

Molecular Characterization of the Asian Soybean Rust Disease in Resistant and Susceptible Soybean Lines

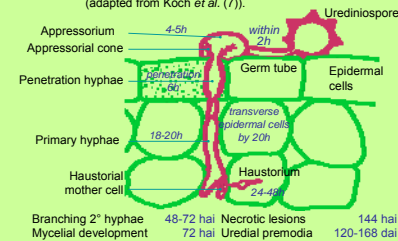
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Introduction Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi*, is a major disease of soybean. Yield losses associated with ASR have been reported to range from 10% under mild disease pressure to 80% during severe epidemics (1,2). In 2004, ASR entered the U.S. and successfully over-wintered the past two years in the southern U.S., spreading slowly throughout the southeastern U.S. in the 2005 and 2006 growing seasons. When ASR infects a field the only current method of control is to apply the appropriate fungicide(s). The most economical way to control the pathogen would be to breed varieties for ASR resistance, but this approach does not appear to be as promising as for many other pathogens. In the field, ASR appears to change very quickly to adapt to new soybean cultivars, U.S. soybean cultivars are highly susceptible to ASR infection and known resistance genes (*Rpp1-4*) have been broken in the field (3,4). Therefore, in the event that breeding programs fail to provide durable resistance to ASR, new ways will be needed to combat the disease if costly scouting and fungicide applications are to be avoided or if fungicides become ineffective. As a starting point, research is needed to provide some basic information about the soybean response in resistant and susceptible soybean cultivars. The work presented here will allow us to assess soybean gene expression in susceptible and resistant cultivars to ASR infection over time.

ASR biology *P. pachyrhizi* is a biotrophic plant pathogenic fungus that rapidly colonizes leaf tissue and to a lower extent also infects stems and pods (3). Its rapid and destructive behavior and the direct penetration of epidermal cells are rather unusual for a biotrophic fungus (Figure 1). After penetration, the fungus colonizes mesophyll cells using specialized fungal structures called haustoria, which are fungal feeding structures (5) and are now also thought to be site of direct control of the host (6). The fungus forms spore producing uredinia within 6-11 days, which are actively disseminating urediniospores for a period of 1 to 3 weeks (7) after which heavily infected leaves are abscised and both the plant and the fungus perish. The period during which we sampled is from 6 hours after inoculation (hai) to 7 days (168 hai) when lesions just become apparent.

Figure 1: Diagram of *P. pachyrhizi* infection process (adapted from Koch et al. (7)).



A few ASR isolates have been reported to give immune reactions on *Rpp1*-containing soybean plants (4). However, most ASR isolates, including the Brazilian greenhouse isolate we used, show lesions on soybean plants carrying known resistance genes and no durable resistance has been identified. Reddish-brown (RB) lesions with no or sparsely sporulating uredinia are considered to be a resistant lesion type (on PI230970 plants) compared to the susceptible TAN lesions (on Embrapa-48 plants) with fully sporulating uredinia (4,9). (Figure 2G-J)

Experimental design

- Infection type [2] Brazilian ASR field isolate and mock inoculation; • Cultivars [2] Susceptible (Embrapa-48) and resistant (PI230970); • Time points [10] 6, 12, 18, 24, 36, 48, 72, 96, 120, and 168 hai; • Replications [3] in a completely randomized, full factorial design. Total number of samples: 120.

Figure 2: Picture of the experiment.

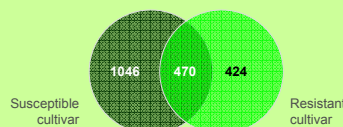
(A) Collection of spores into (B) microcentrifuge tube. (C) *P. pachyrhizi* urediniospores (500x magnification). (D) Inoculation by misting. (E) Inoculated plants after inoculation placed covered for 21 days to create a humid environment conducive to infection. (F) Experiment 168 dai. TAN lesions on susceptible Embrapa-48 leaves; (G) adaxial and (H) abaxial, and reddish-brown lesions on resistant PI230970 leaves; (I) adaxial and (J) abaxial.

RNA analyses Leaflets of the 5th trifoliolate of ASR- and mock-infected plants collected and were immediately frozen in liquid nitrogen. RNA was extracted using RNAwiz (Ambion), purified by a lithium chloride precipitation and RNeasy purification (Qiagen). Levels of gene expression in the purified RNA samples were assessed using GeneChip soybean genome arrays (Affymetrix) at the ISU GeneChip Facility.

Statistical analysis For statistical analysis log base 2 transformed data was median centered for each chip. Mixed model analysis was then performed separately for each gene modeling all three treatments and their interactions as fixed classification variables. Data analysis for both cultivars was performed together to pool the variance and increase the denominator degrees of freedom. This is possible since separate analysis of both cultivars did not significantly change the results. F tests, resulting in p-values, were performed to test for treatment effects. A q-value adjustment, which estimates the False Discovery Rate (FDR), was then computed for each p-value using the method described by Storey and Tibshirani (9).

ASR-regulated genes Probe set lists were compiled by combining probe sets that tested significant in either the infection type by time interaction effect or in the infection type main effect for which we accepted a 5% FDR (q value ≤ 0.05).

Figure 3: Number of probe sets testing significantly different between ASR- and mock-infection in susceptible and resistant cultivar.



Expression of ASR-regulated genes Clustering over the whole time frame was not informative due to the large amount of data points. This also prevents us from classifying ASR-regulated genes in simple up- or down-regulated categories. To effectively analyze the data, we clustered the genes in early, middle, and late time frames (Fig 4).

For changes in expression level (relative to a mock-inoculated sample) to be detected at these early time points, either transcript levels must have been dramatically reduced or elevated or there must be many sites of attack. Examples of the expression profiles of ASR-regulated genes are shown in Fig 5.

Figure 4: Number of significant probe sets that respond to ASR in the susceptible and resistant cultivar (5% FDR).

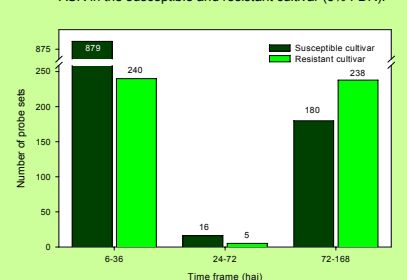
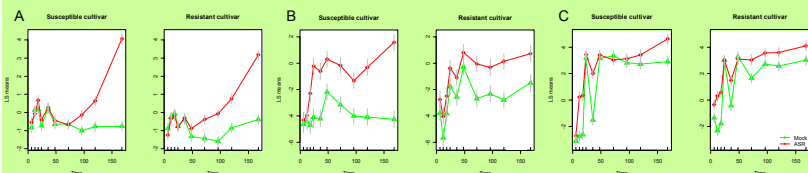


Figure 5: (A) Expression profiles that diverge earlier in the resistant cultivar (48 hai) versus the susceptible cultivar (72 hai). (B) Expression induced earlier in the susceptible cultivar. (C) Expression profile where main divergence occurs during the early time frame in susceptible and resistant cultivars.



Hierarchical clustering analysis of the early and late time periods revealed that 62% of the probe sets in common to both cultivars had similar expression profiles in the early time period, but only 6% of these probe sets had similar expression profiles in the late time period. Similar responses to pathogen infection in compatible and incompatible interactions at early time periods have previously been reported (10) and may be attributed to activation of basal defenses. Observation of individual expression profiles of probe sets in the susceptible and resistant cultivar side by side, suggests that divergent patterns of expression are established earlier in the resistant cultivar.

Functional classification

In order to relate ASR gene expression to biological functions, we used Affymetrix probe set BLAST results and *Arabidopsis* homologs with functional classification kindly provided by the Goldberg lab (UCLA). Highly represented classes of genes that are in common to both cultivars include primary and secondary metabolism and disease/defense-related functions (Table 1). When considering genes that are unique to the resistant cultivar transport functions and transcription are overrepresented, possibly involved in limiting fungal proliferation, while overrepresented functional classes specific to the susceptible cultivar include signal transduction and energy metabolism functions and are likely involved in assisting fungal invasion.

Table 1: Representation of functional classes in ASR-regulated probe sets.

Functional class	Total probe sets in category on GeneChip	Number of ASR-responsive probe sets (and Bonferroni adjusted p-value)*	
		Susceptible cultivar	Resistant cultivar
Metabolism	3316	201 (0.000) ↑	139 (0.000) ↑
Disease & Defense	1078	134 (0.000) ↑	91 (0.000) ↑
Secondary Metabolism	610	63 (0.000) ↑	61 (0.000) ↑
Signal Transduction	2123	120 (0.003) ↑	71 (0.072)
Energy	685	47 (0.006) ↑	8 (0.532)
Transporter	1283	68 (0.410)	48 (0.039) ↑
Transcription	2356	102 (7.154)	97 (0.000) ↑
Transposon	120	1 (1.323)	0 (1.694)
Intracellular Traffic	532	27 (3.963)	6 (0.885)
Protein Destination & Storage	1954	81 (12.449)	29 (0.095)
Cell Structure	1021	26 (0.181)	9 (0.010) ↓
Cell Growth & Division	641	14 (0.206)	4 (0.017) ↓
Protein Synthesis	1012	28 (0.561)	3 (0.000) ↓
Unknown proteins	20223	595 (0.000) ↓	326 (0.000) ↓
Post-Transcription	639	9 (0.003) ↓	1 (0.000) ↓

* ↑, ↓ represents statistically over- and under-represented categories, resp. (p-value ≤ 0.05).

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Summary

- 1516 and 894 probe sets tested significant in the susceptible and resistant cultivar, respectively, of which 470 probe sets are common to both cultivars (Figure 3)
- Many ASR-regulated genes respond early during the infection (6-36 hai) and/or late during the infection (72-168 hai), while only very few genes are regulated in the intermediate time period from 24-72 hai (Figure 4)
- At the earlier time points, expression profiles of genes common to both cultivars are similar, suggestive of basal defenses being activated
- During the late time period, many ASR-responsive genes appear to be induced earlier in the resistant cultivar than in the susceptible cultivar and may be associated with the more limited fungal growth in the resistant cultivar (Figure 5)
- Functional classes that appear overrepresented in both interactions include primary and secondary metabolism and disease/defense-related functions (Table 1)

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