Genome-Wide Association Study for Mid-Salt Tolerance in Rice

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ABSTRACT

Salt stress is one of the environmental constraints that affect crop cultivation worldwide, since more than 800 Mha of land throughout the world suffer from salinization problems. Among cereals, rice (*Oryza sativa* L.) is one of the most sensitive to salt stress, although cultivars can differ in their response to salinity. In Europe, due to scarce water availability and the rise in sea levels, there is a clear tendency toward salinization in the river deltas where rice is grown. Thus, the identification of rice cultivars tolerant to salt stress and the dissection of salt stress tolerance mechanisms are of high interest for European rice breeding. Plant response to salt stress is a complex trait, depending on the combination of many genes and metabolic pathways, and thus difficult to control and engineer. Exploiting natural variation occurring in worldwide genotypes may be a powerful approach to discover new traits to tolerate high salinity conditions. In this context, a phenotyping activity has been performed to study the natural variation of a worldwide *japonica* rice collection in response to mid-salt stress. A greenhouse experiment was carried out on 281 *japonica* rice cultivars subjected to salt stress and the measurement of physiological traits (*i.e.* seedlings emergence rate, plant growth, chlorophyll fluorescence, flowering delay) was assessed. A genome wide association study (GWAS) highlighted the presence of significant *loci* involved in salt tolerance. Analysis of candidate genes significantly associated to these *loci* is in progress.

Key words

Oryza sativa, salt stress, phenomics

1. Introduction

Soil salinity is one of the environmental constraints that affect crop cultivation worldwide. More than 800 Mha of land throughout the world and about 20% of the irrigated areas suffer from salinization problems (FAO, 2008). Among cereals, rice (*Oryza sativa* L.) is one of the most salt-sensitive although cultivars can differ in their response to salt stress (Horie et al., 2012). In European coastal rice areas, salty raining and the salt wedge intrusion phenomenon caused by the rise in the sea levels consequent to the ongoing climate changes are provoking a tendency toward salinization in the adjacent paddy fields where rice is grown.

Moreover, the island apple snail (previously called *Pomacea insularium* and now called *Pomacea maculata*) is becoming one of the major pests problems for rice throughout the world, including the European areas (EFSA, 2014), since it eats the sown seeds and the

rice plantlets, completely destroying paddy fields (Cowie, 2002). To date, the only effective measure to contrast apple snail is the flooding of infested paddy fields with seawater. This treatment successfully destroys apple snail infestations, nevertheless residual salt concentrations negatively affects rice productivity.

Taking into account the above statements, it is clear that the identification of European elite rice varieties tolerant to salt stress and, first of all, the dissection in them of salt stress tolerance mechanisms are of high interest for European rice breeding. Salinity tolerance in rice is a very complex trait, having different components changing as a function of the plant developmental stages or of the intensity of the imposed stress. Genome-wide association study (GWAS) is proving to be an effective approach for identifying loci controlling complex traits in plants (Ingvarsson and Street, 2011).

Recently, a whole-genome genotyping was carried out using the Genotyping-By-Sequencing (GBS) technology on a panel of 391 *O. sativa* varieties from a Rice Germplasm Collection maintained at the CREA-Rice Research Unit in Vercelli, Italy (Biscarini et al., 2016). In the present work, a phenotyping activity for mid-salt stress has been performed on a subset of this panel consisting in 281 *japonica* rice. Then, a GWAS was carried out in order to identify possible *loci* involved in salt tolerance in *japonica* rice.

2. Methods

2.1 Salt stress phenotyping

For phenotyping the mid-salt stress treatment, a greenhouse experiment has been performed on 281 *japonica* rice cultivars. Five *indica* genotypes were used as control, since their phenotype of sensitiveness/tolerance in response to salt stress is known: IR29 and IR64 (sensitive genotypes) and Pokkali, FL478 (IR29 X Pokkali), and IR64-Saltol(tolerant genotypes). The greenhouse is located in Tavazzano (Lodi, ITALY; 45.333449, 9.427294). Plants were sowed on 16th May 2016. The pots containing paddy soil were placed in a bigger bucket, containing water to mimic submerged condition of the soil.

The measurement of four physiological traits was considered: i) seedling emergence percent; ii) plant height, iii) chlorophyll *a* fluorescence on the flag leaf 10 days after flowering, and iv) delay in flowering.

For seedling emergence, the flooded soil in salt treatment was maintained at an electrical conductivity of the saturated paste extract at about 9 dS m^{-1}. Each biological replicate consisted in a control pot (non-saline flooded soil measuring approximately 1 dS m^{-1}) placed close to a treated pot (flooded soil maintained at about 9 dS m^{-1}). Twenty seeds were placed in each pot. Four replicates were used. At 23 days after sowing, the emerging seedlings were counted.

For the measurement of the other physiological traits, the flooded soil in salt treatment was maintained at an electrical conductivity of the saturated paste extract at about 4 dS m^{-1}, a value just above the rice threshold for salt stress (Chinnusamy et al., 2005). Each biological replicate consisted in a control pot (non-saline flooded soil measuring approximately 1 dS m^{-1}) placed close to a treated pot (flooded soil maintained at about 4 dS m^{-1}). Five plants were considered for each pot. Two biological replicates were used.

For measurement of plant growth, the height of three different plants for each of the two replicates was measured weekly during the vegetative stage (*i.e.* from 54 days after sowing for eight weeks). The data referred to 68 days after sowing were used for the association analysis.

Chlorophyll *a* fluorescence transients were determined on dark-adapted leaves kept for 30min at room temperature, using a portable Handy PEA (Hansatech, UK). The measurements were taken on the leaf surface (4 mm diameter) exposed to an excitation light intensity (ultrabright red LEDs with a peak at 650 nm) of 3000 μmol m^{-2} s^{-1} (600W m^{-2})

emitted by three diodes. Leaf fluorescence detection was measured by fast-response PIN photodiode with RG9 longpass filter (Hansatech, technical manual). The parameters measured were F_o(minimal fluorescence), F_m (maximum fluorescence), and F_v/F_m(variable fluorescence/maximum fluorescence). Three measures for each replication were taken on the flag leaf 15 days after flowering. The average level of F_v/F_m in control *vs* treated pots was used for the association analysis.

The delay in flowering time between control and salt-treated plants was also evaluated.

For all these four phenotypic traits, the stress susceptibility index (SSI) was calculated using the following formula: SSI = (1 − Ys/Yp)/D, where Ys = mean performance of a genotype under stress; Yp =mean performance of the same genotype without stress; D (stress intensity) = 1 − (mean Ys of all genotypes/mean Yp of all genotypes) (Fischer and Maurer 1978).

2.2 Genome-wide association analysis

Genotyping-By-Sequencing (GBS) technology was as described by Biscarini et al. (2016). A filtering of SNPs was applied for the present GWAS panel of 281 accessions: only those SNPs, having both missing data <5% (call-rate > 95%) and minor allele frequency (MAF) ≥ 5%, resulting in a final number of SNPs after filtering of 31,421.

For the analysis of the population structure, to estimate the number of subgroups of the panel based on genetic diversity, both Discriminant Analysis of Principal Components (DAPC) with R software, (Adegenet 2.0.0 package) and Evanno methods with STRUCTURE 2.3.4 program were used.

The pairwise SNP linkage disequilibrium (LD) among the 31421 SNPs was estimated as the correlation among pairs of alleles across a pair of markers (r^2) and was calculated with R software (LDcoreSV package). The mean r^2 drops below 0.2 beyond approximately 900 kb inter-marker distance.

For association analysis of genotyping and phenotyping datasets , Tassel 3.0 was used to calculate a Mixed Linear Model (MLM, setting the options no compression and P3D off). The kinship matrix (K) estimated from SNP genotyping data was used to improve statistical power of the association. The critical *p*-values for assessing the significance of SNPs were calculated based on a False Discovery Rate (FDR), by the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995), separately for each trait. A FDR cut-off of α 0.05 was used for determining significance.

3. Results

The evaluation of data about seedling emergence percent highlighted that most genotypes exhibited an intermediate phenotype in response to salt stress. Few varieties resulted very sensitive, showing a very low emergence percent (0-15%) in salt condition compared to control ones. On the other hand, few genotypes resulted very tolerant to salt stress, showing high emergence percentage (65-85%) under salt stress condition.

A similar trend was observed in the analysis of plant height data. Indeed, most genotypes exhibited an intermediate phenotype in response to salt stress. Few genotypes resulted very sensitive, showing a reduction in height ranging from -50% to -20% in treated plants. On the other hand, some genotypes resulted very tolerant, showing no effect or even a higher growth (up to +20%) under salt treatment (data not shown).

Moreover, the rice cultivars exhibited different chlorophyll *a* fluorescence values in response to salt treatment. In particular, the F_v/F_m index was considered, since it mirrors the maximum quantum yield of PSII and it is a valid index of the photoinibition damage. In control conditions, the rice plants exhibited F_v/F_m values between 0.77 and 0.86, as expected

in not-stressed plants, whereas under salt treatment 35 genotypes showed low values (under 0.75) of F_v/F_m, with the lowest ones (around 0.45) in three genotypes, indicating a high level of damage to the PSII.

Considering the time of flowering, just 11 genotypes did not show any effect on flowering time due to the treatment. Most genotypes (more than 200) flowered later in salt conditions than to control ones. In particular, the delay of 21 of these genotypes was more than 10 days. Finally, 33 genotypes showed an early flowering of 1-5 days when subjected to salt stress.

To identify possible *loci* involved in salt tolerance in the considered *japonica* rice accessions, a GWAS analysis was conducted expressing the phenotypic traits as SSI. The Manhattan plots of $-\log_{10}$(p-values) and the Q-Q (quantile-quantile) plots of expected (under a Gaussian distribution) *vs* observed *p*-values for SNP-based genotype-phenotype associations, related to the four salt stress-related traits under examination, are reported in Figs 1, 2, 3, and 4.

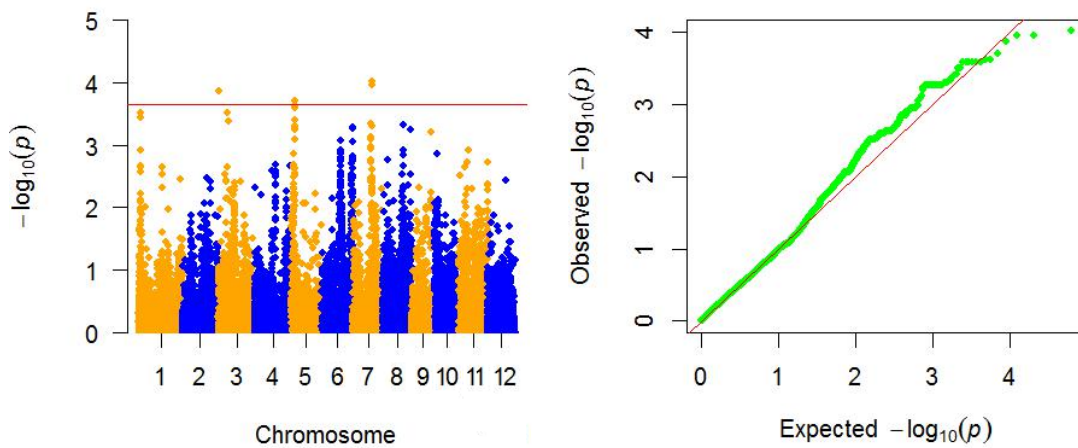Figure 1. Manhattan (a) and Q-Q (b) plots of GWAS results for the SSI related to emergence rate.

Figure 2. Manhattan (a) and Q-Q (b) plots of GWAS results for the SSI related to plant height.
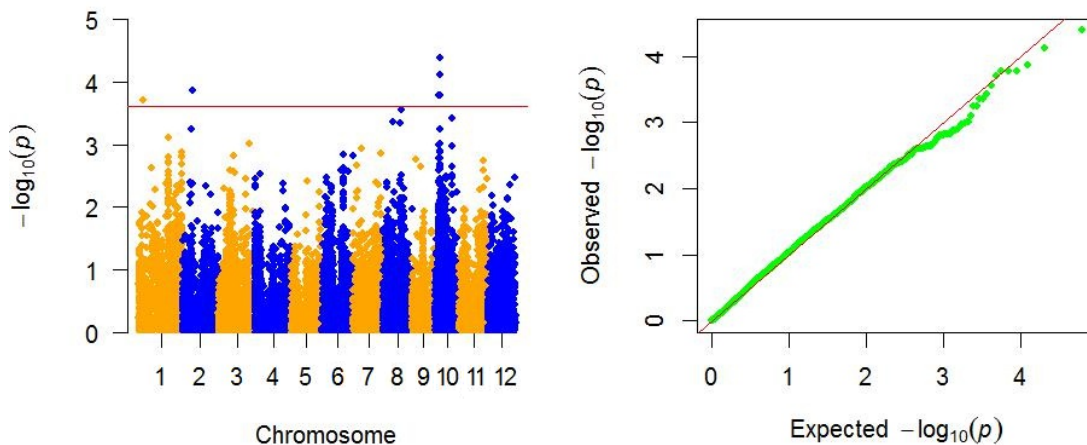
Figure 3. Manhattan (a) and Q-Q (b) plots of GWAS results for the SSI related to F_v/F_m values.
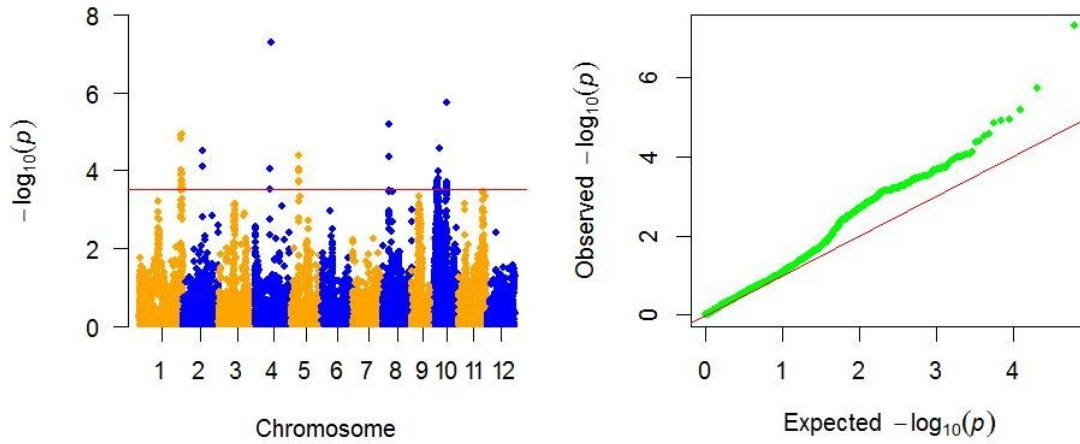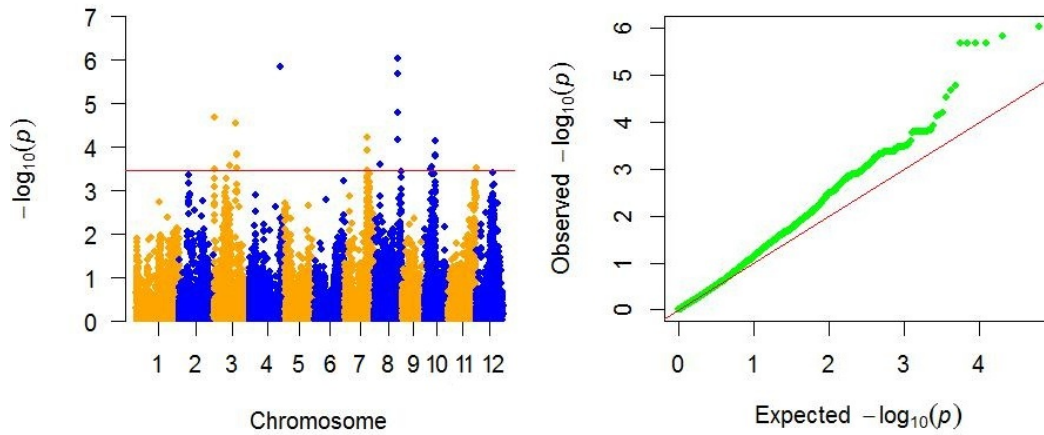

Figure 4. Manhattan (a) and Q-Q (b) plots of GWAS results for the SSI related to flowering delay.

A total of 90 significant associations between SNPs and the analysed salt stress-related traits were identified. In particular, 5 associations were related to seedling emergence rate (Fig. 1), 7 to plant height (Fig. 2), 40 to the values of F_v/F_m (Fig. 3), and 38 to flowering delay (Fig. 4). The most significant associations (-log10(*p*-value) > 4) detected in the GWAS study are listed in Table 1. For the largest peaks, only the most significant SNP is reported, together with the number of additional significant SNPs and the interval they span. The analysis of candidate genes lying in the genomic regions associated to these *loci* is still in progress.

Table 1. Most significant associations between SNP genotypes and salt stress-related traits in the analyzed rice accessions. The SNPs with the highest *p*-value is reported; FDR: false discovery rate(-log$_{10}$ (*p*)).

Traits	Marker	Chr	*p*-value	-log(*p*)	FDR cut off	Interval (bp)	n. SNPs
SSI_Emergence	S7_18728431	7	$9.66e^{-05}$	4.015	3.65	647786	3
SSI_Height	S10_4081529	10	$1.67e^{-04}$	3.776	3.60	195830	3
SSI_Fv/Fm	S1_41860369	1	$1.26e^{-05}$	4.898	3.53	63466	3
SSI_Fv/Fm	S1_41942315	1	$1,16e^{-05}$	4.936	3.53	92798	4
SSI_Fv/Fm	S2_19178445	2	$3.08e^{-05}$	4.511	3.53	841762	2
SSI_Fv/Fm	S4_15873294	4	$5.09e^{-08}$	7.294	3.53	951027	1

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SSI_Fv/Fm	S5_7398498	5	$8.93e^{-05}$	4.049	3.53	37177	5
SSI_Fv/Fm	S8_6315321	8	$4.46e^{-05}$	4.351	3.53	74415	1
SSI_Fv/Fm	S10_1160476	10	$1.84e^{-06}$	5.735	3.53	487839	5
SSI_Delay	S7_22807442	7	$6.14e^{-05}$	4.212	3.46	380718	6
SSI_Delay	S8_24175384	8	$9.48e^{-07}$	6.023	3.46	114057	6
SSI_Delay	S10_10427921	10	$7.32e^{-05}$	4.135	3.46	121677	11

4. Discussion

The response of 281 *japonica* rice genotypes to mid-salt stress was evaluated, through the measurement of some physiological traits related to different growth stage (seedling emergence, vegetative growth and flowering) of the rice plants. The phenotypic data highlighted that most genotypes exhibited an intermediate phenotype in response to salt stress, whereas some varieties showed an extreme phenotype (tolerance or susceptibility), suggesting that this panel might be a good resource for the discovery of traits related to salt stress response.

A total of 12 most significant associations were identified. Interestingly, the most significant associations were found with the two traits (measurement of chlorophyll fluorescence in the flag leaf 15 days after flowering and the delay in flowering time) that were related to reproductive stages. This result suggests that the variability among the 281 considered genotypes in the response to salt stress could be due to specific traits related to this specific growth stage, rather than to differences in the response related to the vegetative stage. The analysis of the genes significantly associated to these *loci* is still in progress. A preliminary analysis showed the presence of putative ion transporters ("similar to low affinity calcium transporter", "similar to potassium transporter", "cation/H^+ exchanger domain containing protein"), which may be related to a different Na^+ uptake and/or exclusion from the cells as possible candidate genes for some of the significant associations detected.

Conclusions

In the present work, a phenotyping activity has been performed to study the natural variation of a worldwide japonica rice collection in response to mid-salt stress. The measurement of phenotypic data highlighted a variability among the genotypes in response to the treatment, suggesting that this panel might be a good resource for the discovery of traits related to salt stress response. Moreover, a genome-wide association study identified significant associations between SNPs and the analysed salt stress-related traits. The analysis of these *loci* might reveal possible candidate genes involved in salt tolerance in *japonica* rice.

Acknowledgements

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