

# Chapter 2

## The Genetic Basis of Abiotic Stress Resistance in Extremophilic Fungi: The Genes Cloning and Application

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### 2.1 Introduction

The major lineage of fungi was believed to have first arisen about 1000 million or so years ago, which was followed by land plants in approximately 700 million years ago (Heckman et al. 2001). From the biological and environmental evolution perspective, fungi are one of the earliest eukaryotes to colonize the ancient earth (Gray and Shear 1992; Horodyski and Knauth 1994). Considering the harsh physical environments on the ancient earth, to ensure the chances of survival, fungi need to be more tolerant or resistant to adverse environmental factors than the latter appeared plants or animals. Indeed, within the last few decades a number of fungal species (halophile, xerophiles, or thermophile) that can live in a variety of extreme environments have been isolated. For example, *Eurotium herbariorum* that can survive in 340 g/L total dissolved salts was isolated from the Dead Sea (Kis-Papo et al. 2001; Yan et al. 2005); *Sarcinomyces petricola* strain A95 (a representative strain of rock-inhabiting fungi) was isolated from a marble rock surface near the Philopappos monument on Musaios Hill in Athens (Gorbushina 2007); a thermophilic fungus *Thermomyces lanuginosus* is able to survive at 62 °C, the highest growth temperature recorded so far, was isolated from horse dung (Prasad and Maheshwari 1978); and in our laboratory, the fungal strain *Chaetomium thermophile* isolated from rotting corn stalk was observed to be capable of growing at 65 °C (Song and Zhang, unpublished data). In contrast, with the exception of plant symbionts harboring mycorrhizal and/or endophytic fungi, it is hard to find plants that are able to survive under any one of the above extreme conditions.

Generally speaking, plants are relatively sensitive to high levels of abiotic stresses such as drought, high salt, extreme low/high temperatures, therefore, they are less able to survive and thrive in extreme environments (Alpert 2000). However,

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when an association between a plant and a mycorrhizal or endophytic fungus is established in an extreme habitat, the plant together with the habitat-adapted fungal endophytes, are able to survive or even thrive in that environment (Rodriguez et al. 2008). Without the fungal endophytes, the same plant is unable to endure habitat-imposed abiotic and biotic stresses (Redman et al. 2011). Therefore, this chapter will address the importance of endophytic fungi to emphasize their potential application in plant stress tolerance biotechnology.

Though great achievements have been made in recent years in understanding the abiotic stress responses and molecular mechanisms in plants (Bhatnagar-Mathur et al. 2008; Gupta and Huang 2014; Zhan et al. 2015), crop-breeding practice of higher resistant varieties remains unsatisfactory due to the lack of abiotic stress resistance germplasms. Wild germplasms are of interests, as wild crops may have retained genetic information before the domestication and artificial selection of modern plants (Lam et al. 2010). Wild germplasms screening, however, is a daunting and time-consuming task; and most likely we will never find certain wild germplasms again because of their extinction due to modern agricultural practices and environmental changes. Considering the survival abilities in extreme conditions, extremophilic fungi may provide special or different resistance mechanism compared to plants. Why are endophyte-free plants so sensitive to extreme adversities? And why do fungal endophytes facilitate and ensure plants to thrive? Both are complicated questions but the genetic advantage retained in fungal endophytes might provide information to enlighten and enable us to use the extremophilic fungi efficiently. The genetic basis of abiotic stress resistance in fungi, particularly in extremophilic fungi, is a unique genetic resource to improve abiotic stress resistance of crops. Here, we highlight the abiotic stress resistance mechanisms and resistant genes in extremophilic fungi. In addition, application strategies for anti-abiostress genetic engineering are also discussed.

## 2.2 The Responsive Pathways to Abiotic Stress in Extremophilic Fungi<sup>1</sup>

Fungi like other eukaryotic organisms (such as plants) depend on signal-receiving and transmitting systems to respond to, survive and thrive under the imposed adverse conditions. In eukaryote microorganisms, the yeast *S. cerevisiae* is known to have only moderate levels of tolerance to salt, drought, extreme temperature and other stressors (Prista et al. 2002, 2005; Serrano and Gaxiola 1994). Thus, a wild type *S. cerevisiae* is not the best model organism, neither for salt tolerance nor for sensitivity to salt. However, *S. cerevisiae* was an excellent tool for genetic

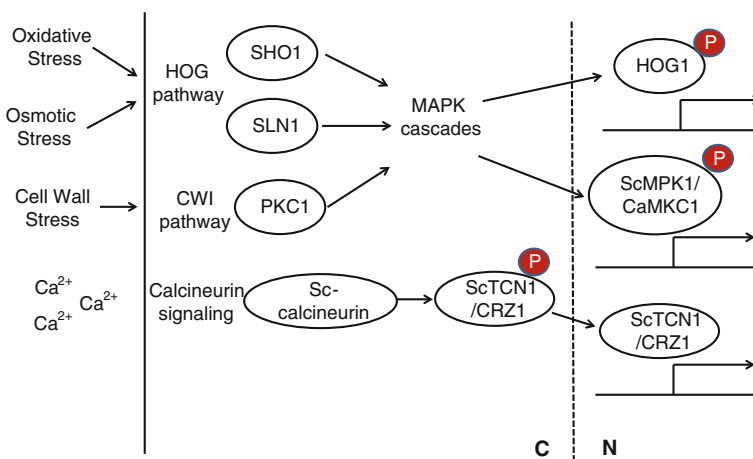
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<sup>1</sup>For additional information on stress responses in acidophilic fungi against heavy metals and metalloids, please see Chap. 9—*Mycoremediation of heavy metal/metalloid-contaminated soil: current understanding and future prospects*.

manipulation (fast growth rate and easy transformation) and has been widely applied in the field of resistance research. The high-osmolarity glycerol (HOG1) pathway which is an essential stress-signaling module has been extensively studied in fungi (Fig. 2.1): from the yeast *S. cerevisiae* to the filamentous fungus *Thichoderma harzianum* (Brewster et al. 1993; Delgado-Jarana et al. 2006). The HOG pathway is responsible for survival of the fungus in periods of high-osmolarity or oxidative stress (San José et al. 1996; Ikner and Shiozaki 2005; Enjalbert et al. 2006; Alonso-Monge et al. 2006). The activation of the HOG pathway leads to an increase in intracellular glycerol which provides protection against osmotic stress (Saito and Posas 2012). The activation of ScHog1 in *S. cerevisiae* can lead to downregulation of ergosterol biosynthesis and adaption to osmotic stress through changes in membrane fluidity (Montañés et al. 2011). In *Candida albicans*, mitochondrial function appears to be required for the activation of CaHog1 in response to oxidative stress (Thomas et al. 2013).

The HOG pathway has high sequence identity across many fungal species (Hayes et al. 2014). HOG1 is also found in extremophilic fungi. Nevo's group testified that *Eurotium herbariorum* HOG1 is highly similar to the homologs from non-extreme fungi such as *Aspergillus nidulans*, *S. cerevisiae*, *Schizosaccharomyces pombe* (Yan et al. 2005). However, it appears that HOG1 is found exclusively in fungi and no homologue gene has been detected in plants.

The HOG1 regulation system in yeast involves two pathways, the low osmolarity SHO1 pathway and high-osmolarity SLN1 pathway. The difference between the two pathways lays at the SHO1 and SLN1 sensors, but both pathways ultimately leads to glycerol biosynthesis and the glycerol concentration for osmotic balance (Brewster et al. 1993). Besides salt, HOG1 also responds to a variety of other stressors (Delgado-Jarana et al. 2006), suggesting cross-talking feature in the HOG pathways. Another stress response is the cell wall integrity (CWI) pathway



**Fig. 2.1** Stress signaling pathways in fungi. C cytoplasm; N nucleus

(Fig. 2.1). The CWI pathway is involved in sensing cell wall stresses (Levin 2011). CWI is activated in response to abiotic stresses, such as osmotic pressure, cell wall damage, alteration of growth temperature, and is responsible for an osmotically stable cell wall as well as cell integrity during cell growth (Navarro-García et al. 1995, 2005; Ikner and Shiozaki 2005; Levin 2011). The function of the CWI pathway has been described in detail for *S. cerevisiae*; it also operates in other fungal species, such as *C. albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Botrytis cinerea*, *Fusarium graminearum* and *Magnaporthe grisea* (Navarro-García et al. 1995; Xu et al. 1998; Hou et al. 2002; Kraus et al. 2003; Rui and Hahn 2007; Valiante et al. 2008). However, there is a dearth of information about this pathway in extremophilic fungi.

An additional stress responsive system required for salt stress tolerance in yeast is calcineurin; this protein phosphatase complex is dependent on calcium ion and calmodulin (Nakamura et al. 1993; Mendoza et al. 1994). Calcineurin is required for the genes transcription of sodium and calcium ion ATPases and a cell wall  $\beta$ -1,3 glucan synthase through regulating CRZ1/TCN1, the downstream zinc-finger transcription factor (Matheos et al. 1997; Stathopoulos-Gerontides et al. 1999). The salt-responsive calcineurin-CRZ1 pathway is also involved in yeast stress responses (Juvvadi et al. 2014). When CRZ1 was overexpressed in the industrial baker's yeast HS13 strain, tolerance to both salt and freezing was increased (Panadero et al. 2007).

The calcineurin pathway (Fig. 2.1), unlike HOG1, is highly conserved in eukaryotes from yeast to animals, which can be searched in the public nucleic acid sequence repository (<http://www.ncbi.nlm.nih.gov/genbank>). In plants, the physiology functions of calcineurin have been clarified (Luan et al. 1993; Allen and Sanders 1995), the osmotic stress resistance is associated with the increased expression of calcineurin pathway genes. In fungi, the calcineurin homologs PsCNA1/PsCNB1 from the wheat rust disease fungus *Puccinia striiformis* have been studied. Results indicated that the calcineurin signaling pathway participates in stripe rust morphogenetic differentiation, especially the formation of haustoria during the early stage of infection and during the production of urediospores (Zhang et al. 2012). Interestingly, calcineurin may be a multifunctional enzyme, because it was required not only for drug tolerance but also hyphal growth and virulence in *Candida tropicalis* (Chen et al. 2014). In contrast, relatively little is known about the calcineurin pathway in extremophilic fungal.

## 2.3 Osmoregulation in Extremophilic Fungi

To remain viable, fungi under extreme conditions must regulate and keep essential cellular processes. The fluidity and components of the plasma membrane play important roles in maintaining the cell membrane physiological functions and the adaption to extreme conditions (Turk et al. 2007; Zhang et al. 2015). Plasma membrane fluidity has been regarded as a typical indicator of fitness for survival in extreme environments (Turk et al. 2007). Unsaturated fatty acids are the key

compounds in plasma membrane and cellular unsaturated fatty acids constitutions are directly controlled by fatty acid desaturases. In *Pichia pastoris* GS115, cellular fatty acids compositions were changed with the increased or decreased expression of desaturases; in addition, deletions of fatty acid desaturases give rise to increased resistance to adverse environmental stress (Zhang et al. 2015). In Turk's study (Turk et al. 2007), all the tested fungi showed increased plasma membrane fluidity in response to increased salt concentrations. However, when salinity exceeded their optimal range, the extremophilic fungi (*Hortaea werneckii*, *Cryptococcus liquefaciens*) showed decreased plasma membrane fluidity, reflecting the limitation of cell membrane remodeling and suggesting extremophilic fungi could have different resistant mechanism.

When responding to osmotic stress, eukaryotic microorganism cells accumulate some metabolites inside the cells to equilibrate the cytoplasm osmotically with the outside of the cells (Brown 1978). Many polyols have been reported to contribute to fungal survival at high-salt concentrations or drought conditions. And among these compatible solutes, glycerol and trehalose have been extensively studied. Glycerol is the major product when extremophilic fungi, such as *Aspergillus glaucus*, grow on glucose-contained medium with high concentrations of NaCl (Liu et al. 2015). In the process of glycerol biosynthesis, a number of key enzymes determine the production of intracellular glycerol and therefore impact on osmotic stress tolerance. Glycerol 3-phosphate dehydrogenase encoded by the gene of GPD1 in *S. cerevisiae* is important for yeast survival under osmotic stress (Albertyn et al. 1994). The yeast glycerol 3-phosphatases gpp1p and gpp2p are also essential for glycerol biosynthesis, but their roles in the cellular responses to osmotic, anaerobic, and oxidative stress are different (Pahlman et al. 2001).

To conquer high-osmotic stress by biosynthesis of glycerol is inefficient and uneconomical; the active retention and uptake of glycerol become necessary when fungi are at high density. Aquaglyceroporins [AQGP; (GlpFs in yeast)] transport glycerol along with water and other uncharged solutes are involved in osmoregulation in myriad species. The two genes encoding AQGPs in the yeast genome, Fps1 (Oliveira et al. 2003; Tamás et al. 1999) and Yfl054 (Hohmann et al. 2000; Oliveira et al. 2003), are functional glycerol facilitators. Fps1 plays a key role in yeast osmoregulation by regulating intracellular glycerol levels during changes in external osmolarity (Luyten et al. 1995; Hohmann et al. 2007; Ahmadpour et al. 2014), whereas the cellular function of Yfl054 remains uncertain (Oliveira et al. 2003). Recently, the AQGP of arbuscular mycorrhizal fungi have received a lot of attention. The aquaglyceroporin GintAQPF2 from *Glomus intraradices*, a member of the  $\gamma$  subgroup (Xu et al. 2013), showed high activity when exposed to polyethylene glycol and high capacity to transport water, which is crucial for transformed yeast cells to survive osmotic stress (Li et al. 2013). Similarly, two AQGPs (Lacbi1:317173 and 391485) in the ectomycorrhizal basidiomycete *Laccaria bicolor*, belonging to the  $\alpha$  subgroup (Xu et al. 2013), enabled much higher water permeability than the orthodox Aquaporins (AQP) Lacbi1:392091 (Dietz et al. 2011). Given the relationship between mycorrhizal fungus and its host plant, AQGPs' function may partially explain the higher stress tolerance in endophyte plant than in endophyte-free plant. In the halophilic fungus

*Aspergillus glaucus*, the aquaglyceroporin gene *AgGlpF* has been demonstrated to be a water/glycerol channel (Liu et al. 2015). Interestingly, *AgGlpF* functions not only in *S. cerevisiae* and *Neurospora crassa* but also in model plants such as *Arabidopsis thaliana*. When *AgGlpF* was expressed in *A. thaliana*, the transgenic lines survived under high osmotic pressure and particularly under drought stress (Liu et al. 2015).

Another metabolite associated with osmoregulation is trehalose, the highly stable disaccharide is commonly found in nature. Trehalose has multiple functions (Elbein et al. 2003) and is well known for osmoprotection where correlations between accumulation of trehalose and high resistance to various stresses have been observed (Crowe et al. 1992). However, an unbiased study carried out by Petitjean et al. (2015) casted doubt on this long-held belief that trehalose is an osmoprotectant. By combining the use of mutant strains expressing catalytically inactive variants of Tps1, MAL<sup>+</sup> yeast strains were able to accumulate trehalose from an exogenous supply, the authors found that the stress-protecting role of trehalose in the yeast was largely overestimated: trehalose actually was unable to protect yeast cells from dying; on the contrary, it is the Tps1 protein, the key enzyme for synthesis of trehalose, that played essential roles for yeast survival in response to temperature, oxidative and desiccation stresses (Petitjean et al. 2015). Though we do not know the concrete molecular mechanism of Tps1 on playing a secondary function, the phenomenon may indicate osmoregulation is not limited to polyols only. To uncover osmoregulation mechanisms, more polyols and moonlighting proteins must be further investigated.

## 2.4 Abiotic Stress Resistance Genes in Extremophilic Fungi

Like *S. cerevisiae*, the yeast *Debaryomyces hansenii* that is usually found in salty environments has been extensively investigated in recent years. As a salt-loving fungus, it is able to accumulate high concentrations of sodium without suffering from any adverse effect. It also grows well under additional stress factors such as high temperature and extreme pH in the presence of 0.25 M NaCl (Almagro et al. 2000). Through screening of *S. cerevisiae* transformants that contain the genomic library prepared from *D. hansenii* (Prista et al. 2002), a series of genes associated with salt tolerance were identified and characterized. For example, the *DhGZF3* gene, which encodes a GATA transcription factor homologues to Dal80 and Gzf3 in *S. cerevisiae*, has been functionally analyzed in *D. hansenii*, but the gene was verified to be a negative transcription factor when it was expressed in *S. cerevisiae* (García-Salcedo et al. 2006). Using the cDNA library from the stress-tolerant basidiomycetes yeast *Rhodotorula mucilaginosa*, more than 100 *S. cerevisiae* transformants that are tolerant to concentrations of various osmolites have been screened by Gostinčar and Turk (2012). Among the sequenced clones, 12 genes mediated increased stress tolerance in the *R. mucilaginosa* transformants. Recently,

from the *D. hansenii* genome database, Pereira et al. (2014) analyzed nine candidates of polyol/H(+) symporters by heterologous expression in *S. cerevisiae*. Five distinct polyol/H(+) symporters were confirmed, among which two symporters were tested to be specific for uncommon substrates as galactitol and D-(+)-chiro-inositol.

Interestingly, the stress tolerance genes in extremophilic fungi are scarcely reported and their functions need more research (Table 2.1). These genes could be of significant importance in transgenic biotechnology. Above all, the abiotic stress resistance genes isolated from extremophilic fungi appear to be more resistant than the homologs from non-extremophiles. *EhHOG*, as mentioned above, is the *E. herbariorum* MAPK kinase gene similar to the HOG1 homologs from *A. nidulans*, *S. cerevisiae*, *Schizosaccharomyces pombe*, and most other fungi; but HOG1 mutant complemented with *EhHOG* outperformed the wild type under high salt and freezing–thawing conditions (Yan et al. 2005), indicating the higher genetic fitness of *EhHOG* in comparison with the corresponding HOG from *S. cerevisiae*. Some genes isolated from the halophilic fungus *A. glaucus* were also found to be more resistant to osmotic stress than the common fungi such as *S. cerevisiae* and *Magnaporthe oryzae*. A yeast expression library containing full-length cDNAs of *A. glaucus* was constructed and used to screen salt resistance transformants in our laboratory. The ribosomal protein L44 (RPL44), one of the proteins of the large ribosomal subunit 60S, was obtained according to its association with salt resistance. In comparison with the homologous sequence from *M. oryzae*, *MoRPL44* in a yeast expression system, the yeast cells with overexpressed *AgRPL44* were more resistant to salt, drought and heavy metals than yeast cells expressing *MoRPL44* at a similar level of stress. In addition, when *AgRPL44* was introduced into *M. oryzae*,

**Table 2.1** Abiotic stress resistance genes in extremophilic fungi

Extremophilic fungi	Protein/function	Resistance gene	Effect on abiotic stress	Reference (s)
<i>Eurotium herbariorum</i>	High-osmolarity glycerol	<i>EhHOG</i>	Tolerance to high salt and freezing–thawing	Yan et al. (2005)
<i>Aspergillus glaucus</i>	Ribosomal protein L44	<i>AgRPL44</i>	Resistant to salt, drought, heavy metals	Liu et al. (2014)
<i>A. glaucus</i>	Ribosomal protein subunit	<i>AgRPS3aE</i>	Resistant to salt, sorbitol	Liang et al. (2015)
<i>A. glaucus</i>	Aquaglyceroporins	<i>AgglpF</i>	Tolerance to salt, sorbitol, CuSO <sub>4</sub>	Liu et al. (2015)
<i>A. glaucus</i>	Chitinase	To be identified	Enhanced salt and drought	Zhang and Liu, unpublished
<i>A. glaucus</i>	Cellulose	To be identified	Enhanced salt and drought	Li et al., unpublished
<i>A. glaucus</i>	Glucanase	To be identified	Enhanced salt and drought	Zhang and Liu, unpublished



the transformants also displayed significantly enhanced tolerance to salt and drought, indicating the unique osmosis resistance ability from the halophilic fungus. Similar results were obtained in the studies of another ribosomal protein subunit of AgRPS3aE (Liang et al. 2015), a aquaglyceroporins of Agg1pF (Liu et al. 2015), a 60S protease subunit and 14 other unknown or predicted genes including the cell wall degrading enzymes such as chitinase, cellulase and glucanase (Zhang and Liu, unpublished). The common features of all these genes are highly conserved, at least not specific to extremophilic fungi, but they obviously support transgenic cells or organisms surviving under stress conditions, suggesting special mechanisms to be uncovered in future and potential values for genetic engineering.

## 2.5 Genetic Application Strategies

Foreign gene transfer from unrelated organisms frequently happened in the course of plant evolution; and indeed, plants can benefit from the presence of foreign genes, such as CtHSR1 from the halophytic yeast *Candida tropicalis* transferring the ability to adapt to adverse environments (Martínez et al. 2015). Up to now, many genes from diverse fungi have been successfully transferred into plants. *T. harzianum* is commonly used as a mycoparasite fungus for agriculture biological control. On the other hand, *T. harzianum* has moderate levels of tolerance to stressors; it is regarded as an active agent with abilities to induce resistance to abiotic stress in plants and to promote plant growth (Dana et al. 2006; Shores et al. 2010). *T. harzianum* provides an excellent genetic pool for cloning multiresistance genes. For example, ThHog1 (Delgado-Jarana et al. 2006), HSP70 (Montero-Barrientos et al. 2008, 2010) and Thkel1 (Hermosa et al. 2011) have been successively characterized to be the genes responsible for resistance to salt or other stressors. Interestingly, some genes generally associated with cell wall degradation were shown to be associated with stress tolerance when they were transferred into plants (Nicolás et al. 2014). This is not dissimilar to the ribosomal protein subunits RPL44 and RPS3aE described above. Considering the moderate levels of tolerance in *T. harzianum*, the homologous genes from extremophilic fungi could be even more resistant. Therefore, it is important and necessary to identify and characterize more genes related to stress resistance regardless of their origin and novelty.

On the other hand, the potential applications of these fungal resistant genes are strengthened according to their functions in transgenic plants. Three major steps are required to obtain a transgenic plant line. Briefly: (i) the identification of a function determined gene; (ii) transfer of the gene into the target plants and (iii) the selection of a tolerance improved line. How to achieve an economical, efficient, genetically stable and biological safety transgenic plant would require further research and optimisation. Crops are often exposed to multiple stresses. One gene with multiple actions such as *Trichoderma* HSP70 is no doubt efficient and economical. Transgenic *Arabidopsis* containing HSP70 showed an enhanced tolerance to oxidative, osmotic and salt stresses (Montero-Barrientos et al. 2010). The highly conserved ribosomal protein subunits like RPL44 and RPS3aE are also promising



candidates for creating tolerance enhanced crops (Liu et al. 2014; Liang et al. 2015). These genes are generally in the downstream of resistant pathway and likely to have direct contribution to stress tolerance. Therefore, other physiological traits in transgenic plants may not be seriously affected, even if all these genes are overexpressed.

As to other genes, the expression levels and patterns of transferred sequence may have many unpredicted impacts on the growth and development of transgenic plants: low levels of expression may have no anticipated function; however, highly levels of expression probably affect the bioassay or productivity of the plant. Thus, timely expression of transferred genes is in need. Inducible promoters are available in stress resistance genetic engineering. In plants, there exist many stress responsive genes, particularly in sensitive plants (Dey et al. 2015). The resistant gene or genes from an extremophilic fungus driven by a plant inducible promoter constitutes the so called “two-component sensor systems” (de Wit 1992). This strategy will solve the problem of excess cellular materials and energy (ATP) consumption.

## 2.6 Concluding Remarks

The three most pervasive environmental problems afflicting crop growth and development throughout the world are saline soils, drought and temperature extremes. Due to the lack of abiotic stress resistance in germplasms, conventional anti-stress breeding in plants is largely not successful. Fortunately, recent researches that focus on molecular mechanisms and genes cloning in extremophilic fungi offer hope in solving these problems. Based on the two significant osmotic-resistance pathways in yeast systems, a series of homolog resistance genes in extremophilic fungi have been characterized. And indeed, these genes were confirmed to be more resistant than their homologs in non-extremophiles, suggesting certain specific genetic base and novel mechanism exist in extremophilic fungi. More promising resistant genes will be exploited with the publication of the whole genome sequences of extremophilic fungi. Therefore, a reliable filamentous genetic model of extremophilic fungi must be established as soon as possible, for to date, yeast is the only system that has been studied extensively.

*T. harzianum* cell wall degrading enzymes have shown to be effective candidates for promoting tolerance to various stresses; and several genes have been used to create multi-resistant plants (Nicolás et al. 2014). These homologous genes in extremophilic fungi should be identified as well, because of their special genetic background. Ribosomal proteins AgRPL44 and AgRPS3aE, which supported plants to survive under high osmosis conditions, are highly conserved in organisms, suggesting these genes can be prospective candidates for creating genetic modified crops without consideration of their biosafety.

As described above, salt tolerance genes are extensively studied in halophilic fungi. However, only a few thermophilic fungi have been examined: *T. lanuginosus*, *Myceliophthora thermophila* and *Thielavia terrestris*; and, these studies

focused mainly on the biomass-degrading application and regulation mechanisms (Singh et al. 2003; Berka et al. 2011; McHunu et al. 2013). Genes contributing to temperature extremes should be uncovered in these thermophiles. Once identified, the high or low temperature resistance genes, with halotolerant genes, could be used to generate multiple resistant plants. In our laboratory, we are cloning and transforming the abiotic stress resistance genes observed from extremophilic fungi to plants (Liu et al. 2014, 2015; Liang et al. 2015). Additional genes and their functions will be elucidated soon.

**Acknowledgments** This work was partially supported by a grant of National Natural Science Foundation of China (31171794) and a project of Ministry of Agriculture of China (2011ZX08009-001). The author is very grateful to Dr. Diane Purchase who reviewed individual sentences and gave tremendous help to the preparation of this chapter. The author also likes to express his thanks to Dr. Liu Shou-An who kindly helped to revise the manuscript of this chapter.

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Purchase, D. (Ed.)

2016, XVII, 405 p. 44 illus., 24 illus. in color., Hardcover

ISBN: 978-3-319-42850-5