Gene Expression Profiling in Rice Infected with Rice Blast Fungus using SAGE

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(Received on July 1, 2008; Accepted on November 5, 2008)

Rice blast disease, caused by the pathogenic fungus Magnaporthe grisea, is a serious issue in rice (Oryza sativa L.) growing regions of the world. Transcript profiling in rice inoculated with the fungus has been investigated using the transcriptomics technology, serial analysis of gene expression (SAGE). Short sequence tags containing sufficient information which are ten base-pairs representing the unique transcripts were identified by SAGE technology. We identified a total of 910 tag sequences via the GenBank database, and the resulting genes were shown to be up-regulated in all functional categories under the fungal biotic stress. Compared to the compatible interaction, the stress and defense genes in the incompatible interaction appear to be more up-regulated. Particularly, thaumatin-like gene (TLP) was investigated in determining the gene and protein expression level utilizing Northern and Western blotting analyses, resulting in an increase in both the gene and the protein expression level which arose earlier in the incompatible interaction than in the compatible interaction.

Keywords: abiotic stress, Magnaporthe grisea, Oryza sativa, pathogenesis-related gene, rice blast, SAGE

One of the most devastating diseases in rice, called rice blast, is caused by Magnaporthe grisea (Hamer and Talbot, 1998; Kim et al., 2001). The rice blast disease is a very serious and recurrent issue in rice-growing regions of the world (Talbot, 2003). The infection usually occurs on rice plant leaves where the fungal spores of M. grisea land and attach themselves using the fungal unique adhesive structure called an appressorium (Hamer et al., 1988) to penetrate rice leaf surface by building high turgor pressure (De Jong et al., 1997). Every year, the rice blast infection has the potential to affect the amount of harvested rice by which about sixty million people can be fed (Zeifler et al., 1991). In an effort to remedy this agricultural crisis, finding the genes that are able to defend against the fungal biotic stress would be of great value. It is an obvious interest to determine which genes would be involved in the regulation and with what gene expression level in response to M. grisea infection. Transcriptomics can be used to determine the gene expression level of the messenger RNA (mRNA) in a given cell population. Transcriptomics is the study of the transcriptome, which is the set of all mRNA or transcripts produced in a population of cells. The transcriptome varies under stress conditions because all mRNA transcripts in a cell are a reflection of the genes that are being actively expressed under any stress conditions. Two technologies can be useful tools for transcriptomics. One of the technologies is microarray analysis, which utilizes labeled cDNAs hybridized to an array of DNA elements as probes affixed to a solid support. Using microarray analysis, high densities are achievable and enable the measurement of over 10,000 genes. Microarray is one of the popular technologies that contain information derived from hundreds or even thousands of samples. As another approach to transcriptomics, serial analysis of gene expression (SAGE) can be used, which has been developed by Velculescu et al. (1995). SAGE technology is also a powerful tool that allows the analysis of overall gene patterns. The level of gene expression of an organism in a variety of normal, developmental, and disease states can be quantified by SAGE technology in the same way as microarray technology. The principle of the SAGE methodology is to use a short sequence tag containing sufficient information to uniquely identify a transcript. The size of the short sequence tag is normally between ten and fourteen base pairs (bp) that will likely be chosen from a unique position within each transcript. The tags will then be linked together to form long serial tags that have multiple pieces of transcript information. After cloning the long serial tags, we can quantify the number of tags, which are demonstrating the gene expression levels of the corresponding transcripts. SAGE tags, therefore, are used not only for gene identification, but also for measuring

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the relative abundance of their cognate transcripts within the mRNA population based on the number of occurrences of a given SAGE tag.

In this study, we prepared three different kinds of rice leaves: leaves that grew under no stress conditions (as a control), leaves that conferred resistance to *M. grisea* infection (called an incompatible interaction) and leaves that displayed susceptibility to the fungal infection (called a compatible interaction). SAGE technology was applied to the prepared leaves in an effort to find the genes up-regulated in response to the *M. grisea* infection. We analyzed the types of genes by grouping them into specific categories, permitting us to identify the resistant genes. Northern and Western blotting analyses for a gene of interest were carried out for detailed information.

### Materials and Methods

#### Plant materials.
Rice (*Oryza sativa*) seedlings with 4-5 leaf stages grown under natural light in a greenhouse at a temperature of 28°C were used for inoculation of whole plants with blast fungus *M. grisea*. *M. grisea* was sprayed onto the rice leaves as described previously (Kim et al., 2004a). The inoculated plants were kept in a humidity chamber at 28°C and harvested at defined time periods.

#### Restriction endonuclease.
The restriction endonuclease Sau3A1 (restriction recognition site: /GATC; the slash (/) represents the cleavage site) was used as an anchor. The type IIa restriction enzyme (also known as a tagging enzyme) FokI cleaves the sites 9-13 base pairs away from the recognition site (recognition site: GGATG (9-13))/.

#### Primer and Linker.
Linkers should contain three important sequences: the appropriate anchoring enzyme overhang, a recognition site for a type IIs restriction enzyme, and a priming site for PCR amplification (Madden et al., 2000). Two SAGE linkers attached with biotinylated oligos (20 dT) (Integrated DNA Technologies) were used. SAGE linker 1 was formed through hybridization of oligonucleotides 1A (forward direction): 5'-TTTGGCCAGGTCACCTC-TTCTCGGATACCTCGTCACACAGAG-3', and 1B (reverse direction): 5'-GATCATCCTGTGACGAGATACCGAG-AGAGTGCACCTG-3'. SAGE linker 2 was obtained from 12% native polyacrylamide gel. The sequence of oligonucleotide on the beads. Oligonucleotides were purified from 12% native polyacrylamide gel. The sequence of cognate PCR primers for linkers 1 and 2 were 5'-GCCAGGCACCTCTTCTCAGGA-3' and 5'-GCTCTGGAACCTCT-TGCTCGT-3', respectively.

#### RNA isolation and cDNA synthesis.
Total RNA was extracted from rice leaves 24 h after inoculation with *M. grisea* by an SDS-phenol extraction protocol (Datson et al., 1999; Munasinghe et al., 2001; Yamamoto et al., 2001). miRNAs containing polyA were isolated using Oligotex-dT column (Qiagen Inc.) as recommended by the manufacturer. Five micrograms of the mRNA were used for double-stranded cDNA synthesis with a cDNA synthesis kit (Life Technologies Inc.). Protocols supplied with the kits were followed, except for the use of biotinylated oligo-dT.

#### SAGE procedure.
SAGE procedure was performed according to the original protocol (Velculescu et al., 1995) with some modifications (Fig. 1). The cDNA was digested with the anchoring enzyme Sau3A1 (New England Biolabs) and captured with the 3' end cDNA through binding to streptavidin-coated magnetic beads (New England Biolabs). The streptavidin-bound cDNA was divided into two fractions. Linkers A and B were ligated to the captured 3' end cDNA, and SAGE tags adjacent to the linkers were released from beads by Fok1 (New England Biolabs) digestion. The released cDNA tags were blunt-ended, and the two fractions were then ligated to each other by using T4 DNA ligase (5 U/µL) purchased from Gibco BRL. Ditags were amplified by PCR using ExTaq DNA polymerase purchased from TaKaRa Co., and specific primers (5'-GCCAGGCACCTCTTCTCAGGA-3' and 5'-GCTCTGGAACCTCTTGCTCGT-3') were used for the biotinylated linker. The sample derived from PCR was loaded on ten lanes of 12% polyacrylamide gel (Margulies et al., 2001). The region around 100 bp was excised across all ten lanes of the gel; we then removed the polyacrylamide using SpinX columns (Costar Co.) (Angelastro et al., 2000). Amplified fragments (24-28 bp) were released from the linkers and purified again from polyacrylamide gels. The purified ditags were purified again using the affinity bead. Tag concatamers were obtained by ligation of purified ditags (Powell, 1998; Kenzelmann and Muhlemann, 1999). Before cloning to the pBluescript SK(+) vector (Stratagene), concatamers were size-fractionated (>300 bp). *E. coli* XL1 blue competent cells were transformed with the subcloned plasmids. Colonies on the Luria-Bertani medium containing 50 µL/mL of ampicillin were screened for the plasmids (van den Berg et al., 1999).

#### Sequencing and analysis of clones.
PCR with vector-