

Use of cell selection tools in the creation of agricultural crop varieties resistant to abiotic stress

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Abstract

Anikina, I., Oves, E., Adamzhanova, Zh. & Kaynidenov, N. (2021). Use of cell selection tools in the creation of agricultural crop varieties resistant to abiotic stress. *Bulg. J. Agric. Sci.*, 27 (3), 505–511

To increase the productivity and sustainability of agriculture, it is necessary to create high-yielding and climate-resistant crops that are resistant to abiotic and biotic stresses. This review presents the role of cell culture as an accessible and effective tool for studying the genetic mechanisms of plant resistance and creating crop varieties that are resistant to abiotic stresses. In addition, this review describes the potential role of the latest genomic tools in decoding combined plant stress tolerance. The review explores integrated strategies that include the vast and diverse traditional and modern (structural, functional, comparative, and epigenomic) approaches, resources, and genomics-based selection methods that agricultural biotechnologists can adopt and use to analyze and decode the molecular and gene regulatory networks involved, complex quantitative indicators of crop yield and stress resistance. The use of the latest tools of biotechnology, namely QTL analysis, genome assessment through sequenced EST and cDNA analysis using biochips, mutagenesis research, as well as transgenic approaches can provide success in understanding the mechanisms of resistance to abiotic stress, which will overcome the barriers to creating varieties that are resistant to negative factors of the growing environment.

Keywords: tissue culture; stress; resistance; salinity tolerance; cell lines; genomic selection

Introduction

The enormous population growth, climate change and global warming are now considered major threats to agriculture and world's food security. To improve the productivity and sustainability of agriculture, the development of high yielding and durable abiotic and biotic stress-tolerant cultivars and climate resilient crops is essential (Kujur et al., 2013; Mitova et al., 2017).

Abiotic stress conditions such as drought, high and low temperature and salinity are known to influence the occurrence and spread of pathogens, insects, and weeds (Scherin & Coakley, 2003; McDonald et al., 2009; Ziska et al., 2010; Peters et al., 2014). Additionally, abiotic stress con-

ditions such as drought enhance competitive interactions of weeds on crops as several weeds exhibit enhanced water use efficiency than crops (Ziska et al., 2010; Valerio et al., 2013).

The effect of combined stress factors on crops is not always additive, because the outcome is typically dictated by the nature of interactions between the stress factors (Atkinson et al., 2013; Prasch & Sonnewald, 2013; Pandey et al., 2015a,b; Choudhary et al., 2016; Ramu et al., 2016). Plants tailor their responses to combined stress factors and exhibit several unique responses, along with other common responses. Therefore, development of plants with enhanced tolerance to combined abiotic and biotic stresses involves identification of physio-morphological traits that are affected by combined stresses.

Cultivation of cells of plants *in vitro* represents a universal experimental model for the production and study of biochemical plant mutants.

Advantages of the *in vitro* system are: 1) the ability to work with millions of cells; 2) use of selective systems; 3) production of regenerant plants suitable for accelerated microclonal reproduction (Butenko, 1984; Trigiano & Gray, 1999; Lutova, 2003).

The list of mutants with important agricultural traits, the selection of which is feasible at the cellular level, is quite large. These include mutants of resistance to stress factors, herbicides, various diseases, overproducers of essential amino acids.

Great practical importance has the selection for the stability of higher plants to the abiotic factors of the environment, in particular, to salinity.

Soil salinization is one of the extreme factors of world – wide crop production. According to the FAO (food and agriculture organization of the United Nations), saline soils occupy a huge area in the world — about 25% of the entire land surface. FAO experts are confident that salinization is a global problem for humanity.

Soil salinity is detrimental to most agricultural crops. Increased salinity in the root zone affects almost all aspects of plant physiology and biochemistry, significantly inhibits their growth and development, and leads to a decrease in yield (Foolad, 2004).

In view of the importance of this problem in the world, many scientists have made great efforts to understand the physiological aspects of plant resistance to salinity and to create salinity-resistant genotypes of important agricultural crops. Despite these huge efforts, only a small number of varieties have been created that are partially resistant to salinity (Cuartero et al., 2006). This is probably due to the fact that salt-tolerant varieties must also have other important economic characteristics and performance attributes corresponding to modern varieties (Grandillo et al., 1999).

In addition, the main reason is that plant resistance is a very complex trait and practically does not lend itself to classical genetic analysis, and in the process of ontogenesis, salt resistance changes significantly (Foolad, 2004; Turhanand & Seniz, 2012).

In the course of research, it was found that the reduction of crop growth parameters under the conditions of salt stress of crops at an early stage of growth can be used as selection criteria, since they correlate with the salt resistance of crops, Bolarin et al. (1991), Foolad (1996), Ibrahim (2003) indicated that the growth of shoots in salinity compared to the control is the best practical indicator of salt tolerance of tomatoes.

Research by Ahsan et al. (2000) showed that the content of ions in tissues and ion selectivity were good selection criteria for selection for salt tolerance of tomatoes. The selectivity of potassium for Na^+ is considered a good indicator of salt tolerance in the study of several genotypes of cultivated and wild tomato species (Cuartero et al., 1992).

The greatest success is achieved when analyzing the inheritance of cellular mechanisms of salt tolerance. It is noted that cellular mechanisms of salt tolerance differ not only between species or genera, but also between varieties of the same culture (Kavi Kishor & Reddi, 1985).

At the present time known that cultured *in vitro* cell lines of glycophyte plants can be “adapted” for growth under salt stress. Passing haploid callus tissues of *N. sylvestris* on a medium with a constantly increasing salt concentration, cell lines capable of growth in the presence of 0.17 M (1%) NaCl were obtained. After preliminary processing of the *N. tabacum* suspension culture with a mutagen (0.15% EMC, 60 min), Cell lines resistant to 0.03 M NaCl were isolated by single-step selection. Resistance to salt was preserved by suspension cultures when they were cultured for several passages on selective media. Flax plants resistant to high salt concentrations (2.5-3%) were obtained through tissue culture. Callus tissues were obtained from hypocotyl segments, planted on a selective medium, and after 32 days, green colonies were selected among the dead cells, from which plants that were resistant to salinity were generated.

In most cases, the selected cell cultures in the presence of salt grew worse than in the usual medium. Some salt-tolerant lines, for example alfalfa and rice, possessed the properties of halophytes, and the growth optimum was noted in the presence of sodium chloride in the medium. Salinity is stable even in the absence of a constant selective pressure and can be manifested at the level of plants regenerates.

Selection for high concentrations (0.43 M) allows obtaining cell lines characterized by both unstable and stable tolerance (Philip, 1990). The reason for reversible unstable adaptation of cells to NaCl is the physiological adaptation caused by changes in gene expression. Changes in gene expression, in particular 24 kD protein synthesis, can be induced by abscisic acid, which accumulates in salt-adapted cells.

Despite the fact that sodium chloride is one of the main stressors of saline soils, other salts also have a toxic effect on plants. Therefore, modeling *in vitro* conditions closer to those encountered in nature, use for selection of a mixture of salts or sea salt. Was received stably stable callus culture of *Brassica napus* to 10 mM Na_2SO_4 was obtained. This culture contained 5-6 times more proline than the control.

Ve et al. (1987), studying barley regenerate plants grown by another culture on sodium sulfate solutions of various concentrations, showed the possibility of selecting salt-resistant barley genotypes in hybrid offspring. It is shown that in the offspring of plants obtained from the pollen of reciprocal hybrids, plants appear that are more resistant to salts in comparison with the parent forms. Information about the role of the cytoplasm in the inheritance of salt tolerance was first reported in 1965 (Granick, 1965), later other confirmations of this fact were obtained (Turhan & Seniz, 2012).

Salt tolerance of plants can also be increased as a result of selection to a single salinization factor – osmotic stress (Deinlein et al., 2014). For example, tomato cells adapted to water stress induced by polyethylene glycol had increased resistance to NaCl. Increased salt tolerance was found in carrot cell lines selected on a medium containing mannitol in high concentration (99-870 mM) as a non-utilizable osmotic. The obtaining of salt-tolerant plant forms is possible through somaclonal variability and chemical mutagenesis.

Significant interest produces the formation of stability of higher plants to ion stress. In all experiments on cell selection of plants for resistance to ion stress, a direct selection method was used in which toxic concentrations of salts were used as a selective agent. However, creating stressful selective conditions *in vitro*, identical to those in nature, is extremely difficult.

In natural conditions, in addition to the toxic effect of ions, other factors are imposed, in particular the presence of various substances, acidic pH values of the medium. For selection at the cellular level, media were proposed that, although not completely in accordance with natural stress conditions, still provided expression of the resistance sign and allowed the selection of the desired variants.

The creation of such special media was necessary, for example, for selection for resistance to aluminum (Shirokih et al., 2011). Aluminum ions precipitate at high pH values in the form $Al(OH)_3$, and at low pH (<5) the agar medium does not solidify. Agar mediums with a standard pH value (5.8) containing aluminum in the form of Al-EDTA, as well as liquid media with a low pH (4.0) were used. So, in 1978, tomato cell lines were isolated, which grew in the presence of $200 \mu M Al^{3+}$ in the form of Al-EDTA. In subsequent experiments with the culture of cells, media with $600 \mu M Al^{3+}$ were used, with low concentrations of phosphate ($10 \mu M$), calcium ($0.1 \mu M$) and pH 4.0.

To isolate resistant variants, direct selection was undertaken in which the untreated mutagenic suspension cells were transferred to an Al-toxic medium and after 123 days were screened selection was made or after a 10-day cultivation of the cells on a selective medium they were planted into

non-selective ones followed by identification of resistant clones. Aluminum-resistant cell lines, as well as regenerate plants, are obtained from sorghum, soy and carrots.

By direct selection *in vitro* selected petunia cell lines resistant to mercury; cell lines of carrots, simultaneously resistant to aluminum and manganese; Suspension cell cultures of *Datura* and tomato, resistant to cadmium, which were selected on medium with $CdCl_2$. Constantly increasing the concentration of $CdSO_4$ in the medium, lines, were isolated, resistant to 500-2000 μM cadmium. After 3 months cultivation on ordinary media, cadmium resistance was maintained, and cross resistance to zinc and copper was detected.

Significant practical interest is the formation of resistance of higher plants to drought (Kosma et al., 2009; Ziska et al., 2010; Valerio et al., 2013). The water regime is one of the main factors of successful crop production, since water is an important component of the “living” substance of plants (Petrova & Kireva, 2016; Gadzhalskaya et al., 2017). To simulate the stress effect of drought *in vitro*, nutrient media are used that are supplemented with osmotically active substances that lower the external water potential. As such a selective agent for breeding selection for drought resistance, PEG is used, which is a non-penetrating osmotically active substance into the cell.

The first report on the isolation of PEG-induced stress-induced tobacco cell lines emerged in 1979. Later, tomato cell lines were used to select for drought resistance and underwent water stress during culturing in the presence of PEG. The selective culture medium for callus culture contained 15% PEG 6000. As a result of the experiments, stable callus lines were selected, but the stability was quickly lost during cultivation of callus on a medium without osmotic, which indicates the physiological nature of adaptation.

Testing of callus lines on growth in the presence of PEG was proposed to identify drought-resistant soybean genotypes. Analysis of the growth of callus tissues of ten varieties of soybeans on media with 0; 15; 20% PEG 8000 indicated a correlation of plant drought tolerance and tolerance to PEG cultured cells. These data confirm the possibility of searching *in vitro* for resistant genotypes among the genetic variety existing in nature.

An equally important aspect of cell selection is the formation of resistance of higher plants to extreme temperatures.

The cause of the stress factor in plants can be relatively high or low temperatures. Cold stress in plants can be caused by temperatures of a large range: from 10-15 to $0^\circ C$. This stress is most affected to plants in tropical and subtropical zones.

Modern theories explain all types of damage caused by cooling, temperature-dependent transformation of mem-

brane lipids from the liquid crystalline state to a solid and temperature-dependent change in the hydrophobic nature of membrane proteins, leading to disturbances in various physiological processes (Kavi Kishor, 1999).

Resistance of plants to cooling is due to the ability of lipid membranes to remain in the liquid state due to the presence of a large proportion of unsaturated fatty acids and/or an increased content of sterols. Damages caused by the freezing of plants (temperature below 0°C) are primarily related to the formation of extracellular ice. In this case, the outflow of water into the extracellular space leads to a secondary effect caused by the stress of the skin.

Adaptation of plants to negative temperatures in most frost-resistant plants occurs with gradual hardening by lowered temperatures during the autumn period. Violations caused by negative temperatures can be prevented by the accumulation of antifreeze substances, a decrease in the amount of unbound water during dehydration and an increase in the ability to supercool (Butenko, 1986).

An important role in plant protection is played by the accumulation of free proline (Delauney & Verma, 1993; Zhu, 2001). As shown by the results of cell selection for hydroxyproline, the obtained callus lines and potato plants resistant to this proline analog accumulated proline and had increased frost resistance and tolerance to NaCl.

The first experiments, which described the possibility of using cultivated plant cells for the selection of hardy to low-temperature cell lines, were published in 1968. As the material for selection, suspension cultures of tobacco and pepper were used, which, after sowing in agarized medium, were subjected to temperatures of 3 and 40°C for 21 days. The surviving colonies were cultivated at 240°C and then re-tested. Among the selected clones, lines were found that stably maintained increased cold resistance (Tumanov et al., 1968).

Work in this direction continues with the use of cell technologies (Tsvetkov et al., 2007; Alcazar et al., 2007).

Significant interest in modern conditions causes the formation of resistance of higher plants to radiation stress (Mittler, 2006).

Under natural or experimental conditions, a stress state in plants can be induced by an increased level of UV or ionizing radiation. The paramount importance of irradiation is associated with its effect on the genetic apparatus of the cell. Different types of radiation can also directly violate many physiological processes: respiration, photosynthesis, growth, active transport, as well as ion balance and protein synthesis. Different types of plants or individual samples have different sensitivity to UV and penetrating radiation. The main role in the protection of plants from irradiation is played by repa-

ration processes, among which the repair of genetic control systems of the cell and the repair of individual cellular structures are distinguished (Philip, 1990).

Irradiation in the work with cell cultures was used as a mutagen. Directed selection in vitro for resistance to high doses of ionizing radiation gave positive results in experiments with bacterial cells.

Resistance to UV radiation, which has a weak penetrating power, can be due to the absorption of incident radiation by epidermal cells, the morphological structure of plants that protects sensitive cells by photo-reparation mechanisms.

A significant role in the protection of plants from UV is played by the accumulation in the vacuole of flavonoid cells, which absorb a significant part of UV radiation (Lutova, 2003). The formation and accumulation of flavonoids was observed in the treatment of UV plants and unorganized growing tissues. Irradiation of parenteral UV protoplasts stimulated the entire phenylpropanoid biochemical pathway for the synthesis of flavonoids.

To isolate UV-resistant lines, a suspension culture of *Rosa damascena* was used, which was exposed to a dose of 250 J/m². The colony found during the sowing of irradiated cells maintained its stability; the cells were 2.2-2.8 times larger in size and had twice the increased DNA content. In the later stages of cultivation, resistant cells accumulated large amounts of flavonoids.

Determination of the nature of changes at the level of somatic cells is carried out according to a number of criteria (Trigiano & Gray, 1999), each of which cannot separately serve as evidence of a mutation:

1. The frequency of the altered cells should be very low ($1 \cdot 10^{-6}$ – 10^{-7}).
2. It increases significantly when using mutagens ($1 \cdot 10^{-4}$ – 10^{-5}).
3. Modified cells are capable of stable long-term growth.
4. The stability of the changed trait is preserved in the absence of selective pressure.
5. The product of the altered gene is found.

Since plant cells in vitro possess totipotency, i.e. the ability to regenerate plants under certain conditions, and then genetic analysis of regenerate plants is carried out to prove the nature of the changes at the somatic level (Butenko, 1982; Yang & Quiros, 1993; Kavi Kishor, 1999)

Although the methods of classical selection, in particular for breeding drought-resistant genotypes are valuable, they do not provide information about the regions of chromosomes that regulate the variation of each quantitative trait; pleiotropic effects of these chromosomes on other traits; and possible cause-and-effect relationships between genes. Some of these difficulties can be partially overcome by using mo-

lecular markers that can identify the QTL loci that define the selected trait and evaluate the effects of the desired QTL locus on other traits. For example, if QTL-locus effects explain a large proportion of variation, genetic markers can be used to select suitable phenotypes.

Despite huge efforts to study QTL loci of resistance to abiotic stress, only a few studies aim to actually isolate the genes included in QTL loci. Several methods, including genetic analysis of expressed marker sequences (EST) on microchips, as well as the creation of mutants with increased or decreased/lost (gene knockout) protein activity, are applicable in the search for candidate genes for subsequent cloning. The combination of these methods and approaches will reduce the number of candidate genes analyzed from hundreds or thousands to several target genes.

Large-scale analysis of cDNA microarrays of abiotic stress resistance gene expression profiles was performed on model plants such as *Arabidopsis* and crystal grass, as well as on agricultural crops such as corn, cabbage, and sugar beet.

Mutants are often used to determine the function of important genes that provide tolerance to abiotic stress. On the example of *Arabidopsis*, a technology for screening mutants with impaired response to low water potential has been developed. Mutants are important for identifying the physiological role of mutated genes and the processes in which they are involved. Cloning these genes provides unique information about their work and the ability to reintroduce them into the plant, thereby enhancing or reducing their expression. Transgenic approaches have also been used to identify biologically important components for increasing stress tolerance. For example, on transgenic tobacco (*Nicotiana tabacum*), which stores fructose polymers, it was found that fructan-producing tobacco has more developed roots and is better adapted to drought. Apparently, the introduction of fructans in varieties that do not produce them can increase drought tolerance.

In general, transgenic approaches use individual genes and constitutively active promoters, which lead to a minimal increase in resistance to environmental stress. This confirms once again that many genes are involved in determining resistance to abiotic stress. The use of combined genomic approaches (mutagenesis, microarrays, and molecular markers) will allow identifying pathways and sets of genes necessary for manipulating resistance.

In recent years, tremendous progress has been made in plant genomics and molecular breeding research pertaining to conventional and next-generation whole genome, transcriptome and epigenome sequencing efforts, generation of huge genomic, transcriptomic and epigenomic resources and development of modern genomics-assisted breed-

ing approaches in diverse crop genotypes with contrasting yield and abiotic stress tolerance traits. Unfortunately, the detailed molecular mechanism and gene regulatory networks controlling such complex quantitative traits is not yet well understood in crop plants (Kujur et al., 2013). Therefore, we propose an integrated strategies involving available enormous and diverse traditional and modern (structural, functional, comparative and epigenomics) approaches, resources and genomics-assisted breeding methods which agricultural biotechnologist can adopt, utilize to dissect and decode the molecular and gene regulatory networks involved in the complex quantitative yield and stress tolerance traits in crop plants. This would provide clues and much needed inputs for rapid selection of novel functionally relevant molecular tags regulating such complex traits to expedite traditional and modern marker-assisted genetic enhancement studies in target crop species for developing high-yielding stress-tolerant varieties.

In the integrated genomic approach, resistance to abiotic stress is considered as a polygenic trait. The DREB study of transcription factors is a good example of applying a combined genomic approach to increase resistance to abiotic stress.

Thus, the transformation of *A. thaliana* by the Durable genome under the control of the 358-promoter of tobacco mosaic virus causes strict constitutive expression of stress-inducible genes and increases resistance to frost, water and salt stress. In one recent study were able to identify genes involved in the increase of expression of DREB. The enzyme spermidine synthase, involved in the biosynthesis of polyamines, increases resistance to the simultaneous action of many stressors and increases the expression of DREB transcription factor. Transgenic plants with overexpression of spermidine synthase show increased spermidine content, as well as increased resistance to various stressors, including cold, salinity, and drought. Analysis of transgenic plants using KDI 1 K microarrays showed that several genes, including DREB1, were more actively transcribed during cold stress.

A few important molecular studies have recently been employed to elucidate the molecular responses of plants against combined drought and pathogen stresses (Thao & Tran, 2016). These studies, according to Prachi Pandey et al. (2017), have not only shed light on a plant's defense mechanism against combined stresses but also revealed some potential candidates for improvement of plant tolerance to combined stresses. Some of the important candidate genes identified so far are methionine homeostasis gene; methionine gamma lyase (AtMGL), rapid alkalization factor-like 8 (AtRALFL8) involved in cell wall remodeling

and azelaic acid induced 1 (AZI1) functioning in systemic plant immunity (Atkinson et al., 2013). The identified candidate genes can be suitably modulated to confer enhanced tolerance against the combined stresses. The modification can be done by genome editing using tools like CRISPR/Cas9 system. CRISPR/Cas9 system can also be used to modulate the transcription of the genes of interest by guiding catalytically inactive dead Cas9 (dCas9) or dCas9 fused with transcriptional repressors/activators to the promoter of a gene.

Further research in this direction using the different functional genomic approaches can, thus, help in uncovering responses of plants to combined abiotic stress.

Genetic improvement of several economically important crops during the 20th century using phenotypic, pedigree, and performance data was very successful Pandey (2017). However, signs of grain yield stagnation in some crops, especially in drought-stressed and semi-arid regions, are evident.

Genomic selection makes it possible to increase the production of agricultural products in less time (Ramu et al., 2016).

International Maize and Wheat Improvement Center (CIMMYT) maize breeding research in Sub-Saharan Africa, India, and Mexico has shown that genomic selection can reduce the breeding interval cycle to at least half the conventional time and produces lines that, in hybrid combinations, significantly increase grain yield performance over that of commercial checks (Kujur et al., 2013).

In conclusion, taking into account the complexity and complexity of the plant response to abiotic stress, as well as to significantly increase resistance to abiotic stress in the field, a multidisciplinary approach is necessary, including genetics, biochemistry, physiology, plant breeding, and crop production. Currently, the attention of breeders is focused on improving the understanding of the genetic basis of resistance to abiotic stress through the use of a set of genomic approaches, as well as evaluating the expression of various genes encoding enzymes and proteins of various biochemical pathways that cause resistance to abiotic stress. This approach, which includes QTL analysis, genome assessment through sequenced EST and cDNA analysis using biochips, mutagenesis research, and transgenic approaches, can provide success in understanding the mechanisms of abiotic stress resistance.

The use of these tools will make it possible to make a breakthrough in optimizing the signs of resistance, which will help to overcome the barriers that stand in the way of creating varieties that are resistant to negative factors of the growing environment.

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Received: October, 17, 2020; Accepted: January, 14, 2021; Published: June, 2021