Stress Tolerance Profiling of a Collection of Extant Salt-Tolerant Rice Varieties and Transgenic Plants Overexpressing Abiotic Stress Tolerance Genes

Ken-ichi Kurotani1, Kazumasa Yamanaka1, Yosuke Toda1,2, Daisuke Ogawa1,3, Maiko Tanaka1, Hirotsugu Kozawa1, Hidemitsu Nakamura3,4, Makoto Hakata3,5, Hiroaki Ichikawa3, Tsukaho Hattori1 and Shin Takeda1,*

1Bioscience and Biotechnology Center, Nagoya University, Chikusa, Nagoya, 464-8601 Japan
2Graduate School of Science, Nagoya University, Chikusa, Nagoya, 464-8601 Japan
3National Institute of Agrobiological Sciences, Kannondai, Tsukuba, 305-8602 Japan
4Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657 Japan
5Lowland Farming Research Division, NARO Kyushu Okinawa Agricultural Research Center, Izumi 496, Chikugo, Fukuoka, 833-0041 Japan
*Corresponding author: E-mail, takeda@agr.nagoya-u.ac.jp; Fax, +81-52-789-5214.
(Received May 3, 2015; Accepted July 10, 2015)

Environmental stress tolerance is an important trait for crop improvement. In recent decades, numerous genes that confer tolerance to abiotic stress such as salinity were reported. However, the levels of salt tolerance differ greatly depending on growth conditions, and mechanisms underlying the complicated nature of stress tolerance are far from being fully understood. In this study, we investigated the profiles of stress tolerance of nine salt-tolerant rice varieties and transgenic rice lines carrying constitutively expressed genes that are potentially involved in salt tolerance, by evaluating their growth and viability under salt, heat, ionic and hyperosmotic stress conditions. Profiling of the extant varieties and selected chromosome segment substitution lines showed that salt tolerance in a greenhouse condition was more tightly correlated with ionic stress tolerance than osmotic stresses. In Nona Bokra, one of the most salt-tolerant varieties, the contribution of the previously identified sodium transporter HKT1;5 to salt tolerance was fairly limited. In addition, Nona Bokra exhibited high tolerance to all the stresses imposed. More surprisingly, comparative evaluation of 74 stress tolerance genes revealed that the most striking effect to enhance salt tolerance was conferred by overexpressing CYP94C2b, which promotes deactivation of jasmonate. In contrast, genes encoding ABA signaling factors conferred multiple stress tolerance. Genes conferring tolerance to both heat and hyperosmotic stresses were preferentially linked to functional categories related to heat shock proteins, scavenging of reactive oxygen species and Ca2+ signaling. These comparative profiling data provide a new basis for understanding the ability of plants to grow under harsh environmental conditions.

Keywords: Abiotic stress • Full-length cDNA overexpression • Jasmonate • Rice • Salt tolerance.

Introduction

Soil salinity, in addition to drought, is one of the most severe problems in agriculture. Absorption of excessive salt inhibits both root and shoot growth, reduces reproductive activity and affects viability of plants. To counter salinity stress, plant cells have several mechanisms. These include excretion of toxic levels of sodium ions from the cytosol to the apoplast or into the vacuole mediated by the Na+/H+ exchanger SOS1 located at the plasma membrane and the vacuolar Na+/H+ antiporter NHX1, respectively (Yamaguchi and Blumwald 2005, Munns and Tester 2008, Takeda and Matsuoka 2008, Pardo 2010, Deinlein et al. 2014). In addition, heat shock proteins (HSPs) or chaperones that catalyze refolding of denatured proteins and compatible solutes or osmoprotectants are synthesized under salt stress conditions (Munns and Tester 2008, Takeda and Matsuoka 2008, Deinlein et al. 2014). Overexpression of SOS1 and NHX1, as well as of HSPs or biosynthetic enzymes of compatible solutes, enhances salt tolerance in several plant species (Yamaguchi and Blumwald 2005, Munns and Tester 2008, Takeda and Matsuoka 2008). Detoxification of reactive oxygen species (ROS) generated by salt stress is another protective mechanism, given that enhanced expression of enzymes involved in protection from ROS, such as superoxide dismutase, glutathione peroxidase and ascorbate peroxidase, improves salt tolerance (Zhu 2001). Many genes involved in abiotic stress tolerance are regulated at the level of transcription, and manipulation of their transcriptional activators to increase the expression of these genes often leads to enhancement of stress tolerance (Kasuga et al. 1999, Nakashima et al. 2009, Deinlein et al. 2014). In addition, some salt tolerance genes confer tolerance to several other abiotic stresses, such as...
drought and freezing stresses (Kasuga et al. 1999, Deinlein et al. 2014).

At the whole-plant level, it is believed that the balance of Na⁺ and K⁺ ions (the cellular Na⁺/K⁺ ratio) is important for salt tolerance (Deinlein et al. 2014). In rice, SKC1 has been identified as a gene corresponding to a major quantitative trait locus (QTL) controlling shoot K⁺ content, using the salt-tolerant variety Nona Bokra and the salt-sensitive variety Koshihikari (Ren et al. 2005). SKC1 encodes HKT1;5, a selective transporter for Na⁺, localized at the plasma membrane. SKC1 is expressed preferentially in parenchyma cells surrounding the xylem vessels, and is suggested to function in unloading of Na⁺ from xylem in the root, thereby affecting the Na⁺/K⁺ ratio in the shoot. A near-isogenic line containing the salt-tolerant SKC1 allele of Nona Bokra in the background of Koshihikari shows salt tolerance and a high K⁺/Na⁺ ratio in the shoot under salt stress conditions. However, the extent to which SKC1 accounts for salt tolerance remains uncertain, given that major but not well characterized QTLs other than SKC1 contribute to seedling survival under saline conditions (Lin et al. 2004).

Among plant hormones, ABA has been well documented as being involved in tolerance to salinity and dehydration (Nakashima et al. 2009, Cutler et al. 2010, Pardo 2010, Raghavendra et al. 2010, Qin et al. 2011). ABA regulates seed maturation and dormancy, including accumulation of storage proteins and protective proteins required for desiccation tolerance (Cutler et al. 2010). ABA also mediates responses to water stress in vegetative tissues by induction of such protective proteins and by controlling stomatal closure (Cutler et al. 2010, Raghavendra et al. 2010, Qin et al. 2011). ABA is perceived by PYR/PYL/RCAR receptor proteins, which in turn inhibit Type 2C protein phosphatase (PP2C) activity, leading SnRK2 family protein kinases to be in an active, phosphorylated state (Ma et al. 2009, Park et al. 2009, Cutler et al. 2010, Raghavendra et al. 2010, Umezawa et al. 2013). This results in activation of ABA-responsive transcription factors by SnRK2s and thereby downstream gene activation (Nakashima et al. 2009, Cutler et al. 2010, Raghavendra et al. 2010, Umezawa et al. 2013). On the other hand, however, it has recently been reported that ABA-associated genes are induced in mature tissues but not in growing tissues under mild osmotic stress conditions (Skirycz et al. 2011). This implies that different tolerance mechanisms operate depending on the developmental stage.

Recent molecular genetic studies of rice have also revealed several factors involved in mechanisms that ensure plant growth under salinity conditions. Rice Salt Sensitive 1 (RSS1) is required for cell proliferative activities in the shoot and root meristem as well as leaf and root primordia under stressful conditions, and loss-of-function mutants of RSS1 are hypersensitive not only to salt stress but also to ion, hypersmotic and high and low temperature stresses (Ogawa et al. 2011, Ogawa et al. 2012). Analysis of another salt-sensitive mutant, rss3, has shown that the suppression of excessive responses to jasmonate (JA) is required for root elongation under salinity conditions (Toda et al. 2013a, Toda et al. 2013b). RSS3 encodes a nuclear protein binding to basic helix–loop–helix (bHLH) transcription factors and jasmonate ZIM domain (JAZ) proteins, and mediates repression of JA-inducible gene expression in root tips (Toda et al. 2013a). The importance of suppression of JA action has also been suggested by the finding that over-expression of the gene encoding CYP94C2b, which promotes deactivation of JA-Ile, results in enhanced salt tolerance (Kurotani et al. 2015b).

Although numerous genes have been reported to confer tolerance to salt and other abiotic stresses, comparative data showing the effects of genes under the same growth conditions are scarce. In this study, we investigated a collection of transgenic rice lines, in each of which a gene expected to confer salt tolerance was overexpressed, by taking advantage of previously prepared full-length cDNA overexpression (FOX) lines (Hakata et al. 2010, Tsuchida-Mayama et al. 2010). We analyzed profiles of the stress tolerance of these lines, together with extant salt-tolerant rice varieties, and selected chromosome segment substitution lines (CSSLs), by exposing them to ion, hypersmotic and heat stresses, in addition to salinity stress, for which tolerance was evaluated under both laboratory and greenhouse conditions. We found that the overexpression of CYP94C2b was most effective to confer salt tolerance under both laboratory and greenhouse conditions, whereas overexpression of factors involved in ABA signaling conferred multiple stress tolerance. The comparative data described here provide a basis for designing stress-tolerant plants, depending on expected environmental conditions.

### Results

**The Nona Bokra allele of SKC1 has limited effects on salt tolerance in the Koshihikari background, depending on the growth conditions**

To obtain a basis for comparison, we evaluated salt tolerance levels in nine salt- or drought-tolerant rice varieties (Nona Bokra, Pokkali, Heitai, Dekiyama, Kyudai-asahi 3, Yaemugura, Shiniriki-Daigaku, Shiniriki 7 and Taikanseiuruchi) and two standard japonica cultivars (Nipponbare and Koshihikari) under two different experimental conditions. We also examined three CSSLs, 501–503, derived from Nona Bokra and Koshiihikari (Takai et al. 2007), carrying the Nona Bokra allele of SKC1 in the Koshihikari background, to evaluate the effects of the SKC1 allele under different salinity conditions. First, shoot explants excised from 1-week-old seedlings were grown on Murashige and Skoog (MS) medium supplemented with an equal volume of 600 mM NaCl (300 mM final) under aseptic conditions (Kurotani et al. 2015b) [Supplementary Fig. S1]. In this test, all of the varieties and CSSLs showed high salt tolerance in comparison with Nipponbare and Koshihikari [Fig. 1, salinity (chamber); Supplementary Fig. S1]. In contrast, when evaluated under hydroponic culture in the greenhouse [Supplementary Fig. S2; Supplementary Data set S2], the levels of salt tolerance of the stress-tolerant varieties and the CSSLs were more highly varied. Nona Bokra, Pokkali and some varieties showed high salt tolerance compared with Nipponbare and Koshihikari [Fig. 1, salinity (greenhouse)].
However, the other varieties and the CSSLs showed no conspicuous salt tolerance in comparison with Nipponbare, even though they were moderately more tolerant than Koshihikari. Thus, the degree of salt tolerance differs greatly depending on growth conditions. The results also suggest that the Nona Bokra allele of SKC1 has quite limited effects on salt tolerance.

Salt tolerance of extant rice varieties correlated with ionic stress tolerance more tightly than osmotic stress tolerance

In plant tissues, high concentrations of NaCl impose ionic and osmotic stresses (Zhu 2001), the latter of which can partly mimic dehydration stress. Plants are often exposed to heat stress under dehydration stress conditions, which cause stomatal closure and reduced transpiration that minimizes water loss, resulting in a rise in temperature at the leaf surface. Considering possible overlap among these stress responses, the ability to adapt to various abiotic stresses may affect the levels of salt tolerance, depending on growth conditions.

We accordingly evaluated tolerance to ionic, hyperosmotic and heat stresses in addition to salinity, using the same lines. Seedlings were exposed to 40 mM LiCl for testing ionic stress tolerance, and to 26% polyethylene glycol (PEG) for scoring hyperosmotic stress tolerance (Supplementary Figs. S3, S4). For treatment with heat stress, seedlings were incubated at 42°C (Supplementary Fig. S5). Notably, Nona Bokra and Pokkali showed high tolerance to all the stresses imposed (Fig. 1). The other salt-tolerant varieties also showed ionic or heat tolerance to some extent. The three CSSLs were not tolerant to these stresses as compared with Koshihikari, which showed moderate levels of hyperosmotic stress tolerance when compared with Nipponbare. By cluster analysis, salinity tolerance in the greenhouse appeared to have higher correlation with tolerance to ionic stress than to other stresses among the varieties and the CSSLs (Fig. 1). This finding was supported by a Pearson’s correlation coefficient of 0.76 between tolerance to ionic stress and salinity in the greenhouse (P < 0.01) (Supplementary Data set S5).
CYP94C2b is a key determinant for viability under salinity conditions.

We next investigated the extent to which stress tolerance genes have varied or overlapping effects, depending on conditions. For this analysis, we selected stress tolerance genes in two ways. First, we screened a population of FOX rice lines individually overexpressing rice full-length cDNAs (Hakata et al. 2010, Tsuchida-Mayama et al. 2010) to identify salt tolerance genes as described (Kurotani et al. 2015b). We found two lines, CU099 and FE047, bearing full-length cDNAs for Os04g0584800 and Os12g0150200, respectively, which showed salinity tolerance under aseptic conditions, and confirmed that overexpression of these genes is responsible for salt tolerance (Supplementary Fig. S6; Kurotani et al. 2015b). In addition to these two genes, we selected 72 genes that have been reported to confer resistance to salt and/or other abiotic stresses, or their homologs (Supplementary Table S1; Supplementary Data set S1). The 74 genes encode proteins that cover a wide range of functional categories.

We then compared the effects of overexpression of the 74 genes on stress tolerance in the background of Nipponbare, by exposing respective FOX lines and additionally prepared gene-overexpressing lines (Supplementary Data set S1) to salt, ionic, hyperosmotic and heat stresses. Of the 74 genes investigated, 19 and 17 genes conferred salt tolerance under chamber and greenhouse conditions, respectively, compared with the non-transgenic wild type (cv. Nipponbare), whereas 25 genes were effective for ionic stress tolerance, 38 for heat tolerance and 50 for hyperosmotic stress tolerance (Fig. 2; Supplementary Table S2; Supplementary Data set S3). The effects of the overexpressed genes on salt tolerance depended greatly on the growth conditions, with only seven genes conferring tolerance under both salinity conditions (Fig. 2, green circles). Among them, the most effective salt tolerance gene was Os12g0150200 encoding CYP94C2b. CYP94C2b is a homolog of the Cyt P450 family protein CYP94C1, involved in deactivation of JA-Ile (Heitz et al. 2012, Kurotani et al. 2015b). Overexpression of CYP94C2b results in lower sensitivity to JA and delay of salinity-induced leaf senescence, suggesting that inactivation of JA signaling is important for enhancement of salt tolerance (Kurotani et al. 2015b). Notably, the levels of salt tolerance in the CYP94C2b-overexpressing line were comparable with those in Nona Bokra and Pokkali, the two most salt-tolerant varieties (Supplementary Data set S4). This suggests that deactivation of JA is one of the key determinants for viability under salinity conditions.

CYP94C2b was highly expressed in salt-tolerant varieties such as Pokkali and Heitai

To explore further the effects of CYP94C2b, we investigated the levels of its expression in the varieties that had exhibited high levels of salt tolerance in the greenhouse (Pokkali, Nona Bokra, Heitai and Taikanseiuruchi). We also examined Shinriki 7, which had shown a stress tolerance profile similar to that of the CYP94C2b-overexpressing line. As shown in Fig. 3, strikingly high levels of CYP94C2b expression were observed in Pokkali and Heitai. Nona Bokra also showed moderately higher levels of CYP94C2b expression. In contrast, Taikanseiuruchi and Shinriki 7 showed nearly the same levels of CYP94C2b expression as those in Nipponbare. Thus, high-level expression of CYP94C2b may in part account for salt tolerance in some but not all the salt-tolerant rice varieties.

Genes encoding ABA signaling factors had effects to confer multiple stress tolerance

We classified the 74 genes according to the stress tolerance profiles of the corresponding gene-overexpressing lines. The results of the four stress tests (salinity (greenhouse), LiCl, heat and hyperosmotic stresses) were subjected to clustering. Salt tolerance in the chamber was not included for this analysis, because many lines were not viable under that condition, resulting in a narrow range of comparison.

As shown in Fig. 2, most of the tested genes were classified into eight groups. Ten genes were classified into cluster II, in which the corresponding gene-overexpressing lines showed enhanced stress tolerance under several different conditions. These genes conferred relatively moderate levels of tolerance to each stress, except for three genes conferring high levels of LiCl tolerance, and did not show apparent negative effects on stress tolerance under any of the tested conditions. Of the 10 genes, only the newly identified Os04g0584800 conferred high levels of salt tolerance under the aseptic condition, thus in all the five conditions tested. Os04g0584800 encodes a protein annotated as GTP1 that possesses putative GTP-binding and adaptin-binding domains (PFAM domains PF08477/PF00071 and PF10199) and is similar to an Arabidopsis protein, AT5G65960, but otherwise not functionally characterized (Supplementary Fig. S6). Most of the genes other than those in cluster II conferred tolerance under only one or two stress conditions.

Cluster II contained three genes encoding proteins involved in the activation of ABA signaling: a protein kinase (SnRK2-8) and two transcription factors (AREB1 and ABF3) (Kobayashi et al. 2004, Fujita et al. 2005, Kobayashi et al. 2005, Fujita et al. 2009, Nakashima et al. 2009, Cutler et al. 2010, Yoshida et al. 2010, Umezawa et al. 2013) (Fig. 2, blue stars). These genes conferred enhanced stress tolerance under at least three different conditions. To investigate whether ABA-induced genes were preferentially categorized in cluster II, we compared profiles of gene expression in response to ABA and other phytohormones with the stress tolerance profiles (Supplementary Figs. S7, S8). Many of the 74 genes were responsive to ABA and/or JA. However, ABA-induced genes were not enriched in cluster II or any other specific cluster, and the same was true for JA-induced genes. These results suggest that ABA signaling contributes to versatile stress tolerance mechanisms by activation of downstream genes, each of which confers tolerance to different types of stresses.

Genes conferring tolerance to both heat and hyperosmotic stresses were preferentially linked to functional categories related to heat shock proteins, ROS scavenging and Ca\(^{2+}\) signaling

Clustering analysis with the gene-overexpressing lines showed a weak but substantial correlation between heat and...
hyperosmotic stress tolerance, but no striking correlation between other stress tolerance patterns (Fig. 2). This was also indicated by Pearson’s correlation coefficient (Supplementary Data set S5). Twenty-nine genes conferred both heat and hyperosmotic tolerance and were classified into three groups (Fig. 4): class A, less tolerant to heat than to hyperosmotic stress; class B, highly tolerant to both heat and hyperosmotic stress; and class C, moderately tolerant to heat and hyperosmotic stress. Among 10 genes in class A, five genes were associated with Ca\(^{2+}\) signaling. This finding shows significant enrichment, given that only eight genes associated with Ca\(^{2+}\) signaling were included in the 74 tested genes (Fig. 4; Supplementary Table S3). Similarly, class C appeared to be associated preferentially with genes encoding HSPs and proteins for ROS scavenging, such as superoxide dismutase or ascorbate peroxidase.
In this study, we compared the patterns of abiotic stress tolerance of a collection of rice varieties, CSSLs and transgenic lines overexpressing stress tolerance genes under the same experimental conditions. Stress tolerance profiling of the extant varieties and the CSSLs showed that salinity tolerance in the greenhouse was associated with LiCl tolerance. This raises the possibility that there might be genes that are fairly effective at conferring both ionic stress tolerance and salinity tolerance in some of the tested varieties. In the case of Nona Bokra, previous findings suggest that a cation transporter, HKT1;5, encoded by SKC1 is involved in salt tolerance (Ren et al. 2005). It should be noted, however, that this selectively transports Na\(^+\) but not Li\(^+\) (Ren et al. 2005). Moreover, we could not observe conspicuous effects of the Nona Bokra allele of SKC1 on salt tolerance in the greenhouse, suggesting that unknown loci other than SKC1 are required for high levels of salt tolerance in Nona Bokra. Yet, under the salinity condition with MS medium, the CSSLs containing the Nona Bokra allele of SKC1 exhibited higher levels of salt tolerance than Koshihikari and Nipponbare. This may imply that the salinity condition in the greenhouse causes additional physiological change(s) in rice plants, leading to masking of the effects of SKC1. For example, under the aseptic condition, seedlings are grown in capped bottles, preserving high humidity, whereas in the greenhouse, seedlings are exposed to lower humidity, possibly causing increased ion uptake along with enhanced transpiration flow. Interestingly, Nona Bokra and Pokkali showed not only the highest levels of salt tolerance, but also high tolerance to ionic, heat and hyperosmotic stresses. This raises the question of whether heat and hyperosmotic tolerance genes, in addition to ionic stress-tolerant genes, are involved in high levels of salinity tolerance in these varieties.

Stress tolerance profiling using the transgenic lines showed various patterns of the effects of abiotic stress tolerance genes. Overexpression of genes encoding ABA signaling factors conferred multiple stress tolerance. This probably reflects that gene regulation that is co-ordinated by ABA is involved in abiotic stress tolerance. However, at least under the two salinity stress conditions, CYP94C2b involved in JA metabolism was the most effective gene conferring salt tolerance by overexpression. Notably, the levels of salt tolerance in the CYP94C2b-overexpressing line were comparable with those in the most salt-tolerant varieties, Nona Bokra and Pokkali. Since CYP94C2b has recently been shown to promote deactivation of JA-Ile (Kurotani et al. 2015b), these findings further emphasize the importance of suppression of JA signaling for salt tolerance. As reported by Kurotani et al. (2015b), salt tolerance is enhanced in the transgenic lines (in the background of Nipponbare), in which 5- to 150-fold increased levels expression of CYP94C2b are observed. In Pokkali and Heitai, the CYP94C2b expression levels were 10- to 45-fold and 5- to 80-fold increased as compared with Nipponbare, respectively. Therefore, it is plausible that higher expression of CYP94C2b contributes to enhanced tolerance at least in these varieties.

In addition to the study of CYP94C2b-overexpressing lines, there have been several studies that point out repressive roles of JA in salt tolerance. For example, after salt stress shock, roots of a JA-insensitive Arabidopsis mutant jai3 enter the recovery phase earlier and exhibit a higher growth rate thereafter than the wild type (Geng et al. 2013). In rice, a defect of RSS3 factor that mediates repression of JA-responsive gene expression results in severe growth inhibition under salinity stress conditions (Toda et al. 2013a). In contrast, however, it has been suggested that JA signaling is involved in stress responses, leading to tolerance to abiotic stresses including salinity stress (Kazan 2015). In rice, for example, Os-bHLH148, a transcription factor induced by JA, ABA and abiotic stresses such as drought and salinity, confers drought tolerance when overexpressed (Seo et al. 2011). Such a difference in effects of JA may be due to differences in dynamic properties of JA signals, in the context of timing and amplitude, whose importance in controlling gene regulation has recently been proposed (Ismail et al. 2014, Kurotani et al. 2015a).

In the greenhouse, the damage symptoms in leaves of the CYP94C2b-overexpressing line were less pronounced. This is probably because overexpression of CYP94C2b alleviates JA responses and causes delay of salinity-induced leaf senescence (Kurotani et al. 2015b). This has also raised the possibility that delayed loss of Chl and photosynthesis via reduced JA-Ile levels contributes to enhanced viability of the CYP94C2b-overexpressing lines under salinity conditions. If so, tolerance to other abiotic stresses might also be enhanced. However, overexpression of CYP94C2b moderately improved tolerance to heat but not to ionic and hyperosmotic stresses, suggesting...
that enhanced viability under salinity conditions in the CYP94C2b-overexpressing lines is not due merely to residual photosynthetic activity. Rather, another effect of repression of JA signalling might also be responsible for salt and heat tolerance. It will be desirable to identify downstream factors involved in salt and heat tolerance.

Among the 10 multiple stress tolerance genes in cluster II, only the newly identified gene (Os04g0584800) conferred stress tolerance under all experimental conditions, including salinity in the chamber. Os04g0584800 (GTP1) encodes a protein that contains a GTP-binding domain related to that of Rho or Rab GTPases and an adaptin-binding domain sharing sequence similarity with α- and γ-adaptin-binding protein p34 (Page et al. 1999). α- and γ-adaptins are components of the tetrameric adaptor complexes AP2 and AP1, respectively. These complexes form clathrin-coated vesicles that mediate membrane traffic and are responsible for protein transport from the plasma membrane and trans-Golgi network, such as endocytosis and vacuolar protein sorting (Robinson and Pimpl 2014, Wang et al. 2014). The p34 protein binding to α- and γ-adaptins may have a chaperone-like function,
probably ensuring vesicle trafficking (Page et al. 1999). It is currently unclear whether the rice putative GTP- and adaptin-binding protein has a similar function, although membrane vesicle trafficking is suggested to be important for salt and osmotic stress tolerance (Mazel et al. 2004, Asaoka et al. 2013). From another viewpoint, Os04g0584800 belonged to class C among the genes that conferred heat and hyperosmotic stresses (Fig. 4). Given that class C was enriched with genes encoding HSPs and ROS-scavenging proteins, Os04g0584800 may have functions similar to these. Future studies may clarify how this protein is involved in multiple stress tolerance.

In our study, most of the 74 examined genes showed effects to confer abiotic stress tolerance. Some transgenes appeared to be less effective than we expected, even though they were selected based on previous studies. This is probably due to the fact that the expression levels of the transgenes would not be suitable to confer substantial tolerance, and/or that levels of stress tolerance often vary depending on growth conditions. Despite such a limitation, we may speculate on the most effective approach to conferring environmental stress tolerance upon plants, based on our profiling results. Enhancement of ABA signaling is a reasonable approach, as previously suggested (Nakashima et al. 2009, Cutler et al. 2010, Pardo 2010, Raghavendra et al. 2010, Qin et al. 2011). Overexpression of SnRK2 and transcription factors that activate ABA-inducible genes (Kobayashi et al. 2004, Fujita et al. 2005, Kobayashi et al. 2005, Fujita et al. 2009, Nakashima et al. 2009, Cutler et al. 2010, Yoshida et al. 2010, Umezawa et al. 2013) indeed conferred multiple stress tolerance. This effect is probably achieved by downstream genes, each of which has different effects on stress tolerance, considering the patterns of stress tolerance conferred by ABA-responsive genes. It is desirable to utilize these genes more effectively. For example, it would be potentially valuable to identify factors complementing the action of ABA. Another effective way of conferring stress tolerance would be to deploy a combination of genes, each of which confers specific and pronounced effects on stress tolerance. In this case, candidate genes can be selected according to their stress tolerance profiles. Such profiles would be useful for improvement not only of rice but also of other crops, when combined with stress tolerance profiles of varieties of interest. Some genes can also be tested for their effects on tolerance to combined stresses, such as ‘heat and salinity’. It will also be interesting to identify the genes responsible for tolerance to various stresses in Nona Bokra and Pokkali. Identification and functional characterization of such genes should contribute to further understanding of complicated stress tolerance mechanisms and crop improvement.

**Materials and Methods**

**Plant materials**

FOX lines of rice (Oryza sativa, background cv. Nipponbare) were prepared previously (Hakata et al. 2010, Tsuchida-Mayama et al. 2010), or in this study, by essentially the same procedure. For some genes, cDNA with protein-coding regions was overexpressed instead of full-length cDNA. DNA fragments were amplified from NIAS cDNA clones or from a cDNA library by PCR with the primers listed in Supplementary Table S4, inserted into pSMAHdN637L-GateA or pActnos-Hm2 (courtesy of Dr. Makoto Matsuoka, Nagoya University), and used for rice transformation as described (Ogawa et al. 2011, Toda et al. 2013a). Insertion of transgenes was confirmed by PCR using the primers listed in Supplementary Table S4, and by subsequent DNA sequencing (for newly prepared lines). Seeds of Heitai, Dekiyama, Kyudai-ashai 3, Yaemugura, Shinriki-Daigaku and Shiriuki 7 were obtained from the Institute of Genetic Resources, Faculty of Agriculture, Kyushu University. Seeds of Nona Bokra, Pokkali, Koshihikari and CSSLs derived from Nona Bokra and Koshihikari were obtained from NIAS, Japan.

**Salt tolerance test under aseptic conditions**

Salt tolerance of rice plants under aseptic conditions was evaluated as described (Kurotani et al. 2015b). Seeds of wild-type and transgenic rice were surface-sterilized and germinated on solid MS basal medium (1% sucrose, 0.25% gelatin gum and 0.05% MES-KOH, pH 5.8) in 13 cm high capped bottles in a chamber at 28°C under a photoperiod of 14 h light (6,000 lux, white light) and 10 h dark. Shoot explants containing the basal region (4 cm long) from 7-day-old seedlings were transferred onto the same medium, and evaluated for salt tolerance by pouring the same volume of 600 mM NaCl over the medium (final, 300 mM NaCl, 0.5 × MS medium). The explants were grown for 5 weeks in the same chamber, and their survival after washing and subsequent cultivation with water for a week was scored. For screening salt-tolerant transgenic lines, T0 or T1 seedlings of rice FOX or gene-overexpressing lines (~2,500 lines in total) were evaluated. The surviving plants were transferred to soil and grown in an isolated greenhouse for seed harvesting. For candidate salt-tolerant lines, the same test was performed with T2 plants, in parallel with salt-tolerant and control varieties. Under these conditions, almost none of the non-transgenic wild-type Nipponbare plants was viable. The corresponding rice cDNAs for candidate genes for salt tolerance were amplified from respective NIAS cDNA clones to prepare independent overexpressing lines. PCR products sub-cloned in the pENTR/D-TOPO vector (Life Technologies) were transferred to pSMAHdN637L-GateA as described (Kurotani et al. 2015b). Among the genes in the five most tolerant lines, Os04g0584800 and Os12g0150200 were confirmed to confer salt tolerance in independently prepared transgenic lines, and were accordingly selected for further analyses. For comparative analysis of transgenic lines, non-transgenic Nipponbare wild-type plants were set in each experiment as the comparison reference.

**Salt tolerance test under greenhouse conditions**

Salinity tolerance using hydroponically cultured seedlings in the greenhouse was evaluated as described (Thomson et al. 2010, Kurotani et al. 2015b). After sterilization with benomyl, seeds were incubated on moist filter paper at 30°C in darkness for 2 d for germination. Seedlings were transferred to seedling floats and grown with deionized water in the greenhouse for 3 d, and then with a 10 liter solution of salinized Yoshida medium (pH 5.0) (Thomson et al. 2010). Electrical conductivity (EC) of the hydroponic culture solution was adjusted to 6 μS cm−1 for the initial 3 d, and then increased to 12 μS cm−1 by addition of NaCl. In the latter solution, the final concentration of NaCl was around 120 mM. EC and pH were adjusted every 2–3 d by addition of water and NaOH. Each float included both salt-sensitive Nipponbare and salt-tolerant Shiriuki 7 as reference for comparison. After 2 weeks, leaf symptoms caused by salinity stress were evaluated as described (Thomson et al. 2010). In each experiment, 20 individuals for each line were evaluated. The median scores of these individuals were then converted into relative values by the following equation, using scores of plants used as comparison reference: relative value = (score of Nipponbare − score of sample)/(score of Nipponbare − score of Shiriuki 7). All values were tested for normality against a standard normal distribution by χ2 test. The relative values were normally distributed and were further converted into Z-scores as described below.

**Evaluation of heat, hyperosmotic and ionic stress tolerance**

For evaluation of heat stress tolerance, rice seeds were germinated on moist filter paper at 30°C in the dark for 3 d. Seedlings were hydroponically cultured
in water for 3 d and then with Yoshida medium (pH 5.0) for 17 d at 28°C. The cultured plants were then placed at 42°C for 7 d. After further cultivation at 28°C for 7 d, survival was scored. In some experiments, heat treatment at 42°C was extended to 10 d until most of the Nipponbare plants were severely damaged. For the hyperosmotic stress test, seedlings were germinated and hydroponically cultured in the same manner, with water for 3 d and then with Yoshida medium (pH 5.0) for 15 d at 28°C. Plants were cultivated in medium containing 26% PEG (mol. wt = 4,000 Da on average) for 10 d, and then without PEG for 7 d before scoring survival. To test ionic stress tolerance, surface-sterilized seeds of rice were germinated and cultivated on MS basal medium for 7 d. Shoot segments were excised from 7-day-old seedlings and cultured plants were then placed at 42°C with Yoshida medium (pH 5.0) for 15 d at 28°C.

The authors thank Taisuke Nishimura for helpful discussion, Toshihiro Kumamaru, Hidemi Kitano, Kazuyuki Doi and Masahiro Yano for providing seeds of the stress-tolerant rice varieties, Keiko Iida-Okada and Etsuko Sugai for preparation of the FOX rice seeds, Akiko Yamamoto and Tomonori Hobo for technical advice, and Miyuki Hattori, Reiko Hisada, Naoko Kominami, Mami Sugii, Chiho Kanayama, Rena Endo, Yoshiko Nagai and Toshiko Komada for technical assistance.

**Disclosures**

The authors have no conflicts of interest to declare.

**References**


Kurotani, K., Hayashi, K., Hatanaka, S., Toda, Y., Ogawa, D., Ichikawa, H., et al. (2015b) Elevated levels of CYP94 family gene expression alleviate...