IMPROVED ABIOTIC STRESS TOLERANCE IN PLANTS BY ACCUMULATION OF OSMOPROTECTANTS - GENE TRANSFER APPROACH

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ABSTRACT

Abiotic stress is the major limiting factor of plant growth and crop yields. Traditional plant breeding has achieved significant results but the process is rather time- consuming and expensive. Plant biotechnology appears to be an attractive alternative in respect of the possibility for direct introduction of single genes into crops. The group of osmoprotectants is of particular interest for improving abiotic stress tolerance of crops. In the recent years, the efforts in the area gained considerable results. The studies performed at ABI will be discussed in relation with the major achievements elsewhere and the possibilities for elucidation of the complex role of osmoprotectants in abiotic stress tolerance and the practical breeding.

Introduction

The beginning of 21 century is marked by global scarcity of water resources, environment pollution and increased salinization of soils and waters. Abiotic stress is already a major limiting factor in plant growth and will soon become even more severe as desertification covers more and more of the world's terrestrial area (1). Serious changes in the agricultural practices all over the world are expected at present when population growth exceeds food supply. Recent advances in marker-assisted selection help traditional breeding to develop new cultivars but the process is rather time- consuming and expensive. Thus, plant biotechnologies aimed at overcoming severe environmental stresses need to be fully implemented. For example, direct introduction of small number of genes into crops seems to be attractive alternative (2).

Not so long time ago, the osmoprotectants have been definitely proven to be among the most important factors to protect plants cells from dehydration (3). They are non-toxic and are accumulated to significant levels without disrupting plant metabolism. The compounds fall into several

groups - amino acids (e.g. proline), quaternary ammonium compounds (glycine betaine), polyols and sugars (mannitol, D-ononitil, trehalose, sucrose, fructan) (4). Serious work was done for identification of metabolc pathways and most of the genes responsible for the synthesis and catabolism were identified and cloned. This paved the road to the massive work on gene transfer.

In this review, we will discuss the results obtained in ABI in regards to the past, present and future of genetic manipulation of osmoprotectants metabolism in respect of improved abiotic stress tolerance in plants.

Polyols

The accumulation of polyols (manitol, sorbitol, inositol and their derivatives) is considered to be related to drought and salinity stress tolerance in many plant species (5). They interact with the membranes, protein complexes or enzymes and protect them by scavenging reactive oxygen species. In this respect, they became attractive candidates for plant transformation.

Manitol

The model species *Arabidopsis* and tobacco are not manitol accumulators and in this respect are attractive candidates for transformation. Tobacco transformed to express *mt1D* into chloroplasts showed oxidative stress tolerance (6). Earlier, transgenic tobacco plants carrying bacterial gene coding for manitol-1-phosphatedehydrogenase (*mt1D*) accumulated manitol in their cytoplasm and were with enhaced biomass growth under salt stress (7, 8). Under normal conditions, however the transformants showed significant growth delay in comparison with the wild type plants. Transgenic *Arabidopsis* seeds carrying the same gene germinated well on high salt (9).

Sorbitol

Transgenic tobacco lines carrying apple cDNA for sorbitol-6-phosphatedehydrogenase accumulated wide range of sorbitol levels (10). The plants with low sorbitol were with normal phenotype. Those with higher amounts however showed necrotic damages on the leaves, sterile flower set and inability to root. The same construct was used to transform other plant species - *Diospyros kaki* (11). Under salt stress the photosynthetic activity of the transgenic plants accumulating sorbitol was higher then that of the wild type plants. It was suggested that this reaction could be considered as stress tolerance

D-Ononitol

Tobacco transgenic plants transformed with *imt1* gene coding for myo-inositol-o-methyltransferase – enzyme taking part in the biosynthesis of ononitol were more drought and salt tolerant than the wild type plants (12).

The studies aiming transfer of genes involved in the biosynthetic pathway of poliols were performed during the first years of abiotic stress tolerance manipulations. In this respect they are contributing to the elucidation of the influence on plant metabolism. A high competitiveness for one and the same products occurs between host and transgenic manitol and sorbitol. This could be the reasonable explanation for the negative effect of high polyols concentrations appearing after plant transformation (13).

Trehalose

Trehalose is a non-reducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress in bacteria, fungi, and invertebrates. It is very rare compound for plant kingdom (14) but in the recent years gene transfer for trehalose accumulation is among the busiest area of scientific interest. The reason for this is the ability of the compound to stabilize enzymes, proteins, and lipid membranes during desiccation. Almost all members of the plant kingdom do not seem to accumulate detectable amounts of trehalose**.** However, homologous genes for trehalose biosynthesis have been recently discovered in several wild

and crop plants (15, 16) which make them attractive candidates for gene transfer along with genes isolated from prokaryotes.

Trehalose-6-phosphate synthase is a key enzyme for trehalose biosynthesis in yeast, encoded by the structural gene *TPS1*. The gene of *Saccharomyces cerevisiae* was constitutively expressed in transgenic potato plants (17). The transformants exhibited various morphological phenotypes in vitro - from normal to severely aberrant growth and shapes. After acclimation in soil mixture, however the plants recovered to normal phenotype. The *TPS1* transgenic potato plants showed significantly increased drought resistance.

Earlier the same gene was expressed in tobacco plants (18). Trehalose accumulated at very low levels in leaf extracts of transformants. They exhibited multiple phenotypic alterations, including reduced sucrose content and improved drought tolerance. It was suggested that trehalose accumulation is influencing the alteration of sugar metabolism, plant development and stress tolerance, rather than leading to an osmoprotectant effect.

Similarly, very low levels of trehalose were traced in transgenic tobacco expressing *otsA* and *otsB* genes of *E. coli*, coding two enzymes of trehaolse biosynthesis pathway (19). The transformants were more drought tolerant than the wild type tobacco.

Abiotic stress tolerance was successfully manipulated in rice by overexpression of *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*) (20). The expression of the transgene was under the control of either tissue-specific or stress-dependent promoters. Compared with nontransgenic rice, several independent transgenic lines exhibited sustained plant growth, less photo-oxidative damage, and more favorable mineral balance under salt, drought, and low-temperature stress conditions. The authors suggest that the primary effect of trehalose is not as a compatible solute. Increased trehalose accumulation correlates with higher soluble carbohydrate levels and elevated photosynthesis capacity. Again, the peak trehalose levels were reported well below 1 mg/g fresh

Recently trehalose synthase (*TSase)* gene of the edible wood fungi *Grifola frondosa* was reported to be expressed in tobacco (21). The transformants accumulated higher levels of products of trehalose compared to many other known transgenic plants (400-fold higher than tobacco co-transformed with *Escherichia coli TPS* and *TPP*, twofold higher than rice transformed with a bi functional fusion gene (*TPSP)* of the trehalose-6-phosphate (T-6-P) synthase (TPS) and T-6-P phosphatase (TPP) of *E. coli*, and 12-fold higher than tobacco transformed with yeast *TPS1* gene). Obvious morphological changes were reported, including thick and deep-coloured leaves, but no growth inhibition. In addition, the morphological changes were restored to normal type in T_2 progenies. Trehalose accumulation in *35S-35S:TSase* plants resulted in increased tolerance to drought and salt, as shown by the results of tests on drought, salt tolerance, and drought physiological indices, such as water content in excised leaves, malondialdehyde content, chlorophyll *a* and *b* contents, and the activity of superoxide dismutase and peroxidase in excised leaves. These results suggest that transgenic plants transformed with the *TSase* gene can accumulate high levels of trehalose and have enhanced tolerance to drought and salt.

Fructans

Fructans are polymers of fructose and serve as main storage carbohydrate in many plant species (22). In the recent years they attracted considerable commercial interest as replacements for high calorie sweeteners and fats because humans lack the fructan-degrading enzymes necessary to digest them. In plants, the fructans accumulate in vacuoles and are thought to be involved in

abiotic stress tolerance (23, 24).

Historically, fructan biosynthesis was elucidated earlier in bacteria than in higher plants. By the early 1990s the bacterial fructan polymerase, levansucrase, had been purified and characterized, antibody probes were available and the genes were isolated, sequenced and cloned. By contrast, the first plant fructosyl transferase genes did not become available until the mid-1990s. It was a logical early step to incorporate the available bacterial gene into the available, transformable, fructan-non-accumulating plants (25).

The two main targets of fructan utilization – as safe food ingredients and as osmorpotectant define the efforts for genetic manipulation of plants: transformation for high quantities of fructans and transformation for improved stress tolerance. For the specific purposes of our review, we will focus our attention only to stress tolerance application.

Transformation for improved abiotic stress tolerance

So far SacB gene is used most extensively in studies. Tobacc and potato were first transformed with the gene of the bacteria *Bacillus subtilis*, coding for the levansucrase enzyme (26, 27). Under the control of 35S constitutive promoter the gene was expressed in the vacuoles. The tobacco transformants accumulated fructans similar to the bacterial ones and showed increased tolerance to drought, mild chilling or salt stress (23). In total, however the growth was insignificantly better than that of the untransformed plants. The same *SacB* gene of *Bacillus subtilis* was used for sugar beet transformation (28). The fructan content of the transgenic plants was lower than that obtained in tobacco and, especially in potato transformants (26, 27) but is comparable with the amounts in transgenic *Arabidopsis*. Under drought stress, the transgenic sugar beet exceeded the control plants in biomass and performance.

It is still unclear how exactly the fructans contribute for the stress toleranceof the transformed plants. Their accumulation is at rather low levels to be osmotically significant (28). It is suggested that like other sugars (manitol, trehalose) they act as regulators or signal molecules, influencing plant metabolism or as scavengers of reactive oxygen species (29).

Proline

The accumulation of proline under abiotic stress conditions has been studied in numerous plant species for half a century (5, 30). Still, however the relationship between this trait and stress tolerance is not clear among species. While Solanaceae species can increase their proline pool by more than two orders of magnitude there are many others that react with only a moderate increase in proline content under stress (31). It was shown that under osmotic stress proline stabilizes proteins, membranes and subcellular structures (32), and protects cellular functions by scavenging reactive oxygen species (33).

Genes encoding most of the enzymes associated with the synthesis and degradation of proline were cloned and partially characterized, but the factors regulating the expression of these enzymes are largely unidentified. During the last decade, several attempts were made to increase the level of praline accumulation in plants by transferring the genes associated with the biosynthetic pathway.

Pathways of proline metabolism

It was postulated that glutamate pathway of proline synthesis is predominant under osmotic stress in plants (34). Later, *d-OAT* transgenic *Arabidopsis* plants were shown to be with higher enzyme activity, higher biomass accumulation and germination rate under osmotic stress thus suggesting that the ornithine pathway plays also an important role at stress (35, 36).

In addition to synthesis, proline catabolism and transport are thought to control endogenous proline accumulation in plants.

Antisense transgenic *Arabidopsis* plants carrying *AtProDH* cDNA encoding proline dehydrogenase (*ProDH*), accumulated proline at higher levels than wild-type plants. The transformants were more tolerant to freezing and high salinity (37). Other *PDH* transgenic plants however did not show significant levels of osmotic stress tolerance (38).

Transgenics for enhancing proline accumulation and abiotic stress tolerance

D1-pyrroline-5-carboxylate synthetase (P5CS)

Plants of the model tobacco cultivar Xanthi were transformed for the first time with the *P5CS* gene isolated from *V. aconitifolia* under the control of 35S promoter (39). Transgenic plants produced high levels of the enzyme and synthesized 14-fold more proline than the controls before stress, but only about 2-fold greater after water stress. The transformants tolerated salt stress and showed enhanced root biomass under glasshouse conditions. The increase of proline in transgenics suggests that P5CS activity is the rate-limiting step. The feed back inhibition by proline as end product was shown to be lost under stress (40, 41, 42).

Despite the fact that some of the issues raised by the report were questioned and discussed critically (43) further success was achieved when the same gene construct was introduced in other plant species.

Elevated levels of proline caused by overexpression of mothbean P5CS in transgenic rice conferred enhanced tolerance to salt stress (44). The same construct pBI-P5CS was introduced into wheat by *Agrobacterium*-mediated gene transfer via indirect pollen system (45). The transgenic wheat plants showed overproduction of proline and increased tolerance to salt stress. Enhanced salt tolerance was achieved also when the same gene was introduced in carrot cell lines (46). *Vigna P5CS* gene was also overexpressed into the green microalga *Chlamydomonas reinhardtii* (47). The transgenic algae had 80% higher proline levels, grew more rapidly in toxic cadmium concentrations and bound four-fold more cadmium than wild-type cells. The promoter of the *P5CS* gene was described as stress-inducible in transgenic *Arabidopsis* subjected to water stress (48). This was confirmed later when P5CS gene isolated from rice was introduced and overexpressed in the same plant (49). The transgenics was found to be salt inducible and is also essential for salt and cold tolerance.

D1-pyrroline-5-carboxylate reductase (P5CR)

When tobacco was transformed with soybean P5CR gene and the enzyme activity was fifty-fold enhanced this did not yield significant increase in proline levels (50). These results were considered as confirmation for the importance of the substrate P5C as a limiting factor for the P5CR activity. Hence, co-expression of *P5CS* and *P5CR* genes under the control of stress inducible promoter might result in enhanced proline accumulation during stress and may bring down the retardation effect of plant growth. When soybean was transformed with antisense *P5CR* gene controlled by an inducible heat shock promoter (IHSP), the reduction of the gene expression resulted in a decline in proline synthesis as well as protein synthesis and osmotic stress sensitivity (51).

Glycine betaine

Glycinebetaine is a quaternary ammonium compound that occurs naturally in a wide variety of plants, animals and microorganisms (52). In plants, its accumulation has been widely recognized as abiotic stress response where it acts as an osmoprotectant by stabilizing both the quaternary structure of proteins and the highly ordered structure of membranes (53).

Glycinebetaine is created from choline. In many plants, it is synthesized through a two-step oxidation of choline, by choline monooxygenase and betaine aldehyde de-

hydrogenase (54). The genes coding for the two enzymes have been cloned from plants (55, 56, 57, 58) and were introduced separately into plants to generate glycinebetaine, but sufficient quantities of the choline substrate and the intermediate betaine aldehyde could not be obtained within the cell (59). This was confirmed later, when transgenic rice plant containing the betaine aldehyde dehydrogenase gene was supplied with sufficient exogenous betaine aldehyde to artificially generate a considerable quantity of glycinebetaine and showed improved tolerance against both salt and low temperatures (60).

A different one-step pathway is available in *E. coli* and *Arthrobacter globiformis* where glycinebetaine is synthesized from choline, by choline dehydrogenase and choline oxidase, respectively. Transformation with *codA* has been performed extensively (61). Enhanced tolerance to abiotic stress has been achieved when the gene was directed to chloroplast or cytosol (62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74). However, glycinebetaine was accumulated in very low often undetectable amounts suggesting that so far the success is limited or that like in other osmorpotectants its type of action is still not clear. In some cases, exogenous supply of glycine betaines helps the engineered compound to improve stress tolerance (75).

Recently, an alternative biosynthetic pathway of betaine from glycine, catalyzed by two *N*-methyltransferase enzymes, was found (76). In cyanobacterium and in *Arabidopsis* was found that the coexpression of *N*-methyltransferase genes caused accumulation of a significant amount of betaine and conferred stress tolerance (77). These first results demonstrate the usefulness of glycine *N*-methyltransferase genes for the improvement of abiotic stress tolerance in crop plants.

Genetic engineering for improved abiotic stress tolerance at ABI

Abiotic stresses are the major environmental challenges for crops in countries with typical continental climate like Bulgaria. Drought, high summer and low winter and spring temperatures restrict plant growing season and decrease the productivity. In this respect, application of biotechnology for development of abiotic stress tolerant crop lines is a long lasting goal for AgroBioInstitute.

Tobacco is a model culture for biotechnology studies and at the same time, very important crop. It is known to be a relatively drought stress tolerant plant *per se* (78). Thus, the establishment of effective screening system to distinguish stress tolerant and sensitive genotypes is of crucial importance. As most of the crops, planted or sown in spring, tobacco is very sensitive to late spring frosts at transplanting stage. In this respect, the reaction to freezing under controlled conditions appeared to be very useful screening procedure providing clear-cut differences between the wild-type and transgenic plants (24, 78, 79).

In our experiments, we used three cultivars, representing the three types of commercially grown tobacco (*Nicotiana tabacum*) - Nevrokop 1146 (oriental-type), Coker 254 (flue-cured) and Burley 21 (Burley type). They are known to be of high quality and wide-spread use in Bulgaria (78). The plants were transformed to accumulate proline, fructan or glycine betaine (78, 80). We used constructs harboring *Arabidopsis*- or *Vigna*-derived genes (*AtP5Cs*, *VacP5Cs*) for D^1-pyroline-5-carboxylate synthetase production, *SacB* gene coding for levansucrase from *Bacillus subtilis* or the *codA* gene coding for choline oxidase from *Arthrobacter globiformis*. The genes were isolated earlier (26, 40; 66, 81) and kindly provided to us. We developed a specific procedure with several steps of selection, including osmotic stress in vitro and freezing of potted seedlings at

transplanting stage, complemented with various physiological studies (24, 78, 79, 80) This procedure allowed us to select stable transgenic lines able to survive freezing stress at controlled and field conditions in several subsequent progenies. The transgenic seeds were able to germinate after long exposure to chilling in vitro better than the wild-type seeds. The transgenic seedlings grew normally while the wild-type seedlings showed reduced growth (82). Our reports were we believe the first for freezing tolerance in proline and fructan transformed plants.

Using the freezing procedure at transplanting stage, we studied the reaction to the oxidative component of the applied stress to our transgenic tobacco lines and their wild type (83). Freezing for 24 h resulted in severe damages for the wild type. A corresponding increase of electrolyte leakage, hydrogen peroxide and malondialdehyde contents, a rise of peroxidase activity and inhibition of catalase activity occurred in the non-transformants. Similar, but significantly lower trend of the same parameters has been found for the transgenic lines. Moreover, the oxidative markers returned to their normal levels when the transformants were able to recover from freezing. We speculated that transfer of genes, coding for accumulation of osmoprotectants, is related to decreased intensity of freezing-induced oxidative processes.

In similar studies we followed the photosynthetic activity of wild type and transformed tobacco plants. JIP test was used to measure chlorophyll fluorescence induction and to analyze the functional activity of photosystem II (84). No significant differences were found among wild type and transgenic plants after short-term freezing and at recovery. Prolonged freezing resulted in strong inhibition of photosynthetic reactions of the wild type plants that could not be restored. At the same time, the evaluated parameters of transgenic plants did not change significantly at long-term freezing and recovery from it.

Conclusions

Understanding the metabolism, transport and roles of the osmoprotectants during stress is vital in developing plants for stress-tolerance. There is an urgent need to identify the signaling components related to the osmolytes biosynthesis and degradation and their coordination in gene expression events under stress and at recovery.

Despite the euphoria resulting from the achievements, most of the results are still questioned with reasonable prejudice (31). In many cases, the convincing molecular evidences for stable integration of the genes coincide with very low concentrations of the relevant compounds which are difficult to be related to the occurring stress tolerance. The relatively minor impact of the organic osmolytes on cellular water relations and the controversy about osmotic adjustment of the transgenic plants (43) leads to the presumption that these substances mainly participate in stabilization and protection. Thus, it appears that the discussion is not about the efficiency of the gene transfer but more for the reasonable explanation of the positive results obtained. In this respect, stable transgenic lines resulting from well-established commercial cultivars (78) provide further opportunities for elucidation of the complex role of osmoprotectants in abiotic stress tolerance and the practical breeding.

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