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Use of EcoTILLING to identify natural allelic variants of rice candidate genes involved in salinity tolerance

S. Negrão[1], C. Almadanim[1], I. Pires[1], K. L. McNally[2] and M. M. Oliveira[1]*
[1]*ITQB (Instituto de Tecnologia Química e Biológica), IBET (Instituto de Biologia Experimental e Tecnológica), Universidade Nova de Lisboa, Avenida da República, 2780-157 Oeiras, Portugal and* [2]*International Rice Research Institute, DAPO Box 7777, Metro Manila, Philippines*

Abstract

Rice is a salt-sensitive species with enormous genetic variation for salt tolerance hidden in its germplasm pool. The EcoTILLING technique allows us to assign haplotypes, thus reducing the number of accessions to be sequenced, becoming a cost-effective, time-saving and high-throughput method, ideal to be used in laboratories with limited financial resources. Aiming to find alleles associated with salinity tolerance, we are currently using the EcoTILLING technique to detect single nucleotide polymorphisms (SNPs) and small indels across 375 germplasm accessions representing the diversity available in domesticated rice. We are targeting several genes known to be involved in salt stress signal transduction (*OsCPK17*) or tolerance mechanisms (*SalT*). So far, we found a total of 15 and 23 representative SNPs or indels in *OsCPK17* and *SalT*, respectively. These natural allelic variants are mostly located in 3′-untranslated region, thus opening a new path for studying their potential contribution to the regulation of gene expression and possible role in salt tolerance.

Keywords: EcoTILLING; genetic variability; rice; salt tolerance

Introduction

Soil salinity existed long before humans and agriculture; however, the problem has been aggravated by agricultural practices such as irrigation (Zhu, 2001). It is estimated that about 20% of irrigated agricultural land throughout the world is adversely affected by salinity. Plants differ greatly in their tolerance to salinity, with rice (*Oryza sativa* L.) being the most sensitive cereal. Rice is the primary food source for over half of the world's population and has been the subject of countless breeding programmes to increase its productivity and tolerance to both biotic and abiotic stresses. Analysis of the molecular mechanisms underlying salinity tolerance is being undertaken to provide practical contributions to food production, particularly to mitigate the effects of increasing salinization and climate change.

In rice, differences among genotypes account for its response towards salinity (Zeng, 2005). The importance of genes hidden in the primary rice gene pool (including low-yielding ancestors and traditional landraces) to enhance rice performance in stress conditions was previously illustrated (Ali *et al.*, 2006). Since the most common forms of genetic variation within natural populations are single nucleotide polymorphisms (SNPs) and small insertions and deletions (indels), a further step can be achieved by investigating such variation in key-responsive genes from diverse germplasm (Raghavan *et al.*, 2007). While we are now in the 'next-generation sequencing' era, many research programmes have limited funding and need more cost-effective, high-throughput techniques. The EcoTILLING method allows SNPs and indels discovery and the delineation of haplotypes at loci of interest (Comai *et al.*, 2004). We are using EcoTILLING to explore the

* Corresponding author. E-mail: mmolive@itqb.unl.pt

natural variability existing in rice germplasm at key genes related to salinity tolerance. Although this large-scale analysis is still ongoing, we have already obtained promising results showing high genetic variability in rice germplasm at salinity tolerance loci and the potential to deliver superior alleles for breeding programmes targeting salinity tolerance.

Materials and methods

We used 375 genotypes that represent the diversity present in *O. sativa*, which were selected from the set genotyped by single sequence repeats in the 'Generation Challenge Program' with a population structure similar to that of Garris *et al.* (2005). Deoxyribonucleic acid (DNA) was extracted from leaf tissue according to Fulton *et al.* (1995). DNA from all samples was brought to a concentration of 0.5 ng/μl. For EcoTILLING, DNA from each genotype was contrasted with either 'IR64' (against *indica*, admixed and Aus accessions) or 'Nipponbare' (against *japonica*, aromatic and admixed) separately, in a 1:1 ratio.

Target genes were *SalT* (LOC_Os01g24710) and *OsCPK17* (LOC_Os07g06740). Primers were designed (based on the 'Nipponbare' genome sequence) with Primer3 software to amplify part of an intron and the 3′-untranslated region (UTR) of *OsCPK17* (forward – AATTGGAGGTTGGGCCATAG; reverse – TGTGAGGTGGAAGAAGCAAAC) and the second exon and the 3′-UTR region of *SalT* (forward – ACCACTCAACACCGGTAGGACACT; reverse – GCAGATTAAACTGGGCTCCTCTGA), corresponding to 979 and 825 bp products, respectively.

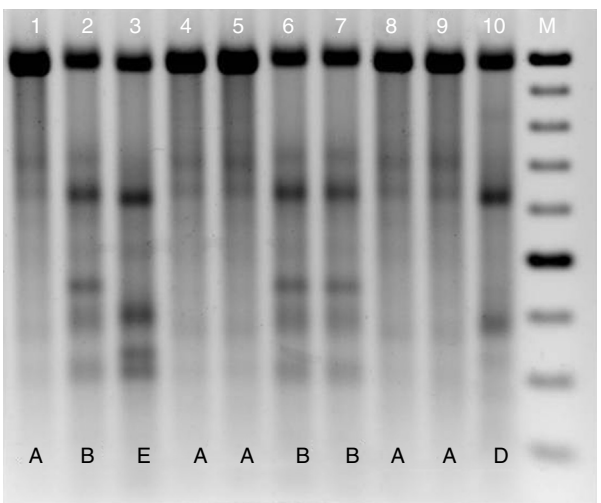

Fig. 1. Analysis of the EcoTILLING digestion patterns in agarose gel for *OsCPK17* in different rice varieties. The varieties shown were contrasted with 'IR64' and created four haplotype groups (A, B, E and D) according to CJE-cleaved products. 1, PATNAI 23; 2, CODE NO. 31293; 3, TOS7564; 4, KHAO PON; 5, MALLIGAI (KOTTAMALLI SAMBA); 6, TUNGHWANPEI; 7, DE ABRIL; 8, LAGEADO; 9, BALA; 10, SINNA SITHIRA KALI; M, molecular marker.

The polymerase chain reaction (PCR) was performed in 14 μl final volume, using 3.5 ng of total DNA as template, 0.4 U/reaction of *Taq* DNA polymerase (Promega, Madison, WI, USA). The PCR cycling conditions were set at 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 61–64°C (depending on primer specificity) for 30 s, 72°C for 30 s and a final extension of 7 min at 72°C. The PCR products were denatured at 99°C for 10 min and renatured initially at 70°C for 20 s followed by 69 cycles with a temperature decrease by 0.3°C per cycle. Celery juice extract (CJE) was produced by the technique of Till *et al.* (2004). The mismatch cleavage (CJE digestion optimized for each primer) and EcoTILLING analysis in agarose gel were performed according to Raghavan *et al.* (2007).

Results and discussion

Rice cultivars with different salt sensitivities have been studied using transcriptomic approaches (Walia *et al.*, 2005, 2007; Kumari *et al.*, 2009; Senadheera *et al.*, 2009), revealing highly significant differences in gene regulatory mechanisms between genotypes. A clear example of the importance of genotypic differences between varieties towards salt stress was given by the allelic differences found in *OsHKT8* gene between two different genotypes (Ren *et al.*, 2005). The six nucleotide substitutions in the coding region leading to four amino acid changes present in 'Nona Bokra' enhanced the overall Na^+ transport activity (Ren *et al.*, 2005), showing the importance of discovering superior alleles in salt stress-related genes.

We are presently evaluating the haplotype groups of *OsCPK17* and *SalT* genes by EcoTILLING. *OsCPK17* encodes a calcium-dependent protein kinase, and its promoter contains *cis* elements responsive to various stress stimuli. Wan *et al.* (2007) showed that *OsCPK17* is down-regulated by salt, cold and drought, indicating its importance in stress signal transduction in rice. The *SalT* gene was first isolated and characterized from the roots of rice plants treated with salt (Claes *et al.*, 1990) and co-localizes with the *Saltol* Quantitative Trait Loci. Although several studies have already been performed (Claes *et al.*, 1990; Zhang and Blumwald, 2001; de Souza *et al.*, 2003), the function of SalT is not clearly understood.

In our EcoTILLING assay, we amplified *c.* 1 kb targets of each gene using specific primers. If the amplicons differ in sequence content between the reference and target germplasm, heteroduplex mismatch molecules will occur on re-annealing. Digestion with *CJE*

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Table 1. Sequence and position of the SNPs and indels found in *OsCPK17* and *SalT* genes

	OsCPK17 position relative to the beginning of locus Os07g06740														
Haplotype	4170	4181	4203	4238	4288	4417	4479	4547	4589	4593	4644	4699	4705	4731	4735
A	C	A	$(CT)_7$	C	$(CT)_5$ T	T	G	$(T)_{10}$	C	$(GT)_4$	A	A	A	G	A
B	C	A	$(CT)_9$	C	$(CT)_{12}$ T	C	C	$(T)_9$	C	$(GT)_4$	A	A	A	G	A
C	C	A	$(CT)_8$	C	$(CT)_5$ T	T	G	$(T)_{10}$	C	$(GT)_4$	A	A	A	G	A
D	C	T	$(CT)_7$	A	$(CT)_9$ T	T	G	$(T)_{10}$	C	$(GT)_4$	A	G	G	A	A
E	C	A	$(CT)_5$	C	$(CT)_7$ C	C	G	$(T)_9$	–	GT GC $(GT)_2$	A	A	A	G	A
F	C	A	$(CT)_8$	C	$(CT)_8$ T	C	G	$(T)_{11}$	C	$(GT)_4$	G	A	A	G	T
G	C	A	$(CT)_9$	C	$(CT)_{11}$ T	C	C	$(T)_9$	C	$(GT)_4$	A	A	A	G	A
H	A	A	$(CT)_{10}$	C	$(CT)_{12}$ T	C	C	$(T)_9$	C	$(GT)_4$	A	A	A	G	A
I	C	A	$(CT)_9$	C	$(CT)_{12}$ T	C	C	$(T)_9$	C	$(GT)_3$	A	A	A	G	A
J	C	A	$(CT)_7$	C	$(CT)_9$ T	T	G	$(T)_9$	C	$(GT)_4$	A	A	A	G	A

	SalT position relative to the beginning of Locus Os01g24710														
Haplotype	1108	1159	1292	1307	1360	1365	1387	1391	1395	1408	1413	1425	1476	1480	1530
A	T	A	$(AT)_2$	T	C	A	C	A	ATGTTGT	C	T	TTA	C	G-	C
B	C	A	$(AT)_2$	G	T	G	T	G	GTTC–	T	C	–	A	TA	C
C	T	C	$(AT)_2$	T	C	A	C	A	ATGTTGT	C	T	–	C	G-	C
D	C	A	AT	G	T	G	T	G	GTTC–	T	C	–	A	TA	T
E	C	A	$(AT)_2$	G	T	G	T	G	GTTC–	T	C	–	A	TA	C
F	C	A	$(AT)_2$	G	T	G	T	G	GTTC–	T	C	–	A	TA	C

Haplotype	1537	1550	1573	1683	1685	1711	1742	1803
A	G	A	G	G	T	T	A	G
B	G	A	A	G	A	C	G	A
C	A	A	G	A	T	T	A	G
D	G	C	A	G	A	C	G	A
E	G	A	A	G	A	C	G	A
F	G	A	A	G	A	C	G	G

endonuclease reveals bands other than full-length products indicating SNPs or/and indels. By comparing the digestion patterns, haplotypes can be assigned, as shown in Fig. 1. The number, position and type of SNPs in the haplotypes were validated by sequencing the PCR amplicons.

The analysis of the EcoTLLING digestion patterns identified ten haplotypes for *OsCPK17* and six haplotypes for *SalT* (Table 1). For *OsCPK17*, haplotypes A and G (which include the reference types 'IR64' and 'Nipponbare', respectively) are more frequent, whereas E, F, I and J are very rare (each represented by one accession). The observed mismatches were explained by 15 SNPs or indels with most of the indels being detected in repetitive sequences: (CT), (T) or (GT). All polymorphisms detected in *OsCPK17* do not seem to interfere with 3′-splice sites. As of now, we have found 23 SNPs or indels in the *SalT* gene. Haplotype A is the most frequent and includes both reference genotypes 'IR64' and 'Nipponbare'. The transition T/C (position 1108) is a silent mutation, whereas the transition A/C (position 1159) leads to a glutamate to aspartate change. Since these two amino acids belong to the same group, this change may have little or no consequence on protein structure/function. The remaining SNPs and indels were located in the 3′-UTR region. These variations may be of significance since it is now recognized that 3′-UTRs play crucial roles in the post-transcriptional regulation of gene expression through the modulation of nucleocytoplasmic messenger ribonucleic acid (mRNA) transport, regulation of mRNA polyadenylation and translation, translation efficiency, sub-cellular localization and messenger stability (Mignone *et al.*, 2005).

Our large-scale analysis is also targeting and analysing other regions of *SalT* and *OsCPK17*, as well as of other genes important in salinity tolerance. We will further assess the relevance of the DNA variations by testing association with salinity tolerance phenotypes. Eventually, we hope to uncover novel superior alleles that can be used in rice-breeding programmes for salt stress tolerance.

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