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ORIGINAL PAPER

# Targeted association analysis identified *japonica* rice varieties achieving $Na^+/K^+$ homeostasis without the allelic make-up of the salt tolerant *indica* variety Nona Bokra

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**Abstract** During the last decade, a large number of QTLs and candidate genes for rice tolerance to salinity have been reported. Using 124 SNP and 52 SSR markers, we targeted 14 QTLs and 65 candidate genes for association mapping within the European Rice Core collection (ERCC) comprising 180 *japonica* accessions. Significant differences in phenotypic response to salinity were observed. Nineteen distinct loci significantly associated with one or more phenotypic response traits were detected. Linkage disequilibrium between these loci was extremely low, indicating a random distribution of favourable alleles in the ERCC. Analysis of the function of these loci indicated that all major tolerance mechanisms were present in the ERCC although the useful level of expression of the different mechanisms was scattered among different accessions. Under moderate salinity stress some accessions achieved the same level of control of $Na^+$ concentration and $Na^+/K^+$ equilibrium as the *indica* reference variety for salinity tolerance Nona Bokra, although without sharing the same alleles at several loci associated with $Na^+$ concentration. This suggests (a) differences between *indica* and *japonica* subspecies in the effect of QTLs and genes involved in salinity tolerance and (b) further potential for the improvement of tolerance to salinity above the tolerance level of Nona Bokra, provided the underlying mechanisms are complementary at the whole plant level. No accession carried all favourable alleles, or showed the best phenotypic responses for all traits measured. At least nine accessions were needed to assemble the favourable alleles and all the best phenotypic responses. An effective strategy for the accumulation of the favourable alleles would be marker-assisted population improvement.

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## Introduction

Rice (*Oryza sativa* L.) is rated as a salt-sensitive species and salinity stress is the most widespread soil problem

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impeding rice production (Greenland 1984). Salinity occurs in coastal areas, in river delta soils and former floodplains around the world (Yeo et al. 1990). In these particular areas, rice is also often the only crop grown since the irrigation water it requires helps leach salt from top soils (Bhumbla and Abrol 1978). This is the case in Europe, where rice is grown mainly in deltaic areas with salinity problems (Ferrero 2007). The problem of salinity is expected to increase in the future due to global warming (Wassmann et al. 2004).

Large genetic variability for tolerance to salinity among rice varieties has been reported (Akbar et al. 1972; Flowers and Yeo 1981; Asch et al. 2000). The most famous tolerant varieties, Pokkali and Nona Bokra, belong to the *indica* subspecies (Yeo et al 1990), but the distribution of salinity tolerance among different *O. sativa* subspecies and varietal groups is not well documented. Rice sensitivity to salt varies with the growth stage (Zeng and Shannon 2000), seedling and reproductive stages being the most sensitive. Varietal tolerance at these two stages is not directly related (Moradi et al. 2003).

High salinity causes both hyperionic and hyperosmotic stress effects. Altered water status affects photosynthesis and metabolic activities leading to the inhibition of cell division and expansion, accelerated cell senescence and, ultimately, reduced growth and diminished grain yield (reviewed by Munns and Tester 2008; Negrão et al.2011). The major factors which govern the growth-stage-dependent response of rice to salt application are responsive stomata which close within minutes after exposure to salt stress but partially reopen after a period of acclimation, high vigour, salt exclusion at the root level, compartmentalization of $Na^+$ ions in structural and older tissues, high tissue tolerance, and up-regulation of antioxidant systems. However, individual mechanisms are only loosely correlated with each other and with the whole-plant survival or with yield under salinity stress (Yeo et al. 1990; Ismail et al. 2007). Whatever the mechanisms in play, plant ability to maintain high $K^+/Na^+$ ratio is a key feature of salt tolerance. Indeed, intracellular $Na^+$ and $K^+$ homeostasis plays a vital role in the growth and development of higher plants (Clarkson and Hanson 1980). Low cytosolic $Na^+$ and high $K^+/Na^+$ ratios help maintain the osmotic and biochemical equilibrium of plant cells. Leaf $K^+/Na^+$ ratio predicts salinity-induced yield loss in rice (Asch et al. 2000).

In recent decades, linkage mapping in populations derived from crosses between varieties with contrasted salinity tolerance has played a critical role in dissecting the genetic architecture of tolerance to salinity in rice. A large number of quantitative trait loci (QTLs) involved in plant survival, relative seedling vigour, plant height, tiller number, root length, and biomass, as well as in root and shoot $Na^+$ and $K^+$ concentrations, have been identified (Ammar et al. 2007; Bonilla et al. 2002; Flowers et al. 2000; Haq et al. 2010; Kim et al. 2009; Koyama et al. 2001; Lee et al. 2007; Lin et al. 2004; Prasad et al. 1999; Sabouri et al. 2009; Takehisa et al. 2004). A list of QTLs involved in rice salinity tolerance can be found in Gramene (http://www.gramene.org). More detailed information on these QTLs has been compiled in the rice module of the TropGene database (http://tropgenedb.cirad.fr), following a procedure identical to that used to compile drought tolerance QTLs (Courtois et al. 2009). Using the Nona Bokra x Koshihikari mapping population, Ren et al. (2005) cloned the gene underlying the major QTL mapped on the short arm of chromosome 1. This gene, *SKC1* (*OsHKT8*), encodes a $Na^+$ transporter of HKT type and is involved in $Na^+$ and $K^+$ homeostasis. Fine mapping work conducted recently in the same area of chromosome 1 suggests that *OsHKT8* may be part of a cluster of salinity tolerance QTLs (Haq et al. 2010). Another QTL, SalTol, has also been fine-mapped on chromosome 1, a few Mb away from OsHKT8. It co-localizes with the *Sal*T gene (Claes et al. 1990) but so far, there is no evidence that the gene and the QTL represent a single genetic factor. Additional fine mapping and the demonstration that SalTol acts to control shoot $Na^+/K^+$ homeostasis seems to support the hypothesis that *OsHKT*8 is the causal gene underlying the QTL (Thomson et al. 2010).

During the same period, a very large number of candidate genes involved in rice response to salinity have also been identified using genomic approaches (reviewed by Ismail et al. 2007; Negrão et al. 2011). These include signalling genes (Boonburapong and Buaboocha 2007; Chen et al. 2006; Martinez-Atienza et al. 2007a; Wan et al. 2007), genes involved in ion homeostasis (Garciadeblas et al. 2003; Golldack et al. 2003; Horie et al. 2007; Fukuda et al. 2010) and in the synthesis of osmoprotective proteins (Wang et al. 2007), as well as transcription factors (Liu et al. 2007; Matsukura et al. 2010) and genes involved in the rapid post-translational regulation of cell proteomes (Martinez-Atienza et al. 2007a; Khan et al. 2005).

So far, little of the tremendous knowledge on the genetic bases of tolerance to salinity accumulated during the last 10 years has been exploited for breeding purposes. Marker-assisted selection for tolerance to salinity in rice is confined to introgression of the favourable alleles of one or two QTLs into elite lines (Jena and Mackill 2008; Lang et al. 2008; Mackill 2007).

Recently, association mapping based on the strength of linkage disequilibrium between markers and functional polymorphisms across a set of diverse germplasm emerged as a powerful tool for gene tagging (Mackay and Powell 2006; Zhu et al. 2008). The approach involves harnessing the genetic diversity of a core collection from a gene bank or of varieties representing the elite germplasm of a breeding

programme. Depending on the scale and focus of the study, association mapping generally falls into two broad categories: (a) targeted or candidate-gene association mapping, which relates polymorphisms in selected candidate genes thought to play a role in controlling phenotypic variations for specific traits and (b) genome-wide association mapping, which checks for genetic variations in the whole genome to locate association signals for complex traits (Risch and Merikangas 1996), but requires considerable genotyping effort to reach the proper marker density.

We report here the implementation of targeted association mapping within a collection of elite germplasm for European rice breeding programmes, with the aim of (a) identifying the most effective salinity tolerance QTLs and candidate genes in this collection and (b) providing the European rice breeding programmes with the best-performing alleles for tolerance to salinity as well as associated donors and molecular markers to accelerate breeding for salinity tolerance.

## Materials and methods

### Plant material

The plant material belongs to the European Rice Germplasm Collection (ERGC), composed of 450 accessions, established by merging the working collections of five European public rice breeding programmes (in France, Greece, Italy, Portugal and Spain) (Chantereau 2001). The ERGC was genotyped with 26 independent SSRs. Two hundred accessions, hereafter referred to as the European Rice Core Collection (ERCC) were selected, using DarWin software (http://darwin.cirad.fr/darwin/Home.php), to represent the diversity of alleles, allelic combinations and geographic origins of the ERGC, while discarding accessions belonging to small exotic groups, such as Basmati, with low adaptation for cultivation under temperate climates (Supplementary table S1).

### Phenotyping for tolerance to salinity stress

The 200 selected accessions of the ERCC were phenotyped for tolerance to salinity in two separate experiments using hydroponic culture under controlled conditions to address tolerance to salinity during the seedling and the tillering stages (conducted in Greece and in France, respectively).

Experiment one (Exp1), focused on salinity tolerance during the seedling stage. The experimental design was an augmented design with two treatments (control and salinity stress), three blocks and eight replicates per treatment, with the susceptible check, IR29, present in each block. 4 days after sowing (DAS), the distilled water was replaced by modified Hoagland and Arnon (1950) nutritive solution supplemented with 3 g/l of NaCl, corresponding to an electrical conductivity (EC) of 6 dS $m^{-1}$. At 7 DAS, the EC of the solutions was raised to 12 dS $m^{-1}$ by adding an extra 3 g/l of NaCl. The pH of the nutritive solution was adjusted to 5.5 daily. The experiment was conducted in a glasshouse maintained at approximately 29°C/22°C day/night with 70% relative humidity. At 16 DAS, salt injury (SIS) was scored visually in individual plants using a 1–9 scale (IRRI, 1997); then, using a chlorophyll-meter (Opti-Science CCM-200), leaf chlorophyll concentration (LCC) was measured at three points on the last expanded leaf of each plant. Finally, plants were dried for 72 h at 60°C and the dry weight of the shoots (SDW16) was measured. Only 140 accessions out of 200 have been phenotyped for LCC and SDW16.

Experiment 2 (Exp2), focused on salinity tolerance during the tillering phase. The experimental design was a split plot with two factors (control and salinity stress), 16 blocks, 4 replications and seven check varieties: two susceptible varieties (IR29 and Aychade), two tolerant varieties (Nona Bokra and Pokali), and three well-known varieties, Giano, Fidji and Nipponbare. Salinity stress (EC of 6 dS $m^{-1}$) was applied at 24 DAS by adding NaCl to the modified Hoagland and Arnon (1950) nutritive solution, and continued until 38 DAS. Air temperature in the culture chamber was 28°C/23°C day/night; relative air humidity was 60%/80% day/night, and 8.0 $MJm^{-2}$ $d^{-1}$ of light was supplied with halogen lamps during a 14-h photoperiod. Plant height (PH), tiller number (TN), leaf number (LN) on the main tiller and maximum root length (MRL) were measured on individual plants, just before the beginning of salinity stress. The same variables, plus root dry weight (RDW), shoot dry weight (SDW), and $Na^+$ and $K^+$ ion concentration in the last fully expanded leaf were measured at 38 DAS. For each measured variable $X$, a response to salinity variable $X\_r$ was computed: $X\_r = ((X\_s - X\_c) \times 100)/X\_c$, were $X\_c$ is the variable $X$ under optimal growth condition or control treatment and $X\_s$ the same variable under salinity stress. For instance, $PH\_r = ((PH\_s - PH\_c) \times 100)/PH\_c$. PH_r provides an evaluation of the reduction of plant height normalised over the existing variability of plant height among the 200 accessions in the absence of salinity stress.

### Selection of target loci and the linked SNP and SSR markers

Seven papers related to rice QTLs for salinity tolerance, published up to early 2008, were curated manually and the relevant information was compiled in http://tropgenedb.cirad.fr/html/rice_QTL.html database. Subsequently the QTLs were projected on the rice physical map to be visualized through a CMap representation of the rice

chromosomes (http://tropgenedb.cirad.fr/cgi-bin/cmap/viewer). Likewise, 62 papers related to rice tolerance to salinity, published up to mid-2008, were curated and relevant information was compiled on 102 genes with known proteins. A similar procedure was used for 54 differentially expressed genes reported by Kumari et al. (2008) and 98 genes reported by Perin (personal communication). Based on their co-localization with QTLs and on the supposed function of the gene family, 240 candidate genes were selected for the SNP search. The OryzaSNP database (http://www.oryzasnp.org/) was investigated for SNPs in the 240 candidate genes that responded to the requirements of the *Illumina* (http://www.illumina.com) genotyping technology. A total of 1,029 SNPs filling these requirements were identified. Of these SNPs, we selected 213 that were polymorphic among the seven *japonica* accessions of the OryzaSNP database, since the European Collection is mainly composed of *japonica* accessions. Finally, after additional quality scoring for *Illumina Bead Xpress* PCR and hybridisation criteria, 124 SNPs covering 47 candidate genes were selected (Table S2).

To overcome selection bias due to the lack of polymorphic SNPs in candidate genes, 22 genes were selected based on their membership of gene families and/or co-localization with salinity QTLs (14 cases), to be investigated using SSR markers. The "Get Sequence" function of OrygenesDB (http://orygenesdb.cirad.fr/) was used to recover 250 kb of sequence in Fasta format on both sides of each chosen gene. Then, the SAT function of Southgreen toolbox (http://southgreen.cirad.fr/) was used to detect all SSR markers (defined as any motif with at least four repeats) in these sequences and to define primers for each SSR for an annealing temperature of 55°C and an amplicon size ranging from 100 to 250 bp. Using the "Primer Blaster" function of OrygenesDB and the Nipponbare genome as reference, the primer pairs were tested in silico for amplification of the right locus and for a low number of hits (<100). The selected SSRs were investigated for polymorphism ($2 <$ number of alleles $< 6$), among six reference temperate *japonica* accessions and 1–4 SSRs were selected for each target gene. Finally, using the Gramene database (http://www.gramene.org/), additional SSRs were selected for one marker/Mb coverage of the confidence interval of each of the 14 above-mentioned QTLs. Overall, 107 SSR markers were tested, and the 52 polymorphic SSRs selected corresponded to 22 target candidate genes and 14 QTLs. These SSRs were used to genotype the core collection (Table S3).

## Genotyping

DNA of the 450 ERGC accessions was extracted at PTPF Genomic platform (Lodi, Italy) from lyophilised leaves of one 4-week-old plant, using an automated 96-well plate method with Tecan liquid handler (Tecan Freedom Evo 150) and magnetic beads to selectively bind DNA (Promega). Purified DNA was quantified using the Picogreen method, normalised to a concentration of 5 mg/ml and distributed to the different research partners. The 450 accessions of ERGC and the 200 accessions of ERCC were genotyped with 26 independent SSR markers and with 62 SSR linked to salinity tolerance genes or/and QTLs, respectively, using the protocol of Risterucci et al. (2000) with the automated infrared fluorescence technology of LICOR IR2 sequencers at Cirad genotyping and robotics platform (Montpellier, France). In ITQB (Oeiras, Portugal), the 200 ERCC accessions were genotyped using a gel-based system (Double Wide Mini-Vertical Gel unit (16 × 33 cm), from CBS Scientific, USA). The products were detected using 6% polyacrylamide gels in 1× Tris-Borate EDTA buffer. After electrophoresis, the gels were stained with ethidium bromide solution and photographed under UV light using Gel-Doc 1000 (Biorad, USA). The molecular size of the amplification products was estimated using a 25 bp ladder (Invitrogen, USA). Differences in molecular weight of products amplified from SSR markers were manually measured using image analysis software Gene Tools (Syngene, UK). SNP genotyping was performed at PTPF Rice Genomics Unit (Lodi, Italy) using the *Illumina* Golden Gate assay with BeadXpress readout. In addition to the 124 SNPs in candidate genes, the ERCC was also genotyped with 66 independent (minimum distance of 1 Mb) SNPs not linked to known salinity candidate genes or QTLs. The genotypic data per accession are available http://eurigen.cirad.fr

## Analysis of phenotypic data

A linear mixed model (genotype, treatment and genotype × treatment interaction as fixed effects; replication and other interactions as random effects) was used under SAS software to compute adjusted means for each measured phenotypic trait and to test the effect of salinity stress and variety × salinity interaction. Principal Component Analysis (PCA) was performed on computed responses to salinity variables (*X*_r) at 38 DAS, using the Pearson matrix of correlation under XLSTAT software. Ascendant Hierarchical Classification (AHC) was performed on the same variables, using a Euclidian dissimilarity matrix and the Ward method under XLSTAT software.

## Population structure

The structure of ERCC was investigated using the genotypic data of the 200 accessions at 66 independent SNPs under "admixture" hypothesis with STRUCTURE software

(Pritchard et al. 2000). The "haploid" option was used to deal with the Hardy Weinberg equilibrium hypothesis. The Monte Carlo Markov chain was run 10 times (with 50,000 burn-in periods and 500,000 iterations) for each of the 1 to 10 options of the number of subpopulations ($Q$) tested. The optimal number of subpopulations was established using the *ad hoc* statistic $\Delta Q$ based on the rate of change in the log probability of data between successive $Q$ values (Evanno et al. 2005).

### Kinship

Kinship among the 200 ERCC accessions was calculated using their genotypic data at 26 independent SSR loci under the "Kin" function of Tassel software (http://www.maize-genetics.net/bioinformatics/tassel/).

### Association analysis

Per accession phenotypic and genotypic data used for association analysis are available at http://eurigen.cirad.fr. Using Tassel, we tested associations between markers and response to salinity variables under three statistical models: (1) a General Linear Model (GLM_$Q$), with the allelic status at the target loci considered as a fixed effect and the matrix of subpopulation membership ($Q$ matrix) used as cofactor to account for population structure; (2) a Mixed Linear Model (MLM_ $K$) in which, in addition to the fixed effects of segregating markers, a multiple background QTLs effect is estimated using the kinship matrix ($K$) and is incorporated as a random effect; (3) a MLM_$K$ + $Q$ model, where in addition to $K$ matrix, false associations are partially corrected using $Q$ matrix.

In order to reduce data unbalance when testing the effect of each marker, SNP markers with rare alleles (frequency < 5%) and individuals carrying the rare alleles (frequency < 5%) of SSR markers were excluded. This brought the final number of tested SNPs to 80 (corresponding to 41 candidate genes). To provide a test of significance that corresponds to experiment-wise error and corrects for multiple comparisons, 1,000 permutations were performed for the calculation of the $p$ value of the $F$ test and the significance threshold of the association tests was fixed at 0.005 for *GLM_Q* and 0.01 for MLM analysis.

## Results

### Genetic diversity for response to salinity stress in the European Rice Core Collection

In Exp1, salinity stress applied between 4 and 16 DAS significantly affected leaf chlorophyll content (LCC_r) and shoot biomass (SDW16_r) of ERCC accessions. Significant differences were also observed in the salinity injury score (SIS). In Exp2, highly significant differences were observed in rice growth and development (PH, TN, LN, MRL), and biomass production (RDW and SDW) between control and salt-stress treatments applied between 24 and 38 DAS. The interaction between genotypes and salinity treatments was highly significant for almost all traits, confirming the existence of genetic diversity in response to salinity among the ERCC accessions (Table 1). Significant varietal differences were also observed for the computed response variables, PH_r, TN_r, LN_r, MRL_r, RDW_r, and SDW_r (Table S4). Likewise, salt stress significantly increased the leaf concentration of $Na^+$ ions (mean increase of 800%) and decreased the leaf concentration of $K^+$ ions (mean decrease of 39%). The correlation between developmental variables and ionic variables was low ($r < |0.3|$) but highly significant ($p < 0.01$) (data not shown). Correlations between variables observed in Exp1 and Exp2 were loose and not significant, except for TN_r and SDW16_r ($r = 0.31$).

**Table 1** Effects of salinity treatment on growth, development and $Na^+$ and $K^+$ ion concentration of the 200 accessions of the European rice core collection (ERCC)

| | Sources of variation | | |
|---|---|---|---|
| | Accession (A) | Salinity treatment (ST) | Interaction A × ST |
| | df = 199 | df = 1 | df = 199 |
| PH | <0.0001*** | <0.0001*** | <0.0001*** |
| TN | 0.0088** | 0.0003*** | 0.2297 |
| LN | 0.0017** | 0.0009*** | 0.0008*** |
| MRL | <0.0001*** | 0.5928 | 0.0074** |
| SDW | 0.0221* | 0.0016** | <0.0001*** |
| RDW | <0.0001*** | <0.0001*** | 0.0008*** |
| RDW/SDW | 0.0303* | 0.2935 | 0.6701 |
| $K^+$ | 0.0691 | <0.0001*** | <0.0001*** |
| $Na^+$ | 0.0104* | <0.0001*** | <0.0001*** |
| $Na^+/K^+$ | 0.0012** | <0.0001*** | <0.0001*** |

*PH* plant height, *TN* tiller number, *LN* leaf number on the main tiller, *MRL* maximum root length, *RDW* root dry weight, *SDW* shoot dry weight, *df* degree of freedom

The computed response variables were subjected to Principal Component Analysis to reduce the complexity of correlations between variables and the high dimensionality of the data into more simple, linearized axes while retaining as much of the original variations as possible. The method summarises the phenotypes by combining correlated traits into single PCA indices. When data from Exp1 and Exp2 were analysed together, the first three axes of the PCA cumulatively explained 58% of the salinity response traits. When data from Exp2 were analysed alone, the cumulative percentage of variation explained reached 75%. In this case, the coordinates of the accessions on the plan of the

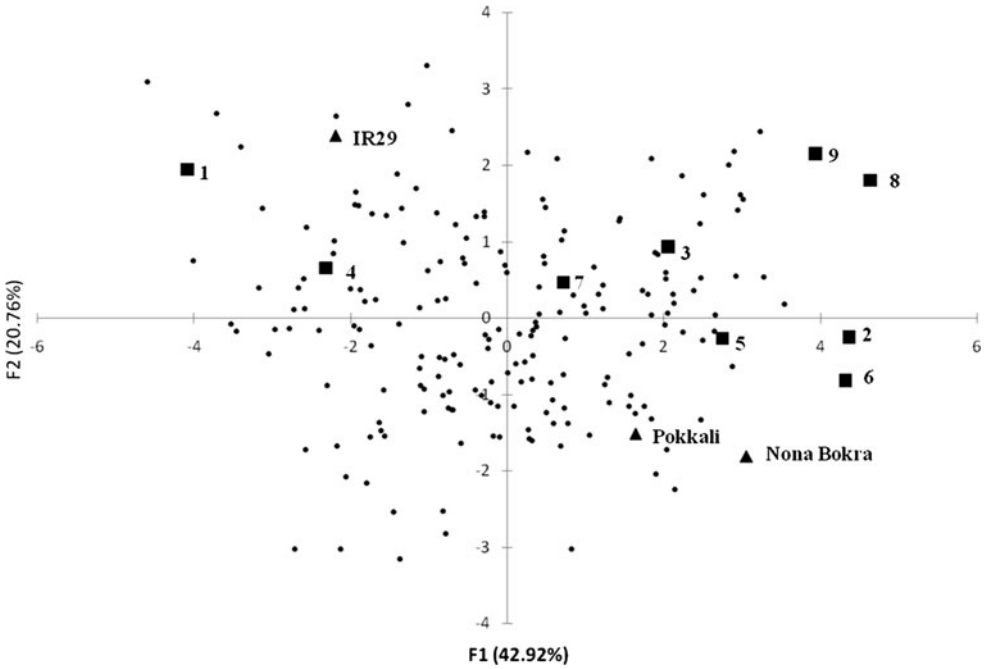


**Fig. 1** Projection of the 200 accessions of the European Rice Core Collection (ERCC) on the plane of the two first axes of the Principal Component Analysis using eight traits associated with response to salinity. Nona Bokra and Pokkali (tolerant check); IR29 (susceptible check), 1–9: respectively, YRL-196, Honduras, Beirão, Plovdiv 22, Slava, Gigante Vercelli, Kulon, CT 36, King

first two axes of the PCA summarised most of the response to salinity of the accessions, as illustrated by the coordinates of the susceptible check IR29 and the tolerant checks Nona Bokra and Pokkali (Fig. 1). Response variables related to growth and development were the major contributors to axis 1 of the PCA, while axis 2 was mainly defined by $Na^+$ and $K^+$ concentrations. The coordinates on the first three axes of the PCA on data from Exp2 were used for the search for associations between phenotypic data and candidate genes. Using the Hierarchical Ascendant Classification, we were able to identify three groups of responses to salinity. Membership of these three groups was also used for the association search.

## Population structure, kinship and linkage disequilibrium

Based on SNP data, the highest likelihood score was observed for three subpopulations. Thanks to the presence of reference accessions, it was possible to assign the three subpopulations to well-known *O. sativa* genetic groups. Subpopulation SP1, which accounted for 63% of the total number of accessions, grouped the typical temperate *japonica* varieties originating from a large range of geographical areas. SP2, which accounted for 23% of the accessions, grouped mainly (70%) temperate *japonica* originating from the American continent (USA and Argentina). SP3 grouped a small number of accessions (4% of the total) belonging to the *indica* sub-species. The remaining 10% were admixed (less than 70% of the genome could be inferred to one of the three subpopulations) mainly between SP1 and SP2 (Table S1). SP1, SP2 and SP3 had a $F_{ST}$ of 0.111, 0.172 and 0.277, respectively. The $F_{ST}$ across the three subpopulations was equal to 0.501, indicating high differentiation: 0.484 for SP1–SP2, 0.801 for SP1-SP3 and 0.684 for SP2-SP3. In this context, we concluded that the ERCC structure was well captured with 66 unlinked SNPs and that we could use membership of a subpopulation as a covariate to account for global structure.

A total of 179 alleles were detected within the ERCC for the 26 independent SSR loci and the $r^2$ parameter of pairwise linkage disequilibrium (LD) estimates among the 26 SSR loci varied from 0.00 to 0.289 with only 8% of $r^2$ values higher than 0.1. Given the higher number of alleles detected with the 26 SSR markers than with the 66 SNPs, we concluded that SSR data were the most relevant for kinship estimation and should be used as random factors in the mixed model for association analysis. The pairwise kinship between the 200 accessions estimated with the SSR data averaged 0.737.

## Association between candidate genes and phenotypic response to salinity stress

Significant associations between markers and phenotypic response to salinity were detected with all three methods of analysis, *GLM_Q*, MLM_*Q* and MLM_*Q* + *K* (Table S5). The significant associations detected with the *GLM_Q* and MLM_*Q* + *K* models were almost identical. The MLM_*Q* model proved to be less stringent; it detected almost all significant associations detected under *GLM_Q* and MLM_*Q* + *K*, plus some new ones that had a *p*-value slightly above the significance threshold of the *GLM_Q*

models. We decided to consider all associations with *p* value below the significance threshold of at least one of the three models.

A total of 15 SNP markers out of 80, representing 10 candidate genes out of the 41 targeted initially, showed significant associations with one or more phenotypic responses to salinity. The 15 SNPs included five cases of two or three tightly linked SNPs revealing the same associations (Table S5). Likewise, nine SSR markers out of 52, representing nine candidate genes/QTLs out of the 22 targeted initially, showed significant associations with one or more phenotypic responses to salinity. Thus, 19 distinct loci significantly associated with one or more salinity response traits were detected. The location on the rice chromosomes of these 19 loci and of the other candidate genes and QTLs for salinity tolerance targeted in this study is given in supplementary figure S1.

All phenotypic traits considered, except LCC_r and SDW16_r, were significantly associated with at least one SNP or SSR marker representing a candidate gene or a QTL (Table 2). RDW_r was associated with five distinct loci, while only one locus was detected for other traits related to growth and development (PH_r, LN_r, TN_r and SDW_r). All these loci had distinct chromosomal locations. Four significant associations were detected for $Na^+$ concentration, of which three were also significant for the $Na^+/K^+$ ratio. Among the synthetic traits derived from multivariate analysis, the highest number of associations was detected for PCA-2, while no association was detected for PCA-1 and only one was detected for PCA-3 and HAC. Two PCA-2 associations co-localised with $Na^+$ and one other with HP_r. Interestingly, $Na^+$ and HP_r were the major contributors to axis 2 of the PCA. Overall, few loci associated with ionic concentrations co-localised with loci associated with traits related to growth and development, suggesting distinct response mechanisms and regulation pathways. The absence of significant association for LCC_r and SDW16_r is probably due to the fact that only 140 accessions out of 200 have been phenotyped for these two traits".

Among the 19 distinct loci significantly associated with one or more salinity response traits, the $r^2$ parameter of pairwise LD varied from 0.000 to 0.379 with only 8% of $r^2$ values higher than 0.1. These results suggest random distribution of the favourable alleles of these loci among the ERCC accessions. Comparison of these favourable alleles with the alleles of the *indica* tolerant variety Nona Bokra at the same loci showed loose similarity (Table 3). The favourable alleles of the ERCC were identical to those of Nona Bokra at only one significant SNP locus out of ten and at only three significant SSR loci out of nine. The SNP locus was associated with SIS and the three SSR loci were associated with a wide range of traits including ion concentration, and growth and development traits. This loose genotypic similarity with Nona Bokra at significant loci suggests the possibility of achieving similar levels of tolerance to salinity with different sets of loci playing the determining role. However, it should be underlined that these results were obtained mainly under the moderate salinity stress (6 dS $m^{-1}$) of Exp2. The absence of Nona Bokra from Exp1, *does not allow to conclude for more severe salinity stress*.

The percentage of variation of a given trait explained by each associated marker was low, 7% on average, and did not exceed 13%. High standard deviations were observed for the phenotypic effect of each allele and, therefore, the genotype at significant loci could not accurately predict the associated phenotypic responses to salinity. Conversely, the genotype at significant loci could be better predicted for the extreme values of the associated phenotypic response, i.e. the 5th and 95th percentiles of the distribution (the 5% lowest and the 5% highest values) of phenotypic responses. No accession carried the favourable allele at all significant loci simultaneously, or had the highest value for all phenotypic traits measured. At least nine accessions were needed to assemble the favourable alleles for the 11 phenotypic traits and the highest phenotypic values of the traits (Table 3).

The localisation of the significant loci detected for each trait was in good agreement with the corresponding QTL information reported in the literature. For instance, the HP_r loci co-localised with QTLs involved in relative biomass or relative seedling vigour (Table 2); the LN_r loci co-localised with salt tolerance and shoot length QTLs; the SIS loci co-localised with QTLs involved in "days from seedling to death" and "shoot length" on chromosome 1 and with "tiller number" QTLs on chromosome 2. Likewise, the $K^+$ locus on chromosome 4 co-localised with a QTL involved in root $K^+$ concentration and the $Na^+$ loci on chromosome 9 with a QTL for root $Na^+$ concentration and $K^+$ uptake.

The significant associations identified covered a wide range of gene families and adaptation mechanisms, some of which are already well documented (Table 2). For instance, the $K^+$ locus on chromosome 4 corresponds to *OsHAK*1, which belongs to a gene family involved in cation transport in the roots; the $Na^+$ locus on chromosome 6 corresponds to *OsSOS*2 (*OsCIPK*24), belonging to a gene family involved in $Na^+$ extrusion from the cells and in long-distance transport; the $Na^+$ locus on chromosome 9 corresponds to a gene from the DREB family, which is a drought and salt-stress-inducible transcriptional activator; the RDW_r and SDW_r loci on chromosome 1 correspond to *OsHKT*8, which is involved in $Na^+$ transport and uptake into cells; the TN_r on chromosome 6 corresponds to *OsNHX*1 gene from the NHX family, $Na^+/H^+$ antiporters which confer high tissue tolerance through $Na^+$ sequestration in vacuoles; and the SIS locus on chromosome 1 corresponds to an ATP-binding

**Table 2** Significant associations identified between traits related to tolerance to salinity and markers targeting salt tolerance QTLs or candidate genes related to salt tolerance

| Type of Marker | Locus | Chr | Position | PH_r | LN_r | TN_r | RDW_r | SDW_r | BDW_r | SIS | $Na^+$ | $K^+$ | $Na^+/K^+$ | PCA_2 | PCA_3 | HAC | Target QTL | Target gene | Description | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP | Os01g07870 | 1 | 3 806 502 | | | | | | | abc | | | | | | | | | ABC transporter | Senadheera et al (2009) |
| SSR | RM572 | 1 | 9 863 371 | | | | b | | | | | | | | | | KCS, KCR, NUP, Na/K | OsGSK1 | Glycogen synthase kinase | Koh et al. (2007) |
| SNP | Os01g20160 | 1 | 11 457 944 | | | | b | b | | | | | | | | | SKC1 | OsHKT8 | Cation transporter | Ren et al (2005) |
| SNP | Os01g62410 | 1 | 36 126 568 | | | | | | | ac | | | | | | | DSD, SLG, NQR | OsMYB3R-2/ | Intracellular $Na^+$ and $K^+$ homeostasis | Dai et al. (2007) |
| SNP | Os02g02410 | 2 | 839 986 | | abc | | | | | | | | | | | | | | Dnak-type molecular chaperone Bip | Le Quang et al. (2008) |
| SNP | Os02g38920 | 2 | 23 524 580 | | | | | | | abc | | | | | | | | | Glyceraldehyde-3-phosphate dehydrogenase | Le Quang et al. (2008) |
| SSR | Os02g42290 | 2 | 25 381 091 | | | | b | | | | | | | | | | TN | OsCLP3 | ATP-dependent Clp protease proteolytic subunit | Kumari et al. (2009) |
| SSR | RM450 | 2 | 28 628 206 | | | | | | | abc | abc | abc | abc | abc | | | TN | Os02g50680 | AAA-type ATPase family protein, putative, expressed | Le Quang et al. (2008) |
| SNP | Os04g32920 | 4 | 19 715 224 | | | | b | | | | | ab | | | | | KCR | OsHAK1 | $K^+$ transporter | Okada et al. (2008) |
| SSR | RM177 | 4 | 22 379 620 | | | | | abc | | | | | | | | | KCR | Os04g40630 | BTBZ4; Transcription factor | Le Quang et al. (2008) |
| SNP | Os05g05270 | 5 | 2 598 496 | | | | | | | | abc | | abc | abc | | | | OsLti6b | Putative membrane protein | Kim et al. (2007) |
| SSR | RM31 | 5 | 28 590 085 | | | | | | | | | | | | | ab | | SOS3 | Activation of the membrane-bound $Na^+/H^+$ antiporter SOS1 | Liu et al. (2000), Martinez-Atienza et al. (2007) |
| SSR | Os06g40370 | 6 | 23 810 742 | | | | | | | | abc | | abc | | | | DSD, RSRL | SOS2 | | |
| SSR | Os06g40370 | 6 | 24 035 433 | | | | | | | | | | | | b | | DSD, RSRL | SOS2 | | |
| SSR | Os06g48810 | 6 | 29 341 541 | abc | | | | | | | | | | abc | | | RSRL, SBM, RSVG | OsHKT1, OsHKT2 | Cation transporter | Horie et al. (2001), Horie et al. (2007) |
| SNP | Os09g07300 | 9 | 3 599 600 | | | | b | | | | | | | | | | | Os09g07300 | BIG, putative expressed | Le Quang et al. (2008) |
| SNP | Os09g11450 | 9 | 6 379 927 | | | ac | | | | | | | | | | | TN | OsNHX5 | $Na^+$ and $K^+$ compartmentation | Fukuda et al. (2010) |
| SNP | Os09g17740 | 9 | 10 846 121 | | | abc | | | | | | | | | | | | Os09g17740 | Chlorophyll a-b binding protein 1 | Le Quang et al. (2008) |
| SSR | RM410 | 9 | 17 642 699 | | | | | | | | abc | | | | | | NCR, KUP | OsDREB1A | Transcription factor | Dubouzet et al. (2003) |

*a*, *b* and *c* significant association under KLM, MLM_*K* and MLM_*K*+*Q* models, respectively. *Chr* chromosome, *Position* physical position according to TIGRv5, *PH_r* plant height response, *TN_r* tiller number response, *LN_r* leaf number response, *MRL_r* maximum root length response, *RDW_r* root dry weight response, *SDW_r* shoot dry weight response, *PCA_2* and *PCA_3* coordinate of second and third axis of Principal Component Analysis, *HAC* class of hierarchical ascendant classification. *KCS* $K^+$ concentration in shoots, *KCR* $K^+$ concentration in roots, *NCR* $Na^+$ concentration in roots, *TN* tiller number, *RSRL* relative seminal root length, *RBM* relative biomass, *RSVG* relative seedling vigour, *DSD* days from seedling to death, *SLG* shoot length, *KUP* $K^+$ uptake, *Na/K* $Na^+/K^+$ ratio

**Table 3** Genotype of nine rice accessions at 19 loci significantly associated with one or more salinity tolerance traits and phenotype of the same accessions for 13 traits related to response to salinity

| | Genotype at loci significantly associated with one or more tolerance traits | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chromosome | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 4 | 4 | 5 | 5 | 6 | 6 | 6 | 9 | 9 | 9 | 9 |
| Accessions | Os01g 07870 | RM572 | Os01g 20160 | Os01g 62410 | Os02g 02410 | Os02g 38920 | Os02g 42290 | RM450 | Os04g 32920 | RM177 | Os05g 05270 | RM31 | Os06g 40370 | Os06g 40370 | Os06g 48810 | Os09g 07300 | Os09g 11450 | Os09g 17740 | RM410 |
| YRL-196 | 3 | 180 | 1 | 2 | 2 | 2 | 255 | 160 | 1 | 214 | 1 | 142 | 175 | 198 | 174 | 2 | 1 | 2 | 206 |
| Honduras | 3 | 180 | 1 | 2 | 2 | na | 253 | 158 | 2 | 212 | 2 | 138 | 180 | 200 | 177 | 1 | 1 | 1 | 202 |
| Beirão | 3 | 180 | 2 | 2 | 2 | 2 | 241 | 158 | 1 | na | 2 | 144 | 180 | 200 | 180 | 2 | 1 | 1 | 202 |
| Plovdiv 22 | 3 | 180 | 2 | 2 | 1 | 2 | 233 | 162 | 2 | 214 | 2 | 144 | 180 | 180 | 177 | 2 | 1 | 1 | 206 |
| Slava | 3 | 180 | 2 | 2 | 2 | 2 | 255 | 162 | 1 | 212 | 1 | 142 | 175 | 180 | 183 | 2 | 1 | 1 | 206 |
| Gigante Vercelli | 3 | 180 | 1 | 2 | 2 | 2 | 255 | 160 | 1 | 212 | 1 | 142 | 180 | na | 180 | 2 | 1 | 1 | 208 |
| Kulon | 3 | 180 | 1 | 2 | 2 | na | 257 | 158 | 2 | 212 | 1 | 138 | na | 198 | 177 | 1 | 1 | 1 | 202 |
| CT 36 | 3 | 178 | 1 | 1 | 2 | 2 | 255 | 162 | 2 | 214 | 2 | 142 | 175 | na | 177 | 2 | 1 | 1 | 208 |
| King | 3 | 178 | 1 | 2 | 1 | na | 253 | 156 | 2 | 214 | 1 | 138 | 175 | 198 | 180 | 1 | 1 | 1 | 206 |
| Nona Bokra | 3 | 200 | 2 | 1 | 1 | 1 | 253 | 158 | 1 | 212 | 1 | 132 | 164 | 172 | 174 | 2 | 2 | 1 | na |
| Pokkali | 3 | 164 | 2 | 1 | 1 | 1 | 253 | 154 | na | 212 | 1 | 138 | 164 | 172 | 174 | 2 | 2 | 1 | na |

| Chromosome | Phenotype for response to salinity | | | | | | | | | | | | | | | Subpopulation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Accessions | $K^+$ | $Na^+$ | $Na^+/K^+$ | PH_r | TN_r | LN_r | RDW_r | SDW_r | PCA_1 | PCA_2 | PCA_3 | HAC | SIS | LCC | SDW16_r | |
| YRL-196 | 2.1 | 1.6 | 0.8 | −18.7 | −15.0 | −18.2 | −32.6 | −46.8 | −4.1 | 2.0 | −2.2 | 2.0 | 7.7 | −36 | −62 | 1 |
| Honduras | 2.8 | 0.7 | 0.2 | −17.6 | 18.7 | −1.3 | 6.6 | 7.8 | 4.4 | −0.2 | −2.0 | 3.0 | 8.0 | −42 | −45 | 2 |
| Beirão | 2.1 | 0.8 | 0.4 | −7.4 | −27.4 | 0.2 | −12.6 | 6.7 | 1.9 | 0.8 | 1.7 | 1.0 | 8.0 | −37 | −24 | 1 |
| Plovdiv 22 | 2.0 | 1.1 | 0.6 | −20.6 | −28.6 | 7.7 | −39.2 | −47.0 | −2.3 | 0.7 | 2.2 | 2.0 | 8.2 | −63 | −49 | 1 |
| Slava | 2.0 | 0.4 | 0.2 | −10.1 | −7.7 | 7.1 | −19.0 | −3.0 | 2.7 | −0.3 | 2.1 | 3.0 | 9.0 | −52 | −48 | 1 |
| Gigante Vercelli | 2.7 | 0.5 | 0.2 | −13.7 | 17.2 | 13.6 | −23.2 | −4.0 | 4.3 | −0.8 | 0.4 | 3.0 | 8.7 | −54 | −41 | 1 |
| Kulon | 2.3 | 0.9 | 0.4 | −11.3 | −7.7 | −3.0 | −22.7 | −18.7 | 0.7 | 0.5 | 0.0 | 1.0 | 3.4 | na | na | 1 |
| CT 36 | 2.4 | 0.7 | 0.3 | −2.7 | 28.1 | 0.5 | 12.0 | 5.7 | 4.6 | 1.8 | −1.0 | 3.0 | 5.1 | −58 | −12 | m |
| King | 2.3 | 0.8 | 0.4 | −8.0 | −1.5 | 8.3 | 48.1 | −18.2 | 3.9 | 2.2 | 0.7 | 3.0 | 8.9 | na | na | 2 |
| Nona Bokra | 2.7 | 0.5 | 0.2 | −7.8 | −11.8 | −2.6 | −19.5 | −14.7 | 3.1 | −1.8 | 0.1 | 1.0 | na | na | na | na |
| Pokkali | 2.5 | 0.7 | 0.3 | −16.7 | −15.4 | −5.2 | −29.2 | −17.8 | 1.6 | −1.5 | −0.4 | 1.0 | na | na | na | na |

At least nine accessions were required to assemble the favourable alleles for the 11 phenotypic traits and the best phenotypic value (among the 5% most favourable responses for tolerance to salinity)

The genotypes at SNP loci are indicated by the numbers 1 and 2. The genotypes at SSR loci are shown with the size of the alleles. The favourable allele at each locus is in grey, *na* not available

The phenotypic response to salinity: $X\_r = ((X\_s - X\_c) \times 100)/X\_c$, were $X\_c$ the variable $X$ under control treatment and $X\_s$ the same variable under salinity stress. *PH_r* plant height response, *TN_r* tiller number response, *LN_r* leaf number response, *MRL_r* maximum root length response, *RDW_r* root dry weight response, *SDW_r* shoot dry weight response, $K^+$ and $N^+$ leaf concentration of $K^+$ and $N^+$, *PCA_1*, *PCA_2* and *PCA_3* coordinates on the first, second and third axes of the Principal Component Analysis, *HAC* Class of hierarchical ascendant classification, *SIS* salinity injury score, *LCC_r* leaf chlorophyll content response, *SDW16_r* shoot dry weight response at 16 days after sowing. Subpopulation: *1*, *2*, *3* and *m* membership of temperate *japonica*, temperate *japonica* from American continent, *indica* and admixed, *na* not available

cassette (ABC) transporter, which translocates a wide range of substrates across extra- and intracellular membranes. The less well-known genes were those differentially expressed under salinity stress during the vegetative stage.

## Discussion

The aim of this study was to provide European rice breeding programmes with the best performing genes and alleles for tolerance to salinity, and to identify donors and molecular makers to accelerate the breeding process. The genetic basis of tolerance to salinity in rice is widely documented (for a detailed review see Negrão et al. 2011) through bi-parental QTL analysis (Prasad et al. 1999; Koyama et al. 2001; Flowers et al. 2000; Lin et al. 2004; Takehisa et al. 2004), differential transcriptome analysis (Walia et al. 2005, 2007; Kumari et al. 2009; Senadheera et al. 2009) and using comparative genomics approaches (reviewed by Sahi et al. 2006). The *SKC1* QTL, which has a major effect on $K^+$ concentration, was cloned and identified as the sodium transporter *OsHTK8* (Ren et al. 2005). However, almost all these studies used *indica* varieties as the source of salt tolerance. Within the *indica* subspecies, the available variation for component traits of tolerance to salinity is scattered among several varieties (Yeo et al. 1990), and little is known about the genetic diversity for tolerance to salinity among *japonica* subspecies. Given the difficulties in harnessing crosses between the two rice subspecies (Oka 1983), especially for the improvement of complex polygenic traits, and since there are very specific varietal requirements for European rice cropping systems in terms of grain quality and adaptation to climatic constraints, we decided to first explore the potential of the ERGC, composed mainly of *japonica* accessions, for the improvement of salinity tolerance. In this context, analysing the phenotypic diversity for tolerance to salinity within the ERGC and looking for correlations between the phenotypic performance of individuals and their genotype at loci known to be involved in tolerance mechanisms, seemed the most straightforward approach to detect markers and donors. The advantages of using this association mapping approach in plants were first described by Jannink et al. (2001). In particular, information derived from association analysis can be directly used in breeding programmes provided the experimental population is representative of the population for which inference is desired. This is exactly the case of ERGC and the European breeding programmes. However, the presence of closely related accessions within the experimental population could inflate the rate of false positive detection. The ERGC includes a large number of elite accessions representing the expansion of a rather small number of founders. Although we did not have exhaustive pedigree information for the ERGC, we were able to reduce the number of closely related accessions by discarding the most similar ones when establishing the ERCC. As a result, significant LD within the ERCC was not observed either among the 26 independent SSR markers or between the 66 SNPs, except for 2 couples of SNP separated by less than 2 Mb.

### Selection of candidate genes and QTLs

Once the general approach had been decided, the next step was the selection of the relevant candidate targets, with at least two options: (a) focusing on a small number of genes known for their high phenotypic effects, and looking for causative polymorphism through resequencing (usually the case in candidate gene association mapping) (Zhu et al. 2008); (b) or targeting a large number of loci, either QTLs or candidate genes, and looking for the most relevant candidates. Given the large number of QTLs and candidate genes with rather small individual effects on rice tolerance to salinity reported in the literature, we chose the second option with the aim of identifying the most relevant candidates within *japonica* subspecies. This decision was justified by the fact that the average extent of LD within the *japonica* subspecies is estimated at about 500 kb (Mather et al. 2007), offering a reasonable probability of identifying significant associations with a small number of SNPs within each candidate gene. The first round of selection of candidate genes was strongly influenced by the existence of polymorphic SNP within each gene among the seven *japonica* accessions of OryzaSNP database while responding to the requirement of the SNP genotyping technology. This skewed selection was rectified by the remaining candidate genes co-localising with salt tolerance QTLs through the use of SSR markers. The density of one SSR every 250 kb along the confidence interval of the QTL was consistent with LD decay; however, the use of SSR also faced the problem of limited polymorphism within the ERCC. An additional limitation for the detection of associations arose from either a very high number of alleles (>10) and/or the presence of low-frequency alleles (<5%). A larger collection would both increase detection power and allow quantification of the effect of low-frequency SNP and SSR alleles. However, increasing the size of the collection would also make it less manageable for phenotyping and could increase the number of related accessions. Thus, the selection of the candidate genes and QTLs undergoes the funnel effect of availability of polymorphic markers within the extent of the LD. Despite this constraint, association analysis was possible for the most important candidate genes and QTLs.

## Association analysis

The most important risk in association analysis is an inflated number of false positives due to unaccounted subdivisions in the population, creating covariance among individuals and leading to biases in the estimation of allele effects. We explicitly included the population membership in the *GLM_Q* model. Moreover, we estimated the pairwise relatedness coefficient or kinship (*K*-matrix) and included the combined information of *K* and *Q* in the MLM model of analysis. Working with a sample of 277 diverse maize inbred lines with complex family relationships and population structure, Yu et al. (2006) demonstrated improved control of both type I and type II error rates when the *K* and *Q* information was combined in the mixed model. Zhao et al. (2007) extensively tested the MLM approach of Yu et al. (2006) in their global set of 95 highly structured *Arabidopsis* accessions and concluded that the $K + Q$ MLM model performed better than any other model which used *K* or *Q* matrices alone. In our case, the MLM_$K + Q$ model detected two additional associations ($P < 0.01$) which were not detected with the MLM_*K* model ($P < 0.005$). However it did not reject any association detected with the latter model, suggesting the absence of false positives.

The MLM_*K* model inflated the number of significant associations compared to the MLM_$K + Q$ model (31 vs. 23) while it did not detect three significant associations detected with the latter model. This is consistent with the result of simulations performed by Zhao et al. (2007) and suggests that *K* and *Q* capture different levels of relatedness. In addition, we found that the phenotypic covariance between individuals is not directly proportional to their relative relatedness and the random effect of kinship does not account for the membership of a subpopulation. This difference appears to be so important that it remains true even when kinship is estimated rather roughly. Indeed, (a) we did not estimate kinship as the fraction of shared genome identical by descent between related individuals, but instead as identical in state between unrelated individuals, (b) we used a rather small number of SSR markers compared to the "several hundreds" recommended by Lynch and Ritland (1999) and, (c) given the high mutation rate of SSR markers and homoplasy phenomenon, identity in state does not imply identity by descent. *K* and *Q* capture different levels of relatedness, probably because the ERCC includes a rather large number of elite lines, resulting from the expansion of a few founders.

Given the differences between the results obtained with different methods of analysis, which include (or not) membership of the subpopulation (GLM_*Q* and MLM_$K + Q$ versus MLM_*K*), the most relevant criterion for the selection of associations worthy of further study was consistency across methods. However, we instead decided to consider all loci revealed to be significant using one or more methods. This conservative decision was justified by the fact that: (a) additional associations detected by MLM_*K* may not be false positives (indeed it can be argued that the causal polymorphisms of these loci were more tightly correlated with the underlying population structure, and removing the latter with the *Q* matrix would also remove the former). One example of such a phenomenon is the case of the three SNP loci on chromosome 4: the frequency of the favourable alleles at these loci is clearly related to membership of a subpopulation (0% in SP3, 36% in SP1 and 95% in SP2) and the association between these loci and $K^+$ ion concentration was significant only under the MLM_*K* model; (b) for almost all associations which were significant only under the MLM_*K* model, the *p* values were close to the threshold of significance under the other models, or the same loci were associated with several phenotypic traits and, in one case, three loci with very tight genetic linkage were involved.

A total of 19 distinct loci significantly associated with one or more phenotypic traits were detected. The absence of LD between these loci, indicating random distribution of the favourable alleles among the ERCC, suggests that the material has undergone little selection pressure for tolerance to salinity and that there is thus high potential for the improvement of tolerance to salinity by pyramiding the favourable alleles at different loci. However, during the first step in the process (choice of donors), the same importance should be given to phenotypic and genotypic information because the marker alleles are correlated with phenotypic performance but are not entirely predictive of the phenotype.

## Mechanisms of tolerance to salinity

Like in other plants, rice tolerance to salinity stress is based on mechanisms that prevent salt accumulating in the cytoplasm (Yeo 1998; Ismail et al. 2007; Munns and Tester 2008). These mechanisms include (a) control of salt uptake and salt exclusion from growing tissues, (b) tissue tolerance through efficient compartmentalization of $Na^+$ in the vacuoles or in particular cell types where the damage to metabolism is kept to a minimum and (c) osmotic tolerance or ability to cope with low water potential and with toxic molecules.

Control of salt uptake relies first on rapid stomatal closure in response to an increase in salt concentration in the root medium, in order to limit the passive uptake of $Na^+$ via the transpirational apoplastic bypass flow. Moradi and Ismail (2007) reported a faster stomatal response in salt-tolerant rice varieties than in susceptible varieties, leading to a lower $Na^+$ concentration in their leaves, with plants gradually recovering their stomatal conductance within a few

days. We did not phenotype the ERCC for this early response to salinity.

The second mechanism of control of salt uptake is the impediment of $Na^+$ influx into the root system, either passively or involving active pathways. Large genetic variability for $Na^+$ uptake and accumulation in the roots and/or shoots has been reported (Yeo and Flowers 1986; Yeo et al. 1990; Moradi et al. 2003). Our data confirmed the existence of this variability within the ERCC. However, the specific properties of root cellular membranes that control $Na^+$ influx are poorly understood. Membrane transporter gene families, particularly the $K^+$ transporter (HKT) family have been shown to play important roles in $Na^+$ and $K^+$ uptake and homeostasis in *Arabidopsis* (Uozumi et al. 2000; Rus et al. 2004) and rice (Golldack et al. 2003; Horie et al. 2007). For instance *OsHKT8* was identified as the causal gene of the rice QTL *SKC1* (Ren et al. 2005) responsible for $Na^+$ concentration in rice leaves, and *AtHKT1* was found to be involved in $Na^+$ recirculation from shoots to roots (Berthomieu et al. 2003) which removes large amounts of $Na^+$ from the shoot. Another important gene family involved in $K^+$ and $Na^+$ transport via roots and uptake into cells is the High-Affinity $K^+$ Transporters (HAK/KUP). By mediating the influx of multiple cations, especially $K^+$, this gene family prevents $Na^+$ influx at high $Na^+$ concentrations. Within the ERCC, no direct association was found between $Na^+$ concentration and the HKT gene family; however we detected an association between $K^+$ concentration and the *OsHAK1* gene on chromosome 4. As for HKT genes, *OsHKT1* and *OsHKT2* (located at the same locus) were associated with PCA-2 and PH_r, and *OsHKT8* with RDW_r and SDW_r. Interestingly, we found that $Na^+$ concentration was the main contributor to axis 2 of the PCA.

Tissue tolerance to salinity is the plant's ability to maintain growth and development processes, at least partially, in spite of abnormal salt concentrations in leaves. Genetic variability for tissue tolerance to salinity was reported in rice (Yeo and Flowers 1986; Yeo et al. 1990). The existence of such variability within the ERCC is illustrated by the shape of the cloud of accessions on the first plan of the PCA (Fig. 1): similar coordinates on axis 2, determined mainly by the $Na^+$ concentrations in leaves and by the $Na^+/K^+$ ratio, led to different coordinates on axis 1, mainly determined by LN_r, TN_r, RDW_r and SDW_r. Differences in tissue tolerance are attributed to mechanisms that re-establish cytoplasm ion homeostasis, mainly $Na^+/K^+$ equilibrium, for normal metabolism. The $Na^+$ ions are removed from the cytoplasm by either sequestrating $Na^+$ in vacuoles or rejecting it from the cell. Two gene families are reported to play an important role in these mechanisms. The $Na^+/H^+$ antiporter family (NHX), with *OsNHX*1 in rice, compartmentalizes excessive $Na^+$ in the vacuoles (Fukuda et al. 2004) and the Salt Overly Sensitive pathway (SOS), with *SOS*1, *SOS*2 and *SOS*3 rice genes, contributes to ion homeostasis by extruding $Na^+$ ions from the cell and by mediating their long-distance transport (Martinez-Atienza et al. 2007). The two gene families play significant roles in the ERCC responses to salinity stress. We found that locus *SOS*2 (chromosome 6) is strongly associated with $Na^+$ concentration and with the $Na^+/K^+$ ratio, while locus *SOS*3 (chromosome 5) is associated with HAC (a global score of salinity tolerance) and *OsNHX5* locus with TN_r (a parameter related to tillering maintenance despite high $Na^+$ concentrations in leaves).

Osmotic tolerance is related to the plant's ability to produce (a) organic solutes which disturb metabolism less than $Na^+$ ions and stabilize macromolecules under low water potential and (b) osmoprotectants or scavengers which protect the plant against oxidative damage due to excessive production of reactive oxygen species. The transcription factor family DREB is well known for controlling the expression of dehydration-inducible genes which encode either enzymes required for biosynthesis of various osmoprotectants, antifreeze, or detoxification proteins (Yamaguchi-Shinozaki and Shinozaki 1994; Mie et al. 1999). It has been reported that the expression of *OsDREB*1A and *OsDREB*1B is induced by cold, whereas the expression of *OsDREB*2A is induced by dehydration and high-salt stresses (Dubouzet *et al*. 2003). Within the ERCC, we detected a significant association between the *OsDREB*1A locus on chromosome 9 and the concentration of $Na^+$.

When screening rice genotypes for physiological characters contributing to tolerance to salinity in rice, Yeo et al. (1990) found that "there was no grouping of the resistance traits in single genotypes and the most resistant varieties (Nona Bokra and Pokkali) did not have high scores in more than two of the four traits considered", namely: vigour, shoot $Na^+$, tissue tolerance (estimated through relative chlorophyll concentration), and leaf to leaf compartmentalisation. These authors concluded that "the salinity resistance of rice could be increased above the resistance of Nona Bokra variety". Our data indicate that the three major categories of mechanisms of tolerance to salinity are present in the ERCC, although the useful level of expression of the different mechanisms is scattered among different accessions. We also found that under the moderate salinity stress some accessions achieve the same level of control of $Na^+$ concentration and $Na^+/K^+$ equilibrium as Nona Bokra and Pokkali without sharing alleles with these two varieties at several loci associated with $Na^+$ concentration. We do not know if they can achieve the same control under more severe stress and we do not know to what extent those allelic differences contribute to membership to the *japonica* or *indica* subspecies, but their existence suggests further potential for the improvement of tolerance to salinity above

the tolerance level of Nona Bokra, provided the underlying mechanisms are complementary at the whole plant level.

We identified the best performing genes and alleles for tolerance to salinity present in the ERCC, as well as the associated donors and molecular makers. Given the high number of loci and donors involved, an effective strategy for the accumulation of the favourable alleles into a unique line would be a marker-assisted population improvement approach relying on a male sterility gene for the recombination cycles (Courtois et al. 2005). On the other hand, the set of best performing loci could be investigated for causative SNPs through sequence comparisons between accessions with extreme (the 5% lowest and the 5% highest values) phenotypic responses and contrasting genotypes at the significant loci we identified.

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