Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

JZUS

Identification of candidate genes for drought stress tolerance in rice by the integration of a genetic (QTL) map with the rice genome physical map*

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Received Sept. 15, 2004; revision accepted Dec. 26, 2004

Abstract: Genetic improvement for drought stress tolerance in rice involves the quantitative nature of the trait, which reflects the additive effects of several genetic loci throughout the genome. Yield components and related traits under stressed and well-water conditions were assayed in mapping populations derived from crosses of Azucena×IR64 and Azucena×Bala. To find the candidate rice genes underlying Quantitative Trait Loci (QTL) in these populations, we conducted in silico analysis of a candidate region flanked by the genetic markers RM212 and RM319 on chromosome 1, proximal to the semi-dwarf (sd1) locus. A total of 175 annotated genes were identified from this region. These included 48 genes annotated by functional homology to known genes, 23 pseudogenes, 24 *ab initio* predicted genes supported by an alignment match to an EST (Expressed sequence tag) of unknown function, and 80 hypothetical genes predicted solely by *ab initio* means. Among these, 16 candidate genes could potentially be involved in drought stress response.

Key words: Rice genome sequence, Candidate genes, Drought stress, Quantitative Trait Loci (QTL)
doi:10.1631/jzus.2005.B0382 **Document code:** A **CLC number:** Q943

INTRODUCTION

Drought stress is a major constraint to rice (*Oryza sativa*) production and yield stability in rained ecosystems (Dey and Upadhyaya, 1996). Rice must be made more drought tolerant, but this is a somewhat contradictory objective considering that rice is most commonly grown under flooded conditions. Achieving drought tolerance in rice will require a deeper understanding of the possible physiological mechanisms available for water stress tolerance and the identification of favorable alleles for introgression into rice varieties that otherwise suit specific environments. Quantitative Trait Loci (QTL) are useful starting points for identifying such alleles. QTL relating to drought tolerance have been identified in rice, barley, and grain sorghum (Price *et al*., 2002a).

However, positionally cloning a QTL gene locus normally requires fine scale mapping with large mapping populations across many seasons. Availability of the whole rice genome sequences (Goff *et al*., 2002; Yu *et al*., 2002; Sasaki *et al*., 2002; Feng *et al*., 2002) provides a new tool for this task, along with a means of characterizing their associated molecular functions. In this study, we exploited this new source of data by anchoring drought stress tolerance QTL maps reported by other researchers (Lafitte *et al.*, 2002; Price *et al*., 2002a; 2002b; 2002c; Causse *et al*.,

* Project supported partly by the Rockefeller Foundation thesis dissertation training grant and the National Hi-Tech Research and Development Program (863) of China

1994; Harushima *et al*., 1998) to a rice physical and sequence map using sequence based markers. We then identified genes as candidates with respect to position.

Drought stress most severely impacts yield when applied during the reproductive stage of the rice plant. The sd1 is one of the most important genes in rice, whose recessive character results in a shortened culm with improved lodging resistance and a harvest index (Jennings, 1964). The rice height-related QTL was shown to be tightly linked to restricted fragment length polymorphism (RFLP) markers RZ730 and RG810 on the long arm of chromosome 1 (Li *et al.*, 2003), consisting of the QTL region of drought resistance (Lafitte *et al.*, 2002). As further evidence for drought tolerance gene candidacy, we aligned EST sequences from an available drought stressed panicle library onto our candidate gene structures. We also assessed gene candidacy based on literature support.

MATERIALS AND METHODS

Genetic map for IR64×Azucena and Bala×Azucena QTL mapping populations

Published genetic mapping data from a double haploid mapping population, IR64×Azucena (Temnykh *et al.*, 2001) and Bala×Azucena (Price *et al*., 2002b) were used as starting points for this study. IR64×Azucena map included 93 amplified fragment length polymorphism (AFLP), 481 simple sequence repeat (SSR), 15 'known' genes, 1 isozyme, a restriction fragment length polymorphism (RFLP) and 13 random amplification of polymorphic DNA (RAPD) markers adapted from oats. The Bala×Azucena population includes 7 SSR, 105 RFLP and 34 AFLP markers, as well as one marker derived from oats.

Consensus map construction

To identify genetic map regions containing multiple overlapping QTL across populations, we computationally merged the maps of the two aforementioned mapping populations with common anchor markers using the NCGR ISYS comparative mapping tool (NCGR; http://www.ncgr.org/isys; Siepel *et al*., 2001).

Rice genome sequence map

Identification of candidate genes corresponding to a QTL interval requires a contiguous genome sequence map. Although chromosome 1 and chromosome 4 were essentially completed by the International Rice Genome Sequencing Project (IRGSP) during the latter part of this project, early in the project we needed to assemble our own sequence map using available public data. This assembly work focussed on rice chromosome 1. The input data for map assembly included the list of clone names, GenBank accession numbers and the public rice physical map (Chen *et al*., 2002). We assembled the bacterial artificial chromosome (BAC) clone sequences into a pseudo-molecule using a suite of Perl scripts graciously provided by Dr. David Beare of the Sanger Centre, United Kingdom.

Anchoring sequence-based genetic markers to the sequence map

We used a local implementation of the "electronic polymerase chain reaction" approach ("ePCR"; Schuler, 1997) using Perl regular expression alignment of primer sequences with orientation and threshold distance constraints to identify putative PCR amplicons in target sequences. The primers for the analysis were obtained retrieved from either the Gramene database (Ware *et al*., 2002; www.gramene.org) or the Japan Rice Genome Project (RGP) database (Sasaki, 2001). Markers were correlated with the physical map by ePCR, run against the rice genome BAC sequences, retrieval from the Gramene database and by BLAST (Altschul *et al*., 1990) alignment searches of the rice BAC sequences.

Database storage and visualization of maps

The resulting genetic (QTL), physical and sequence maps compiled for the study were stored in the International Rice Information System (IRIS; www.iris.irri.org; Bruskiewich *et al*., 2003) and visualized using a comparative mapping tool developed at the National Center for Genome Resources. To facilitate identification of candidate genes, we also have developed QTL2Gene (http://ibi.zju.edu.cn/qtl2gene/qtl2gene.htm) as a flexible database using the public rice genome sequence and RFLP and SSR markers. The database provides an interface for searching the genes underlying one QTL using two

flanking markers' name. Other information related with this paper has been published on website (http://ibi.zju.edu.cn/publish/QTL2gene/drought.htm).

Choice of candidate QTL region for detailed analysis

The choice of candidate QTL region on rice chromosome 1 was chosen based on a survey of drought QTL (Price *et al*., 2002c; Lafitte *et al*., 2002) and International Rice Research Institute (IRRI) fine mapping work ([1]Zhikang Li, personal communications). This region is flanked by RM212 and RM319, which were shown to be located on 148.7 cM (CentiMorgan) and 150.5 cM of IR64×Azucena genetic map respectively, and spans a map distance of approximately 1.8 cM across the sd1 gene locus.

Gene and functional annotation

We downloaded gene annotation for the RM212-RM319 candidate region from the Japan Rice Genome Project (RGP) sequence data found in GenBank (www.ncbi.nlm.nih.gov) and from the rice databases of The Institute for Genomic Research (TIGR; www.tigr.org). These annotations were assessed using the *gbrowse* sequence browser from the Generic Model Organism Database project (http://www.gmod.org; Stein *et al*., 2002; Lewis *et al*., 2002).

Drought panicle cDNA library

An IR64-based drought stressed rice panicle cDNA library was constructed by researchers at the International Rice Research Institute (Arumugam *et al*., 2005). Briefly, the normalized library was constructed from pooled mRNA obtained from the rice panicles collected from control (well watered) and water stressed plants at 2 d before heading, at heading, 50% flowering and 4 d after 50% flowering. Water stress was applied by not watering for several consecutive days. Expressed sequence tags (EST) obtained by 5′ and/or 3′ end sequencing of the clones were clustered and annotated using standard in silico approaches (Altschul *et al*., 1990).

RESULTS

Alignment of the rice genetic map onto the physical map and gene identification

The chromosome 1 consensus map for IR64×Azucena and Bala×Azucena was anchored using sequence-based markers to the assembled rice physical map. The candidate sequence interval spanning the candidate QTL flanked by RM212 to RM319 was then identified. The assembly of seven BAC clones in this interval yielded a contiguous sequence region of 855008 base pairs within which we identified 175 predicted gene structures. Fig.1 shows a synopsis of gene annotation categories. The RGP annotation included 48 (27%) genes annotated by functional homology to known genes, 23 (13%) pseudogenes, 24 (14%) *ab initio* predicted genes supported by an alignment match to an EST of unknown function, and 80 (46%) hypothetical genes predicted by *ab initio* algorithms.

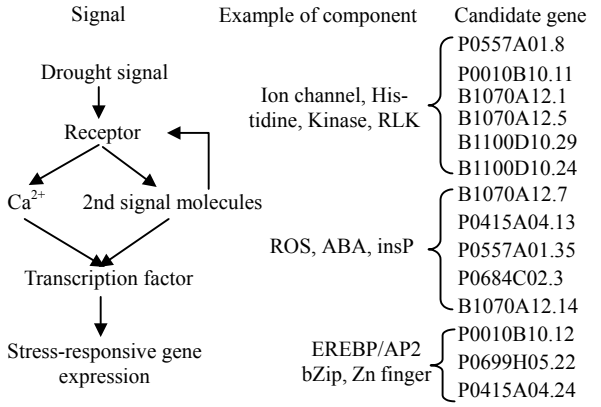

Fig.1 Possible assignment of candidate genes identified in candidate interval as components in a generic pathway for the transduction of drought stress signals in plants

Mapping drought stressed panicle library EST into the region

To collect further supporting evidence for the function of the predicted genes in the region, EST sequences obtained from the IRRI drought stressed panicle library were aligned by BLAST (Altschul *et al*., 1990) against candidate gene sequences. This analysis revealed that 30 candidate gene predictions match EST sequences in the library (Table 1).

Candidate genes placed into drought signal pathways

In our study, we found 16 candidate genes in the region with some literatures support (Shinozaki and Yamaguchi-Shinozaki, 1996; Ingram and Bartels, 1996; Yamaguchi-Shinozaki *et al*., 2002) potentially involved in drought stress response (Table 2).

[1]Li, Z.K., 2002

Table 1 EST alignments to candidate genes

Gene name	Gene		BLAST score	Percent identity	EST GenBank accession ID	EST		Annotation
	Start	Stop				Start	Stop	
B1011A07.16	607	852	690	75.61	CA762217	2	247	Putative pectinesterase 2.1 precursor
B1070A12.1	2131	2424	1380	96.6	CA765415	1	294	Putative serine/threonine kinase
B1070A12.17	1305	973	1655	99.7	CA760294	352	685	Hypothetical protein
P0010B10.6	1221	1024	990	100	CA760259	488	685	Hypothetical protein
P0010B10.7	511	344	831	99.4	CA763140	493	660	Zinc protease
P0010B10.12	1	249	1245	100	CA762979	439	687	Helicase-like transcription factor
P0010B10.21	458	570	330	77.19	CB096315	212	325	Hypothetical protein
P0010B10.21	458	570	321	76.32	CB096974	175	288	Hypothetical protein
P0010B10.22	861	443	1663	88.54	CA759417	267	685	Secretory carrier membrane protein
B1100D10.21	582	1	2868	99.31	CA762908	135	715	Acyl-CoA:1-acylglycerol-3-phosphate acyltransferase
B1100D10.25	223	38	513	76.72	CB096516	86	271	Hypothetical protein
B1100D10.33	1790	1918	304	71.76	CA764883	1	130	Serine/threonine kinase receptor precursor (EC 2.7.1.37) (S-receptor kinase)
P0458E05.33	354	47	720	74.14	CA759325	282	592	Hypothetical protein
P0482C06.17	483	227	638	74.81	CA760143	176	429	Hypothetical protein
P0682C06.15	330	1	1533	96.06	CA766823	326	655	Putative prolyl endopeptidase
P0682C06.16	360	2	1375	87.19	CA763742	143	500	Unknown protein
P0699H05.4	1520	2043	2593	99.43	CA766000	2	525	Putative subtilase
P0699H05.7	2136	1602	2524	97.01	CB096421	317	851	Putative subtilase
P0699H05.7	1678	2136	2286	99.78	CA766686	1	459	Putative subtilase
P0699H05.7	378	926	2414	93.88	CA763105	162	714	Putative subtilase
P0699H05.9	120	44	162	74.07	CA766969	204	279	Hypothetical protein
P0699H05.17	292	1	1436	99.32	CB096487	1	291	Hypothetical protein
P0699H05.17	323	1	1606	99.69	CA766130	35	357	Hypothetical protein
P0699H05.22	670	708	195	100	CA767024	2	40	Ethylene-responsive element binding factor 3
P0699H05.22	708	194	1977	87.4	CA759952	183	696	Ethylene-responsive element binding factor 3

Table 2 Genes encoding proteins of known function possibly that may possibly be responsive to drought stress

Classes	Gene name	Putative functional description	Protein_id	Reference
Receptor	P0557A01.8	Kinase-like	BAB89770.1	Fujimoto *et al*., 2000
	P0010B10.11	Kinase-like protein receptor	BAB63567.1	Zhu, 2002
	B1070A12.1	Serine/threonine kinase	BAB92578.1	Xiong and Zhu, 2001
	B1070A12.2	Receptor kinase	BAB92579.1	Xiong *et al.*, 2002
	B1070A12.5	Receptor kinase	BAB92581.1	Xiong *et al*., 2002
	B1100D10.29	Diacylglycerol kinase	BAB92552.1	Arisz *et al*., 2003
Ca^{2+}	B1100D10.24	Cyclic nucleotide and calmodulin-regulated ion channel		Urao *et al*., 1994
Signalling	B1070A12.7	1,4-benzoquinone reductase	BAB92583.1	Choi *et al*., 2002
	P0415A04.13	Peroxidase-like protein contains EST AU075654 (E1982)		Kim *et al*., 2003
	P0557A01.35	Polyphenol oxidase	BAB89784.1	Zhang and Kirkham, 1994
	P0684C02.3	Polyphenol oxidase	BAB89047.1	Zhang and Kirkham, 1994
	B1070A12.14	Auxin-responsive GH3	BAB92590.1	Kovtun *et al*., 2000
Transcript	P0010B10.12	Helicase-like transcription factor	BAB63568.1	Zhu, 2002
	P0699H05.22	Ethylene-responsive element binding factor 3		Fujimoto *et al*., 2000
	P0415A04.24	Nuclear transport factor 2	BAB90110.1	Seki *et al*., 2002
Vacuolar	P0010B10.27	Vacuolar sorting-associated protein		Gaxiola *et al*., 2001

DISCUSSION

In this study, we anchored rice QTL maps to the rice physical map to identify drought stress tolerance candidate genes based on candidate QTL position. We chose the sd1 region of chromosome 1 for our attempt to link drought stress tolerance genotype to phenotype using bioinformatics because of strong combined genetic evidence for the existence of a large effect QTL for stress tolerance and because of the relatively complete rice genome sequences in the region. A set of candidate genes of known or inferred function were identified in this region using rice genome annotation. Published literature supports candidacy of some of these genes in drought stress response.

Fig.1 shows one generic drought signal pathway (Zhu, 2002) against which the candidate genes identified in this study could potentially be assigned. This pathway has three classes of genes: (1) signal perception, such as P0557A01.8; (2) generation of second messengers, such as B1070A12.7; and (3) gene expression. Candidate genes potentially implicated in this pathway are listed on the right hand side of the Fig.1.

Another possible pathway for drought signals is the Salt-Overly-Sensitive (SOS) pathway (Xiong *et al*., 2002), as shown in Fig.2. Hypothetically, a myristoylated calcium-binding protein encoded by SOS3 may sense a drought stress or salt-elicited calcium signal and translate it downstream; SOS3 interacts with and activates SOS2, a serine/threonine protein kinase. SOS2 and SOS3 regulate the expression level of SOS1, a salt tolerance effecter gene encoding a plasma membrane Na^+/H^+ antiporter. In our study, we found several genes that could act in this pathway: a vacuolar protein, a serine/threonine kinase, three kinds of putative receptor kinase genes and a putative diacylglycerol kinase gene.

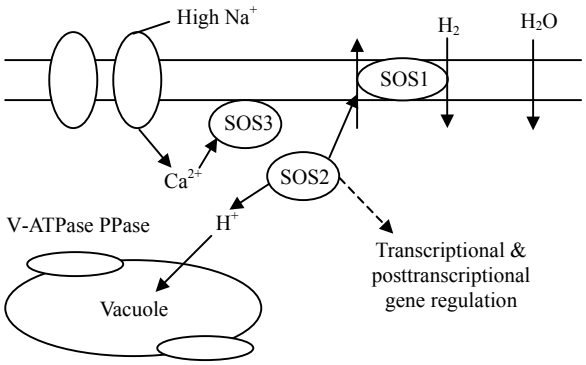

Fig.2 Regulation of ion homeostasis by the SOS pathway

Endogenous abscisic acid (ABA) levels have been reported to increase as a result of water deficit in many physiological studies, and therefore ABA is thought to be involved in the signal transduction. ABA-dependent and ABA-independent signal pathways in the activation of stress-inducible genes under dehydration conditions have been reported (Shinozaki and Yamaguchi-Shinozaki, 1997), as shown in Fig.3. We identified an ethylene-responsive element binding factor (EREBP) gene (P0699H05.22) that could be a candidate related to this pathway. EREBP-like genes are known to be induced by a variety of abiotic stresses, including drought and chilling stresses (Gilmour *et al*., 1998).

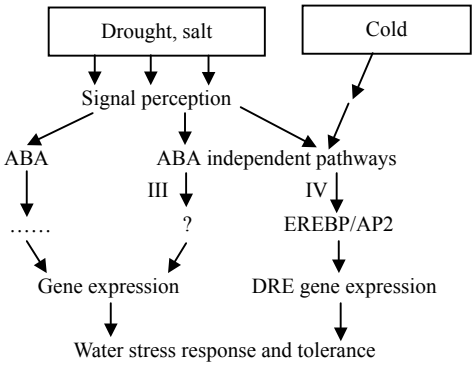

Fig.3 ABA-dependent and ABA-independent pathway

A few of the smaller gene clusters were composed of a single family of closely related genes underlying one QTL, and most of these are within 1–2 cM. Thus, we investigated this region for gene family and found that 4 rust resistance protein clusters on BAC clone (Accession number: B1100D10). These genes have above 89% identity to proteins as shown by ClastalW tool with default parameters. The rust resistance family in this region indicated that it may related to disease resistance.

One major challenge is to discover the drought candidate genes of not only those genes for which functions are already known, but also those with still unknown functions. Clearly, for such genes, fine mapping and functional genomic experimentation will be required to clarify their candidacy.

ACKNOWLEDGMENTS

The authors of this paper would like to ac-

knowledge the kind advice of Renee Lafitte, Zhikang Li and John Bennett in this project. We thank Aixia Ren for assistance with the pathway studies. Some of the drought panicle EST sequences and annotation were obtained from the Hans Bonhert laboratory at the University of Illinois. Additional drought panicle EST sequence gene annotation was generated by S. Rudd of the Munich Information on Protein Sequences (MIPS) center.

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