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Examinations of the Physiological Background of Drought Hardiness in *Arabidopsis* and Production of Transgenic Plants

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INTRODUCTION

Nowadays drought tolerance of crop plants is a relevant problem. Water supply for irrigation is continuously decreasing, due to scarcity of rainwater and global warming, which increasingly imposes drought stress on plants. It is more and more important to understand the mechanisms of transpiration and water balance systems of plants.

The phenotype of drought tolerance is the result of rather complex, harmonized physiological processes. Along with the development of science, there have been several ways scientists tried to increase drought tolerance of plants. By the methods of conventional breeding – cross-breeding and selection strategies – plants were created with advantageous properties in this respect (e.g. see Hungarian patent No 218 309.).

With the development of molecular biology, the significance of transgenic techniques increased, which helped to understand the molecular processes influencing drought tolerance in plants.

During our work, we dealt with model species to analyze transpiration and water loss in plants. Stomatal closure is the most efficient way to quickly reduce transpirational water loss. As a result of transpiration through stomata and the cuticle the resulting decrease in water potential moves the transpirational water flow up from the roots to the leaves and finally to the atmosphere. The opening and closing of stomata have large impacts on the plants' water retention. Abscisic acid (ABA) is a stress hormone in plants that regulates stomatal closure, its signal transduction however is not well understood.

The aim of our work was to analyze drought stress response mechanisms and fitness of the ABA hypersensitive *Arabidopsis thaliana* cv. Columbia *cbp20* mutant, along with effects of this mutation on the water balance of the plant.

Preceding our work, the *cbp20* mutant was shown to display increased drought tolerance and ABA sensitivity. The development of the mutant is slightly delayed, it has a more compact stature, but generally shows similar morphology to the wild type. The mutation affects the *Arabidopsis* At5g44200 gene that encodes the Cap Binding Protein 20 (CBP20). CBP20 is part of the (nuclear) Cap Binding Complex (nCBC) that binds the cap structure of nascent nuclear mRNA's. nCBC has two subunits; CBP20 (20 kiloDalton, kD), and CBP80 (80 kD weight). These two subunits together can bind the cap structure. nCBC has a regulating function in mRNS splicing, processing and 3' end maturation, as well as in the export of some mRNAs from the nucleus.

According to results in scientific literature, loss-of-function of nCBC is not lethal in *Arabidopsis*, but the plant's physiological functions change in a way towards ABA hypersensitivity and drought tolerance. The *abh1* and *cbp20* mutants preclude germination at low concentrations of ABA that do not block the development of wild type plants. These mutant plants show higher drought tolerance and transpirate less water. This favorable phenotype has mainly attributed to faster stomata closure.

The changed RNS metabolism and water retention has only slight effect on other properties of plants. As pleiotrop mutant traits of the nCBC mutants are so mild, practical utilization of *cbp20* crop plants might be conceivable.

FINAL GOALS

- The aim of our work was a comprehensive analysis of the *Arabidopsis thaliana* cv. Columbia *cbp20* mutant with physiological and molecular methods, and we started to introduce this mutation in tomato. In cooperative efforts, we would have liked to make *CBP20* gene silencing constructions also in potato and rice.
- Our molecular examinations aimed at revealing the mechanism beyond the drought tolerant phenotype. We were trying to analyze the role of the mutation in alternative splicing, based on information from earlier scientific literature.
- In our physiological experiments we were trying to monitor the effect of lower transpiration rate of the *cbp20* mutant on physiological parameters, such as photosynthetic activity and the efficiency of the photosynthesis. We tried to examine physiological parameters in drought stressed plants compared to controls. In addition, our aim was to compare the sensitivity and the usefulness of the instruments used in detecting physiological responses during drought stress.
- Besides physiological monitoring, our other goal was to characterize ecophysiological properties of mutant plants. Our aim was to establish whether the mutation proved to be advantegous for the plant against competitors (e.g. wild type plants) in a heterogenous population in water stress situation.
- Another goal of our work was to find the orthologues of the *Arabidopsis CBP20* gene in tomato, potato and rice. Having found those, we planned to build gene silencing constructs for the *CBP20* orthologous genes in these crops.
- As a result of our research we tried to adapt a tomato gene transformation system, which can be used routinely. To compare efficiency and practical usability, two different transformation systems were assessed.

RESULTS AND DISCUSSION

Comparison of *Arabidopsis thaliana* cv. Columbia wild type and *cbp20* mutant fitness parameters

The plants were grown up in soil perlite mixture followed by terminal drought stress treatment with no water supply. In the beginning water potential of plants were on the same level, but on the 4th and the 5th day after the water supply was stopped, there was a big difference between control (wild type plants) and the mutants (*cbp20*). The water potential of the mutants did not change compared to the unstressed (watered) level, but in the wild type plants water potential decreased to –5 Bar.

We analyzed the photosynthetic activity of the plants with an LCi instrument. In well watered condition there was no difference between the plants. During drought stress however, from the 6th day on a big difference was found between them. Photosynthetic activity stayed high in the mutant compared to wild type plants, that showed dramatic decrease.

Photochemical quenching in drought stress - measured by an FMSII device– decreased in mutant and wild type too, but at a higher rate in wild type plants starting with the 3rd day on till the end of the experiment. There was no significant difference between the samples however after the 7th day. The Fv/F_M value is an important parameter of plant photosystems but in our experiments this value decreased significantly only in the control plants and only as a result of strong drought stress. It was not helpful in detection of drought stress at an early stage. The Φ_{PSII} parameter showed some difference between the mutant and the wild type plants from the 5th day on, but deviation values of data were rather high. With the help of statistical methods, we could establish a difference between these data sets starting with the 7th day after water deprivation. The non-photochemical quenching had a higher level from the begining in the *cbp20* mutants. This value increased in all samples from the 4th-5th day on after drought stress. Serious water deficit – after the 6th day of treatment – drew this value to the same level in mutant and wild type plants.

We measured termoluminscence (TL) with a Hammatsu photomultiplier. We recorded termoluminescence in wild type and mutant plants in well watered state, and after drought stress, on the 8th day. TL curves did not show variance among the plants in well watered state. After water deprivation, the *cbp20* mutants' curve was around 3000 TL unit, that is 1500 TL unit less than at the steady state. In wild type plants this curve was more flat, with a relative linear curve around 800 TL unit.

The gravimetric water content of soil (GWC) was determined daily by the weight of the pots. Already on the third day of water retention measured values showed significant difference between the mutant and control plants. GWC of mutant plants decreased to a smaller extent than that of the wild type - control - plants.

Comparison of seed-yield, thousand-seeds weight, fresh weight, and capacity for germination of *Arabidopsis thaliana* cv. Columbia wilde type and *cbp20* mutant plants

To determine whether the *cbp20* mutation has an effect on germination and/or yield, we compared wild type *Arabidopsis thaliana* cv. Columbia, and *cbp 20* mutant seeds. The seed-yield of wild type plants was higher than that of the mutant, however the variance was also higher. The thousand-seeds weights and the fresh weights were equal in both the wild type and the mutant plants. However there was a deflection in germination: 57% of the wild type seeds germinated, while altogether only 28% of the mutant seeds germinated under our experimental conditions.

Arabidopsis wild type and cbp20 mutant plants under competitive circumstances

In the course of our experiments we planted wild type and *cbp20* mutant plants into the same pot, in standard arrangements and into the same weight of soil mixture. To analyze competition, we planted 9 *cbp20* mutants into a pot, 9 wild type Col-0 plants into a different one, while there were 5 wild types and 4 *cbp20* mutants mixed in the third one.

We stressed the grown-up plants by witholding water for 8 days, and examined physiological consequences of the treatment and stress tolerance achieved. Similarly to our previous results, when plants were grown separately wild types ran dry after 8 days of water retention, while mutants did not. It was found that there was no capital difference between mutant and wild type plants regarding the weight of the roots. When we planted mutant and wild type plants separately, just like in case of our previous observations, the water-loss of mutants was slower, so they desiccated later than wild type plants. However when we planted them into the same pot the signs of desiccation appeared at the same time. From the parameters we measured, the water potential value decreased the same way in case of the mutant and wild type plants during drought stress. We also measured the gravimetric water content of the pots. The water content of pots with only mutant plants was highest at the end of the stress, while the pots containing wild type or mixed plants lost more water.

We analyzed *Arabidopsis thaliana* cv. Columbia *era-1* mutants under the conditions mentioned above to confirm that our results with *cbp20* mutants were not due to some special characteristic of this mutant. We planted *era-1* mutant and wild type plants in the same arrangements as with the *cbp20* mutant. When the plants grew up, we stopped supplying water and followed the water potential of the plants. By the end of the experiment the water potential values were highest in the pots that contained *era-1* mutants only, while in the pots that contained mixed or wild type plants only, it decreased. The visible changes that we observed in case of wild type plants didn't show up on the *era-1* mutants after water stress, their leaves remained green and turgid.

The effect of limited water supply in competition

Under natural circumstances drought periods do not always mean absolute lack of water; inadequate water supply could also mean a stress situation for the plants. We tried to analyze the effect of the competition described above closer to natural circumstances. To model this, we planted *Arabidopsis thaliana* cv. Columbia wild type and *cbp 20* mutant plants and followed their interaction in case of limited water supply. Here again wild type and mutant plants in the same pot went dry together, while separated mutants did not change significantly after a 2 week treatment period. Mutants planted among wild type plants did not show increased drought resistance. Gravimetric water content of the pots that contained *cpb20* mutants only were around 50% on the 12th day, while pots containing mixed or wild type plants only retained around 20-30% of their original water.

Following up water potential we got the same results as in the case of fast drought stress treatment. In the separated mutants water potential did not change significantly, while it decreased in case of mixed or wild type plants.

The role of the CBP20 gene in alternative splicing

In animal, yeast and plant systems the *CBP20* and *CBP80* gene functions influence processes of mRNA maturation, export and stability. Based on studies with *cbp* mutants these functions profoundly affect morphology and physiological traits of the plant.

Dispite the seemingly basic nature of the function fulfilled, there is only detectable difference in mRNA quantity of just a few genes in these mutants, revealed by microarray technology. In an attempt to shed light for the molecular regulatory mechanism, we hypothesized that alternative splicing might be modulated by the CBP20 protein.

To support our idea we tried to PCR amplify relevant intron spanning regions in gene products with known alternative mRNA splicing events from wild type and mutant *Arabidopsis* plants.

We chose a few members of stress signaling genes, for which we designed primers spanning the exon to be spliced out from the primary transcript. Unfortunately we could analyse just a few genes, and those did not show specific difference between wild type and mutant in alternative splicing.

According to recent results of other laboratories however the *CBP20* and *CBP80* genes are indeed found to modulate the expression of other genes via aternative splicing and miRNA regulation.

Identification of CBP20 gene homologs in tomato and potato, designing gene silencing construct for this gene

We searched for homologs of the *Arabidopsis CBP20* gene in the tomato EST database and succceed to identify an EST sequence which showed substantial homology with the *CBP20* gene. To silence this gene we designed, amplified and cloned an inverted repeated gene construction built on the relevant cDNA sequences (EST No TC 171884 and TC187934). This DNA sequence when transcribed into mRNA is able to initiate a natural gene silencing process of the target gene in tomato, and also an entirely (100%) homologous potato gene.

The construction was introduced into the pCP60 binary vector, which was later transformed into the *Agrobacterium tumefaciens* strain LBA4404.

Identification of a CBP20 homolog in rice and building a gene silencing construct targeting this gene

Using a bioinformatical method (BLAST algorithm) we identified the rice sequence Os02g39890 showing strong homology with Arabidopsis thaliana *CBP20*. Appropiate gene segments (one containing an intron) were amplified from genomic DNA by using PCR reaction and an inverted repeat construction was again made.

This construct was cloned into a pCAMBIA1300 based expression vector and transformed to *Agrobacterium tumefaciens LBA4404* strain.

János Györgyey's research group at BRC (Szeged) transformed this construct succesfully into rice, with biological tests for drought tolerance still in progress.

The results of tomato transformation

Leaf transformation

We performed tomato leaf transformation with the first true leaves. Leaf discs were transferred to MS media after bacterial infection. 35% of the transferred leaves developed into calli, and these were resistant against the antibiotics used. Only a small fraction of these started shoot growth, few developed roots and we could raise only one whole transformed plant in the greenhouse.

Cotyledon transformation

When we used cotyledon transfomation, more calli developed compared to the leaf tansformation. Plant regeneration also turned out to be more efficient. 70% of the infected parts developed into a callus resistant against the antibiotics used, and from these we succeeded to raise and root plants with higher efficiency. Altogether 28 antibiotics resistant plants were selected and planted out in the greenhouse. We conducted molecular biological experiments on these plants to prove the success of transformation.

Confirmation of the tomato transformation with PCR reactions

We prepared genomic DNA from the putative transformant tomatoes. To verify the quality of the DNA samples and the amplification process we designed primers specific for a tomato ubiquitin gene and made control PCR reactions. We made PCR reactions based on the NPTII marker gene to confirm transformations. These results quite specificly show the presence of the NPTII transgene in the plant samples, making very likely that the silencing construct has also been present. We repeated our analysis several times to confirm results. Altogether we got positive results in 20 plant lines, giving a transformation efficiency of 8%.

NEW SCIENTIFIC RESULTS

- We demonstrated the applicability of different methods and instruments in the observation of physiological consequences of drought stress. We showed that gravimetric water content, water potential and photosynthetic activity are the most sensitive tools for an early detection of water stress.
- We observed – with the detailed analysis of the *cbp20* mutant – that decreased transpiration of this mutant does not have a negative effect on the photosynthesis of the plant.
- Lower transpiration rate of the *cbp20* and *era-1* mutants do not represent an advantage against wild type plants in a competitive situation for water.
- We identified CBP20 homologs in a number of crop species. Based on publicly available sequences we built gene silencing constructs for CBP20 homologs in tomato, potato and rice.
- We adapted a cotyledon transformation system in tomato, and used it for the introduction of a CBP20 gene silencing construct into the plant genome. We confirmed transgenesis in several independent transformated lines.

SUMMARY

The *Arabidopsis thaliana* cv. Columbia *cap binding protein 20 (cbp20)* is a loss-of function mutant which is increasingly sensitive to abscisic acid, with decreased transpiration and elevated drought tolerance. The mutant contains a T-DNA insertion in a subunit of the nuclear cap binding complex.

As a result of our research it became clear that despite decreased transpiration *cbp20* mutation does not impose a negative effect on the photosynthesis of the plant, which could have been a possible result of decreased gas exchange. This result is hopeful regarding cultivation of *cbp20* mutant crop plants. The mutation had however negative effect on the germination rate and seed quantity in the model plant *Arabidopsis*. These characteristics need to be analyzed in detail if genetic modification or targeted breeding of crop plants may be planned. Other yield parameters, such as kernel weight and fresh weight did not change in the mutant in case of normal water supply. When deficient amount of water is provided however, the advantage of the mutant over wild type is obvious; it survived substantially longer in case of persistent water deprivation in our experiments.

As scientific literature describes several different parameters and physiological characteristics in realtion to plant water economy, we compared the usability of these parameters to monitor drought stress in *Arabidopsis*. Our findings show that parameters measured with the applied instruments are suitable to a different degree. The gravimetric method based on measuring pot weight, water potential value and photosynthetic activity proved to be relatively sensitive indicators of drought stress. We found the Φ_{PSII} parameter of fluorescent induction less sensitive, while the F_V/F_M value, the photochemical and non-photochemical extinctions showed significant changes only in case of serious water deprivation.

To analyse whether decreased transpiration rate gives the mutant plants an advantage in competition for water we examined the plants' response to drought stress in competitive circumstances. It appeared that witholding water doesn't give an advantage against plants with a more vigorous transpiration (the wild type in this case) in a competitive situation. This is presumably due to the fact that wild type plants can use up the remaining water from the soil fast and effectively. This caused the mutant plants to desiccate at the same rate as the wild type. We got similar results when we supplyed a low amount of water as well as in case of another ABA supersensitive *Arabidopsis* mutant (*era-1*) with decreased transpiration rate, but with a different genetic background. Our results bring attention to a weakness of transpiration as a biotechnological and breeding goal set to be attained, according to which water saver mutants may not show their favorable characteristics when competing for water. A conclusion can be drawn from our results regarding effective selection of drought tolerant mutants from a screening population as well.

The aim of our work – apart from the detailed fitness analysis of the *Arabidopsis thaliana* cv. Columbia *cbp20* mutant – was the examination of the nCBC regulation mechanism, and investigate this mechanisms in crop plants. Therefore we set out to try silencing orthologs of the *Arabidopsis CBP20* gene in some species. Three different crops were targeted in transgenic experiments: tomato, potato and rice. We searched for the assumed orthologues of the *Arabidopsis CBP20* gene in EST and genomic sequences of these species. Potato and tomato sequences were found to be 100% homologuous, therefore we used the same DNA construct for silencing in these species. We prepared the necessary gene constructions based on publicly available database sequences.

Plant transformation was successful in all three species. Tomato transformation took place in our laboratory, potato transformation was performed by Dr. Zsófia Bánfalvi in the Agricultural Biotechnology Center (Gödöllő), while rice transformation was carried out by Dr. János Györgyey's team at the Biological Research Center (Hungarian Academy of Sciences, Szeged).

So far only evaluation of the gene silenced potato's phenotype has been completed. In this case plant transformation resulted in decreased transpiration rate of potato (Zsófia Bánfalvi, personal communication). This proves that functionality of the nCBC complex can be changed in a crop plant in a similar way as in the model species *Arabidopsis*. Investigation of the transformed tomato and rice lines are currently underway.

The practical utilization of the results is a task for the future. We do not suggest direct involvement of the genetically modified (GM) plants we produce in breeding programs. Commercial use of GM crop species raises many questions and problems. On the other hand, the useful phenotype of the *cbp 20* mutant is based on loss of function of a gene. Biotechnological methods are known to abolish expression of a targeted gene without a need for genetic modification. A mutant plant line for a given gene can be selected with molecular methods in a traditionally mutagenized population with the help of the TILLING method. Theoretically there is an opportunity to produce non-genetically modified *cbp20* lines in any species with this non-transgenic technology.

A further aim of our work is investigation of the transgenic tomato and potato lines obtained. These gene silenced lines – aside from their biotechnological significance – may help us to discover the molecular mechanism underlying the mutant phenotypes, which is a primary interest in our research.

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