J.I. (2008) mRNA cap binding proteins: effects on abscisic acid signal transduction, mRNA processing, and microarray analyses. In: *Nuclear pre-mRNA Processing in Plants: Current Topics in Microbiology and Immunology* (Reddy, A.S.N. and Golovkin, M., eds), pp. 326. Berlin, Heidelberg: Springer-Verlag.
Kung, Y.J., Lin, S.S., Huang, Y.L., Chen, T.C., Harish, S.S., Chua, N.H. and Yeh, S.D. (2012) Multiple artificial microRNAs targeting conserved motifs of the replicase gene confer robust transgenic resistance to negative-sense single-stranded RNA plant virus. *Mol. Plant Pathol.* **13**, 303–317.
Laubinger, S., Sachsenberg, T., Zeller, G., Busch, W., Lohmann, J.U., Rascht, G. and Weigel, D. (2008) Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and microRNA processing in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **105**, 8795–8800.
Mac, A., Krzymowska, M., Barabasz, A. and Hennig, J. (2004) Transcriptional regulation of the gluB promoter during plant response to infection. *Cell. Mol. Biol. Lett.* **9**, 843–853.
MacKerron, D.K.L. and Jefferies, R.A. (1988) The distributions of tuber sizes in droughted and irrigated crops of potato. I. Observations on the effect of water stress on graded yields from differing cultivars. *Potato Res.* **31**, 269–278.
Mir, R.R., Zaman-Allah, M., Sreenivasulu, N., Trethowan, R. and Varshney, R.K. (2012) Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. *Theor. Appl. Genet.* **125**, 625–645.
Nicot, N., Hausman, J.F., Hoffmann, L. and Evers, D. (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *J. Exp. Bot.* **56**, 2907–2914.
Ossowski, S., Schwab, R. and Weigel, D. (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J.* **53**, 674–690.
Pallardy, S.G. (2008) *Lipids Terpenes, and Related Substances. Physiology of Woody Plants*, 3rd edn. San Diego, California, USA: Elsevier Inc., 217–232.
Papp, I., Mur, L.A., Dalmadi, A., Dulai, S. and Koncz, C. (2004) A mutation in the cap binding protein 20 gene confers drought tolerance to Arabidopsis. *Plant Mol. Biol.* **55**, 679–686.
Pei, Z.M., Kuchitsu, K., Ward, J.M., Schwarz, M. and Schroeder, J.I. (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and abi1 and abi2 mutants. *Plant Cell*, **9**, 409–423.
Raczynska, K.D., Simpson, C.G., Ciesiolka, A., Szewc, L., Lewandowska, D., McNicol, J., Szweykowska-Kulinska, Z., Brown, J.W.S. and Jarmolowski, A. (2010) Involvement of the nuclear cap-binding protein complex in alternative splicing in *Arabidopsis thaliana*. *Nucleic Acids Res.* **38**, 265–278.
Razem, F.A., El-Kereamy, A., Abrams, S.R. and Hill, R.D. (2006) The RNA-binding protein FCA is an abscisic acid receptor. (Retracted Article See vol 456, pg 824, 2008). *Nature*, **439**, 290–294.
Reyes, J.L. and Chua, N.H. (2007) ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. *Plant J.* **49**, 592–606.
Riederer, M. and Schreiber, L. (2001) Protecting against water loss: analysis of the barrier properties of plant cuticles. *J. Exp. Bot.* **52**, 2023–2032.
Savvides, A., Fanourakis, D. and van Ieperen, W. (2012) Co-ordination of hydraulic and stomatal conductances across light qualities in cucumber leaves. *J. Exp. Bot.* **63**, 1135–1143.
Schwab, R., Ossowski, S., Riester, M., Warthmann, N. and Weigel, D. (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell*, **18**, 1121–1133.
Shin, D., Moon, S.J., Han, S., Kim, B.G., Park, S.R., Lee, S.K., Yoon, H.J., Lee, H. E., Kwon, H.B., Baek, D., Yi, B.Y. and Byun, M.O. (2011) Expression of StMYB1R-1, a novel potato single MYB-like domain transcription factor, increases drought tolerance. *Plant Physiol.* **155**, 421–432.
Simko, I., Haynes, K.G. and Jones, R.W. (2006) Assessment of linkage disequilibrium in potato genome with single nucleotide polymorphism markers. *Genetics*, **173**, 2237–2245.
Szarzynska, B., Sobkowiak, L., Pant, B.D., Balazadeh, S., Scheible, W.R., Mueller-Roeber, B., Jarmolowski, A. and Szweykowska-Kulinska, Z. (2009) Gene structures and processing of *Arabidopsis thaliana* HYL1-dependent pri-miRNAs. *Nucleic Acids Res.* **37**, 3083–3093.
Turner, N.C. and Singh, D.P. (1984) Responses of Adaxial and Abaxial Stomata to light and water deficits in sunflower and sorghum. *New Phytol.* **96**, 187–195.
Xie, F.L., Frazier, T.P. and Zhang, B.H. (2011) Identification, characterization and expression analysis of MicroRNAs and their targets in the potato (Solanum tuberosum). *Gene*, **473**, 8–22.
Yoo, C.Y., Pence, H.E., Jin, J.B., Miura, K., Gosney, M.J., Hasegawa, P.M. and Mickelbart, M.V. (2010) The Arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. *Plant Cell*, **22**, 4128–4141.
Zhang, X., Li, H., Zhang, J., Zhang, C., Gong, P., Ziaf, K., Xiao, F. and Ye, Z. (2011) Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. *Transgenic Res.* **20**, 569–581.

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 $\Delta\Delta C_T$ method in the Desiree and amiRNA80.2-14 plants after 14 days without watering.

Table S1 The oligonucleotide primers and probes used.

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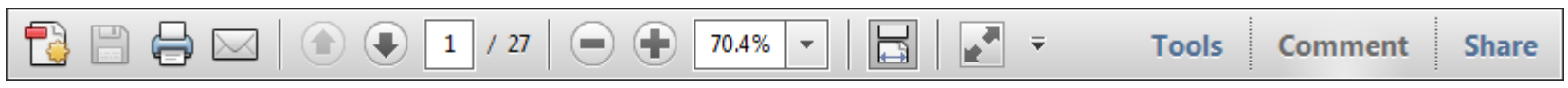

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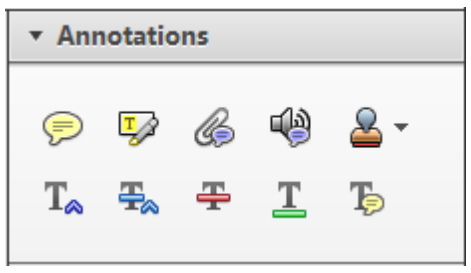

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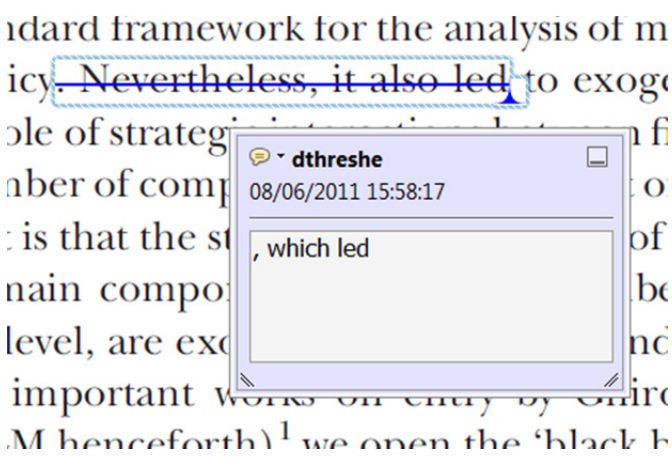

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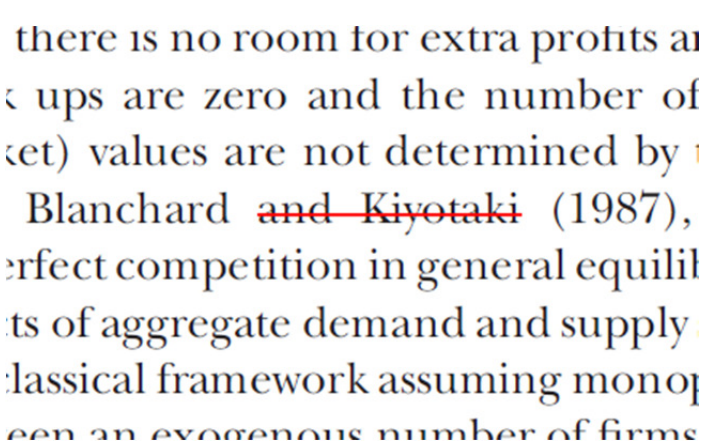
How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

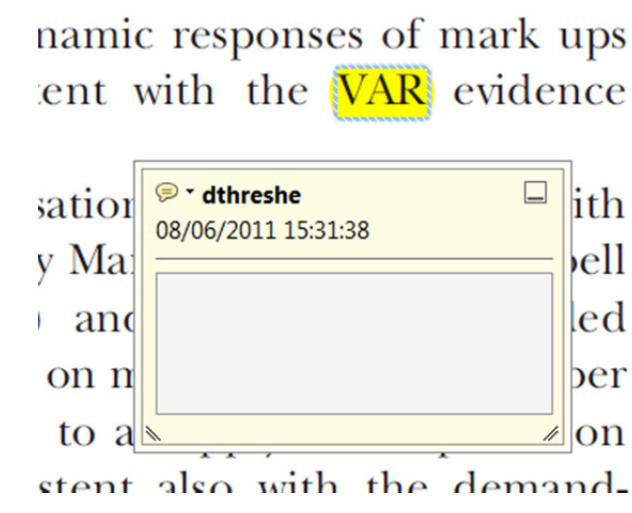


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

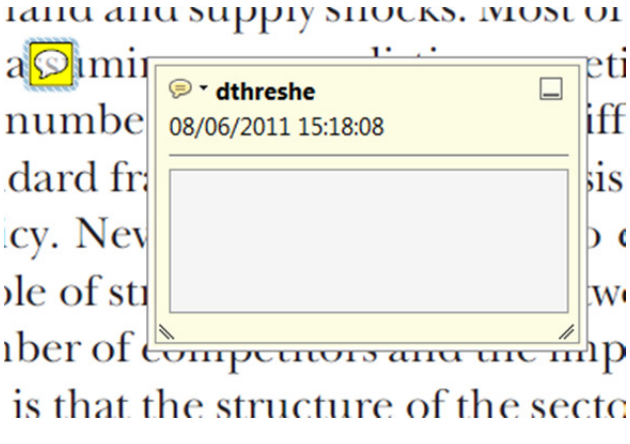


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



WILEY-BLACKWELL

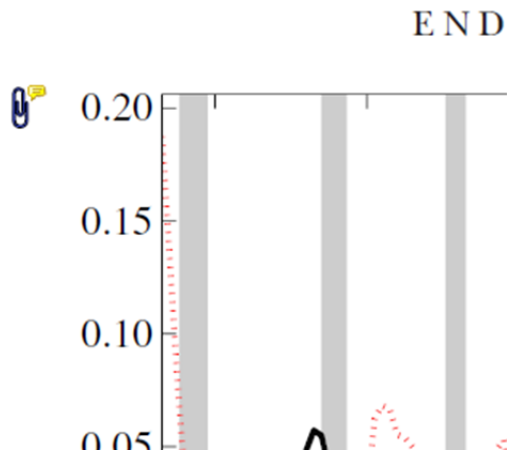
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

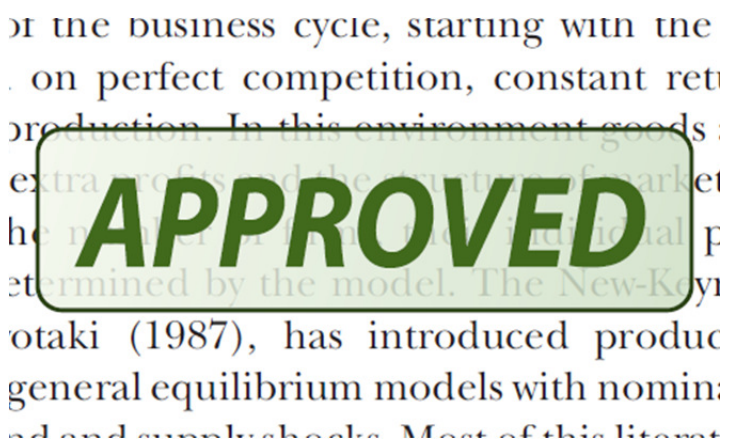


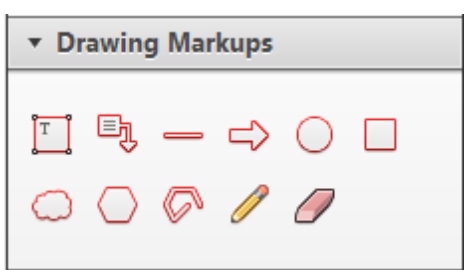
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



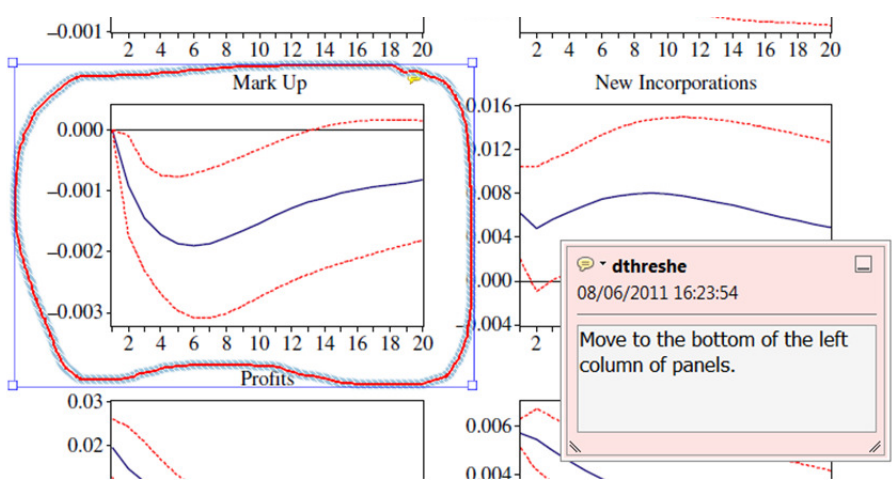


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: