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A meta-analysis of potential candidate genes associated with salinity stress tolerance in rice

Sukhdeep Kaur [a], M.A. Iquebal [a], Sarika Jaiswal [a], Gitanjali Tandon [a], R.M. Sundaram [b], R.K. Gautam [c], K.P. Suresh [d], Anil Rai [a], Dinesh Kumar [a,*]

[a] Centre for Agricultural Bioinformatics, ICAR-Indian Agricultural Statistics Research Institute, New Delhi 110012, India
[b] Crop Improvement Section, ICAR- Indian Institute of Rice Research, Rajendranagar, Hyderabad 500030, Telengana, India
[c] Field Crop Improvement & Protection Division, ICAR-Central Island Agricultural Research Institute, Port Blair 744101, Andaman & Nicobar Islands, India
[d] ICAR-NIVEDI, Hebbal, Bengaluru 560024, Karnataka, India

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ABSTRACT

Even though cultivated rice is highly sensitive to salinity, significant variability exists in the primary and secondary gene-pool of rice with respect to traits of salinity tolerance. Breeding salinity tolerance rice varieties is imperative due to climate change and increasing rice demand for global population. A meta-analysis of plethora of genomic data and published literature available on various genes/factors associated with response to rice salinity and tolerance can be used to enlist selected candidates genes affecting salinity. Such genes can be utilized to identify potential candidate salinity resistance genes from donor rice genotypes and facilitate their transfer to high yielding varieties of rice through marker-assisted breeding. This approach has tremendous advantage over transgenic approach as no bio-safety or regulatory issues are involved in exploiting the variability.

Meta-analyses were performed on three datasets viz., rice microarray data of 166 series comprising of 2586 samples, 1228 published research literature in the last one and half decades and RNA-Seq data of 454 and Illumina from Sequence Retrieval Archive (SRA) at NCBI. Among microarray dataset, six salinity related series were finally selected and multi experiment analysis revealed 2289 differentially expressed genes belonging to 44 gene families. Out of these, 13 families viz., AP2-EREBP, AUX/IAA, bZIP, C2H2, bHLH, C3H, HB, HSF, MYB, MYB-related, NAC, Tify and WRKY were selected. Applying various parameters on the published literature data, 13 genes were selected, of which five were common to the different microarray datasets. From RNA-Seq data, total of 751 differentially expressed genes were obtained from 21 gene families, out of which 11 genes were common with those obtained from microarray data and five genes, viz., AP2-EREBP/DREB, MYB, HSF, bZIP and NAC were common to all the three data sets. Based on the results obtained, a total of 31 meta-analyzed genes have been selected and recommended for use in genetic improvement programs aimed at salinity resistance in rice.

The meta-analysis of microarray, RNA-Seq and published literature has been successfully used to select 31 best salinity tolerance associated genes which can be exploited by candidate gene approach for targeted introgression through marker assisted breeding. This approach has multi-fold advantages, as it obviates statutory and ecological issues. Such endeavors are more warranted for combating the key abiotic stresses like salinity, whose effects are increasing due to a changing climate.

1. Introduction

Rice is one of the leading food crops in the world, grown over 154 million ha, 85% of total rice produced is consumed by humans. It provides 21% of global human per capita energy and 15% of per capita protein. Rice also provides minerals, vitamins, and fiber, although all constituents except carbohydrates are reduced by milling. Rice eaters and growers constitute the bulk of the world's poor

Abbreviations: TF, Transcription Factor; DEGs, Differentially Expressed Genes.

* Corresponding author.
E-mail addresses: sukhii.deep@gmail.com (S. Kaur), jiqubal@gmail.com (M.A. Iquebal), aijaiswal@gmail.com (S. Jaiswal), gitanjali.tandon@gmail.com (G. Tandon), rms@drricar.org (R.M. Sundaram), rgautampb@gmail.com (R.K. Gautam), sureshkp97@gmail.com (K.P. Suresh), anilrai645@gmail.com (A. Rai), dineshkumarbhu@gmail.com (D. Kumar).

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(UNDP Human Development Report, 1997), approximately 70% of the world's 1.3 billion poor people live in Asia, where rice is the staple food. The salt-affected soils are estimated to cover about 1.0 billion ha in the world (Massoud, 1974; Ponnamperuma, 1984). It has also been estimated that about 20% of all irrigated lands in the world are affected by excessive salts (Pitman and Läuchli, 2002). Salt affected soils encompass two types i.e. sodic and saline soils which together occupy about 6.73 m ha land in India and thus adversely affect crop productivity (Sharma et al., 2004). Though rice (*Oryza sativa* L.) is the most important crop and feasible option to start with crop cultivation and reclamation in such soils (Tyagi, 1998; Ismail et al., 2007; Singh et al., 2010), rice is also expected to suffer the most due to salt stress conditions particularly in countries with long sea shore line (Swaminathan and Kesavan, 2012).

It has been found that if root zone salinity exceeds from its threshold (i.e., lower to upper ranging from 3 dS/M to 11.3 dS/M, respectively), then plant growth ceases leading to yield loss of 12% per dS/M (Raes et al., 2012), with seedling and flowering stages being the most sensitive stages. Rice happens to be one of the most susceptible crop species especially to salinity (Grattan et al., 2002; Munns and Tester, 2008) and even with little amount of 50 mM NaCl (Yeo and Flowers, 1986) can affect adversely. Rice productivity in salt-affected areas is as low as <1.5 t/ha (Hairmansis et al., 2014).

The total rice requirement of world population is going to be 800 mT by 2025 (Kubo and Purevdorj, 2004), while in Asia, rice demand is expected to increase by 70% in coming 30 years coupled with population growth (Muthayya et al., 2014). This is likely to face twin facet problem, i.e., increase in the area under salinity and decrease in yield per unit area. Salinity reclamation has its own limitation, thus genetic improvement of rice for better salinity tolerance is imperative. Rice genome has been deciphered, and is recorded to possess >35 K genes (Goff et al., 2002). In order to exploit candidate gene approach, genes related to salinity response and tolerance must be enlisted and rationally prioritized. In the last decade, more than thousand research papers have been published across the globe related to salinity stress in rice and other crops and related data with respect to differential gene expression experiments are enriched in various public databases. Availability of such plethora of data in knowledge discovery research has led to enigmatic question that how many genes can be enlisted with prioritization indices in order so that they can be targeted for genetic improvement of rice for salinity tolerance through marker-assisted breeding.

There are two methods of genetic improvement viz., transgenic approach and candidate gene approach. Both the approaches have been used in rice salinity resistance breeding programs. In transgenic approach, for example e.g. SOD genes from yeast (Tanaka et al., 1999) and catalase genes (Motohashi et al., 2010) from E coli have been used to increase salinity resistance. Among candidate genes approach, for example, FL478 was used as a donor parent to introgress the Saltol QTL conferring salt tolerance into BT7 (Linh et al., 2012) and the candidate genes underlying the QTL have been identified and validated (Thomson et al., 2010). In case of candidate gene approach, there are no regulatory or other issues concerning environment, food and feed safety as the donor and recipients belong to the rice gene pool with hybridization being the key factor in mobilization of genes. There are well known salt tolerant donors varieties like Pokkali, Nona Bokra etc. (Moons et al., 1995). If a set of potential candidate salinity tolerance associated genes can be identified through meta-analysis, they can be validated and utilized in the rice breeding programs.

Meta-analysis, which is quantitative review of related but independent studies (Normand, 1999; Hunter and Schmidt, 2004) can be used as an important tool for knowledge discovery. Meta-analysis approach can resolve the issues related to identification of key of salinity tolerance associated genes and their prioritization so that marker assisted introgression programs can be gainfully be initiated. Though limited attempt of meta-analysis of abiotic stress in rice has been carried out for different traits like drought (Trijatmiko et al., 2014; Swamy et al., 2011; Khowaja et al., 2009; Shaik and Ramakrishna, 2013), biomarker search (Zimmermann et al., 2008), biotic stresses like rice blast (Ballini et al., 2008) and bacterial blight (Shaik and Ramakrishna, 2013), meta-analysis of rice salinity tolerance associated genes has not been attempted. A meta-analysis platform of rice is now available (McLaren et al., 2005). Thus there is a need to identify salinity tolerance related key candidate genes by meta-analysis using all existing datasets (both published literature and public databases).

The present work aims at meta-analysis of potential rice salinity tolerance associated candidate genes from publicly available experimental data (both microarray and RNA-Seq datasets) and published literature.

2. Materials and methods

Microarray data for salinity stress was retrieved from Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/). The microarray datasets from affymetrix platform Rice genome Array (GPL2025) were downloaded. Out of 166 series, 9 series were available for salt stress viz., GSE3053, GSE4438, GSE6901, GSE11175, GSE13735, GSE14403, GSE16108, GSE21651, GSE28209. The microarray dataset workflow is represented in Fig. 1. To maintain the uniformity of dataset, only 6 series datasets were used in the present study, excluding GSE3053, GSE4438 and GSE28209 from the working dataset (Table S1a). The RNA-Seq data (454 and Illumina) of rice salinity with accessions DRX000191, DRX000192, DRX000194, SRX145290, SRX144646, SRX144647, SRX145289, SRX144645, SRX145288, SRX144644, SRX145287 from Sequence Retrieval Archive (SRA) at NCBI were used, details of which are given (Table S1b). These were further used for meta-analysis of rice salinity, the workflow of which is illustrated in Fig. 2.

2.1. Identification of differentially expressed genes from microarray dataset

After data collection, data normalization was done using quantile method and for summarization step (Irizarry et al., 2003), median polish method was applied at RMAExpress (Bolstad, 2012). The RMA preprocessed files were used to identify differentially expressed genes using RankProd method in MultiExperiment Viewer (Saeed et al., 2003), which is a non-parametric method. In this method, genes were ranked according to their up and down regulation, fold change and p-values (Breitling et al., 2004). Rank product method combined the datasets from various studies that were carried out with different criteria like number of permutation set as 250, p-value cutoff <0.05, FDR (Benjamini and Hochberg, 1995) at 0.05 and fold change >2. Genes that satisfied all the cutoff thresholds were considered as differentially expressed genes (DEGs) (Hong and Breitling, 2008). Different number of DEGs were identified from each experiment and collected in single non redundant stress signal file. This file contains 2289 genes which were considered for further analysis.

2.2. Gene set enrichment analysis for microarray dataset

Functional enrichment analysis was done by AgriGO (Du et al., 2010b) followed by transcription factor database RiceSRTFDB (Priya and Jain, 2013). Enrichment analysis was done to characterize and combine specific pathways, domains in genes. Salinity responsiveness genes were searched. Gene ontology study was performed on the obtained 2289 genes in non-redundant stress signal file to find out enriched GO terms in molecular function, biological process and cellular components contributing in salinity response. To further prove salinity response we collected all salinity responsive transcription factors from RiceSRTFDB and comparatively analyzed them with the enriched GO IDs to annotate the all genes. Annotating genes and factors were further analyzed and selection was made for those with frequency/occurrence greater than five times. Analyzing 2289 genes, shows 44 types of

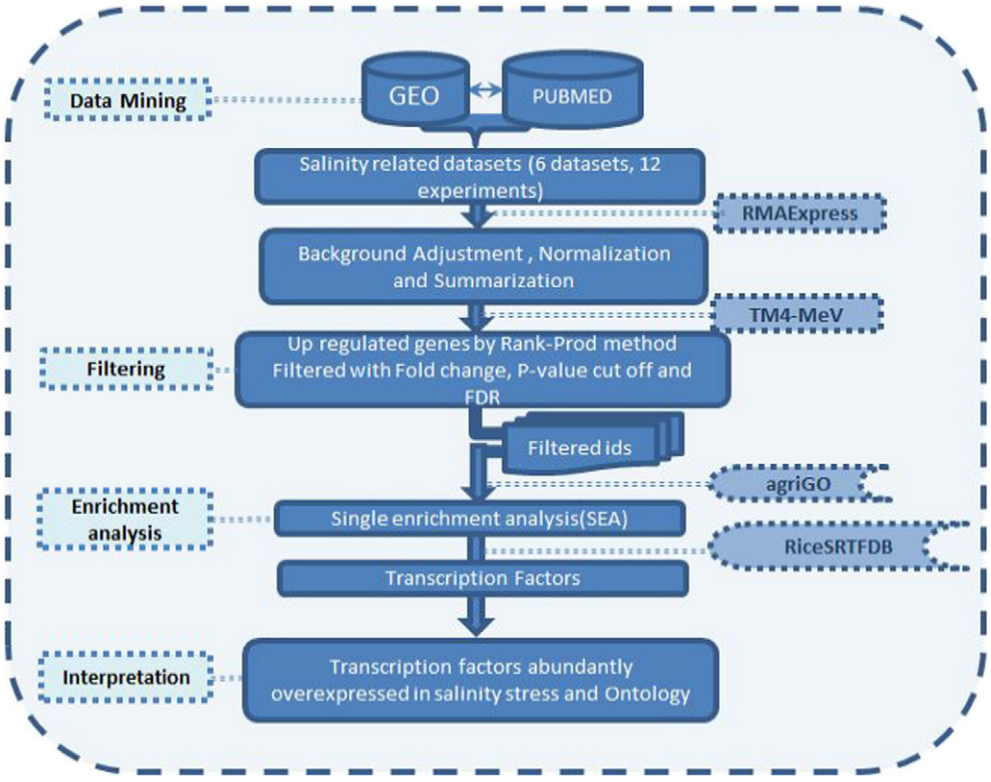

Fig. 1. Microarray dataset meta-analysis workflow.

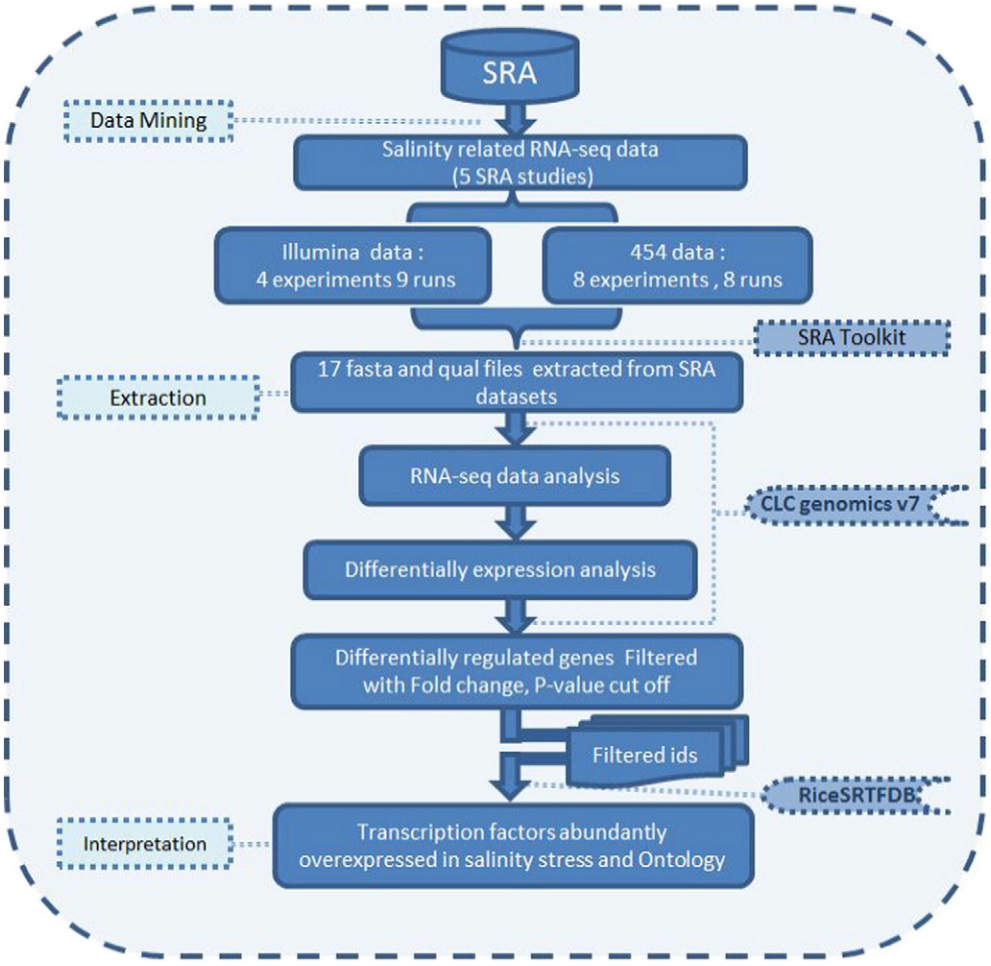

Fig. 2. RNA-Seq dataset meta-analysis workflow.

Year	Salinity Publications	Total Publications	Salinity Publications Normalized
2000	30	496776	6.038939079
2001	30	538235	5.573773538
2002	34	602544	5.642741443
2003	56	607718	9.214800286
2004	49	628228	7.799716027
2005	54	688084	7.847879038
2006	76	707992	10.73458457
2007	90	744307	12.09178471
2008	98	787195	12.44926606
2009	108	797166	13.54799377
2010	102	832244	12.25602107
2011	126	886103	14.2195659
2012	160	920555	17.38081918
2013	167	972888	17.165388
2014	48	NA	NA

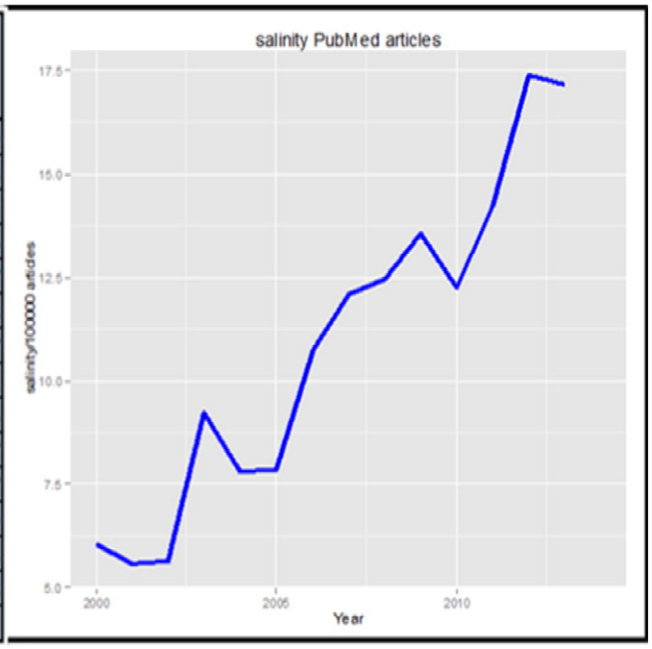

Fig. 3. Year wise distribution of salinity related studies published in PubMed from 2000 onwards.

gene/gene family, out of which 13 were the most significant based on above criteria.

The RNA-Seq dataset retrieved from SRA at NCBI were taken for reference based RNA-Seq analysis using CLC Bio Workbench V 7.0.4. The analysis resulted in total of 751 significant differentially expressed genes at 1% probability and fold value 2. These differentially expressed genes were further used for meta-analysis and it was found that it belonged to 21 gene families. For this dataset also,

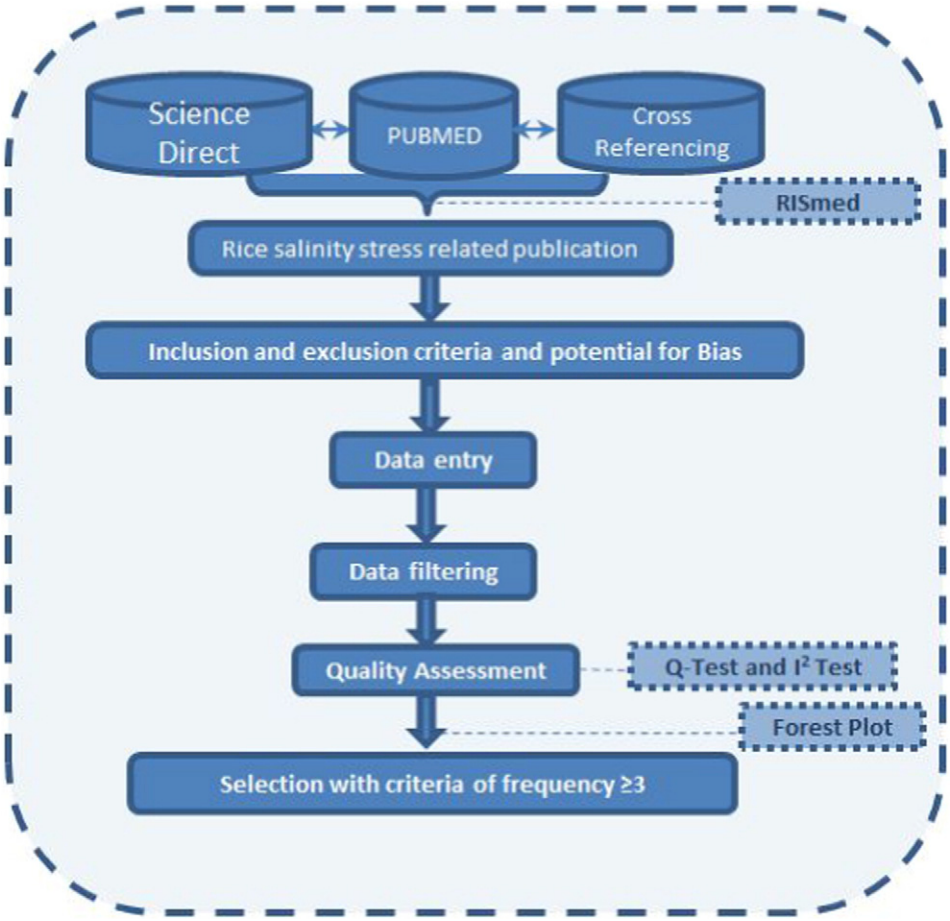

Fig. 4. Workflow of systematic review of published studies for meta-analysis.

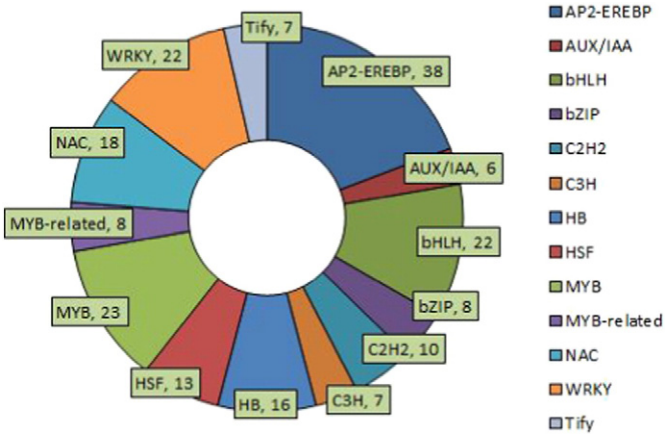

Fig. 5. Distribution of gene families in microarray analysis for salinity stress.

same procedure of gene enrichment with GO IDs and their annotation was applied.

A systematic review i.e. scientific summary of all available evidence of rice salinity stress related literature was extensively made. These were carried out at public repositories of published literature viz. Science Direct (www.sciencedirect.com), PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and by cross referencing citations of selected publications (Fig. 3). This information were imported to an R package, RISmed (Kovalchik, 2014) for meta-analysis. Out of the 1228 published manuscripts related to rice salinity stress collected from year 2000 onwards, a total of 200 published manuscripts were filtered based on the criteria of study viz., rice, salinity genes, type and degree of salt stress and salinity transcription factor. After thorough study of these articles, the data were collected related to author, year, title, source, development stage, salt concentration, stress duration, availability of articles and variety of rice used in experiment. Fig. 4 shows the workflow of systematic review of published studies for meta-analysis. Quality assessment of the collected published literature In order to check the quality of selected publication and control its biasness, a series of statistical tests were applied (Wells et al., 2000). Under the null hypothesis of existence of homogeneity in reported publications, the homogeneity test was applied. The effect size was calculated, followed by standard error, variance and weight of study and Q-test. Further, I2 (Neyeloff et al., 2012) was also calculated to get the total variance across studies. These two tests helps to confirm the homogeneity of data collected under study.

2.3. Selection of final key genes from all the three analyzed dataset

The collected 44 genes from microarray experiments with occurrence >5, 21 genes from RNA-Seq dataset and 13 genes from publication search with occurrence >2 were considered further. The genes from all the three sets were pooled together to look for the genes common to microarray experiment, RNA-Seq experiments and literature collection.

3. Results and discussion

3.1. Results from analysis of microarray and gene enrichment datasets

All the six series of datasets, viz., GSE6901, GSE11175, GSE13735, GSE14403, GSE16108, SE21651, were successfully analyzed simultaneously in order to minimize variation within the dataset. Microarray analysis of rice salinity responsive genes showed 2289 differentially expressed genes (Table S2a). These 2289 DEGs belonged to 44 gene families, out of which, 13 gene families that are significantly present and occurs more than five time were selected. These include AP2-EREBP, AUX/IAA, bZIP, C2H2, bHLH, C3H, HB, HSF, MYB, MYB-related, NAC, Tify and WRKY. We found a family AP2-EREBP consisting of at least 38 transcription factor encoding genes (Fig. 5). Maximum percentage of genes (Approximately 68%) showed enriched GO terms for ion binding followed by catalytic activity (approximately 52%; represented in Fig. 6). This is expected, particularly when higher salt content and ion concentration stimulates the ion transport mechanism for balancing of ion charge as described (Brini and Masmoudi, 2012; Wang et al., 2011, 2012). The collected salinity responsive transcription factors from RiceSRTFDB were comparatively analyzed with the enriched GO IDs to annotate the all genes (Table S3a).

3.2. Results from analysis of available RNA-Seq data

All the RNA-Seq data retrieved from SRA at NCBI from 454 and Illumina were taken for reference based RNA-Seq analysis using CLC Bio Workbench V 4.0. The analysis resulted in total of 751 significant differentially expressed genes at 1% probability and fold value 2 (Table S2b). These differentially expressed genes belonged to 21 gene families. For this dataset also, similar procedure was applied as in case of microarray dataset for gene enrichment with GO IDs and their annotation (Table S3b).

3.3. Results from analysis of published literature

The meta-analysis of 1228 published literature was carried out based on the parameters related to author, year, title, source, development

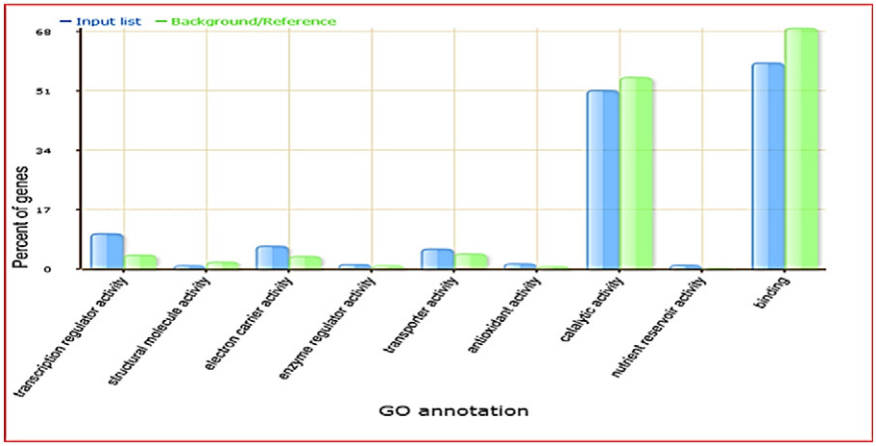

Fig. 6. Enriched GO distribution of salinity responsive genes from microarray dataset.

Table 1
Information extracted from rice publications based on salinity stress.

Type of information	Category	Essential information
Source of publication	PubMed	No
Author name	–	No
Title	–	Yes
Year of publication	–	Yes
Type of publication	–	Yes
Salt concentration	Salt conc. in mm	Yes
Developmental stage	–	Yes
Stress gene	–	Yes
Transcription factors	–	Yes
Stress duration	Hours	Yes
Varieties	–	Yes
Plant species	Landrace/cultivar	No
Origin of plant species	Indica/Japonica	Yes
Availability of publications	Free/paid	No

stage, salt concentration, stress duration, availability of articles and variety of rice used in experiment as detailed in Table 1.

After applying the homogeneity test on the published literature to remove any bias related to the effect size, standard error, variance and weight of study and Q-test (Table S4a). For the data analyzed in the present study, table value at confidence interval 0.05 was 21.026 and calculated Q at (k-1), the degree of freedom was 15.699. I2 was found to be 23.5%. These two tests confirm the data collected under study to be homogenous (Table S4b).

Genes which are over-represented in publications, microarray datasets as well as RNA-Seq data sets are listed along with their respective occurrences in Tables 2, 3 and 4, respectively.

The common gene families obtained in meta-analysis of microarray, RNA-Seq and publication data set along with the selected other gene/families are presented as Venn diagram (Fig. 7). Interestingly, it was found at least eight gene families viz., MYB-related, WRKY, BHLH, C3H, C2H2, AUX/IAA, HB and Tify after analysis of microarray data, were not described in literature. There were five genes (AP2-EREBP, MYB, HSF, bZIP and NAC) common to all the datasets viz., microarray, publication and RNA-Seq as depicted from Venn diagram (Fig. 7).

After pooling the selected genes from the meta-analyses viz., microarray data-set, RNA-Seq dataset and published literature, a total of 31 genes were selected based on score related to salinity resistance in rice. The role of these genes in salinity resistance pathways, known to contribute in both ABA dependent and ABA independent pathway have been well described (Kumar et al., 2013) which is summarized in Table 5. The role of such genes are found in both abiotic stresses (salinity and drought), as depicted in the Table 5 along with respective references.

Table 2
List of genes from published literature in salinity stress of rice, frequency of publication and gene family/transcription factor.

Salinity genes	Frequency of publication	Gene family/transcription factor
OsDREB	9	AP2-EREBP
OsbZIP	4	bZIP
OsCYP	3	CYP
OsHSF	5	HSF
OsCAM	4	CAM
OsABP	4	ABP
OsCIPK	4	CIPK
OsNHX	8	NHX
Rab16/OsLEA	11	LEA
OsNAC	13	NAC
OsP5CS	3	P5CS
TPSP	3	TPSP
OsMYB	7	MYB

Table 3
List of genes from microarray analysis in salinity stress of rice.

Rice TF families/regulators	Rice salinity (frequency)
ABI3VP1	2
AP2-EREBP	38
ARF	1
AUX/IAA	6
bHLH	22
bZIP	8
C2C2-CO-like	2
C2C2-Dof	4
C2H2	10
C3H	7
CCAAT	1
CPP	1
DBB	1
DBP	1
EIL	1
FHA	1
G2-like	2
GNAT	2
GRAS	3
GRF	4
HB	16
HMG	1
HSF	13
LOB	5
MADS	3
MBF1	1
MYB	23
MYB-related	8
NAC	18
NF-X1	2
OFP	3
Orphans	3
PHD	1
Pseudo_ARR-B	1
RB	1
SBP	3
SWI/SNF-BAF60b	1
TCP	1
Tify	7
TRAF	1
Trihelix	1
TUB	1
ULT	1
WRKY	22

Table 4
List of genes from RNA-Seq data analysis in salinity stress of rice.

Rice TF families/regulators	Rice salinity (frequency)
AP2-EREBP	16
WRKY	14
bHLH	11
Tify	9
MYB	7
MYB-related	6
C2H2	4
HB	3
HSF	3
ABI3VP1	2
bZIP	2
DBB	2
DBP	2
EIL	2
FAR1	2
GRAS	2
LOB	2
NAC	2
SNF2	2
C2C2-CO-like	1
G2-like	1

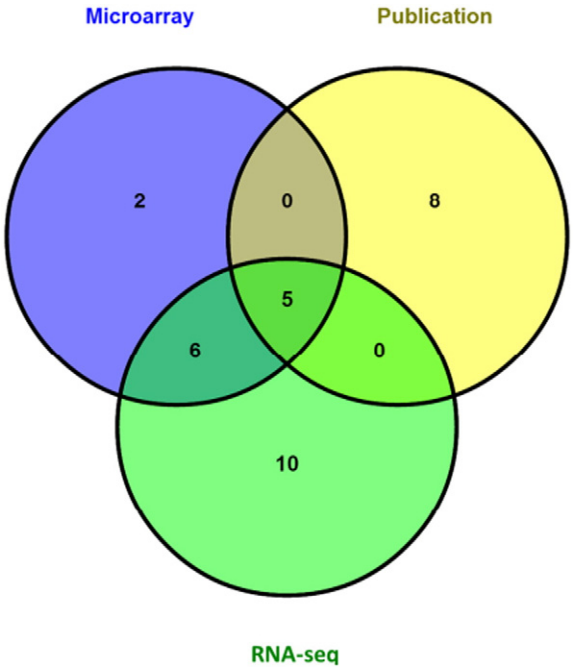

Fig. 7. Venn diagram showing common five genes from datasets under study.

4. Conclusion

The meta-analyses of the three sets of salinity tolerance related data (viz., microarray, RNA-Seq and published literature dataset) of rice has enlisted 31 rationally selected gene families. These 31 panel of gene families were represented by microarray (Du et al., 2010a), RNA-Seq (Guo et al., 2014) and literature (Du et al., 2010a). Out of 31 gene families, literature has reported only 13 families so far thus there is a greater need for evaluation of salinity tolerance by doing molecular introgression by conventional breeding. We found RNA-Seq analysis as much more sensitive technique yielding highest number of differentially expressed gene families (Guo et al., 2014).

Allele mining can be carried out to identify sequence perturbations associated with tolerance to salinity and they can be validated in mapping population. Markers targeting the allelic variations in these candidate genes can be gainfully used in breeding programs through marker-assisted selection. Even if such interventions may be of limited magnitude, they may still boost rice productivity over larger area fetching better return to rice growers.

Conflict of interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aggene.2016.08.001.

Table 5
Top 31 selected salinity tolerant candidate genes and their role.

S.N.	Gene/transcription factor/gene family	Function	References
1	OsDREB/AP2-EREBP	Salinity tolerance (ABA independent)	Zhang et al., 2013; Wang et al., 2008; Mallikarjuna et al., 2011; Dubouzet et al., 2003
2	OsABP/ABP	Salinity tolerance with cellular responses (ABA independency proved by homologous AvDH1)	Macovei et al., 2012; Amin et al., 2012
3	OsbZIP/bZIP	ABA dependent Salt inducible	Nijhawan et al., 2008
4	OsMYB/MYB	ABA dependent Salinity Tolerance	Yang et al., 2012
5	MYB-related	ABA dependent Salinity Tolerance	Xiong et al., 2014
6	OsNAC/NAC	ABA dependent Salinity Tolerance	Hu et al., 2006
7	WRKY	Abscisic acid signaling and salt stress tolerance (ABA dependent)	Tao et al., 2011; Qiu et al., 2008
8	Rab, OsLEA/LEA	ABA dependent Salinity tolerance	Duan and Cai, 2012
9	bHLH	Osmotic balance for salt tolerance (ABA dependent)	Chen et al., 2013
10	C3H(CCCH)	leaf senescence (ABA dependent)	Kong et al., 2006; Ay et al., 2014
11	OsP5CS	Osmoprotectant; proline biosynthesis (ABA dependent)	Zhu et al., 1998
12	C2H2	Abscisic acid-induced antioxidant defense and oxidative stress tolerance	Zhang et al., 2014
13	TPSP	Trehalose accumulation (ABA dependent)	Elbein et al., 2003
14	OsHSF/HSF	Signaling and ion homeostasis	Schmidt et al., 2012
15	OsCYP/CYP	Salinity Tolerance(ROS pathway)	Ruan et al., 2011
16	OsCAM/CAM	Salinity tolerance (Ca2+/PLC pathway)	Du et al., 2010a, Saeng-ngam et al., 2012
17	OsCIPK/CIPK	Salinity tolerance via ca2+ binding (SOS Pathway)	Xiang et al., 2007
18	OsNHX/NHX	Salinity tolerance (Ionic Pathway)	Bassil et al., 2012; Blumwald and Poole, 1987
19	AUX/IAA	Auxin mediated and abiotic stress signaling pathway for salinity response	Wang et al., 2009; Jain and Khurana, 2009
20	HB	Auxin responsive salinity tolerance	Itoh et al., 2008
21	Tify	Jasmonaic acid mediated signaling (ABA dependent and independent)	Zhang et al., 2012
22	AB13VPI	ABA dependent Salinity Tolerance	Li et al., 2015
23	DBB	ABA insensitive Salt tolerance	Kiełbowicz-Matuk et al., 2014
24	DBP	ABA insensitive Salt tolerance	Xu et al., 2014
25	EIL	Salinity tolerance by Na/K homeostasis (ethylene signaling pathway)	Peng et al., 2014
26	FAR1	ABA induced salt sensitivity	Tang et al., 2014, Domergue et al., 2010
27	GRAS	ABA independent salinity tolerance(GA insensitive)	Golldack et al., 2014
28	LOB	Auxin inducible salinity tolerance	Ariel et al., 2010
29	SNF2	Salinity Tolerance(ROS scavenging)	Guo et al., 2014
30	C2C2-CO-like	ABA dependent Salinity tolerance	Min et al., 2015, Lagercrantz and Axelsson, 2000
31	G2-like	ABA dependent Salt inducible	Richardt et al., 2010

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