# MOLECULAR STUDIES OF SUNFLOWER RESPONSES TO ABIOTIC STRESSES

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## ABSTRACT

Sunflower (*Helianthus annuus* L.) is the third major crop for vegetable oil production worldwide among *Asteraceae* species. Mutant resources or routine protocols to transform genes to sunflower are not available as sunflower genome has not been completed yet. Abiotic stress conditions like drought, extreme temperatures, and high salt causes series of biochemical, physiological and morphological changes. These conditions lead to the production of excess reactive oxygen species (ROS) and osmotic imbalances that limit the productivity and growth of plants. In a scientific literature search, it was found that several genes were characterized in abiotic stress tolerance of sunflower. For instance, sunflower *HaWRKY6* shows functional response in temperature stress, and it is regulated by a miRNA. Sunflower HD-Zip I members *HaHB4, HaHB1* and *HaHB11* have critical functions in drought, freezing, and submergence tolerance, respectively. The functions of many sunflower regulatory genes and transcription factors in abiotic stresses are still unclear due to divergent genes encoding for transcription factors. For further studies, outstanding experimental strategies can be applied to overcome difficulties of studying divergent genes encoding for transcription factors in sunflower in abiotic stress tolerance. Understanding of plant responses to abiotic stresses is essential for structural and functional characterization of environmental stress-induced genes. Here we present the current molecular studies of sunflower responses to abiotic stresses.

**Keywords:** sunflower, abiotic stress, drought, salinity, heat, low temperature

## INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the significant oil crops in the world and is North America's native crop. It is used in medicine ad as food and is first domesticated by Indians (Kaya, Jocić, & Miladinović, 2012). Sunflower is also used as an ornamental plant and grown commercially. High oil and protein containing commercial sunflower hybrids are used for oil crop breeding (Cvejić, 2016). It is cultivated area is over 22 million ha and annual production of sunflower is over 9 million tonnes in the world (Fernández-Martínez et al., 2009). Sunflower production is mainly concentrated in Ukraine, Russia, Argentina and India (Gulya 2014). According to Turkish Statistical Institute, sunflower production is generated as 2.6% and became 1.7 million tonnes in Turkey (Tuik, 2015). Development of varieties including high oil content became a milestone in oil seed sunflower breeding in the world. Another milestone in the development of sunflower was the discovery of cytoplasmic male sterility. This highly reliable method paved the road to the production of commercial hybrid seeds with inherent advantages (Leclercq, 1969). In early efforts, breeders tried to cope with parasitic weeds (broomrape, *Orabanche cumana*) and insects (Homeosome electellum, sunflower moth) (Fick, 1997) by genetic control. In 1910-1912, Krasnodar by Vasilii Stepanovich Pustovoit started a scientific sunflower breeding program from locally developed varieties. Sunflower is more tolerant to abiotic stresses compared to other field crops because its main organs such as stem, leaves, head and roots have developed specific

structures able to grow under negative conditions or in marginal soils in semiarid zones. To increase the genetic tolerance of cultivated sunflower against abiotic stresses, diversity of the wild *Helianthus* species has been used with good reactions (Škorić, 2009). *H. argophyllus* and *H. paradoxus* showed the best results as wild sunflower species in sunflower breeding against drought and salinity, respectively. Integration of molecular breeding techniques is essential to provide the genetic tolerance mechanisms of wild Helianthus species towards enhancing the abiotic stress tolerance in sunflower breeding program. More progress has been carried out about heat tolerance compared to cold tolerance in sunflower breeding. On the other hand, special breeding programs are needed to be develop in sunflower to deal with mineral toxicities and deficiencies.

Under abiotic stress conditions, transcription factors (TFs) (bZIP, MYC, MYB and DREB), protein kinases and proteases are essential for the regulation of transcriptional changes under adverse environments such as abiotic stress conditions (Pradeep et.al. 2006). Transcription factors are induced by abiotic stress conditions to activate transcription machinery (Figure 1). Cold stress, salinity and drought cause production of reactive oxygen species (ROS) in photosynthesis pathway, limit the availability of $CO_2$ for the dark reaction and this, in turn, leaves oxygen as the main reductive product of photosynthesis (Mitter, 2002). Abiotic stresses such as drought, salt and cold lead to the accumulation of hydroxyl radicals, hydrogen peroxide, and superoxide in the cells (Hasegawa et.al. 2000). Because of the accumulation of these products along the oxidative stress, many expressed sequence tags (ESTs) from leaf and stem cDNA libraries express catalases, thioredoxins, oxygen-evolving enhancer proteins and peroxidases (Kawasaki et. al., 2001). Due to oxidative stress and accumulation of ROS, most of those proteins are up-regulated in stress conditions (Kawasaki et al., 2001).

In literature, several TFs of *Asteraceae* species are defined as essential in abiotic stress tolerance. During the last five years, characterized *Asteraceae* TFs include sunflower *HaWRKY6* that is regulated by a miRNA in temperature response; chrysanthemum *DgWRKY3* that is involved in salt tolerance; sunflower HD-Zip I members *HaHB4*, *HaHB1* and *HaHB11* that are functional in drought, freezing and submergence tolerances, respectively; chrysanthemum DREB subfamily member of the AP2/ERF family *CgDREBa* and the bHLH member *CdICE1* that are essential in freezing, salt and drought stress tolerance; chrysanthemum MYB TF, *CmMYB2* that is involved in salinity and drought stress; chrysanthemum NAC *DgNAC1* that confers salt tolerance; chrysanthemum zinc finger protein *DgZFP* bringing about salt tolerance (Table 1).

**Table 1.** *Asteraceae* genes encoding transcription factors under abiotic stress conditions.

| **Gene name and source** | **Function** | **Reference** |
|---|---|---|
| Sunflower *HaWRKY6* | High temperature tolerance | Giacomelli et al., 2012 |
| Chrysanthemum *DgWRKY3* | Salt tolerance | Liu et al., 2013 |
| Sunflower *HaHB4* | Drought tolerance | Dezar et al., 2005 |
| Chrysanthemum *CgDREBa* | Freezing, salinity and drought tolerance | Chen et al., 2012 |
| Sunflower *HaHB1* | Freezing and drought tolerance | Cabello et al., 2012 |
| Chrysanthemum *CdICE1* | Freezing, salt and drought tolerance | Song et al., 2014 |
| Chrysanthemum *CmMYB2* | Salinity and drought tolerance | Shan et al., 2012 |
| Chrysanthemum *DgNAC1* | Salinity and drought tolerance | Liu et al., 2011 |

From drought and salinity stress samples, microsatellites located within ESTs in *H. annuus* are analyzed from populations from arid desert and salty areas. Test statistics of lnRV and lnRH were used to select candidate genes that have a wide variety of functions. 17 significant loci of included genes were analyzed based on BLAST hits with homology search. According to the results, genes were categorized as five transcription factors, three cellular components, four genes with catalytic or metabolic functions, four genes of unknown homology or function and one DNA-repair gene (Kane et al. 2007).

A large quantity of ESTs from *Helianthus* spp. are available in public databases, but they are not studied well (Giacomelli, 2010). Giacomelli et al. (2010) estimated 97 sunflower WRKY members derived from EST databases. They report that *Asteraceae* WRKY family can be the source of specific new functions with a particular diversification. Additionally, they suggest that the sunflower *WRKY*s can be used as markers of tolerance to necrotrophic pathogens because they could have a significant function in biotic stress response. Furthermore, specific features of the sunflower WRKY family are identified. For instance, they suggest that *HaWRKY4* may function in senescence (Giacomelli, 2010).

Flooding is one of the environmental abiotic stress conditions that affect food production negatively (Boyer, 1982). Cabello et al. (2016) studied on sunflower transcription factor *HaHB11*, which is a member of the sunflower homeodomain-leucine zipper I subfamily of transcription factors. According to their results, overexpression of HaHB11 in transgenic Arabidopsis plants led to larger rosettes, wider stems and significantly increased biomass compared to wild type plants. Transgenic Arabidopsis plants expressing *HaHB11* showed enhanced tolerance to flooding stress. Additionally, transgenic plants produced twice the amount of seeds that the wild type plants produced (Cabello, 2016).

## DROUGHT TOLERANCE IN SUNFLOWER BREEDING

Quartacci and Navari-Izzo (1992) indicated that sunflower seedlings exposed to water deficiency accumulated lower levels of soluble proteins, chlorophyll, and total and polar lipids compared to control plants. Under water stress, root growth extension is observed into moist soil regions. To escape from desiccation tolerance, there are available mechanisms, pathways and reactions, including the accumulation of intracellular proteins such as late embryogenesis-abundant (LEA) proteins. They stabilize other proteins and membranes against drying. Dehydrins are among drought stress induced proteins in D-11 subgroup of LEA family (Giordani, 1999).

Mayrose et al. (2011) analyzed protein phosphatase 2C and the HD-Zip transcription factor ATHB8 under drought stress conditions. Protein phosphatase 2C gene is from a group of serine /threonine protein phosphatases. These proteins are negative regulators in plant responses under abiotic stress conditions such as drought (Schroeder et al. 2001; Tahtiharju&Palva, 2001). HD-Zip transcription factor ATHB8 induces developmental reactions to the environmental conditions. *ATHB8* expression decreased under drought stress such that 1.3 fold repression in native plants, 2.6 fold repression in weeds were observed, while the highest repression was found in crops as 3.2 (Mayrose et al., 2011). Interestingly, they showed that no control plant has the expression of the *ATHB8* gene. Additionally, *ATHB8* transcription factor is available in reduced growth and weedy plants under drought conditions.

Members of the sunflower (or other *Asteraceae* species) WRKY family are not clear completely so far. HaWRKY76 is a sunflower transcription factor whose biological role is not found yet because the WRKY family is highly diversified in the *Asteraceae* (Giacomelli et al. 2010). Raineri et al. (2015) indicated that HaWRKY76 is a divergent sunflower WRKY transcription factor. It enhances the dehydration and submergence tolerance in Arabidopsis when expressed in transgenic plants. It is suggested that HaWRKY76 could be potential tool to make drought tolerant plants (Raineri, 2015).

**SALINITY TOLERANCE IN SUNFLOWER BREEDING**

Mineral salt accumulation in global arable lands leads to abiotic stresses. After moisture stress, salinity is in the second rank in causing agricultural problems. Accumulation of excess amount of soluble salts, mineral toxicity or deficiency may cause this stress (Singh, 2006). Salinity stress limits plant growth and productivity (Khan et al., 2014). Khan&Asim (1998) evaluated that limited cell division resulted from salt stress causes cell volume reduction. Salt stress negatively affects biochemical and physiological changes, placement of solute dissolved proteins, nutrient uptake, ion-uptake and carbon assimilation (Schroeder et al., 2013; Naz&Bano, 2015). Selectivity of root membranes is impaired by excess amount of $Na^+$ and $Cl^-$ that are predominant ions causing high ionic imbalances (Bohra&Dörffling, 1993). To examine the comparative differences of salinity effect, different physiological characters such as compartmentation of $Na^+$ and $Cl^-$ ions, osmotic adjustment, selectivity for $K^+$ ahould be taken into consideration regarding to the salt tolerance in crops (Wyn Jones&Storey, 1981). Ahmed et al. (2005) explained that sunflower cultivars grown in saline environment show crucial reductions in height, leaf area and stem girth. These growth limitations cause oil percentage reductions. In salinity conditions, plant cell turgor pressure is reduced and then this causes stomatal closure, which limits carbon fixation and photo-assimilation rate (Gale & Zeroni 1984).

Fernandez et.al (2008) studied eighty genes isolated from organ-specific cDNA libraries under salinity (NaCl) and low temperature conditions. They looked at microarray profiling of chilling and NaCl-treated sunflower leaves, and indicated significant changes in transcription factors, defense/stress related proteins, transcript abundance and effectors of homeostasis under both stresses. They categorized results of differentially expressed genes according to their functions (Table 2). In Table 2, down-regulated and up-regulated number of genes in categorized metabolism are given under salinity stress.

**Table 2.** Number of genes involved in different functional categories (Fernandez, 2008).

| | **Cold** | | | **Salinity** | | |
|---|---|---|---|---|---|---|
| **Functionally classified proteins** | **NC** | **Up** | **Down** | **NC** | **Up** | **Down** |
| Central metabolism/Photosynthesis | 1 | 2 | 7 | 2 | 2 | 6 |
| Translation machinery | 2 | 3 | 1 | 1 | 3 | 2 |
| Transcriptional machinery | 2 | 2 | - | 2 | 2 | - |
| Signaling machinery | - | 1 | 1 | - | 1 | 1 |
| Protein turnover/folding/interactions | | 3 | 2 | 2 | 1 | 2 |
| Transport | - | 3 | - | - | 2 | 1 |
| Secondary metabolism | 1 | - | 2 | - | 1 | 2 |
| ROS machinery | - | 5 | 2 | 2 | 3 | 2 |
| Total | 6 | 19 | 15 | 9 | 15 | 16 |

NC: No change.

First genetic map of sunflower was constructed by the help of quantitative traits controlling physiological characters regarding to the oil yield and the adaptive responses of sunflower to abiotic stresses (Tang, 2003). This type of genomics-based approach allows the development of low-cost

procedures that will be used further by researchers in breeding programs whose goals are enhancing sustainability and yield stability under abiotic stress conditions.

Fernandez et al. (2008) analyzed that EST T411, similar to a plastidic aldolase is up-regulated under salinity stress. Plastidic aldolase genes are indicated in *Nicotiana* plants and are grouped as AldP1 and AldP2. Yamada et.al. (2000) firstly reported that AldP2 was up-regulated under salt stress while AldP1 was suppressed in salt stress conditions. EST H136 (similar to a chloroplast drought-induced stress protein) is down-regulated under chilling and salinity stresses (Fernandez et al., 2008). CDSP (CHLOROPLAST DROUGHT-INDUCED STRESS PROTEIN) is a type of thioredoxins, which play role in oxidative stress (Broin et al., 2000)

It is found that salinity induces transcription of the *MIPS* (*MYO-INOSITOL-1-PHOSPHATE SYNTHASE*) during biosynthesis pathway of myo-inostol and its derivatives (Nelson, 1998; 1999). Myo-inositol-1l-phosphate synthase (MIPS) is functional in *de novo* inositol biosynthesis pathway (Loweus and Loweus, 1983). In *M. crystallinum*, salinity stress induces higher expression of *MIPS* mRNA as 5-folds, resulting in free inositol accumulation of 10-folds (Ishitani et al., 1996).

Understanding of genetic mechanism to salty environment will improve plant responses to changing conditions and develop insights to long-standing questions. Edelist et al. (2009) reported constitutively under- or over-expressed genes regarding to potassium and calcium transport (homologues of *KT1*, *KT2, ECA1*) in hybrid species of *H. paradoxus.* They found that salinity treatment induced over-expression of homologues of the potassium transporter *HAK8* and its transcriptional regulator.

In sunflower, a small family of three genes (*HAS1*, *HAS1.1* and *HAS2*) encodes asparagine synthetase (AS; EC 6.3.5.4) (Herrera-Rodríguez et al., 2007). They are regulated differentially by nitrogen, carbon and light availability. Gene specific probes are used in Northern analysis under osmotic stress, heavy metal stress and salt stress. They reported that stress treatments did not induce any changes in the expression of *HAS2.* Osmotic and salt stresses decreased the expression of *HAS1* and *HAS1.1* genes in light conditions (Herrera-Rodríguez et al., 2007).

SALT OVERLY SENSITIVE2 (SOS2) and PLASMA MEMBRANE PROTEIN3-1 (PMP3-1) are functional in homeostasis. They were analyzed in two salinity-contrasting sunflower lines, Hysun-38 (salt tolerant) and S-278 (moderately salt tolerant) (Saadia, 2013). In sunflower root tissues from both tolerant and moderately tolerant lines, *SOS2* expression showed gradual increase under salt stress. A gradual increase of *SOS2* expression was observed in leaf tissues of tolerant variety compared to moderately tolerant one. They observed highest level of *PMP3-1* expression in the roots of tolerant sunflower line in the post-salinity level (6 and 12 h of stress treatment). Higher expression of *PMP3-1* was observed in moderately tolerant line at 12 and 24 h of salt treatment (Saadia, 2013).

NAC family transcription factors in plants are functional in abiotic stress responses (Jeong, 2010). In tolerance to abiotic stresses, only a few stress-responsive NAC proteins are characterized (Nakashima, 2011). Manjunath et al. (2013) developed a simple and effective screening methodology to identify transformants under salt tolerance. They created leaf discs of *EcNAC1* gene transformants. They analyzed *EcNAC1* gene with *HPT II* specific primers and *Sac I* restriction enzyme is used to digest the amplified *EcNAC1* gene product. They suggest that initial identification of promising transformants result from *in vitro* screening strategy at plant level based on the target gene (Manjunath, 2013).

HEME OXYGENASE1 (HO1) is functional in protecting mechanisms against environmental stress responses (Zhu, 2014). It is a stress responsive antioxidant enzyme that cleaves heme to biliverdin IXα (BV). BV functions in concominant release of carbon monoxide (CO) and production of free iron ($Fe^{2+}$) (Shekhawat 2010). Zhu et al. (2014) cloned sunflower *HaHO1* gene, which is required for sunflower salinity acclimation. They showed the induction of *HaHO1* was closely associated with the sunflower salinity acclimation.

## HEAT TOLERANCE IN SUNFLOWER BREEDING

Heat stress is defined as high temperature lasting in enough duration that cause important yield reduction compared to control plants (Singh, 2004). Emissions of heat stress in environment resulting from automobiles, industry and urbanization cause temperature increase that endangers diversity of fauna and flora (Singh et al. 2006). High temperature causes heat stress in plants that affects physiological, morphological and physiological traits negatively (Table 3).

High temperatures may cause stomatal closure, a rise in respiration rate, leaf, or canopy temperature, cell membrane injuries, disruption of the photosynthetic apparatus, and the induction of stress-specific growth regulators, which shorten the total growth period due to changes in crop phenology, biomass, fruiting sites, gamete sterility, seed fruit, seed size, and seed quality (Moriondo & Bindi 2006; Moriondo et al. 2011).

In the growing environment, plants are more vulnerable to heat stress in their flowering stages. Under such conditions, large quantities of pollens are selected by breeders. To obtain the best pollination and seed formation, it is necessary to maintain pistil, stigma or disk flowers that are tolerant to high temperature (Škorić, 2012).

**Table 3.** Effects of heat stress on sunflower.

| **Traits** | **Effects** | **LITERATURE** |
|---|---|---|
| Leaf growth period (d) | Decreased by 1.04 days per °C above 36°C | Rawson and Hindmarsh (1982) |
| Grain weight /yield (g) | Grain weight was reduced up to 21% and final grain yield reduced by 10% at 38°C | Ploschuk and Hall (1995) |
| Grain-filling duration (d) | Reduced by 2–6 days at 38°C | Ploschuk and Hall (1995) |
| Grain weight (g) | 40% decrease when temperature is >35°C during early grain development | Rondanini et al. (2003, 2006) |
| Respiration rate (mmol $m^{-2}$ $s^{-1}$) | Increased 19% when night temperature 5°C higher than control | Manunta and Kirkham (1996) |
| Oleic acid (%) | Increased oleic acid production at the expense of linoleic acid | Harris et al. (1978); Fernández-Moya et al. (2002) |
| Leaf temperature | 1–2°C higher than ambient air temperature (42°C) in susceptible lines | Kalyar et al. (Forthcoming 2013) |
| Heat stress injury (%) | Decreased 10–65% in sunflower germplasm with variable resistance evaluated at 40°C | Kalyar (2013) |

WRKY transcription factors are functional in plant stress responses. The sunflower *HaWRKY6* contain a target site for the binding of miR396. Giacomelli et al. (2012) analyzed the possible post-transcriptional regulation of *HaWRKY6* by miR396 in the *Asteraceae*. They found that the silencing of *HaWRKY6* due to miR396 accumulation is responsible for high-temperature protection in sunflower (Giacomelli, 2012).

## LOW TEMPERATURE TOLERANCE IN SUNFLOWER BREEDING

Low temperature limits crop productivity in many environments. When the temperature is above freezing level (> 0 °C), it is called as chilling; if it is below 0 °C, it is called as freezing. Kalaydzhyan et al. (2009) developed sunflower genotypes that are tolerant to cold after mutagenizing the plants by dimethyl sulfate (DMS) as chemical mutagen. 44.000 seeds of about 2.000 mutagenic progenies were screened under low temperatures by planting them in early and late winter. 499 plants from 72 mutagenic progenies were able to grow under harsh winter and low temperature conditions (down to −20°C).

*HaF455* involved in ribosomal activity is induced by cold and salinity stresses in sunflower (Fernandez et al. 2008). Fernandez et.al. (2008) showed that the expression of EST H123 [GenBank: BU672069] having high identity with myo-inositol phosphate synthase (MIPS protein, isomerase involved in inositol metabolism) was decreased by chilling and salinity stresses.

## CONCLUSION

There are many reports on molecular mechanism of sunflower abiotic stress tolerance. However, molecular attempts to sunflower abiotic stress tolerance have not been enough as compared to the molecular studies performed with other crops. Especially, the use of molecular techniques such as QTL identification and associating mapping will enable a faster and more efficient breeding program in sunflower abiotic stress tolerance. For further studies, the application of different molecular methods such as transcriptomics will help development of new sunflower cultivars that are more tolerant to abiotic stress conditions. In further studies, array based cDNAs will contribute to the understanding of functions of more sets of genes functioning in sunflower abiotic stress tolerance.

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