RESEARCH PAPER

The 2′-O-methyladenosine nucleoside modification gene OsTRM13 positively regulates salt stress tolerance in rice

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Abstract

Stress induces changes of modified nucleosides in tRNA, and these changes can influence codon–anticodon interaction and therefore the translation of target proteins. Certain nucleoside modification genes are associated with regulation of stress tolerance and immune response in plants. In this study, we found a dramatic increase of 2′-O-methyladenosine (Am) nucleoside in rice seedlings subjected to salt stress and abscisic acid (ABA) treatment. We identified LOC_Os03g61750 (OsTRM13) as a rice candidate methyltransferase for the Am modification. OsTRM13 transcript levels increased significantly upon salt stress and ABA treatment, and the OsTrm13 protein was found to be located primarily to the nucleus. More importantly, OsTRM13 overexpression plants displayed improved salt stress tolerance, and vice versa, OsTRM13 RNA interference (RNAi) plants showed reduced tolerance. Furthermore, OsTRM13 complemented a yeast trm13Δ mutant, deficient in Am synthesis, and the purified OsTrm13 protein catalysed Am nucleoside formation on tRNA-Gly-GCC in vitro. Our results show that OsTRM13, encoding a rice tRNA nucleoside methyltransferase, is an important regulator of salt stress tolerance in rice.

Key words: 2′-O-methyladenosine, ABA, modified nucleoside, rice, salt stress tolerance, tRNA.

Introduction

Modified nucleosides are derivatives of the four common nucleosides, adenosine (A), guanosine (G), uridine (U), and cytidine (C). They are particularly important for transfer RNA (tRNA), since more than 85% of all modified nucleosides are present on tRNA molecules (RNA modification database, http://rna-mdb.cas.albany.edu/; Cantara et al., 2011). Modified nucleosides influence the decoding process of tRNA, and therefore affect protein translation and cellular metabolism (Björk et al., 1987; Bjork, 1995; Urbonavicius et al., 2001). Approximately 25–30 different modified

Abbreviations: Ψ, pseudouridine; A, adenosine; ac4C, N4-acetylcytidine; AdoMet, S-adenosyl-methionine; Am, 2′-O-methyladenosine; C, cytidine; Cm, 2′-O-methylcytidine; D, dihydrouridine; G, guanosine; Gm, 2′-O-methylguanosine; I, inosine; m1A, 1-methyladenosine; m2A, 2-methyladenosine; m3A, N3-methyladenosine; m6C, 6-methylcytidine; m7G, 7-methylguanosine; m2G, N2-methylguanosine; m2,G, N2,N2-dimethylguanosine; m2,G, 7-methylguanosine; m1,G, 1-methylinosine; m1,G,A, N1-methyl-N6-threonylcarbamoyladenosine; m2,G,U, 5-methyluridine; ncm5U, 5-carbamoylmethyluridine; t6A, N6-threonylcarbamoyladenosine; U, uridine; Um, 2′-O-methyluridine.
nucleosides can be found in tRNAs across organisms, and on average each tRNA molecule contains at least five to six modified nucleosides (Jühling et al., 2009; Machnicka et al., 2013). At present, more than 600 sequenced tRNAs are available from archea to eukaryotes, including cytosolic, mitochondrial and chloroplast tRNAs (Modomics, http://modomics.genesilico.pl/; Dunin-Horkawicz et al., 2006). The modified nucleosides in tRNAs can change in response to alterations of environmental conditions, and across developmental stages, including aging, starvation, and different stress conditions (Dirheimer et al., 1995; Suzuki and Nagao, 2011b; Dedon and Begley, 2014). Therefore, it has been suggested to work as a ‘sensing system’ to link environmental and developmental stimuli to cellular translational machinery and metabolism (Chan et al., 2012; El Yacoubi et al., 2012; Zinshteyn and Gilbert, 2013).

Recently, a mechanism in which tRNA-derived nucleoside modifications control the translation of stress-related proteins was proposed, and termed ‘MoTT’ (modification of tRNA tuned) (Chan et al., 2012; Gu et al., 2014). According to this model, cells respond to stresses (e.g. oxidative stress) by changes of tRNA nucleoside modifications, which subsequently influence decoding of certain codons and therefore the translation of proteins (Dedon and Begley, 2014). Nucleoside modification changes were therefore not only manifested as a ‘signal’ for stress, but may also be used as a ‘regulatory module’ to timely adapt the cell to environmental changes, in a fast, broad and effective manner (Chan et al., 2010, 2012). Such a regulation also plays an important role in the development of some human diseases (Dirheimer et al., 1995; Kirino et al., 2004; Rodriguez et al., 2007; Suzuki and Nagao, 2011a).

Knowledge of tRNA nucleoside modification genes in higher plants is rather limited. Among 642 sequenced tRNAs in the Modomics database, only 78 are from land plants. According to the PlantRNA database (http://plantrna.ibmp.cnrs.fr/; Cognat et al., 2013), more than 600 tRNA genes are present in the Arabidopsis genome coding for ca 200 tRNA unique sequences, of which none is associated with information of modified nucleosides. Nevertheless, several mutants of tRNA nucleoside modification genes have been described in Arabidopsis, many of which result in retarded plant growth (Chen et al., 2006; Hu et al., 2010; Zhou et al., 2013), impaired immune response (Wang et al., 2013) or abrogated abiotic stress tolerance (Zhou et al., 2013; Burgess et al., 2015).

Rice is an important cereal crop as well as a monocot model plant, and stress tolerance is a very important trait for breeding purposes. Rice is one of the most salt sensitive cereal crops, contrasting with, for example, barley, which is one of the most salt tolerant ( Munns and Tester, 2008). Different plant species develop specific strategies to combat abiotic stress, and insights into these strategies have been obtained via metabolite comparisons between stress-tolerant and stress-sensitive accessions ( Hasegawa et al., 2000; Zhu, 2002). Plants accumulate several osmolytes in response to drought and salt stress, including soluble sugars (glucose, sucrose, trehalose), non-digestible carbohydrates (e.g. raffinose, stachyose, and verbascose), polyols (e.g. mannitol and sorbitol), amino acids (e.g. proline), quaternary ammonium compounds (e.g. glycine betaine) and polyamines (e.g. putrescine, spermidine and spermine) (Golldack et al., 2014; Jorge et al., 2016). These compounds, in one way or another, help the cell to maintain turgor pressure and avoid water loss, and also to deal with ROS and re-establish redox balance ( Munns and Tester, 2008). The response of amino acids, soluble sugars and TCA cycle intermediates from different rice cultivars to salt does in general coincide with the core metabolite adaptation in most glycophylic plants, such as Arabidopsis thaliana (Jorge et al., 2016). Abscisic acid (ABA) plays an important role in abiotic stress signaling (Zhu, 2002). The connection between ABA and abiotic stress, mostly drought and salt stress, is well established in Arabidopsis but less well in rice. Many genes and proteins are involved in the ABA-mediated stress-signaling pathway, including ABA biosynthesis (ABA1, AAO3, ABA3, NCED5), ABA receptor (PYR/PYL/RCAR) and PP2C (e.g. SnRK2), AP2 (e.g. DREB2A, ABI4) and bZIP transcription factor (ABFs, ABI5) genes (Ye et al., 2012; Nakashima and Yamaguchi-Shinozaki, 2013).

Although several studies have shown that tRNA nucleoside modification genes can influence plant growth, anthocyanin biosynthesis, hormone homeostasis and immune response in Arabidopsis (Chen et al., 2006; Zhou et al., 2009; Hu et al., 2010; Nelissen et al., 2010; Lehne et al., 2011; Xu et al., 2012), similar studies are lacking for crop plants, including rice. Indeed, no sequenced tRNA was available for rice in the PlantRNA, Modomics or RNAmod databases, nor for tRNA nucleoside modification genes. In this study we found a significant increase of 2′-O-methyladenosine (Am) nucleosides during salt stress and ABA treatment in rice. We further identified LOC_Os3g61750 (OsTRM13) as the gene responsible for Am modification. In accordance with the elevated Am nucleoside levels, OsTRM13 transcript levels increased upon salt stress and ABA treatment. In addition, transgenic rice overexpressing OsTRM13 showed improved salt tolerance. These data indicate that OsTRM13 is responsible for tRNA nucleoside modification, and that this function is important for salt stress and ABA hormone responses in rice.

Materials and methods

Plant material and bacterial strains

Nipponbare rice ( O. sativa L. spp. japonica) was used throughout this study. Minghui63 (MH63) and Zhanhan97 (ZS97) rice were kindly provided by Dr Liangcai Peng from Huazhong Agricultural University, and Brachypodium distachyon (L.) was provided by Dr Lingqiang Wang in Huazhong Agricultural University. Yeast strains Y07126 and Y27126 were purchased from EUROSCARF (www.euroscarf.de). Arabidopsis thaliana Columbia ecotype, and hybrid poplar 717 (Populus tremula × P. alba 717-1B4 genotype) were maintained in our own lab.

Sampling of rice tissues upon abiotic stress or ABA hormone treatment

Seeds of Nipponbare rice (NBP) were sterilized with 70% ethanol followed by 2.5% sodium hydrochloride, washed and soaked in distilled water for 2 d. Seeds were grown hydroponically in a climate chamber with 16 h–8 h light–dark photoperiod at 28 °C. Ten-day-old
rice seedlings were used as starting material for salt stress and ABA treatment. Cold stress was applied by transferring seedlings into pre-equilibrated 4 °C cold distilled water in a cold room for up to 7 d, drought/air-drying stress was performed by transferring seedlings onto Whatman 3MM filter paper (GE Healthcare Life Sciences) in a growth chamber for up to 7 d. Salt stress was applied by changing distilled water to 200 mM NaCl solution for rice seedling cultivation. ABA treatment was performed by applying 100 µM ABA in culture medium. Samples were taken at specified time points in triplicates, flash-frozen in liquid nitrogen and stored at –80 °C until further use.

tRNA isolation and nucleotide analysis by liquid chromatography–mass spectrometry

Small tRNAs were extracted using microRNA Extraction Kit (Omega Bio-tek Inc.). RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). About 20 µg tRNA was digested with 2 units of P1 nuclease (Sigma-Aldrich) and 1.5 unit of calf intestine alkaline phosphatase (Toyobo) in 20 mM Hepes–KOH (pH 7.0) at 37 °C for 3 h (Noma et al., 2006). Samples were diluted with Milli-Q water (Millipore Synergy) to a concentration of 10 µg ml⁻¹.

Detailed settings for each nucleotide are summarized in Supplementary Table S1 at JXB online. An API 4000 Q-Trap mass spectrometer (Applied Biosystems) was used with an LC-20A HPLC system and a diode array UV detector (190–400 nm). Electrospray ionization mass spectrometry was conducted in positive ion mode. The nebulizer gas, auxiliary gas, curtain gas, turbo gas temperature, entrance potential, and ion spray voltage were 80 psi, 20 psi, 550 °C, 10 and 5500 V, respectively. An Inertisil ODS-3 column (2.1 mm×150 mm, 5 µm particle size; Shimadzu) was used for nucleoside separation. The mobile phase gradient was the following (Yan et al., 2013): 0–10 min, 0–50% solvent B; 10–13 min, 50–100% solvent B; 13–23 min, 100% solvent B; 23–23.1 min, 100–5% solvent B; 23.1–30 min, 5–0% solvent B. The flow rate was 0.6 ml min⁻¹ at ambient temperature. The injection volume was 10 µl. Multiple reaction monitoring mode was performed to determine parent-to-product ion transitions. Uridine, cytosine, adenosine, guanosine, 7-methylguanosine, 5-methyluridine, 5-methylcytidine, and 2′-O-methylguanosine nucleoside standards were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Protein purification and tRNA in vitro methylation

OsTRM13 full length cDNA was amplified and cloned into pGEX-6P-3 (GE healthcare Life Sciences, Shanghai, China) using BamHI and NotI sites, resulting in a fusion protein with glutathione S-transferase (GST) at the N-terminus. The recombinant vector was transformed into BL21 cells; expression of fusion protein was induced with 0.5 mM IPTG and purified with ProteinIso GST Resin (Transgen Biotech). The N-terminal GST tag was cleaved off by ProScission Protease (Genscript Biotechnology Co. Ltd, Nanjing, China). Yeast strains carrying the recombinant plasmid (Y07126+OsTRM13), Y07126 and Y27126 were analysed further for growth phenotype on YPD medium, and Am nucleoside level by liquid chromatography–mass spectrometry (LC-MS). OsTRM13-eGFP vector was constructed for subcellular localization. The enhanced green fluorescent protein (eGFP) tag was fused in frame to the 3′-end of the OsTRM13 gene sequence. Primers are listed in Supplementary Table S2. The construct was transformed into Agrobacterium strain GV3101 and infiltrated into tobacco leaves for confocal microscopy. The subcellular localization of GFP was visualized using a confocal laser scanning microscope (Leica SP5 CLSM) with ×63 objective lens. 4',6-Diamidino-2-phenylindole (DAPI) staining was used as a nuclear marker.

Subcellular localization

The full-length OsTRM13 cDNA sequences were amplified and cloned with KpnI and XbaI sites into a pd1301s-eGFP binary vector. This vector also carried hygromycin as a plant selection marker, and the eGFP gene between SalI and PstI restriction sites. The recombinant pd1301s-OsTRM13-eGFP was used both for overexpression and subcellular localization. For RNAi construction, a 162 bp fragment was amplified and cloned into gateway RNAi destination vector pH7GW1WG2(II) (http://gateway.psb.ugent.be/) via pENTR/D-TOPO entry vector (Thermo Fisher Scientific, China) (Karimi et al., 2007). Overexpression and RNAi destination vector was introduced into Agrobacterium tumefaciens strain EHA105 and transformed into Nipponbare rice. Positive T0 transgenic lines were selected using hygromycin (Hyg) as plant selection marker, and segregating T1 plants were screened by PCR and verified by qRT-PCR.

Chlorophyll and proline measurement in transgenic rice before and after salt stress

Flag leaves at bolting stage were cut into pieces and submerged in distilled water or 200 mM NaCl solution for 3 d. Leaf sample was ground and extracted for chlorophyll content determination (Inskeep and Bloom, 1985; Srivastava et al., 2016). For proline content determination before and after salt stress, 2-week-old transgenic or NPB seedlings cultivated in a greenhouse were subjected to 200 mM NaCl for 3 d, and proline content was measured according to Bates (1973).

Enzymatic activity of superoxide dismutase and peroxidase in transgenic rice before and after salt stress

Two-week-old pd1301s-OsTRM13-eGFP (overexpression) or pH7GW1WG2(II)-OsTRM13 (RNAi) transgenic seedlings cultivated in a greenhouse were subjected to salt stress in 200 mM NaCl for 5 d, samples were taken at 0, 3, and 5 d, and three biological replicates were used from each line. Peroxidase (POD) assay kit (Nanjing Jiancheng Bioengineering Institute, China) was used for POD enzymatic activity determination, following the method of Amako et al. (1994). A superoxide dismutase (SOD) assay kit (Nanjing Jiangcheng Bioengineering Institute) was used based on the hydroxylamine method (Zhang, 2008). One unit of SOD activity is defined as the amount of SOD enzyme required for 50% inhibition.
of superoxide anion radicals determined by colorimetric method in a 1 ml reaction (Zhang et al., 2008). The POD or SOD activities were presented as U mg\(^{-1}\) proteins.

Quantitative RT-PCR

Total RNA was extracted by the RNAprep pure Plant Kit (Tiangen Biotech, Beijing, China). M-MLV RTase (TaKaRa, Dalian, China) was used to generate cDNA. qRT-PCR was conducted using a Bio-Rad IQ\textsuperscript{TM} real-time PCR system (Life Science, Wuhan, China). LOC\_Os06g48970 (UBQ) and LOC\_Os06g11170 (ACTIN) were served as reference genes (Narsai et al., 2010). The ΔΔC\textsubscript{T} method was used for quantification of relative expression (Livak and Schmittgen, 2001).

At least three biological replicates were used for each line analysed, and mean and standard deviation (SD) were calculated. Student's \(t\) test was performed to judge the difference significance level (*, statistically significant at \(P \leq 0.05\); **, statistically significant at \(P \leq 0.01\)).

Results

Salt stress and ABA treatment induced a significant increase in 2′-O-methyladenosine nucleosides in rice

Modified nucleosides in tRNAs may change dramatically during various stress conditions in animal and yeast systems (Chan et al., 2012; Dedon and Begley, 2014); however, similar studies in plants are lacking. To assess modified nucleosides in tRNAs, 9-day-old seedlings of Nipponbare rice (NPB) were subjected to drought (air dry), cold, and salt stress or abscisic acid (ABA) hormone treatment, and were analysed by LC-MS for modified nucleosides (see Supplementary Table S1). Nucleoside levels were calculated by their abundance in stress conditions divided by that in control condition (Fig. 1). A prominent increase of 2′-O-methyladenosine (Am) nucleosides was observed during salt stress and ABA treatment (Fig. 1A, B). Am nucleoside level increased from 3 to 7 d during salt stress, and its abundance peaked at 7 d where it was 10 times higher than that of control (Fig. 1A). The Am nucleoside profile showed a similar trend for ABA-treated samples, which also showed highest abundance after 7 d treatment (Fig. 1B). In contrast, Am nucleoside levels did not change significantly during cold or drought stress conditions (Fig. 1C, D).

Identification of a putative 2′-O-methyladenosine modification gene in rice

Am nucleoside may be found at position 4 in eukaryotic tRNAs (Wilkinson et al., 2007). Trm13p of Saccharomyces cerevisiae (baker’s yeast) was the first enzyme identified for Am4 and Cm4 modifications (Wilkinson et al., 2007). Trm13p belongs to the Rossman fold (RFM) group of S-adenosyl-methionine (AdoMet)-dependent methyltransferases (MTases) (Schubert et al., 2003). Using AdoMet as a methyl donor, Trm13p catalyses Am or Cm formation at position 4 on tRNA-His, tRNA-Pro and tRNA-Gly in yeast (Wilkinson et al., 2007). Trm13 proteins are unique for eukaryotes, and amino acid residues critical for AdoMet binding and conformation of the catalytic domain have been suggested (Tkaczuk, 2010).

No components for Am and/or Cm nucleoside modification have been reported for plants. Therefore, we used the yeast Trm13p protein sequence to find Am modification candidate genes in the rice genome. LOC\_Os03g61750 was the only candidate with a blastp value below 1.0 × 10\(^{-6}\) (data not shown), and we therefore tentatively named this gene OsTRM13. Indeed, OsTrm13 was annotated as methyltransferase-Trm13-domain-containing protein in the RGAP database (http://rice.plantbiology.msu.edu/). In addition to the TRM13 MTase domain, OsTrm13 also contained two zinc-finger domains, zf-Trm13-CCCH and zf-U11-48K (Fig. 2A). The OsTrm13 showed 28.9% sequence similarity to Trm13p, and 54.5% to a putative Arabidopsis homolog (At4g01880). In a phylogenetic tree of eukaryotic Trm13p homologs, the mammal and yeast Trm13s were sparsely separated from the plant Trm13 homologs. Plant Trm13p homologs were divided into two clades, where the Arabidopsis Trm13p homolog At4g01880 was in group I and OsTrm13 was in group II (Fig. 2B).

OsTrm13 protein is located in the nucleus

An eGFP tag was fused to the C-terminus of OsTrm13 protein to investigate the subcellular localization of the protein...
in transient transfected tobacco leave cells (Fig. 3). As eGFP fluorescence signal was observed in a compartment reminiscent of the nucleus (Fig. 3B, G), DAPI staining was used to verify the nuclear localization of OsTrm13-eGFP (Fig. 3C, H). Although OsTrm13 was predicted to be localized to the chloroplast by TargetP (http://www.cbs.dtu.dk/services/TargetP/), we did not find any overlap between the eGFP signal and chlorophyll autofluorescence (Fig. 3E, J). Our data are, furthermore, in agreement with the function of Trm13 in yeast and animal cells where it acts as tRNA nucleoside modifier in the cytosol and nucleus (Wilkinson et al., 2007; Tkaczuk, 2010).

OsTrm13 catalyses formation of 2′-O-methylated nucleosides in vitro

Am nucleoside is only found in tRNA-His-GUG in S. cerevisiae; however, no tRNA-His-GUG from Arabidopsis or rice contains an adenosine at position 4 (Table 1). Since tRNA-Pro-UGG and tRNA-Gly-GCC also are Trm13p substrates (Wilkinson et al., 2007), yeast tRNA-Gly-GCC was used as substrate for in vitro methylation assays.

OsTrm13 was expressed as an N-terminal fusion protein with GST. Expression of this fusion protein (~66 kDa) was induced by IPTG and tag-free protein (~30 kDa) was purified after ProScission Protease cleavage (Fig. 4A). Yeast tRNA-Gly-GCC was in vitro transcribed as a naked/unmodified transcript from linearized pGEM-T vector (Fig. 4B). Purified OsTrm13 protein was added to the methylation reaction, with AdoMet as methyl donor (Wilkinson et al., 2007). The products were digested into nucleosides and analysed by LC-MS. As shown in Fig. 4C, Cm production was positively correlated with OsTrm13 protein amount and input of tRNA. When the cytidine at position 4 was mutated to adenosine, OsTrm13-dependent Am nucleoside was formed instead (Fig. 4D). In the absence of OsTrm13 protein, neither Cm nor Am was found, and the reaction needed AdoMet as methyl donor (Fig. 4C, D). These results corroborated that OsTrm13 is an AdoMet-dependent MTase that could methylate A to Am or C to Cm nucleoside on yeast tRNAs in vitro.

Complementation of yeast Δtrm13 mutant by OsTRM13

To corroborate the in vitro activity of OsTrm13, we expressed the OsTRM13 gene in yeast strains deficient in Am nucleoside formation. Y07126 and Y27126 represent Δtrm13
### Table 1. DNA sequences of tRNA-His-GTG, tRNA-Gly-GCC and tRNA-Pro-TGG in yeast, Arabidopsis and rice

AA, amino acid; AC, anticodon; Acc, acceptor; Ac, anticodon; V, variable loop; T, TψC loop; Sc, S. cerevisiae; At, A. thaliana, Os, O. sativa.

| Species | Chr | AA      | AC     | p-1 | Ac | D | D | V | T | T | Acc | p73 | CCA |
|---------|-----|---------|--------|-----|----|---|---|---|---|---|-----|-----|-----|
| Sc      |     | His GTG | GCCATCT|     | TA | GTAT | AGTGGTTA | GTAC | A  | CATCG | TCTGG | GCCATG | AAC | CTCGG | TTTGATT |
| At      |     | His GTG | GGTGGGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | mito   | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | nucleus | His GTG | GGTGGGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | nucleus | His GTG | GGTGGGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | nucleus | His GTG | GGTGGGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | chloroplast | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | chloroplast | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| At      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Os      |     | mitochondrion | His GTG | GCCATGC | TA | GTTT | AGTGGTTA | GAAAT | T  | CCACG | TCTGG | GCCTGG | AGAC | CTCGG | CTCGAAT |
| Sc      |     | Gly GCC | GCCAAGA | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| Sc      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| At      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| Os      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| Os      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| Os      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
| Os      |     | Gly GCC | GCCAATGC | TA | GTTT | AGTGGTTA | AAAT | C  | CAAGG | TGGATTG | GGCCTG | AGAC | CTCGG | CTCGAAT |
OsTrm13 can methylate tRNA-Gly-GCC in vitro. (A) In vitro expression of GST-tagged OsTrm13 and purification of tag-free OsTrm13 protein. Protein sizes are shown on the left. Lane 1: total protein from cell extract. Lane 2: total protein after IPTG induction. Position of GST-OsTrm13 is indicated with arrow. Lane 3: purified GST-OsTrm13 protein from the GST column. Lane 4: purified tag-free OsTrm13 protein after ProScission Protease digestion. The expected size of GST-OsTrm13 is indicated with an arrow, and tag-free OsTrm13 protein with an arrowhead. Dotted lines indicate separate gels. (B) Cloverleaf structure of substrate tRNA: yeast tRNA-Gly-GCC. Adenosine at position 4 is highlighted. (C) Cm nucleoside level during in vitro methylation with tRNA-Gly-C4 (cytidine at position 4) as substrate. (D) Am nucleoside level during in vitro methylation with tRNA-Gly-A4 (adenosine at position 4) as substrate. Error bars represent standard deviation from two technical replicates.

mutant and congenic wild-type, respectively, and defects of Am nucleoside were verified in Y07126 (Fig. 5). A full length OsTRM13 cDNA clone in yeast chromosomal integrating vector was introduced into Y07126 to test whether OsTRM13 could restore Am nucleoside. Three positive yeast clones were tested (OsTRM13-6, -11, -29) for the presence of the OsTRM13 gene (Fig. 5B). tRNA was extracted subsequently from these lines, together with tRNAs from Y07126 and Y27126, and analysed for nucleoside modifications (Fig. 5C).

Increased expression of OsTRM13 correlated with elevated Am nucleoside abundance during salt stress or ABA treatment

To test if the increase of Am nucleoside was specific to Nipponbare rice accession, or if we could also observe such changes in other rice accessions, two other cultivated rice species, MH63 and ZS97, as well as Brachypodium distachyon as another monocot plant, were subjected to salt stress and analysed (Fig. 6C–H). For comparison, Arabidopsis and hybrid poplar were also tested. The results indicated that Am nucleoside increased during salt stress in all three rice accessions.
as well as hybrid poplar (Fig. 6C–H). However, changes of Am nucleoside were not as pronounced in Brachypodium and Arabidopsis (Fig. 6F, G).

OsTrm13 affects the endogenous Am nucleoside levels and impacts plant growth

To investigate the impact of OsTrm13 on rice growth and development, we generated OsTRM13 overexpression (OE) or RNAi transgenic rice. Since the endogenous OsTRM13 expression is low in rice seedlings, RNAi plants only showed transcript reduction up to 40% (Fig. 7A). When tRNA nucleoside modifications were analysed, we found that the Am nucleoside levels were two to three times higher in the OE lines than that of control plants; however, the levels were not significantly different in the RNAi lines (Fig. 7B). This may be due to the fact that the Am nucleosides are not solely present on tRNAs, but also on snRNA, snoRNA and 5s rRNAs (Modomics and RNAmod databases). When seedling growth and root growth of the OE and RNAi lines were compared against wild-type, a slight increase in root length in 2- to 5-day-old OsTRM13 OE seedlings was observed (Fig. 7C–E). Meanwhile, a significant decrease of root length was observed in the RNAi plants (Fig. 7C–E). Therefore, although a decrease of Am nucleoside in tRNAs from OsTRM13 RNAi lines might be ‘masked’ by its presence on other small RNAs, the reduced root length in RNAi-8 lines (Fig. 7C, D) indirectly suggested that the tRNA-derived Am nucleosides was reduced. However, no differences in plant height were observed in later developmental stages, suggesting that the difference of Am nucleoside mainly affected early vegetative growth in rice.

Fig. 6. Salt stress and ABA treatment cause increase in OsTRM13 expression and in tRNA nucleoside methylation. (A) OsTRM13 transcript levels during salt stress (light gray) or ABA treatment (dark gray). (B) Fitting curve of Am nucleoside levels vs OsTRM13 transcript levels under salt stress or ABA treatment. Pearson correlation coefficient ($R^2$) was calculated with SPSS software. (C–H) Am nucleoside levels during salt stress in NPB (C), MH63 (D), ZS97 rice (E), Brachypodium (F), Arabidopsis (G) and hybrid poplar (H). For rice, Brachypodium and Arabidopsis, 10- to 14 day-old seedlings were used. For hybrid poplar, 1-month-old seedlings from tissue culture (ca 10 cm high) were used. Samples were taken at 0, 3, 5 and 7 d during salt stress treatment. tRNAs were extracted and nucleoside modifications were analysed. Error bars represent standard deviation from three biological replicates. *$P<0.05$ and **$P<0.01$ by Student’s t-test.
OsTrm13 is important for rice stress tolerance

To assess how the changed levels of OsTrm13 and of Am nucleosides affected plant stress tolerance, we investigated the impact of salt stress on OsTRM13 OE and RNAi transgenic plants (Fig. 8). Chlorophyll content was measured from flag leaves before or after salt stress (Srivastava et al., 2016). As shown in Fig. 8A, B, NPB rice suffered ca 55% decrease of chlorophyll in response to 200 mM NaCl as compared with H2O treatment; however, the decrease of chlorophyll was much smaller in the OE lines, i.e. 33% and 11% in OE-3 and OE-8 lines, respectively (Fig. 8A, B). Moreover, the chlorophyll decrease in RNAi lines was more severe than the wild-type NPB rice (Fig. 8B).

Proline is an important osmolyte protectant for cells to mitigate osmotic stress, including drought and salt stress. P5CS is a key proline biosynthetic gene (You et al., 2012) and its expression is, together with those of LEA and POD genes, an indicator of cell stress (Hasegawa et al., 2000; Zhu, 2002; Duan and Cai, 2012; Nounjan et al., 2012). Similarly, elevated enzymatic superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were also used as markers for cellular stress (Hasegawa et al., 2000). These parameters, i.e. proline content, transcript level of OsP5CS, OsLEA3, and OsPOD5, and SOD and POD enzymatic activities, were investigated in OsTRM13 OE and RNAi transgenic plants that had been exposed to salt stress (Fig. 8C-H). We found that the proline levels in the OE plants were higher than in wild-type NPB, and that the OsP5CS transcript levels were higher, after 3 d of salt stress (Fig. 8C, D). OsLEA3 expression also increased shortly after salt stress (Fig. 8E). In contrast, the expression of OsPOD5 was induced later (Fig. 8F), suggesting a different regulatory window of the POD, LEA, and P5CS genes. Oxidative enzymes, such as SOD and POD, showed increased activity during the salt stress experiment, and the enzyme activities were generally higher in the OE lines than wild-type NPB, and lower in RNAi lines (Fig. 8G, H).

ABA plays an essential role in abiotic stress, especially drought and salt stress (Fujita et al., 2011; Nakashima and Yamaguchi-Shinozaki, 2013). We selected some of the salt stress marker genes (SOS1, HKT1, and NHX1) (Yokoi et al., 2002; Hamamoto et al., 2015), as well as genes involved in ABA synthesis, perception, and signaling (ABA1, AAO3, PYL/PYR/RCAR1, ABI5, and SnRK2.1) (Finkelstein and Lynch, 2000; Seo et al., 2000; Xiong et al., 2002; Yoshida et al., 2002; Melcher et al., 2009; Miyazono et al., 2009; Santiago et al., 2009), and measured their transcript levels. As shown in Fig. 9A, the transcript of OsHKT1 (K+/Na+ influx) was down-regulated by salt stress in all three genetic backgrounds, but to a lesser extent in the OsTRM13 OE-3 line (Fig. 9A). To the contrary, the transcript of OsSOSI (for Na+ efflux) was up-regulated in all three genetic backgrounds, and the most in the OsTRM13 OE-3 line (Fig. 9B). Similarly, mRNA levels for OsNHX1, coding for a tonoplast-located Na+/K+ exchanger, were also increased (Fig. 9C). Here, the induction of OsNHX1 transcript in the OsTRM13 OE-3 line was comparable with NPB, but less in RNAi-8 lines (Fig. 9C). In contrast, the ABA signaling genes, OsABI5, OsSAPK2, were up-regulated in OsTRM13 RNAi-8 lines, but less or slightly repressed in NPB.
and OE-3 lines (Fig. 9D, E). As for the ABA synthesis genes, the transcript level of OsABA1 was slightly up-regulated in OsTRM13 OE-3 lines as in the control, but down-regulated in RNAi-8 lines (Fig. 9F). OsAAO3 transcript was up-regulated in all three backgrounds but the most in RNAi-8 lines (Fig. 9G), and OsRCAR1 (ABA receptor) transcript levels were down-regulated in all, but also the most repressed in OsTRM13 RNAi-8 lines (Fig. 9H).

Taken together, the expression data of ABA- and salt stress-related genes revealed a complex picture, but a partial explanation for how the OsTRM13 OE-3 or RNAi-8 lines differed in salt stress tolerance. These differences appear to, at least in part, be due to variations in the expression of ion transporters (HKT1, NHX1, and SOS1), as well as genes in the ABA signaling pathway (ABA1, AAO3, RCAR1, ABI5, and SnRK2.1).

**Discussion**

Rice is the staple food for a large fraction of the world's population, and is thus an important cereal crop and a monocot model plant (Havukkala, 1996; Coudert et al., 2010; Lo et al., 2016). A basic understanding of how stress tolerance may be improved in rice is therefore of the utmost importance. We report that salt and ABA treatments induced Am nucleoside levels, and that OsTRM13 is involved in Am nucleoside formation in rice. Furthermore, we show that changes in the OsTRM13 expression levels contribute to salt stress tolerance of rice.

We found that OsTrm13 is an AdoMet-dependent MTase, which can catalyse Am and Cm modification at position 4 of yeast tRNA-Gly-GCC. The yeast Trm13p can use tRNA-Gly-GCC, tRNA-His-GUG, and tRNA-Pro-UGG as substrates (Wilkinson et al., 2007). To find out the plant substrate tRNAs, we checked the tRNA genomic sequences from the Modomics and PlantRNA databases for yeast, Arabidopsis and rice. We found 40 tRNA-Gly, tRNA-His, and tRNA-Pro sequences in these species (Table 1). Rice contained 43 tRNA-Gly genes, coding for 18 unique tRNA-Gly sequences. Six of the tRNA-Gly-GCC sequences contained a cytidine at position 4, and one chloroplast gene had adenosine, making it a possible substrate candidate (Table 1). The three rice tRNA-His-GUG sequences all contained guanosine at position 4, and one chloroplast gene had adenosine, making it a possible substrate candidate (Table 1). The three rice tRNA-His-GUG sequences all contained guanosine at position 4, and finally, all rice tRNA-Pro-UGG genes have either a cytidine or a guanosine at position (Table 1). These data suggest that tRNA-Gly-GCC and possibly tRNA-Pro-UGG may be in vivo substrates for the OsTrm13. However, it is of course also possible that OsTrm13 may have extended its substrate repertoire and that also other tRNA nucleosides may be affected. Indeed, the OsTrm13 protein had a shorter MTase domain compared with Trm13p and AtTrm13, perhaps indicating a change in substrate recognition and activity.
Another major finding in this story is that OsTRM13 transgenic rice influences salt stress tolerance. We analysed chlorophyll content, proline and MDA contents before and after salt stress in NPB control and OsTRM13 OE or RNAi lines. We also measured enzymatic activities of oxidative enzymes, and relative expression of ABA and salt stress marker genes. The qRT-PCR analysis of ABA (ABA1, AAO3, RCAR1, ABI5, and SnRK2.1) and salt stress marker genes (HKT1, NHX1, and SOS1) gave a partial explanation for why OsTRM13 OE or RNAi lines differed in salt stress tolerance (Figs 8 and 9). SOS1, as a Na⁺ efflux transporter, has a central role in salt stress tolerance, and the higher induction of OsSOS1 in the OsTRM13 OE-3 line (Fig. 9B) therefore supports an improved salt stress tolerance (Fig. 8A, B). These data are further supported by a lower reduction of OsNHX1 in the OsTRM13 RNAi-8 line (Fig 9C), which may reduce the buffering capacity of excess Na⁺ ions in the vacuole, leaving more Na⁺ in the cytosol and consequently a reduced salt tolerance phenotype (Fig. 8A, B). We also found differences in the expression of assorted ABA-related genes, e.g. SnRK2.1, ABA1, ABI5, AAO3, and RCAR1, in the OsTRM13 transgenic lines as compared with wild-type (Fig. 9D–H). Taken together, these data suggest that OsTRM13 impacts the transcriptional regulation of ABA- and salt stress-related genes. Notably, our samples for gene expressions were taken 3–5 d after the salt stress treatment (Figs 8 and 9). Hence, our data are more likely related to the acclimation of the different lines to the salt stress response.

Stress is known to induce changes of tRNA nucleoside modifications in both prokaryotes and eukaryotes (Chan et al., 2010). Here, we showed that Am nucleoside in tRNAs increased during salt stress in three rice accessions and poplar, but less in Brachypodium and Arabidopsis (Fig 6). A study by Kim et al. (2007) reported an increase of methylation index with salt stress. They found that the ratio of AdoMet and S-adenylyl-l-homocysteine increased during the early phase (first 12 h) of salt stress, accompanied by an induction of the biosynthesis of aromatic amino acids and lignin (Kim et al., 2007). However, during long-term exposure to salt stress, methylation-related metabolites were repressed (Kim et al., 2007). Since AdoMet is a methyl donor for Trm13p-like MTases, an increase of methylation during the early phase of salt stress might be associated with elevated levels of Am nucleosides presented in this study.

Our knowledge of how tRNA nucleoside modifications contribute to stress tolerance in plants is still largely lacking. We showed that both Am nucleoside levels and Am and/or Cm nucleoside MTase OsTRM13 were up-regulated during salt stress or ABA treatment. Moreover, up- or down-regulation of OsTRM13 influences the expression of ABA- and salt stress-related genes and therefore salt tolerance in rice. However, the primary effect of modified nucleosides on tRNA
and translation might be translational instead of transcriptional. tRNA nucleoside modification could influence protein translation in three ways: (i) the location of the modified nucleoside on the tRNA molecule—in contrast to nucleosides within the anticodon loop that directly affect codon–anticodon recognition, Am on position 4 is more likely to influence the stability of the tRNA; (ii) the abundance of the tRNAs carrying the modification; and (iii) the codon composition of the target protein sequences. Proteomics data would be helpful to illustrate which proteins are affected by the presence or absence of Am nucleosides in OsTRM13 transgenic plants during salt stress conditions. While we show that an increase of Am nucleoside during salt stress seems widespread in land plants, and that the protein sequences for the corresponding methyltransferase (Trm13p orthologs) are conserved (see Supplementary Fig S1), it is unclear whether the translational/transcriptional regulation behind the methylated nucleosides of tRNAs in ABA signaling- and salt stress-related proteins is similar in monocot and dicot plants.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. Multi-sequence alignment of yeast Trm13p and plant TRM13 orthologs.

Table S1. LC-MS parameters for nucleoside analysis.

Table S2. Primers used in this study.

Table S3. Statistics of data for qRT-PCR and nucleoside abundance analysis.

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Author contributions
YW performed most of the experiments; DL provided technical assistance for LC-MS analysis; XL conceived all bioinformatics analyses; JG, XJ and RZ performed vector construction, qRT-PCR and enzymatic activity experiments; ZH conceived transgenic plant analysis; RZ analysed the data; PC conceived the project and wrote the article with contributions of all the authors; SP supervised and complemented the writing.

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