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Molecular Abiotic Stress Tolerans Strategies: From Genetic Engineering to Genome Editing Era

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Abstract

In last decades, plants were increasingly subjected to multiple environmental abiotic stress factors as never before due to their stationary nature. Excess urbanization following the intense industrial applications introduced combinations of abiotic stresses as heat, drought, salinity, heavy metals etc. to plants in various intensities. Technological advancements brought novel biotechnological tools to the abiotic stress tolerance area as an alternative to time and money consuming traditional crop breeding activities as well as they brought vast majority of the problem themselves. Discoveries of single gene (as osmoprotectant, detoxifying enzyme, transporter protein genes etc.) and multi gene (biomolecule synthesis, heat shock protein, regulatory transcription factor and signal transduction genes etc.) targets through functional genomic approaches identified abiotic stress responsive genes through EST based cDNA micro and macro arrays. In nowadays, genetic engineering and genome editing tools are present to transfer genes among different species and modify these target genes in site specific, even single nucleotide specific manner. This present chapter will evaluate genomic engineering approaches and applications targeting these abiotic stress tolerance responsive mechanisms as well as future prospects of genome editing applications in this field.

Keywords: GMO, abiotic stress, genetic engineering, genome editing

1. Introduction

Before the first examples of the crop domestication by late hunter-gatherer man approximately 10.000 years ago, plants evolved most of their traits following the most basic rule of evolution: success in reproduction and producing next generations. To achieve this goal, plants evolved various adaptation mechanisms against harsh environmental conditions described as abiotic stress factors as well as biotic stress factors like diseases and herbivores [1, 2]. The early domesticated agronomical traits desired by the primal farmers were mostly controlled by the limited number of genes. Traits as panicle size (rice, pearl millet), fruit size (tomato, eggplant), seed size (rice, sorghum, maize, common bean), seed dispersal (cereals in general) are easily controlled by small number of genes [3, 4]. Traits as bitter

taste which is one of the adaptation mechanism of plants against herbivores can be controlled by single or multiple genes due to the complexity of the biochemical pathway of the compound. In the absence of genetic knowledge, first breeders domesticated almond as its bitterness is controlled by single gene, while the similar bitter taste in oak acorns is controlled by multiple genes and it was never domestication target [5]. Human intervention to natural selection of plants helped plants to achieve their reproduction goal at the cost of losing most of the gene pool diversity including abiotic stress tolerance and disease resistance genes in favor of human desired traits. Until Gregory Mendel's breakthrough findings, plant breeding was based on easily made crosses, observations and mass selections without knowing sexual recombination of alleles recombined in meiosis to produce new traits for hundreds of years. Even after the knowledge of Mendelian segregation of traits, crossing of distantly related plant species for desired traits as disease resistance became problematic due to the abnormal chromosome pairing and infertile hybrids not to mention horizontal gene transfer between different kingdoms as bacteria and viruses. Following the studies on molecular structure and function of genes during 1940s and the discovery of the structure of DNA by Watson and Crick in 1953, technical developments as PCR and use of restriction enzymes led to the first transfer of bacterial antibiotic resistance gene to plant cells in 1983. Foreign bacterial gene introduced to petunia cell cultures and plantlets derived from transformed cells retained antibiotic resistance [6]. Following these developments, tomato was the first genetically engineered plant which was intended to be used in commercial practices in 1982. Commercial use of genetically engineered crops started in 1996 in US, since four genetically engineered plants were allowed by USDA for field testing in 1985. Technical and technological research kept improving on this field. Certain trends in engineered plants divided the use of this technology in three categories. Targeted traits of the first generation genetically engineered plants were mostly oriented according to farmer's benefit as abiotic stress tolerance or herbicide resistance. Second generation of genetically engineered plants emphasized more on commercial benefits as shelf life or nutritional value. The third generation of genetically engineered plants represent the idea of functional foods which were enhanced with pharmaceutical product that are not present in the plant itself [7].

Use of genetic engineering techniques in abiotic stress tolerance of plants requires knowledge on physiology and biochemical processes to identify key gene targets. Functional genomic approaches utilize transcriptome analysis of plants under abiotic environmental stress factor by using techniques as quantitative, real-time PCR, microarray and high-throughput RNAseq. Gene expression profiles of specific tissues under development stage of choice can be searched between previously submitted Expressed Sequence Tags (ESTs) in various cDNA libraries. Abiotic stress responsive genes in particular abiotic stress tolerance mechanisms can be evaluated before progressing further in experimental design [8]. Molecular regulation type of the targeted trait is also important in genetic engineering approach. Abiotic stress tolerance is mostly regulated by multiple genes depending on stress and targeted tolerance mechanism. One of the early effects of the abiotic stresses on plants is disruption of osmotic balance in cells. Therefore, biosynthesis and accumulation of osmoprotectant molecules as proline, glycine betaine, polyamines, mannitol, trehalose can be targeted as they are regulated by single individual genes. Likewise, reactive oxygen species scavenging genes (superoxide dismutase, glutathione reductase, ascorbate peroxidase), late embryogenesis abundant (LEA) proteins, ion transport genes are some single gene targets used in genetic engineering approach for abiotic stress tolerance which will be further detailed in this chapter. Targeting a tolerance mechanism which is regulated by multiple genes is more complicated. Still, there are many successful genetic engineering applications

targeting heat shock proteins (HSPs), transcription factors (TFs), signal transduction genes for abiotic stress tolerance [9, 10]. After all of these decision making process which requires intense precision, promoter type is one of the key factors since all transferred genes requires expression regulation. Calliflower Mosaic Virus 35S (CaMV35S), maize ubiquitin-1 (Ubi-1) and rice actin-1 (act-1) are some of the mostly used strongly expressed constitutive promoters which were used in abiotic stress tolerant genetically engineered plant studies. Despite their advantages to plants as strong expression in all developmental stages in all tissues without any stimulation from the environment, they are not economical for plants in contexts of energy and biochemical source consumption. Signal transduction genes as SAPK4, OsITPK2, OsCPKI2, transcription factor genes as JERF3, ZFP182, antioxidant genes as katE, CAT1, GST, OsMIOX, osmotic homeostasis genes as codA, ion homeostasis genes as AgNHX1, nhaA, OshAK5 are some examples which are regulated by CaMV35S promoter in abiotic stress tolerant genetically engineered plants while Ubi-1 promoter is utilized for DSM1, OsCPK21, OsCPK4 signal transduction genes, OsWRKY45-1, OsWRKY45-2, ZmCBF3, OsZIP46 transcription factor genes, Sod1 antioxidant gene, otsA, otsB, adc osmotic homeostasis genes and OsKAT1 ion homeostasis genes [11]. Constitutive and avoidable use of biochemical sources in stress free periods or environments leads these genetically engineered plants to reduced growth and loss of yield in some cases. Therefore, regulation of abiotic stress induced or tissue/developmental stage specific promoters are more viable options. General aspects of desired stress induced promoters are described as: (i) having basal expression level under stress free conditions, (ii) strongly promoting expression of resistance genes, (iii) having dose dependent and dose sensitive stress induction, (iv) being stress specific and (v) having reversible induction which rapidly reduce to basal level as removal of stress factor. Arabidopsis rd29A promoter is the most frequently used stress inducible promoter in genetically engineered plant studies. Some stress inducible promoters may lack some desired features as stress induced rab16A, LIP9, OsNAC6 which have higher promoting regulation under basal conditions or some may need further characterization and knowledge as HVA1s, Dhn8s and Dhn4s from barley and DGP1 from tobacco [12].

Most of the abiotic stress tolerant genetically engineered plant trials are limited to laboratory experiments and do not have commercial uses due to the regulations or inadequate field performance. Commercial genetically engineered plants need to tolerate or resist to multiple biotic and abiotic stresses in different combination, duration and concentrations. Tolerance or resistance should not be limited only to developmental stages but also reproductive stage which plants are more vulnerable to abiotic stresses.

As an alternative to gene transfer approach, many studies utilize genome editing techniques as they allow researchers to modify the genome even in a few nucleotide level as well as they can be used to alter or replace alleles, silence or insert new gene(s) to targeted sites in genome. Genome editing by using site specific endonucleases is not a new concept in developing crop plants by means of biotic and abiotic stress tolerance. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) is the most recent site specific genome editing technique which dominated the alternatives as transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) due to the accuracy, cost and time efficiency and by the means of application advantages as allowing multiple site editing at the same time. Even this new approach evolved in short time due to the occurring limitations during application and new alternatives to Cas9 as Cpf1 are seriously in consideration recently. Also transgene free genome editing applications are introduced lately as the law regulations and public acceptance on this topic are crippling for researchers [13, 14].

Plant	Transformation Event / Developer	Resistance	Expressing	Common Use
<i>Zea mays L.</i>	MON87427 x MON87460 x MON89034 x TC1507 x MON87411 x 59122 / Monsanto	insect resistance, drought and herbicide (glyphosate and specifically glufosinate ammonium) tolerance	cp4 epsps, cspB, cry2Ab2, cry1A.105, cry1F, pat, cry34Ab1, cry35Ab1, cry3Bb1, dvsnf	Food Feed Biofuel
	MON87460 (Genuity® DroughtGard™) / Monsanto	cold / heat drought tolerance	cspB, nptII	Food Feed Biofuel
	MON87460 x MON88017 / Monsanto	insect resistant (coleopteran and lepidoptera), drought and herbicide (glyphosate) tolerant	cspB, cp4 epsps, cry1A.105, cry2Ab2, cry3Bb1, nptII	Food Feed
	MON87460 x MON89034 x MON88017 / Monsanto	cold / heat, drought, herbicide (glyphosate) tolerance and insect resistance (lepidoptera and coleopteran)	cspB, cry1A.105, cry2Ab2, cp4epsps and cry3Bb1	Food Feed
	MON87460 x MON89034 x NK603 / Monsanto	cold / heat, drought, herbicide (glyphosate) tolerance and insect (lepidoptera) resistance	cspB, cry1A.105, cp4 epsps, cry2Ab2, nptII	Food Feed
	MON87460 x NK603 / Monsanto	drought and herbicide (glyphosate) tolerance	cspB, cp4 epsps nptII	Food Feed
	MON89034 x MON87460 / Monsanto	drought tolerance and insect (lepidoptera) resistance	Cry2Ab2, cry1A.105 and cspB	Food Feed
<i>Glycine max L.</i>	HB4 / Verdeca	drought and hyper salinity tolerance	Hahb-4	Food Feed
	HB4 x GTS 40-3-2 / INDEAR	drought, salinity, herbicide (glyphosate) tolerance	Hahb-4 and cp4 epsps	Food Feed
<i>Saccharum sp.</i>	NXI-1T / PT Perkebunan Nusantara XI (Persero)	drought tolerance	EcBetA, nptII, aph4 (hpt)	Food
	NXI-4T / PT Perkebunan Nusantara XI (Persero)	drought tolerance	RmBetA	Food Feed
	NXI-6T / PT Perkebunan Nusantara XI (Persero)	drought tolerance	RmBetA	Food

Table 1. Commercial genetically engineered abiotic stress tolerant plant varieties [15, 16].

Consumers and farmers are interested to the traits as taste, productivity, yield, shape of commercial genetically engineered and genome edited varieties more than just survival under abiotic stresses. Therefore, most of the abiotic stress tolerant plant strategies mentioned further in this chapter are laboratory applications. **Table 1** represents successful commercial transformation events of abiotic stress tolerant maize, soybean and sugarcane developed by Monsanto, Verdeca and Persero Companies.

2. Genetic engineering applications in plant abiotic stress tolerance

One of the key points in breeding better crops under various stress conditions is understanding the cellular, biochemical and molecular changes that occur in response to stress [17]. Understanding the mechanisms underlying plants responses to abiotic stress is an important step for genetic engineering that focuses on improving or enhancing tolerance to these stresses (i.e. salinity, cold, dehydration). Identifying key genes that positively affect tolerance within these mechanisms and introduction and overexpression of them allow improvement of genetically modified plants tolerant to abiotic stress. Additionally, understanding the mechanisms by which plants perceive and transmit stress signals is another important point for genetic engineering of crops [18, 19]. Growing number of molecular and biochemical studies on this subject provide an understanding of the signal transduction network involved in the response to abiotic stress and the pathways associated with this network. The development of plants by conventional breeding methods has certain limitations as transfer of a gene requires repeated cycles of crossing and selection. Moreover, the classical breeding process is limited to species with sexual reproduction. Another disadvantage of classical breeding is that genes with undesirable characteristics are transferred together with target genes in this process. With the advent of modern biotechnology tools, it has become possible to modify genetic structure of plants using genes from other living organisms, and recombinant DNA technology has provided an effective alternative to traditional approach. In addition, it has become possible to exchange genetic material between sexually incompatible species. Genes encoding proteins known to play a role in resistance and tolerance to biotic (virus, bacteria, nematode, fungus, herbivores) and abiotic stresses (drought, salinity, temperature, cold) are widely used in the obtaining genetically modified plants. Plants have developed a signal transduction pathway that regulates various stress-response genes such as kinases, molecular chaperones, osmoprotectants, transcription factors, and thus gives an idea of how tolerance to environmental factors is achieved. Since it is known that this signal transduction and associated physiological, biochemical and molecular pathways are governed by more than one gene, it is extremely difficult for only one gene to achieve complete abiotic stress tolerance. Among the molecules known to have protective roles against abiotic stress are proline, which acts as an osmoprotectant, metal chelator, antioxidative defense molecule and signal molecule [20]; trehalose, which acts as an osmoprotectant and is involved in ROS scavenge during abiotic stress [21]; heat shock proteins (HSPs) that serve as molecular chaperones responsible for the folding, assembly, translocation and degradation of proteins [22]; Late embryogenesis abundant (LEA) proteins with antioxidant functions involved in protein protection, membrane protection, and ion binding [23]; aquaporins involved in the transport of water and neutral molecules [24]; calcineurin B-like proteins that act as calcium sensors and play a role in signal transmission. Transcription factors (NAC, WRKY, MYB, bZIP, DREB/CBF), kinases and phosphatases serve a function in stress perception and signal transduction and in the regulation of stress-inducible genes.

In addition to the identification, isolation and characterization of genes involved in all this abiotic stress response, introduction of these genes to plants is an effective molecular approach to understand the roles of genes and products in plant tolerance, behavior and phenotypes.

2.1 Late embryogenesis abundant proteins

Late embryogenesis abundant proteins, which were first discovered in the late developmental stages of plant seeds, are among the proteins most commonly used by plants in their response to abiotic stresses such as drought, high salt, extreme temperature and oxidative stress. Most of the LEA proteins and their mRNAs accumulate in high amounts in the embryo tissues in the final stages of seed development when drought initiates [23]. In addition, LEA proteins also accumulate in plant tissues exposed to dehydration, osmotic and low temperature stress. There is strong evidence that LEA proteins or their genes classified into 6 groups according to their specific domains are correlated with stress resistance [25, 26]. Many studies have reported that stress tolerance occurs as a result of the introduction of LEA genes to various plants such as *Arabidopsis*, tobacco, rice, barley, and corn by genetic engineering techniques (**Table 2**). Amara [30] reported that the Rab28 LEA gene was overexpressed in maize plants in which they transferred the Rab28 LEA gene with a constitutive maize promoter by particle bombardment method. With the expression of the Rab28 transcripts, the Rab28 protein accumulated in transgenic plants and the transgenic plants continued their growth in the dehydration condition in medium containing polyethyleneglycol (PEG) compared to wild-type controls. These results showed that LEA Rab28 (belonging to the 5th subgroup of the LEA protein family) protein is one of the important candidates that can be used to increase stress tolerance in maize plants. In another transformation study performed with the LEA genes, the barley (HVA1) gene encoding the late embryogenesis abundant protein was transferred to mulberry, which is an important industrial plant used in silk production, by *Agrobacterium tumefaciens*. The HVA1 gene expressing with the rd29a promoter increased the tolerance to cold, drought and salinity in transgenic mulberry [28]. Similarly, overexpression of the ZmLEA3 gene in the tobacco (*Nicotiana tabacum*) plants plays important roles in tolerance to osmotic and oxidative stresses. ZmLEA3 protein plays an active role in protecting plants from damage by preserving the protein structure and holding metals under osmotic and oxidative stress [32]. Late embryogenesis abundant genes have been isolated and characterized from many different plants. Thus, along with transgenic approaches, new gene sources that can be used in abiotic stress tolerance are discovered. The overexpression of the gene responsible for the JcLEA protein (belonging to the 5th subgroup of the LEA protein family) isolated from a tropical plant, *Jatropha curcas*, provided increased resistance to both drought and salt stress compared to the wild-type in transgenic *Arabidopsis* plants [31]. Transgenic *Arabidopsis thaliana* and *Setaria italica* (foxtail millet) seedlings showed higher tolerance to salt and osmotic stress than the wild-type with overexpression of the SiLEA14 gene [35]. After transformation of plants with LEA genes, chlorophyll content increases, while lipid peroxidation rates, as indicator of ROS decrease. Higher chlorophyll and low lipid peroxidation values obtained by the expression of the AdLEA (belonging to the 5th subgroup of the LEA protein family) gene isolated from the perennial *Arachis diogeni* plant in the tobacco plant (*Nicotiana tabacum*) is a representing study. These transgenic plants have been shown to tolerate dehydration, salinity and oxidative stress. Chlorophyll fluorescence measurements have shown that tobacco plants in which AdLEA is overexpressed can maintain their photosynthetic performance under drought conditions [36].

Gene Action	Gene Source	Promoter	Target Traits	Trans-formed Plant	Transfer Method	Ref.
LEA	ROB5 from Bromegrass, DHN4 from <i>Hordeum vulgare</i> SOD3.1 from <i>Triticum aestivum</i>	CaMV35S Cor78	Drought Heat Cold	<i>Solanum tuberosum</i>	Agrobacterium	[27]
LEA	Hva1 from <i>Hordeum vulgare</i>	rd29A	Salinity Drought Cold	<i>Morus indica</i>	<i>Agrobacterium tumefaciens</i> Ag11	[28]
LEA	OsLEA3-2 from <i>Oryza sativa</i>	CaMV35S	Salinity Drought	<i>Oryza sativa</i> <i>Arabidopsis thaliana</i>	Agrobacterium EHA105	[29]
LEA	LEA Rab28 gene from <i>Zea mays</i>	UBQ	Water stress	<i>Zea mays</i>	Biolistic system	[30]
LEA	JcLEA from <i>Jatropha curcas</i>	CaMV35S	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> LBA4404 floral dip	[31]
LEA	ZmLEA3 from <i>Zea mays</i>	CaMV35S	Osmotic, Oxidative stress	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> strain LBA4404	[32]
LEA	WCI16 from <i>Triticum aestivum</i>	CaMV35S	Freezing	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> floral dip	[33]
LAE	ZmLEA5C from <i>Zea mays</i>	CaMV35S	Osmatic stress Cold	<i>Nicotiana benthamiana</i>	<i>Agrobacterium tumefaciens</i> strain LBA4404	[34]
LAE	SiLAE from <i>Setaria italica</i>	Super promoter	Osmotic stress Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> strain GV1301	[35]
LEA	AdLEA from <i>Arachis diogeni</i>	CaMV35S	Salinity Oxidative stress	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[36]

Table 2. Summary of transgenic plants over-expressing late embryogenesis abundant (LEA) genes for abiotic stress tolerance.

2.2 Aquaporins (AQPs)

Aquaporins (AQPs) are a family of membrane water channel proteins that control the osmotic movement of water and other molecules such as carbon dioxide, glycerol, urea, and ammonia [24]. This system, which has high osmotic water permeability, operates with lower activation energy [37]. Since environmental conditions such as high temperatures and winds encountered by plants living on land cause an increase in the rate of transpiration. These plants have developed various strategies such as controlling transpiration with hormones, cellular signaling mechanisms, ion channels, and transporters to protect homeostasis [38, 39]. However, the current situation worsens when osmotic pressure increases, water is absent and root hydraulic conductivity (L_{pr}) decreases [18]. In order to overcome

this negative situation, the isolation of water channel genes and their introduction to the desired plant by using genetic transformation emerges as one of the most important solutions. Thus, this situation can be reorganized by transgenic expression of water channels that ensure the continuity of processes such as transcellular water transport, stomatal closure, osmoregulation, vacuolar differentiation and plant cell expansion (**Table 3**). Under saline conditions, plant growth is suppressed due to osmotic stress, and in addition, this suppression is further increased due to reduced photosynthesis and gas exchange due to salt accumulation in the leaves. Aquaporins are thought to be extremely effective in plant growth thanks to their effects on leaf gas exchange as well as root water intake. While overexpression of plasma membrane intrinsic proteins (PIP) causes an increase in leaf gas exchange under normal conditions, it increases assimilation and development under salt stress [43]. Moreover, it has been reported that shoot length and fresh weight significantly increased in PIP introduced plants under salt stress [43]. They indicated that transgenic plants developed by transferring the GmPIP1;6 genes to soybean plant (*Glycine max*) are more tolerant of salt stress than wild-type plants. In another study, overexpression of GhPIP2;7 in *Arabidopsis* increased the tolerance of the transgenic plant to drought stress [41]. It has also been reported that the introduced plasma membrane intrinsic gene may play a role in leaf development and in absorption in remaining water under drought stress and carry certain substrates instead of water to protect plants from drought. Additionally, seed germination rates increased in most of the transgenic plants produced by introducing plasma membrane intrinsic genes [44]. Transgenic *Arabidopsis thaliana* plants developed with introduction of BvCOLD1 gene from *Beta vulgaris* have been observed to increase in germination and early development rates at 10°C and under different abiotic stress conditions such as NaCl, LiCl, and sorbic acid [44]. Plant aquaporins function by settling in different subcellular locations. Liu [42] reported that one of the most abundant plasma membrane proteins in transgenic rice leaves and roots is OsPIP1 and that its overexpression causes changes in many physiological properties of transgenic plants depending on the stress dose. Rice seed yield, salt resistance, root hydraulic conductivity and seed germination rates increased with the moderate expression of OsPIP1 [42]. Beside plasma membranes, they can be localized in mitochondria, endoplasmic reticulum, chloroplast and vacuole [45].

2.3 Osmoprotectants

Trehalose is a non-reducing disaccharide sugar found in bacteria, fungi, invertebrates, including plants. The accumulation of trehalose, which is an important component of plant growth, affecting sugar metabolism and causes an osmoprotectant effect under abiotic stresses [46]. Trehalose is considered as an osmoprotectant as it counteracts against the effects of dehydration of plants caused by drought, salinity or low temperature [47]. Studies have shown that enzymes, proteins and lipid membranes can be stabilized by trehalose and that biological structures can be protected from damage by trehalose during abiotic stress [18]. Trehalose is an osmolite that both regulates osmosis and protects macromolecules. In higher plants, overexpression of trehalose-6-phosphate synthases (TPS) and/or trehalose-6-phosphate phosphatases genes originated from microorganisms has led to tolerance to various abiotic stresses. In addition, several genes involved in trehalose metabolism have been used together to improve the stress tolerance of some plants. The results show that the increased production of trehalose with overexpression of the bifunctional fusion gene (TPSP) of the trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase genes increases the soluble sugar content and increases the tolerance to drought and salt stress in rice [48]. As a result of the expression of

Gene Action	Gene Source	Promoter	Target Traits	Trans-formed Plant	Transfer Method	Ref.
Aquaporin (plasma membrane intrinsic proteins)	TdPIP1 and TdPIP2 from <i>Triticum turgidum</i>	CaMV35S	Osmotic stress Salinity	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i>	[40]
Aquaporin (plasma membrane intrinsic protein)	GhPIP2;7 from <i>Gossypium hirsutum</i>	GhPIP2;7	Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i>	[41]
Aquaporin (plasma membrane intrinsic protein)	OsPIP1 from <i>Oryza sativa</i>	UBQ	Salinity	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> EHA101	[42]
Aquaporin (plasma membrane intrinsic protein)	GmPIP1;6 from <i>Glycine max</i>	CaMV35S	Salinity	<i>Glycine max</i>	<i>Agrobacterium tumefaciens</i>	[43]
Aquaporin (plasma membrane intrinsic protein)	BvCOLD1 from <i>Beta vulgaris</i>	CaMV35S	Cold	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> C58C1	[44]

Table 3.
 Summary of transgenic plants over-expressing aquaporin genes for abiotic stress tolerance.

the TPSP fusion gene, tomatoes that accumulate high amounts of trehalose in their leaves exhibited a tolerance to drought and salt stress, and showed higher photosynthetic rates under salt stress conditions than wild-type plants [49]. Proline, another osmoprotectant, is an amino acid that plays a role in plant functions exposed to various abiotic stresses. Proline accumulates in response to many environmental stresses such as salinity, drought, heavy metals, and there is a positive correlation between plant stress and proline accumulation. Proline protects proteins and other molecules in the cell against denaturation by stabilizing them. In addition to being an excellent osmolyte, it also functions as a metal chelator, antioxidant defense molecule and signal molecule [20]. In addition, it plays an important role in the protection of subcellular structures, adjustment of cell turgor and osmotic balance, prevention of electrolyte leakage by stabilizing membranes, and balancing the concentration of reactive oxygen species (ROS). Proline synthesis in plants is carried out by two important pathways; glutamate pathway and ornithine pathway. However, among these, the glutamate pathway is the primary pathway of proline accumulation during osmotic stress [50]. This reaction is catalyzed in most plants by the enzyme Δ^1 -pyrroline-5 carboxylate synthetase (P5CS) encoded by two genes and the enzyme Δ^1 -pyrroline-5-carboxylate reductase (P5CR) encoded by a gene. In addition to the effects of proline on the physiology and biochemical processes of various plants, a high amount of endogenous proline has been shown to act as a regulator and/or signaling molecule that can alter the transcription levels of stress-related genes such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) [51, 52]. A P5CS gene that can be transferred in addition to the P5CS genes present in plants by genetic transformation helps plants to adapt

to osmotic conditions by regulating proline accumulation, and also supports legume plants for optimum nodule formation and maintenance of nodule function [53]. Ghanti [54] reported that when they transferred the *Vigna aconitifolia* P5CS gene to *Cicer arietinum* plant, more proline accumulation in the leaves and roots of the plants and higher chlorophyll content under salt stress, as well as increased tolerance to salinity. It has also been shown that a single copy of P5CS transferred to plants can be effective in the development of tolerance under stress, regardless of the integration position or copy number [54]. P5CS-transferred transgenic plants, when exposed to high salt stress, grew to maturity by showing good growth and flowering, but control plants died within a certain period of time under the same conditions [55]. Moreover, introduction of a mutagenized version (P5CSF129A) of the *Vigna aconitifolia* P5CS gene to pigeonpea (*Cajanus cajan*), both proline accumulation in plants increased and tolerance to salt stress enhanced. In addition to improved growth performances and higher chlorophyll and relative water content, especially transgenic plants also showed lower levels of lipid peroxidation under 200 mM NaCl.

Plants synthesize and accumulate another osmoprotectant, glycine betaine (GB), in response to environmental stresses. Glycine betaine, which also plays a role in osmoregulation, protects the activity of macromolecules and the integrity of the membranes against stresses and scavenge the ROS. Glycine betaine synthesis reactions are catalyzed in two steps by the enzymes choline monoxygenase (CMO) and NADC-linked betaine aldehyde dehydrogenase. Many studies have been carried out showing that the abiotic stress tolerance of transgenic plants obtained as a result of isolating these genes in the biosynthetic pathway and transferring them to other plants has developed. Several osmoprotectant target for gene transfer approach are listed in **Table 4**.

2.4 Calcineurin B-like proteins (CBLs), calcium-dependent protein kinases (CDPKs)

Plants perceive the stimuli from the external environment through receptors in their membranes. After transmitting this signal, they respond to this stimulus. Ca^{2+} acts as one of the secondary messengers in the ABA signaling pathway, which plays an active role in this process. Calcium acts as a central link in numerous signaling pathways, and thus external signals such as hormone, light, biotic and abiotic stresses cause changes in Ca^{2+} concentration in the cell. The fluctuation in the cytosolic calcium concentration (calcium signatures) initiates various biochemical and physiological processes in the cell. Cellular calcium sensor molecules such as calcineurin B-like proteins (CBLs), calcium-dependent protein kinases (CDPKs) and calcium-dependent protein kinases (CIPKs), calmodulin (CaM) detect Ca^{2+} signals and enable Ca^{2+} ions to be transmitted to downstream pathways. Especially, calcineurin family of B-like proteins (CBLs) interacts and activates calcium-dependent protein kinases (CIPKs). Many studies have shown that this regulatory pathway plays a role in plants in response to environmental stresses (**Table 5**). It has been reported that overexpression of GmCBL1 in Arabidopsis increases tolerance to both high salt and drought stress in transgenic plants. It has also been shown that hypocotyl elongation is promoted under light conditions by overexpression of GmCBL1. Calcineurin B-like proteins (CBLs) can regulate stress tolerance by activating stress-related genes such as DREB1A, DREB2A, RD29A and KIN1, while also controlling hypocotyl development by changing the expression of genes related to gibberellin biosynthesis [68]. BdCIPK31, a CIPK gene belonging to *Brachypodium distachyon*, decreased under polyethylene glycol, NaCl, H_2O_2 and abscisic acid (ABA) treatments. Transgenic tobacco plants overexpressing BdCIPK31 developed tolerance to drought and salinity and lost less water than control plants under dehydration conditions. BdCIPK31 affects the expression of some ion channels and transporter

Gene Action	Gene Source	Promoter	Target Traits	Trans-formed Plant	Transfer Method	Ref.
Pyrroline-5-carboxylate synthetase	P5CS from <i>Arabidopsis thaliana</i>	CaMV35S	Salinity	<i>Solanum tuberosum</i>	<i>Agrobacterium tumefaciens</i> C58C1	[53]
Pyrroline-5-carboxylate synthetase	P5CS from <i>Vigna aconitifolia</i>	CaMV35S	Salinity	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> strain LBA 4404	[56]
Pyrroline-5-carboxylate synthetase	P5CS from <i>Vigna aconitifolia</i>	CaMV35S	Salinity	<i>Cicer arietinum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[57]
Pyrroline-5-carboxylate synthetase	VaP5CSF129A from <i>Vigna aconitifolia</i>	CaMV35S	Drought	<i>Swingle citrumelo</i>	-	[54]
Pyrroline-5-carboxylate synthetase	MtP5CS3 from <i>Medicago truncatula</i>	CaMV35S	Salinity	<i>Medicago truncatula</i>	-	[58]
Pyrroline-5-carboxylate synthetase	P5CSF129A from <i>Vigna aconitifolia</i>	CaMV35S	Salinity	<i>Cajanus cajan</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[59]
Trehalose-6-phosphate synthase	OsTSP1 from <i>Oryza sativa</i>	CaMV35S	Salinity Drought Cold	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> AGLO	[60]
Trehalose-6-phosphate synthase/ phosphatase	Recombinant fusion TPSP from <i>E. coli</i>	UBQ	Drought Salinity	<i>Oryza sativa</i>	-	[51]
Trehalose-6-phosphate synthase	TPS1 from yeast	StDS2	Drought	<i>Solanum tuberosum</i>	<i>Agrobacterium tumefaciens</i> strain C58C1	[61]
Trehalose-6-phosphate synthase/ phosphatase	Recombinant fusion TPSP from <i>E. coli</i>	CaMV35S	Drought Salinity	<i>Solanum lycopersicum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[52]
Glycine betaine	codA (Choline oxidase) from <i>Arthrobacter globiformis</i>	SWPA2	Cold Salinity	<i>Solanum tuberosum</i>	<i>Agrobacterium tumefaciens</i> strain AGLO	[62]
Glycine betaine	BADH from <i>Spinacia oleracea</i>	CaMV35S	Salinity Cold	<i>Ipomoea batatas</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[63]
Glycine betaine	betaine aldehyde dehydrogenase gene (AhBADH) from <i>Atriplex hortensis</i> ,	CaMV35S	Salinity	<i>Poncirus trifoliata</i>	<i>Agrobacterium tumefaciens</i> LB4404	[64]

Gene Action	Gene Source	Promoter	Target Traits	Trans-formed Plant	Transfer Method	Ref.
Glycine betaine	betA gene from <i>E. coli</i>	UBQ	Salinity	<i>Triticum aestivum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[65]
Glycine betaine	betA gene from <i>E. coli</i>	UBQ	Drought	<i>Triticum aestivum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[66]
Glycine betaine	Mpgsmt and Mpsdmt from <i>Methanohalophilus portucalensis</i>	CaMV35S	Drought Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101	[67]
Glycine betaine	codA from <i>Arthrobacter globiformis</i>	-	Heat	<i>Solanum lycopersicum</i>	<i>Agrobacterium tumefaciens</i> EHA101	[68]
Glycine betain	BADH betaine aldehyde dehydrogenase from <i>Spinacia oleracea</i>	CaMV35S	Heat	<i>Lycopersicon esculentum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[69]
Glycine betaine	codA from <i>Arthrobacter globiformis</i>	CaMV35S	Salinity	<i>Solanum lycopersicum</i>	<i>Agrobacterium tumefaciens</i> EHA101	[70]

Table 4.
Summary of transgenic plants over-expressing osmoprotectant-related genes for abiotic stress tolerance.

Gene Action	Gene Source	Promoter	Target Traits	Trans-formed Plant	Transfer Method	Ref.
CIPK	OsCIPK23m from <i>Oryza sativa</i>	UBQ	Drought	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> EHA105	[71]
CBL-CIPK	BnCBL1-BnCIPK6 from <i>Brassica napus</i>	CaMV35S	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> floral dip method	[72]
CBL protein	GmCBL1 from <i>Glycine max</i>	CaMV35S	Salinity Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> C58C1	[68]
CBL protein	PeCBL10 from <i>Populus euphratica</i>	CaMV35S	Salinity Drought Cold	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101	[73]
CBL protein	PtCBL10A PtCBL10B from <i>Populus trichocarpa</i>	CaMV35S	Salinity	<i>P. davidiana</i> × <i>P. bolleana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[74]
CBL protein	NsylCBL10 from <i>Nicotiana sylvestris</i>	CaMV35S	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[70]
CBL protein	SpCBL6 from <i>Stipa purpurea</i>	CaMV35S	Cold	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> strain GV3101	[75]
CIPK	BdCIPK31 from <i>Brachypodium distachyon</i>	CaMV35S	Drought Salinity	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> EHA105	[69]

Table 5. Summary of transgenic plants over-expressing calcineurin B-like (CBL) and CBL-interacting protein kinase (CIPK) genes for abiotic stress tolerance.

genes under high salinity stress. Scavenging of reactive oxygen species and osmolite accumulation has been increased with the overexpression of BdCIPK31, thereby mitigate oxidative and osmotic damages [69]. In another transformation study performed with CBL, overexpression of SpCBL6 gene isolated from *Stipa purpurea* has been shown to increase tolerance to cold stress in transgenic *Arabidopsis thaliana*. In further analysis, it was reported that SpCBL6 overexpressing plants had increased water potential and photosynthetic efficiency (Fv/Fm) and decreased ion leakage compared with wild-type plants after cold application. In another study, NsylCBL10, a CBL gene belonging to *Nicotiana sylvestris*, has increased tolerance to high salt stress by maintaining Na⁺ balance. Ion content analyzes revealed that transgenic plants maintained the lower Na⁺/K⁺ ratio in the roots and the higher Na⁺/K⁺ ratio in the shoots with the overexpression of NsylCBL10 [70]. Similarly, overexpression of PtCBL10A or PtCBL10B in transgenic poplar plants under salinity stress played an important role in tolerance to salt stress by maintaining ion homeostasis in shoot tissues [74]. Overexpression of BnCIPK6, its activated form BnCIPK6M and BnCBL1 CBL genes also increased tolerance to high salinity and low phosphate conditions in transgenic *Arabidopsis* plants, demonstrating how important the interaction of BnCBL1 and BnCIPK6 is for signaling pathways.

2.5 Heat shock protein

Heat shock proteins (HSPs) were originally identified as proteins that respond to high temperature conditions and involve in eukaryotes and prokaryotes. However,

many studies have revealed the relationship of these proteins with various abiotic stresses. Heat shock proteins are powerful chaperones produced in response to many physiological and environmental stresses. In general, heat shock proteins function in the cytoplasm, while they are also located in organelles such as the nucleus, mitochondria, chloroplasts, endoplasmic reticulum [22]. These proteins that are evolutionarily conserved in prokaryotes and eukaryotes are also abundant in plants. HSPs divided into 5 different groups including HSP100, HSP90, HSP70, HSP60 and sHSP (small heat shock protein) according to their molecular weights. Among these groups, small heat shock proteins are also induced by other stresses such as drought, salinity and cold stress, and also play an active role in processes such as seed germination, embryogenesis and fruit development [76]. Transgenic *Arabidopsis thaliana* plants introduced with the chloroplastic sHSP26 gene from wheat (*Triticum aestivum*) show tolerance to higher temperatures than wild-type plants. In addition, these plants show more photosynthetic pigment accumulation and higher biomass and seed yield [76]. Another sHSP26 gene belonging to rice (*Oryza sativa*), which encodes small heat shock protein localized in chloroplast, provided less electrolyte leakage in transgenic plants under heat stress, while it played a role in the accumulation of thiobarbituric acid reactive substances in plants. Transgenic plants showed more photosystem II (PSII) (Fv/Fm) photochemical activity under temperature stress at 42°C than control plants [77]. There is much evidence that small heat shock proteins are also associated with scavenging reactive oxygen species. Thermo tolerance of the plants increased in correlation with ROS scavenging by overexpression of the OsHSP18.6 in transgenic plants. In addition, with the overexpression of OsHSP18.6, malondialdehyde (MDA) levels decreased and CAT and SOD activities increased under heat and drought stress [78]. HSP70, another heat shock protein, stabilizes the proteins and also prevents their denaturation and aggregation. In addition, it maintains protein homeostasis by taking part in processes such as the transport of certain proteins in the cell, folding of newly synthesized proteins, denaturation of unwanted proteins, and the formation and separation of protein complexes [79]. In rice plants produced by introducing anti-apoptotic genes associated with programmed cell death (PCD) such as AtBAG4 (*Arabidopsis thaliana*), Hsp70 (Citrus tristeza virus) and p35 (Baculo virus), the salinity tolerance of rice plants has increased and along with this, ROS production and plant damage have been reduced [79]. In transgenic sugarcane (*Saccharum spp.* Hybrid) plants transformed with the HSP70 gene from *Erianthus arundinaceus*, the expression of stress-related genes increases with the overexpression of the HSP70 gene. These transgenic plants have higher germination ability and higher chlorophyll content under salt stress [80]. HSP90 is one of the proteins that have been well preserved in the evolutionary process and found in large amounts. As one of the important components of the stress response, they are actively involved in various processes such as signal transduction, protein degradation, cell cycle control and protein traffic (**Table 6**). Xu [83] reported that overexpression of HSP90 genes (GmHsp90A2, GmHsp90A4, GmHsp90B1, GmHsp90C1.1 and GmHsp90C2.1) in *Arabidopsis thaliana* plants reduced the damage caused by abiotic stress. In addition, high proline accumulation has been detected in HSP90 transgenic events and it has been reported that HSP90 genes may affect proline synthesis in relation to the AtP5CS1 (pyrroline-5-carboxylate synthases 1) gene responsible for proline synthesis [83]. Polyamine homeostasis is regulated by many different regulatory mechanisms such as transport, turnover and modification of amino groups. At the same time, these molecules affect the extent of heat shock proteins synthesis under increasing temperatures. It has been reported that silencing of HSP90 genes in *Arabidopsis thaliana* plant increased the levels of soluble spermidine (S Spd), acetylated Spd (N8-acetyl-Spd) and acetylated spermine (N1 acetyl-Spm). Moreover,

Gene Action	Gene Source	Promoter	Target Traits	Trans-formed Plant	Transfer Method	Ref.
Heat Shock	GHSP26 from <i>Gossypium arboreum</i>	CaMV35S	Drought	<i>Gossypium hirsutum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[81]
Heat Shock	OsHSP6 from <i>Oryza sativa</i>	CaMV35S	Oxidative stress Heat	<i>Festuca arundinacea</i>	<i>Agrobacterium tumefaciens</i> EHA105	[77]
Heat shock	TaHSP26 from <i>Triticum aestivum</i>	CaMV35S TaHSP26	Heat	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> AGL1	[76]
Heat Shock	ZmHSP16.9 from <i>Zea mays</i>	CaMV35S	Heat	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[82]
Heat Shock	GmHsp90s from <i>Glycine max</i>	CaMV35S	Osmotic stress Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[83]
Heat Shock	EaHSP70 from <i>Erianthus arundinaceus</i>	Port Ubi 2.3	Salinity Water deficiency	<i>Saccharum spp. hybrid</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[80]
Heat Shock	HSP70 from <i>Citrus tristeza virus</i> (CTV)	UBQ	Salinity	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> AGL1	[84]
Heat Shock	sHSP18.6 from <i>Oryza sativa</i>	CaMV35S	Heat Drought Salt Cold	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> EHA101	[78]

Table 6.
 Summary of transgenic plants over-expressing heat shock genes for abiotic stress tolerance.

HSP90s and polyamine (PA) oxidases (PAOs) organize the acetylation, oxidation and PA/H₂O₂ homeostasis of polyamines [85].

2.6 Transcription factors

The characterization and identification of genes involved in the stress response of plants is a prerequisite for producing genetically modified plants that are tolerant of various stresses. The fact that these identified genes are regulatory genes responsible for regulation become prominent as an effective approach in terms of ensuring the efficient control of many other genes involved in stress management. The most important regulatory gene candidates that can be used in the development of stress-tolerant plants are transcription factors (TFs) that also regulate the expression of stress genes. Transcription factors belonging to many different families such as AP2/EREBP, NAC, WRKY, MYB, bZIP are involved in various abiotic and biotic stress processes.

NAC proteins stand out as one of the largest family of transcription factors in plants. In particular, they perform vital tasks in the developmental processes of plants and their response to environmental stresses. They play a role in the regulation of many processes such as cell division, flower development, lateral root development, senescence, phytohormone homeostasis, secondary cell wall formation, abiotic and biotic stress responses. There are many studies that develop tolerance to abiotic stress as a result of transformation of plants with NAC transcription factors. Huang [86] transferred the TaNAC29 gene, which they isolated from wheat

(*Triticum aestivum*), to *Arabidopsis thaliana* plants. It has been reported that plants have developed tolerance to high salt and dehydration as a result of the overexpression of this gene. Transgenic plants accumulated less MDA under stress, but showed higher amounts of SOD and CAT activities. This shows that ABA-signaling pathway and antioxidant enzyme mechanisms are involved in NAC29-mediated stress tolerance. Similarly, overexpression of the TaNAC67 gene isolated from wheat plant increased the expression of many multiple abiotic stress response genes such as DREB1A, DREB2A, RD29A, RD29B, RD22, COR15 and Rab18 simultaneously in transgenic plants. Thus, improved cell membrane stability, higher chlorophyll content and Na⁺ efflux rates, and enhanced tolerance to drought, salinity and cold stress were observed in plants [87, 88].

Another important transcription factor family that is widely distributed in plants is MYB TFs. Many MYB transcription factors function in many physiological and biochemical processes, including cell development, cell cycle, hormone cycle, primary and secondary metabolism and signal transduction, biotic and abiotic stresses [89]. There are many studies reporting that transgenic plants enhance tolerance by overexpression of MYB transcription factors (**Table 7**). GmMYB1 introduced transgenic *Arabidopsis* plants have been shown to have an increased tolerance to drought and salinity compared to wild-type plants. At the same time, these plants showed higher plant height and less water loss rate when exposed to dehydration and cold stresses. Moreover, less MDA accumulation was detected in transgenic plants under stress with the overexpression of MYB [115]. TaMYB3R1 (*Triticum aestivum*) transcription factors provided drought tolerance by promoting the closure of stomata and limiting transpiration in *Arabidopsis* plants under stress [116].

The basic leucine zipper (bZIP) is a class of transcription factors with a conserved bZIP domain consisting of a region for DNA binding and nuclear localization at the N-terminus and a lysine-rich region for dimerization at the C-terminus. Similar to other TFs, it not only plays a role in developmental processes but also plays a regulatory role in the response to various abiotic stresses. In this context, *Nicotiana tabacum* plants in which the LrbZIP (*Nelumbo nucifera*) transcription factor was transferred have developed tolerance to salt stress by showing less electrolyte leakage and higher chlorophyll content under salt stress [137]. Another bZIP, group S transcription factor CaBZ1 (*Capsicum annuum*), reduced the rate of water loss of *Solanum tuberosum* plants under drought stress and increased the expression of ABA and stress-related genes with faster closure [110].

Large regulatory WRKY transcription factors protein family contain two conserved WRKY domains, consisting of approximately 60 amino acids containing the amino site sequence WRKYGQK at the N-terminus and zinc-finger motif at the C-terminus [138]. WRKYs manage many developmental and physiological processes including leaf senescence, regulation of biosynthetic pathways, hormone signaling, embryogenesis, trichome development. Various WRKY transcription factors are also known to be involved in abiotic stress responses. Overexpression of transcription factors, such as ZmWRKY33 (*Zea mays*), that activate various stress-induced genes plays important roles in the acquisition of stress tolerance [131]. Moreover, WRKY TFs increased physiological characteristics such as seed germination, root length and chlorophyll content in transgenic plants under stress conditions [132, 134]. Transgenic *Arabidopsis thaliana* plants developed with the transfer of TaWRKY79 (*Triticum aestivum*) gene have gained tolerance to salinity and ion stress with the improvement in their capacity to elongate their primary roots under salt stress. Transgenic plants developed by the transfer of the GhWRKY34 (*Gossypium hirsutum*) gene have similarly increased tolerance to salt stress, on the other hand, they contain a lower rate of Na⁺/K⁺ in their leaves and roots [134]. They also act as regulators in the ABA-related pathway [132].

Gene Action	Gene Source	Target Traits	Transformed Plant	Transfer Method	Ref.
AP2-ERFBP	GmERF3 from <i>Glycine max</i>	Salinity Drought	<i>Nicotiana tabacum</i>	Agrobacterium-mediated transformation	[90]
DREB1	OsDREB1D from <i>Oryza sativa</i>	Cold Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101	[90]
AP2-ERFBP	SbDREB2 from <i>Sorghum bicolor</i>	Drought	<i>Oryza sativa</i> subsp <i>indica</i> ,	<i>Agrobacterium tumefaciens</i> LBA4404	[91]
DREB1A	AtDREB1A from <i>Arabidopsis thaliana</i>	Drought	<i>Glycine max</i>	Particle-bombardment transformation	[92]
AP2-ERFBP	LcDREB2 from <i>Leymus chinensis</i>	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[93]
AP2-ERFBP	OsERF4a from <i>Oryza sativa</i>	Drought	<i>Oryza sativa</i> ,	<i>Agrobacterium tumefaciens</i> LBA4404	[94]
AP2ERFB	EaDREB2 from <i>Erianthus arundinaceus</i>	Drought Salinity	<i>Saccharum spp. hybrid</i> ,	<i>Agrobacterium tumefaciens</i> LBA4404	[95]
DREB1A	AtDREB1A from <i>Arabidopsis thaliana</i>	Drought	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[96]
AP2-ERFBP	LcERF054 from <i>Lotus corniculatus</i>	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101 floral dip	[97]
AP2-ERFBP	TaPIE1 from <i>Triticum aestivum</i>	Freezing	<i>Triticum aestivum</i>	Biolistic bombardment	[98]
AP2-ERFBP	VrDREB2A from <i>Vigna radiata</i>	DroughtSalinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105 floral dip	[99]
AP2-ERF	OsEREBP1 from <i>Oryza sativa</i>	Drought	<i>Oryza sativa</i> ,	<i>Agrobacterium tumefaciens</i> EHA105	[100]
AP2-ERFBP	SsDREB from <i>Suaeda salsa</i>	Salt Drought	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> EHA105	[101]
bZIP	PtRABF from <i>Poncirus trifoliata</i>	Drought	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> EHA105	[102]
bZIP	GmbZIP1 from <i>Glycine max</i>	DroughtSalinity Cold	<i>Nicotiana tabacum</i> - <i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[103]
bZIP	OsZIP39 from <i>Oryza sativa</i>	ER stress	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> EHA105	[104]
bZIP	MsbZIP from <i>Medicago sativa</i>	Drought Salinity	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[105]
bZIP	ZmbZIP72 from <i>Zea Mays</i>	Drought Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101	[106]
bZIP	LrbZIP from <i>Nelumbo nucifera</i>	Salinity	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> GV3101 leaf disc method	[107]

Gene Action	Gene Source	Target Traits	Transformed Plant	Transfer Method	Ref.
bZIP	OsZIP71 from <i>Oryza sativa</i>	Drought Salinity	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> AGL1	[108]
bZIP	TabZIP60 from <i>Triticum aestivum</i>	Drought Salinity Freezing	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101 floral dip	[109]
bZIP	CaBZ1 from <i>Capsicum annuum</i>	Drought	<i>Solanum tuberosum</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[110]
bZIP	AtTGA4 from <i>Arabidopsis thaliana</i>	Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> floral dip	[111]
MYB	AtMYB15 from <i>Arabidopsis thaliana</i>	Drought Salinity	<i>Arabidopsis thaliana</i>	Agrobacterium-mediated floral dip	[112]
MYB	TaPIMP1 from <i>Triticum aestivum</i>	Drought Salinity	<i>Nicotiana tabacum</i>	Agrobacterium-mediated leaf disc method	[113]
MYB	LcMYB1 from <i>Leymus chinensis</i>	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[114]
MYB	GmMYB1 from <i>Glycine max</i>	Drought Cold	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[115]
MYB	TaMYB3R1 from <i>Triticum aestivum</i>	Drought Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[116]
MYB	LeAN2 from <i>Lycopersicon esculentum</i>	Heat	<i>Lycopersicon esculentum</i>	<i>Agrobacterium tumefaciens</i> LBA4404 leaf disc method	[117]
MYB	SbMYB2 and SbMYB7 from <i>Scutellaria baicalensis</i>	Salinity	<i>Nicotiana tabacum</i>	-	[118]
NAC	ONAC063 from <i>Oryza sativa</i>	Salinity Osmotic stress	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101 floral dip	[119]
NAC	GmNAC20 GmNAC11 from <i>Glycine max</i>	Salt Freezing	<i>Arabidopsis thaliana</i>	Agrobacterium-mediated vacuum infiltration	[120]
NAC	ZmSNAC1 from <i>Zea mays</i>	Cold Salinity Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101 floral dip	[121]
NAC	TaNAC2a from <i>Triticum aestivum</i>	Drought	<i>Nicotiana tabacum</i>	Agrobacterium-mediated transformation	[122]
NAC	AhNAC3 from <i>Arachis hypogaea</i>	Drought	<i>Nicotiana tabacum</i>	-	[123]
NAC	SNAC1 from <i>Oryza sativa</i>	Drought Salinity	<i>Triticum aestivum</i>	Bombardment with a biolistic gun	[124]
NAC	OsNAP from <i>Oryza sativa</i>	Cold Salinity Drought	<i>Oryza sativa</i>	<i>Agrobacterium tumefaciens</i> EHA105	[125]

Gene Action	Gene Source	Target Traits	Transformed Plant	Transfer Method	Ref.
NAC	TaNAC67 from <i>Triticum aestivum</i>	Cold Salinity Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> floral infiltration	[87]
NAC	TaNAC29 from <i>Triticum aestivum</i>	Drought Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[86]
NAC	MLNAC5 from <i>Miscanthus lutarioriparius</i>	Drought Cold	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105 floral dip	[126]
NAC	TaNAC47 from <i>Triticum aestivum</i>	Salt Cold Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101	[127]
WRKY	OsWRKY45 from <i>Oryza sativa</i>	Drought Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> LBA4404	[128]
WRKY	VvWRKY11 from <i>Vitis vinifera</i>	Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101	[129]
WRKY	AtWRKY28 from <i>Arabidopsis thaliana</i>	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105 floral dip	[130]
WRKY	ZmWRKY33 from <i>Zea may</i>	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> EHA105	[131]
WRKY	TaWRKY79 from <i>Triticum aestivum</i>	Drought	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> GV3101 floral dip	[132]
WRKY	BdWRKY36 from <i>Brachypodium distachyon</i>	Drought	<i>Nicotiana tabacum</i>	<i>Agrobacterium tumefaciens</i> EHA105 leaf disc method	[133]
WRKY	GhWRKY34 from <i>Gossypium hirsutum</i>	Salinity	<i>Arabidopsis thaliana</i>	<i>Agrobacterium tumefaciens</i> LBA4404 floral dip	[134]
WRKY	MtWRKY76 from <i>Medicago truncatula</i>	Drought Salinity	<i>Medicago truncatula</i>	<i>Agrobacterium</i> -mediated transformation	[135]
HomeoBox 4	HaHB4 from <i>Helianthus annuus</i>	Drought	<i>Glycine max-Triticum vulgare</i>	-	[136]

Table 7. Summary of transgenic plants over-expressing transcription factor genes for abiotic stress tolerance.

The Apetala2/Ethylene responsive factor family is characterized by the highly conserved APETALA2 (AP2)/Ethylene Responsive Element Binding Factor (EREB) domain containing 40–70 amino acid sequences involved in DNA binding. AP2/ERFs are divided into four main groups; Apetala2 (AP2), related to abscisic acid intensive 3/Viviparous (RAV), dehydration-responsive element binding protein (DREB), ethylene responsive factor (ERF). These transcription factors play an important role in the regulation of both plant growth and the response to various

stresses [139]. The AP2 family of Ethylene Responsive Element Binding Factors supports the survival of plants under stress by activating jasmonate and abscisic acid signaling pathways. The amount of α linolenate, some jasmonate derivatives and abscisic acid increased in rice plants, in which the EREB1 transcription factor gene from AP2/ERF transcription factor family was transferred. The role of this gene family in the response to both biotic and abiotic stress is very important for the regulation of multiple stress tolerance for genetic engineering [100]. Dehydration responsive element-binding proteins (DREB) cooperated with the genes involved in polyamine biosynthesis to enable plants to tolerate salt stress [93]. Thanks to the transfer of another subgroup of ERF transcription factor LcERF054 (*Leymus chinensis*) gene, an increase in relative moisture content, dissolved sugar and proline amount occurred in Arabidopsis plants. Moreover, these plants have developed salinity stress tolerance. At the same time, the expression of the hyperosmotic salinity stress response genes COR15A, LEA4-5, P5CS1 and RD29A increased with the expression of the LcERF054 gene [97]. It has been reported that DREB transcription factor is used effectively in stress tolerance like other transcription factors belonging to the Apetala2/Ethylene responsive factor family [96].

3. Genome editing applications for abiotic stress tolerance in plants on emphasis to recent CRISPR applications

Since the bacterial defense mechanism against biotic agents as viruses based on the detection and elimination of invader nucleic acids suggested as novel tool for site specific genome editing tool by Jinek [140] in 2012, CRISPR/Cas9 is used in developing crop plants for abiotic stress tolerance as well as many other plant biotechnology applications. All of the abiotic stress tolerance mechanisms mentioned earlier in this chapter which were targets for gene transfer applications can be targeted also by CRISPR/Cas9 system as it allows both induction (CRISPRactivation) or repression (CRISPRinterference) of genes. Therefore, it can be used for activation of tolerance genes (T genes) as well as suppressing sensitivity (S genes) genes [141].

Targeting hormonal regulation for abiotic stress tolerance is one of the viable options. Abscisic acid (ABA) regulates physiological processes as seed dormancy, stomatal closure, plant development, as well as plant responses to environmental stimuli and multiple stresses. 9-cis-epoxycarotenoid dioxygenase (NCED), which is responsible from regulation of ABA in rice, targeted for multiple abiotic stress tolerance including salinity, drought and subsequent H₂O₂ stress. *nced3* mutant rice plants were very susceptible to these stresses as they express very low level of ABA. Overexpression of *OsNCEB3* in rice by CRISPRa system increased ABA accumulation and tolerance to salinity and drought stresses [142]. Downstream genes of the ABA signaling pathway in response to drought stress are regulated by ABA-responsive element binding proteins/ABRE binding factors (AREB/ABFs). CRISPRa system, set by CRISPR/dCAS9 fusion with histone acetyltransferase which promotes gene expression, is used to enhance drought resistance of Arabidopsis plants through activation of endogenous promoter of *AREB1* [143].

Transcription factors (TFs) regulate the expression patterns of the genes on promoter regions. Therefore, they are important targets for both genetic engineering and genome editing applications in abiotic stress tolerance of plants. CRISPRi system designed for 696-amino acid B-type response regulator transcription factor encoding *OsRR22* gene successfully improved salinity tolerance of rice plants since its involvement in cytokinin signal transduction and metabolism [144]. In maize, ARGOS genes regulate ethylene signal transduction. It is known to be a negative

regulator of ethylene responses. ARGOS genes are reported to increase ethylene sensitivity of Arabidopsis and maize when they were overexpressed through transgenic approach and enhance drought tolerance in plants [145]. Likewise, 400 inbred lines of maize were evaluated for ARGOS8 mRNA expression comparing to the transgenic ARGOS8 events. All inbred lines presented less expression than transgenic events. Therefore, overexpression of ARGOS8 also achieved by using CRISPRa approach and improved traits as drought tolerance and grain yield were generated [146]. Auxin response factors (ARFs) undertake important regulation roles in auxin response gene expression. Differential expression of ARFs were shown to involve in many physiological processes and abiotic stress responses. Downregulation and loss of function applications of *SLARF4* mediated by CRISPR/Cas9 presented salinity and osmotic stress tolerance in tomato [147].

Antioxidant scavenging is also very important in abiotic stress tolerance. Hence, elevation of antioxidant enzyme capacities in plants is a legitimate approach to improve tolerance. There are several successful transgenic application as described before. *Oryza sativa* stress-related RING Finger Protein 1 RING finger (OsSRFP1) which is a E3 ligase is responsive to cold, dehydration, salt, H₂O₂ and abscisic acid. However, overexpression of OsSRFP1 leads increased sensitivity to all of these factors. Transgenic RNAi silenced OsSRFP1 mutant plants obtained enhanced salinity and cold tolerance due to increased antioxidant capacity. It is also proposed as potential candidate target for CRISPRi applications [148, 149].

Ion homeostasis is another key factor in abiotic stress tolerance in plants especially against salinity. In Cucurbitaceae family, salt sensitive cucumber and salt tolerant pumpkin varieties were compared in context of K⁺ uptake during salt stress and salt tolerant pumpkins were found superior to cucumber plants for this trait. CRISPRi knocking out of NADPH oxidase (respiratory burst oxidase homolog D; RBOHD), which was previously tested by its inhibitor diphenylene iodonium, presented decrease in salinity tolerance. On the contrary, ectopic expression of pumpkin RBOHD in Arabidopsis enhanced the salinity tolerance [150].

Along with CRISPRa and CRISPRi application to enhance tolerance to abiotic stresses, it can also be used to identify roles of particular genes in abiotic stress tolerance. Nonexpressor of pathogenesis-related gene 1 (NPR1) is well known gene in plant pathogen response. To evaluate its involvement in drought stress CRISPRi derived tomato *slmpr1* mutants presented reduced drought tolerance which presented that gene's role in tolerance [151]. Likewise, CRISPRi application on mitogen-activated protein kinases (MAPKs) presented role of these signal molecules on drought tolerance of tomato plants through generating *slmapk3* mutants with altered drought stress responsive gene expression including SILOX, SIGST, and SIDREB [152]. Ca²⁺-dependent phospholipid-binding proteins called annexins were targeted by CRISPRi system. *OsAnn3* knockout rice mutants generated by this method were susceptible to cold stress indicating their role in mechanism [153]. Tomato is also very susceptible plant for cold and chilling stress. C-repeat binding factors (CBFs) were tested their potential role in chilling resistance. *slcbf1* mutants generated by CRISPRi system presented symptoms of chilling more severely comparing to the wild-type relatives [154].

Despite all of these present efforts and obviously more to come in near future, there are some bottlenecks on this topic beyond the genome editing technique itself. The topic of law regulations and public opinion on genetically engineered plants still have many uncertainties. In these days, when many countries regulated or starting to regulated their laws for genetically engineered plants, CRISPR/Cas9 derived products started another debate due to the lack of standardized detection methods and difficulty to distinguish these changes from naturally occurring mutations. Court of Justice of the European Union (ECJ) ruled their opinion on genome edited products

in 25 July 2018 as they are to be subjected under the same stringent regulations previously determined for genetically engineered products in 2001 directives. On the other hand, United States Department of Agriculture (USDA) announced their opinion as regulation is not needed for genome edited mutations since they can already occur naturally. Likewise, some countries as Brazil, Argentina and Australia shared the same opinion. In 2016, France inquired to ECJ about reviewing 2001 directive in favor of genome editing techniques as many researchers share their opinion as this method should be evaluated similar to use of radiation in mutation breeding allowing random mutations on genome. But the deliberate and intentional nature of the genome editing mutations brings the opposition. Still, Calyxt, an agricultural biotechnology company in Minnesota, announced the first US sale of high-oleic-acid oil product generated from gene edited soybeans in February, 2019. Following this development, Intrexon Company in Maryland announced start of commercial non-browning gene edited lettuce trials [13, 14].

Woo [155] suggested a novel approach to overcome this dilemma as DNA-free genome editing. In contrary to existing CRISPR/Cas9 system which is based on delivery of RNA guided nucleases into plants either by *Agrobacterium tumefaciens* or transfecting plasmids, they suggested delivery of pre-assembled Cas9-gRNA ribonucleoprotein (RNPs) into protoplast cells of *Arabidopsis thaliana*, tobacco, lettuce and rice plants instead of plasmids encoding these required components. They modified six genes in four different plants and these targeted mutations were maintained in plants regenerated from these protoplasts. Since, this system is free from involvement of recombinant DNA, they shared their opinion as this gene editing should be exempt from current genetically engineered plant regulations. This, direct delivery of purified CRISPR/Cas9 RNPs system is also adopted for other plants. MLO-7, a susceptible gene responsible from increasing resistance to powdery mildew disease in grapevine and DIPM-1, DIPM-2, DIPM-4 which are responsible from increased resistance to fire blight disease in apple were edited through DNA-free genome editing approach [156]. Due to the fact that regeneration of whole plant from the protoplast cells can be challenging in some plants especially in monocots as hexaploid bread wheat. Liang [157] reported use of particle bombardment delivery system into immature embryo cells for CRISPR/Cas9 RNPs system. The Cpf1 protein was suggested as an alternative to type II CRISPR-Cas system in 2015 [158]. Unlike Cas9 protein which recognizes proximal 3'-G-rich PAM sequences, Cpf1 recognized 5'-T-rich PAM sequences. Cpf1 is also a ribonuclease which processes precursor crRNAs and does not require trans-acting crRNA for guidance. Merging these two approaches is also achieved as CRISPR/Cpf1-mediated DNA-free plant genome editing. Soybean and tobacco protoplast genomes were successfully edited by this approach. Researchers suggested three potential advantages of this method as: no foreign DNA insertion, larger deletions in target site comparing to the Cas9 and different cleavage pattern of Cpf1 which may assist NHEJ-mediated insertion of donor DNAs [159].

In conclusion, following the footsteps of the data generated by the genetic engineering application for abiotic stress tolerance in crop plants, the most recent developments in genome editing techniques as transgene free approach combined with superior endonucleases provides promising results on this topic from many perspectives as law regulations, public acceptance, technical issues.

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