Salt tolerance involved candidate genes in rice: an integrative meta-analysis approach

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Raheleh Mirdar Mansuri
Agricultural Biotechnology Research Institute of Iran

Zahra-Sadat Shobbar
Agricultural Biotechnology Research Institute of Iran

shobbar@abrii.ac.ir Corresponding Author
ORCiD: https://orcid.org/0000-0002-7011-5415

Nadali Babaeian Jelodar
Sari Agricultural Sciences and Natural Resources University

Mohammadreza Ghaffari
Agricultural Biotechnology Research Institute of Iran

Seyed Mahdi Mohammadi
Agricultural Biotechnology Research Institute of Iran

Parisa Daryani
Agricultural Biotechnology Research Institute of Iran

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Abstract
Background: Salinity, as one of the main abiotic stresses, critically threatens growth and fertility of main food crops including rice in the world. To get insight into the molecular mechanisms by which tolerant genotypes respond to the salinity stress, we propose an integrative meta-analysis approach to find the key genes involved in salinity tolerance. Herein, a genome-wide meta-analysis, using microarray and RNA-seq data was conducted which resulted in the identification of differentially expressed genes (DEGs) under salinity stress at tolerant rice genotypes. DEGs were then confirmed by meta-QTL analysis and literature review.

Results: A total of 3449 DEGs were detected in 46 meta-QTL positions, while 1286, 86, 1729 and 348 DEGs were observed respectively in root, shoot, seedling and leaves tissues. Moreover, functional annotation of DEGs located in meta-QTLs suggested some involved biological processes (e.g. ion transport, regulation of transcription, cell wall organization and modification as well as response to stress) and molecular function terms (e.g. transporter activity, transcription factor activity and oxidoreductase activity). Remarkably, 20 potential candidate genes were detected in Saltol and hotspot-regions overlying original QTLs for both yield components and ion homeostasis traits; among which, there were many unreported salinity-responsive genes. Some promising candidate genes were detected as pectinesterase, peroxidase, transcription regulator, high-affinity potassium transporter, cell wall organization, protein serine/threonine phosphatase, and CBS domain containing protein.

Conclusions: The obtained results indicate that, the salt tolerant genotypes use qualified mechanisms particularly in sensing and signalling of the salt stress, regulation of transcription, ionic homeostasis, and ROS scavenging in response to the salt stress.

Background
Currently, rice ranked as the first most important food crop in the world before wheat and maize supplying a major source of calorie for more than 3.5 billion people all over the world[1, 2]. However, rice is classified as a very sensitive crop to salinity in both seedling and reproductive stages, while soil salinity is one of the most widespread abiotic stresses in Asia and somewhat river deltas in Europe[3, 4]. Salinity challenge at the seedling stage causes the growth arrest or death of rice plant, that
reduces significantly the yield [5, 6]; therefore, increasing the salinity tolerance at the seedling stage would be effective to improve the environmental adaptation and yield maintenance in rice. It is necessary to understand the mechanisms underlying salinity stress tolerance because of increasing the population, limited arable land and changing the climates and can provide us a better perspective of how to manage the increasing demand for high-yielding rice [2, 7]. Salinity tolerance is a complicated trait both genetically and physiologically [8]. Rice, as a well-studied model organism, is particularly rewarding for investigating the salinity stress responses [7]. Many QTLs have been identified in rice breeding programs [9-16], including a major locus on chromosome 1, namely Saltol, involved in Na/K homeostasis derived from Pokkali and SKC1 (OsHKT1; 5) from Nona Bokra [17]. Isolation of the identified QTLs related to salt tolerance seems to improve greatly the world agriculture and global food security but is a challenging task [18]. Although many QTLs have been found, but we have still limited knowledge of the gene networks related salinity tolerance in rice.

Technologies such as microarray and gene expression profiling based on sequencing approaches result in advancing the progress toward a comprehensive understanding of the genetic mechanisms related responses to environmental stresses [19, 20]. Fast advances and decreased price of high-throughput sequencing technology has caused to use RNA sequencing widely in various species in recent years [21]. Therefore, a large amount of differentially expressed genes (DEGs) was identified between contrasting samples through mentioned technologies. Researchers have recently used the integration of DEGs and QTLs as a confident method to identify the potential candidate genes [22]. Currently, a great and varied set of genomic data has become publicly available; subsequently combination of numerous existing studies can increase the consistency and generalizability of the results. Combining the results caused by the independent but related studies is called “meta-analysis (MA)”; Researchers can obtain more exact estimation of gene expression differentials by increasing the statistical power in MA [23, 24]. Breeding by introgression of the identified QTLs is limited due to the inconsistency of QTLs in various genetic backgrounds and environments [25]; while meta-QTL analysis suggests an opportunity to use QTL information from multiple mapping populations with different genetic backgrounds to detect the accurate location of QTLs [26]. Several studies have
identified accurate meta-QTLs of various traits for mining candidate genes in rice and other crop plants [26–30]. However, an integrative meta-analysis approach was done in this study and resulted in finding several promising genes involved in salinity tolerance, among which, some of the important genes/gene families with sufficient evidence to support their candidacy in rice are listed and discussed later. We used all data produced in the previous studies to identify candidate genes related to salt tolerance in rice, and the candidate genes were also then confirmed using meta-analysis. This study provides valuable information on the regulation of salinity tolerance in rice.

Results

**Salinity tolerance associated Meta-QTLs in rice**

A total of 265 QTLs using SSR markers related to 32 traits were collected in this study (Table S1, S2), among which, 126 QTLs in normal conditions and 139 QTLs in the salinity conditions were selected for further analysis (Table S3). Most of the QTLs were belonged to salinity tolerance score (STS) (27 QTLs), shoot potassium concentration (KS) (26 QTLs), shoot sodium concentration (NS) (21 QTLs), chlorophyll content (CHL) (19 QTLs) and shoot dry weight (DSW) traits (19 QTLs) (Fig.S1). In contrast, the rare QTLs were attributed to the number of sterile spikelets (NSS) [20], dead seedling rate (DSR), leaf potassium concentration (KLV), reduction of seedling height (RSH) and reduction of leaf area (RLA) traits (Fig.S1). The highest number of QTLs were observed on chromosome 1 (37 QTLs) and 2 (36 QTLs) followed by chromosome 7 (29 QTLs), while chromosome 8 (12 QTLs) and 11 (12 QTLs) had the lowest number of QTLs (Fig.S2). The phenotypic variance described by the original QTLs varied from 0.7% to 33.25% and the confidence interval (CI) of markers was different from 0.99 to 84.36 cM (Table S3). After the integration of all the collected QTLs on the consensus map, 46 meta-QTLs were identified in 12 chromosome of rice (Fig.1). There were meta-QTLs with a CI of 95% based on the lowest Akaike information criterion (AIC) values. Remarkably, second meta-QTLs on Chr7: M-QTL2, Chr2: M-QTL2, and Chr1: M-QTL2 included the highest number of initial QTLs (17, 16 and 12, respectively), which covered a relatively narrow CI (4.78, 1.82 and 2.84 cM, respectively) (Table S4).

These meta-QTLs support the important traits; for example, ratio of the shoot sodium and potassium concentration (NKS), number of fertile spikelets (NFS), root length (RTL) and chlorophyll content
Chr12: M-QTL4, Chr 9: M-QTL3 and Chr3: M-QTL2 had the highest mean percentage of phenotypic variation ($R^2$), which can be considered to be the main effective QTLs for the involved traits (Table S4). A total of 9366 genes were detected in 46 meta-QTL positions, among which, Chr8: M-QTL2 contained the highest number of genes (868 genes); while, Chr12: M-QTL2 contained the lowest number of genes (14 genes) (Table S4). Moreover, the proportion of functionally characterized annotated genes (27%) is actually limited compared to the about 73% of unannotated genes with allocated putative functions. It is interesting to note that, 81 genes were identified on Chr1: M-QTL2 which were located in Saltol region.

**Expression profiling analyses in the salinity tolerant genotypes of rice**

In our previous study, the DEGs were recognized under salinity stress compared to control conditions in the salinity tolerant genotypes. A total of 1714 DEGs were observed in roots of FL478 as a salinity tolerant genotype, among which, 927 were up- and 787 down-regulated under salinity conditions [31]. To get a deeper insight regarding the salt responsive genes in the salinity tolerant rice genotypes, DEGs from multiple RNA-seq datasets were combined and the DEGs were classified into root, shoot, seedling, and leaves. A total of 3030, 396, 703 and 723 DEGs were merely recognized respectively in root, shoot, seedling and leaves (Fig.S3). Also, raw microarray data from 9 independent experiments were downloaded (Table S5) and analyzed uniformly. Microarray meta-analysis suggested 11694 DEGs, among which, 4121, 13, 6247 and 1199 DEGs were exclusively expressed in root, shoot, seedling and leaves, respectively (Fig.S4). In addition, a total of 4763 and 5862 DEGs were merely up- and down-regulated, respectively, in the salinity tolerant genotypes.

**Integration of DEGs from two Meta-Analysis approaches**

Identified DEGs in both RNA-Seq and microarray meta-analysis were combined to confirm the consistency of the obtained results. A list of overlapping DEGs were detected in 4 tissues, separately after removing all the duplicate genes.

Comparative transcriptome analysis indicated that 227, 2, 311 and 84 DEGs were commonly detected by RNA-Seq and microarray respectively in root, shoot, seedling and leaves tissues (Fig.2). A total of 4255 and 10980 DEGs were exclusively identified by RNA-Seq and microarray meta-analysis, while
only 156 DEGs were previously reported in the literature (Fig.2).

**DEGs detection in meta-QTL positions**

There were a total of 1345, 86, 1729 and 552 DEGs in the meta-QTL positions respectively in root, shoot, seedling and leaves (Fig.3). Among the identified DEGs in the meta-QTL positions, 664 and 2359 DEGs were respectively identified by RNA-Seq and microarray meta-analysis, while only 82 located DEGs in the meta-QTL positions were previously reported in the literature (Fig.3).

**Functional annotation of DEGs located in meta-QTL positions**

Gene ontology enrichment analysis was performed to achieve the biological roles of the DEGs located in meta-QTL positions. Carbohydrate metabolic process, regulation of cellular process, regulation of transcription, response to stress and regulation of nitrogen compound metabolic process were indicated as dominant terms in biological processes (BP) (Fig.S5). Moreover, some BP terms including regulation of transcription, inorganic anion transport, anion transport, ion transport as well as regulation of gene expression, cell wall organization and modification were significantly enriched (Fig.S5). The most significant over-represented molecular function (MF) terms were nucleotide binding, ATP binding, anion transmembrane transporter activity, inorganic anion transmembrane transporter activity, transcription factor activity and oxidoreductase activity (Fig.S5). In terms of cellular component (CC) ontology, the most significant enriched terms were intrinsic to membrane and integral to membrane (Fig.S5).

**Mining potential candidate genes in meta-QTL positions**

Exploring meta-QTL regions for the common genes were resulted in finding 60 potential candidate genes in root (Table S6), among which, only 4 genes were previously reported associated to the salinity response. Remarkably, LOC_Os01g20980.1 (coding Pectinesterase) was found in Chr1: M-QTL2 located in Saltol region (Table S6). Ion homeostasis related QTLs were also placed in Chr1: M-QTL2 which control KLV, NS, NKS, KS and RN traits (Table S4). Overall, identified potential candidate genes were classified in several terms in root tissue, for example, transcription factor (e.g. *TIFY*, *GRAS*, *HOX*, *WRKY* and *MYB* family), signaling (e.g. *OsWAK125*, *pectinesterase*, *OsMKK1*, *CHIT15*), transporter (e.g. *OsHKT1* and some genes coding transmembrane transport and anion transporter).
and some other functions (e.g. NUDIX family, genes coding aspartic protease ) (Table S6).

Four genes in meta-regions on Chr2, 3 and 8 were identified as potential candidate genes in shoot, which has been also discussed in the literature; for instance, TIP2-1 (LOC_Os02g44080.1) in Chr2: M-QTL4 (Table S6). Chr2: M-QTL4 was integrated with 7 initial QTLs controlling RTL and some other related traits (e.g. S, KS, NKS, SIS and NS) (Table S4). Moreover, two transcription factors (LOC_Os03g08310.1 and LOC_Os08g15050.1) were identified respectively as possible candidate genes in Chr3: M-QTL1 and Chr8: M-QTL2 (Table S6); supporting root length and photosynthesis related traits respectively (Table S4). It is interesting to note that, LOC_Os03g08310.1 (coding TIFY11A) was identified as common candidate gene in root and shoot (Table S6).

Our results indicated 98 potential candidate genes in seedling, including 84 DEGs located in M-QTLs that were not reported yet. However, 14 genes had previously been considered in the literature (Table S6). Functional classification of these potential candidate genes further suggested that they were belonged to transcription regulation (e.g. AP2, WRKY, HOX and GRAM family), signal transduction (e.g. CIPK24, GDSL) and there were some genes with another functions including kinase, phosphatase, and transporter terms under salinity stress in seedling tissue (Table S6). Remarkably, LOC_Os01g20830.1 (coding a transporter protein) and LOC_Os01g21144.1 (unknown function) were found in saltol region on Chr1: M-QTL2 (Table S6). As well, there were some potential candidate genes in hotspot-regions; for example, WRKY70 (LOC_Os05g39720.1) in Chr5: M-QTL4 and PP2C (LOC_Os06g48300.1) in Chr6: M-QTL4 (R^2 = 10.31%) (Table S4, S6). Moreover, some genes were identified as potential candidate genes in Chr2: M-QTL1, Chr8: M-QTL1, Chr10: M-QTL3 and Chr11: M-QTL1; these Meta-regions were integrated the importance of the initial QTLs for photosynthesis, straw dry weight, yield components (e.g. QGW, DF and NFS) and RTL traits (Table S4, S6).

We identified 28 potential candidate genes in leaves, of which 14 genes were found in the literature. We identified LOC_Os01g22249.1 (coding peroxidase) located in saltol region in Chr1: M-QTL2 as another leading candidate gene. It is interesting to note that, there were OsHKT1 (LOC_Os06g48810.1) and PP2C (LOC_Os06g48300.1) in hotspot-regions in Chr6: M-QTL4 (Table S4, S6).
The obtained results indicated that, 20 genes were located on hotspot-regions containing original QTLs for both yield components and ion homeostasis traits, which could be suggested as promising candidate genes (Fig.4, Table 1). The promising genes were belonged to the following functions: pectinesterase, peroxidase, transcription regulation, high-affinity potassium transporter, protein serine/threonine phosphatase, cell wall organization and a CBS domain containing gene, among which, 2 genes were found in salttol region (Table 1).

Validation of differential gene expression using qRT-PCR

To further validate the potential candidate genes, 15 genes were selected for qRT-PCR in FL478 as a salt tolerant genotype (Fig.5). The qRT-PCR results were confirmed the outcome of the meta-analysis (Fig.S6).

Discussion

Rice is highly affected by salinity stress at seedling and reproductive stages. High salinity concentrations cause the ionic imbalances, dehydration, osmotic stress and oxidative damage. It is therefore important to identify most accurate QTLs and the involved candidate genes. We provide a panel of potential candidate genes both located on meta-QTL regions and differentially expressed under salinity stress conditions in the tolerant genotypes (Fig. 6).

Sensing and Signaling

Plants tolerance to abiotic stresses including salinity is initiated by complex multicomponent signaling pathways to return cellular homeostasis and promote survival [32]. The plant cell wall is one of the first layers for biotic and abiotic stimuli perception, and cell wall remodeling provides a general response mechanism to stresses [33]. There were several genes coding integral components of membrane and cell wall organization in the hotspot-regions. There was OsWAK125 in Chr12: M-QTL1 and up-regulated in roots (Table S6, Fig.6), which is belonged to wall-associated kinase family and has been mainly examined as a potential candidate for the cell wall “sensor” [34, 35]. WAKs are firmly bound to the pectic network of the cell wall, protrude the membrane, and link it to the cytoplasm where a Ser/Thr kinase domain is responsible for further signaling [34, 35]. We also observed a drought and salinity responsive class of cell wall-related genes (represented by pectinesterase) in
Saltol region stress which was up-regulated in roots (Table S6, Fig.6). Various crops such as soybean, wheat, tomato exhibited higher levels of pectin remodeling enzymes in tolerant cultivars than susceptible genotypes under salinity and drought stress [33]. Several serine/threonine phosphatase genes were differentially expressed in leaves at seedling stage in hotspot-regions (Table S6). Serine/threonine phosphatases play significant roles to regulate the adaptive stress responses and signaling pathways in various crops such as, potato, wheat and rice [36-40].

OsMKK1 in Ch6: M-QTL12 and OsCHIT15 in Chr10: M-QTL3 were also detected, which up-regulated in roots, and mediates salinity signaling in rice (Table S6, Fig.6) [41]. Plant chitinases play an important role in abiotic stress response; it is also reported that hydrolysis of the carbohydrate chains by chitinases indicates its possible role in signaling or osmotic adjustment functions [34]. Moreover, 7 hydrolase coding genes involved in signaling pathways were among the DEGs and located on meta-QTL regions (Table S6), of which 2 GDSL-like lipase/acylhydrolase in Chr5:M-QTL2 and Chr6:M-QTL1 were up-regulated in seedlings under salinity stress (Table S6, Fig.6). We also observed OsCIPK24 (SOS2) in Chr6:M-QTL3 and OsCIPK10 in Chr3:M-QTL2 up-regulated in seedlings (Table S6, Fig.4). CIPK (CBL- interacting protein kinases) pathway is appearing as a main signaling pathway and modulates salt tolerance in rice [42, 43]. A generic signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g., inositol phosphates and ROS) followed by transcription factors controlling specific sets of stress-regulated genes [44].

**Transcription Regulation**

Transcription factors are important to emerge any phenotype, as they are able to regulate the expression of all the related genes [32]. HSFA6B (located in Chr1:M-QTL3, up-regulated at seedlings) performs as a positive regulator downstream of ABA signaling directly bound to the promoter of Dehydration-responsive element-binding (DREB) and increase its expression (Table S6, Fig.6). Upregulation of DREB2A can activate various genes related to stress tolerance in different plant species [45]. It is also explained that over-expression of OsTIFY11 (located in Chr3:M-QTL1, up-regulated at shoot and root) increased the tolerance to salinity stress through JA signaling and through modulating potassium homeostasis (Table S6, Fig.6) [46]. There were OsHOX22 and
OsHOX24 from homeobox family in Chr3:M-QTL1 and Chr4:M-QTL3, respectively, both up-regulated in seedlings (Table S6, Fig.6). OsHOX24 were the most up-regulated gene under 150 mM NaCl in the salt tolerant genotype (FL478); while it was highly down-regulated in the salt sensitive genotype (IR29) [31]. Also, the role of OsHOX24 was already detected as regulation of abiotic stress responses through fine tuning the expression of stress-responsive genes in rice [47]. Moreover, there was OsWRKY70 in Chr5:M-QTL4 and up-regulated in seedlings (Table S6, Fig.6). It is reported that OsWRKY70 as positive regulator increases the plant tolerance to osmotic stress [48]. Moreover, GRAS (located in Chr5:M-QTL4 and down-regulated in roots) proteins belong to a plant-specific transcription factor family, which are involved in many plant processes including plant growth and development as well as abiotic stress responses (Table S6, Fig.6) [49, 50]. It is also reported that MOC1 encodes a nuclear transcription factor from GRAS family. MOC1 acts as a positive regulator of lateral branching or increased tiller number [51].

**ROS Inhibition**

One of the key mechanisms to increase the plants adaptation to detrimental environmental conditions including high salt concentrations is regulation of the toxic reactive oxygen species levels [33, 52]. There was Nudix hydrolase in Chr4: M-QTL3 and up-regulated in roots (Table S6, Fig.6), whose general function is to remove the excess toxic metabolites or to control the accessibility of intermediates in metabolic pathways [53]. Also, there was a peroxidase coding gene belongs to antioxidant system in Chr1:M-QTL2 and up-regulated in leaves (Table S6, Fig.6). Transgenic Arabidopsis expressing cytosolic peroxidase genes have been reported to show higher salt tolerance [20]. In addition, there was a hydrolase coding gene belongs to alpha/beta fold family domain containing protein in Chr3:M-QTL3; and up-regulated in seedling (Table S6, Fig.6). It is reported that overexpressing of a gene coding α/β-hydrolase fold enzyme showed significantly higher salinity tolerance compared to the wild-type because of protecting the membrane integrity and increasing the reactive oxygen species scavenging capacity in sweetpotato [54].

**Ionic Homeostasis**

Under salinity stress, regulation of ion flux is necessary for cells to keep low the concentrations of
toxic ions and to collect the essential ions. Salinity stress up-regulates trasporter encoding genes such as Na\(^+\) and K\(^+\) transporters and vacuolar Na\(^+\)/H\(^+\) exchangers [55]. We detected several transporters in meta-QTL positions, among which, there was HKT1 in Chr6: M-QTL4; down-regulated in leaves and up-regulated in roots (hotspot-region, Table S6, Fig.6). High affinity K\(^+\) transporter known as Na\(^+\)/K\(^+\) co-transporters which reduce the transport of Na\(^+\) to shoots and positively regulate salinity tolerance in rice and Arabidopsis [56]. We then identified 2 genes encoding vacuolar protein with signal peptide domain in Chr1: M-QTL3 and Chr3:M-QTL2 (Table S6); up-regulated in seedling. We also observed genes coding sodium/calcium exchanger (NCX) in Chr12:M-QTL4; up-regulated in seedling (Table S6, Fig.6), which play significant roles in Ca\(^{2+}\) signaling and ion homeostasis. Sodium/calcium exchangers use the Na\(^+\) electrochemical gradient through the plasma membrane to extrude intracellular Ca\(^{2+}\) [57, 58].

**Other salt tolerance involved potential candidate genes**

We also found 23 unknown potential candidate genes, among which, 5 genes possess the CBS or cupin domain(s) in their sequence. For instance, a gene containing CBS domain were located in Chr2:M-QTL1 and up-regulated in roots. Previous reports indicated that, it plays a role in salinity and oxidative stress tolerance through affecting chloride channels (Kushwaha et al. 2009). It is also reported that overexpression of *OsCBSX4* exhibited improved the tolerance against salinity and oxidative stress in tobacco transgenic lines[59].

We further observed 4 genes possess the cupin domain(s) in their sequence in various M-QTL positions (Table S6) while there were up-regulated in seedlings. According to the previous reports, cupin domain might play a role to improve the seed germination in rice under salinity stress because proteins with the cupin domain(s) were observed near the position of QTLs for seed dormancy, seed reserve utilization, and seed germination [60].

**Conclusions**

To inspect the molecular mechanisms by which tolerant genotypes respond to the salinity stress, we employed an integrative approach to identify candidate genes related to salt tolerance in rice. Based
on the achieved results, the salt tolerant genotypes utilize more effectual mechanisms in response to
the salt stress (Fig.6) particularly in; 1) Sensing and signalling of the salt stress; Several genes coding
cell wall organization, pectinesterase, serine/threonine phosphatase, chitinase, CBL-interacting
protein kinases were observed in the hotspot-regions and differentially expressed in the tolerant
genotypes. 2) Regulation of transcription; Several salinity responsive transcription factors (TFs)
belonged to different families including TIFY, MYB, HSF, HOX, WRKY, AP2 and GRAS families were
found both in meta-QTL regions and among the DEGs, which have known essential roles in salinity
tolerance in rice. 3) Ionic and osmotic homeostasis; Some transporters were also among promising
candidate genes such as HKT1 (Na/K transporter), NCX (sodium/calcium exchanger) and TIP2-1
(aquaporin). and 4) ROS scavenging; there were many important genes involved in detoxification
such as hydrolase, oxidoreductase, and peroxidase among the DEGs and located in meta-QTL
positions. Further research on these promising candidate genes can be lead to beneficial information
which would be used to improve salt tolerance of given genotypes through genetic engineering or
molecular breeding.

Methods

Meta-analysis of QTLs

Preparing QTL data- All reported QTLs related to salinity tolerance in rice, from 2009 to 2018, were
collected including 15 previously published studies [9-14, 16, 61-68]. The QTLs data including
parental lines, the QTL mapping population type and size, and the number of QTLs per trait was
provided. Moreover, we also evaluated flanking molecular markers, confidence interval (CI), QTL
position, LOD score and proportion of phenotypic variance explained (PVE or R2) in terms of each
QTL. These QTLs were used in this study derived from various population types (including: F2,
backcrossed lines (BC3F4), recombinant inbred lines (RILs)), and size (from 87 to 285 plants) from
different tissues at seedling and reproductive developmental stages (Table S1).

Consensus map and QTL projection- The consensus QTLs was identified using the BioMercator
software [69]. A consensus map contained 1677 SSR markers was used as a reference map for Meta-
QTL analysis (http://archive.gramene.org/db/cmap/download_data?map_set_acc=irmi-2003). Prior to
projecting the QTLs on the consensus map, the 95% CI of the initial QTL was computed using the following formulas:

\[ CI = \frac{530}{N \times R^2} \]

i. For F2 lines:

\[ CI = \frac{287}{N \times R^2} \]

ii. For Double Haploid (DH) lines:

\[ CI = \frac{163}{N \times R^2} \]

iii. For RILs:

Where, \( N \) is the population size and \( R^2 \) is the percentage of phenotypic variation explained by the related QTL. The scaling rule between the marker intervals of the initial QTLs was used for the QTL positions on the consensus chromosome map.

**QTLs Meta-Analysis** - Meta-analysis was performed using the default parameter settings of BioMercator. BioMercator calculated the consensus QTL as models 1, 2, 3, and n. The Akaike Information Criterion (AIC) was used to select QTL models on each chromosome [70]. According to the AIC value, the QTL model with the lowest AIC value was considered as a significant model.

**RNA -sequencing**

RNA-Seq data was obtained from our previous study on two contrasting genotypes of *Oryza Sativa* under salinity stress [31]. Briefly, the young seedlings of FL478 (Salt tolerant) and IR29 (Salt sensitive) were treated with 150 mM NaCl and the root samples were collected 24 h after inception of the salt stress. The purified RNA was used to construct the cDNA library; the qualified libraries were subsequently sequenced using IlluminaHiSeq™ 2500 sequencer. The quality of datasets was conducted using FastQC [71]. TopHat was used to map eight paired-end sequencing libraries of two rice genotypes against the reference genome sequences IRGSP 1.0 (ftp://ftp.ensemblgenomes.org/pub/plants) [71]. Raw sequencing reads were then assembled through Cufflinks and Cuffmerge meta assembler utilities [71]. Finally, differentially expressed genes (DEGs) were determined using Cuffdiff utility, Q-value cut-off of \( \leq 0.05 \) and log2 fold change \( \geq 2 \) (up-regulated genes) and \( \leq (-2) \) (down-regulated genes).

**Meta-analysis of gene expression data according to tissues**
RNA-seq Meta-dataset

The available (by the time of this analysis) transcriptome datasets of rice plants exposed to salinity stress were collected from the National Center for Biotechnology (NCBI) (Table S7). The genes with log2 fold change ≥ 2 (up-regulated genes) and ≤ (-2) (down-regulated genes) with Q-value cut-off of ≤ 0.05 were separately determined as DEGs from these RNA-seq datasets in each tissues.

Microarray Meta-Analysis

Expression data of rice plants subjected to salinity stress were obtained from the NCBI’s Gene Expression Omnibus repository [71, 72]. Totally, 9 GEO dataset from affymetrix platform Rice Genome Array (Affymetrix or Agilent microarray platforms) were downloaded (Table S5). Expression data from each source study was preprocessed distinctly as individual microarray datasets. The package LIMMA in the R program was used to analyze Agilent microarray data [73], while affymetrix platforms were processed in the R program using the package Affy. The raw data from both sources were preprocessed using Robust Multi-Array Average background correction and quantile normalization. Then, non-informative and low-intensity probes were removed from the program standard settings; therefore, probes were transformed into their related genomic locus. The values normalized by RMA were used for the subsequent MA. A contrast between each treatment and its control was estimated with the LIMMA package. A simple empirical Bayes model was used to revise the standard errors, after fitting the data into a linear model. Moderated t-statistic and log-odds of differential expression were calculated for each contrast for each gene. Genes with significant Q-value (FDR ≤ 5%) were considered as DEGs and were classified in four tissues (e.g. shoot, root, seedling and leaves). Log2-fold-change values of each experiment/dataset were saved for further analysis.

Integration of significant gene expression and literature citations for the DEGs

A novel data processing pipeline was proposed in this research integrating different data types to identify promising candidate genes related to salt tolerance in rice (Fig. 4). On one hand, DEGs in response to salinity stress in rice from both microarray and RNA-Seq technologies was integrated. On the other hand, the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) literature was searched to identify the published reports related to the salinity tolerance genes in rice.
In this research, 111 papers were examined and all the salinity tolerant involved reported genes in rice were collected. All the identified genes were classified into 4 tissues (including shoot, root, seedling and leaves) (Table S8). Venn diagram (using the R package) was used to compare the overlaps in detected genes for each tissue using different approaches (including RNA-seq, microarray and literature review) and the common genes were detected. Finally, salinity tolerance associated meta-QTLs regions were explored to find the DEGs, which are coincided with the meta-QTL positions.

Functional annotation and pathway analysis

Enrichment analysis of the DEGs were performed using the AgriGO public web tool [74]. The over-represented GO terms in the three main categories, “Biological Process”, “Molecular Function” and “Cellular Component” were filtered using Fisher's exact test (Q-value < 0.05) and corrected by the FDR method <0.05.

Identification of salinity tolerance-related candidate genes in meta-QTL region

The genes which were found at least by two approaches (from the three applied methodologies including RNA-seq, microarray and literature review) called as common genes in this manuscript. To find the potential candidate genes, the common genes were sought in the salinity tolerance associated meta-QTLs regions. The potential candidate genes that were located on hotspot-regions overlying original QTLs for both yield components and ion homeostasis traits were assumed as promising candidate genes (Fig.4).

Plant material and salinity stress treatment

The salt tolerant rice (Oryza sativa L.) seeds of FL478 cultivar were obtained from International Rice Research Institute (IRRI). Sterilization of seeds were performed by 3 - 6% sodium hypochlorite solution (with one drop of Tween 20 per tube) and sterilized seeds were then germinated in the dark at 28°C in the germinator. The young seedlings of FL478 were transferred in the growth chamber (14 h light/10 h dark at temperature 28 ± 2 °C) and were cultured in hydroponic system (plastic trays on the Styrofoam sheet containing 4 L of distilled water) for 4 days. Then, seedlings were grown in Yoshida solution for 21 days [75]. The hydroponic experiments were done in a factorial arrangement based on a complete randomized block design with 3 biological replicates containing 10 samples
each. The 21-day-old seedlings were treated 150 mM NaCl and Root and shoot samples were then collected 24 h after the onset of salinity stress and immediately placed in liquid nitrogen and stored in at -80 °C in order to RNA extraction.

**RNA extraction and cDNA library synthesis**

Total RNA from leaves and roots tissues were extracted from 100 mg of each tissues using an RNeasy Plant kit (Qiagen). The RNA integrity and quality was then checked by a NanoDrop ND-1000® spectrophotometer and agarose gel electrophoresis.

**Validation of salinity tolerance-related candidate genes by qRT-PCR assay**

Based on initial meta-analysis, second experiment was performed a focused on FL478 as a salt tolerant rice genotype. A sum of 16 genes were randomly selected from the list of possible candidate genes in each tissues (Table S6) for validation using quantitative real-time PCR (RT-qPCR). Specific primer pairs for each gene (Table S9 for list primer) were designed using Oligo 7.0 (ver. 5.0; National Bioscience Inc., Plymouth, USA). cDNA was synthesized using iScript™ cDNA synthesis kit (BioBasic) according to the manufacturer’s protocol. The qRT-PCR was performed for three independent biological replicates using a LightCycler® 96 Real-Time PCR System (Roche Life Science, Germany) and SYBR Premix Without ROX based on manufacturer’s instructions. Rice actin gene (OS03G0836000) was used as an appropriate internal control gene. Transcript levels of the genes from three biological replicates were computed as 2- ΔΔCt [76].

**Abbreviations**

DEGs: differentially expressed genes; QTLs: Quantitative trait locus; MA: meta-analysis; ROS: Reactive oxygen species; KLV: K+ in leaf vegetative; NS: Shoot sodium concentration; NKS: Ratio of the shoot sodium and shoot potassium concentration; KS: Shoot potassium concentration; RN: Root Na+ concentration; SIS: Salt injury score; QGW: 1000-grain weight (g); DF: Days to flowering; NFS: Number of fertile spikelets; BP: Biological Processes; GO: Gene Ontology; MF: Molecular Function; CC: Cellular Component; M-QTLs: Meta-QTLs; CI: Confidence Interval; LOD: logarithm of the odds ratio; AIC: Akaike Information Criterion.

**Declarations**
**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Accession codes: All primary sequence read data has been deposited in NCBI database under BioProject ID: PRJNA493951 and PRJNA493923. All data supporting the conclusions of this article are provided within the article and its supplementary (Additional file 1: Table S1, Table S2, Table S3, Table S4, Table S5, Table S6, Table S7, Table S8, Table S9, Fig S1, Fig S2, Fig S3, Fig S4, Fig S5, Fig S6).

**Competing interests**

The authors declare that they have no competing interests.

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**Authors ‘contributions**

RMM conducted the experiments, and drafted the manuscript. Z-SS conceived the project, supervised and coordinated the research, also revised the manuscript. RMM analyzed RNA-seq data, RMM and PD performed the meta-QTL analysis, RMM and S-MM analyzed Microarray data. NBJ and MRG checked the final manuscript. All the authors read and approved the manuscript.

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**Tables**

Table 1. The promising genes associated with salinity tolerance. The differentially expressed genes detected by more than one approach (common genes) and located on meta-QTLs regions overlying original QTLs for both yield components and ion homeostasis traits were assumed as promising candidate genes in this study (the pipeline is presented in Fig.4).
| Gene ID                  | Gene function                                       | Meta position | Tissue(s) |
|-------------------------|-----------------------------------------------------|---------------|-----------|
| LOC_Os01g20980.1        | Pectinesterase                                      | Chr1: M-QTL2  | R         |
| LOC_Os01g22249.1        | Peroxidase                                          | Chr1: M-QTL2  | L;L;S     |
| LOC_Os02g06410.1        | CBS domain containing membrane protein              | Chr2: M-QTL1  | R         |
| LOC_Os02g06640.1        | Ubiquitin family protein, putative, expressed       | Chr2: M-QTL1  | L;L;S     |
| LOC_Os04g3810.1         | OsSub38                                             | Chr4: M-QTL1  | R         |
| LOC_Os04g26870.1        | Oxidoreductase, aldo/keto reductase family          | Chr4: M-QTL2  | S         |
| LOC_Os04g06910.1        | Expressed protein                                   | Chr4: M-QTL1  | S         |
| LOC_Os04g10750.1        | Inorganic phosphate transporter                      | Chr4: M-QTL1  | S         |
| LOC_Os05g42130.1        | MOC1, Transcription regulation, GRAS family         | Chr5: M-QTL4  | R         |
| LOC_Os05g39720.1        | WRKY70                                              | Chr5: M-QTL4  | S         |
| LOC_Os05g39770.1        | Aminotransferase, putative, expressed               | Chr5: M-QTL4  | L;L;S     |
| LOC_Os05g38660.1        | Expressed protein                                   | Chr5: M-QTL4  | S         |
| LOC_Os05g40010.1        | LTPL17                                              | Chr5: M-QTL4  | S         |
| LOC_Os05g41670.1        | Expressed protein                                   | Chr5: M-QTL4  | S         |
| LOC_Os05g39990.1        | Plant-type cell wall organization                    | Chr5: M-QTL4  | R         |
| LOC_Os05g39250.1        | Phosphatidylethanolamine                            | Chr5: M-QTL4  | R         |
| LOC_Os06g48860.1        | OsSAUR28                                            | Chr6: M-QTL4  | R         |
| LOC_Os06g48810.1        | OsHKT1                                              | Chr6: M-QTL4  | R         |
| LOC_Os06g48300.1        | PP2C                                                | Chr6: M-QTL4  | R         |
| LOC_Os06g49190.1        | LTPL154                                             | Chr6: M-QTL4  | S         |
identified by RNA-seq meta-analysis in 4 tissues (including shoot, root, seedling and leaves). **Fig. S4.**

Number of differentially expressed genes (DEGs) identified by microarray meta-analysis in 4 tissues (including shoot, root, seedling and leaves). **Fig. S5.** GO term assignment of the identified DEGs located in meta-QTL positions to 3 main categories of cellular component, molecular function and biological process. **Fig. S6.** Graph illustrating of the melt curves from qRT-PCR of the selected potential candidate genes in FL478.

Figures
Figure 1

Meta-QTL positions for traits associated with the salt tolerance (Table S1) on 12 chromosomes of rice. Vertical lines on the left of the chromosomes show the confidence interval of each QTL. Marker names and positions (in cM on the consensus map) are indicated on the left. The colors indicate Meta-QTL positions for traits associated with the salt tolerance.
The comparison between differentially expressed genes at salt stress conditions in the tolerant genotypes revealed by RNA-Seq and microarray data analysis or through literature review in a) root, b) shoot, c) seedling and d) leaves.
Figure 3

The number of differentially expressed genes identified by RNA-Seq and microarray data analysis or through literature review, which are located on meta-QTL positions in each tissue (roots, shoots, seedlings and leaves).
Flowchart showing different steps of meta-analysis pipeline used to identify the promising candidate genes involved in salinity tolerance. The differentially expressed genes detected by more than one approach called common genes in this manuscript. To find the potential candidate genes, the common genes were sought in the salinity tolerance associated meta-QTLs regions. The potential candidate genes that were located on hotspot-regions overlying original QTLs for both yield components and ion homeostasis traits were assumed as promising candidate genes.
Validation of selected genes using qRT-PCR in root and shoot tissues of FL478 (tolerant genotype). Bar graphs depict the relative transcript abundance of the selected potential candidate genes in FL478 under different conditions. Data points are represented as log2 fold change values.
The schematic representation of the molecular response to salt stress in the tolerant genotypes. Some candidates are depicted, whose coding gene were differentially expressed under salt stress conditions and also located on meta-QTLs.

Supplementary Files
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