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Microarray Gene Expression Profiling for Salt Tolerant Gene Selection

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ABSTRACT

Salt stress is serious threat to agriculture and sustainability of world food supply. The generation of transgenic crops with increased salt tolerance would contribute to address the problem. In this article, we argue that the microarray expression profiling for global salt-tolerant gene selection towards a generation of transgenic plants that can tolerate high levels of salinity. Although there have been several successes in producing transgenic plants with increased salt tolerance, no genes so far have been from microarray gene selection. Microarray systematic gene selection can be a rapid and effective way to identify the gene that confers high salt tolerance. We especially focus on salt tolerant study on halophytes. Most of salt tolerant studies have been on glycophyte, lacking the genetic basis of high-salinity tolerant. Study of halophyte may be instructive. The gene resource of halophytes may have unique salt tolerant determinants that are absent in model glycophyte plants, and are expected to have novel genes that could increase salt tolerance in transgenic plants.

Keywords: halophytes, high-throughput, salt stress, salt tolerance, transcriptome, transgenic plants

CONTENTS

INTRODUCTION

Salinity is an ever-present threat to crop yields, especially in countries where agriculture relies on irrigation. Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss by 2025, and up to 50% by 2050. On the other hand, world population is increasing to reach about six billion by the end of the 2050. Salt stress threatens the sustainability of world food supply. The breeding for salt tolerant crops may contribute to address the problem. Complex salt tolerance mechanism have been elucidated gradually (for reviews; Blumwald *et al.* 2000; Hasegawa *et al.* 2000; Zhu 2001, 2002, 2003; Yamaguchi and Blumwald 2005). In parallel, genetic modification approaches to enhance salt tolerance, including both introducing novel genes and altering expression levels of the existing genes, have been started to bear fruit (for reviews: Wang *et al.* 2003; Flowers 2004; Zhang *et al.* 2004; Vinocur and Altman 2005; Yamaguchi and Blumwald 2005; Teixeira da Silva 2006). The overexpression of transcription factor DREB1A (GenBank accession no. NM_118680) from stress inducible RD29A promoter increased drought, cold and salt tolerance without plant growth inhibition (Kasuga *et al.* 1999). The overexpression of AtNHX1 (GenBank accession no. NM_122597), a vacuolar Na^+/H^+ antiporter considered to compartment Na^+ into vacuole resulted to avoid cytosolic Na^+ toxic effect, enabled transgenic plants to grow in 200 mM NaCl (Apse *et al.* 1999). Several other regulatory genes, such as CDPK (Saijo *et al.* 2000), Alfin1 (Winicov and Bastola 1999), and NPK (Kovtun *et al.* 2000), have also been expressed in transgenic plants to enhance stress tolerance. Genes encoding key enzymes of the synthesis or degradation of compatible solute such as glycinebetaine, mannitol, ononitol, proline, trehalose, ectoine, and fructane have been engineered in transgenic plants to improve salt tolerance or water stress (Kishor *et al.* 1995; Pilon-Smits *et al.* 1995; Sheveleva *et al.* 1997; Nakayama *et al.* 2000).

None of these transgenic studies so far has been based on global microarray gene selection. Functional genomics is the study of gene function through the parallel expression measurements (expression profiling), most commonly using microarray technology. The transcription of genomic DNA generates an mRNA collection that is referred to as the transcriptome. The transcriptome is one of the major determinants of cellular phenotype and function. Unlike the genome, the transcriptome is highly dynamic, and changes rapidly and dramatically in response to environmental stimuli. Differential regulation of gene expression leads to morphological and phenotypic changes. Monitoring and analyzing when, where, and to what extent a gene is expressed contributes to understanding the activity and biological roles of its encoded protein. Moreover, expression profiling can provide the clues about regulatory mechanisms, broader cellular functions, and biochemical pathways. A genome-wide microarray study has the potential to provide new insights into plant salt response mechanisms, and to identify the novel candidate gene(s) that confers salt tolerance in trans-

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genic plants. In this article, we argue that a system-level gene selection based on microarray expression profiling towards a rapid and effective way to identify the gene that confers high salt tolerance and a comprehensive understanding of plant stress response and a sustainable improvement of salt stress tolerance in plants.

Furthermore, we focus on gene resources of salt-tolerant plants (halophytes). Recent advances in expressed sequence tag (EST) sequencing technology (Adams *et al.* 1991; Rudd 2003) creates an opportunity to obtain gene resource of halophytes. The study of halophytes and comparative study with salt-sensitive plant species (glycophytes) may be instructive for the understanding of plant salt tolerant mechanisms. The existence of halophytes and differences in salt tolerance between genotypes within glycolphytes indicates that there is a genetic basis to salt response. The genes of halophytes – absent in glycophytes – may have the potential to confer salt tolerance.

MICROARRAY EXPRESSION PROFILING IN RESPONSE TO SALT STRESS

Microarray gene-expression profiling has been adapted to expression profiling in response to salt stress (**Table 1**). Microarray experiments detect differential transcriptome regulation between a stressed and non-stressed state. The detected differentially expressed genes were expected to be concerned with a salt-tolerant mechanism. Salt stress-specific responses are often observed alongside the shared stress responses in response to various abiotic stresses, such as osmotic, drought, dehydration, heat, and cold. To distinguish stress specific- and shared-responses, salt stress responses were often studied with other stress. Previously, microarray with 1300 full-length Arabidopsis cDNAs was used to survey drought- and cold-inducible genes (Seki *et al.* 2001). They focused especially on the *cis*-element of DREB1A/CBF3, a transcription factor that confers salt tolerance in transgenic plants (Kasuga *et al.* 1999). This study showed that full-length cDNA microarray was useful to study the target of transcription element.

Microarray containing Arabidopsis 7000 full length cDNAs was used to expression profiling under drought, cold (4°C) and high salinity (250 mM; Seki *et al.* 2002b). 53, 277 and 194 genes were induced more than 5-fold after cold-, drought and high-salinity stress treatment over non-treatment. The relationships between stresses were assessed with a Venn diagram. 22 genes (0.3%) were up-regulated over three stresses. 141 genes were upregulated in drought and salt stress, 30 genes were drought and cold, and 24 genes were cold and salt. Among the 194 highly (>5-fold) salt responsive genes, 70% were also responsive to drought stress. These results indicated that these stresses partially triggered common signal transduction pathways, and that the cross-talk between drought and salt stress signaling pathways was greater than that between other stress pairs. A gene encoding DREB1A, a transcription factor that confer salt tolerance in transgenic plant (Kasuga *et al.* 1999), was upregulated that expression peaked at 2 h after cold stress treatment and the target genes of DREB1A were upregulated subsequently.

Another pilot study showed a more stress-specific response. The Arabidopsis responses to salt (100 mM NaCl), osmotic (200 mM mannitol; iso-osmotic to 100 mM NaCl) and cold (4°C), were monitored at 3 and 27 h after stress treatment with the Arabidopsis GeneChip containing 8100 Arabidopsis genes (Kreps *et al.* 2002). 2409 (29.7%) genes out of 8100 genes were responsive with a greater than 2-fold over the non-treatment. These results showed the majority of these responsive genes were stimulus specific, and the content of sharing response over three stresses reduced over time. At 3 h, 5% (118 genes) were responsive over three stresses. At 27 h, the percentage decreased to 0.5%. The decrease indicates the progress towards a more stimulus-specific response.

Generally, salt stress causes both osmotic and ionic stress. Salinity imposes an water-deficit that results from the relatively high solute concentrations in the soil (osmotic stress), and causes ion stress results from altered cytosolic K^+/Na^+ ratios through over-accumulation of salt in the cells (ion stress). Although Na^+ is required in some plants, especially in halophytes, a high concentration of NaCl is detrimental to plants (Glenn *et al.* 1999). Comparing the expression profiles of 460 salt responsive barley (*Hordeum vulgare* L. cv. 'Haruna-nijyo') EST genes using microarrays, a relatively small amount of genes were regulated in an identical manner under salt and osmotic stress (Ueda *et al.* 2004). During the 24 h after stress treatment, 52 genes showed differential expression under osmotic stress (20% w/v PEG). During the same period, 92 genes showed differential expression under salt stress (200 mM NaCl). 18 genes out of 62 salt responsive genes were also up-regulated by osmotic stress, while 16 out of the 30 genes showed down-regulation under both stress treatments. Only 4 of the up-regulated genes and 6 of the down-regulated genes had a truly similar pattern (location, timing and extent of induction) under both stress conditions (Ueda *et al.* 2004). These results suggest that the expression profiles under salt stress and osmotic stress were highly differentiated, and that osmotic stress response is not a subset of salt stress response.

Another study of barley (*Hordeum vulgare* L.) transcriptome under salt stress was conducted (Walia *et al.* 2006) using the GeneChip containing 22,750 probe sets with a moderate stress level (100 mM) and a gradual stress-imposing method designed to reduce the sudden impact of osmotic shock. NaCl concentrations were brought up to 100 mM by increments of 25 mM NaCl per day. A total of 339 and

Table 1 Transcriptome profiling of salinity stress responses using microarray.

Plant	Stress	Gene no. on microarray	Reference
Glycophyte			
Rice	Salt (150 mM)	1728	Kawasaki *et al.* 2001
Arabidopsis thaliana	Salt (250 mM NaCl), drought and cold (4°C)	7000	Seki *et al*. 2002
A. thaliana	Salt (100 mM NaCl), hyperosmotic (200 mM mannitol) and Cold (4°C)	8100	Kreps *et al*. 2002
Barley (*Hordeum vulgare* L. cv. 'Haruna-nijyo')	Salt (200 mM NaCl) and hyperosmotic (PEG)	460	Ueda *et al*. 2004
Sorghum bicolor	Salt (150 mM), hyperosmotic (20% PEG) and Abscisic acid (125 μM)		Buchanan *et al.* 2005
Potato	Salt (100 mM), cold (4°C) and heat (35°C)	11,243	Rensink *et al.* 2005
Barley (*Hordeum vulgare* L.)	Salt (100 mM)	22,750	Walia *et al*. 2006
Common wheat (*Triticum aestivum* cv. 'Norin 26')	Salt (150 mM NaCl)	22,000	Kawamura *et al*. 2006
A. thaliana (Roots)	Salt (150 mM NaCl)	23,686	Jiang and Deyholos 2006
Halophyte			
Salt cress (*Thellungiella halophila*), *A. thaliana*	Salt (250 mM NaCl)	7000	Taji *et al.* 2004
Salt cress (*T. halophila*), *A. thaliana*	Salt (150 mM NaCl)	25,000	Gong *et al.* 2005
Burma mangrove (*Bruguiera gymnorhiza*)	Salt (500 mM NaCl)	7029	Miyama and Hanagata 2007

331 genes were up-regulated and down-regulated respectively under salt stress (100 mM) at any of the three time points (3, 8, and 27 h). These salt responsive genes include a number of the genes of the jasmonic acid pathway and genes known to be abiotic stress-responsive genes, supporting the notion of cross talk among abiotic stresses like heat, low temperature, and dehydration stress. Barley plants are known to accumulate Na^+ at the rate of 5 mM per day (Munns 2002). Unexpectedly, Na^+/H^+ antiporters that successfully conferred salt tolerance in transgenic plants (Apse *et al.* 1999) were not up-regulated significantly (>1.5-fold). This unexpected result is consistent with other studies (Fukuda *et al.* 2004; Gong *et al.* 2005). This is a quite important lesson that genes that can be used to increase salt tolerance in transgenic plants are not always induced under salt stress.

Salinity stress has also been studied in other systems. With rice microarrays containing 1728 cDNA from salt-stressed rice roots, salt-tolerant rice ('Pokkali') showed differentially regulated expression only 15 min after salt treatment (Kawasaki *et al.* 2001). Within 1 h, 10% of genes were differentially regulated. These initial responses continued for hours but gradually disappeared as the plant adapted to salinity over time. Increased protein synthesis was observed early in time, followed by the up-regulation of known stress-responsive genes within hours, and finally the up-regulation of known defense-related genes. Analysis of saline-sensitive rice (IR29) showed a delayed response compared to 'Pokkali' and later resulted in down-regulation and death.

Study of salt response in common wheat roots and shoots using a 22k oligo-DNA microarray (Kawaura *et al.* 2006) showed that 1,811 (9%) genes were highly up-regulated at 6 and 24 h after stress treatment. No clones out of seven genes encoding Ca^{2+} dependent protein kinase (CDPK), and only one clone of five genes encoding dehydration-responsive binding protein (DREB) were up-regulated. These transcription factors at the most upstream of the signal transduction pathways responded quickly and transiently (Saijo *et al.* 2000; Seki *et al.* 2002b). 6 and 24 h were considered to be too late to detect the response of these transcription factors. On the other hand, 21 DHN/LEA/RAB genes were upregulated, suggesting that they are downstream of DREB signaling pathways.

STUDIES ON HALOPHYTES

Studies of salt response have been conducted mainly on glycophytes lacking a genetic basis to survive in a high salinity environment. Halophytes potentially have novel genes absent in glycophytes that increase salt tolerance. Despite the potential, gene discovery efforts in halophytes have been limited. Halophytic model plants, including common ice plant (*Mesembryanthemum crystallinum*) and Burma mangrove (*Bruguiera gymnorhiza*; Sugihara *et al.* 2000; Takemura *et al.* 2000; Banzai *et al.* 2002a, 2002b; Takemura *et al.* 2002; Banzai *et al.* 2003), had been studied without a genome-scale gene resource. EST sequencing enables cost-effective and rapid gene discovery (Bogouski *et al.* 1993; Ohlrogge and Benning 2000). Genetic resources of halophytes have gradually increased (**Table 2**): common ice plant (Kore-eda *et al.* 2004; 27,348 ESTs dbEST), Burma mangrove (Miyama *et al.* 2006; 20,373 ESTs dbEST), *Suaeda maritima* subsp. *salsa* (Zhang *et al.* 2001; 1000 ESTs dbEST), and *Thellungiella halophila* (salt cress; Zhu 2001; 2851 ESTs dbEST), all March 9, 2007 release. Despite entire genome of halophytic species not to be sequenced in the near future, EST sequence information and cDNA microarray of close related species enables us to monitor the global gene expression profile of halophytes effectively.

A microarray study with 7000 Burma mangrove cDNA microarrays was conducted under sea-water level salinity (500 mM NaCl; Miyama and Hanagata 2007). As a result, 287 genes were significantly up-regulated (more than 5-fold). A number of well-known salt responsive genes in glycophytes, such as CDPK, peroxidase and pathogenesis related (PR) proteins (Hoffmann-Sommergruber 2002), were identified as salt responsive. These results indicate that salt tolerant mechanisms of Burma mangrove and glycophytes are common in part. On the other hand, several genes such as BURP-domain containing protein (RD-22 homologue; Banzai *et al.* 2002b) showed completely opposite results to former studies in Arabidopsis (Seki *et al.* 2002b). A number of mangrove-specific genes were also up-regulated in response to salt stress. These results suggest the existence of unique salt responsive mechanisms.

Comparative expression profiling was conducted on salt cress with Arabidopsis using a full-length Arabidopsis cDNA microarray (Taji *et al.* 2004). Salt cress is a close relative of Arabidopsis and has 90% cDNA sequence and 95% amino acid sequence identities with Arabidopsis respectively (Bressan *et al.* 2001). The high sequence conservation allowed for a comparative expression profiling study between salt cress and Arabidopsis. As a result, only 6 genes were significantly induced (more than 1.5-fold) after 250 mM NaCl treatment in salt cress, whereas 40 genes were induced in Arabidopsis. Comparative analysis of the expression profile between non-stressed salt cress and non-stressed Arabidopsis showed that a number of genes were highly expressed even under a non-stressed state in salt cress compared to Arabidopsis. One-half of these genes were highly induced in Arabidopsis by various stress treatments, including drought, salt, cold, abscisic acid (ABA) and oxidative stress (Seki *et al.* 2002a, 2002b; Narusaka *et al.* 2003). These results indicate that salt tolerance in salt cress may be due to constitutive overexpression of genes that are induced in glycophytes after stress treatment, implying that salt cress dose not respond to stress immediately at the transcriptional level.

CONCLUSIONS AND PERSPECTIVES

Microarrays have been used to monitor abiotic stress responses in various species. The diversity of microarray platforms used, and the diversity of data analysis methods makes it difficult to perform a global comparison and comprehensive analysis. Nevertheless, the microarray studies teach us fundamental features of stress response: i) the existence of cross-talk in signal transduction pathways between salinity and other abiotic stresses is detectable through expression profiling (Seki *et al.* 2002b). It is suggested that there are numerous points of potential cross-talk within the signaling pathways mediating abiotic stress responses (Chinnusamy *et al.* 2004). ii) The gene (ex. *AtNHX*) that successfully confers salt tolerance in transgenic plants is not always responsive to salt stress (Gong *et al.* 2005; Walia *et al.* 2006). iii) Constitutive and highly expressed genes, even in a non-stressed condition, may contribute salt tolerance in halophytic plants (Taji *et al.* 2004). The identification of these genes will require a comparative microarray study between close relative species. The gene selection by identi-

Table 2 Representative gene resource of halophytes.

Plant	No. of ESTs[a]	Reference
Common ice plant (*Mesembryanthemum crystallinum)*	27,348	Kore-eda *et al.* 2004
Burma mangrove (*Bruguiera gymnorhiza*)	20,373	Miyama *et al.* 2006
Salt cress (*Thellungiella halophila*)	2851	Zhu 2001
Sueda maritima subsp. *salsa*	1000	Zhang *et al.* 2001

[a] dbEST release March 9, 2007.

fying differentially regulated (up-regulated) after stress treatment may be invalid in this case. iv) Salt stress-responsive genes of halophytes contain known stress-related genes together with novel halophytic genes (Miyama and Hanagata 2007) along with novel halophytes specific genes, suggesting that the salt-responsive mechanism of halophytes is partly common with that of glycophytes. v) Although salinity stress leads to ionic and osmotic stress, the expression profile in response to osmotic stress is different from the profile under salt stress, at least not a subset of the profile of response to salt stress (Kreps *et al.* 2002; Seki *et al.* 2002b; Ueda *et al.* 2004).

Now the several essential issues have emerged, is this a valid strategy for developing stress-tolerant crops to transform the most up-regulated genes in stressed state comparing to non-stressed state? Are the most up-regulated genes the most important for stress tolerance? Can stress treatment induce stress-tolerant gene expression if the stress tolerant genes are constitutively expressed even in a non-stressed state? Do model plants have the genetic resources that can be used to increase salt tolerance? Do halophytes have novel genes that confer crop salt tolerance? Although there have been several success in producing transgenic salt-tolerant plants, no genes so far have been from microarray systematic selection. Here are genes that have been selected as stress responsive. The next challenge is evaluating the selected genes that would confer salt tolerance in transgenic plants.

Several developments have enabled us to analyze microarray data in a sophisticated way. These include: i) the increasingly available microarray data in public repositories, such as NCBI Gene Expression Omnibus (GEO; Edgar *et al.* 2002; Barrett *et al.* 2007), and EBI Array Express (Brazma *et al.* 2003), which enable us to conduct *in silico* analysis. For example, comparative genomics including comparing gene expression of orthologs among different species, and determining the stress specific- or shared-responsive genes through comparing the expression profile of various stress treatments. ii) Several statistical methods for identifying significantly differentially expressed genes have been applied to microarray data analysis, such as Significant Analysis of Microarray (SAM; Tusher *et al.* 2001) and ANOVA (Kerr *et al.* 2000). These statistical methods enable us to identify the statistically significant differential expression without simple natural variations within populations sampled.

Functional genomics based on sophisticated expression profiling combined with increasing sequence information including novel gene resource of halophyte, analysis information of physiological, biochemical, and metabolic studies will contribute to the identification of transgenes that confer transgenic lines with high-salinity tolerance without unsatisfactory agricultural performance.

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