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Differential Gene Expression of Soybean [*Glycine max* (L.) Merr.] in Response to *Xanthomonas axonopodis* pv. *glycines* by Using Oligonulceotide Macroarray

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Abstract

Xanthomonas axonopodis pv. *glycines (Xag)* is a pathogen that causes bacterial leaf pustule (BLP) disease in soybeans grown in Korea and the southern United States. Typical and early symptoms of the disease are small, yellow to brown lesions with raised pustules that develop into large necrotic lesions leading to a substantial loss in yield due to premature defoliation. After *Xag* infects PI 96188, only pustules without chlorotic haloes were observed, indicating the different response to *Xag*. To identify differentially expressed genes prior to and 24 hr after *Xag* inoculation to PI 96188 and BLP-resistant SS2-2, an oligonucleotide macroarray was constructed with 100 genes related to disease resistance and metabolism from soybean and *Arabidopsis*. After cDNAs from each genotype were applied on the oligonucleotide macroarrays with three replicates and dye swapping, 36 and 81 genes were expressed as significantly different between 0 hr and 24 hr in PI 96188 and SS2-2, respectively. Six UniGenes, such as the leucine-rich repeat protein precursor or 14-3-3-like protein, were selected because they down-regulated in PI 96188 and up-regulated in SS2-2 after *Xag* infection, simultaneously. Using tubulin and cDNA of Jangyeobkong (BLP-susceptible) as controls, the oligonucleotide macroarray data concurred with quantitative real-time RT-PCR (QRT RT-PCR) results in most cases, supporting the accuracy of the oligonucleotide macroarray experiments. Also, QRT RT-PCR data suggested six candidate genes that might be involved in a necrotic response to *Xag* in PI 96188.

Key words: bacterial leaf pustule, disease resistance, macroarray, soybean, *Xanthomonas axonopodis* pv. *glycines*

Introduction

Bacterial leaf pustule (BLP) caused by *Xanthomonas axonopodis* pv. *glycines (Xag)* is a soybean (*Glycine max* (L) Merr.) disease that occurs in Korea and the southern United States, where hot and humid weather conditions are prevalent (Narvel et al. 2001). Typical and early symptoms of the disease are small, yellow to brown lesions with a raised pustule, which develop into large necrotic lesions that potentially cause substantial losses in yield through premature defoliation (Hartwig and Johnson 1953; Groth and Braun 1986; Kennedy and Tachibana 1973; Weber et al. 1966). A single recessive gene (*rxp*), linked to the malate dehydrogenase (*Mdh*) locus, controls BLP resistance in a soybean cultivar, CNS (Bernard and Weiss 1973; Hartwig and Johnson 1953; Palmer et al. 1992). Two independent genetic mapping studies revealed that the *Rxp* locus was tightly linked with Satt372 on LG D2 (Narvel et al. 2001; Van et al. 2004).

After *Xag* 8ra infected PI 96188, this soybean genotype showed only pustules without chlorotic haloes, although PI 96188 was susceptible to BLP (Han et al. 2001). Regardless of the races of *Xag* (SL1017, SL1018, SL1045, SL1157, and SL2098), this response was observed only in PI 96188. The previous study revealed that this necrotic lesion developed slowly as the pustule grew larger (Han et al. 2001). Five SSR markers on LG D2 were not associated with this novel necrotic symptom in PI 96188 and no co-segregation between *rxp* and this response was

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observed in recombinant inbred lines (RILs) derived from a cross of PI 96188 and BLP-susceptible Jinju 1 (Park 2003). Thus, these results suggest that a new gene other than *rxp* must be responsible for this necrotic response to *Xag* in PI 96188.

The genes involved in the pathway for response to *Xag* infection including *Rxp* were not characterized at the molecular level, although many BLP-resistant soybean genotypes have been developed. This interaction between plants and pathogens at the transcriptional level is a major step towards understanding plant defense responses against the pathogen (Kazan et al. 2001). In particular, genome-wide transcriptional profiling can be helpful in the identification of the genes responding to the plant pathogen (Kazan et al. 2001; Rushton and Somssich 1998). Microarrays or 'chips', along with high-density arrays on nylon filters and serial analysis of gene expression (SAGE), are techniques to study global differences in gene expression (Vodkin et al. 2004). Many cDNA and oligonucleotide microarray experiments have studied plant responses to pathogens (De Boer et al. 2002; Katagiri 2004; Kazan et al. 2001; Lee et al. 2004; Lopez et al. 2005; Sato et al. 2007; Tao et al. 2003; van Wees et al. 2003), and oligonucleotide microarray analysis has previously been used to study transcriptional regulation and functional genomics (Giles and Kipling 2003; Glazebrook et al. 2003; Kane et al. 2000).

To identify differentially expressed genes before and 24 hr after *Xag* inoculation in PI 96188, which displayed the only necrotic symptom to BLP infection and in BLP-resistant SS2-2, an oligonucleotide macroarray was constructed with 100 genes related to disease resistance and metabolism from soybean and *Arabidopsis*. Quantitative real-time RT-PCR was also performed on genes that were differentially expressed against *Xag* to validate the macroarray results.

Materials and Methods

Plant materials and preparation of inoculum

Two soybean genotypes were used for the oligonucleotide macroarray experiments: PI 96188, collected in Korea showing only necrotic symptom to *Xag* and SS2-2, a BLP-resistant and supernodulating genotype developed from Sinpaldalkong 2 by EMS mutagenesis (Lee et al. 1997). Additionally, the BLP-susceptible cultivar Jangyeobkong, originally Suweon 86, was included in quantitative real-time RT-PCR as a control (Choi et al. 1979). All plants were grown in pots at 25 °C in a greenhouse.

The *Xag* 8ra strain was cultured on peptone-sucrose agar (PSA; 10 g L^{-1} bacto-peptone, 10 g L^{-1} sucrose, 1 g L^{-1} Na-glutamate, and 15 g L^{-1} agar) with rifampicin (100 μg ml^{-1}) at 28 °C for two days (Hwang et al. 1992). After the bacterial culture was diluted with 10 mM $MgCl_2$ and adjusted to 5 x 10^8 cell formation units (CFU) ml^{-1}, the suspension was sprayed on fully expanded, tagged young leaves of one-month-old soybean plants using an atomizer. These inoculated plants were kept in a greenhouse with a high relative humidity to aid development of BLP symptoms.

Oligonucleotide design and macroarray printing

To specify which genes are differentially expressed in response to bacterial pathogens instead of surveying whole expressed genes, 100 UniGenes from soybean were selected from the NCBI UniGene database (Table 1). Of the 100 oligonucleotides, 45 were designed from disease resistance genes and 35 were selected from genes related to metabolism. In addition, oligonucleotides from other sources such as the cell cycle, nodulation, genes related to stress responses and heat shock, calmodulin, and lipoxygenase were synthesized, including [a] DNA as a control. After sequences of these 100 ESTs were obtained, RepeatMasker (http://ftp.genomewashington.edu/RM/webrepeatmakerhelp.html) was used to remove repeats, and 50-mer oligonucleotides were designed from the regions of low-complexity sequence defined by RepeatMasker (Table 2). All oligonucleotides were filtered by the following parameters: 1) each oligonucleotide is in the 3' end of the available gene sequences; 2) the ratio of any single nucleotide (A, T, G, or C) is less than 50% of the total length of each oligonucleotide; 3) each oligonucleotide is scanned for less than seven contiguous single nucleotide base repeats and potential secondary structures; 4) the ratios of contiguous AT or GC should not exceed 25%; 5) a probe is selected if the score is less than 30 in the BLAST search to remove redundancy; 6) T_m values of all oligonucleotides fall within ±5 °C of each other. Tm values of oligonucleotides ranged from 72.7 to 90.1 °C with an average of 75.7 °C.

The oligonucleotides were synthesized and stored in 384-well plates. Samples were spotted on UltraGAPS™ coated slides (Corning Inc., Corning, NY, USA) with three replicates as adjacent spots per glass slide using a Gen III array spotter (Amersham Biosciences, Piscataway, NJ, USA). Blocking, quality control, and other processes were according to the manufacturer's instructions.

Probe synthesis and hybridization

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from *Xag* non-inoculated (0 hr) or inoculated (24 hr) soybean leaves. Fifty μg of total purified RNA was mixed with 1 ng of control λmRNA and 1.5 μg of oligo(dT)15 primer (Genotech Co. Ltd., Daejun, Korea). The resulting annealing mixture was incubated at 70 °C for 5 min and chilled immediately on ice for 2 min. The components of the labeling reaction mix were 3 μl of 1 mM Cy3(C)/Cy5-dUTP(T) (Amersham Biosciences, Piscataway, NJ, USA), 8 μl of 5x AMV reverse transcriptase (RT) buffer, 4 U/μl of 10x low dT dNTP (5 mM dATP, 5 mM dGTP, 5 mM dCTP, and 2 mM dTTP), 1 μl of RNase inhibitor (40 U/μl), and 200 U of AMV

Table 1. List of selected genes related to disease resistance and metabolism in plants.

UniGene Cluster_ID	Description	Source
Gma.2	*G. max* aminoacyl peptidase mRNA, complete cds	Other
Gma.3	*G. max* seed maturation protein (GmPM3) mRNA, complete cds	Metabolism
Gma.16	*G. max* mRNA for glycinamide ribonucleotide transformylase	Other
Gma.36	*G. max* maturation-associated protein (MAT1) mRNA, complete cds	Metabolism
Gma.48	*G. max* heat shock protein (SB100) mRNA, complete cds	Other
Gma.66	*G. max* clone c27 resistance protein mRNA, partial cds	R gene
Gma.67	*G. max* clone 19.2 resistance protein mRNA, partial cds	R gene
Gma.171	*G. max* mRNA for resistance protein, partial	R gene
Gma.197	*G. max* asparagine synthetase mRNA, complete cds	Other
Gma.219	*G. max* chlorophyll a/b-binding protein (cab3) mRNA, nuclear gene encoding chloroplast protein, complete cds	Metabolism
Gma.289	*G. max* Ribulose-1,5-bisphosphate carboxylase small subunit rbcS3	Other
Gma.325	*G. max* mRNA for urease (Ure gene)	Other
Gma.338	*G. max* peroxidase (Prx2b) mRNA, complete cds	Other
Gma.383	ESTs, Weakly similar to leucine rich repeat protein, putative [*Arabidopsis thaliana*]	R gene
Gma.384	ESTs, Weakly similar to disease resistance protein EDS1 [*A. thaliana*]	R gene
Gma.399	ESTs, Weakly similar to disease resistance protein, putative [*A. thaliana*]	R gene
Gma.483	Dihydrofolate reductase-thymidylate synthase, bifunctional enzyme [*G. max*, seedling, mRNA, 1794 nt]	Other
Gma.596	*G. max* 14-3-3-like protein mRNA, complete cds	Metabolism
Gma.1043	ESTs, Weakly similar to NBS/LRR disease resistance protein, putative [*A. thaliana*]	R gene
Gma.1458	ESTs, Moderately similar to pectinesterase, putative [*A. thaliana*]	R gene
Gma.1460	ESTs, Moderately similar to HMD1_ARATH 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (HMGR1) [*A. thaliana*]	Metabolism
Gma.1555	*G. max* early light-induced protein (ELIP) mRNA, complete cds	Metabolism
Gma.1689	*G. max* mRNA for 6-phosphogluconate dehydrogenase, complete cds	Other
Gma.1778	ESTs, Weakly similar to unknown protein [*A. thaliana*]	R gene
Gma.1814	*G. max* (Rab1p) mRNA, complete cds	Other
Gma.1872	*G. max* sucrose binding protein (sbp) mRNA, complete cds.	Other
Gma.1950	*G. max* isoflavone reductase homolog 2 (IFR2) mRNA, complete cds	Other
Gma.1965	*G. max* calmodulin (SCaM-3) mRNA, complete cds	Other
Gma.2019	*G. max* lipoxygenase (vlxC) mRNA, complete cds	Other
Gma.2044	*G. max* maturation-associated protein (MAT9) mRNA, complete cds	Metabolism
Gma.2102	ESTs, Weakly similar to putative disease resistance response protein [*A. thaliana*]	R gene
Gma.2178	ESTs, Weakly similar to disease resistance protein-like [*A. thaliana*]	R gene
Gma.2253	*G. max* seed maturation protein PM34 (PM34) mRNA, complete cds	Metabolism
Gma.2308	*G. max* Isoflavone synthase 2 (ifs2)	Other
Gma.2359	*G. max* beta-1,3-endoglucanase mRNA, complete cds	Metabolism
Gma.2371	ESTs, Highly similar to transcription factor, putative [*A. thaliana*]	Metabolism
Gma.2440	ESTs, Moderately similar to Cf-5 disease resistance protein - like [*A. thaliana*]	R gene
Gma.2693	*G. max* arginine decarboxylase mRNA, complete cds	R gene
Gma.2718	ESTs, Moderately similar to unknown protein [*A. thaliana*]	R gene
Gma.2744	ESTs, Weakly similar to putative protein [*A. thaliana*]	R gene
Gma.2837	ESTs, Weakly similar to disease resistance protein [*A. thaliana*]	R gene
Gma.2854	*G. max* mRNA for uricase (Nod-35), complete cds	Other
Gma.2855	ESTs, Moderately similar to putative translation initiation factor [*A. thaliana*]	Metabolism
Gma.3149	ESTs, Weakly similar to disease resistance RPP5 like protein (fragment) [*A. thaliana*]	R gene
Gma.3221	ESTs, Weakly similar to putative protein [*A. thaliana*]	R gene
Gma.3717	ESTs, Moderately similar to acyl CoA thioesterase, putative [*A. thaliana*]	Metabolism
Gma.3858	*G. max* farnesylated protein GMFP5 mRNA, partial cds	R gene
Gma.4045	*G. max* mRNA for glyoxalase I	Metabolism
Gma.4206	*G. max* Isoflavone synthase 1 (ifs1)	Other
Gma.4300	*G. max* Chalcone synthase	Other
Gma.4332	ESTs, Weakly similar to disease resistance response protein-like [*A. thaliana*]	R gene
Gma.4564	ESTs, Weakly similar to T48928 disease resistance protein-like [A. thaliana]	R gene
Gma.4589	ESTs, Moderately similar to HMD1_ARATH 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (HMGR1) [*A. thaliana*]	Metabolism R gene
Gma.4639	ESTs, Weakly similar to non-race specific disease resistance protein (NDR1) [*A. thaliana*]	Metabolism
Gma.4699	ESTs, Moderately similar to putative protein [*A. thaliana*]	
Gma.4954	ESTs, Weakly similar to disease resistance protein-like [*A. thaliana*]	R gene
Gma.5064	ESTs, Moderately similar to 1-aminocyclopropane-1-carboxylate oxidase [*A. thaliana*]	Other
Gma.5085	ESTs, Weakly similar to disease resistance protein, putative [*A. thaliana*]	R gene
Gma.5099	ESTs, Weakly similar to dirigent protein, putative [*A. thaliana*]	R gene
Gma.5550	ESTs, Weakly similar to TMV resistance protein-like [*A. thaliana*]	R gene
Gma.5625	ESTs, Moderately similar to ubiquitin-conjugating enzyme-like protein [*A. thaliana*]	Metabolism
Gma.5672	*G. max* mRNA for Mg chelatase subunit (46 kD), complete cds	Other
Gma.5784	ESTs, Weakly similar to putative disease resistance response protein [*A. thaliana*]	R gene
Gma.6077	ESTs, Weakly similar to T50662 UVB-resistance protein UVR8 [imported] [*A. thaliana*]	R gene
Gma.6101	*G. max* calmodulin (SCaM-1) mRNA, complete cds	Other
Gma.6289	ESTs, Moderately similar to fumarylacetoacetate hydrolase-like protein [*A. thaliana*]	Metabolism
Gma.6450	ESTs, Moderately similar to guanine nucleotide regulatory protein, putative [*A. thaliana*]	Metabolism
Gma.6692	ESTs, Moderately similar to T01855 probable chaperonin-containing TCP-1 complex gamma chain F9D12.18 [*A. thaliana*]	Metabolism
Gma.6797	ESTs, Moderately similar to putative UDP-glucose:glycoprotein glucosyltransferase [*A. thaliana*]	Metabolism
Gma.6883	ESTs, Weakly similar to RPP1 disease resistance protein - like [*A. thaliana*]	R gene
Gma.7199	ESTs, Moderately similar to T09913 X-Pro dipeptidase homolog T16L4.10 (fragment) [*A. thaliana*]	Metabolism
Gma.7831	*G. max* proteasome IOTA subunit mRNA, complete cds	Metabolism
Gma.8162	ESTs, Moderately similar to prolyl endopeptidase, putative [*A. thaliana*]	Metabolism
Gma.8292	ESTs, Weakly similar to disease resistance protein-like [*A. thaliana*]	R gene
Gma.8293	ESTs, Highly similar to T47613 ABC transporter-like protein [*A. thaliana*]	Metabolism
Gma.8416	*G. max* nitrate reductase (BCNR-A) mRNA, partial cds	Other
Gma.8424	*G. max* mRNA for late nodulin, complete cds	Other
Gma.8436	*G. max* mRNA for mitotic cyclin	Other
Gma.8443	*G. max* functional candidate resistance protein KR1 (KR1) mRNA, complete cds	R gene
Gma.8456	*G. max* functional resistance protein KR2 mRNA, partial cds	R gene
Gma.8467	*G. max* Shi-shi 51 kDa seed maturation protein (pGmPM10) mRNA, complete cds.	Metabolism
Gma.8474	*G. max* putative resistance protein (L33) mRNA, complete cds	R gene
Gma.8478	*G. max* resistance protein MG13 mRNA, partial cds	R gene
Gma.8479	*G. max* resistance protein MG23 mRNA, partial cds	R gene
Gma.8507	*G. max* clone NTN5c NBS-type putative resistance protein mRNA, partial cds	R gene
Gma.8508	*G. max* clone NTN4c NBS-type putative resistance protein mRNA, partial cds	R gene
Gma.8520	*G. max* mRNA for root nodule acid phosphatase	Other
Gma.8701	ESTs, Moderately similar to argininosuccinate synthase -like protein [*A. thaliana*]	Metabolism
Gma.8781	ESTs, Moderately similar to A49318 protein kinase (EC 2.7.1.37) tousled [*A. thaliana*]	Metabolism
Gma.8782	ESTs, Weakly similar to disease resistance protein, putative [*A. thaliana*]	R gene
Gma.8815	ESTs, Weakly similar to putative disease resistance protein [*A. thaliana*]	R gene
Gma.8866	ESTs, Moderately similar to replication factor C - like [*A. thaliana*]	Metabolism
Gma.9251	ESTs, Highly similar to kinesin-like protein [*A. thaliana*]	Metabolism
Gma.9560	ESTs, Weakly similar to resistance protein RPP13, putative [*A. thaliana*]	R gene
Gma.9601	ESTs, Weakly similar to disease resistance like protein [*A. thaliana*]	R gene
Gma.9636	*G. max* resistance gene analog LM1 pseudogene, mRNA sequence	R gene
Gma.9712	ESTs, Moderately similar to putative hydroxymethyltransferase [*A. thaliana*]	Metabolism
Gma.9827	*G. max* resistance protein KR4 mRNA, complete cds	R gene
Gma.10594	ESTs, Moderately similar to T47840 multi resistance protein homolog [*A. thaliana*]	R gene
Gma.11474	ESTs, Similar to disease resistance protein RPP1-WsB - like protein [*A. thaliana*]	R gene

RT (DCC-BIONET, Sungnam, Korea) in an 18.5-μl total volume. The samples were fluorescently labeled with Cy3 (0 hr) or Cy5 (24 hr after inoculation) dye with three replicates. With the same RNA sample, three additional hybridization experiments were repeated for PI 96188 and SS2-2 with the dyes reversed so that the total mRNA from 0 hr was labeled with the Cy5 dye and the total mRNA from 24 hr was labeled with the Cy3 dye.

After the annealing reaction mixture was mixed with the labeling reaction mixtures and stored at 42 °C for 1 hr with an additional 200 U of AMV RT, the reaction was terminated by addition of 5 μl of 0.5 M EDTA. RNA was hydrolyzed with 10 μl of 1 M NaOH at 37 °C for 10 min and neutralized with 25 μl of 1 M Tris-HCl (pH 7.5). Each reaction mixture was cleaned with a Chromaspin TE100 column (BD Biosciences Clontech,

Mountain View, CA, USA) and 300 μl of 100% EtOH and 10 μl of 3 M NaOAc were added to precipitate the eluted probe. An equal amount (25-30 μg) of each labeled probe was prepared as pellets after dissolving in 5x fragmentation buffer (20 mM Tris-Acetate (pH 8.1), 50 mM KOAc, and 15 mM MgOAc).

The dried pellets were resuspended in 55-60 μl of hybridization buffer [50 mM Na-phosphate (pH 8.0), 50% formamide, 6x SSC, 5X Denhart's solution and 0.5% SDS], denatured at 95 °C for 3 min, and placed on ice for a maximum of 3 min. The labeled probes were applied to pre-hybridized (5x SSC, 0.1% SDS and 0.1% BSA at 42 °C for 1 hr) macroarray slides and incubated in a moist chamber at 42 °C for 24 hr. After hybridization, the slides were washed in 2x SSC/0.1% SDS, 1x SSC, and 0.5 x SSC for 5 min each at 30 °C. The arrays were dried by centrifugation at 1,500 rpm for 2 min.

Macroarray data analysis

Macroarray slides were scanned with a Gen III scanner (Amersham Biosciences, Piscataway, NJ, USA) to visualize the hybridization images. The signal was first normalized using the average ratio between the two channels as visualized in the control spots. The 'R' package (http://lib.stat.cmu.edu/R/CRAN/) was used for background correction along with several additional normalization steps. The fluorescent signals of the spots were scored only after normalization, correction for low intensity, and replicates and dye-swap filtering were applied.

A total of six hybridizations, three replicates, and dye swapping (Quackenbush, 2002) were performed and analyzed using the R language (http://www.r-project.org/). Significant differences between 0 hr and 24 hr stages were detected using paired t-tests in the Statistical Analysis System Software (SAS Institute Inc. 2001) with $\alpha = 0.05$. To calculate adjusted p-values, the false discovery rate (FDR) multiple testing correction (Benjamini and Hochberg 1995) was performed by using 'R' statistical software (http://www.cran.r-project.org/).

Quantitative real-time RT-PCR (QRT RT-PCR)

QRT RT-PCR (iScript™ One-Step RT-PCR Kit with SYBR Green, Bio-Rad Laboratories, Hercules, CA, USA) was used on differentially expressed genes identified by the oligonucleotide macroarray experiments. Primers for QRT RT-PCR were designed and selected using Gene Runner, version 3.05 (Hasting Software, Hastings on Hudson, NY, USA) according to primer criteria described by Bio-Rad protocol. The QRT RT-PCR primer sequences for the internal control were 5'-AAC CTC CTC CTC ATC GTA CT-3' and 5'-GAC AGC ATC AGC CAT GTT CA-3' for the soybean tubulin gene (Tian et al. 2004). Before QRT RT-PCR was conducted to analyze gene expression levels, SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, Carlsbad, CA, USA) was used to test the gene specificity of each primer. The reaction mixtures were made to

Table 2. 50-mer sequence information for oligonucleotide macroarray construction.

UniGene Cluster_ID	Sequence 5' 3'
Gma.2	ACAGACTCTTGGGAGCCATATACTATGTGAGAGAGGCTTTATGCGCTATA
Gma.3	ATCCAATGCACAAGCCTGCTACTGCAGGAACTCGTGTCTAAGTGCCACTG
Gma.16	GTTCCTCTCATCCAAAGCAAAGAAAACCCCAATGAGTTTCGCTGATAATC
Gma.36	GAGGACACAGTGACAAGTAGATGCATCGTGCATGCATGCATATAGTATAT
Gma.48	CTGGGCAGAAGTCCGATATCTTGATTCAGATACCTAATGGACATGCACCT
Gma.66	GGAGTGCATCTGACTTGTAAGGCTAAAGCCTTAGCCCAAAATGAAGCCCT
Gma.67	GCCATTGGGTAGCACTGAGTCATTCAAGCTATTTTGCTCAAGGGCTTTCC
Gma.171	AAAGTTCAAGAAAGGAACCAGGGAAGCGGAGTCGATTGTGGTTTCATGAG
Gma.197	GCACCTGACCTAAAGGCAGCAAAGGAAGTAGCAGACTACATAGGTACTGT
Gma.219	TATGGCTCTTCTACTCACATCCACACTCAGCGCTACTACACATCTCCTGT
Gma.289	CTGGGTATACAAGCCCGCCATGCCTTCACTTGCACCTTATGCATGACGCC
Gma.325	GAGCTATGGGACTGAAGCTGCATGAGGACTGGGGAACTACACCGGCTGCA
Gma.338	AAGCACCATGATCAGGACCCTTGAATTTTCCTCCCCTTGTCTATGATCGA
Gma.383	GGTTCGATGATACATCCAAGTCCGCAAGCCGCTTCATTTTGGACACGGAT
Gma.384	GTACTAGTGTGCTACTCAGTGTGCTTTTTTAAGCCAACTCAGCATATGGT
Gma.399	CCTCCGGTAAGCATTGGAGCCTGGGGCAGTTAGAAAGATGCAATTTCTTG
Gma.483	GGCTGCTGATTTCAAGCTCATAGGCTATGATCCTCACCAGAAGATTGATA
Gma.596	GAAGCCTCTATCTGAATGGTGCACTGCACCTCATCAACTTTGAGTCATTC
Gma.1043	GGGCGGAGATGTTATCCCATTGGAAACTGATCTCAACTCTGCCATGCAAA
Gma.1458	GACCTGCCATACAAAACACAATTTTAGTGGGTGAATGAGGACCCACCTTT
Gma.1460	ACCGGAGGTGCTTGGGCAGACCAATAAAGGGCAGGGCTCTATTCTCTGTG
Gma.1555	CCCCGTACTCCATTACGCCTATATGTAAGTCTATTACATGCCGATCAACT
Gma.1689	GACATAGAGGGGTCTTACCATACTGAGTGGTTCAAGCTTGCCAAACAGTC
Gma.1778	GGCATACGATCTAGCATATCATTGTGCGATCATCCCTTAGAGCCCCTCGA
Gma.1814	TGAGTCCCTCTTGGCGGTTAAAGATGAAACGTCATACCTTACTCCTTTTT
Gma.1872	AATTGATAGGAAGACTCTACCACTTGCCTCATAAGGACCGAAAGGAGAGT
Gma.1950	CCGCACCACCCGTGAATGTGATCCTGTCAATTAACCACTCTTCTTATGTA
Gma.1965	TTCCGGGTTTTCGACAAGGACCAGAATGGGTTCATCTCTGCTGCTGAGCT
Gma.2019	GGCAATACCCTTATGGAGGTTATATCGTGAACCGTCCAACTCTAGCCAGA
Gma.2044	ATAGACACCGATAGGCAACAACATGGGACTACTGGTGGCTATGCCGGTGA
Gma.2102	ACTTATTCGGCTTCAGCACATCCCCTTTTGCCAGTTGGCGTAATACGAAA
Gma.2178	TTGGTAGAACTTTCTAGCTAAGACTGCTTGACCTCTCGAACTGGATAAGC
Gma.2253	TTAATGCATGGCTAACTCACTCAGGTCCTCTCTGCACTGTTAGAGGTGGG
Gma.2308	GTGCTGGGTCCACAAGGACAGATATTGAAGGGTGGTGACGCCAAAGTTAG
Gma.2359	GAACCCCTAAAAGGCCTGGTGCACCCCTTGAAACTTATGTGTTTGCCATG
Gma.2371	CCATCGACTCCGGCAGTTACTTTGTTGCTAAATGTAGTATCTCCTTTGAA
Gma.2440	CTGGAGGAATACCTGCACAACTTGCTAACCATACCAGTCTTGAGATGTTG
Gma.2693	CCCTTGAATGGGCATAAGCGCGGGGGTGGTTTTTACCCACGGAACGGTAA
Gma.2718	CAGCTGCAGGAAGAATTCTTCCTCAAATCTTAGCAGACAGCAACATTGAA
Gma.2744	TTACTTAAAAGACTCATGCCGCAGTCGGTAACCTCGCACATACAAGTCGG
Gma.2837	CATCCATTTGGTGGGCCTCTATGTTCGTACTTTGCATTGTGTCTTGATGG
Gma.2854	TCATCCCTTCTGGTGGATTGCTAGTGCAGCCTAAACTAGCAAACCTGGCC
Gma.2855	ATTGACGGGAACAGAGCTAATCGTGGGTGGGATGTCCTGAGGGAGCAACC
Gma.3149	CTTGCAGGGTCAAATGGACAAGGTGCAAAAGTGGAGGGTGGCTCTCAGAG
Gma.3221	AAAATCCAAAGGCCAGGAACTCCTCAAGAGCAGCAATACGCAAAGGCTCG
Gma.3717	GGTTTTCTTTTTAGTTCTGGCTTGGTCTATAAGGCATGGTGAGACATCTC
Gma.3858	TTGAACGGCTATATACCGCGGGAGTGGGGATTGGGACCGAGATGAGAATT
Gma.4045	CGCTTAGGCTTAAGATACACCTTCAAGGTCGGGTCTTCTTGTGAACGATC
Gma.4206	GTTCCACTTGCAAGGATCGGCGTTGCATCTAAACTCCTTTCTTAATTAAG
Gma.4300	GATAAAAAAGGCCCCCTCCTTCCTAAATTGCCTTAAGTAGAGGCCCCCCT
Gma.4332	AGCAAATGCCCAAAAGAGTGAGAGGATTCCCCCTGAGCCCCGGGATTCAT
Gma.4564	ACGTAGTATTCAGCCGCAGCAGTTTTAAGGTGCAATTGTGGAGGTGTAGT
Gma.4589	ACTGTCTGCTATGATATCATCGCCACTCCGGACGGCGATCCTATGCGCTT
Gma.4639	TACTTCACCGCAGTAGCATAGTCCACACGGTACCACACTTTCCCATCCAG
Gma.4699	CTGCAGCTGTGTGTGATTTTGTAACAAGTCCTTTTGTGACCTGTATATCC
Gma.4954	GACCATATCATGCTTATGCTGACCACTATAGAGATAGTGCATAGTACCGT
Gma.5064	CACCAGTATTGTTGGATCACAAGGCAGAAGATACATGACATGTGTATCCA
Gma.5085	CCCTGGAGCTGCTTCTGCGTCATGGGAGAATCAATTCTCAGATTTCAATG
Gma.5099	AACCATAAGCGTGACAACCTTTTCCGTGGGATAAAAAGGCCCCATCTCTC
Gma.5550	TCCATAACCTTGTGGGCTGATGATGGAAACAGAGACTAACGAGGCCTTCA
Gma.5625	GTCCTTATTAGCTATCGCTAACCTGTACGGCGCTTTATATTGCGTTTATC
Gma.5672	GCTGAGGATCATAGATGTAACACGGTTTGTATTCTGTGGTATTCCATGAG
Gma.5784	CAGGTTTGGTGCAATGGTGTGAACATGATCAGCAATCATGAATGTGTTGA
Gma.6077	ATTTGTCACACATGAGCCAAGCTTTCCCCTCAAACCTCTAAATAGAGACT
Gma.6101	GTGGACTTGTGGATAGTACAACTAGCCTTGGGCTAATGCAATACGGCTAT
Gma.6289	ACCCATTCCCCCCTTATGTATCATACGCCCTAAATTGGGCGGTTTTACAC
Gma.6450	GCTGGACTGCCCTTATTGTGTTGCTGTATATGGGTTTCCTAGGGCTATGA
Gma.6692	CTGCCCTAGCTTTTGAGTCATACCACGAACTTTGCACAAAATTTGTGAGT
Gma.6797	AGGTGCATTGAAAGAGGACTTGGAATCCCAGGCAGAGTTGTGAACATGAT

Table 3. List of significantly[a] and differentially expressed genes in PI 96188 and SS2-2 responsive to *Xanthomonas axonopodis* pv. *glycines (Xag)*.

Condition	Source	UniGene Cluster_ID	Description	Ave Ratio[b] ± STD[c]	
				PI 96188	SS2-2
Up-regulated (Increased level at 24 hr after inoculation)	R gene (Disease resistant gene)	Gma.66	*G. max* clone c27 resistance protein mRNA, partial cds		1.92 ± 0.92
		Gma.67	*G. max* clone 19.2 resistance protein mRNA, partial cds		1.23 ± 0.17
		Gma.171	*G. max* mRNA for resistance protein, partial		1.54 ± 0.21
		Gma.338	*G. max* peroxidase (Prx2b) mRNA, complete cds		1.28 ± 0.17
		Gma.384	ESTs, Weakly similar to disease resistance protein EDS1 [*A. thaliana*]		1.28 ± 0.31
		Gma.399	ESTs, Weakly similar to disease resistance protein, putative [*A. thaliana*]		1.31 ± 0.16
		Gma.1043	ESTs, Weakly similar to NBS/LRR disease resistance protein, putative [*A. thaliana*]		1.64 ± 0.51
		Gma.1458	ESTs, Moderately similar to pectinesterase, putative [*A. thaliana*]		1.64 ± 0.41
		Gma.2102	ESTs, Weakly similar to putative disease resistance response protein [*A. thaliana*]		1.17 ± 0.22
		Gma.2440	ESTs, Moderately similar to Cf-5 disease resistance protein - like [*A. thaliana*]		1.54 ± 0.49
		Gma.2693	*G. max* arginine decarboxylase mRNA, complete cds		2.53 ± 1.44
		Gma.2718	ESTs, Moderately similar to unknown protein [*A. thaliana*]		1.51 ± 0.19
		Gma.2744	ESTs, Weakly similar to putative protein [*A. thaliana*]		1.58 ± 0.40
		Gma.2837	ESTs, Weakly similar to disease resistance protein [*A. thaliana*]		1.16 ± 0.04
		Gma.3149	ESTs, Weakly similar to disease resistance RPP5 like protein (fragment) [*A. thaliana*]		1.09 ± 0.05
		Gma.3221	ESTs, Weakly similar to putative protein [*A. thaliana*]	1.59 ± 0.65	2.06 ± 1.14
		Gma.3858	*G. max* farnesylated protein GMFP5 mRNA, partial cds	1.19 ± 0.08	1.18 ± 0.08
		Gma.4332	ESTs, Weakly similar to disease resistance response protein-like [*A. thaliana*]		2.23 ± 1.35
		Gma.4564	ESTs, Weakly similar to T48928 disease resistance protein-like [*A. thaliana*]		1.18 ± 0.09
		Gma.4639	ESTs, Weakly similar to non-race specific disease resistance protein (NDR1) [*A. thaliana*]		1.37 ± 0.39
		Gma.4954	ESTs, Weakly similar to disease resistance protein-like [*A. thaliana*]		2.89 ± 1.61
		Gma.5085	ESTs, Weakly similar to disease resistance protein, putative [*A. thaliana*	1.16 ± 0.08	1.21 ± 0.16
		Gma.5099	ESTs, Weakly similar to dirigent protein, putative [*A. thaliana*]		2.72 ± 1.61
		Gma.5550	ESTs, Weakly similar to TMV resistance protein-like [*A. thaliana*]		1.24 ± 0.13
		Gma.5784	ESTs, Weakly similar to putative disease resistance response protein [*A. thaliana*]	1.18 ± 0.10	
		Gma.6077	ESTs, Weakly similar to T50662 UVB-resistance protein UVR8 [imported] - [*A. thaliana*]		1.27 ± 0.28
		Gma.6883	ESTs, Weakly similar to RPP1 disease resistance protein-like [*A. thaliana*]		4.06 ± 2.34
		Gma.8443	*G. max* functional candidate resistance protein KR1 (KR1) mRNA, complete cds	1.18 ± 0.06	
		Gma.8456	*G. max* functional resistance protein KR2 mRNA, partial cds	1.18 ± 0.13	1.18 ± 0.07
		Gma.8474	*G. max* putative resistance protein (L33) mRNA, complete cds	1.12 ± 0.10	1.30 ± 0.24
		Gma.8478	*G. max* resistance protein MG13 mRNA, partial cds		1.46 ± 0.22
		Gma.8479	*G. max* resistance protein MG23 mRNA, partial cds		2.02 ± 0.98
		Gma.8507	*G. max* clone NTN5c NBS-type putative resistance protein mRNA, partial cds		2.05 ± 1.03
		Gma.8508	*G. max* clone NTN4c NBS-type putative resistance protein mRNA, partial cds	1.13 ± 0.10	1.16 ± 0.10
		Gma.8782	ESTs, Weakly similar to disease resistance protein, putative [*A. thaliana*]		1.29 ± 0.15
		Gma.8815	ESTs, Weakly similar to putative disease resistance protein [*A. thaliana*]		1.37 ± 0.29
		Gma.9560	ESTs, Weakly similar to resistance protein RPP13, putative [*A. thaliana*]	1.21 ± 0.16	1.25 ± 0.15
		Gma.9601	ESTs, Weakly similar to disease resistance like protein [*A. thaliana*]		1.30 ± 0.36
		Gma.9636	*G. max* resistance gene analog LM1 pseudogene, mRNA sequence	1.15 ± 0.07	1.16 ± 0.10
		Gma.9827	*G. max* resistance protein KR4 mRNA, complete cds		1.15 ± 0.08
		Gma.10594	ESTs, Moderately similar to T47840 multi resistance protein homolog [*A. thaliana*]	1.16 ± 0.07	1.15 ± 0.07
		Gma.11474	ESTs, Similar to disease resistance protein RPP1-WsB - like protein [*A. thaliana*]		1.65 ± 0.63
	Metabolism	Gma.3	*G. max* seed maturation protein (GmPM3) mRNA, complete cds		1.49 ± 0.24
		Gma.36	*G. max* maturation-associated protein (MAT1) mRNA, complete cds	1.50 ± 0.85	1.68 ± 0.36
		Gma.219	*G. max* chlorophyll a/b-binding protein (cab3) mRNA, nuclear gene encoding chloroplast protein, complete cds		1.15 ± 0.08
		Gma.1460	ESTs, Moderately similar to HMD1_ARATH 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (HMGR1) [*A. thaliana*]	1.48 ± 0.76	2.34 ± 0.51
		Gma.2044	*G. max* maturation-associated protein (MAT9) mRNA, complete cds		2.75 ± 1.17
		Gma.2855	ESTs, Moderately similar to putative translation initiation factor [*A. thaliana*]		1.26 ± 0.17
		Gma.3717	ESTs, Moderately similar to acyl CoA thioesterase, putative [*A. thaliana*]		1.40 ± 0.28
		Gma.4589	ESTs, Moderately similar to HMD1_ARATH 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (HMG-CoA reductase 1) (HMGR1) [*A. thaliana*]		2.08 ± 1.05
		Gma.4699	ESTs, Moderately similar to putative protein [*A. thaliana*]		1.17 ± 0.11
		Gma.6450	ESTs, Moderately similar to guanine nucleotide regulatory protein, putative [*A. thaliana*]	1.24 ± 0.10	
		Gma.6797	ESTs, Moderately similar to putative UDP-glucose:glycoprotein glucosyltransferase [*A. thaliana*]	1.18 ± 0.13	
		Gma.7199	ESTs, Moderately similar to T09913 X-Pro dipeptidase homolog T16L4.10 (fragment) [*A. thaliana*]		1.96 ± 0.50
		Gma.7831	*G. max* proteasome IOTA subunit mRNA, complete cds	1.38 ± 0.39	1.69 ± 0.85
		Gma.8162	ESTs, Moderately similar to prolyl endopeptidase, putative [*A. thaliana*]		2.02 ± 0.74
		Gma.8293	ESTs, Highly similar to T47613 ABC transporter-like protein [*A. thaliana*]	1.21 ± 0.16	1.44 ± 0.47
		Gma.8467	*G. max* Shi-shi 51 kDa seed maturation protein (pGmPM10) mRNA, complete cds	1.50 ± 0.39	2.72 ± 1.36
		Gma.8701	ESTs, Moderately similar to argininosuccinate synthase -like protein [*A. thaliana*]	1.49 ± 0.50	1.89 ± 0.79
		Gma.8866	ESTs, Moderately similar to replication factor C-like [*A. thaliana*]	1.11 ± 0.08	1.19 ± 0.11
		Gma.9251	ESTs, Highly similar to kinesin-like protein [*A. thaliana*]	1.22 ± 0.10	1.19 ± 0.09

Table 3. continued

Condition	Source	UniGene Cluster_ID	Description	Ave Ratio[b] ± STD[c]	
				PI 96188	SS2-2
	Other	Gma.2	*G. max* aminoacyl peptidase mRNA, complete cds		1.34 ± 0.31
		Gma.16	*G. max* mRNA for glycinamide ribonucleotide transformylase		1.55 ± 0.32
		Gma.48	*G. max* heat shock protein (SB100) mRNA, complete cds		1.42 ± 0.30
		Gma.197	*G. max* asparagine synthetase mRNA, complete cds		1.83 ± 0.41
		Gma.289	*G. max* Ribulose-1,5-bisphosphate carboxylase small subunit rbcS3	1.19 ± 0.18	1.15 ± 0.08
		Gma.325	*G. max* mRNA for urease (Ure gene)	1.22 ± 0.12	1.19 ± 0.09
		Gma.483	Dihydrofolate reductase-thymidylate synthase, bifunctional enzyme [*G. max*, seedling, mRNA, 1794 nt]		1.40 ± 0.22
		Gma.1689	*G. max* mRNA for 6-phosphogluconate dehydrogenase, complete cds		1.31 ± 0.14
		Gma.1872	*G. max* sucrose binding protein (sbp) mRNA, complete cds		3.28 ± 1.81
		Gma.1950	*G. max* isoflavone reductase homolog 2 (IFR2) mRNA, complete cds	1.20 ± 0.09	1.23 ± 0.19
		Gma.1965	*G. max* calmodulin (SCaM-3) mRNA, complete cds	1.16 ± 0.14	
		Gma.2308	*G. max* isoflavone synthase 2 (ifs2)	1.24 ± 0.24	
		Gma.2854	*G. max* mRNA for uricase (Nod-35), complete cds.		1.50 ± 0.32
		Gma.5064	ESTs, Moderately similar to 1-aminocyclopropane-1-carboxylate oxidase [*A. thaliana*]		2.88 ± 1.69
		Gma.5672	*G. max* mRNA for Mg chelatase subunit (46 kD), complete cds		1.54 ± 0.47
		Gma.6101	*G. max* calmodulin (SCaM-1) mRNA, complete cds.		1.64 ± 0.29
		Gma.8416	*G. max* nitrate reductase (BCNR-A) mRNA, partial cds	1.25 ± 0.16	1.50 ± 0.35
		Gma.8424	*G. max* mRNA for late nodulin, complete cds		3.62 ± 2.01
		Gma.8520	*G. max* mRNA for root nodule acid phosphatase		1.76 ± 1.07
Down-regulated (Decreased level at 24 hr after inoculation)	Metabolism	Gma.1555	*G. max* early light-induced protein (ELIP) mRNA, complete cds	0.24 ± 0.17	
		Gma.5625	ESTs, Moderately similar to ubiquitin-conjugating enzyme-like protein [*A. thaliana*]		0.47 ± 0.31
		Gma.9712	ESTs, Moderately similar to putative hydroxymethyltransferase [*A. thaliana*]	0.72 ± 0.26	
	Other	Gma.1814	*G. max* (Rab1p) mRNA, complete cds	0.33 ± 0.05	

[a] Paired t-test was used for determining statically significant gene expressions between 0 hr and 24 hr after inoculation at $P < 0.05$.
[b] Ave ratio was estimated by ratio of signal strength at 24 hr to 0 hr after inoculation.
[c] STD = standard deviation.

half the total volume (50 μl) recommended by the manufacturer's protocol, and 1μg of template total RNA was used. Reverse transcription was performed as the manufacturer's recommendations. QRT RT-PCR was performed in a total volume of 25 μl containing 100 ng of template total RNA. Each reaction mixture was amplified in the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The products were analyzed through a melting curve analysis by applying 95 °C for 1 min, 55 °C for 1 min, and 55 °C for 2 sec, followed by an increase in temperature from 50 to 90 °C (0.5 °C/cycle for 80 cycles) and continuous fluorescence recording. The results from the tubulin gene were then used to normalize the gene expression levels observed by QRT RT-PCR.

Results

Phenotype of PI 96188 responding to *Xag* infection

The phenotypes of three soybean genotypes were observed ten days after inoculation with 1 x 10^8 CFU ml^{-1} of *Xag* 8ra on fully expanded trifoliate leaves (Fig. 1). Jangyoebkong exhibited typical symptoms of *Xag* infection: small pustules surrounded by yellow chlorotic haloes. No pustules were detected on the sprayed leaves of BLP-resistant SS2-2. However, PI 96188 exhibited a different response to *Xag* infection than the other soybean genotypes. Necrotic lesions lacking yellow haloes were observed, a clearly distinguishable difference from symptoms typically observed 12 days following inoculation (data not shown).

Gene expression in PI 96188 and SS2-2

Paired t-tests and FDR were used to identify genes with statistically significant ($P < 0.05$) altered expression following *Xag* infection, rather than using the fold-change cutoffs provided by the software program. A total of 90 genes showed significant gene expression changes 24 hr after inoculation compared with non-inoculated leaves, regardless of genotype (Tables 3 and 4). Table 3 summarizes the observed gene expression patterns, the genotype in which it was observed, and the category (metabolism, disease resistance, or otherwise) to which the gene belongs. And, ratio data were shown only if a significant change from 0 hr to 24 hr was detected. A list of the six genes that were down-regulated in PI 96188 and up-regulated in SS2-2 is presented in Table 4.

Eighty genes were found to be up-regulated at 24 hr after *Xag* infection in at least one of the genotypes. Twenty-one of the 80 sequences were co-expressed in both PI 96188 and SS2-2 (Table 3). Interestingly, nine genes were uniquely expressed in PI 96188 after *Xag* infection. Most of the up-regulated genes were expressed in SS2-2 alone. A different trend was observed for genes with decreased mRNA abundance following *Xag* infection (Table 3). Only four down-regulated genes were detected in PI 96188 and/or SS2-2 24 hr after *Xag* inoculation. In addition, no genes were found

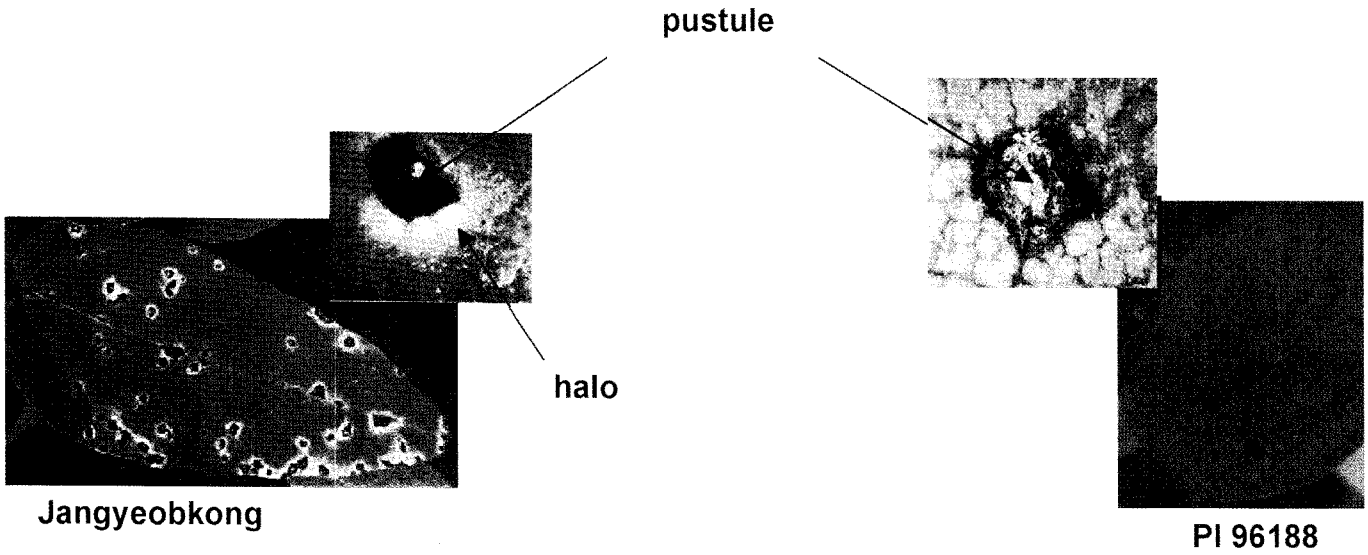

Fig. 1. Phenotypes caused by *Xag* infection in three soybean genotypes used in this study. 10 day-old leaves inoculated with *Xag* in BLP-susceptible Jangyeobkong, BLP-resistant SS2-2 and PI 96188, in which its novel symptom against *Xag* is demonstrated. Pustules and halo were indicated by arrows.

to be co-expressed in both genotypes following *Xag* infection. Among the 80 genes listed in Table 3, the 42 genes up-regulated by *Xag* infection were predominantly from the 'R gene' category. However, no genes from this category appeared to be down-regulated by *Xag* infection.

Gene expression patterns after *Xag* infection may vary between PI 96188 and BLP-resistant SS2-2, as a necrotic symptom was shown in PI 96188. Interestingly, six UniGenes showed genotype-dependent gene expression patterns 24 hr after *Xag* inoculation (Table 4). These six genes were down-regulated in PI 96188, but up-regulated in SS2-2, simultaneously. One sequence (Gma.1778) was from the 'R gene' class, showing similarity to a protein of unknown function in Arabidopsis. Since this gene was similar to Arabidopsis sequences, homology searches were conducted to identify tentative consensus (TC) sequences of soybean at the TIGR Soybean Gene Index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=soybean) (Table 4). This gene (Gma.1778) of unknown function was identified as TC217113, which is similar to a leucine-rich repeat protein precursor. Five UniGenes that were down-regulated in PI 96188 and up-regulated in SS2-2 after *Xag* infection were from the 'Metabolism' class. Two genes (Gma.596 and Gma.8781) encoding proteins related to signal transduction were differentially expressed. Also, genes involved in seed development (Gma.2253), cell cycle (Gma.2371), and tyrosine catabolism (Gma.6289) were differentially expressed 24 hr after *Xag* inoculation in this study. The similarity of these TC sequences and their putative functions to UniGenes supports the validity of further study to verify the macroarray data by QRT RT-PCR.

Table 4. List of genes down-regulated in PI 96188 but up-regulated in SS2-2 simultaneously from 0 hr to 24 hr after *Xag* inoculation.

UniGene Cluster_ID	UniGene title	Ave Ratio[a] ± STD[b]		Genbank accession number/ TIGR_TC_ID	Tentative Annotation
		PI 96188	SS2-2		
R gene				R gene	
Gma.1778	ESTs, Weakly similar to unknown protein [*A. thaliana*]	0.58 ± 0.35	2.17 ± 0.79	TC217113	Similar to UP|Q708X5 (Q708X5) Leucine rich repeat protein precursor, partial (96%)
Metabolism					
Gma.596	*G. max* 14-3-3-like protein mRNA, complete cds	0.59 ± 0.24	1.75 ± 0.77	TC216198	UP|Q9M5K7 (Q9M5K7) 14-3-3-like protein, complete
Gma.2253	*G. max* seed maturation protein PM34 (PM34) mRNA, complete cds	0.75 ± 0.21	1.56 ± 0.32	AF169018	*G. max* seed maturation protein PM34 (PM34) mRNA, complete cds
Gma.2371	ESTs, Highly similar to transcription factor, putative [*A. thaliana*]	0.72 ± 0.44	1.28 ± 0.33	TC228516	Similar to UP|Q6QNH2 (Q6QNH2) Minichromosomal maintenance factor, partial (40%)
Gma.6289	ESTs, Moderately similar to fumarylacetoacetate hydrolase-like protein [*A. thaliana*]	0.64 ± 0.35	1.89 ± 1.10	TC205011	Similar to (Q6H7M1) Putative fumarylacetoacetate hydrolase, partial (93%)
				TC205014	Similar to (Q6H7M1) Putative fumarylacetoacetate hydrolase, partial (23%)
Gma.8781	ESTs, Moderately similar to A49318 protein kinase (EC 2.7.1.37) tousled - [*A. thaliana*]	0.53 ± 0.30	4.10 ± 1.60	TC210972	Similar to (Q6RK06) Tousled-like kinase 2, partial (41%)

[a]Ave ratio was estimated by ratio of signal strength at 24 hr to 0 hr after inoculation.
[b]STD = standard deviation.

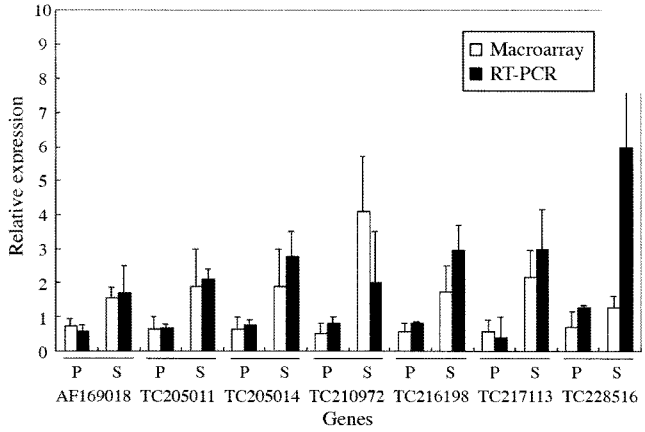

Fig. 2. Comparison of differential gene expression between oligonucleotide macroarray (24 hr / 0 hr) and quantitative real-time RT-PCR in PI 96188 (P) and SS2-2 (S) against *Xag*, with genes shown down-regulation in PI 96188 but up-regulation in SS2-2, simultaneously. Each primer set was confirmed by non-quantitative RT-PCR, showing the production of one PCR amplicon. The amount of SYBR Green product in the tubulin product was used as a control and the fold ratio calculated as $2^{-\Delta\Delta Ct}$ to convert the logarithmic value of C_t to a linear value.

Validation of macroarray data by quantitative real-time RT-PCR

With three replicates, QRT RT-PCR was performed to confirm the reliability of the oligonucleotide macroarray experiments. The templates were cDNAs synthesized from the total RNA samples used in the oligonucleotide macroarray hybridizations. Six UniGenes that were down-regulated in PI 96188 and up-regulated in SS2-2 24 hr after *Xag* inoculation (Table 4) were chosen as templates in QRT RT-PCR since these genes might be responsible for differential gene expression between PI 96188 and SS2-2. Since one UniGene (Gma.6289) showed similarity to two TC sequences, a total of 7 TC sequences were included for designing primers. The primer information of selected genes is listed in Table 5. As a control, BLP-susceptible Jangyeobkong, which displays both pustules and haloes after *Xag* infection, showed very low ratios in all genes tested. QRT RT-PCR confirmed our macroarray experiments in nearly all cases (Table 6). The transcriptional levels at 24 hr in PI 96188 were 1-fold lower than 0 hr after *Xag* inoculation, with the exception of one TC sequence (TC228516). In SS2-2, all genes showed higher transcriptional levels 24 hr after *Xag* infection compared to 0 hr. The same pattern of gene expression, down-regulation in PI 96188 and up-regulation in SS2-2, was conserved for the majority of the genes (Fig. 2). Although the magnitude of the ratios from QRT RT-PCR results were greater than those obtained by the macroarray experiments, the trends of mRNA changes identified by the QRT RT-PCR were in accordance with the macroarray data (Fig. 2).

Discussion

BLP is a severe disease caused by a bacterial pathogen in soybean. A typical symptom of this pathogen *Xag* is the appearance of small, yellow haloes to brown lesions with a raised pustule in the center (Van et al. 2004). However, PI 96188 is the only soybean genotype demonstrating a necrotic response against *Xag*, shown by the lack of chlorotic haloes in infected plants (Fig. 1). This novel symptom in PI 96188 is different than the typical hypersensitive response, as the lesion and pustule develop in a different manner compared with the symptoms observed in other soybean genotypes (Han et al. 2001). Also, *Xag* multiplied in PI 96188 10^{-1}- to 10^{-2}-fold slower than in the susceptible genotype. But the same bacteria growth in PI 96188 was observed to be 10 times faster than in the resistant genotype (unpublished data, Sunggi Hue). In SSR marker analysis, this symptom was not co-segregated with *rxp* on LG D2 (Park 2003). Therefore, the gene responsible for this symptom could be different than *rxp*, conditioning the typical resistance response to *Xag*.

Since the genes involved in the defense mechanism response to *Xag* have not been characterized, microarray experiments would be helpful for comparison of differential gene expression between the two soybean genotypes during infection. The genes identified by microarray analysis might affect signaling networks and could thus be subjected to detailed functional analyses (Glazebrook et al. 2003; Kazan et al. 2001). To increase specificity, flexibility, cost-effectiveness, and uniformity (Denef et al. 2003; Hessner et al. 2004), an oligonucleotide macroarray was printed with genes related to disease resistance, metabolism, and housekeeping. In previous studies, rapid changes in gene expression were detected after pathogen inoculation (Scheideler et al. 2002; Tao et al. 2003). Using cDNA microarray and cDNA-AFLP, the cassava-X. *axonopodis* pv. *manihotis* interaction was observed by changing gene expression profiles at 12, 24, 48 hr, and seven and 15 days after post-infection (Lopez et al. 2005; Santaella et al. 2004). Therefore, soybean leaves were used 24 hr after *Xag* infection to observe differential gene expression profiles between uninfected and infected plants. Each *Xag*-inoculated leaf was also tagged to avoid dilution effects. To maximize the reliability, comparability and reproducibility of the macroarray data, three replicates with dye-swap were performed. Significance analysis of microarray (SAM) has previously been used to identify differentially expressed genes in replicated microarray experiments (Tusher et al. 2001). However, other microarray studies performed statistical analyses, such as a linear model analysis, to identify differentially expressed genes more reliably (Kerr et al. 2000; Puthoff et al. 2003). Thus, a paired *t*-test (α = 0.05) and FDR were used as our statistical analysis rather than SAM, which is generally considered more appropriate for larger studies.

Previous studies have shown that in whole-genome microar-

rays, only 10% of genes were differentially expressed in response to pathogens (Ramonell and Somerville 2002). However, 90% of genes showed statistically significant, differential gene expression between 0 hr samples and 24 hr samples following *Xag* inoculation. This is due to the small set of 100 soybean UniGenes printed on an oligonucleotide macroarray used for this study (Table 3). Although it is hard to compare between whole-genome microarrays and this experiment, our macroarray study suggests that a considerably large number of genes may be involved in the response to *Xag* infection. Additionally, the elimination of potential dilution effects through tagging leaves may have also contributed to the high percentage of genes differentially expressed after *Xag* inoculation. Although 90 genes were differentially expressed between 0 hr and 24 hr after *Xag* infection, six UniGenes were particularly unique because they were down-regulated in PI 96188 and up-regulated in SS2-2 simultaneously, representing possible candidates for genes involved in a necrotic response against *Xag*.

Genes related to disease resistance are clustered in regions on several soybean chromosomes (Jeong et al. 2001), and the encoded proteins of these R genes from plant species such as *Arabidopsis*, rice, soybean, and tomato, tend to share one or more similar motifs, including leucine-rich repeat regions, nucleotide-binding sites, and kinase domains (Century et al. 1997; Sato et al. 2007; Tao et al. 2003). These motifs are considered to have specific roles in the signal transduction pathways that respond to various pathogens. In *Arabidopsis*, the *NDR1* gene confers resistance to both bacterial and fungal pathogens (Century et al. 1997; Sato et al. 2007; Tao et al. 2003). In this study, the resistance gene motifs were also found to be involved in the signal transduction pathway for *Xag* attack (Table 4). One UniGene (Gma.1778) in the 'R' class showed similarities to the motifs mentioned above, as they were down-regulated in PI 96188 and up-regulated in SS2-2 simultaneously. The up-regulation of these four genes in SS2-2 may be due to its BLP-resistant genotype, whereas PI 96188 showed reduced transcriptional levels of these genes 24 hr after inoculation, possibly leading to susceptibility to BLP in PI 96188. Thus, these four genes were differentially expressed as a result of their potential involvement in the defense mechanism against *Xag*.

Five UniGenes in the 'Metabolism' class were identified as differentially expressed between PI 96188 and SS2-2 (Table 4). The transcriptional level of the soybean 14-3-3-like protein mRNA (Gma.596) decreased after *Xag* infection in PI 96188 and increased in SS2-2. Through direct protein-protein interactions, plant 14-3-3 proteins regulate a variety of events in intracellular signaling cascades, transcription, and carbon and nitrogen metabolism (Hajduch et al. 2005; for review, see Roberts 2003). In addition, plant 14-3-3s were involved in pathogen defense through transcriptional regulation. Pathogen-resistance responses, such as the formation of necrotic lesions and the accumulation of defense-related *PR1* and *GST6* transcripts, were observed in *Arabidopsis* transformed with an antisense *AKR2* gene that interacted with the 14-3-3 protein (Yan et al. 2002).

Gma.8781, which is similar to tousled-like kinase 2 (TLK2), also displayed a genotype-dependent expression pattern following *Xag* infection. TLK, a member of the Ser/Thr protein kinase family that specifically targets DNA damage, was observed to reduce damage from ionizing radiation in mouse cells overexpressing *TLK1* (Ehsan et al. 2004; Li et al. 2001). If *TLK* genes are mutated in *Arabidopsis* and fruit flies, abnormalities are observed in the flowers and leaves, and the development of the embryo is arrested (Ehsan et al. 2004; Roe et al. 1993). Puthoff et al. (2003) supported the theory that the activity of cell cycle-related genes is altered in *Arabidopsis*-cyst nematode interactions. In this study, the cell cycle-related gene minichromosomal maintenance factor (Gma.2371) also showed an altered expression pattern 24 hr after *Xag* inoculation.

Xag infection also affects seed development (Gma.2253). BLP was also observed on the surface of PI 96188 seeds. Down-regulation of PM34 mRNA in PI 96188 might be linked to the presence of BLP on the surface of seeds. However, there was an increased level of PM34 mRNA correlating with the response to the pathogen on seeds of SS2-2, although no disease was detected. *SC24*, which is related to other soybean seed proteins in the seed coat, was also induced in vegetative tissue by pathogen infection and wounding (Dhaubhadel et al. 2005).

Gma.6289, which was up-regulated in SS2-2 but down-regu-

Table 5. Primer sequences used for quantitative real-time RT-PCR.

Genbank accession number/ TIGR_TC_ID	Primers	
	Forward 5' 3'	Reverse 5' 3'
AF169018	TTG GAC GAG CGG TGT GTA ACT TG	GCA CTC ACG ACC TCA TCA ACC AC
AF314550	GCT TGG GTT TGT GTT TCG GAT G	GCG GAG TTA GCA CAG CTT CCC AC
TC204619	AGG GCG TGG CAA GTC TGT GG	GGA GGG CAT GGT CAC GGA AAC
TC204620	CAA GTG GTT CGG ACG CTG ATT C	AAG GAA GCG GAA GGA GTT GTC G
TC205011	GGT ATC GCC TGC CTG TTG CC	TCC CAT GCC TGA ATA TCT CGA GC
TC205014	TGA GCC AGA GTC CCG TGC ATG	AGC TGC GGG TAC AAT CTT GCC TG
TC206595	TTT GGA CAG GTT GGA GTT GGT GAC	TTG CCC ATT TGT GCC TCT TCC
TC206596	GCC GGA CGA AAC TGT CTC AGG	TCC AAT GAA CTG ATG AGG CAG AGA C
TC210972	CCG AGA GGG AGG CTA AGG TCA TC	AAT TCC AGC AGA CCA GAC ATC AAC C
TC216198	CAG GGA TTA CAG GAA CAA GGT GGA G	TTA GAG CCA GAC CCA AAC GAA TAG G
TC216510	GGG AAT GAA GGT GTA TCG TAA AGC C	CCC GTG CTT AGA TTC AAA CTC GTG
TC217113	CGC TGA TTG GAA AGA CAT TGC C	AGG TCC AGG TGT TTG AGT GAG CC
TC228516	TCT GCC GTG GGT CTT ACT GCC	GCC GCA GCA ATA ACA GAA CAG C

Table 6. Primer sequences used for quantitative real-time RT-PCR and QRT RT-PCR results for 13 selected sequences in soybean. Values were the average of three replicates. C_t = cycle threshold number, $\Delta\Delta C_t$ = difference in cycle number between 0 hr and 24 hr after inoculation, respectively.

Genbank accession number/TIGR_TC_ID	Ratio ($2^{-\Delta\Delta Ct}$)		
	Jangyeobkong	PI 96188	SS2-2
AF169018	0.07 ± 0.02	0.59 ± 0.19	1.72 ± 0.79
TC205011	0.18 ± 0.05	0.66 ± 0.13	2.12 ± 0.30
TC205014	0.25 ± 0.07	0.78 ± 0.14	2.77 ± 0.74
TC210972	0.23 ± 0.09	0.85 ±0.18	2.03 ± 1.47
TC216198	0.29 ± 0.04	0.82 ± 0.03	2.95 ± 0.76
TC217113	0.07 ± 0.03	0.40 ± 0.06	2.97 ± 1.20
TC228516	0.14 ± 0.04	1.28 ± 0.07	5.96 ± 3.10

lated in PI 96188, is similar to fumarylacetoacetate hydrolase, the last enzyme in the tyrosine catabolic pathway. Glutathione transferase (GST), known to be a key enzyme in secondary stress metabolism and signaling, also plays a role upstream of fumarylacetoacetate hydrolase in tyrosine catabolism (for review, see Dixon et al. 2002). Thus, all six UniGenes that were up-regulated in SS2-2 and down-regulated in PI 96188 are related to plant defense mechanisms in some way, even though some genes were in the 'Metabolism' class. Consequently, SS2-2 may be resistant to *Xag* because these six UniGenes are up-regulated. Similarly, the down-regulation of these genes in PI 96188 may be related to its BLP susceptibility.

QRT RT-PCR was performed to validate oligonucleotide macroarray data, specifically the six UniGenes displaying differential expression patterns. Many researchers have used this method to confirm microarray analysis because gene expression patterns/mRNA levels in different sampling groups can be recognized and compared through RT-PCR based assays. To normalize mRNAs extracted from PI 96188 and SS2-2 at two different time points, housekeeping genes like tubulin were included in this method (for review, see Bustin 2002). Generally, our QRT RT-PCR results were in agreement with the oligonucleotide macroarray data (Table 6). Gene expression increased very rapidly in SS2-2 after *Xag* infection, whereas transcriptional levels of each gene in PI 96188 were decreased in response to the pathogen. However, the magnitude of the changes was smaller in the macroarray analysis (Fig. 2). The ratios of gene expression could differ between the two methods depending on the location of oligonucletides design in the gene because potential secondary structures of mRNA flanking the oligonucleotides can potentially lead to variations in gene expression levels (Lu et al. 2005).

Using specially designed oligonucleotide macroarrays, we identified genes with statistically significant differences in expression levels. These gene expression patterns differed depending on soybean genotypes used for evaluating responses to *Xag* infection. QRT RT-PCR confirmed the transcriptional changes of 10 selected genes showing down-regulation in PI 96188, and up-regulation in SS2-2. These results are indicative of the reliability and reproducibility of these macroarray experiments.

A necrotic response to *Xag* in PI 96188 is a new resistant gene source against *Xag* descriptor. Mapping studies also supported the idea that this new gene controls the specific symptom in PI 96188. In our macroarray experiments for examining gene expression profiles according to soybean genotype after *Xag* inoculation, six genes were expressed differently; down-regulation in PI 96188 showed the necrotic response to *Xag* and up-regulation in BLP-resistant SS2-2. These six genes might also be involved in the signal transduction pathway for response to *Xag* infection at upstream/downstream levels. The *Glycine max* nodule autoregulation receptor-like protein kinase precursor (*GmNARK*) is responsible for determination of nodulation phenotypes (normal or hyper-/supernodulation) in soybean, but *GmNARK*, along with more than 100 interesting genes detected by cDNA-AFLP, might be potential components of the different signal transduction pathway for symbiosis (Lestari et al. 2006). Accordingly, functional analyses of the six interesting genes identified in this study would be the next logical step, although a single gene identified by a preliminary mapping study generally controls a necrotic response. These gene expression patterns could lead to the identification of genotype-specific components of signal transduction pathways for plant-pathogen interactions.

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