Microarrays as a tool to identify untagged deletions in rice

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Introduction

The rice lesion mimic mutant *spl1,* which spontaneously exhibits necrotic lesions, shows enhanced resistance to important rice pathogens, including *Magnaporthe grisea* (rice blast) and *Xanthomonas oryzae* pv. *oryzae* (bacterial blight). Many lesion mimic mutants, which have been discovered in plant species including *Arabidopsis*, *Zea mays*, *Hordeum vulgare* and *Triticum sp.*, show enhanced resistance to pathogen invasion. To study the molecular basis for the broad spectrum resistance exhibited by the rice *spl1* mutant, we examined changes in gene expression and initiated work to identify the gene causing the phenotype.

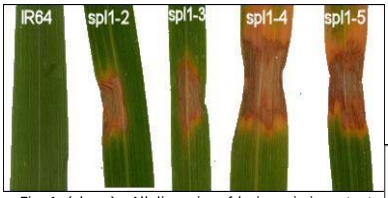
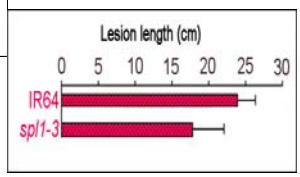


Fig. 1 (above) – Allelic series of lesion mimic mutant *spl1*. Orange/brown necrotic lesions of irregular shape are a hallmarks of the mutation. Shown are the wild type (IR64) and mutants generated by fast neutron (*spl1-2,* N-1856), gamma ray (*spl1-3*, GR-650), and diepoxybutane (*spl1-4,* D6-1137 and *spl1-5,* D6-2943).

Fig. 2 (below) – Lesion mimic mutant *spl1-3* shows enhanced resistance to the bacterial blight pathogen *X. oryzae* pv. *oryzae* as evidenced by the overall decrease in lesion length (cm). Plants were inoculated using the leaf-clipping method.



In a screen of >30,000 chemically and physically induced mutations in indica cultivar IR64, more than ten mutants with the *spl1* phenotype were found. The four mutations in Fig. 1 were shown to occur in the same gene by complementation analyses. Inoculation of the *spl1* mutants with *X. o.* pv. *oryzae* showed reduced lesion lengths compared to IR64, the wild type (Fig. 2) suggesting the lesion mimic mutants may provide valuable insight into rice defense response pathways.

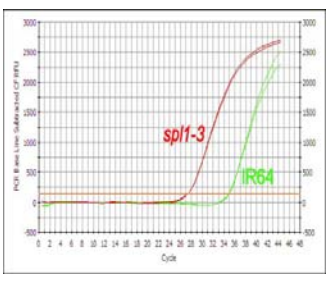
Changes in gene expression

Expression studies of *spl1-3* using Agilent's Rice Oligo Microarray (representing more than 22,000 full length cDNAs from the KOME full length cDNA database, http://cdna01.dna.affrc.go.jp/cDNA/) showed many genes differentially regulated across different leaf ages. To model changes in gene expression as a function of lesion development, the experiment included three sequential leaf positions from 45 day-old plants, with leaf one being the youngest fully expanded leaf.

Differentially expressed genes included membrane associated kinases, defense response genes, genes responsible for maintenance of cell walls, sterol metabolism enzymes, transporters and photosynthetic genes. Our results indicate that the mutation causes aberrant signaling or constitutive activation of salicylate-mediated (SA) defense response pathways, indicated by significant up-regulation of genes like PR1 (Fig. 3).

Another utility for the expression profiles is their potential for identification of deleted genes. Volcano plots of the data show genes that may be deleted, as indicated by large, negative $\log_2$ ratios of mutant to wild type and highly significant p-values for tests on multiple technical replicates (Fig. 4).

Fig. 3 (below) – Real time PCR amplification of PR1 – an indication of SA-pathway activation. Lesion mimic mutant spl1-3 (GR-650) in red and wild type IR64 in green. The data indicate approximately 7-fold up-regulation in the mutant.



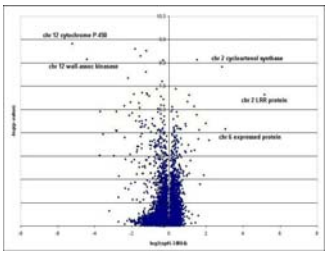


Fig. 4 (above) – Volcano plot of expression data. Log2 ratios of mutant to wild type signal are plotted on the x-axis and -log of p-value for 4 technical replicates are represented. This data represents expression in the youngest fully expanded leaf in 45 day old plants.

Detecting deletions by microarray

Genomic DNA from the allelic series of spl1 mutants was purified by CsCl-gradient centrifugation, labeled by random prime labeling and hybridized to the Affymetrix Rice GeneChip® microarray. In preliminary experiments, we determined that the DEB-generated mutants were usually too small to detect by this method. For this reason, data presented here is based on the confirmed allelic radiation-induced mutants (N-1856 and GR-650 – spl1-2 and spl1-3 respectively) and putative alleles N-2045 and GR-9799.

To discover potentially deleted genes, we compared perfect match(PM) probe intensities to their corresponding mismatch (MM) probe intensities to indicate presence or absence of the probe in the genomic DNA. Ratios were calculated that counted the number of probes in a probe set such that the PM intensity was less than or equal to MM intensity divided by the total number of probes in the probe set. The same ratio calculated from the wild type arrays was subtracted from the ratio from the mutant arrays.

No statistical tests are involved, but the data can be quickly validated by attempting to amplify fragments potentially called deleted in the mutants. Using this protocol, we identified deleted regions throughout the throughout the genome (Fig. 5). Using data from rice lines harboring different mutant alleles, we found a cluster of potentially deleted genes on chromosome 12 in the position indicated by mapping experiments as containing the gene responsible for the spl1 phenotype (Fig. 6).

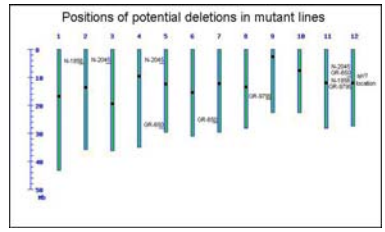


Fig. 5 – Chromosomal positions of potential deletions. While other genomic deletions are predicted in the allelic mutants, the only overlapping region of predictions is on chr. 12 in the region previously identified as containing the *spl1* gene. Additional mutants showing phenotype also have predicted overlapping deletions.

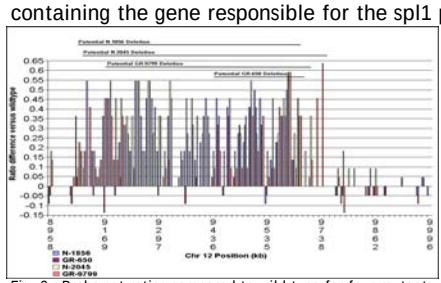


Fig. 6 – Probe set ratios compared to wild type for four mutants – two confirmed as *spl1* alleles (N-1856 and GR-650) and two showing *spl1* phenotype (N-2045 and GR-9799). Values are calculated as (# PM ≤ MM /# of probes in probe set)$_{mutant}$ - (# PM ≤ MM /# of probes in probe set)$_{wildtype}$. Though the mutants showing the *spl1* phenotype had not been confirmed as alleles by complementation, the cluster of chr. 12 deletions overlaps with the other mutants.

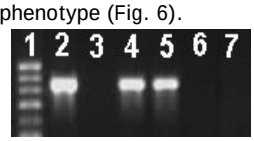


Fig. 7 – Confirmation of deletion. Lane 1 – 1kb+ ladder, Lane 2 – IR64 wild type, Lane 3 – negative control, Lane 4 – D6-1137 *spl1* allele, Lane 5 – D6-2945 *spl1* allele, Lane 6 – GR-650 *spl1* allele and Lane 7 – N-1856 *spl1* allele. Amplification fails in the last two mutants. This gene was indicated as deleted in these two mutants. However, it does amplify in the diepoxybutane mutants. This gene is a zinc finger transcription factor previously implicated as a candidate for *spl1* in a mapping study of the mutant allelic series (Liu et al, 2004).

Conclusions

- From the expression studies, we conclude that the gene responsible for the *spl1* phenotype is likely a negative regulator of cell death. The mutant plants appear to be constitutively signalling through the SA-mediated response pathways, as indicated by consistent observation of SA marker *PR1* up-regulation.
- Expression profiling by micorarray may be useful for detecting deleted genes, especially when there are multiple replicates available for analysis. Volcano plots give a rapid visual analysis of differentially expressed genes and may quickly identify candidates for deletions.
- Hybridizing genomic DNA to Affymetrix arrays allows detection of large deletions. Unlike expression arrays, which depend on relative amounts of transcript, the screen for deletions by genomic DNA hybridization is qualitative. Our approach to analysis allows for rapid data validation by PCR (Fig. 7).
- While complementation tests have not confirmed some of the mutants as allelic, the microarray data suggest that they harbor a cluster of deletions in the same region on chr. 12 as those confirmed allelic.

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