Chapter 3
Transcriptomics of Heat Stress in Plants

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Abstract High-temperature stress is a major abiotic stress that affects various biological processes of plants such as biochemical and physiological response, growth, development, and yield. High-temperature stress has critical effects at cellular and molecular levels also. The increased concentration of regulatory proteins such as heat shock transcription factors (Hsfs) is a major molecular response that occurs during heat stress. These regulatory proteins in turn regulate the expression of heat shock protein (HSP) genes that act as critical players during stress to maintain cell homeostasis. Besides HSPs, the other metabolic and regulatory genes, signaling compounds, compatible osmolytes, and antioxidants too play an important role during heat stress in plants. Apart from the protein-coding genes, recent studies have shown that noncoding microRNAs (miRNAs) also play a key role during heat stress by modulating the gene expression at the transcription and post-transcriptional level. The transcriptome approaches are important to understand the molecular and cellular changes occurring in response to heat stress. The approaches rely mostly by adopting the traditional methods like Northern blot/RNA blot and reverse transcription PCR (RT-PCR), where the expression of the genes can be studied in different tissues and cells, whereas the extent of their expression can be achieved by quantitative PCR or real time PCR. Further, the genome-wide expression profiling tools such as microarray analysis, next-generation sequencing, and RNA sequencing offer a great potential in this direction. This chapter primarily provides the current understanding on the role of regulatory genes (transcription factors), HSP genes, metabolic genes, signaling compounds, osmolytes, reactive oxygen species,

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and miRNAs as well as other small RNAs of plants under high temperature. In addition, it gives a brief account of various transcriptome approaches to study the expression profiling of genes during heat stress.

Keywords High temperature · HSPs · Strategies · Transcription factors · miRNAs

1 Introduction

Elevation of global mean temperature beyond the optimum by various means is termed as high-temperature stress or heat stress. In a changing climate scenario, heat stress is considered as a serious threat for food crop productivity (Hall 2001) and is a major challenge in attaining food security. The extent of rise in temperature in specific climatic zones depends on the period of high temperature occurring during the day and the night. Apart from the day temperature, increase in night temperature also shows a serious effect on crop productivity. A considerable rise in gaseous emissions is thought to be one of the reasons for increasing concentrations of greenhouse gases like carbon dioxide, methane, nitrous oxides, chlorofluorocarbons, etc. It is predicted that rise in these greenhouse gases will ultimately increase the global ambient temperature (Wahid et al. 2007). A report of the Intergovernmental Panel on Climatic Change (IPCC) suggested global mean temperature rises between about 0.15 and 0.3 °C per decade for 1990–2005. It says that "continued greenhouse gas emissions at or above current rates would cause further warming and induce many changes in the global climate system during the 21st century that would very likely be larger than those observed during the 20th century."

Heat stress can cause severe damage to almost all the stages of crop growth and may lead to death of the plants if it causes an irreversible damage in cellular homeostasis, destruction of metabolic pathways, and degradation of structural and functional proteins/metabolites and membranes which ultimately lead to cell death (Vinocur and Altman 2005; Bohnert et al. 2006). As plants are sessile in nature, they have adapted different mechanisms to tolerate stress caused by high temperature. Avoidance mechanism is one of the important mechanisms adapted by the plants to cope with the stress which includes change in leaf orientation, transpirational cooling, alteration of membrane lipid profile, and excessive rooting (Lehman and Engelke 1993; Bonos and Murphy 1999). The other mechanism called tolerance mechanism includes activation of free radical scavengers, osmoprotectants, ion transporters, increased concentration of heat shock transcription factors (Hsfs), heat shock proteins (HSPs), late embryogenesis abundant (LEA) proteins, switching on different signal transduction cascades, production of metabolites, etc. (Wahid et al. 2007). In several crop plants, it is reported that under high temperature, plants attain the reproductive stage faster with compromise in total yield which is described as an escape mechanism (Adams et al. 2001).

Morphologically, high temperature causes significant reduction in relative growth and dry weight. Reduced size of internodes, more tillering, early senescence, and reduced biomass in sugarcane plants was observed during high temperature (Ebrahim et al. 1998). It may lead to production of polymorphic leaves to reduce water loss by transpiration (Sayed 1996). Anatomical changes such as cell size reduction, closing of stomata, and condensed water loss are reported during heat stress (Banon et al. 2004). Crop plants affected by heat during anthesis and grain filling stage show yield reduction. In wheat, heat stress increases the period of grain filling with kernel growth reduction that leads to the reduction in density and weight of kernel by 7% (Guilioni et al. 1997) and ultimately a reduction in grain number (Ferris et al. 1998). Similarly, in maize, decreased accumulation of proteins, starch, and lipid content of the kernel was observed (Wilhelm et al. 1999). In rice, heat stress affects anthesis causing irreversible effects on reproductive tissues and leads to spikelet sterility (Prasad et al. 2006; Oh-e et al. 2007; Jagadish et al. 2008). Yield loss due to heat stress was reported in groundnut (Vara Prasad et al. 1999) and Phaseolus (Rainey and Griffiths 2005).

Heat stress affects the physiological and biochemical processes of plants. Plant hormones such as cytokinins and ethylene play regulatory roles in heat stress tolerance (Veselov et al. 1998; Musatenko et al. 2003; Xu and Huang 2009). Stress shows significant impact on protein biochemistry such as proteins synthesis, folding, posttranslational modifications, targeting, and degradation depending on the level and duration of high temperature. Further, heat injury causes loss of membrane integrity and inactivates chloroplast and mitochondrial enzymes (Howarth 2005). Increased respiration during high temperature causes synthesis of reactive oxygen species (ROS) in plants (McDonald and Vanlerberghe 2005). Heat stress damages the mesophyll cells and increases the plasma membrane permeability (Zhang et al. 2005). At the subcellular level, major changes can be observed in chloroplasts such as reduced photosynthesis by alteration in the thylakoids structural organization (Karim et al. 1997). It also affects microtubule organization and spindle formation in mitotic cells (Smertenko et al. 1997).

Biological membrane integrity and function are sensitive to high temperature which changes the tertiary and quaternary structures of membrane proteins. Regular function of biological membranes under stress is crucial for important physiological processes such as photosynthesis and respiration (Blum 1988). Rapid movement of molecules across membranes during heat stress disrupts the chemical bonds present in cellular membranes. Fluidity of the lipid bilayer in membranes also occurs by protein denaturation and increased concentration in unsaturated fatty acids (Savchenko et al. 2002). These changes increase the permeability of membranes resulting in electrolyte leakage. Thus, cell membrane thermostability (MTS) is a measure of increased electrolyte leakage and has been used in a wide variety of species like wheat (Blum et al. 2001), rice (Mohammed and Tarpley 2009), cotton (Ashraf et al. 1994), soybean (Martineau et al. 1979), sorghum (Marcum 1998), barley (Wahid and Shabbir 2005), etc. to understand the heat tolerance mechanism. The positive correlation between MTS and yield was observed in crops like wheat (Reynolds et al. 1994) and sorghum (Sullivan and Ross 1979). Early effects of

high-temperature stress on the plasmalemma leads to fluidity of the membrane lipid bilayer which in turn increases Ca^{2+} influx and reorganization of cytoskeleton. This helps in transducing the activation of various signal molecules (Sung et al. 2003).

The heat stress-induced morphological, cellular, physiological, and biochemical changes described above are ultimately governed by the expression of a set of genes or transcripts. Change in osmotic level, ion concentrations, and membrane fluidity during heat stress activates several genes encoding transcription factors and signaling molecules to activate different pathways involved in the production of various compounds to maintain cellular homeostasis. The study of transcriptome during heat stress provides information about the regulatory roles of different genes involved in stress tolerance and susceptibility. Transcriptomics can be defined as a study of gene expression through mRNA profiling. Increase in the availability of genome and transcriptome sequence data for model crop species has helped in understanding the molecular events leading to various pathways of stress response. Complete genome sequences are available for several plants species. *Arabidopsis thaliana* was the first plant genome sequenced (Kaul et al. 2000) followed by rice (Barry 2001; Yu et al. 2002; International rice genome 2005) and sorghum (Bedell et al. 2005; Paterson et al. 2009). Whole genome sequencing has been done in other important food crops like pigeonpea and soybean. The advancement of genomic tools, particularly next-generation sequencing (NGS) technologies, has helped in understanding the genome structure and information of many plant species. Besides the genomic structure and genes organization, understanding the expression pattern of the genes or transcriptomics is very crucial to fully appreciate the usefulness of genome sequence information. Unlike genomics, transcriptomics is highly dynamic in nature as the expression of genes is unique to tissue, stage of plant growth, environmental stimuli, etc. Further, the complexity of transcriptomics is increased due to different levels of regulations at the posttranscriptional level, particularly alternate splicing, alternate polyadenylation, genes/transcripts fusion, RNA editing, and posttranscriptional gene silencing (PTGS).

Traditional methods like northern blot analysis and RT-PCR have helped to understand the gene expression of plants to a certain extent. The efforts on this direction were further augmented with the advent of semiquantitative and qPCR / real-time PCR. Gene expression can be quantified in terms of relative and absolute numbers by using real-time qPCR. However, these methods can help in understanding the expression of only few known genes at a time. Genome-wide transcription profiling is an important and powerful tool to understand the regulation of genes at the molecular level during stress which can be achieved by using medium- and high-throughput methods. Medium-throughput methods include complementary DNA (cDNA) clones and expressed sequence tags (ESTs) for transcriptomic study, whereas microarray and NGS are high-throughput methods. EST and cDNA sequences provide a direct evidence for all the generated transcripts and they are the most important resources for transcriptome study (Nagaraj et al. 2007). Microarray technology provided a boost to plant biology in understanding genome-wide gene expression in several plant species. With the help of this tool, many stress-associated genes could be discovered in the last decade. Microarray analyses have been

demonstrated in investigations of transcriptional networks occurring in a variety of developmental processes (Lee et al. 2002; Anisimov 2008). It provides a pattern in gene expression as well as metabolic network models (Xiang et al. 2011). However, microarray was difficult to use in crops where genome sequence data were not available. Further, it could identify the expression of only known genes, hence several novel and rare genes playing important roles in plant metabolism were unattended while studying transcriptome using microarray.

In addition, microarray could not specify the posttranscriptional changes in genes which are very important to understand transcriptomics. One step further in this direction, RNA-Seq technologies provide genome-wide information of transcriptomics where limitations of microarray could be sorted out. These tools facilitate the investigations of structural and functional complexity of the transcriptome. The expression level of almost all transcripts in a sample is quantified by measuring the number of individual mRNA molecules transcribed from each gene. RNA-Seq is a more reliable technique for comparative gene expression profiling studies as it gives an overall view of transcripts expression (Wang et al. 2009; Garber et al. 2011). This technology has been utilized in transcriptome studies of various crops such as maize, rice, soybean, etc. (Eveland et al. 2010; Zhang et al. 2010; Severin et al. 2010). This book chapter provides an overview of important heat-stress-regulated genes/transcripts encoding proteins such as chaperones, Hsfs, antioxidants, signaling molecules, osmolytes, etc. Also, the genes influencing the photosynthesis process during high temperature are discussed. In addition to protein-coding mRNAs, this chapter describes the regulatory role of small RNAs (sRNAs) during heat stress. Further, different transcriptomic approaches to study the expression of transcriptomics during stress are also given.

2 Gene Expression During Heat Stress

High-temperature stress induces the alteration of transcriptome in different tissues and stages of plant growth. Heat-responsive genes have been studied in various crops which constitute a significant proportion of the genome. With the progress in genomic technologies, a remarkable progress has been made towards deciphering the transcriptional networks during heat stress in plants. Heat stress response (HSR) in plants and other organisms is generally characterized by the induced expression of a set of proteins known as molecular chaperones or HSPs. Expression of the HSPs is in turn governed by another important heat-stress-induced gene family encoding Hsfs. Plant genome possesses a highly complex multigene family encoding HSPs and Hsfs. In addition to HSPs and Hsfs, transcripts encoding enzymes involved in ROS scavenging, osmolyte accumulation, synthesis of signaling molecules, and photosynthesis machinery are greatly influenced by heat stress. Recently, 25, 29, 26, 9, and 10 genes have been identified in the rice genome encoding Hsfs, small HSP (sHSP), HSP70, HSP90, and HSP100 families, respectively (Hu et al. 2009).

2.1 Heat Shock Proteins

Synthesis and accumulation of HSPs are ascertained during high-temperature stress in plants. Biosynthesis of HSPs during temperature stress is a common response in all living organisms starting from bacteria to plants and human beings (Vierling 1991; Gupta et al. 2010). Acquired thermo-tolerance due to the accumulation of HSPs has been studied (Bowen et al. 2002). HSPs are induced in plants at all the stages of development during heat stress (Vierling 1991). These HSPs are categorized into gene families based on their molecular weight, sequence homologies, and functions: HSP60, HSP100, HSP90, HSP70, and small HSP family (Gupta et al. 2010). These HSPs act like molecular chaperones by inhibiting irreversible aggregation of other proteins as well as participating in refolding of proteins during high-temperature stress to maintain cellular homeostasis (Tripp et al. 2009). Further, HSPs help in preventing the denaturation of other proteins caused by heat stress. Also, HSPs help in shuttling and transporting of other proteins inside the cell. Plants differ significantly in the expression of different kinds and number of HSPs (Hamilton et al. 1996). Not only individually but also in combinations with other proteins the HSPs play a crucial role during heat stress. For example, in many plant species, HSP100 family proteins acquired thermo-tolerance due to the induction of HSP70 as well as HSP101 during heat stress (Schoffl et al. 1999).

2.1.1 HSP60

HSP60 are also called as chaperonins which are evolutionarily homologous to the GroEL protein of *Escherichia coli*. These proteins are present in prokaryotes as well as eukaryotes. In eukaryotes, HSP60 proteins are found in cytosol, mitochondria, and chloroplasts. Chaperonins present in bacteria, mitochondria, and chloroplasts are categorized under group I, such as GroE chaperonins and chCpn60, whereas in Archaea and the cytosol of eukaryotes, they are categorized into group II, such as CCT (chaperonins containing t-complex polypeptide 1; TCP1) chaperonins (Ranson et al. 1998). Generally, these chaperonins assist plastid proteins like Rubisco (Wang et al. 2004) and play an important role in folding and aggregation of other proteins. HSP60 helps in the translocation of proteins to different organelles such as chloroplasts and mitochondria (Lubben et al. 1989). In a mutant of *Arabidopsis,* chloroplast chaperonin Cpn60α showed defects in development of chloroplast (Apuya et al. 2001).

2.1.2 HSP70

Role of HSP70 in different plant species has been studied. These proteins are expressed constitutively as well as in response to environmental stimuli. Constitutively expressed HSP70 helps in the folding of newly synthesized proteins and the

transport of precursor proteins. Environmentally induced HSP70 chaperones along with other co-chaperones (e.g., GrpE and DnaJ/HSP40) act as a protein complex involved in protein folding processes and in preventing aggregation of nonnative proteins under abiotic stress conditions in almost all cellular compartments (Hart 1996; Wang et al. 2004). Furthermore, it has a wide variety of functions in important phases of protein metabolism such as protein synthesis, transport, degradation, folding, and activation of denatured proteins (Zhang et al. 2005). They facilitate the lysosome- or proteasome-mediated degradation of unstable proteins (Wang et al. 2004). HSP70 together with sHSPs act as molecular chaperones to protect the plant cells from deleterious effects caused by heat stress (Rouch et al. 2004). HSP70 participates in adenosine triphosphate (ATP)-dependent protein assembly to prevent protein denaturation during high-temperature stress (Iba 2002). The cells where HSP70 synthesis was blocked were more susceptible to heat injury (Burke 2001). A recent study in *A. thaliana* showed that chloroplast HSP70 is involved in heat tolerance (Su and Li 2008). Schroda et al. (1999) showed that chloroplast HSP70B participates in photo-protection by repairing photosystem II (PSII) proteins during photo-inhibition. HSP70 also plays a key role in the expression of other stress-associated genes and in the modulation of signal transducers. The genes encoding HSP70 are constitutively expressed in plants cells; however, they get upregulated during stress.

2.1.3 HSP90

HSP90 class proteins are one of the most abundant proteins present in a cell which acts on proteins associated with signal transduction such as kinases and steroid hormone receptors. These proteins work in an ATP-dependent fashion and mainly regulate the cellular signals of glucocorticoid receptor activity (Pratt et al. 2004). Besides their major role as molecular chaperones to assist protein folding, these proteins are also associated with signal transduction cascades, cell-cycle regulation, degradation, and trafficking of proteins (Pratt and Toft 2003). In plants, HSP90 is present in the cytosol, ER, and plastids. In *A. thaliana,* it helps in stress adaptation. It forms a multiprotein complex with HSP70 and other co-chaperones to perform its function. Yamada et al. (2007) suggested that under normal conditions, cytoplasmic HSP90 inhibits the activity of Hsfs, while during heat stress, the temporary suspension of HSP90 activity leads to the activation of Hsfs.

2.1.4 HSP100

HSP100, also called Clp proteins, are ATP-dependent chaperones. They solubilize aggregated proteins and help in protein degradations (Boston et al. 1996). In order to maintain cellular homeostasis, degradation of nonfunctional and harmful polypeptides synthesized due to misfolding, denaturation, or aggregation is important (Wang et al. 2004). These proteins in association with HSP70 (ATP-dependent

chaperone system) perform the function of protein disaggregation and refolding. HSP100 plays an important role in plants during severe heat stress (Hong and Vierling 2000). Induced expression of HSP100 proteins have been reported in several plant species, such as *Arabidopsis* (Schirmer et al. 1994), rice (Pareek et al. 1995), maize (Nieto-Sotelo et al. 2002), soybean (Lee et al. 1994), and lima bean (Keeler et al. 2000). Similar to other HSP proteins, HSP100 family chaperones are constitutively expressed in general; however, their expression gets upregulated during stress. In rice, the immunological homologue of yeast HSP104 gets accumulated during heat shock (Singla and Grover 1993). The disappearance of protein granules in yeast was associated with the production of rice HSP100. Moreover, dissolution of electron-dense granules by HSP101 was shown after heat stress, implicating its role during recovery of cell stress (Agarwal et al. 2003). Thermo-tolerance activity of HSP100 family proteins was proved through genetic approaches also (Lee et al. 1994; Schirmer et al. 1994). Increased expression of mitochondrial HSP68 in maize, soybean, tomato, and barley was observed during heat stress (Neumann et al. 1993).

2.1.5 Small HSPs

Low molecular weight (LMW) HSPs of about 12–40 kDa are designated as sHSPs; Vierling 1991; Sun et al. 2002). In comparison to other HSPs, these proteins are more diverse in terms of sequence similarity, localization, and functions. sHSPs cannot refold nonnative proteins by themselves but they can provide stability and prevent nonnative protein aggregation through binding to nonnative proteins, thereby helping ATP-dependent chaperones for subsequent refolding (Wang et al. 2004). During normal conditions, sHSPs are usually not detectable in plant tissues, but during stress conditions, they are induced to impart acquired stress tolerance (Scharf et al. 2001; Zhang et al. 2008a). Based on the abundance and diversification of sHSPs, probably plants have the differential ability to adapt during heat stress conditions. Plant sHSPs are encoded by six nuclear multigene families which are present in different cellular compartments. Class I and class II gene products are present in cytosol, whereas others are present in the chloroplast, endoplasmic reticulum, mitochondria, and membranes (Waters et al. 1996). In *A. thaliana* and *Lycopersicon esculentum,* sHSPs were divided into three subclasses such as subclass CI, CII, and CIII (Scharf et al. 2001; Siddique et al. 2003). A recent study on *A. thaliana* reported other groups in the cytoplasm that were categorized into CIV, CV, CVI, and CVII. Each subclass has its own distinct characteristic specified role (Siddique et al. 2008). Higher plants usually possess up to 20 sHSPs and each different species may contain up to 40 sHSPs (Vierling 1991). It was reported that during heat stress conditions, the expression of class I sHSPs in soybean can increase up to 1% (Hsieh et al. 1992). In the absence of stress, the expression of some sHSPs in plants is confined to certain developmental stages (Sun et al. 2002).

3 Heat Shock Transcription Factors

In plants, the expression of HSPs is a common phenomenon observed during heat stress. The expression of HSPs is in turn regulated by Hsfs and the process is termed as HSR. Generally, eukaryotes possess one to four HSF family members, whereas plants possess a large number of Hsfs. For instance, *Arabidopsis* has 21 HSF members, while rice genome possesses 25 HSF members (Nover et al. 2001). Hsfs control the expression of HSPs in plants by binding specifically to the heat shock element (HSE), a highly conserved region having palindromic motifs of nGAAn (Miller and Mittler 2006). Plant Hsfs are characterized by possessing a DNA-binding domain at the N-terminal (helix-turn-helix type) followed by two hydrophobic heptad repeats (HR-A and HR-B) known as the oligomerization domain, a nuclear localization signal (NLS) for nuclear uptake of the protein, and a nuclear export signal (NES) for exportation. Apart from this, a C-terminal activation domain (CTD) rich in hydrophobic, aromatic, and acidic amino acid residues commonly known as AHA motifs, is essential for the activation of HSF (Nover et al. 2001). Based on the protein structures, three classes of Hsfs have been identified in plants, namely HsfA, HsfB, and HsfC (Nover et al. 2001). HsfA and HsfC have a long HR-A/B region. HsfA possesses the insertion of 21 amino acids, whereas HsfC possess the insertion of 7 amino acids between the hydrophobic regions HR-A and HR-B. HsfB and HsfC do not possess AHA motifs at their C-terminal ends (Nover et al. 2001; Kotak et al. 2004).

Hsfs are transcriptional regulators, having a well-defined role in heat stress signaling and regulation of several downstream target genes. Expression of genes encoding Hsfs is induced during elevated temperature (Liu et al. 2005, 2009). Upregulation of Hsfs in *Arabidopsis* was reported in response to high-temperature stress (Swindell et al. 2007). Overexpression of HSF genes in transgenic plants resulted in the upregulation of heat-stress-associated genes as well as enhanced thermo-tolerance, whereas the downregulation of HSF genes results in the reduction of thermo-tolerance (Schramm et al. 2008). Accumulation of HSPs during the overexpression of HsfA1 suggested a unique function of this HSF as the master regulator for induced thermo-tolerance in tomato (Mishra et al. 2002). The expression of OsHsfA2a gene was greatly stimulated by high-temperature stress in root and shoot tissues of rice (Chauhan et al. 2011a). In *Arabidopsis,* HsfA1 was shown to be the primary regulator of heat stress, while HsfA2 was essential for prolonged heat stress (Charng et al. 2007). Ogawa et al. (2007) reported that the expression of HsfA2 showed higher expression among all 21 *Arabidopsis* Hsfs in response to heat stress. In tomato, constitutive expression of LeHsfA1 showed improved tolerance to high-temperature stress, whereas silencing of LeHsfA resulted in decreased thermo-tolerance (Mishra et al. 2002). Similarly in *Arabidopsis,* overexpression of AtHsfA2 gene in transgenic plants increases tolerance to the environmental stresses and altered expression of some heat-responsive genes. In addition, acquired thermotolerance by using the knockout mutant of AtHsfA2 (Li et al. 2005; Schramm et al. 2006; Charng et al. 2007) was also demonstrated. In the same way, overexpression of rice Hsf OsHsfA2e in transgenic *Arabidopsis* responded to high temperature and showed acquired thermo-tolerance.

4 Reactive Oxygen Species

ROS are reactive chemicals derived from molecular oxygen either by energy transfer or by electron transfer reactions (Gill and Tuteja 2010). These are produced as normal metabolites during cellular metabolism in chloroplasts, mitochondria, and peroxisomes. ROS play very important role in various aspects of plant metabolism such as growth, development, cell cycle, hormone signaling, stress response, and programmed cell death. The production and removal of ROS is tightly regulated; however, the equilibrium gets disturbed during heat stress and other abiotic as well as biotic stresses. In *Arabidopsis,* at least 152 genes are involved in maintaining the steady-state level of ROS (Mittler 2002). Increased concentration of ROS in plants causes oxidative damage by damaging the cellular and membrane proteins, lipids, carbohydrates, and DNA. ROS include free radicals such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2), alkoxy radical ($RO^{\bullet}$), peroxy radical ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), and excited carbonyl ($RO^{\bullet}$). Under steady-state conditions, these free radicals are scavenged by different antioxidative defense mechanisms (Foyer and Noctor 2005). Stress-induced ROS are efficiently scavenged by either enzymatic or nonenzymatic antioxidants defense system. Major enzymes involved in ROS scavenging are superoxide dismutase (SOD), ascorbic peroxidase (APX), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), and guaiacol peroxidase (GOPX). Nonenzymatic antioxidant defense system includes ascorbic acid, glutathione, alkaloids, phenolic compounds, α-tocopherols, and nonprotein amino acids (Apel and Hirt 2004; Gill and Tuteja 2010).

4.1 Super Oxide Dismutase

SOD is a metalloenzyme present in subcellular compartments of all aerobic organisms that catalyzes the dismutation of superoxide into O_2 and H_2O_2, which is a vital antioxidant defense mechanism. This enzyme provides the first line of defense mechanism against the toxic effects produced by ROS and imparts crucial role in stress tolerance (Apel and Hirt 2004; Gill and Tuteja 2010). SODs have been classified into three groups based on the metal cofactor: Fe-SOD (ferrous SOD), Mn-SOD (manganese SOD), and Cu/Zn-SOD (copper/zinc SOD). Fe-SOD, generally not detected in plants, is localized in chloroplasts, while Mn-SOD is localized in mitochondria and peroxisomes. Cu/Zn-SOD has been detected in fractions of chloroplasts and cytosol (Del Rio et al. 1996; Gill and Tuteja 2010). Increased activities of SODs have been shown in plant cells under stress conditions, which is a part of the defensive mechanism under oxidative stress (Ushimaru et al. 1995). In the *A. thaliana* genome, three Cu/Zn-SOD genes, namely CSD1, CSD2, and CSD3, three Fe-SOD genes, namely FSD1, FSD2, and FSD3, and one Mn-SOD gene (MSD1)

have been identified (Kliebenstein et al. 1999). In rice seedlings, the expression of cytosolic Cu/Zn-SOD was stimulated under heat stress (Shah and Nahakpam 2012).

4.2 Catalase

Decomposition of H_2O_2 into H_2O and O_2 is catalyzed by the heme-containing enzyme called catalase. It has the highest turnover rate by converting ~6 million of H_2O_2 molecules per minute. It plays a very crucial role during oxidative stress by removing H_2O_2 produced by oxidases during β-oxidation of fatty acids, photorespiration, and purine catabolism in peroxisomes (Mittler 2002; Vellosillo et al. 2010). It is a light-sensitive enzyme that has different isoforms. *Arabidopsis* possesses three genes, namely CAT1, CAT2, and CAT3, encoding polypeptides that associate to form at least six isozymes (Frugoli et al. 1996). In *Brassica,* 12 isozymes and in *Helianthus annuus* cotyledons, four isozymes were reported (Azpilicueta et al. 2007). Three isoforms of maize are differentially expressed and independently regulated. CAT1 and CAT2 are present in peroxisomes and cytosol, whereas CAT3 is present in mitochondria (Scandalias et al. 1990). In order to improve salinity tolerance of *Oryza sativa,* the rice cultivar Nipponbare was genetically transformed with a catalase gene of *E. coli*. The transgenic rice plants showing constitutive expression of catalase gene could grow for > 14 days in the presence of 250 mM NaCl (Nagamiya et al. 2007). Expression of wheat catalase gene in transgenic rice showed tolerance to cold stress, which may be attributed to the effective detoxification of H_2O_2 by increased catalase activities (Matsumura et al. 2002).

4.3 Ascorbate Peroxidase

APX performs a similar function as catalase. APX performs its action in chloroplast, glyoxisome, and cytosol of plant cells in scavenging ROS and protecting cells in higher plants during stress. It uses ascorbic acid as a hydrogen donor to break down H_2O_2 to form H_2O and monodehydroascorbate (Asada 2000) and is also involved in the electron transport through the ascorbate–glutathione cycle (Foyer and Noctor 2005). It has five different isoforms showing higher affinity towards its substrate when compared to CAT. In cytosol, it has two different isoforms (cAPX), while in chloroplasts soluble (sAPX) and thylakoid-bound forms (tAPX) are reported (Asada 2000, 2006). Another form in the membrane of glyoxisomes (gmAPX) is also present. It was reported that cytosolic APX1 plays an important role in protecting the plants from heat stress (Koussevitzky et al. 2008). Overexpression of the APX-like 1 gene (CAPOA1) of *Capsicum annuum* in transgenic tobacco plants conferred tolerance to oxidative stress (Sarowar et al. 2005). Hsu and Kao (2007) showed that pretreatment of rice seedlings with H_2O_2 resulted in enhanced APX activity and protected seedlings from cadmium (Cd) stress.

4.4 Monodehydroascorbate Reductase and Dehydroascorbate Reductase

MDHAR is a flavin adenine dinucleotide (FAD)-containing enzyme having high specificity towards its substrate monodehydroascorbate (MDHA). Its isoforms are located in chloroplast, cytosol, mitochondria, and peroxisomes. It is involved in the regeneration of reduced ascorbate. MDHA is a very good electron acceptor (Noctor and Foyer 1998; Asada 2000) that accepts electrons from nicotinamide adenine dinucleotide phosphate (NADPH). Reduction of MDHA to ascorbate is attained by using electrons derived from the photosynthetic electron transport chain. It has been reported that increased concentration of MDHAR activity scavenges harmful ROS (Karuppanapandian et al. 2011) and confers chilling stress tolerance in tomato (Stevens et al. 2008). Dehyroascorbate (DHA) is an oxidized form of DHAR which regenerates ascorbic acid. Overexpression of DHAR conferred abiotic stress tolerance in *Arabidopsis* and tobacco (Ushimaru et al. 2006; Eltayeb et al. 2006).

4.5 Glutathione Reductase

GR, mainly present in chloroplast, is a tripeptide flavoprotein oxidase. It catalyzes the reduction of glutathione (GSH) by disulfide bond formation in glutathione disulfide (GSSG) and is crucial in maintaining the equilibrium of GSH via NADPH-dependent reaction. Enhanced GR activity in plants due to the accumulation of GSH provides stress tolerance (Rao and Reddy 2008). Further, GR plays an important role in the regeneration of GSH that provides resistance against oxidative stress (Ding et al. 2009b). Expression of GR is greatly affected by various stresses including high temperature, chilling, exposure to heavy metals, etc. (Apel and Hirt 2004; Karuppanapandian et al. 2011). Sharma and Dubey (2005) reported increased activity of MDHAR, DHAR, and GR in rice seedlings under drought stress.

4.6 Ascorbic Acid

Ascorbic acid is the most abundant water-soluble antioxidant present in almost all types of plant cells. Biosynthesis of ascorbic acid from L-galactono-γ-lactone dehydrogenase and regeneration from oxidized ascorbate takes place in mitochondria. It detoxifies ROS by donating its electrons to a wide range of reactions. It has the ability to scavenge $O_2^{\bullet -}$, $OH^{\bullet}$, and $^1O_2$ directly, and can reduce the concentration of H_2O_2 through APX. Its concentration is maximum in matured leaves with developed chloroplasts and high chlorophyll content. It gives protection to membranes by regenerating α-tocopherol from tocopheroxyl radical. High content of ascorbic acid showed improved tolerance to oxidative stress in tobacco and *Populus* (Aono et al. 1993; Foyer et al. 1995).

4.7 Glutathione

GSH (γ-glutamyl cysteinyl glycine) is an important metabolite involved in scavenging ROS. It occurs as a reduced form localized in cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes, and in apoplast (Mittler and Zilinskas 1992; Jimenez et al. 1998) and plays a crucial role in several physiological events such as detoxification of xenobiotics, transport of sulfate, conjugation of metabolites, and signal transduction (Xiang et al. 2001). A central nucleophilic cysteine residue is critical for the high reductive potential of GSH. It potentially scavenges cytotoxic H_2O_2 along with other ROS molecules, such as $^1O_2$, $O_2^{\bullet-}$, and $OH^{\cdot}$ (Noctor and Foyer 1998). Additionally, GSH plays a major role in the antioxidative defense system in combination with ascorbate through the AA–GSH cycle (Foyer and Halliwell 1976). Plants with increased GSH concentrations were found to be more tolerant to oxidative stress (Pietrini et al. 2003).

4.8 Tocopherols and Carotenoids

Tocopherols (TOCs) are lipophilic antioxidants mainly present in the thylakoid membrane of chloroplasts of plants. In higher plants, chloroplast membranes containing tocopherols protect lipids and other components of membrane from physical quenching and ROS, thus guarding the structure and function PSII (Igamberdiev et al. 2004). Among the four isoforms (α, β, γ, and δ), α-TOC is having the highest antioxidative activity.

Carotenoids are also lipophilic in nature and play multifunctional roles during stress in plants. They act as detoxifying agents during various environmental stresses and protect photosystem complexes (Karuppanapandian et al. 2011). Carotenoids like β-carotene and zeaxanthin play important role by dissipating excess excitation energy as heat and by scavenging ROS. β-Carotene inhibits oxidative damage by preventing the formation of $^1O_2$through direct quenching of triplet sensitizer, Chl^{3*} (Collins 2001).

5 Signaling Compounds

High-temperature stress in plants induces various molecules and ions for sensing and signaling. Rise in cytosolic Ca^{2+} is the primary signal during temperature stress (Larkindale and Knight 2002), which leads to several changes in the plant gene expression and metabolism. Induced concentration of Ca^{2+} along with calcium-dependent protein kinases (CDPK) regulates the expression of HSPs (Sangwan and Dhindsa 2002). Further, increase in cytosolic Ca^{2+} content facilitates plant cells to better survive during heat injury by increasing the activity of antioxidants and turgor maintenance of the guard cells (Webb et al. 1996; Gong et al. 1997). It activates

other signaling pathways through mitogen-activated protein kinase (MAPK) cascade system (Larkindale and Knight 2002). In plants, MAPK is most the important signal transduction cascade that responds to external signal (Kaur and Gupta 2005). Gong et al. (1997) reported that high-temperature stress induces Ca^{2+} uptakes and activates calmodulin (CaM)-related genes in plants. Other signaling compounds known to be involved in HSR are CaM, inositol-3-phosphate (IP3), abscisic acid (ABA), and ethylene. Specific groups of signaling molecules like salicylic acid, calcium chloride ($CaCl_2$), ABA, H_2O_2, and 1-aminocyclopropane-1-carboxylic acid (ACC) may enhance heat stress tolerance by protecting from oxidative damage (Larkindale and Huang 2004).

6 Osmolyte Accumulation

Genes associated with osmolytes synthesis and accumulations are involved in HSR. The increased accumulation of compatible solutes (osmolytes) helps in osmoprotection through maintaining cellular turgidity. In addition, it facilitates antioxidation and chaperoning through direct stabilization of membranes and/or proteins (Yancey et al. 1982; Hare et al. 1998). Different plants may accumulate different types of osmolytes such as sugars, sugar alcohols, proline, tertiary sulfonium compounds, and ammonium compounds (Sairam and Tyagi 2004). Accumulation of these solutes confers heat stress tolerance to the plants. These osmolytes may function as buffering agents during heat stress conditions and other abiotic stresses (Wahid and Close 2007). Glycine betaine (quaternary amine), a compatible solute, plays a crucial role under different abiotic stresses (Sakamoto and Murata 2002). Increased concentration of glycine betaine was observed in maize (Quan et al. 2004) and sugarcane (Wahid and Close 2007) during high-temperature and water-logging conditions.

Proline is an osmolyte which usually accumulates under environmental stress in plants (Kavi Kishore et al. 2005). Proline is synthesized from L-glutamic acid through Δ^1-pyrroline-5-carboxylate, which is catalyzed by two enzymes Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and reductase (P5CR) in plants (Verbruggen and Hermans 2008). Free proline plays the role of osmoprotectants for scavenging different ROS species (Ashraf and Foolad 2007; Trovato et al. 2008). Transgenic tobacco transformed with proline dehydrogenase gene showed more proline accumulation and enhanced osmotolerance (Teteishi et al. 2005). In rice, transgenic plants possessing P5CS gene showed the accumulation of proline that conferred tolerance during water deficit and high salt conditions (Su and Wu 2004). In addition to proline, accumulation of soluble sugars under high-temperature stress was observed in sugarcane (Wahid and Close 2007). Sugars like trehalose, fructans, or mannitol get accumulated during stressed conditions. Several efforts have been made for the production of these sugars through transgenic approaches (Hare et al. 1998).

7 Photosynthesis and Heat Stress

Photosynthesis is considered as one of the most heat-sensitive phenomena of plants. The effect of heat on photosynthesis may occur through reduction in chlorophyll accumulation, disruption in chloroplast membrane, and proteins/enzymes and stomata functioning. The expression of transcripts/genes associated with the photosynthetic machinery of plants is altered during heat stress.

7.1 Effect on Photosystem II and Oxygen Evolving Complex

Heat stress affects greatly the PSII as well as the electron transport chain (Mathur et al. 2011). Primary effect of heat stress is observed on photosynthetic reactions of thylakoid membrane and stroma of chloroplast (Wise et al. 2004). Heat stress may result in the dissociation of oxygen evolving complex (OEC) that leads to imbalance in the flow of electrons from OEC to PSII (De Ronde et al. 2004). The repair mechanism of PSII also gets damaged due to the production of ROS during heat stress that results in the reduction of carbon fixation (Allakhverdiev et al. 2008). Sites in the PSII reaction center were damaged due to high temperature and excessive light in wheat (Sharkova 2001). In barley, damage of PSII units leads to the loss of activity of OEC and restriction of electron transport which was aborted completely after 4 h (Toth et al. 2005).

7.2 Chlorophyll Fluorescence and Reduction

The relation between heat tolerance and fluorescence patterns has been revealed in several plant species (Moffatt et al. 1990). Maximum quantum efficiency of PSII (Fv/Fm) is used to measure the stress in various abiotic stresses including heat stress (Baker and Rosenqvist 2004). It gives information of the maximum energy input into leaf and functions upstream of other potential stress responses. Decrease in Fv/Fm reflects the reduction of maximum quantum yield in photosynthesis (Ogren 1988). Chlorophyll a and b degradation during high temperature was reported more in matured leaves (Karim et al. 1997, 1999). Moreover, decrease in chlorophyll to carotenoids ratio and increase in chlorophyll a to b ratio was reported in tomato implicating that these changes would make the plants tolerant to high temperatures (Camejo et al. 2005; Wahid and Ghazanfar 2006). Chlorophyll a quantification is one of the important methods to measure plant response to heat stress (Maxwell and Johnson 2000; Baker and Rosenqvist 2004).

7.3 Photosynthetic Enzymes

High-temperature stress causes thermal instability of Rubisco (Feller et al. 1998) and impairs the process of carbon assimilation which in turn affects carbohydrate reserves. This leads to the shortening of grain filling duration by fastening the rate of development, and early induction of flag leaf senescence, thus causing reduction in total yield (Yang et al. 2002). Effect of high temperature is observed more in the photosynthetic activity of C3 plants than in C4 plants. Heat stress also affects the synthesis of other metabolites such as starch and sucrose, which are in turn greatly influenced by reduced activity of enzymes such as sucrose phosphate synthase (SPS; Chaitanya et al. 2001), invertase (Vu et al. 2001), and ADPglucose pyrophosphorylase (AGPase). The instability of AGPase, a key regulator of starch biosynthesis, results in starch accumulation during high-temperature stress (Singletary et al. 1994; Linebarger et al. 2005; Yamakawa et al. 2007). Other enzymes like branching enzymes and starch synthases are negatively regulated by heat (Singletary et al. 1994; Yamakawa et al. 2007).

8 Small NonCoding RNAs

Endogenous noncoding sRNAs of 21–25 nts in size are also involved in the plant stress response through a silencing mechanism. These are broadly classified into four categories: microRNAs (miRNAs), natural antisense transcripts small interfering RNAs (nat-siRNAs), trans-acting siRNAs (ta-siRNAs), and repeat-associated siRNAs (Jamalkandi and Masoudi-Nejad 2009). Mostly, sRNAs are produced from long precursor RNAs of double strands or single strand forming a self-complementary hairpin structures. These precursor RNAs are used as a substrate to produce sRNAs by four different dicer-like proteins (DCL) proteins, DCL1, 2, 3, and 4 (Chapman and Carrington 2007). miRNAs are generated from DCL1 protein; nat-siRNAs are produced by DCL1 and DCL2, while DCL3 is involved in the production of heterochromatic siRNAs. DCL4 is involved in the formation of ta-siRNAs. It has been well established that besides the protein-coding mRNAs, noncoding sRNAs play a key regulatory role in abiotic stress response in plants.

8.1 MicroRNAs

miRNAs are endogenous single-stranded sRNAs of 21–25 nt in size produced from single-stranded primary transcript termed as pri-miRNAs (Tang et al. 2008). These sRNAs regulate gene expression. The miRNAs get into the posttranscriptional gene silencing pathway, leading either to degradation of the target mRNA or to

translational repression. miRNAs suppress the expression of target mRNA with the help of a protein complex known as RNA-induced silencing complex (RISC). Since the discovery of the first miRNA lin-4 in *Caenorhabditis elegans,* which regulates the larval timing during development (Lee et al. 2003; Reinhart et al. 2000), a large number of miRNAs have been reported and characterized in both plants and animals. Plant miRNAs were first reported in early 2002 (Llave et al. 2002; Park et al. 2002; Reinhart et al. 2002). Initially, miRNAs were considered to regulate largely transcription factor genes involved in a variety of plant developmental processes (Llave et al. 2002; Reinhart et al. 2002; Rhoades et al. 2002). A number of reports suggested a regulatory role of miRNAs in plant growth and development such as leaf and flower differentiation, flowering time, floral identity, and auxin response (Sunkar et al. 2005; Mallory and Vaucheret 2006). Further research in this area showed that these miRNAs play a vital role in regulating genes associated with abiotic and biotic stresses also (Sunkar and Zhu 2004; Sunkar et al. 2006; Mishra et al. 2009).

Plant miRNAs have been shown to regulate gene expression under heat stress also. Solexa sequencing revealed differential expression of miRNAs in wheat in response to heat (Xin et al. 2010). Out of the 32 identified miRNA families in wheat, 9 were conserved miRNAs and found to be putatively heat responsive. miRNAs miRs156, 159, 160, 166, 168, 169, 393, and 827 were upregulated under heat stress, whereas miR172 showed significant downregulation. In *Brassica rapa,* sRNA library was constructed from the seedlings exposed to high temperature. By deep sequencing, 35 miRNA families were found to be conserved with *A. thaliana*. Within those families, five miRNA families were heat stress responsive. Two miRNAs, miR398a and bra-miR398b, were downregulated during heat stress, whereas the corresponding targets genes—BracCSD1 (copper superoxide dismutase)—were upregulated. Similarly, miR156h and miR156g were upregulated by heat and their putative target BracSPL2 was downregulated (Yu et al. 2011). In *Populus,* solexa sequencing of two sRNA libraries generated from heat stress and control tissue revealed 52 heat-stress-responsive miRNAs from 15 families that included 16 novel miRNAs (Chen et al. 2012). In rice, 62 libraries of sRNAs were constructed and sequenced using Illumina sequencing. These sRNA libraries were constructed from control as well as stress tissues subjected to various stress treatments. Approximately 94 million reads matched with genome resulting in 16 million diverse sRNA sequences. Out of these, 400 were annotated miRNAs, 150 were siRNA like, and 76 new miRNAs were found. In this study, miRNAs involved in regulation in response to water, nutrient, and temperature stress were identified. miR397b.2 expression was upregulated and its target gene, L-ascorbate oxidase, was downregulated during heat stress (Jeong et al. 2011). Another miRNA, mir444, targets the Hsf-type transcription factor (Koskull-Doring et al. 2007). Similarly, miR169 targets nuclear transcription factor Y subunit (a drought-induced protein) and CCAAT-binding transcription factor (Li et al. 2010; Zhou et al. 2010).

8.2 Small Interfering RNAs

siRNAs are sRNAs involved in transcript silencing from which they originate (Bartel 2004). Double-stranded RNAs (dsRNAs) of diverse origins such as viruses, transposons, transgenes, etc. are cleaved into 21–24-nts siRNAs by multiple DCL proteins. These siRNAs are then loaded into RISC complex containing the argonaute protein. This complex binds to the mRNA from which they originate and silences its expression. Further, siRNAs bind to mRNA and convert single-stranded RNA into dsRNA by RNA-dependent RNA polymerase (RDRP), thus amplifying siRNA production. Change in the expression of four siRNAs in wheat seedlings during cold, heat, salt, and drought stresses was reported. Furthermore, siRNA007927_0100_2975.1 was downregulated by all stresses except heat stress (Yao et al. 2010) and the remaining three siRNAs—siRNA002061_0636_3054.1, siRNA 005047_0654_1904.1, and siRNA080621_1340_0098.1—were downregulated during heat stress conditions.

8.3 Trans-acting siRNAs

Ta-siRNAs (21-nt RNAs) are generated by processing of miRNA from TAS gene transcript, with respect to the miRNA cleavage site. In plants, the target mRNA expressed from ta-siRNA loci is cleaved by miR173 and miR390. After cleavage, they are modified into dsRNA by RDRP enzymes and processed into siRNAs which ultimately target the degradation of mRNA different from the transcript of ta-siRNA from which they originated. In *Arabidopsis,* miR173 recognizes TAS1 and TAS2 transcripts, whereas TAS3 and TAS4 are recognized by miR390 and miR828, respectively (Allen et al. 2005). Ta-siRNAs showed enhanced expression in hypoxia-treated tissues of *Arabidopsis* (Moldovan et al. 2009).

9 Approaches/Tools to Study Transcriptomics

Increase in the availability of complete genome sequence information from several plant species such as *Arabidopsis,* rice, *Populus,* chickpea, pigeonpea, etc. has paved the way to perform genome-wide function analysis of plant genes contributing to heat stress tolerance. Transcriptomic study of an organism can be performed by using different throughput methods like low, medium, and high (Fig. 3.1). Low-throughput method comprises single gene expression analysis, medium-throughput methods are ESTs and cDNA clones, and high-throughput methods include microarray and deep sequencing approaches. In order to perform single gene expression analysis, various techniques like northern blot analysis, RT-PCR, and qPCR can be used (Lockhart and Winzeler 2000). Genome-wide transcriptome analysis can be performed using high-throughput technologies such as microarray and NGS platforms. This has an advantage over individual gene analysis tools as interactions

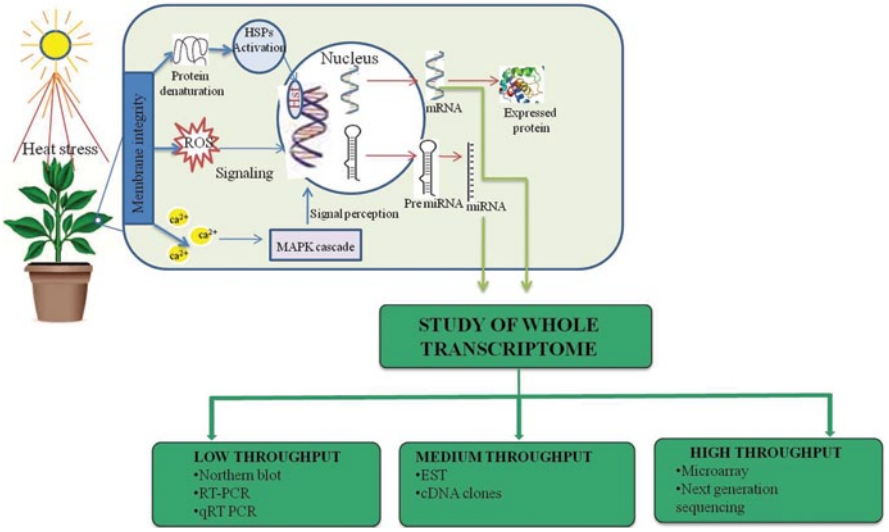

Fig. 3.1 An overview of molecular regulation of heat stress response of plants and approaches of transcriptomics

between different genes, pathway analysis, and action of regulatory elements can easily be understood by using high-throughput technologies. Several studies have used these methods in different plants to study the effect of heat stress on transcriptome (Table 3.1). In addition, recently, these tools have been successfully utilized in sRNA profiling of plants during abiotic stresses (Table 3.2).

9.1 Northern Blot Hybridization

Study of the expression of a particular gene during stressed conditions in plants can be achieved by using northern blotting. It involves the separation of RNA on agarose gel, transfer of RNA from the electrophoresed gel to Hybond-N^+ membrane, and detection by means of a hybridized probe, sequence which is complementary to a part or the entire target gene. This technique is generally used to detect the expression of targeted mRNA as well as sRNAs. It can be used to study the expression of only those genes for which sequence information is available. Northern blot analysis was used to demonstrate that OsBADH1 mRNA expression was upregulated by different abiotic stresses, whereas downregulated by heat stress (Hasthanasombut et al. 2011). The expression of heat-inducible genes such as Hsf (HsfB2a), HSP (HSP101), and cytosolic ascorbate peroxidase (APX1) was reduced in wrky25 mutants. Overexpression of WRKY25 enhanced the expression level of heat-responsive genes HsfA2, HsfB1, HsfB2a, and HSP101 (Li et al. 2009). Also, expression pattern of glutathione reductases was studied in rice, wheat, barley, and maize during photooxidative stress using northern blot analysis (Melchiorre et al. 2009).

Table 3.1 Heat-stress-induced transcriptomics studies of plants using various approaches/tools

S. No	Plant species	Approach used	Differentially regulated genes	Reference
1	Rice	RT-PCR	Spl7 (rice spotted leaf gene)	Yamanouchi et al. 2002
2	Rice	RT-PCR	*OsHsfA4b, OsHsfA5, OsHsfA7, OsHsfA4d, OsHsf A2a, OsHsfA2c, and OsHsfA2d*	Liu et al. 2010
3	Rice	RT-PCR	*OsHSP80.2, OsHSP74.8, OsHSP71.1, OsHSP26.7, OsHSP24.1,OsHSP17.0, OsHSP58.7, OsHSP50.2, and OsHSP23.7*	Zou et al. 2009
4	Rice	RT-PCR	*HSP70* gene	Goswami et al. 2010
5	Rice	Microarray	Hsfs, HSPs, chitinase, cellulase, cell wall invertase 2, beta-expansin, chalcone synthase, and isoflavone reductase family protein genes	Zhang et al. 2008b
6	Rice	Microarray	Hsfs, sHSPs, members of HSP70, HSP90, and HSP100 gene families	Hu et al. 2009
7	Rice	Microarray	*OsHsfA1a, OsHsfA2a, OsHsfA2c, OsHsfA2d, OsHsfA2f, OsHsfA4b, OsHsfB2a, OsHsfB4b,* and *OsHsfC1a* genes	Mittal et al. 2009
8	Rice	Microarray	*HSP17.4-CI, HSP17.9B-CIX, HSP23.2-ER, HSP18.6-CI, HSP24.0-MI, HSP26.2-MI, HSP16.6-CVIII, HSP16.9C-CI,* and *HSP18.0-CII*	Sarkar et al. 2009
9	Rice	Microarray	*OsClpB-cyt, OsClpB-m,* and *OsClpB-c*	Singh et al. 2010
10	Rice	Microarray	*OsHsfA2a, OsHsfA2c, OsHsfA2d, OsHsfB2a, OsHsfB2b, OsHsfB2c,* and *OsHsfC1a*	Chauhan et al. 2011a
11	Rice	Microarray	Hsfs, bZIP TFs, *HSP10s,* and *HSP20s*	Jung et al. 2013
12	Rice	Microarray	*sHSP (HSP17.4), HSP30, HSP70, HSP90, cytochrome P450,* and *CBL-1* gene	Mittal et al. 2012
13	Rice	Microarray	*HSP20, HSP40, HSP70, HSP90, clpB 1, HSP101, OsSTI1, OsSTI2a,* and phosphosulfolactate synthase related	Jung and An 2012
14	Rice	Microarray	*HsfA2a, HsfA2d, HsfA2f, HsfA3, HsfB2a, Hsfb, Hsfc, DREB, ERF,* and members of HSP70, HSP90, and HSP100	Zhang et al. 2012
15	*Arabidopsis*	Microarray	*HsfHsfA2, HsfB1, Hsf-A4a, HsfB2a, HsfB2b,* and *HsfA7a*	Busch et al. 2005
16	*Arabidopsis*	Microarray	*DREB2A, DREB2B, DREB2C,* and *DREB2H*	Lim et al. 2006

Table 3.1 (continued)

S. No	Plant species	Approach used	Differentially regulated genes	Reference
17	*Arabidopsis*	Microarray	*HSP90, HSP70, and HSP101*	Yamada et al. 2007
18	*Arabidopsis*	Microarray	Members of *HSP20, HSP70, HSP90,* and *HSP100*	Swindell et al. 2007
19	*Arabidopsis*	Microarray	*HSP101, HSE7, APX2, HSFA7, NF-XI, Pro oxidase, SGT1a HSP110* (HSP70-15), and choline kinase	Larkindale and Verling 2008
20	*Arabidopsis*	Illumina sequencing	*SR45a*	Gulledge et al. 2012
21	*Arabidopsis*	qRT-PCR	Rubisco's Chaperone activase (RCA), *AtRCAβ2, AtRCAα,* and *AtRCAβ*	Deridder et al. 2012
22	*Arabidopsis*	Illumina	*HSP25.3-P* and *HSP22.0-ER*	Li et al. 2013
23	Wheat	Microarray	*Hsfs, HSPs, DREB2B and DREB6A, ERETC,* and member of *MBF1*	Qin et al. 2008
24	Wheat	Microarray	b-ZIP transcription factors and TaCAM3-1(zinc finger with calmodulin)	Chauhan et al. 2011b
25	Wheat	Microarray	HSPs, transporters, protein modifiers, and signaling molecules	Khurana et al. 2011
26	Maize	qRT-PCR	*ZmHsf-01, ZmHsf-03, ZmHsf-04, ZmHsf-06, ZmHsf-10, ZmHsf-11, ZmHsf-14, ZmHsf-15, ZmHsf-19, ZmHsf-20, ZmHsf-21, ZmHsf-22, ZmHsf-23, ZmHsf-24, and ZmHsf-25*	Lin et al. 2011
27	Maize	EST	*HSP22*	Lund et al. 1998
28	Tomato	Microarray	sHSP genes, members of *HSP70, HSP101,* and *HSP90* families, *HSFA2* and *HSFA3*	Frank et al. 2009
29	Tomato	Microarray	Class I sHSP 17.6, class II sHSP 17.6, class III sHSP, DNA-J and mitochondrial sHSP, protein similar to AthHSP22.3, and cytosolic ascorbate peroxidase	Bita et al. 2011
30	Barley	Microarray	Raffinose synthase 1, UDP-D-glucose 4-epimerase 1, UDP-D-glucose 4-epimerase 3, trehalose-6-phosphate synthase, trehalose-6-phosphate phosphatase, invertase inhibitor, heat shock transcription factor A2d, hexokinase 2, and SNF1-related protein kinases 2.6	Mangelsen et al. 2011
31	*Populus*	RT-PCR	*Hsf-03, Hsf-13, Hsf-15, Hsf-2, Hsf-22,* and *Hsf-23*	Wang et al. 2011

Table 3.2 Expression studies of plant microRNAs during abiotic stresses using different transcriptomic approaches

S. No	Stress	Plant species	Approach	Expressed miRNAs (up-/downregulated)	References
1	Heat	Wheat	Solexa sequencing	miR156, miR159, miR160, miR166, miR168, miR169, miR172, miR827, and miR2005	Xin et al. 2010
2	Heat	*Brassica*	Illumina sequencing	miR156h, miR398a, miR398b, miR399b, and miR827	Yu et al. 2011
3	Heat	*Populus*	Illumina sequencing	miR156, miR166, miR167, miR168, miR396, miR397, miR408, miR1444, miR473, miR530, miR160, miR394, miR395, miR408, miR472, miR482, miR530, and miR1450	Chen et al. 2012
4	Heat	*Arabidopsis*	RNA sequencing	miR156/miR157 and miR172	May et al. 2013
5	Heat	Rice	MPSS	miR397b	Jeong et al. 2011
6	Cold	*Brachypodium*	Deep sequencing	miR169e, miR172b, and miR397	Zhang et al. 2009
7	Cold	*Arabidopsis*	Microarray	miR156, miR159, miR164, miR165, miR169, miR172, miR393, miR394, miR396, miR397, and miR398	Zhou et al. 2008
8	Cold	Rice	Microarray	miR156k, miR166k, miR166m, miR167a/b/c, miR168b, miR169e, miR169f, miR169h, miR171a, miR535, miR319a/b, miR1884b, miR444a.1, miR1850, miR1868, miR1320, miR1435, and miR1876	Lv et al. 2010
9	Drought	Rice	Microarray	miR169g, miR171a, and miR393	Jian et al. 2010; Zhou et al. 2010
10	Drought	*Arabidopsis*	Microarray	miR157, miR167, miR168, miR171, miR408, miR393, and miR396	Liu et al. 2008
11	Drought	*Medicago*	Microarray	miR398a/b and miR408	Trindade et al. 2010
12	Drought	*Populus*	Microarray	miR1446a-e, miR1444a, miR1447, and miR1450	Lu et al. 2008

Table 3.2 (continued)

S. No	Stress	Plant species	Approach	Expressed miRNAs (up-/downregulated)	References
13	Drought	*Prunus persica*	Deep sequencing	miR156, miR157, miR159, miR160, miR165, miR167, miR168, miR169, miR171, miR390, miR393, miR395, miR396, miR397, miR398, and miR408	Eldem et al. 2012
14	Drought	*Populus*	Illumina sequencing	miR159a-c, miR472a, miR472b, miR473a, miR160a-d, miR164a-e, miR394a/b-5p, miR408, and miR1444b-c	Shuai et al. 2013
15	Salinity	Rice	Northern blot	miR169g, miR169n, and miR169o	Zhao et al. 2009
16	Salinity	Maize	Microarray	miR162, miR168, miR395, and miR47	Ding et al. 2009a

9.2 Reverse Transcription PCR

RT-PCR technique is commonly used to detect RNA expression. Here, the amplification using cDNA derived from mRNA template can be highly specific, fast, and sensitive (McDowell et al. 1996; Wang et al. 1999). It is useful to detect gene expression qualitatively through synthesizing cDNA from RNA. To know the presence and expression level of rice spotted leaf gene (spl7), a transgene in rice, RT-PCR was performed which suggested an increase in spl7 mRNA levels with increase in temperature (Yamanouchi et al. 2002). During the study of effect of abscisic acid-insensitive$_3$(ABI$_3$) on HsfA9 and HSP accumulation in *Arabidopsis,* RT-PCR revealed that HsfA9 transcripts are not inducible by heat stress and could not be detected in mutant line, whereas the synthesis of other HSP transcripts such as HSP17.4-CI, HSP17.6A-CI, HSP17.6-CII, HSP17.7-CII, and HSP101 was induced during heat stress (Kotak et al. 2007). In *Arabidopsis,* expression analysis of different members of HSP70 in response to temperature stress was reported using RT-PCR analysis (Sung et al. 2001).

9.3 Real-Time PCR Analysis

Real-time PCR, also called qPCR, is used to detect a gene quantitatively, where amplification and simultaneous quantification of a particular target gene can be achieved. In other words, it enables both detection and quantification. Expression and quantification of both mRNA and sRNAs can be achieved by using this

technique. Plenty of reports have utilized real-time expression analysis of genes during heat stress. Overexpression of HsfA1 gene in transgenic *Arabidopsis,* soybean, and tomato showed upregulation of stress-regulated genes and increased thermo-tolerance (Lohmann et al. 2004; Zhu et al. 2006). Expression of OsHsfA2a gene was greatly induced by heat stress. During high temperatures and high light conditions, it has been reported that the expression of HsfA2 was maximum among all the class A Hsfs in *Arabidopsis* (Schramm et al. 2006; Nishizawa et al. 2007). Apart from these transcription factors, expression of other heat-stress-responsive genes has been reported using qPCR. In rice, increased expression of nine OsHSPs during heat shock was demonstrated using real-time PCR (Zou et al. 2009). Further, expression of HSP70 genes was increased by 2- to 20-fold in *Arabidopsis* during heat stress (Sung et al. 2001). Downregulation of miR398a and b and upregulation of their corresponding targets CSD1 and CSD2 was shown in *B. rapa* and *Populus* under heat stress (Yu et al. 2011; Chen et al. 2012). Elevated expression of miRNAs under high-temperature stress was shown in plants. Expression of miR169j was upregulated in Populus, whereas in wheat, the members of miR156 family were upregulated during heat stress. Similar expression pattern of miR156s and its corresponding target SPL2 was recorded in *B. rapa* (Yu et al. 2011).

9.4 cDNA Clones and ESTs

ESTs are generated by high-throughput single-pass sequencing of cDNA clones (Adams et al. 1991). RNA (mRNA) from desired tissue or organism has to be isolated and transcribed to cDNA, using reverse transcriptase enzyme. cDNA thus formed is cloned to make libraries (cDNA libraries) representing the set of transcribed genes of our interest. Further, these cDNA clones are sequenced for obtaining ESTs. In rice, cDNA-AFLP analysis of heat-tolerant and heat-sensitive rice lines revealed 54 differentially expressed transcript-derived fragments (TDFs) playing an important role during heat stress in tolerant cultivar. Functions of 28 of the 54 TDFs were annotated based on their homologous genes. These genes were classified into different groups: metabolism, biosynthesis, transport, transcriptional regulation, oxidation, and signal transduction (Liao et al. 2012). To date, more than 2 million plant-derived ESTs are available at public databases from various species and these data are useful as a rich source for gene discovery and annotation (Rudd 2003). In addition, in some cases, genome sequencing is less attractive for plants such as maize due to genome complexity and abundance of repetitive sequence elements (Bennetzen 2002). In such cases, EST data set sources can be treated as an alternative to the complete genome sequencing (Rudd 2003). Plant ESTs are versatile and have multiple functions. They are very much useful in gene discovery, the expression of a particular gene, and the extent of its expression during particular stress. In addition, plant ESTs are a very useful resource for the identification of gene families which are conserved among different plant species, to detect alternative splicing, genome structure, discovery and characterization of SNPs, and mapping

of gene-based site markers (Dong et al. 2005; Nagaraj et al. 2007). An efficient and faster way to identify novel genes induced by various environmental stresses using EST approach has been demonstrated (Markandeya et al. 2005; Gorantla et al. 2007). In maize, mitochondrial small heat shock protein HSP22 was induced during heat stress which was identified and cloned using this approach (Lund et al. 1998). cDNA library was constructed and more than 30,000 ESTs were sequenced in *Populus* subjected to different environmental stresses. This helped in analyzing cDNAs encoding for the ERF/AP2 domain transcription factor (Nanjo et al. 2004). Recent study in *Jatropha curcas,* two HSP genes—HSP-1 and HSP-2—were identified and characterized from developing seeds using the EST approach (Omar et al. 2011). In rice, high-density physical maps were developed based on the availability of EST resources of rice cultivar N22 (Markandeya et al. 2005). Another study in rice identified 589 candidate genes sharing the drought response through EST approach by comparative analysis of plants species. Comparative analysis with five EST libraries revealed that expression of tentative Uni Genes (TUGs) was significantly overexpressed in *Zostera noltii* (seagrass) during recovery from heat shock exposure. These were identified as molecular chaperones (Massa et al. 2011).

9.5 Microarray

Microarray is a hybridization-based technique where nucleic acid is hybridized to a probe. Basically, two microarray-based approaches are available—one is oligonucleotide based (in situ synthesis of oligonucleotides) and the other is cDNA based (depositing of DNA fragments on solid surface). The former is termed as the gene chip method commercially prepared by Affymetrix having 25-bp-long oligonucleotides complementary to the 3′ end of the expressed sequence in the genome (Aharoni and Vorst 2001), whereas the later one consists of PCR-amplified cDNA fragments spotted on the glass surface. After the discovery of microarray, significant progress has been achieved in plant biology to characterize transcript expression profiling during stress. To decipher the changes in plant transcriptome under heat stress, microarray studies were carried out in many of the model crop species such as *Arabidopsis,* rice, wheat, maize, soybean, and tomato. Transcript profiling of *A. thaliana* revealed that a significant number of HsfA1a/1b-regulated genes were expressed during the study of mutants and wild species during heat stress (Busch et al. 2005). Hu et al. (2009) studied the regulation of Hsfs and HSPs during different abiotic stresses including heat. The important role of Hsfs in regulation of downstream targets including HSPs under heat stress was revealed using microarray (Mittal et al. 2009). Genome-wide expression profiling of Hsfs demonstrated an important role of OsHsfA2a in HSR in root, shoot, and panicle tissues of rice (Chauhan et al. 2011). In *Arabidopsis,* differential response of genome to photoperiod and thermal induction was demonstrated using microarray. The study suggested that a slight rise in ambient temperature beyond the optimum laboratory conditions triggers flowering of *Arabidopsis* in the absence of photoperiodic cues which is in turn governed

by a set of genes (Balasubhramanian et al. 2006). While exposing barley seedlings to high temperature, many of the genes were found to be upregulated during the seedling stage rather than the panicle stage (Oshino et al. 2011). A similar study in wheat revealed that the altered gene expression was more during short-term stress rather than prolonged heat stress (Qin et al. 2008). Microarrays were also used extensively to study the gene response mechanisms in other abiotic stresses such as drought, cold, and salinity. Expression profiling of sRNAs using microarray has been reported in many crop species like *Arabidopsis* (Zhou et al. 2008), rice (Lv et al. 2010), *Populus* (Lu et al. 2008), *Medicago* (Trindade et al. 2010), maize (Ding et al. 2009), etc. Thus, the application of microarrays in the area of plant transcriptome is very wide. It is a highly useful tool to study the expression, regulation, and role of different genes/pathways involved in stress tolerance mechanisms.

9.6 Next-Generation Sequencing

Sequencing refers to the identification of nucleotides in DNA or RNA and it provides a better option for gene expression studies. Sequencing of whole transcriptome includes isolation of total RNA from tissue of interest and enrichment of mRNA by reducing rRNA abundance followed by construction of cDNA library from total mRNA, sequencing using PCR amplification from either one end (single-end) or both ends (paired-end). The sequenced read length may vary from 30 to 400 nucleotides depending on the sequencing platform used. The sequencing technologies available for transcriptome sequencing include Roche 454 (pyrosequencing-based), Illumina/Hiseq, and Sequencing by Oligo Ligation and Detection (SOLiD) sequencing (Varshney et al. 2009). In addition to above-mentioned NGS technologies, single-molecule sequencing (SMS) can also be used to sequence the nucleic acids. More number of reads helps in the accurate measurement of transcript expression. Usually, shorter read platforms provide deep sequencing and good coverage, which is highly useful in detecting rare transcripts (Jain 2012). NGS is a very useful tool to decipher sRNA profiling also. Furthermore, identification of novel transcripts, transcript isoforms, alternative splice variants, and differentially expressed transcripts in a genome during stress can be identified using this tool. It is very useful in few other applications like single nucleotide polymorphism (SNP) detection, mutational analysis, transcriptional regulation, and identification of RNA editing sites.

Development of the NGS technology has provided a novel method of transcriptome studies under stress conditions. The availability of the complete genome sequence for several plant species facilitates expression profiling of the whole transcriptome using this tool. Whole genome transcript profile of two subspecies of rice was unraveled through sequencing technology, where 15,708 novel transcriptional and 3,464 differentially expressed genes were reported (Lu et al. 2010). Furthermore, whole genome transcript expression (including sRNAs) and integrated epigenome analysis were unraveled in two subspecies of rice and their reciprocal

hybrids using Illumina technology. This was aimed to study epigenetic variations in different genetic backgrounds that lead to phenotypic variability (He et al. 2011). NGS has great potential to be utilized in understanding the transcriptome and gene regulation during heat stress in various plant species. Recent study using NGS revealed that stress-regulated miRNAs play a crucial role during the inflorescence stage in rice (Barrera-Figueroa et al. 2012). Using sequencing technology, heat-stress-responsive miRNAs have been studied in different crops like rice (Jeong et al. 2011), *Brassica* (Yu et al. 2011), *Populus* (Chen et al. 2012), and wheat (Xin et al. 2010). Apart from the heat-stress-regulated miRNAs, sRNAs involved in other abiotic stresses were also studied using NGS (Eldem et al. 2012; Shuai et al. 2013). The revolution in sequencing technology enables the researchers to resolve the ambiguity towards the plant genome in understanding genomic, development, physiological, and evolutionary processes.

10 Conclusion and Future Perspectives

Heat stress shows serious impact on plant growth and development. It affects physiological, morphological, biochemical, and cellular mechanisms of plants. High temperature may lead to cell membranes disintegration, disturbing leaf water relations, and retarded photosynthesis due to the increased concentrations of ROS. In response to heat stress, plants show various adaptation strategies through the induction of Hsfs signaling and accumulation of HSPs, increased production of antioxidant compounds, synthesis of various osmolytes, and activation of signal transduction cascades through induction of signaling molecules. However, not all the plants are equally efficient in employing these adaptation strategies. Plant species show a high degree of variation in stress tolerance and they differ significantly in adaptation capability during high-temperature stress. The difference in heat stress tolerance at cellular or biochemical level is ultimately a response of different genetic constitution of different organisms/species. Further, difference in genetic constitution leads to change in the expression of genes and sRNAs involved in synthesis of metabolites associated with heat stress tolerance. Hence, to understand the heat response at molecular level, transcriptome analysis is very much crucial to unravel the complexity of mechanisms involved in heat stress tolerance. Study of the transcriptome may be aimed at specific genes or whole transcript level which can be achieved by various low- and high-throughput approaches. The progress in transcriptome analysis has enabled researchers to identify entire gene sets and pattern of expression, regulation, and function of potential transcripts. Use of NGS tools in transcriptome analysis has facilitated the identification of SNPs, splice junction sites and splice variants, differentially expressed genes (DEGs), and novel genes. The fast progress in transcriptome studies has helped biologists to understand the regulatory mechanisms of HSR. Particularly, transcriptome studies could establish the overlapping response of plants to various abiotic and biotic stresses. The integration of gene expression data with proteomic studies and sRNA profiling will help in understanding

the network of biological processes operated during stress. With the progress in transcriptome tools, such as direct RNA sequencing, heat-stress-associated gene expression and regulation mechanisms will be further advanced.

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