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A mutation in the *Cap Binding Protein 20* gene confers drought tolerance to *Arabidopsis*

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Abstract

In a genetic screen for *Arabidopsis* mutants displaying pleiotropic alterations in vegetative development and stress responses we have identified a T-DNA insertion mutation in the *Cap Binding Protein 20 (CBP20)* gene, that encodes the 20 kDa subunit of the nuclear mRNA cap binding complex (nCBC). Plants homozygous for the recessive *cbp20* mutation show mild developmental abnormalities, such as serrated rosette leaves, delayed development and slightly reduced stature. Loss of the *cbp20* function also confers hypersensitivity to abscisic acid during germination, significant reduction of stomatal conductance and greatly enhanced tolerance to drought. Expression of the wild type cDNA by CaMV35S promoter provides full genetic complementation of the pleiotropic *cbp20* phenotype. Phenotypic characteristics of the *cbp20* mutant are very similar to those of recently described *abh1* mutant that is defective in the 80 kDa subunit of nCBC. Our data thus confirm that both genes are dedicated to the same function. CBP20 provides a new target for breeding efforts that aim at the improvement of drought tolerance in plants. Our results also show that screening for pleiotropic phenotypes in mutant plant populations may be a fruitful strategy to isolate genes for agronomically important traits.

Abbreviations: ABA, abscisic acid; CBP, cap binding protein; eIF, eukaryotic translation initiation factor; nCBC, nuclear cap binding complex

Introduction

Identification of determinants of specific traits of agronomical importance is generally achieved by genetic screening for mutations that affect the targeted trait qualitatively or quantitatively. However, alternative breeding strategies do exist, which exploit wider screening procedures for pleiotropic mutation effects (Boyes *et al.*, 2001). First, subtle phenotypic changes are sought in a mutagenized population, and then the selected mutants are subjected to rigorous screening to assess their responses to different environmental stimuli (biotic or abiotic stresses) in a search for favorable traits. We have used the latter strategy to screen an *Arabidopsis* T-DNA insertion mutant population to identify mutants showing subtle, but well-defined pleiotopic alterations in development and various stress responses. This approach led to the characterization of a knockout mutation in a gene encoding the CBP20 subunit of nuclear cap binding complex (nCBC).

Eukaryotic nCBC binds to the 5′ cap structure of nascent mRNAs transcribed by RNA polymerase II contributing to their splicing, export and maturation (Colot *et al.*, 1996; Lewis *et al.*, 1996;

Flaherty *et al*., 1997; Lewis and Izaurralde 1997). The nCBC complex consists of a handful of proteins, including the cap binding proteins CBP80, CBP20 and eIF4G (Izaurralde *et al*., 1994; Kataoka *et al*., 1994; McKendrick *et al*., 2001). CBP80 and CBP20 interact, forming the core of nCBC, which is able to bind the 5′ cap structure. Both proteins are essential for nCBC function (Izaurralde *et al*., 1994, 1995). Mutations in either of these genes affect vegetative growth rate in yeast, but the mutants are viable (Fortes *et al*., 1999). Recently, a mutation in the *Arabidopsis CBP80* gene *(ABH1)* has been identified by screening for abscisic acid (ABA) oversensitive mutants (Hugouvieux *et al*., 2001). Studies on CBP80 have raised the intriguing question whether CBP20, another subunit of nCBC, would also contribute to the regulation of ABA and water stress responses in plants. The characterization of the *cbp20* mutant here demonstrates that CBP80 and CBP20 perform overlapping regulatory functions and shows that CBP20 is also essential for regulation of plant responses to ABA and water deficit. The role of CBP20 in RNA metabolism and its potential in biotechnology applications are also discussed.

Materials and methods

Plant material, growth conditions and T-DNA mutants

Arabidopsis thaliana ecotype Columbia (Col-0) was used in all experiments as wild type control. Seeds were sown in soil, imbibed at 4 °C for 2 days in the dark. Large scale screening of the mutant population was performed in a greenhouse at long day (LD; 16 h light, 8 h dark) conditions. In all other experiments plants were grown under controlled environmental parameters in growth chambers (Conviron CMP3246) at 21 °C with 10 and 16 h light periods for short day (SD) and LD, respectively, at photon flux density of 100 μmol m^{-2} s^{-1}. Relative humidity was kept at 65%. For porometry, intact leaves of 8-week-old *Arabidopsis* plants were measured at LD light conditions using a Delta-T AP4 porometer (Delta-T Devices, Cambridge, UK).

For *in vitro* culture, seeds were surface sterilized with $Ca(OCl)_2$, washed extensively and grown on half strength MS medium supplemented with 3% sucrose, 8 g l^{-1} agar and ABA at the concentration indicated. Petri dishes were placed in a culture chamber (SANYO MLR350) under SD condition. In the genetic screen, T2 families were used from an *Arabidopsis* T-DNA insertion mutant population, which was transformed with the T-DNA of *Agrobacterium* binary vector pPCV6NFHyg (Koncz *et al*., 1989, 1990; Rios *et al*., 2002). Plant transformation was done by vacuum infiltration, as described by Clough and Bent (1998).

Cloning of the T-DNA mutated cbp20 locus and wild type CBP20 cDNA

Standard cloning procedures were according to Sambrook et al. (1989). To rescue-clone T-DNA insertion from the plant genome, total genomic DNA was prepared by a DNeasy kit (Qiagen). 2 μg genomic DNA was digested with *Eco*RI or *Hind*III restriction enzymes (Fermentas), religated and transformed into competent *Escherichia coli* (TOP10, Invitrogen). Plant DNA fragments flanking the T-DNA insertion in the CBP20 gene were sequenced using specific primers complementary to the pPCV6NFHyg T-DNA ends. Total and $polyA^+$ RNA samples were prepared from wild type and mutant plants using the RNeasy and Oligotex mRNA kits (Qiagen), respectively. For RT-PCR, the first strand cDNA was synthetized with SuperscriptII reverse transcriptase (Invitrogen). To amplify the full-length CBP20 cDNA, Pfu polymerase (Stratagene) was used with the following gene specific primers:

CBP1 5′CACCTGCAGCGATGGCTTCTTT GTTCAAGGAGCAAG3′

CBP2 5′CTCGGATCCTTAAGATCTTCTCT TCCGATCATC3′

The amplified cDNA fragment was cloned in pBluescript vector (Stratagene) to verify its sequence, and then inserted between the *Eco*RI and *Bam*HI cloning sites of the binary vector pPCV702 (Koncz *et al*., 1994) for genetic complementation experiments. Sequences were determined by using a BigDye cycle sequencing kit (Applied Biosystems) and an ABI3100 automated sequencer.

Northern hybridization

Total RNA was prepared from 6-week-old wild type and mutant plants using Tri-Reagent (Sigma) according to the manufacturer's instructions. A

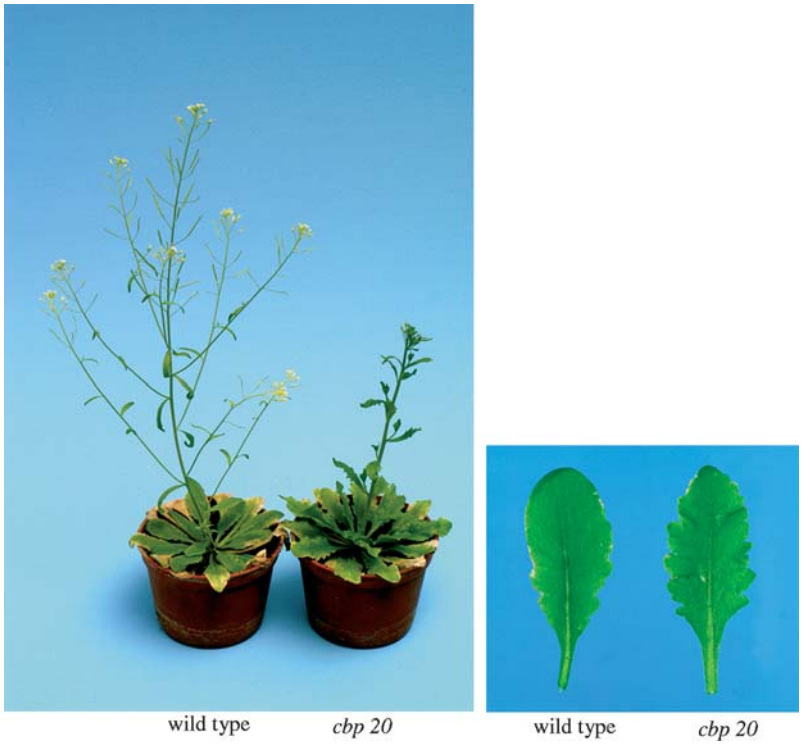

Figure 1. Comparison of wild type and *cbp20* mutant phenotypes. The *cbp20* mutation is characterized by delayed development and serrated rosette leaf margins.

total of 10 μg aliquots of RNA samples were run on denaturing formaldehyde-agarose gel, blotted onto HybondN membrane (Amersham-Pharmacia) and hybridized according to the instructions of the manufacturer. A full-length CBP cDNA fragment was labeled with ^{32}P by a Ready-To-Go kit (Amersham-Pharmacia) and used as probe.

Results

Isolation of the cbp20 *T-DNA insertion mutant*

Approximately 10,000 individual T2 Arabidopsis plants, derived from about 800 T1 parents transformed with the T-DNA of *Agrobacterium* binary vector pPCV6NFHyg, were screened for pleiotropic alterations of developmental traits and responses to abiotic and biotic stress stimuli. Mutant plants segregating in the T2 families were inspected weekly to precisely define their developmental alterations, and the stability of their phenotypes were confirmed by similar analysis of their T3 progeny obtained by self-pollination. This screen identified a mutant, which showed subtle but characteristic pleiotropic changes, resulting in about 10 days delay in development, a somewhat more compact stature in comparison to wild type and serrated leaf margins (Figure 1).

To characterize the mutant genetically, the *cbp20* line was backcrossed with the wild type parent (Col-0). The F2 generation from this cross gave a segregation ratio 38(mutant):188(wild type), the mutant phenotypic traits always co-segregated. Under the conditions used wild type *Arabidopsis* germinated at 95%, whereas the homozygous mutant at 65% frequency. These data corresponded to a single recessive mutation, causing the phenotype observed.

From the F2 plants total DNA was prepared and T-DNA specific PCR reactions were performed. All mutants and two-third of the phenotypically wild type plants tested (24 out of 24 and 16 out of 24 plants, respectively) contained the T-DNA insertion. Thus, the *cpb20* mutant carried a single T-DNA insertion which co-segregated with the mutant phenotype.

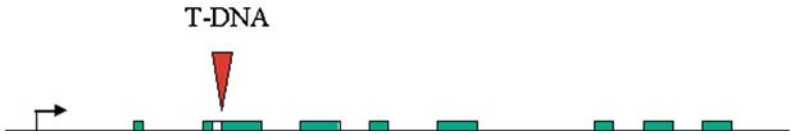

Figure 2. In the *cbp20* mutant the T-DNA has integrated into chromosome V causing a 36 base pair deletion in the second exon of At5g44200 gene (between positions 62,147 and 62,183 in the MLN1 P1 clone). Boxes represent exons of the gene, arrow indicates start of transcription.

To identify the T-DNA tagged gene, genomic DNA was prepared from the homozygous mutant and the T-DNA was rescued along with flanking plant DNA sequences. The plant DNA sequence disrupted by the T-DNA insert was identical to an exon of the *Arabidopsis* At5g44200 gene (Figure 2).

To ascertain that the transcription of the *CBP20* gene was abolished in the T-DNA insertion mutant, RNA hybridization analysis was performed. Total RNA was prepared from wild type and *cbp20* plants, blotted and probed with a gene-specific probe. In comparison to the wild type, the hybridization failed to reveal synthesis of CBP20 mRNA in the knockout mutant (Figure 3).

Characterization of the cbp20 *mutant*

In assays for biotic and abiotic stress-responses, such as resistance to bacterial infections (*Pseudomonas syringae* pv. tomato) or heat shock, the *cbp20* knockout mutant failed to expose any significant difference in comparison to wild type (data not shown). However, analogously to the *cbp80/abh1* mutation, the *cbp20* knockout showed specific alteration of responses to the plant hormone ABA. Thus, unlike wild type, in germination assays the *cbp20* mutant failed to germinate on MS medium containing 0.3 μM ABA (Figure 4a), indicating hypersensitivity to ABA, whereas in drought tolerance assays it displayed an increased tolerance to water deprivation (Figure 4b). When tested on individual plants of the F2 generation described above, this phenotype co-segregated with the morphological traits of the mutant (data not shown).

According to these observations, measurements of stomatal conductance of leaves from wild type and *cbp20* plants by porometry (Figure 5) indicated that stomata of the *cbp20* mutant allowed significantly less gas exchange than those of the wild type.

To assess whether either water stress or the ABA hormone signal would regulate the expression of the *CBP20* gene, Northern RNA hybridization analysis was performed on wild type plants treated either with ABA or subjected to dehydration. Neither desiccation nor ABA treatment did influence the expression of *CBP20* (data not shown).

Complementation of the cbp20 *mutant*

To confirm unequivocally that inactivation of the *CBP20* gene indeed resulted in all phenotypic alterations observed in the knockout mutant, a genetic complementation test was performed. The *CBP20* cDNA was amplified by RT-PCR from first strand cDNA with gene-specific primers. The cDNA was cloned into the binary vector pPCV702 under the control of the 35S promoter and *cbp20* mutant plants were transformed with this construct. The transformants showed wild type leaf phenotype (Figure 6), higher stomatal conductance than the mutant (Figure 5) and no improved drought tolerance (data not shown).

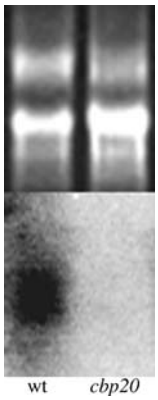

Figure 3. Northern hybridization of total RNA from wild type and *cbp20* knockout mutant with a *CBP20* specific probe.

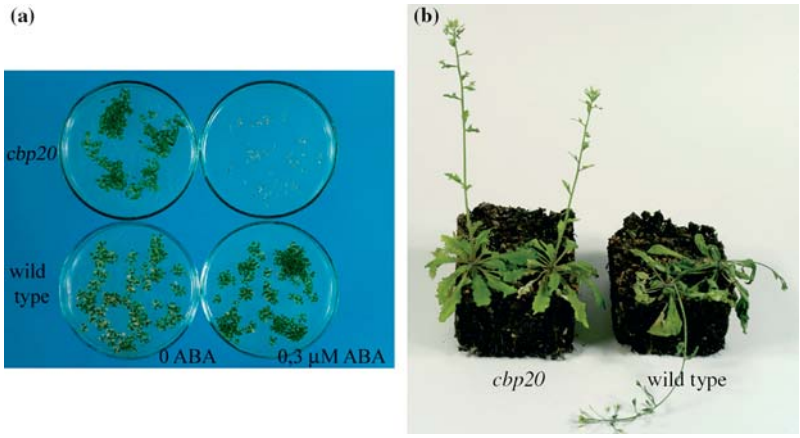

Figure 4. Seeds carrying the *cbp20* mutation fail to germinate on 0.3 μM ABA, which does not affect the germination of wild type seeds (A). Wilting phenotype of *cbp20* and wild type plants after lack of irrigation for 10 days (B).

Discussion

We have performed phenotypic screening of about 800 T-DNA mutagenized T2 *Arabidopsis* families in a search for mutants causing subtle pleiotropic alterations in development and stress responses. A mutant line was identified, which displayed delayed development, slightly reduced stature and serrated leaf margins. Genetic analysis indicated that these phenotypes were caused by a single recessive mutation, which also resulted in hypersensitivity to ABA and significantly increased the level of drought tolerance. Molecular analysis of the mutant locus demonstrated that the T-DNA insertion co-segregating with the mutant phenotype was located in the *Arabidopsis* gene At5g44200 that codes for the cap binding protein CBP20, a subunit of the *Arabidopsis* nCBC.

In other eukaryotes, nCBC is implicated in splicing, export and maturation of mRNAs transcribed by RNA Polymerase II through binding the 5′ ^{7}methylguanosine cap structure. In mammalian *in vitro* system depletion of either of two subunits of nCBC (CBP20 or CBP80) prevents its function (Izaurralde *et al*., 1994, 1995). These two proteins are also known to interact each other. In yeast, loss of either of them results in growth retardation, although the mutants are still viable. Interestingly, the double mutant has milder growth defect than either of the single mutants, pointing to a possibility

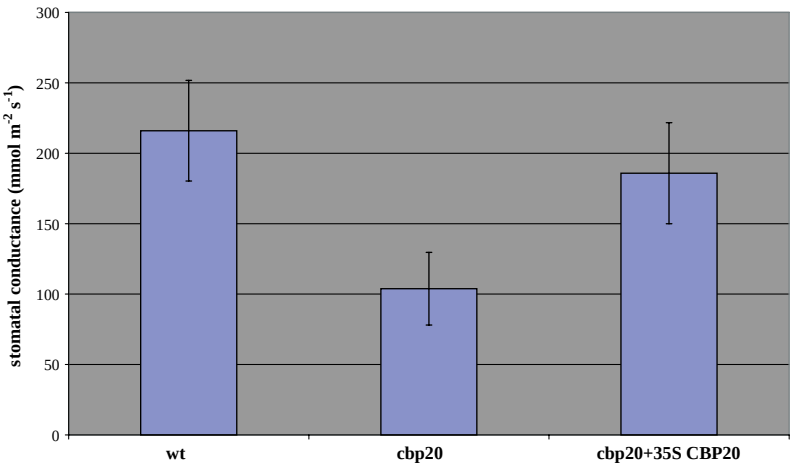

Figure 5. Stomatal conductance of wild type, *cbp20* mutant and genetically complemented *cbp20* mutant plants expressing the wild type CBP20 cDNA under the contol of 35S promoter of Cauliflower Mosaic Virus (CaMV).

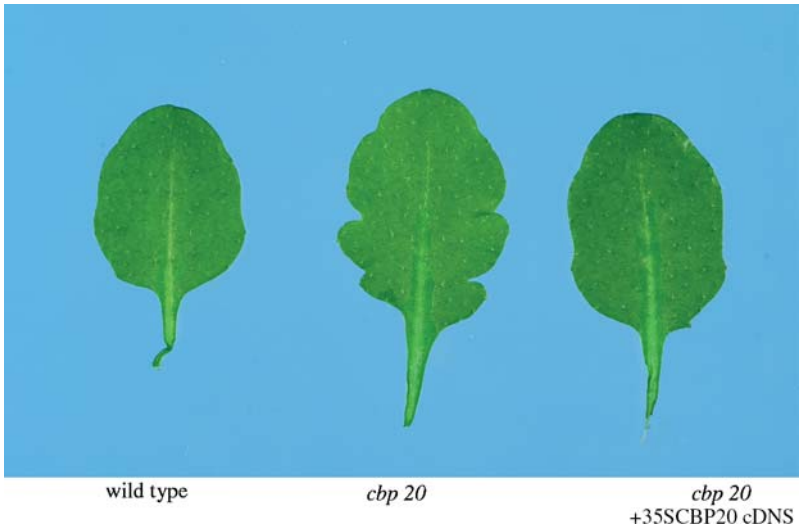

Figure 6. Genetic complementation of serrated leaf phenotype of *cbp20* mutant.

of dominant negative effects (Fortes *et al*., 1999). As in yeast, nCBC function is not essential for viability in *Arabidopsis* either. A loss of function mutation (*abh1*) affecting CBP80 has been described recently (Hugouvieux *et al*., 2001, 2002). Expression of *CBP20* and *CBP80*/*ABH1* in *Arabidopsis* was detected in all organs of the plant (Hugouvieux *et al*., 2002, Kmieciak *et al*., 2002). More specifically, an *ABH1* promoter β-glucuronidase fusion transgene showed tissue and developmental specific expression pattern, indicating strict spatial and temporal regulation of *CBP80* expression and probably nCBC function (Hugouvieux *et al*., 2002).

The data available on mammalian nCBC functions suggest that the role of nCBC in mRNA turnover is regulated by external factors (Fedoroff, 2002). In animal cell culture growth factors and UV stress stimulate nCBC activity by phosphorylation (Wilson *et al*., 1999, 2000). This parallels the regulation of the cytoplasmic form of CBC, which may also be stimulated by extracellular stimuli (reviewed in Wilson and Cerione, 2000). How nCBC is regulated in plants is not yet known. *CBP20* and *CBP80*/*ABH1* are not controlled at the level of transcription by ABA or water stress (this work, Hugouvieux *et al,* 2002). However, there is an intriguing possibility that subunits of the plant nCBC might be regulated by phosphorylation, as are CBP80 and cytoplasmic CBC in mammalian cells.

Given the fact that *Arabidopsis* mutants in two known nCBC subunit genes (*cbp80*/*abh1* and *cbp20*) are both oversensitive to ABA, show abnormal stomatal conductance and increased drought tolerance, we assume that nCBC may participate in the transmission of stress signals mediated by ABA. Based on the function of the nCBC complex, this regulation is likely to take place at either posttranscriptional or posttranslational levels. Posttranscriptional control has been demonstrated to affect a considerable proportion of stress-regulated mammalian genes (Fan *et al*., 2002). nCBC could modulate effector or regulatory mRNA species by either up or down regulating their levels. In fact, Hugouvieux *et al*. (2001) found that some transcripts are more abundant, whereas others are less represented in the *cbp80*/*abh1* mutant in comparison to wild type plants. A potential way of this regulation of mRNA turnover by nCBC may be through affecting either splicing or mRNA degradation in the nucleus (Clark *et al*., 2002; Das *et al*., 2003). In yeast, Clark *et al*. (2002) used splicing-specific microarrays to detect the effects of known splicing-related mutations on spliced and unspliced RNAs. They found that the *gcr3* and *mud13* mutations (i.e. mutations in genes corresponding to yeast homologues of plant *cbp80*/*abh1* and *cbp20*, respectively) have similar effect on intron accumulation, but distinct effects on the spliced RNA set of yeast. This implicates different genome-wide

effects by these mutations and thus at least partially different functions in yeast. In *Arabidopsis,* we found that the *cbp20* mutation results in a phenotype closely similar to that of *cbp80*/*abh1* mutant, suggesting that both proteins are dedicated to the same function, probably through their participation in the nCBC.

Interestingly, mutations in genes of other RNA binding proteins (HYL1 and SAD1) were also found to influence ABA signaling (Lu and Fedoroff, 2000; Xiong *et al*., 2001). This strengthens the view that RNA turnover is interconnected with ABA responses, and probably more generally with stress signaling in plants. As for the biotechnological application of the favorable trait revealed by the analysis of the *cbp20* mutant, more detailed studies are necessary. It has to be further established whether similar genetic determinants define nCBC functions in crop plants and how the pleiotropic phenotype caused by these mutations affect fitness and other agronomically important traits of crops carrying *cbp* mutations in the field.

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