Enhancing drought tolerance in cotton through manipulating stress resistance genes

Muhammad Nadeem Hafeez[1,2], Shumaila Zulfiqar[3], *Qurban Ali[1,2], Kausar Malik[1]

[1]Centre of Excellence in Molecular Biology, University of the Punjab Lahore, Pakistan
[2]Institute of Molecular Biology and Biotechnology, University of Lahore, Lahore, Pakistan
[3]National Institute for Biotechnology and Genetic Engineering Faisalabad, Pakistan
Corresponding email: chnadeemhfz@gmail.com, saim1692@gmail.com

Abstract

Drought stress affects the normal growth of plant by influencing Physiological, morphological molecular and biochemical traits at cellular level. It is a polygenic trait, controlled by multiple genes, which makes its manipulation difficult by genetic engineering. It seems drought could be major threat in future to high yield of cotton in Pakistan as well around the globe because it is spontaneous and can't be controlled with manuring and skilled agricultural practices. Gene manipulation could be a solution of this threat by producing transgenic cotton plants. As it is polygenic trait, so, understanding about cellular mechanism of drought tolerance is crucial to impart tolerance by controlling gene expression under stressed conditions. Universal Stress Proteins (USP) genes have already been identified in drought stressed leaves of *Gossypium arboreum* which make this variety of cotton a rich source of stress tolerance genes. USP genes could be manipulated for drought tolerant transgenic cotton with high yielding as well and it is most important family of proteins in this regard. This family encompasses a conserved group of proteins that has been reported in different organisms which are activating under various abiotic stress conditions. USP is also a regulatory protein; its activity can be increased by manipulating its interactions.

Keywords: *Gossypium arboreum*, polygenic traits, drought stress, universal stress proteins, transgenic

Introduction

Unfavorable environmental conditions usually inhibit the normal plant growth. There are several abiotic factors, which interrupt the bumper growth of crops, among them drought is worth to be mentioned (Farooq et al., 2009). Drought means loss of water either at elevated or normal temperature. It triggers biochemical and physiological modifications at cellular level, among them loss of turgor pressure, changes in membrane fluidity, membrane composition, solute concentration, protein-lipid and protein-protein interactions are need to be studied. Plants respond to drought stress at physiological level as well as at molecular level. They activate

diverse set of metabolic activities to defend themselves against drought stress and initiate their struggle to be survived at the onset of this extreme unfavorable condition (Fahramand et al., 2014). At genetic level, multiple genes are involved, which are responsible to initiate the defense system of plants. Hence, involvement of multiple genes makes it complex to control the drought resistivity of plants by using genetic engineering. However, different studies like transcriptomics, proteomics and expression of genes have identified the activation and regulation of several stress-related genes at cellular level. It has been observed that expression level of different genes in plants is changed under the spell of drought stress (Ribas et al., 2006).

Plants' responses against stresses are mounted and co-ordinated by two types of signaling pathways namely ABA-independent and ABA-dependent pathways. The products of these genes are classified as regulatory and functional proteins which are responsible to activate stress related pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). Functional and regulatory proteins include enzymes for biosynthesis of osmolyte, water channel proteins, LEA-proteins, chaperones, detoxicating enzymes, proteinases, transcription factors, phospholipases and protein kinases. These functional and regulatory proteins are playing their role to regulate signal transduction mechanism and stress related gene expression (Sivamani et al., 2000).

The interaction studies of the genes responsible for plants' biological processes are important to comprehend the mechanism of abiotic stresses in plants. Plants express a multitude of proteins containing USP domains. An analysis of different plant taxa shows that a plant contains an average of 200 different USP domain containing proteins (Isokpehi et al., 2011, Maqbool et al., 2009). Two genes have been identified in cotton which encodes USP proteins named GUSP1 and GUSP2 (Maqbool et al., 2009, Fahramand et al., 2014). These genes are up-regulated during drought stress. Studies conducted on the role of USP in tomato shows that USP activates two gene clusters. One cluster produces LHCB (light harvesting chlorophyll a & b binding proteins) and reduction in aperture of stomata aperture while second cluster produces osmo-protective compounds i.e. proline. Furthermore, USP is also shown to interact with annexin proteins but the details about how actually the USP performs its functions are still to be elucidated (Loukehaich et al., 2012, Fahramand et al., 2014).

Cotton crop is grown around the world especially at tropical and subtropical regions. Currently, China is the largest producer of cotton where as Pakistan is at 4th position after China, India and USA. Its contribution to our national economy is significant because it provides raw

material to our local textile industry. Its share in GDP is 1.6% and its value addition in agriculture is 7.8%. Its productivity is highly vulnerable to several biotic and abiotic factors, however in Pakistan erratic rain fall and non availability of irrigation water is the biggest factor behind its low productivity (Pakistan, 2016-17). It has been observed that plants respond increasing osmo-protectant production, detoxification of ROS, biosynthesis of chlorophyll, increasing water uptake and stabilizing its proteins (Hayano-Kanashiro et al., 2009), all these processes are triggered by several genes. It is obvious from the previous studies that manipulation of drought responsive genes and maintenance of cellular components has remained as major target of attempts to produce plants having enhanced drought tolerance. Cotton (*Gossypium* spp.), is genetically diverse plant, it has four domesticated species. *G. arboreum* has many remarkable benefits over *G. hirsitum,* it has considerable resistance against biotic and abiotic stresses especially drought stress which makes it priceless gene pool to improve future cotton cultivars (Huang and Liu, 2006, Liu et al., 2006, Mehetre et al., 2003).

Several stress proteins and soluble sugars have been reported to act as protectants during cell dehydration in many plants (Padmalatha et al., 2012, Kosmas et al., 2006). The universal stress protein (USP) is one of the most important family of protein for this purpose. USP is a cytoplasmic protein present in bacteria; its expression is enhanced, when cellular viability is challenged by drought spell and other abiotic stresses (Sousa and McKay, 2001, Maqbool et al., 2009, Zahur et al., 2009). The USP super family encompasses a conserved and ancient group of proteins that are present in archaea, bacteria, fungi, plants and flies (Kvint et al., 2003). *E. coli* contains six USP proteins (USPa, USPb, USPc, USPd, USPe, USPf), which are sub divided into two sub-groups on the basis of their sequence similarity (Gustavsson and Nyström, 2002). It has been reported the presence of *USP*-genes in different organisms, where they are playing role in response to heat shock, cold shock, DNA management, metabolic control (Isokpehi et al., 2011, Mbah et al., 2013, Persson et al., 2007, He et al., 2012). Furthermore, USP is a regulatory unit protein; its activity can be increased by manipulating its interactions.

This review will cover the role of drought tolerance related gene in plants and their effect on cellular mechanism in response to drought stress (Maqbool et al., 2009, Zahur et al., 2009). A comprehensive screening of Universal Stress Protein gene-2 will enhance our basic knowledge about key metabolic pathways relating to drought and open ways for future engineering of drought stress tolerance. The functional and cellular characterization of USP genes would be

investigated to ascertain its potential role in drought stress tolerance mechanism in plants especially in cotton. Understanding the mechanism of interaction of universal stress protein genes and its role in drought tolerance will enable us to breed economically important cotton varieties which are adapted to drought stress. The review will cover the major metabolic pathways in connection with drought tolerance, which will be helpful in providing direction for future metabolic engineering for drought-stress tolerance.

What is Abiotic Stress?

Negative influence of non living things of environment on living things in an ecosystem is called abiotic stress (Degenkolbe et al., 2009, Ali et al., 2017). The common stressors are easiest to identify, but some other are less recognizable stress factors which are affecting environment constantly (Kogan et al., 2013). Extreme temperature, drought, salinity, wildfire and sporadic floods are well known abiotic stresses in our ecosystem for plants. Hence, Scarcity of water resources, environmental pollution, salinity, intensified erosional problems are marked in the beginning of 21st century. All these offer abiotic stress to the plant growth, which is the limiting factor in crop yield around the world (Wang et al., 2003). Roots are first line of defense against any type of abiotic stress in plants, if soil is healthy, porous, well aerated and contain all essential nutrients then survival rate of plant is automatically increased. Furthermore, abiotic stress not only limits the crop productivity, (Jaleel et al., 2009) but also distribution of plants.

Drought Stress

Drought stress is major threat among abiotic stresses, which has drastic affects on yield and growth of plants. Drought is a metrological phenomenon which can be defined as a depletion of moisture to the economic injury level, because of prolong period of meager rain fall (Kramer, 1979). Drought is one of the major threats to low productivity in Pakistan. Pakistan has 796096 km^2 areas and available for agriculture is 24.4 mega hectares. However only 18.03 mega hectares is suitable for agriculture with irrigation, the rest is subjected to rain fed agriculture. Pakistan has drawn irrigation water 148 MAF (Million Acre Feet) from Indus river system, 94 MAF is diverted to the canals for irrigation. 50 MAF is allowed to fall in Arabian Sea without any use. However, Pakistan needs 205 MAF water for its irrigated agriculture. 50 MAF shortage of water is compensated with tube well irrigation (Anonmyous, 2016-17). According to the recent World Bank record, Pakistan may face shortage of water in 2050 because of rapid changing in climate.

Total renewable water resources in Pakistan are decreasing continuously since 2000 and water availability is also decreased from 1349 $m^3capita^{-1}$ in 1996 to 1241 $m^3capita^{-1}$ in 2016 (UN

report, 2015). Studies also revealed that if current trend continue than reduction would be at 550 m^3 in 2025. So, water security is required for irrigated agriculture of Pakistan. In short, 4.76million hectare area (MHA) is extremely affected by water deficiency. It is also learnt that every year 3% suitable land for agriculture is converted into the wild land because of shortage of water (UN report, 2016). In this scenario, Pakistan is at the edge of food security problems because of reduction in the productivity of its major crops initiated by the drought stress.

Effects of extreme water deficiency on Plant Growth

Water deficiency results in loss of turgor pressure and osmotic stress in plants (Mansoor et al., 2003). The osmotic stress results in reduction of plant height, decrease in root length, yellowing of leaves because of reduction in chlorophyll content and overall growth rate of plant is reduced. Deficiency in nutrient distribution is another negative effect of drought stress. Reduction in water uptake from the soil to roots also results in slow entry of essential minerals such as nitrate, phosphorus, calcium and potassium into plant and in turn growth rate is ultimately reduced (Zhu et al., 2000).

Secondary effect of water deficiency is oxidative stress because of the accumulation of excessive ROS (Reactive Oxygen Species) like hydroxyl radicals, hydrogen peroxide and superoxide anions. Usually ROS are found to be involved in normal metabolic reactions of cells, however, when plants are growing under water deficient environment, the amount of ROS increases to protect the cells from oxidative injuries (Altaf-Khan et al., 2002). Similarly, water deficiency results in the reduction of root and shoot length and their fresh and dry biomass, therefore roots and shoots may become thinner or thicker (Ashraf and Foolad, 2007). Studies have been revealed that water deficiency is a chief limiting aspect at initial stages of plant growth. Both cell elongation and cell division are severely affected by the drought stress (Shao et al., 2008, Bhatt and Rao, 2005, Koroleva et al., 2005). Various physiological and biochemical processes like ion uptake, respiration, photosynthesis, nutrient metabolism and translocation are severely affected by the reduction in plant growth as a result of drought stress. Similarly studies of Farooq *et al*., (Farooq et al., 2009) observed changes in chlorophyll a and b because of drought stress. All cash crops are affected by drought stress among them rice as a submerged crop is more susceptible to the drought stress (Jaleel et al., 2009).

Another effect of water deficiency is reduction in the stem length (Specht et al., 2001). Similarly, 25% reduction in plant height was observed in water stresses citrus seedlings (Wu et

al., 2008). Significant reduction in the stem length of potato plant was also reported under drought stress (Heuer and Nadler, 1998). The height of other plants like, *Petrosolinum crispum* (Jaleel et al., 2007), *Abelmoschus esculentus* (Manivannan et al., 2007), and *Vigna unguiculata* (Petropoulos et al., 2008) were reported to be decreased. In short, effects of drought stress are characterized by diminished water potential, reduction of total water content, loss of turgor pressure, closing of stomata and reduction of cell division along with cell elongation.

Cotton production in Pakistan

Cotton is an important cash crop for Pakistan known as "white gold". It accounts for 8.2 percent of value added in agriculture and about 3.2 percent to GDP, around two thirds of the country's export earnings are from the cotton and textiles which adds over $2.5 billion to the national economy, while hundreds of ginning factories and textile mills in the country heavily depends upon cotton. Life of millions of farmers is dependent on this crop, in addition to millions of people employed along the entire cotton value chain, from weaving to textile and garment exports (Anonmyous, 2016-17).

Pakistan is the fourth largest producer of cotton in the world, the third largest exporter of raw cotton, the fourth largest consumer of cotton, and the largest exporter of cotton yarn. 1.3 million Farmers (out of a total of 5million) cultivate cotton over 3 million hectares, covering 15 per cent of the cultivable area in the country. Cotton and cotton products contribute about 10 per cent to GDP and 55 per cent to the foreign exchange earnings of the country. The domestic consumption of cotton is about 30 and 40 % (Anonmyous, 2016-17). The remaining cotton is exported as raw cotton, cloth, garments and yarn. Cotton production supports Pakistan's largest industrial sector, comprising 7 million spindles, 27,000 looms in the mill sector,400 textile mills,650 dyeing and finishing units, 4,000 garment units 700 knitwear units, nearly 1,000 ginneries, 300 oil expellers and 15,000 to 20,000 indigenous, small scale oil expellers (kohl-us).

The area under the cultivation of cotton crops has been increased significantly in the last 30 years, around 7.85 million acres in 2005-06 as compared to 7.2 million acres in 2002-03. Besides, being the world's fourth-largest cotton producer in the world our yield per acres ranks 13th in the world, as a result Pakistan annually imports around 1.5-2.00 million bales of cotton to meet growing demand from local textile mills, therefore it has become vital for Pakistan to increase its yield per acre (Anonmyous, 2016-17). If we look at the Pakistan scenario, one of the

major yield limiting factors is water deficiency, therefore understanding of the drought tolerance mechanism in crops is needed to be explored.

Drought stress and cotton yield

Cotton is a cash crop of Pakistan primarily known for oil seed and fiber, its contribution to agriculture is 8.62% and 1.83% to the GDP (Anonmyous, 2016-17). *G.arboreum* is diploid and possesses several desired characteristics (resistance to diseases & insect pests and tolerance to drought and salinity), while *G.hirsutum* are lacking these characters but it has high crop yield and fiber quality. *G.arboreum* provides space to cultivate it in semiarid and arid regions with minimum farming inputs. Besides this, it is also considered a vital gene pool source because of its distinctive qualities for improvement of other cotton cultivars via genetic engineering.

Cost of irrigated cotton production is increasing continuously because of ground water supplies are decreasing in Pakistan; it forces to select drought tolerant cultivars as well as other agricultural commodities. Drought stress at very early stage affected the expansion of leaves in cotton and roots are less sensitive as compared to shoot growth (Malik and Srivastava, 1979, Quisenberry et al., 1985). It was found under drought stress leaf expansion was inhibited which results in decrease in utilization of energy and carbon finally result larger portion of assimilates are translocated to plant roots. Hence, characteristics plant roots can be used as important indicator to drought stress.

The consequence of drought stress on total yield of cotton depends upon severity and timing of drought spell. Krieg (Krieg, 1983) reported that crop yield was decreased under drought stress because of reduction in size and number of leaves and decrease in photosynthetic activity. It has also been observed that initiation of square to first flowering stage is the most susceptible development stage affected by water deficiency. The peak flowering stage was the more susceptible to drought stress which results in heavy losses to crop yield (Golldack et al., 2014). Cotton seeds yield was also decreased because of reduction in total number of bolls per plant under drought stress (Hamada, 2000) and also affected quality of lint in several ways, particularly fiber elongation; maturity and length was reported (Krieg, 1983).

Sensitivity of cotton plant to drought stress

Cotton plant is sensitive to drought stress during boll development and flowering stage (Isokpehi et al., 2011, Turner, 1981). The pollen tube formation in cotton is extremely sensitive to drought stress (Kawakami et al., 2010, Burke et al., 1985). There are several stages of flowering and boll formation in cotton because of perennial growth pattern so, the uncertainty

has exists owing to conflicting reports about the sensitive stage of development to water deficiency (Gazanchian et al., 2007). The early flowering period in cotton, according to Umbeck (Umbeck et al., 1987), is sensitive to drought stress while Vereyhen (Vereyken et al., 2001) reported that peak flowering stages is more susceptible to drought stress which results in yield reduction of cotton. The production of fructans in phospholipids initiated under drought stress condition of plant cells (Vierling and Kimpel, 1992)

Cotton bolls appear to be less sensitive to drought stress than the leaves since they are significantly resistant to water loss and are considered essentially non-transpiring (Quisenberry et al., 1985, Kawakami et al., 2010). A number of researchers however, have reported that limited supply of water during boll development can result in significantly lower yields (Koroleva et al., 2005). In support of these observations, (Quisenberry et al., 1985) it was observed that if drought stress occurs during the first fourteen days after anthesis (on set of flowering), young bolls generally abscise (fall off). Chaves *et al*., conducted growth chamber experiments where bract and capsule wall water potential of 5-, 20-, and 30-day old bolls was monitored along with leaf water potential under a moderate and a severe drought stress regime. They reported that mild drought stress had no effect on bract and capsule wall water potentials while leaf water potentials were significantly decreased (Chaves et al., 2009).

A similar pattern was observed under severe drought stress conditions with the exception of the dark respiration rates of the capsule wall that were significantly decreased under drought stress conditions. Bayely *et al.,* (Bayley et al., 1992) reported that the inverted water potential gradient that was observed for the petals was also present in 20-day after anthesis (on set of flowering) bolls. Water and osmotic potential of bracts and subtending to the bolls leaves compared to the bolls. This was attributed to the xylem connections of the fruits being immature and, hence non-functional, until three weeks post anthesis (on set of flowering), and it was concluded that since the water potential gradient is directed from the fruits to the leaves, the main entrance of water in cotton bolls is through the phloem (Vierling and Kimpel, 1992).

Response of plants to drought stress

Morphological and physiological responses of plants

It is reported that drought stress has drastic affects on plant growth while influencing various biochemical, morphological and physiological reactions, like photosynthesis, fluorescence of chlorophyll, stomatal conductance, ion uptake, respiration, translocation, nutrient metabolism, promoters of growth, carbohydrates, proline & malondialdehyde (MDA) contents

and cellular integrity, (Shao et al., 2008, Jaleel et al., 2008, Filippou et al., 2011, Farooq et al., 2009). Susceptibility of crops to various environmental stresses frequently changes with growth stages of plant and requirements for best possible and development growth (Specht et al., 2001). In soybean shoot length and dry biomass was decreased under drought stress (Kawakami et al., 2010). Almost 25% decrease in the length of citrus seedlings observed (Wu et al., 2008) under drought stress. In potato, shoot length of plant was considerably affected because of drought stress (Heuer and Nadler, 1998), *Vigna unguiculata* (Manivannan et al., 2007) and *Abelmoschus esculentus* (Jaleel et al., 2008). Water uptake is also observed to be reduced under deficiency of which results in the decrease of water contents and elongation of cell is also inhibited because of decrease in turgor pressure.

Leaf water potential

Water potential is a potential energy of water per unit volume relative to pure water. It means to quantify the tendency of water to move from one place to another because of osmosis, gravity and mechanical pressure. Relative water potential is useful to understand the water movement within plants. The measurement of leaf water potential is a reliable indicator of drought stress (Fahramand et al., 2014, Farooq et al., 2009). Morgan, (Morgan, 1984) revealed in a study that plants of drought tolerant cotton variety, *G.arboreum* have more number of cells and stomata per unit of leaf relative to the plants of drought intolerant cotton variety, *G. hirsutum*. Ackerson, (Ackerson, 1981) described while comparing 7 drought resistance and drought prone varieties of cotton that drought resistant varieties have minimum leaf water potential and have ability to maintain turgor at lower relative water potential than that of drought prone cotton plants. Because of turgor maintenance, photosynthesis continues in drought tolerant plants while drought intolerant plants are failed in doing so. Photosynthesis in drought tolerant varieties remain at maximum level because chloroplast of fully turgid leaves contain numerous starch granules and has minimum damage to thylakoid membrane structures. So, Quisenberry and McMicheal, (Quisenberry and McMichael, 1991) used the leaf turgidity for the selection of drought tolerant cotton plants.

Relative water content (RWC)

Water content and moisture content is quantity of water contained in soil, rocks, ceramics, fruits and in different tissues of the plants. Relative water content is used in wide range of scientific and technical area. Along with other indicators relative water content can also be used for the identification of drought resistant plants. It has been observed that the older leaves

of *Gossypium arboreum* have relatively low water potential than that of younger leaves. It was further found that older leaves absorb relatively less water than that of younger one which results in the higher relative water content (Knipling, 1967). Similarly, it has been reported that progressive decline in RWC is because of drought stress in plants especially in *Gossypium hirsutum* (Ferreira et al., 1979). In another experiment Assaad and Signer found a positive relationship between RWC and leaf water content. They also co-relate it with genotype of cotton plants especially desi cotton. However, when the stress is disappeared, RWC progressively recovered within 48 hours (Assaad and Signer, 1992).

Cell membrane permeability

Cell membrane is a biological membrane that separates the interior of cell from outside environment. It is a selectively permeable membrane which controls the movement of ions, organic molecules and other important substances (Choffnes et al., 2001). Its basic function is to protect the cell. Various other biochemical reactions are taking place on the interior surface of the cell membrane so, its stability is imperative for all metabolic reactions of the cell. Both biotic and abiotic stresses affect the stability of cell membrane (Kramer, 1979).

Cell membrane stability is influenced by age of the plant, growing season, development of stage, degree of hardening, type of tissue culture and plant species. However, it is observed injury to plasma membrane because of drought stress in maize plants is much less severe in developing leaves as compared to mature leaves (Nath et al., 2005). It was also measured an increase in the saturated fatty acid under stress (Singh et al., 2015), which alleviates the melting point of plasma membrane and in turn reduce the stress tolerance in plant. Somerville and Browse, revealed that total lipid content of leaves in the membrane of *Arabidopsis* plant, growing under high temperature are decreased up to 1/2 and ratio of the unsaturated to saturated fatty acid is also decreased up to 1/3 of the normal temperature. It must be noted, here, that some species can't co-relate with the degree of lipid saturation (Somerville and Browse, 1991). It was concluded that other factors for membrane stability are also involved along with the fluctuation in temperature. The relationship between the cell membrane stability and crop yield under drought condition may vary from plant to plant (Tanou et al., 2012). For example Showler, described such kind of relationship in few plants especially in sorghum (Showler, 2002). However, before Showler, Martin *et al.,* were failed in defining such kind of relationship in soya

bean plants. In short, it can be said that the major cause of yield suppression under drought stress is still obscure and deserve further experimentation (Martin et al., 1993).

Biochemical response of plants under drought stress

Proline content

Proline is an essential amino acid, which is biosynthetically is derived from the glutamate. It is a major osmoregulant in plant tissues under drought conditions. Proline is produced in larger amount as compared to the normal conditions (Alamillo et al., 1995). It is considered as a compatible solute as well as osmo-protectant, which protects the plant tissues by producing stress responsive protein comparative analysis between CIM-496 *G.hirsutum* and FDH-786-*G.arboreum* (Khedr et al., 2003) in plants of *G.arboreum*. Kumar and his coworkers revealed that when water potential becomes the amount of osmolytes which are imperative for osmoregulation, allows additional water from environment. This helps in minimizing the immediate effect of drought stress (Kumar et al., 2003). Similarly, Unyayer and his coworkers, while studying the characteristics of *Helianthus annus* under drought condition observed a strong correlation between proline content and water deficiency (Ünyayar et al., 2004).

In another experiment the over production of proline in transgenic tobacco was reported, which resulted in increase in biomass of roots (Quisenberry et al., 1985). Similarly, Zhu and his colleagues performed experiment on transgenic rice plants, the conclusion showed that drought stress condition causes the decrease in biomass (Zhu et al., 1998). Other scientists around the world like in sorghum (Yadav et al., 2005), bell pepper (Nath et al., 2005), *Gossypium hirsutum* (Massacci et al., 2008), wheat (Hamada, 2000) and in *Catharanthus roseus* (Jaleel et al., 2009) found that amount of proline content increased under drought stress condition.

Chlorophyll content

Chlorophyll is a green pigment present in chloroplast of all green plants and tissues. It is essential for photosynthesis which has ability to absorb light energy and responsible for the carbohydrate metabolism. By measuring the chlorophyll content of a plant tissue, a reliable estimate of photosynthetic rate in green tissues of a plant can be gagged (Ackerson, 1981, Mahmood et al., 2017, Ali et al., 2017). Various studies by different scientists revealed that photosynthetic activity is decreased under drought stress. To prove this notion, Arnon and Whatley, performed experiment on *G. barbadense*, *G. arboreum*, *G. herbaceum* and *G. hirsutum*. It was also found that chlorophyll content, soluble sugar content and photosynthetic ratio is

higher in *G. barbadense,* which is followed by *G. arboreum, G. herbaceum* and significantly by *G. hirsutum* (Arnon and Whatley, 1949).

Krasichkova and his colleagues observed that rate of photosynthetic activity and chlorophyll content is higher in high yielding cotton varieties (Krasichkova et al., 1989). It is observed that total chlorophyll content in *G.arboreum* is decreased with decreasing the soil water potential (Kvint et al., 2003). Similarly, it was also found that content of the chlorophyll b is higher as compared to the chlorophyll a content in various cotton genotypes in drought condition (Burke et al., 1985). Kar and his colleagues while performing experiment on various lines of *G. hirsutum* plants; they maintained that chlorophyll b has affinity to clear weather condition. They also concluded that moisture deficit condition affects the total chlorophyll as well as proline content in *G. hirsutum* (Kar et al., 2001).

Antioxidant enzymes

Antioxidant enzymes in plant tissues i.e. super oxide dismutase, catalases, glutathione, peroxidase and methadone reductase. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a metabolic reaction in which free radicals are produced. In turn these can start chain reactions which can damage or death to the cell. Antioxidant terminates these chain reactions by removing free radicals intermediates and inhibits other oxidation reactions (Wanner and Junttila, 1999). So, antioxidants are reducing agents. Drought stress in addition to dehydration also induces oxidative stress such as generation of active oxygen species (ROS) including super oxide radical (O^{2-}), nasent oxygen (O), hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-). Their production is injurious to the cell (Nepomuceno et al., 1998). Xu and his colleagues revealed that antioxidant species cause the auto-catalytic oxidation of membrane lipids and pigments then leading to the loss of membrane semi permeability and modification in its functions. Among antioxidant species superoxide radical (O^{2-}) is regularly synthesized in a chloroplast and mitochondria (Xu et al., 2006). However, some of its quantity is also produced in micro-bodies. The quenching of super oxide radical by super oxide dismutase (SOD) results in production of hydrogen peroxide (H_2O_2). However, both O^{2-} and H_2O_2 are not toxic to the cell as OH^- is injurious to cell, which is formed by the combination of O^{2-} and H_2O_2 in the presence of trace amount of Fe^{2+} and Fe^{3+} (Monk et al., 1989). The O^{2-} can damage chlorophyll, protein, DNA, lipid and other important micro-molecules. Thus affect the plant metabolism and limit the crop yields.

Sairam and Tyagi, found that plants have, developed a series of both enzymatic and non-enzymatic detoxification systems to counteract activated oxygen species (AOS), thereby protecting the cells from oxidative damage (Sairam and Tyagi, 2004). Similarly, it was also found by Kosmidou and his colleagues that various physiological and metabolic reactions have been affected by the over expression of super oxide dismutase (SOD) (Voloudakis et al., 2002).

Molecular response of plants to drought stress

The molecular details of a plant's response against stresses are complicated. These involve receptors, transcription factors, genes, noncoding RNAs, ions, and enzymes etc. Despite of the variability the plant response against a stress condition begins with the perception of the signal by specific receptors. Plants perceive dehydration by one of the following two mechanisms (Chaves et al., 2003):

1. Through changes in osmotic potential; the membrane protein AtHK1 (Histidine Kinase 1) senses the change in osmotic potential produced inside the cell, while EcHKT1 works in the similar for sensing changes in extracellular environment.
2. Through changes in membrane texture; the dehydration results in interaction of cationic and anionic amphiphilic substances which changes the membrane texture which is sensed by membranes proteins like OpuA.

The above mentioned proteins initiate a series of signaling events. These involve many molecular respondents. Urao and his colleagues identified three phospho-relay intermediates (ATHP1-3) and four response regulators (ATRR1 -4). These molecules are supposed to play role in post perception events; however, their function is not yet clear (Urao et al., 2000). Post perception events are shown to include phosphorylation and dephosphorylation of phosphatases and changes in cytoplasmic Calcium concentration (Luan, 1998). These events then result in activation of various signaling cascades. These relative unclear cascades divide dehydration response in to branching one that involves ABA and the other which works independent of ABA.

ABA dependent responses are major cellular responses against stress. Two enzymes of ABA biosynthesis have been shown to respond to the cellular perception of stress namely these enzymes are Zeaxanthin epoxidase (ZEP) and 9-cis-epoxycarotenoid dioxygnase (NCED) (Taylor et al., 2000, Qin and Zeevaart, 1999). Stomata closure, maintenance of root growth and restricted leaf expansion are some of the many consequences of ABA activities in different organs of the plants. ABA mediated signaling cascades that make the mentioned things to

happen were reviewed (Bray, 2002). ABA independent pathways are of limited importance for stress response. Mostly these involve genes which have conserved dehydration response element (DRE) in their promoters (Luan, 1998).

Cotton responds to stresses by bringing a large number of changes in its morphology and physiology. Tracking these changes down to molecular level leads to the conclusion that the basic framework of cotton response is similar to other plants but for the large part the specific effectors used by cotton are unique to this plant. ABA mediated responses remain an important part of cotton cells' response to osmotic stress; however, osmotin is shown to be a very important downstream target of ABA in cotton. This protein has binding sites of several TFs in its promoter and has capability to interact with different proteins hence working as a hub in molecular response to osmotic stress (Wilkinson et al., 1995). Trehalose 6 phosphate synthase gene is believed to be important in stress signal transduction suggesting that Trehalose 6 phosphate has a vital role as a secondary messenger in cotton (Kosmas et al., 2006). ABA mediated responses, for the large part, work by the involvement of calcium. In stressed cotton cells calcium based activities are mostly driven by calmodulins. Owing to the importance of calmodulins cotton produce a specific heat shock protein named Heat Shock Protein Camodulin Binding (HSPCB). This protein has the duty to bind with calmodulin and keep it active so that it's able to play its role efficiently (Voloudakis et al., 2002).

Contribution of genetic engineering to drought tolerance

Drought tolerant genes have been identified while investigating the molecular mechanisms of plants response to drought stress. These genes were isolated and characterized by transferring them into drought prone plant species. This approach in some cases has been found successful to increase agronomic performance and crop yield. A good example of this success story is transgenic wheat expressing *HVA1* gene from barley, encoding late embryogenesis abundant (LEA) proteins. Results showed that the *HVA1* protein confers a significant protection from drought stress (Bahieldin et al., 2015). Aquaporins mediate symplastic water transportation in plants could be a limiting factor for growth under unfavorable environmental conditions. Differential expression of these genes during plant development that encode for aquaporins has been observed to be associated with various physiological processes. Such processes include opening and closing of stomata, cell elongation, cell division and organ movement (Berriman et al., 2009). The *SITIP-2* gene coding aquaporin protein was found predominantly effective to

improve drought stress tolerance in tomato plants (Hajheidari et al., 2005). Another successful gene is *OsNAC10,* introduced in rice plants under the control of *GOS2*constitutive promoter and *RCc3* root-specific promoter (Tang and Page, 2013).

Gene cloning and expression

Gene cloning and expression makes it possible to transfer biological properties from one organism to another. This exciting field of research owes its spectacular development to emergence of tools for DNA manipulation, enhancements and extensions in the existing knowledge, novel ways that investigators are using to apply the available technologies and finally the rapid pace with which research is being carried out in this field.

The idea of transferring a gene between organisms was first conceived and materialized in the decade of 1970. Phages have the ability to transfer portions of DNA between bacteria through generalized or specialized transduction mechanisms. It was reported in early 1970s that some linear phage DNAs contain sticky ends on their terminals and that in some abnormal conditions a linear phage DNA may be separated into two or more pieces while retaining the sticky in their original positions. In their first effort to attach two DNA fragments investigators used TdT, an enzyme which adds poly A or poly T tails to 3' blunt ends, and DNA ligase. A small part of bacterial DNA was isolated and it was treated with TdT similarly two fragments of phage DNA were treated with complementary TdT and finally these three fragments were ligated with DNA ligase. This experiment resulted in the creation of a circular phage DNA containing a bacterial DNA fragment in it. The hence produced circular phage DNA was found to be the target of restriction enzyme EcoRI which was observed to cut this circular DNA on one place and make it linear. In final step this EcoRI cut linear phage DNA was ligated with a similarly prepared phage DNA containing antibiotic resistance gene. The resulting circular DNA which consisted of two phage DNAs and two fragments from different bacteria was transformed in *E. coli* where it was propagated. Figure 1 shows a schematic representation of the various steps in the creation of first chimeric DNA (Berg and Mertz, 2010).

Universal Stress Protein (USP)

A protein that contains a USP domain (a characteristic USP structure) is be referred to as a universal stress protein (USP). A USP may contain one or more USP domains a fact that allows USPs to perform a board range of functions (Isokpehi et al., 2011). USP gene was first discovered in bacteria via 2D gel electrophoresis and was named as C-13.5 based on its migration during 2D experiment. Later studies recognized these proteins as part of all stress and

starvation stimulus known at that time and thus these were renamed as Universal Stress Proteins (Kvint et al., 2003). USP genes are involved in wide range of metabolic activities. These have been described to play role in bacterial virulence, heat shock, cold shock, DNA management and metabolic control (Persson et al., 2007, Loukehaich et al., 2012). In most cases the mechanisms of functions performed by USPs are still undiscovered but for a few functions some information is gathered about the mechanisms.

When a plant is subjected to water stress, ABA level is increased which resulted in expression of USP genes. This is consistent with the finding that USPs can bind with transcription factors (Gury et al., 2009). One of the two activated gene clusters produces LHCB (Light Harvesting Chlorophyll a/b Binding) proteins. LHCB keeps the chloroplasts intact and reduces the stomatal aperture to preserve water during water stress. The second gene cluster produces some osmo-protective solutes e.g. proline which protects the cells from harmful effects of ROSs (Loukehaich et al., 2012). In water stress USP is believed to interact with Annexin protein but the details about how actually the USP performs its functions are still to be elucidated.

E coli responds to salt stress by producing an ion transporter called (KdpFABC) which transports the extra salt ions out of the bacterial cell. The production of this transporter comes after induction of its gene with a complex of KdpD and KdpE. This complex is only formed when KdpE is phosphorylated. In excess ions KdpE phosphorylation is inhibited. Here USP comes to play its part such as USP phosphorylates the KdpE and then hold it with KdpD forming the complex which induces the production of KdpFABC (Heermann et al., 2009). Another similar mechanism is also described in *Halomonas elongate*. Here the USP instead of inducing the expression of transporter binds itself with the transporter named TeaABC making it active. The study on *H. elongate* TeaABC and USP interaction stresses on the assertion that ATP binding USP does not play any role in transcription regulation (Huang et al., 2012). Owing to their importance several studies have been conducted to find out the structure of USPs. These investigations reveal that the structure of USP remains similar in different organisms. A USP contains an ATP binding motif at its N-terminal while the C-terminal region takes different forms depending upon the context of the protein. In many proteins the C-terminal region also binds with an ATP molecule making USP capable of binding with two ATPs at a time (Gonzali

et al., 2015). The N-terminal ATP binding motif in various USPs have high % of glycine amino acids which allow USP-proteins to attach with ATP molecules (Drumm et al., 2009).

Plant transformation

Conventional breeding methods were slow and laborious; to beat these limitations plant transformation methods were developed for the production of genetically engineered plants. Through transformation gene of interest can be introduced into plants without altering their vital characters. Plant transformation method is the set of events used to introduce a fragment of DNA, having specific trait, into host plant. By utilizing this method plants are engineered to produce new varieties with desirable traits. It can be achieved either by *Agrobacterium tumefacien*-mediated transformation or by gold particle bombardment. In the first method plant cells are infected with pathogenic *Agrobacterium tumefaciens* bacterium possessing the desired gene. In the later procedure gene gun is used for the gene coded bombardment of particles. Both of these methods are extensively used in research applications (Tinland, 1996, Tzfira et al., 2004, Somerville and Browse, 1991).

***Agrobacterium*-mediated transformation**

Agrobacterium tumefaciens-mediated transformation is leading technology used for production of transgenic plants. The genus *Agrobacterium* has been classified into various species because of its disease symptomology and host range (Otten et al., 1984). *A tumefacien,* naturally present in soil, it penetrates in plants at wound sites and initiates the formation of tumor, disease commonly known as crown gall (Smith and Townsend, 1907). The crown gall disease has been observed because of the transfer of T-DNA (transfer DNA) from tumor-inducing (Ti) plasmid from *A. tumefaciens* to plant cells (Zaenen et al., 1974) and integrated into plant genome (Chilton and Que, 2003). Two genetic elements are required for transfer of T DNA to plants. The first element is 25bp direct repeats defining and flanking region of T-DNA border sequence (Zambryski et al., 1983). The second element virulence genes (*vir*) encoded by the Ti-Plasmid in a region present outside of the T-DNA region. The *vir* genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome. In plant transformation, use of T-DNA process is because of three facts. Firstly, the tumor is formed that resulted from integration of T-DNA and its subsequent expression Secondly, the T-DNA genes do not play role during their transfer process, they are only transcribed inside plant cells. Thirdly, any gene of interest placed between T-DNA borders can be transferred to plant cell.

Cellular localization of gene expression

Eukaryotic cell organelles are membrane bounded there for various cellular activities are restricted to specific well defined organelle inside the cell. These cell organelles have been studied via cell fractionation method and by analyzing samples of fixed tissues. Information about localization of sub-cellular protein is first footstep towards understanding its function (Kokkirala et al., 2010) and this process direct the retention and transportation of protein complexes into tissue specific location. It is imperative to understand complex metabolic processes in various plant tissues such as fruits, roots stem and leaves. To study the metabolism in abundant plant tissues is comparatively easy, because the whole tissue can be used as the sample while, less abundant plant tissues are difficult to be used as sample because their basic metabolic reactions are masked by more abundant plant tissues (Carrigan et al., 2011). It is also difficult in case of plants to understand the specific function of single protein due to the presence of multi gene families. So, it is imperative to compare among different patterns of multi gene families at sub-cellular level (Hanson and Köhler, 2001).

The cellular location of different regulatory proteins and enzymes in plant cells during different stages of development, under diverse environmental circumstances is indicated its functional pathway. Mostly, prediction models of bioinformatics are used for location of different proteins. Moreover, localization of several plant proteins has been found at numerous cellular regions (Small et al., 1998). Green fluorescent protein is green light emitting protein, when it is excited with lower wavelength light. Light emitting proteins also know as fluorescent proteins (FPs), they are classified as brand range family of fluorescent proteins and GFP-proteins belong to this family. Now GFPs are being used in various applications of molecular biology (Zhang et al., 2002). Common use of fluorescent protein has one main advantage of its normal light emitting process with involvement of any enzyme or substrate (Ei-Shemy et al., 2009).

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abolishing the GFP function. This signifies that most of the structure of GFP is important for development and maintenance of fluorescence.

GFPs (like all FPs) consist of 11 beta sheets, small alpha helices and some irregular peptides. The beta sheets come together and form a rigid structure which is known as "Beta Barrel". The beta sheets in a barrel are connected with each other through small helices and flexible proline rich peptides. In addition an alpha helix is present in the center of the beta barrel. This helix contains a highly conserved sequence of three amino acids which come together and form the structure which is responsible for the production of fluorescence (Khedr et al., 2003). The structure thus formed is termed as chromophore (sometimes as fluorophore). The location of chromophore in GFP is very important for its function. The beta barrel has polar amino acids branching out towards the chromophore and hold water molecules hence producing an environment for chromophore to exhibit fluorescence. In addition the barrel also protects the chromophore from the outside environment (Foolad, 2004, Zhang et al., 2002). The native GFP which was isolated from *Aequorea Victoria* is found to be of just a little usage. Research carried out on GFP resulted in modification of native GFP to produce a broad range of derivatives which can be used more readily in the molecular biology applications. These new derivatives of GFP have some properties to make them suitable. The modifications introduced in GFPs along with the resultant properties can be categorized into following categories:

The modification made in the chromophore sequence result in the production of different coloured GFP derivatives. Such changes in the sequence produce different energy states of chromophore as compare to the native, which are capable of emitting light of different wavelengths (producing colours other than green). By using same mechanism, the chromophore modifications can also alter the excitation wavelength of the native GFP hence producing derivatives which absorb different wavelengths while producing same colour (Foolad, 2004). This kind of shift is especially helpful in fine tuning the GFP for the fluorescence detection system present in place. In addition, change in chromophore sequence also is shown to be associated with increased emission of a particular wavelength i.e. increased luminescence (Berriman et al., 2009).

Site directed mutagenesis

Point mutation or site directed mutagenesis has been used to ascertain the function of unknown gene; this technique causes alteration at specific point in the sequence of a gene. This

is also known as oligonucleotide directed mutagenesis. Point mutations can be randomly inserted in the whole sequence of gene at multiple locations or it can be specifically integrated at predetermined location by site directed mutagenesis (Sturm, 2009). It can be carried out both *in-vivo* and *in-vitro*. Model organisms are used in case of *in-vivo* while plasmid constructs are used in case of later. To ascertain the importance of amino acids and their function in protein structure, site directed mutagenesis has been used extensively (Ishii et al., 1998, Kunkel et al., 1991). It can be utilized to study protein function and structural relationship, protein binding sites, active sites present in enzymes, gene characterization and protein-protein interaction.

To alter the sequence of gene of interest, synthetic oligonucleotides are extensively used in research. Numerous protocols have been used and for this purpose while PCR-mediated site directed mutagenesis was found efficient most common method (Saiki et al., 1985). For this purpose two complementary mutagenic primers (40bp long) with mutated nucleotide in its center are designed by using online software (Zhang et al., 2015). Laible and Boonrod carried out site directed mutagenesis of whole plasmid by using non-PCR thermo cycling reaction. This technique was used to produce mutated enzyme to wipe out its enzymatic activity to utilize as experimental model (Laible and Boonrod, 2009). Dipetarudin protease is potent inhibitor of thrombin it inhibits the normal function of trypsin and plasmin (Lopez-Molina et al., 2002). When single amino acid (Arginine-10) was replace by histidine then mutant form, dipetarudinR10H, had lostits activity to inhibit plasmin and trypsin as compared to wild type. In the beginning, artificial oligonucleotides were being used for the rectification of site directed mutagenesis in β-globin gene which causes sickle cell anemia (Saiki et al., 1985). Rectification of sickle cell anemia was a turning point in commercializing this technology (Beetham et al., 1999, Saiki et al., 1985, Zhang et al., 1995, Zhu et al., 2000).

Initially, site directed mutagenesis was used in tobacco and corn, model species. Point mutation in acetohydroxy acid synthase I &III genes, at specific sites made them resistant agaist herbicide both in corn and tobacco (Zhang et al., 2015). Endo and his colleagues mutated acetolactate synthase (ALS) gene of rice at specific point by using site directed mutagenesis and developed resistance against bispyribac herbicide. They reported that change in two amino acids separately in two different clones causes tolerance in bispyribac herbicide. These two amino acids are Serine and Tryptophan which replaced with Isoleucine and Leucine respectively (Endo et al., 2007). However, if both amino acids will change at a time then it confers bispyribac

herbicide resistance to plant. This technique has broad range of applications because it can create several mutations, insertions and deletions. This can also be used for the characterization of unknown genes responsible for fatal diseases. Now commercial kits are easily available which makes it faster, reliable and efficient (Carrigan et al., 2011). This technique has been utilized for the customization of various crops for introduction of desired traits and for increase per capita yield.

Conclusion

In view of above discussion it can be concluded that drought stress affect morphological, physiological, biochemical and molecular traits of cotton plants which became the major cause of yield reduction. Drought stress is important threat among abiotic stresses, which has drastic affects on normal growth of plants. It is considered as major reason to low productivity of cotton in Pakistan and situation could worse in future because depleting irrigation capacity. Drought stress is natural and spontaneous it can't be controlled with either synthetic chemicals or skilled agricultural practices. Modern view about control of drought stress is production of transgenic crops having tolerance towards drought stress. Drought tolerance mechanism is controlled by multiple genes, so, manipulation of one or two drought stress related gene could not be much effective. It is need of hour to understand the cellular mechanism of drought tolerance for future engineering of tolerant plants. USP genes have been identified in one variety of cotton which could be manipulated for drought tolerant transgenic cotton plants with high yielding as well. Several soluble sugars and stress proteins have been reported to act as protectant under drought stress and universal stress protein (USP) is the most important family of proteins in this regard. This family encompasses a conserved and ancient group of proteins that are present and has been reported in different organisms including cotton, where they are playing role in response to heat shock, cold shock, DNA management, metabolic control. Furthermore, USP is a regulatory unit protein; its activity can be increased by manipulating its interactions.

Author contribution statement

MNH wrote the initial draft of manuscript. SZ edited the manuscript and make minor corrections. QA make final editing and corrections in manuscript to make it in its final version to be published. All of the authors have proof-read the manuscript before submission. The final approval for publication was given by KM.

Conflict of interest

The authors declared that there is no any conflict of interest for manuscript.

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