



Molecular evolution of rice oxalate oxidases -- candidate genes for quantitative resistance to rice blast

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Oxalate oxidases (OXOs) in cereals have been implicated to play a role in defense response to pathogen infection. While many studies have focused on the rapid evolution of major resistance genes involved in pathogen recognition, relatively little is known about the molecular evolution of defense genes in plant-pathogen coevolution. Here, we analyzed the molecular changes in members of rice OXO genes mapped to chromosome 3 that are associated with resistance to blast. There are four tandemly duplicated oxalate oxidases (*OsOxo*) in chromosome 3 as well as 70 related sequences forming the cupin superfamily of proteins in the rice genome. These four genes (*OsOxo1*, *OsOXO2*, *OsOxo3*, and *OsOxo4*) exhibit >90% similarity at the nucleotide and amino acid levels. *OsOxo4* have been shown to be expressed in rice-*Magnaporthe oryzae* interaction. In this study, we analyzed the OXO gene family in rice from 62 rice cultivars belonging to six isozyme groups – *indica*, *japonica*, *aus/boro*, aromatic, deepwater subtype III, and deepwater subtype IV. Analyses of the *OsOxo* from 62 rice cultivars showed that synonymous substitution rates often exceeded nonsynonymous rates, suggesting that purifying selection is the major factor that maintains OXO protein homogeneity. The average frequency of SNPs (single nucleotide polymorphisms) was 1 per 24, 19, 18, and 31 bp across the coding region for *OsOxo1*, *OsOXO2*, *OsOxo3*, and *OsOxo4*, respectively. Haplotype and nucleotide diversities were moderate with an average of $Hd = 0.76 \pm 0.05$ and $\pi = 0.03 \pm 0.005$, respectively. Each locus was made up of one to two major haplotypes that clearly distinguish *indica* and *japonica* groups from each other.

Materials and methods

Primer design



Fig. 1. Gene-specific primers to amplify the four OXO genes in chromosome 3 were designed using the PRIMER3 program. Three pairs of overlapping primers were designed that would encompass the 1-kb upstream region, the gene-coding region, and the 3'UTR region of each gene. The expected product sizes were 800–1000 bp.

Genetic materials

Sixty-two rice cultivars belonging to six subgroups –*indica*, *japonica*, *aus/boro*, aromatic, deepwater subtype III, and deepwater subtype IV– were obtained from the Genetic Resources Center of the International Rice Research Institute and the Rural Development Administration of Korea.

Sequencing

PCR products were analyzed on agarose gel, purified using a Qiaquick PCR purification kit (Qiagen, Inc. Valencia, CA), and quantified prior to DNA sequencing. PCR products were sent to Macrogen (<http://www.macrogen.com>) for sequencing.

Data analyses

Multiple sequence alignments and neighbor-joining phylogenetic trees were constructed using MEGA 3.1. Synonymous and nonsynonymous sites, haplotype and nucleotide diversity estimates, parsimony informative sites, and neutrality test statistics (Tajima's D, Fu and Li's D* and F*) were calculated using DnaSP.

Table 1. Nucleotide variation in the gene-coding region of oxalate oxidases from 62 rice cultivars.

Parameter	OsOxo1	OsOxo2	OsOxo3	OsOxo4
Gene-coding region (bp)	684	690	684	690
A. SNPs				
Total polymorphic sites	28	36	38	22
Average frequency of SNPs (bp/SNP)	24	19	18	31
SNP	23	33	17	19
Dinucleotide polymorphism	5	3	0	3
Trinucleotide polymorphism	0	0	1	0
B. InDels				
Total InDels	6	5	11	12
Average frequency of InDels (bp/InDel)	114	138	62	62
Single-base InDel	6	4	8	11
Dinucleotide InDel	0	1	1	1
Trinucleotide InDel	0	0	2	0
Number of synonymous sites	15	45	15	20
Number of nonsynonymous sites	27	28	10	18
Parsimony Informative Sites				
Ratio of Transition/Transversion Pairs	0.4	1.2	0.7	1.6
dN = dS test for neutrality	0.7	0.8	0.8	0.0

Nucleotide polymorphisms and insertions/deletions (InDels) were observed in all four genes. Nucleotide changes occurred every 20.75 bp on average, compared with indels which occurred every 94 bp in the gene-coding regions. Dinucleotide polymorphism was observed in *OsOxo1*, *OsOxo2* and *OsOxo4*, while trinucleotide polymorphisms were observed only in *OsOxo3*. There were more indels >3 nucleotides in the 1-kb upstream region. At the extreme end is a 457-bp transposon insertion common to cultivars Rayada, Dular, and ARC10177. The dN/dS for each locus showed that these genes were under neutral evolution consistent with the tests for neutrality above.

These results are in contrast with the evolutionary behavior of genes involved in defense response such as *R* genes and the chitinase gene family in *Arabidopsis* in plants. The *R* gene evolution has been extensively studied in different species and a common theme is that these genes are under positive selection pressure.

Haplotype relationships of OXOs from 62 rice cultivars.

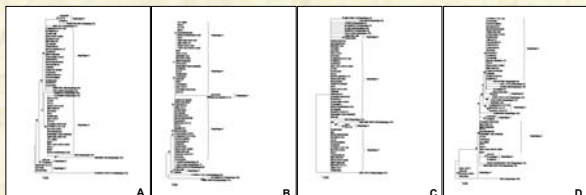


Fig. 2. Unrooted neighbor-joining phylogenetic tree representing haplotype relationships of OXO from 62 rice cultivars. A) There were two major haplotypes for *OsOxo1* that accounted for 72.5% of haplotype diversity. B) There were three major haplotypes for *OsOxo2*. Together, these accounted for 84% of the haplotype diversity in *OsOxo2*. C) Majority of the *OsOxo3* sequences grouped into one haplotype ($n = 51$) and the rest into one to two sequences per haplotype. D) Majority of the *OsOxo4* sequences were in haplotypes 1 ($n = 24$) and 2 ($n = 13$). The rest of the haplotypes were composed of 1-4 *OsOxo4* sequences each. Except for *OsOxo3*, the major haplotypes can be divided into *japonica* and *indica* groups with exceptions, and these correlate well with previously established groupings in rice using isozymes and microsatellite markers. Both nuclear and chloroplast data showed a closer relationship of the *aus* group with the *indica* groups while the aromatic rice showed a closer relationship with the tropical and temperate *japonica* groups. In the current study, two aromatic rice cultivars – Basmati 370 and Chhote Dhan have the same haplotypes as the *japonica* cultivars for *OsOxo1*, *OsOxo2*, and *OsOxo4*. Both cultivars were also in the major haplotype of *OsOxo3*.

Phylogeny of 62 rice cultivars based on the four OXO contiguous gene-coding region sequence

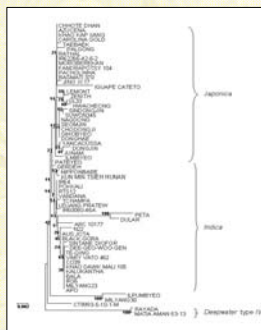


Fig. 3. Neighbor-joining phylogenetic tree of 62 rice cultivars based on the four OXO contiguous gene-coding region. Nucleotide sequences of each rice OXO gene were combined to form a contiguous sequence from *OsOxo1* to *OsOxo4*. There were two major clusters consisting of *indica* and *japonica* groups with the *aus* cultivars interspersed in the *indica* cluster. The aromatic rice clustered with the *japonica*. Matia-Aman 53-13, and Rayada, classified as deepwater type IV formed a separate cluster from the other groups. Two cultivars, Pacholinha and Jing Xi 17, with undetermined isozyme grouping, were clustered in the *japonica* branch of the tree and is consistent with their grouping in the individual phylogenetic tree.

Analyses of the 1-kb upstream region of OXO genes in rice

cis-element	Position (bp)	
	Momberekan	Vandana
TATA Box	-71	-66
WRKY710s	-208 -202	-408 -349
WBOXNERF3	-737 -721	-683 -687
WBOXNTH48	-737 -732	-684 -687
WENTPR1	-208	-202
GCCCORE	-373 -367	-402 -496

*cis-elements were identified from the Plant Cis-Acting Elements (PLACE) database.
† Position in base pair is downstream from transcription start site ATG.

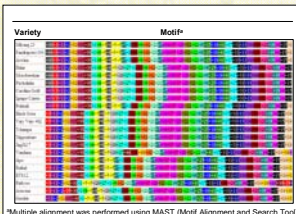


Fig. 4. Multiple alignment of the 1kb upstream region of *OsOxo4* from 62 rice cultivars. This region was highly similar for all sequences (>90% similarity). Motif 1 represents the CAAT box motif common in eukaryotes. Motifs 4 and 41 represent W boxes common in pathogen-responsive genes.



Fig. 5. A 26-bp deletion in Vandana *OsOxo4* leads to deletion of putative cis-elements related to bacterial nodulation-responsive genes. IR64, Leuang Pratew, and Peta also contain this deletion.

Association of SNPs and haplotypes to phenotype

- The 62 cultivars used in this study were evaluated for reaction to three rice blast isolates in the greenhouse and lineages occurring in the blast nursery.
- Results showed that these cultivars have varying degrees of resistance to rice blast isolates PO6-6, CA89, and M64-1-3-9-1 and to field blast.
- Single-marker analyses did not show significant association between haplotypes and reaction to blast. Cultivars in the same haplotype group did not necessarily have the same reaction to a blast isolate. This may be due to epistatic effects brought about by major resistance genes or combination of other defense genes present in the cultivars.

Summary

The rice OXOs occur as tandemly duplicated genes in chromosome 3. Genetic and expression studies have shown their association with resistance to the rice blast pathogen. Overall, our data suggest that, contrary to the divergent evolution of *R* genes, OXOs in rice are under purifying selection. This observation is consistent with the reported roles of OXOs in general defense against pathogens.