PCP
PLANT & CELL PHYSIOLOGY

Induced and Constitutive DNA Methylation in a Salinity-Tolerant Wheat Introgression Line

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Cytosine methylation is a well recognized epigenetic mark. Here, the methylation status of a salinity-tolerant wheat cultivar (cv. SR3, derived from a somatic hybridization event) and its progenitor parent (cv. JN177) was explored both globally and within a set of 24 genes responsive to salinity stress. A further comparison was made between DNA extracted from plants grown under control conditions and when challenged by salinity stress. The SR3 and JN177 genomes differed with respect to their global methylation level, and methylation levels were reduced by exposure to salinity stress. We found the genetic stress- (triggered by a combination of different genomes in somatic hybridization) induced methylation pattern of 13 loci in non-stressed SR3; the same 13 loci were found to undergo methylation in salinity-stressed JN177. For the salinity-responsive genes, SR3 and JN177 also showed different methylation modifications. C methylation polymorphisms induced by salinity stress were present in both the promoter and coding regions of some of the 24 selected genes, but only the former were associated with changes in transcript abundance. The expression of both *TaFLS1* (encoding a flavonol synthase) and *TaWRSI5* (encoding a Bowman–Birk-type protease inhibitor), which showed both a different expression and a different DNA methylation level between SR3 and JN177, enhanced the salinity tolerance of *Arabidopsis thaliana*. C methylation changes appear to be a common component of the plant response to stress, and methylation changes triggered by somatic hybridization may contribute to the superior salinity tolerance of SR3.

Introduction

Methylation at the cytosine C5 position is the most abundant modification of DNA, with up to 25% of the cytosines present in the plant genome affected in this way (Steward et al. 2002). C methylation in mammalian genomes is restricted to CpG dinucleotides, but in plant genomes it also occurs at CpNpG and CpNpN trinucleotide sites (where N is A, T or C) (Meyer et al. 1994). In *Arabidopsis thaliana*, CpG and CpNpG methylation is maintained largely through, respectively, the methyltransferase MET1 and the plant-specific chromomethylase CMT3 (Cao and Jacobsen 2002), while de novo C methylation is carried out by DRM1 and DRM2 (Henderson and Jacobsen 2007), with some contribution from MET1 and CMT3 (Gehring and Henikoff 2008). DNA methylation is thought to influence a wide range of biological processes (Bird 2002, Bender 2004, Goll and Bestor 2005, Zhang et al. 2006). More than one-third of all *A. thaliana* gene sequences experience a degree of methylation, and its occurrence has been correlated with differential gene expression, either temporally or spatially (Zilberman et al. 2007). CpG methylation in promoter sequences has been implicated in the regulation of expression, while that at trinucleotide sites is probably more strongly associated with transposon inactivation (Bird 2002, Cao and Jacobsen 2002).

Some aspects of gene expression during abiotic stress episodes have been shown to be affected by differential methylation (Labra et al. 2002, Aina et al. 2004, Choi and Sano 2007). The absence of *Met1* expression in tobacco has been shown to induce the expression of a large number of stress-response genes (Wada et al. 2004), while in the halophyte *Mesembryanthemum crystallinum*, the switch from C_3 to CAM (Crussulacean acid metabolism) photosynthesis triggered by either drought or salinity stress is associated with the hypermethylation of satellite DNA (Dyachenco et al. 2006). A further agent of C methylation gain and loss is genetic stress (such as the intra- or interspecific hybridization which combine different genomes into the same nucleus), as evidenced in newly synthesized allopolyploids between species of *Triticum*, *Brassica* and *A. thaliana* (Comai et al. 2000, Shaked et al.

2001, Lukens et al. 2006). Re-arrangement of the epigenome in these wide hybrids could explain at least some of the observed non-additive patterns of gene expression (Kashkush et al. 2002). Wide hybrids generated by somatic hybridization are also characterized by genome-wide changes in methylation (S.W. Liu, unpublished). Although DNA methylation is a common genomic response to both environmental and genetic stress, the mechanistic basis of these epigenetic modifications and how they determine greater tolerance to stress is hardly understood. The bread wheat cultivar SR3 is a high yielding, salinity-tolerant derivative of an asymmetric somatic hybrid (Xia 2009) between a salinity-sensitive cultivar Jinan 177 (JN177) and salt-resistant tall wheatgrass (*Thinopyrum ponticum*) (Weimberg and Shannon 1988) which has proven phenotypically and cytogenetically stable over many selfing generations (Wang et al. 2004). The SR3 genome possesses <1.5% genomic DNA putatively derived from tall wheatgrass estimated by GISH (genomic in situ hybridization) (Wang et al. 2004). A series of analyses has uncovered a range of both genetic and epigenetic differences between the genomes of different tall wheatgrass somatic hybrid lines and the parent wheat cv. JN177 (Liu et al. 2007, Liu et al. 2012, unpublished data). For several salinity-responsive genes, there are clear differences in transcript levels between stressed SR3 and JN177 which cannot be explained by differences in either the promoter or the coding sequence of these genes (Shan et al. 2008, Liu et al. 2012, Xu et al. 2013). Here, the focus was to investigate the epigenetic variations induced by somatic hybridization and salinity stress. Our data will help to understand how plants use epigenetic modifications to counteract both genetic and environmental stress.

Results

Global levels of DNA methylation in SR3 and JN177 as assessed by HPLC and MSAP

HPLC analysis indicated that the global proportion in SR3 and JN177 of methylated to non-methylated cytosine was similar; in total approximately 22% under non-stressed and 19% under salinity-stressed conditions (**Fig. 1a**). The distribution of C methylation, as explored using methylation-sensitive amplification polymorphism (MSAP), illustrated a number of differences between SR3 and JN177, both in control plants and in those subjected to salinity stress (**Fig. 1b**, **c**). Of the 360 CpCpGpG sites assayed, 21 were hypermethylated and 30 hypomethylated in SR3 compared with JN177 plants grown under non-stressed conditions; however, in salinity-stressed conditions, 11 and 19 sites were hypermethylated and 16 and 21 were hypomethylated in SR3 and JN177, respectively, compared with non-stressed SR3 and JN177 plants (**Supplementary Table S1**). Moreover, SR3 plants grown in the absence of salinity stress and the JN177 plants grown in its presence shared the same methylation status at 13 (five hyper- and eight hypomethylated) sites (**Supplementary Table S1**); since SR3 showed more salinity tolerance than JN177 (Peng et al. 2009, Liu et al.

2012), such genetic stress-induced methylation modifications may underlie some of the enhanced salinity tolerance shown by SR3.

Methylation status of salinity stress-responsive genes

A set of 24 genes was selected from a microarray-based transcriptomic data set which compared the response of SR3 and JN177 to salinity stress (Liu et al. 2012). Each of these genes was up- or down-regulated by at least 4-fold in plants subjected to salinity stress and also showed different expression levels between SR3 and JN177, and covered a range of functional categories, including transcription factors, signal transduction factors, channel transporters, metabolism, protein modification or degradation, and reactive oxygen species homeostasis (**Supplementary Table S2**). The sequences of the coding and promoter regions of the set of 24 salinity stress-responsive genes were analyzed via bisulfite sequencing. The efficiency of the sodium bisulfite treatment in converting non-methylated cytosine to thymine was about 99.6% (**Supplementary Fig. S1**). The scan involved 1,127 C sites in JN177 and 1,126 for SR3 (since there was a C to T polymorphism in the *TaCP450* coding region). In JN177, 13.7% of the sites were methylated, while the proportion in SR3 was 12.4%. C methylation was detected at seven of the 24 coding sequences (*TaGBF1*, *TaTBP2*, *TaPMSR1*, *TaSRO1*, *TaFLS1*, *TaAQP1* and *TaHKT1;5*), and at nine of the 16 promoter sequences (*TaTIP2;2*, *TaWRSI5*, *TaCaml1*, *TaSRO1*, *TaFLS1*, *TaCHP*, *TaHKT1;5*, *TaCambp* and *TaAQP3*). All the coding region methylation sites occurred at a CpG dinucleotide; however, in the promoter regions about 70% of the sites were CpG, 20% CpNpG and 10% CpNpN (**Fig. 2a**). Of the coding region CpG dinucleotides, 12.3% were methylated in JN177 and 13.2% in SR3, while in the promoter regions the proportions were, respectively, 63.3 and 62.4%. The frequency of methylated trinucleotide sites was 24.8 and 6.3% (CpNpG and CpNpN) in JN177 and 22.5 and 6.2% in SR3 (**Fig. 2b**). The 21 somatic hybridization-induced demethylation sites which were methylated in JN177 but non-methylated in SR3 comprised eight CpG dinucleotides, five CpNpG trinucleotides and eight CpNpN trinucleotides (**Supplementary Table S3**). For example, the cytosine at position −354 in the *TaHKT1;5* promoter was highly methylated in JN177 but not in SR3 (**Fig. 2c**). Of the 10 sites methylated in SR3 but not in JN177, five involved a CpG dinucleotide, three a CpNpG trinucleotide and two a CpNpN trinucleotide (**Fig. 2c**; **Supplementary Table S3**). The coding region of *TaGBF1* included two further examples (**Fig. 2c**). Methylation changes were induced in some of the 24 salt-responsive genes; bisulfite sequencing results indicated that the overall methylation levels of CpG, CpNpG and CpNpN contexts in the promoter region were all reduced under salt treatment, while the overall methylation level was rarely changed in the coding region (**Fig. 2d**).

Methylation changes were induced in the coding regions of *TaTBP5*, *TaPMSR1*, *TaGBF1* and *TaSRO1* in response to the
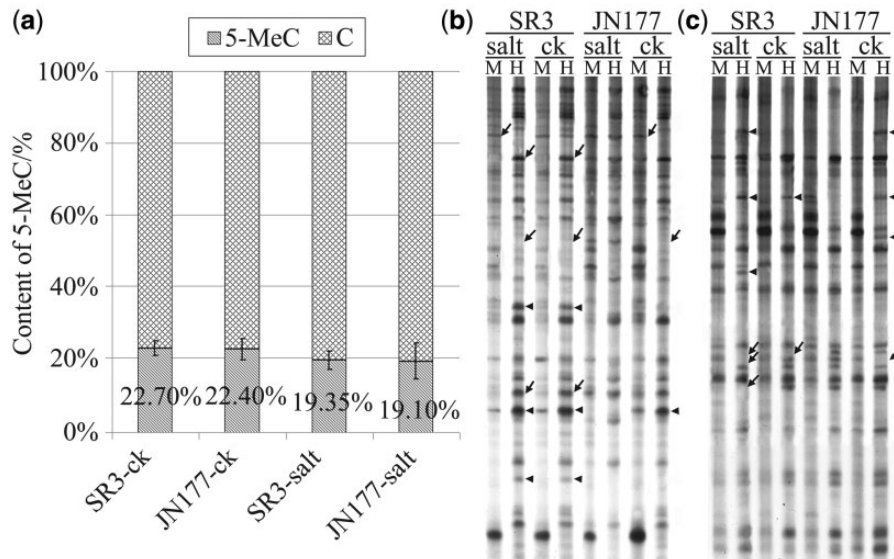
Fig. 1 Global methylation status of cvs. SR3 and JN177 plants exposed to salinity stress (SR3-salt, JN177-salt) or grown in non-stressed conditions (SR3-ck, JN177-ck). (a) HPLC analysis and (b, c) MSAP profiling. In (a), methylated cytosines are shown in the lower column and non-methylated cytosines in the upper column. Data are given as the mean ± SD. In (b) and (c), arrows indicate hypermethylated loci and triangles indicate hypomethylated loci in SR3-ck, SR3-salt and JN177-salt compared with JN177-ck. M, DNA restricted by *Eco*RI + *Msp*I, H, DNA restricted by *Eco*RI + *Hpa*II.

imposition of salinity stress (**Fig. 3**). For example, in *TaTBP5*, positions +801 and +820 became hypermethylated, and positions +808 and +843 hypomethylated (**Fig. 3a**), and the level of C methylation in the *TaPMSR1* CG island (+600 to +720) was similarly affected (**Fig. 3b**). Though there were methylation changes at some specific sites in the coding regions of these genes, the overall methylation levels of coding regions rarely varied, except for *TaGBF1* (**Fig. 3**). The methylation profile of the *TaFLS1*, *TaHKT1;5*, *TaTIP2;2* and *TaWRSI5* promoter regions all changed as a result of the salinity stress treatment. Although the overall methylation level of the *TaFLS1* coding region and the 3′ non-coding region (3′-untranslated region) was unaffected by the imposition of stress (**Supplementary Fig. S2**), the majority of the cytosines in its promoter between positions −1,031 and −396 became hypomethylated, and the level of methylation in the JN177 gene was overall higher than in the SR3 gene under both non-stressed and salinity-stressed conditions (**Fig. 4a**). The level of C methylation in the *TaHKT1;5* promoter between positions −300 and −93 was increased by the stress treatment in both SR3 and JN177, with the overall level being lower in JN177 than in SR3 under both conditions (**Fig. 4b**). In the *TaTIP2;2* promoter CG islands 1 (−570 to −415) and 2 (−2190 to −1930), the methylation status of almost all the cytosines present as CpG dinucleotides was stable (data not shown), but that at CpNpG and CpNpN trinucleotides was clearly increased in both SR3 and JN177 plants exposed to stress (**Fig. 4c**). In the *TaWRSI5* promoter (−1,459 to −359), the exposure to salinity stress decreased the level of C methylation in SR3, but not in JN177 (**Fig. 4d**); no methylation modification was detected in the coding region and 3′-non-coding region of *TaWRSI5* either in SR3 or in JN177.

The relationship between C methylation and transcript abundance under salinity stress

The relationship between methylation and transcript abundance under salinity stress was explored by comparing the outcomes of applying salinity stress and treating with the methyltransferases inhibitor 5-azacytidine (5-azaC). To determine the correct concentration for 5-azaC treatment, we detected the methylation status and transcript abundance of *TaFLS1* after exposure to 10, 50 and 100 μM 5-azaC for 0 h, 24 h and 7 d. The results revealed that 10 μM 5-azaC could not remove the methylation and induce *TaFLS1* expression effectively, while exposure to 100 μM 5-azaC increased *TaFLS1* expression in 24 h when the DNA methylation modification was not removed, which suggested that this concentration was a severe stress for wheat. Treatment with 50 μM 5-azaC for 7 d could remove the methylation and induce *TaFLS1* expression effectively (**Supplementary Fig. S3**); thus, we chose 50 μM 5-azaC treatment to explain the relationship between DNA methylation and the relative expression of genes. When bisulfite sequencing was used to determine the DNA methylation status in the coding regions of *TaTBP5*, *TaPMSR1*, *TaGBF1* and *TaSRO1* and the promoter regions of *TaFLS1*, *TaHKT1;5*, *TaTIP2;2* and *TaWRSI5* following exposure to 5-azaC, the proportion of methylated cytosines fell to <30% (**Supplementary Fig. S4**). No evidence for any change in transcript abundance was noted for *TaTBP5*, *TaPMSR1*, *TaGBF1* or *TaSRO1* in plants treated with 5-azaC (**Fig. 5**); all these genes did, however, experience up- or down-regulation of transcript abundance and also alterations in their DNA methylation status as a result of the imposition of salinity stress. *TaFLS1*, *TaHKT1;5*, *TaTIP2;2*
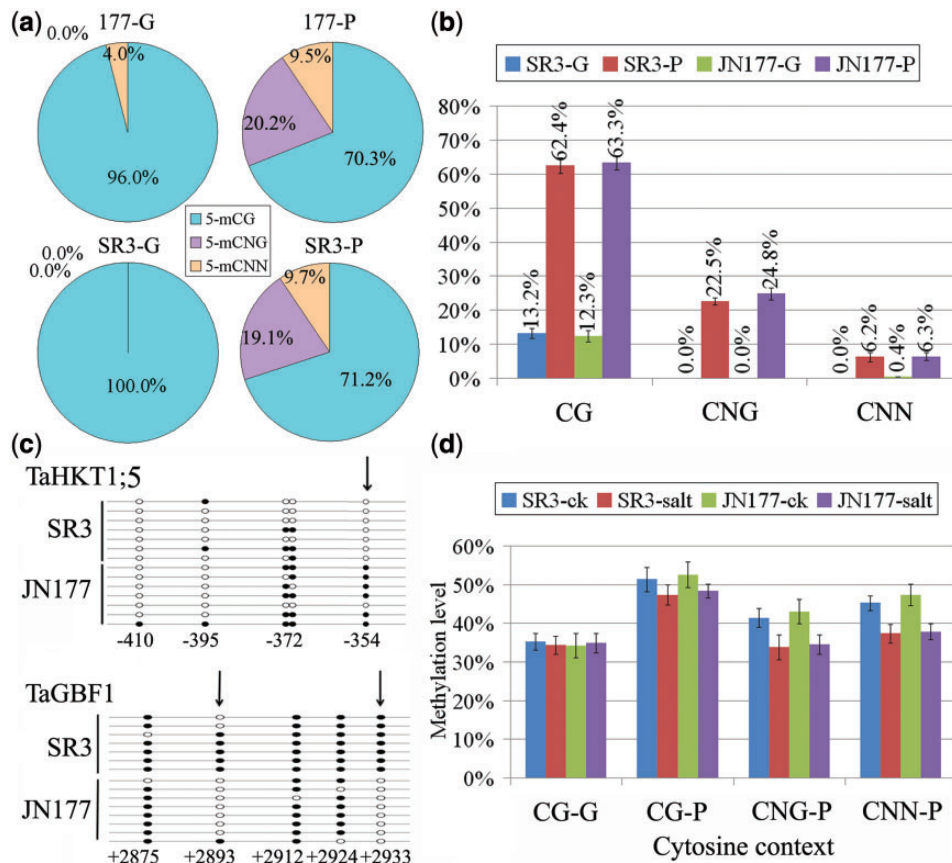
Fig. 2 C methylation in salinity stress-responsive genes of cvs. SR3 and JN177. (a) The distribution of methylated cytosines between the three contexts CpG, CpNpG and CpNpN sites. (b) The methylation frequency of the various cytosine sites in the coding (JN177-G, SR3-G) and promoter (JN177-P, SR3-P) regions. (c) C methylation status in the *TaHKT1;5* promoter and the *TaGBF1* coding region, with rings representing non-methylated cytosines and black dots methylated cytosines; arrows indicate cytosine sites polymorphic between SR3 and JN177 in terms of methylation status. (d) Overall methylation level of different cytosine contexts in the coding region (CG-G) and promoter region (CG-P, CNG-P, CNN-P) of control (SR3-ck, JN177-ck) and salinity-stressed (SR3-salt, JN177-salt) SR3 and JN177 seedlings. Data are shown as the mean ± SD.

and *TaWRSI5* were all up-regulated by the 5-azaC treatment. *TaFLS1* transcript abundance was higher in SR3 than in JN177 (**Fig. 6a**), in contrast to the somatic hybridization-induced lower methylation level in its promoter region (**Fig. 4a**). *TaHKT1;5* was also up-regulated by the 5-azaC treatment, and also the lower expression level was negatively related to a somatic hybridization-induced higher DNA methylation ratio in SR3 than in JN177, under both control and salinity treatment (**Figs. 4b**, **6b**). Exposure to 5-azaC had a pronounced positive effect on the transcript abundance of *TaTIP2;2* (**Fig. 6c**). In contrast, *TaWRSI5* was noticeably up-regulated in SR3 but not in JN177 (**Fig. 6d**), coincident with the decreased promoter methylation in SR3 but unchanged methylation in JN177 by the imposition of salinity stress (**Fig. 4d**). Since DNA methylation is generally negatively related to histone acetylation (Fuks 2005), the behavior of the same genes was also explored in response to treatment with sodium butyrate (NaB), which is generally used as an inhibitor of histone deacetylases to promote histone acetylation. As for the 5-azaC treatment, exposure to NaB

had no effect on the transcription of the four genes which responded to salinity stress by an alteration in their coding region methylation status (**Fig. 5**). However, the NaB treatment did markedly up-regulate *TaFLS1*, *TaHKT1;5* and *TaTIP2;2* (**Fig. 6a–c**), but not *TaWRSI5* (**Fig. 6d**). All these results indicated that the somatic hybridization-induced epigenetic variations participate in regulating the transcript abundance of some salinity-responsive genes. DNA methylation and histone deacetylation of the promoter but not the gene body region was responsible for negatively regulating the transcript abundances of these genes.

The expression of *TaFLS1* and *TaWRSI5* enhanced the salinity tolerance of *A. thaliana*

To understand the roles of the epigenetic-regulated salt-responsive genes in plant abiotic stress responses, two of these genes, *TaFLS1* and *TaWRSI5*, were separately constitutively expressed in *A. thaliana* by placing them under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. The response to
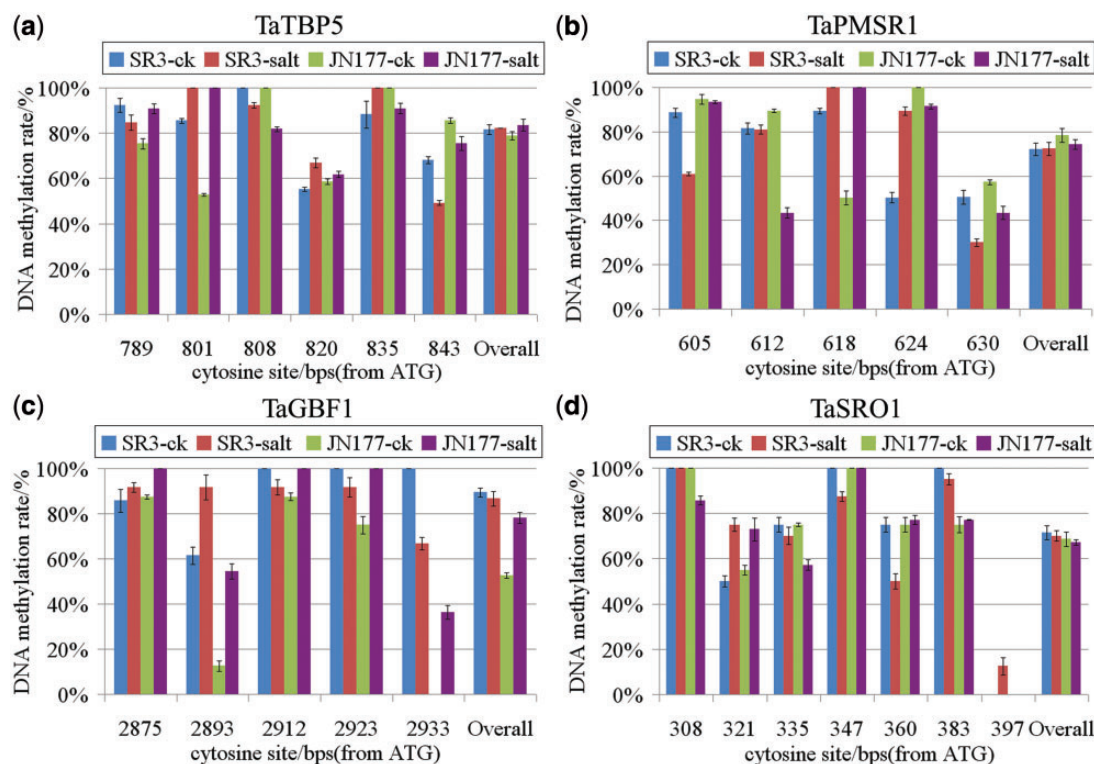
Fig. 3 C methylation in the coding regions of (a) *TaTBP5*, (b) *TaPMSR1*, (c) *TaGBF1* and (d) *TaSRO1* in control (SR3-ck, JN177-ck) and salinity-stressed (SR3-salt, JN177-salt) SR3 and JN177 seedlings. Data are given as the mean ± SD.

salinity stress of two independent *TaFLS1* transgenic lines [flavonol synthase-overexpresser 1 (FLS-OE1) and FLS-OE2], chosen on the basis that they generated a similar level of transgene transcript, was compared with that of a transgenic line harboring only an empty vector (VC). The OE and VC plants did not differ with respect to either their leaf or root growth when grown under non-stressed conditions. However, when challenged with 100, 150 or 200 mM NaCl, the primary roots of the OE plants grew more vigorously than did those of the VC plants (**Fig. 7**). These results indicate that the expression of *TaFLS1* was able to enhance root growth of *A. thaliana* under salinity stress. Additionally, the responses of transgenic plants to salinity stress during the germination stage were also investigated; however, no difference was observed in the germination rate and speed between FLS-OE seeds and VC seeds with or without exposure to NaCl (**Supplementary Fig. S5**). Therefore, *TaFLS1* was able to enhance the salinity tolerance of *A. thaliana* at the seedling stage but not at the germination stage. Transgenic seedlings expressing *TaWRSI5* are known to be able to tolerate salinity stress at the seedling stage (Shan et al. 2008); here we further detected whether the overexpression of *TaWRSI5* enhanced the salinity tolerance of the transgenic plants during the germination stage or not. Both VC and the two selected transgenic lines, WRSI-OE1 and WRSI-OE2, showed the same germination rate in the presence of either 0 or 50 mM NaCl (**Fig. 8a, b**); however, in the presence of 100 or 150 mM NaCl, the germination of WRSI-OE seeds was more

rapid and more complete than that of VC seeds (**Fig. 8c, d**), which confirmed the promoter effect of the transgene for germination in the presence of salinity.

Discussion

Methylation of salinity stress-responsive genes

The imposition of salinity stress induced modifications of the C methylation status in the coding sequence of seven of the 24 genes monitored, a proportion similar to that observed in both *A. thaliana* and rice (Zhang et al. 2006, Li et al. 2008). More than half (9/16) of the promoter regions analyzed became modified, in contrast to the global proportions of just 5% in those of *A. thaliana* and 9% in those of rice (Zilberman et al. 2007, Li et al. 2008). Thus, while DNA methylation is a common feature of plant genes, the indication is that stress-responsive genes appear to be more commonly regulated by DNA methylation than are genes in general. The C methylation in the promoter regions occurred in all three contexts (CpG, CpNpG and CpNpN), while in the coding regions it was almost completely confined to CpG sites. The three contexts responded differently to salinity stress. The level of *TaTIP2;2* transcription was reduced by salinity stress; the CpNpG sites in its promoter became more heavily methylated in response to the salinity stress, while the proportion of CpGs experiencing methylation was hardly affected. The gene's up-regulation by 5-azaC
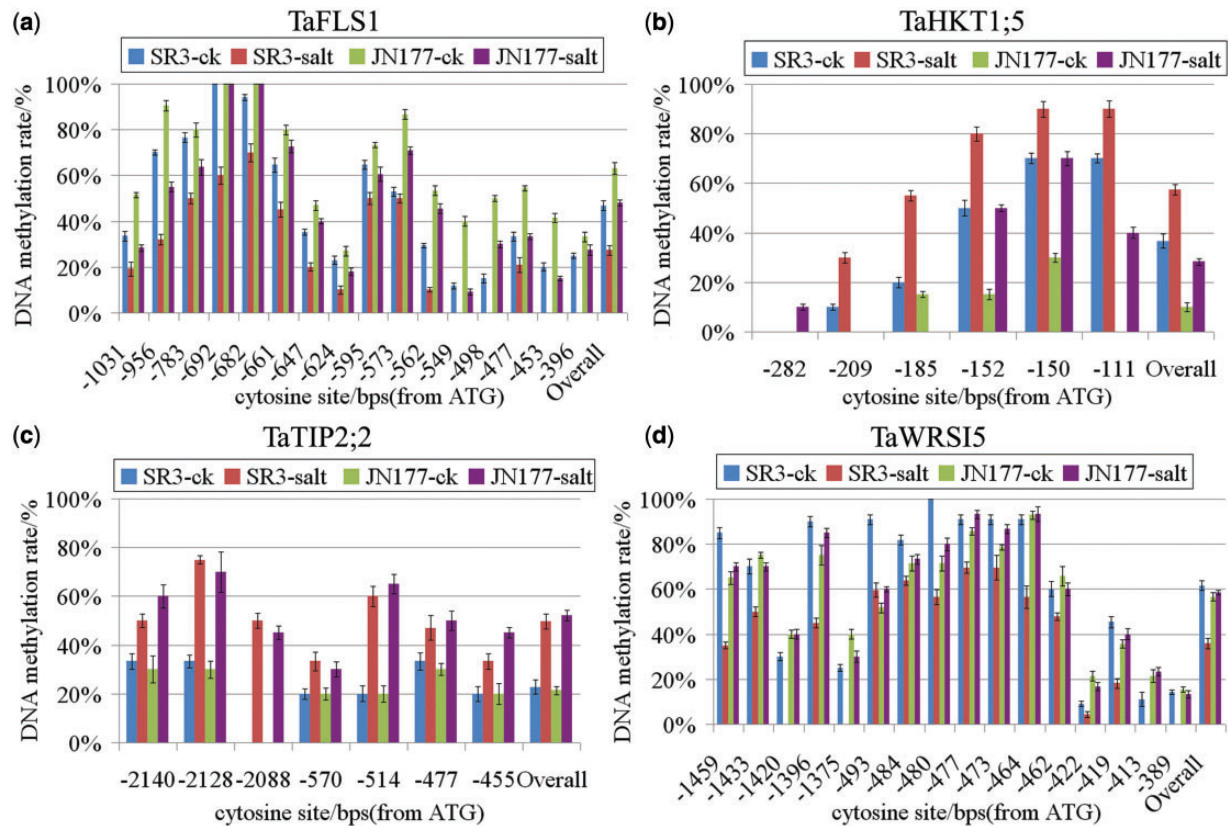
Fig. 4 C methylation in the promoter regions of (a) *TaFLS1*, (b) *TaHKT1;5*, (c) *TaTIP2;2* (with only CpNpG and CpNpN sites present) and (d) *TaWRSI5*. Control (non-stressed) seedlings, SR3-ck, JN177-ck; salinity-stressed seedlings, SR3-salt, JN177-salt. Data are given as the mean ± SD.

treatment implied that it was the hypermethylation of the CpNpGs rather than of CpGs which was primarily responsible for its salinity stress-induced repression. The result chimes well with the observation that in *A. thaliana*, CpNpG methylation makes a greater contribution to gene regulation than that at CpGs (Zhang et al. 2006).

The role of DNA methylation in regulating gene expression

Of the 24 coding and 16 promoter regions analyzed, four of each experienced alterations in their DNA methylation profile as a result of the imposition of salinity stress. Some cytosine positions in the coding regions became demethylated and others became methylated, with the overall methylation level rarely varied. Neither these changes nor the demethylation induced by 5-azaC treatment had any noticeable effect on transcript abundance. Genes which feature C methylation in their coding sequence have a tendency to be transcribed at a moderate or high level, and have proved to be less likely than non-methylated genes to exhibit tissue-specific transcription (Zhang et al. 2006, Zilberman et al. 2007). As a result, the prediction is that methylation in the coding sequence will more probably be associated with the fine-tuning of

transcription (Zilberman et al. 2007). What the function (if any) of stress-induced methylation changes in coding sequence could be remains as yet unresolved.

On the other hand, promoter sequence methylation is known to be intimately associated with gene regulation (Boyes and Bird 1991, Siegfried et al. 1999). The salinity stress-induced gain of methylation in the *TaTIP2;2* and *TaHKT1;5* promoters and its loss in the *TaFLS1* promoter were related, as would be expected, to the pattern of transcription. Their 5-azaC treatment-induced demethylation resulted in a marked up-regulation of all three genes. The overall methylation level within the SR3 *TaFLS1* promoter was lower than in the JN177 promoter, opposite to the higher level of *TaFLS1* transcript present in SR3. The higher methylation level within the SR3 *TaHKT1;5* promoter was also opposite to the lower level of transcript in SR3 compared with that present in JN177. *TaWRSI5* behaved in an unexpected way: the gene was substantially up-regulated by salinity stress in both cultivars, but while the DNA methylation level of the JN177 promoter was largely unaffected, that of the SR3 promoter was reduced. At the same time, *TaWRSI5* transcription was strongly induced by 5-azaC treatment in SR3 but not in JN177. The implication was that DNA demethylation was responsible for the salinity
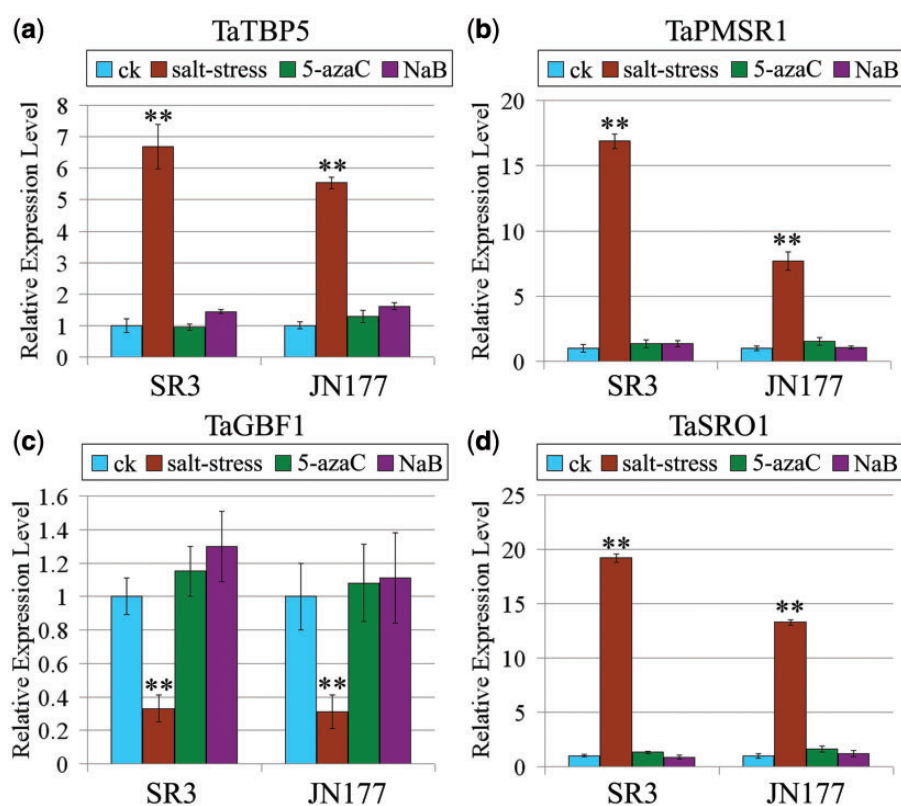
Fig. 5 Transcript abundances of (a) *TaTBP5*, (b) *TaPMSR1*, (c) *TaGBF1* and (d) *TaSRO1* in SR3 and JN177 seedlings grown without any treatment (ck), exposed to salinity stress or treated with either 5-azaC or NaB. The wheat *TaActin* gene was used as an endogenous control. Data are shown as the mean ± SD. Asterisks indicate significantly different means, as determined by the Student's *t*-test, at *$P < 0.05$ and **$P < 0.01$, respectively.

stress-induced up-regulation of the gene in SR3, while another regulation mechanism(s) must have been operating in JN177.

How promoter C methylation interacts with gene expression has been the topic of intensive research. A well recognized mechanism operates through the recruitment of methyl CpG-binding proteins (MBPs); although these differ from one another in how they interact with their target, they all utilize histone deacetylase activity to remodel chromatin locally (Jones et al. 1998, Nan et al. 1998, Ng et al. 1999, Zhang et al. 1999). Thus, the silencing of *TaTIP2;2*, *TaHKT1;5* and *TaFLS1* very probably relied on this mechanism, consistent with the ability of both 5-azaC and NaB treatment to up-regulate each of these three genes. An alternative route to achieve silencing via promoter DNA methylation is offered by the direct inhibition of transcriptional activator binding (Watt and Molloy 1988, Bird 2002). Some of the salinity stress-induced alterations in C methylation occurred at sites located within known *cis*-elements, such as ABRE in *TaCaml1* and a MYB binding site in *TaFLS1* and *TaTIP2;2* (**Supplementary Table S4**). In *A. thaliana*, MYB transcription factors may regulate *FLS* expression by binding to the promoter sequence

(Winkel-Shirley 2002), but whether or not the methylation status of the MYB binding sites affects the binding capacity of the transcription factors, and thereby regulates the expression of *TaFLS1* under salinity stress, will need further investigation.

Changes in C methylation status is a common response to stress

Examples of stress-induced methylation/demethylation have been documented in a number of plant species (Dyachenko et al. 2006, Boyko et al. 2007, Choi and Sano 2007). Since DNA methylation/demethylation is intimately involved in gene regulation, it is logical to expect that the induction of methylation/demethylation represents an important component of the stress response (Angers et al. 2010). Here, salinity stress induced a range of methylation profile changes in the promoter regions of the salinity stress-responsive genes. Two of these genes (*TaFLS1* and *TaWRSI5*), when expressed in *A. thaliana*, enhanced the level of salinity tolerance at the seedling or even the germination stage (**Figs. 7**, **8**; Shan et al. 2008); while another gene *TaTIP2;2*, which is down-regulated under salinity stress, has been shown to enhance sensitivity to salinity stress
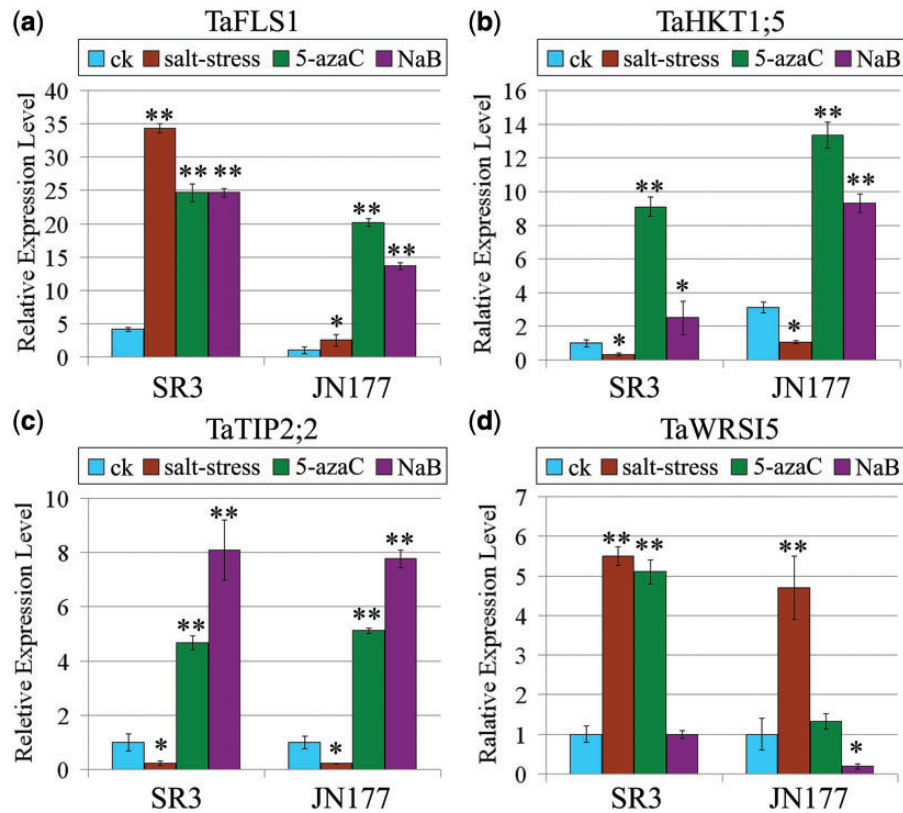
Fig. 6 Transcript abundances of (a) *TaFLS1*, (b) *TaHKT1;5*, (c) *TaTIP2;2* and (d) *TaWRSI5* in SR3 and JN177 seedlings grown without any treatment (ck), exposed to salinity stress or treated with either 5-azaC or NaB. The wheat *TaActin* gene was used as an endogenous control. Data are shown as the mean ± SD. Asterisks indicate significantly different means, as determined by the Student's *t*-test, at *$P < 0.05$ and **$P < 0.01$, respectively.

when expressed in *A. thaliana* (Xu et al. 2013). Thus, methylation-regulated gene expression appears to make a significant contribution to protecting plants against stress-induced damage.

While McClintock's (1984) proposal that genomic restructuring is a likely outcome of de novo widespread hybridization, the more recent ability to distinguish between methylated and non-methylated DNA has led to a recognition that part of this restructuring involves genome-wide alterations in C methylation (Comai et al. 2000, Shaked et al. 2001, Lukens et al. 2006). Here, the analysis of the cultivars JN177 and SR3 via MSAP profiling and bisulfite sequencing has shown that the SR3 and JN177 genomes vary considerably at the epigenetic level, both globally and within the promoter (and coding) region of a selection of salinity stress-responsive genes. In contrast to JN177, 21 sites were demethylated while only 10 sites showed novel methylation modifications in SR3. With some exceptions, the JN177 promoter regions of these genes tended to be more heavily methylated than those of SR3 (**Fig. 2**). The lower methylation level of the SR3 *TaFLS1* (salinity tolerant in OE plants) sequence may be responsible for the higher transcript abundance present. Together, these effects may be, in part at least, responsible for the superior salinity stress tolerance shown by SR3. Given that SR3 is genetically very similar to JN177 and yet is clearly more salinity tolerant, it is tempting to suggest that some of the methylation changes triggered by the somatic hybridization process could have made a significant contribution to the salinity tolerance shown by SR3 (Liu et al. 2012). The conclusion is that C methylation changes represent a common component of the plant response to stress.

Materials and Methods

Wheat materials and growing conditions

Grains of cvs. JN177 and SR3 (the 10th selfed generation) were germinated on wet filter paper for 2 d at 20°C, then transferred to a hydroponic solution containing half-strength Hoagland's liquid medium (Hoagland and Arnon 1950) with minor modification [KH_2PO_4 68 mg l^{-1}, KNO_3 252.5 mg l^{-1}, $Ca(NO_3)_2$ 410 mg l^{-1}, $MgSO_4$ 120 mg l^{-1}, H_3BO_3 1.43 mg l^{-1}, $MnCl_2·4H_2O$ 0.95 mg l^{-1}, $ZnSO_4·7H_2O$ 0.11 mg l^{-1}, $CuSO_4·5H_2O$ 0.04 mg l^{-1}, $NaMoO_4·H_2O$ 0.01 mg l^{-1}, $FeSO_4·7H_2O$ 62 mg l^{-1}, Na_2EDTA 83 mg l^{-1}] under a 12 h photoperiod (light/dark temperature 22/20°C), a relative humidity of 50% and 300 µmol $m^{-2} s^{-1}$ PAR (photosynthetically active radiation) in the growth chamber. The solutions were changed every day to avoid anoxia. At the three-leaf stage (21 d after germination),
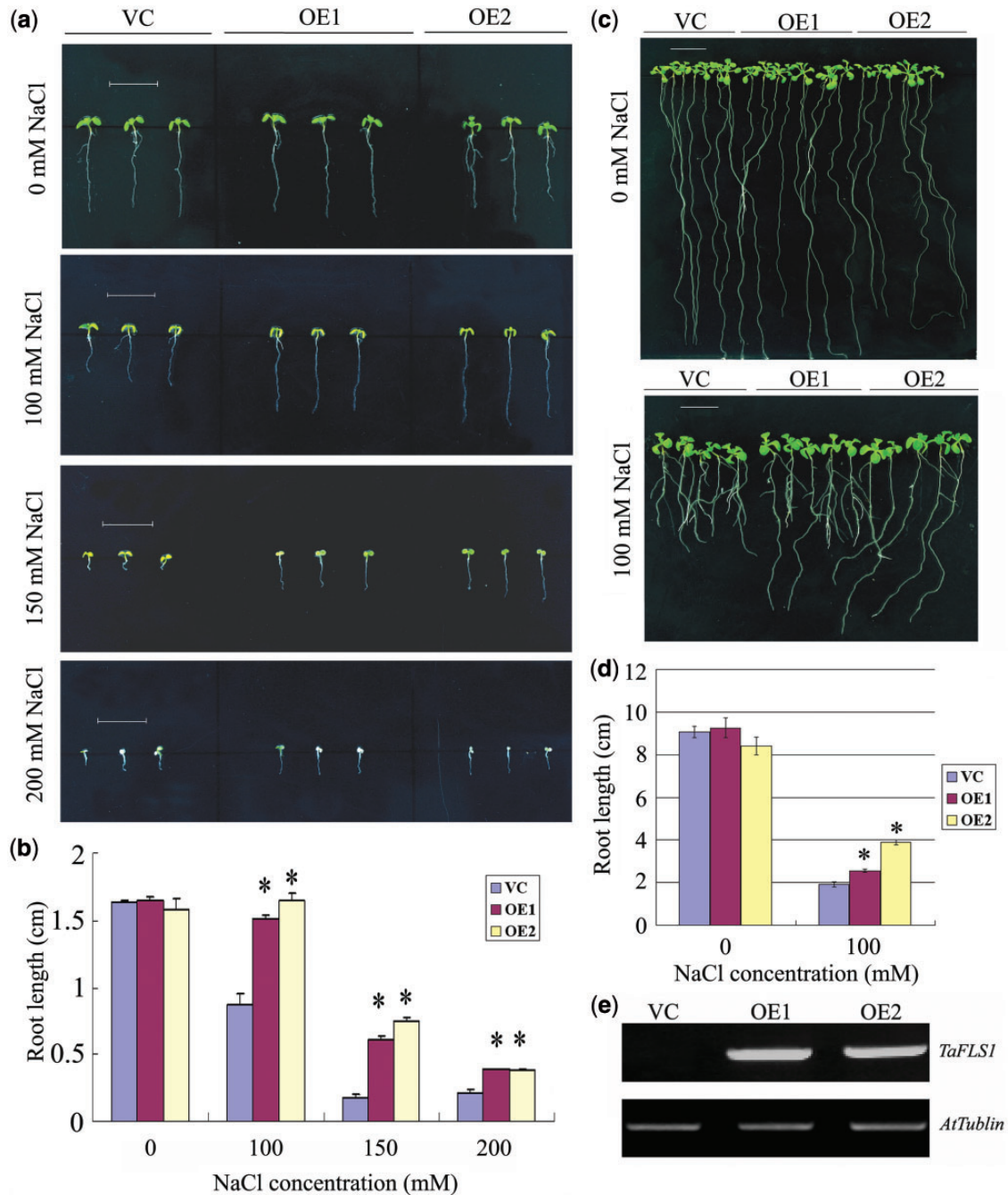
Fig. 7 The expression of *TaFLS1* in *A. thaliana*. (a) OE (carrying the transgene) plants produced longer primary roots than those of the VC (empty vector) plants after a 5 d exposure to NaCl; OE and VC plants were indistinguishable under non-stressed conditions. (b) Root length of OE and VC lines grown under non-stressed and salinity-stressed conditions for 5 d. (c) OE plants produced longer primary roots than the VC plants after a 12 d exposure to 100 mM NaCl. (d) Root length of OE and VC lines grown under non-stressed and salinity-stressed conditions for 12 d. (e) Transcription of *TaFLS1* in OE and VC lines. Data are shown as the mean ±SD. An asterisk indicates significantly different means, as determined by the Student's *t*-test, at $P < 0.05$.

the hydroponic solution was supplemented with either 200 mM NaCl (to impose salinity stress, added in daily increments of 50 mM NaCl), 50 μM 5-azaC (to promote DNA demethylation) or 50 μM NaB (to promote histone acetylation). A fourth treatment where there was no addition of supplements was used as a control. After 24 h exposure to 200 mM NaCl, the roots and the leaves of seedlings in the salinity treatment and control were harvested for DNA and RNA extraction; the treatment for the 5-azaC and NaB exposure was 3 d earlier than salinity treatment (50, 100, 150 and 200 mM NaCl each for 24 h) and the treatment period was 7 d, thus sampling at the same time as those of the salinity treatment and control. Each treatment was replicated
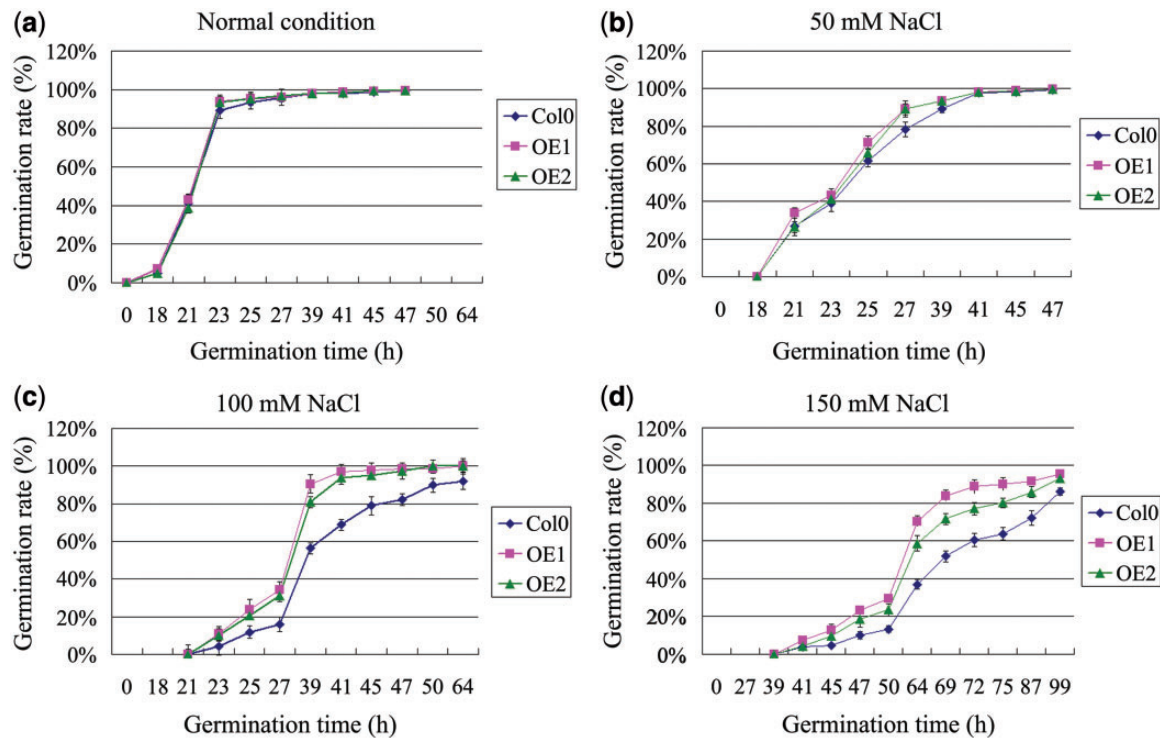
Fig. 8 The expression of *TaWRSI5* in *A. thaliana* enhanced salinity tolerance during germination. The germination rate of OE (carrying the transgene) and VC (empty vector) seeds was comparable under normal conditions (a) or 50 mM NaCl (b), but was more rapid and more complete when the seeds were germinated in the presence of 100 mM (c) or 150 mM NaCl (d). Data are shown as the mean ± SD.

three times, and each replicate comprised three seedlings in one pot (5.5 cm in diameter and 9 cm deep). All seedlings were randomly placed in the growth chamber.

DNA extraction, DNA hydrolysis and HPLC analysis

Genomic DNA was isolated following Doyle and Doyle (1987). For the purpose of HPLC analysis, 40 μg of DNA was added to 50 ml of 70% (v/v) perchloric acid and held at 100°C for 1 h. The pH was adjusted to between 3 and 5 using 1 M KOH. After KClO$_4$ precipitation, the sample was centrifuged at 13,000×g for 5 min and the supernatant was used for HPLC analysis, performed according to Demeulemeester et al. (1999) with minor modifications. The eluent was a mixture of 5% (v/v) methanol, 4.75 mM sodium hexanesulfonate, 0.2% (v/v) triethanolamine and tri-distilled water, adjusted to pH 5.5 with phosphoric acid and vacuum filtered. The sample was first passed through a 4 mm sterile syringe filter with a pore size of 0.2 μm and then injected into an LC-10AT VP device (SHIMADZU) fitted with a Venusil ASB C$_{18}$ (5 μm, 4.6×250 mm, Agela Technologies) column, applying a flow rate of 0.7 ml min^{-1}. The signal was detected at 273 nm. Retention times were compared with those obtained from a standard preparation of cytosine and methylated cytosine. The proportion of methylated cytosine present was calculated from the formula (concentration of methylated cytosine×100%)/(concentration of methylated cytosine + concentration of cytosine).

MSAP profiling

The MSAP procedure followed that of Shaked et al. (2001) and was based on 24 primer combinations. The amplicons were separated by electrophoresis through a 6% denaturing polyacrylamide gel and visualized by silver staining. Each MSAP reaction was amplified in triplicate, and only intensely staining fragments >100 bp were scored.

Identification of genes responsive to salinity stress

The full-length coding region of all 24 salinity-responsive genes was extracted where possible from cDNA libraries made from SR3 and JN177 mRNA; otherwise, it was obtained by RACE (rapid amplification of cDNA ends) PCR applied to partial cDNA sequences. The corresponding genomic sequences were PCR amplified from SR3 and JN177 genomic DNA, and 16 of the 24 promoter sequences were obtained by using a BD GenomeWalker Universal kit (BD Biosciences Clontech) in combination with the wheat cv. Chinese Spring draft genome assembly database (www.cerealsdb.uk.net). Relevant primers are listed in **Supplementary Table S5**.

RNA extraction and real-time quantitative PCR

Total RNA was extracted using the TRIzol Reagent (Invitrogen), and converted to cDNA using the M-MLV reverse transcriptase kit (Invitrogen). The real-time quantitative PCR procedure was based on the iCycler iQ™ real-time PCR detection

system (Bio-Rad), following the manufacturer's instructions. For normalization, the wheat *TaActin* gene (AB181991) was used as an endogenous control. Each reaction was repeated three times for each of three independent biological samples. Relevant primers are given in **Supplementary Table S5**.

Bisulfite sequencing

Genomic DNA extracted from the same seedlings used to extract RNA was processed with an EpiTect Bisulfite kit (Qiagen). CG islands were detected and relevant primers designed using MethPrimer software (Li and Dahiya 2002). Primer sequences are given in **Supplementary Table S5**. The PCR products were ligated with the pEASY-T Vector (TransGen), and at least 30 clones per insert were processed for sequencing. Sequences for which the cytosine transformation efficiency was <97% were removed using Biq Analyzer software (Bock et al. 2005). The ratio of C methylation at each CpG dinucleotide, and CpNpG and CpNpN trinucleotide was calculated and transformed into a percentage using CyMATE software (www.gmi.oeaw.ac.at/research-groups/cymate/cymate/).

Transformation of *A. thaliana* and the imposition of salinity stress

The coding regions of *TaFLS1* and *TaWRSI5* were inserted separately into the pSTART vector under the control of the CaMV 35S promoter. The resulting construct (or the empty pSTART vector) was transformed into *A. thaliana* ecotype Col-0 using the floral dip method (Clough and Bent 1998). Seeds harvested from both homozygous transgenic plants carrying *TaFLS1* (FLS-OE lines) and the empty pSTART vector (VC line) were surface-sterilized, plated on half-strength Murashige and Skoog agar medium, held at 4°C in the dark for 2 d to break seed dormancy, and finally exposed to a 16 h photoperiod at 22°C for 2 d. The seedlings were challenged with salinity by transferring them onto a fresh plate supplemented with either 0, 100, 150 or 200 mM NaCl, where they were left to grow for a further 5 or 12 d. All experiments were run as three independent replicates. The germination assay for the homozygous transgenic plants carrying the respective *TaFLS1* and *TaWRSI5* (WRSI-OE lines) and VC lines comprised approximately 120 surface-sterilized seeds per line placed on half-strength Murashige and Skoog agar medium containing either 0, 50, 100 or 150 mM NaCl. The plates were held at 4°C for 2 d, and then changed to a 16 h photoperiod at 22°C. The emergence of the radicle was taken as representing a successfully germinated seed. The germination rate was given by the number of germinated seeds as a percentage of the total number of seeds plated. Each data point represented the mean of three replicates.

Supplementary data

Supplementary data are available at PCP online.

Disclosures

The authors have no conflicts of interest to declare.

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