Study of Host Resistance of Soybean Against *Phakopsora pachyrhizi* the Causal Agent of Soybean Rust Using *Rpp* Near Isogenic Lines

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Md. Zakir HOSSAIN

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Md. Zakir HOSSAIN

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Abbreviations

Rpp	*Resistance to Phakopsora pachyrhizi*
NILs	*Near-isogenic lines*
ASR	*Asian soybean rust (Phakopsora pachyrhizi)*
GO	*Gene ontology*
FDR	*False discovery rate*
KOG	*Eukaryotic cluster of orthologus group*
KEGG	*Kyoto encyclopedia of genes and genomes pathway*
PFAM	*Protein family database*
Glyma	*Glycine max*
PAMP	*Pathogen-associated molecular pattern*
NLR	*Nucleotide-binding domain, leucine-rich repeat receptors*
PRR	*Pattern recognition receptor*
DEGs	*Differentialy expressed genes*
In silico	*Computar based*
PCR	*Polymerase chain reaction*
RT-qPCR	*Reverse transcription- quantitative polymerase chain reaction*
PAL	*Phenyl ammonia lyase*
G4DT	*Glycinol 4-dimethylallyltransferase*
ADT6	*Arogenate dehydratase 6*
C4H	*Cinnamate 4-hydroxylase*
CHS	*Chalcone synthase*
CHR	*Chalcone reductase*
IFS	*Isoflavone synthase*
IFR	*Isoflavone reductase*
ispG	*4-hydroxy-3-methylbut-2-enyl diphosphate synthase*
PT3	*Phytyltransferase 3*
HR	*Hypersensitive response*

CHAPTER 1: INTRODUCTION

Soybean (*Glycine max* (L.) Merr,) is very important economic crop in the world. The people of China, Japan, Korea, Manchuria, Philippines, and Indonesia, for centuries, has earned title for soybean as "Cow of the field" or "Gold from soil" (Horvath, 1926). It is often called the "miracle crop". As early as 6,000-9,000 years ago, farmers in China first grew soybean (Kim et al., 2012; Sedivy et al., 2017). In 1804, a Yankee clipper ship from China brought soybeans to the USA and in 1829, US farmers first grew soybeans (Hymowitz and Shurtleff, 2005). It is the foremost provider of vegetable protein and oil for human consumption. Another increasing demand of soybean is for poultry and animals feed (Hartman et al., 2011), recently as a biodiesel feedstock (Kurki et al., 2010) and functional food (Bratton et al., 2015; Fritz et al., 2013; Kwon et al., 2010). The soybean production is increasing day by day in the world. It was increased 4.8% annually from 1960s and reached 337.1 million tons in 2015-17 (OECD/FAO, 2018). It is predicted to increase by 2.1% annually to 406.8 million tons by 2027 (OECD/FAO, 2018). There has been an overall increase in the world soybean harvested area: from 20 million ha in 1960 to 120 million ha in 2013 (Sly, 2017). In 2027, the total soybean cultivation area is projected 136.7 million ha (OECD/FAO, 2018). Now it is a popular global crop. United States (USA), Brazil and Argentina are the world top soybean producing countries (OECD/FAO, 2018; Sutton et al., 2005).

Phakopsora pachyrhizi Syd. & P. Syd is responsible for Asian soybean rust (ASR) disease. It was first described in Japan in 1902 (Hennings, 1903). Over the next 90 years, ASR was reported in tropical and subtropical regions (Bromfield, 1984). In Australia, it was detected in 1934 (McLean and Byth, 1976), China in 1940 (Gustavo et al., 2011), India in 1951 (Sharma and Gupta, 2006), and Russia in 1957 (Gustavo et al., 2011). By 1994, ASR

had spread into Hawaii (Killgore et al., 1994) and, within a few years later, it was reported many countries of Africa and South America. ASR was reported in African countries, such as Kenya, Rwanda, and Uganda in 1996, followed by Zambia and Zimbabwe in 1998, Nigeria in 1999, Mozambique in 2000, South Africa in 2001, Ghana and the Democratic Republic of Congo in 2007, Ethiopia in 2011(Tesfay et al., 2017), and Malawi and Tanzania in 2014 (Murithi et al., 2015). The ASR first reports in South America were in Paraguay, followed by Brazil, in 2001 (Yorinori et al., 2008). In 2004, soybean leaf samples from a location north of the equator in Columbia tested positive for ASR (Isard et al., 2005) and, on 6 November 2004, the ASR was discovered in Louisiana, USA (Schneider et al., 2005). ASR was also recorded in Mexico in soybean and jicama (*Pachyrhizus erosus* L.) production regions (Kelly et al., 2015) and at a single location on soybean in Ontario, Canada (Isard et al., 2011; Sarah et al., 2007).

ASR is one of the top twelve most virulent plant pathogens as observed by plant pathologists (Dean et al., 2012). It devastates 89 different plants on all the continents of the world (Tremblay et al., 2013). The soybean producers mostly face 20 to 90% yield loss during environmental conditions conducive for *P. pachyrhizi* infection. The most severe damage is largely in world famous soybean producing areas including Asia, North America, and South America (Akamatsu et al., 2013; Akamatsu et al., 2017). Fungicides have detrimental effects on the environment and are not economically viable, however, without fungicide treatment agronomic practices alone cannot limit the damage of this fungus (Ivancovich et al., 2007). A superior variety with great yield and long-lasting resistance is one of the most efficient ways to control ASR in soybean and other crops (Vuong et al., 2016).

The assessment of resistance is coincided with the measurement of lesion colour, the presence or absence of lesions, uredinial number per lesion and sporulation levels since 1970s (Bromfield, 1984). Several recent studies have evaluated resistance using not only lesion colour but also quantitative traits (Bonde et al., 2006; Walker et al., 2014, 2011). During compatible interaction (in susceptible plant), abundant sporulation are produced to tan colour lesions (TAN) in soybean. In resistance genes bearing plant (incompatible interaction) produce reddish-brown lesions (RB) with less sporulation. In some cases of soybean-ASR combinations, immune reactions (IM) with no visible lesions are also observed (Bromfield, 1984). However, Yamanaka et al., (2015) pointed out that numbers of uredinia per lesion and sporulation level were not necessarily correlated with lesion colour. Yamanaka et al. (2010) compared five criteria for resistance, i.e. lesion colour, number of uredinia per lesion, frequency of lesion that had uredinia, frequency of open uredinia and sporulation level. Their results showed that correlation among these characteristics excluding lesion colour was very high. Yamanaka et al. (2017) selected number of uredinia per lesion, frequency of lesion that had uredinia and sporulation level to assess the degree of resistance.

Host resistance or susceptibility studies of ASR- soybean interaction are concentrated on understanding the defence response. Regarding this, the soybean host resistance studies are continuing. Breeding effort to know the physical location of *Rpp* (Resistance to *Phakopsora pachyrhizi)* genes is major advancement of host resistance study of soybean against ASR. Till today, eight major resistance genes [(*Rpp1-7*), *Rpp1-b*] were mapped (Table 1) (Childs et al., 2018). But these *Rpp* gene-mediated resistances against ASR have been overcome in nature several times (Table 2). Despite the recent release of the soybean genome (Schmutz et al., 2010), no *Rpp* genes was cloned yet. Soybean is a palaeopolyploid,

and presence of multiple copies of soybean *R* genes is responsible for specific genes cloning difficulties (Liu et al., 2015).

Why the *Rpp* gene mediated resistance can overcome in nature by ASR? How it would be possible to know the reason? Based on the soybean genome information, cloning of *Rpp* gene was not possible due to many copies of same type of *Rpp* gene. Meyer et al., (2009) showed that *Rpp4* candidate gene have not only a cluster candidacy in chromosome 18 but also in chromosome 9 due to the gene duplication in soybean genome. Recently, Pedley et al., (2019) revealed the presence of three novel *Rpp1* gene candidates and the NBS-LRR protein with a novel Ubiquitin-like-specific protease 1 (ULP1) domain confirmed a role in resistance to *P. pachyrhizi* by VIGS. Their findings suggested that *Rpp1* silencing altered the IR, but not defense. It may be due to the reduced levels of *Rpp1* expression, or components of the *Rpp1* signaling pathway. The closely related plant species resistance resources screening and transformation may be another option to resistance study, expression analysis of a non-orthologous (Cc=*Cajanus cajan*) Cc*Rpp1* transgene to soybean demonstrates the greater expression in homozygous soybean plants. It also concludes that expression levels greatly influenced on the efficacy of the *R* gene (Kawashima et al., 2016). The *Rpp1* gene segregated as single locus (Hyten, 2007; McLean and Byth, 1980) but the locus contains multiple tightly linked resistance genes (Pedley et al., 2019). Furthermore, little is known about pathogen perspective. Some protein mass spectra come from pathogen to plant that contributes to modulation of fungus fitness (Cooper et al., 2011). Effectors from diverse pathogens may target common host proteins (Pedley et al., 2019). Active or inactive domain of that protein alters the recognition and/or signaling (Malik and Van der Hoorn, 2016; Mukhtar et al., 2011; Wessling et al., 2014). For example, complementation with *Rpp1* candidate transgenes with

inactive ULP1 domains functions by *P. pachyrhizi* effectors (Pedley et al., 2019). Finally the lacking of plant resistance fitness may be one of the reasons to compromise resistance.

Plants display their immunity after a series of consecutive reactions of recognition, signal transduction, and signalling pathways to downstream defence responses (e.g., the production of antimicrobial compounds like phytoalexin) (Dodds and Rathjen, 2010). The ability of a plant to defend itself is mostly dependent on the recognition of potential pathogens (Montesano et al., 2003). The first layer of perception is related to the typical molecular signatures of microbial elicitors called pathogen-associated molecular patterns (PAMPs). Stimulation of pattern recognition receptors (PRRs) leads to PAMP-triggered immunity (PTI). The second layer of perception involves recognition of pathogen virulence molecules called effectors by intracellular immune receptors (Dodds and Rathjen, 2010). The immune receptors are often called the NOD-like receptor (NLR) type (Macho and Zipfel, 2014) or nucleotide-binding leucine-rich repeat (NB-LRR) proteins (Sarris et al., 2016). This layer performs the effector triggered immunity (ETI). Higher plants also have a multilevel net of structural-like plant cell wall strengthening and chemical barriers, which mark the synthesis of phytoalexins and pathogenesis-related (PR) proteins in their plant defence mechanism (Berger et al., 2007). They evoke effective means of communication between cells regarding adjustment to new environmental conditions via hormonal signalling pathways, the phytoalexin biosynthesis pathway, and pathways related to the biosynthesis of other phenolic compounds such as callose deposition.

Near-isogenic lines termed as NILs have an almost homogeneous genetic background except for a single fragment or a small number of genomic fragments. Breeding efforts are focused on introgressing *Rpp* genes from a donor soybean parent into elite soybean germplasm of a common susceptible genetic background such that the subsequent generations develop the

Rpp genes containing NILs (Hassan et al., 2014; Yamanaka et al., 2015, 2013). Most recently, many efforts have concentrated on revealing the mediated defence mechanisms of *Rpp* genes. For example, research pertaining to the *Rpp2* resistant genotype PI230970 and the greatly susceptible genotype Embrapa-48 against the ASR transcriptome was conducted by van de Mortel et al., (2007). In the *Rpp2* resistant genotype they observed the expression profiles of 424 differentially expressed genes (DEGs) were increased their expression within the first 12 hai (Hours after inoculation), which had mostly returned to the mock inoculated levels at 24 hai only in resistance genotype. These DEGs were further increased in expression again at 72 hai, whereas in the susceptible genotype, gene expression remained unaffected until 96 hai. mRNA transcript profiling of variety, Ankur (PI462312) carrying the *Rpp3* gene was done by Schneider et al., (2011). They categorized the 54 over-represented GO biological process into seven broad groups that were important in *Rpp3* gene mediated defence mechanisms (Schneider et al., 2011). The microarray datasets compared the *Rpp2, Rpp3,* and *Rpp4* lines with respect to transcription factors (Morales et al., 2013). All the efforts were unable to clarify the mechanism of *Rpp* gene mediated resistance. In addition, the defence responses against ASR in NILs containing *Rpp* genes have no individual or comparative study. This study aims to clarify the question that " Is there any specific gene or sets of genes or a specific pathway are involved in *Rpp* gene mediated resistance mechanism in soybean NILs? This study also represents the first elucidation of variation in *Rpp* gene mediated resistance to *P. pachyrhizi* focusing on major genes of a phenylpropanoid pathway of different *Rpp* gene carrying NILs.

Table1. Soybean varieties carrying resistant genes for ASR defense. [1]Dominance or recessiveness is affected by genetic background (Garcia et al. 2011).

Plant line or variety	Resistance Gene	Origin	Original Name	*Rpp* Gene Reference
PI 200492	*Rpp1*	Japan	Komata	McLean & Byth (1980)
PI 368039	*Rpp1*	Taiwan	Tainung No. 4	McLean & Byth (1980)
PI 547875	*Rpp1*	USA	L85-2378	Walker et al. (2011)
PI 561356	*Rpp1*	China	Jin Yun Dou	Kim et al. (2012)
PI 594177	*Rpp1*	Japan	Himeshirazu	Yamanaka et al (2015)
PI594760B	*Rpp1, Rpp1* [1]	China	Gou Jiao Huang Dou	Garcia et al. (2011)
Xiao Jing Huang	*Rpp1*	China	Xiao Jing Huang	Yamanaka et al. (2015)
PI 587886	*Rpp1-b*	China	Bai Dou	Ray et al. (2009)
PI 587855	*Rpp1-b*	China	Jia Bai Jia	Yamanaka et al. (2016)
PI 587880A	*Rpp1-b*	China	Huang Dou	Ray et al. (2009)
PI 587905	*Rpp1-b*	China	Xiao Huang Dou	Hossain et al. (2015)
PI 594538A	*Rpp1-b*	China	Min Hou Bai Sha Wan Dou	Chakraborty et al. (2009)
PI 594767A	*Rpp1-b*	China	Zhao Ping Hei Dou	Hossain et al. (2015)
PI 197182	*Rpp2*	Malaysia	Raub 16.1422	Laperuta et al. (2008)
PI 224270	*Rpp2*	Japan	Hougyoku	Garcia et al. (2008)
PI 230970	*Rpp2*	Japan	No. 3	Hartwig & Bromfield (1983)
PI 230971	*Rpp2*	Japan	No.4	Laperuta et al. (2008)
PI 417125	*Rpp2*	Japan	Kyushu 31	Laperuta et al. (2008)
PI 416764	*Rpp3*	Japan	Akasaya	Hossain et al. (2015)
Iyodaizu B	*Rpp2*	Japan	Iyodaizu B	Yamanaka et al. (2015)
PI 462312	*Rpp3*	India	Ankur	Hartwig & Bromfield (1983)
PI 567099A	*Rpp3*	Indonesia	MARIF 2740	Ray et al. (2011)
PI 628932	*Rpp3*	Brazil	FT-2	Brogin (2005)
D86-8286	*Rpp3*	USA	D86-8286	Bonde et al. (2006)
PI 459025	*Rpp4*	China	Bing Nan	Hartwig (1986)
PI 459025B	*Rpp4*	China	Bing Nan	Hartwig (1986)
PI 200487	*Rpp5*	Japan	Kinoshita	Garcia et al. (2008)
PI 200456	*Rpp5*	Japan	Awashima Zairai	Garcia et al. (2008)
PI 200526	*Rpp5*	Japan	Shiranui	Garcia et al. (2008)
PI 471904	*Rpp5*	Indonesia	Orba	Garcia et al. (2008)
PI 567102B	*Rpp6*	Indonesia	MARIF 2767	Li et al. (2012)
UG-5	*Rpp1/Rpp3*	Uganda	UG-5	Paul et al. (2015)
PI605823	*Rpp7*	Vietnam	Sample 87	(Childs et al., 2018)
PI506764	*Rpp?*	Japan	Hyuuga	(Kendrick et al., 2011)

Table2. Characterization of soybean varieties carrying resistant genes and their defence status against ASR

Origin of isolates	Year	*Rpp1 & Rpp1-b*			*Rpp2*			*Rpp3*			*Rpp4*			*Rpp5*			*Rpp6*			References
		RB	IM	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	
Taiwan	1966	PI200492	-	-	-	-	-													Lin 1966
Taiwan	1980	PI587886, PI587905, PI594767A	PI587886 PI587905 PI594767 A	- -	-	-	PI23 0970	-	-	-	PI45 9025	-	-	-	-	-	PI5671 02B	-	-	Bromfield et al. 1980, Pham et al. 2009, Ray et al. 2009, Paul et al. 2015
Taiwan	1983	-	-	PI2004 92	PI230971	-	-	-	-	PI4623 12										Yeh 1983
Taiwan	1972-1 980	-	-	PI2004 92	PI230970	-	-		D86- 8286	PI4623 12										Bonde et al. 2006, Pham et al. 2009, Ray et al. 2009, Paul et al. 2015
Thailand	2001	-	-	PI2004 92	PI230970	-	-		D86- 8286	PI4623 12	PI45 9025									Bonde et al. 2006, Pham et al. 2009, Ray et al. 2009, Paul et al. 2015
India	1973	PI200492 PI587886 PI587905 PI594767A	- PI587886 6, PI587905 PI594767 A	- - -	PI230970	-	-	-	D86- 8286	-	PI45 9025	-	-	-	-	-	PI5671 02B	-	-	Bromfield et al. 1980, Bonde et al. 2006, Pham et al. 2009, Ray et al. 2009, Paul et al. 2015
India, Thailand, Taiwan, Brazil and Paraguay	2009	-	-	-	-	-	-	-	PI56 7099 A	-										Li & Young 2009
Philipines	1977	-	-	-	PI230970	-	-			PI4623 12	PI45 9025									Bromfield et al. 1980, Bonde et al. 2006, Pham et al. 2009
Australia	1979	PI200492 PI587886 PI587905 PI594767A	- PI587886 PI587905 PI594767 A	-	PI230970	-	-	PI46 2312	-	-	-	-	PI45 9025							McLean & Byth (1980), Akamatsu et al. 2013
Japan	1990	-	-	-	PI230970	-	PI23 0970	PI46 2312		PI4623 12										Yamaoka et al. 2002
Japan	1993-1 997	-	-	PI2004 92	-	-	-	-	-	-	PI45 9025									Yamaoka et al. 2002
Japan	2007-2 009	PI200492 - PI587880A	- - -	- PI5878 866 -	PI417125	-	-	PI46 2312	-	-	PI45 9025	-	-	PI20 0526	-	-	-	-	-	Yamaoka et al. 2014 Akamatsu et al. 2013,
Vietnam	2005-2 009	PI200492	-	PI2004 92	-	-	-	-		-	-	-	-	-	-	-	-	-	-	Pham et al. 2010
Brazil	2001-2 002	PI200492	-	-	-	PI23 0970	-	-	-	-	-		-	-	-	-	-	-	-	Yorinori 2008, Bonde et al. 2006

Table 2. Continued

Origin of isolates	Year	*Rpp1* & *Rpp1-b*			*Rpp2*			*Rpp3*			*Rpp4*			*Rpp5*			*Rpp6*			References
		RB	IM	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	
Brazil	2002-2003	-	-	PI200492	-	-	PI230970	-	-	-	-		-	-	-	-	-	-	-	Yorinori 2008
Brazil	2002, 2009	-	-	-	PI230970	-	-	-	-		-	-	-	-	-	-	-	-	-	Yorinori 2008, Pham et al. 2009
Brazil	2010-2015	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	PI567102B	-	Kato et al. 2015
Brazil, Paraguay	2001	- -	- -	PI587886 PI587905	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Pham et al. 2009
Brazil, Paraguay	2006	-	-	-	-	-	-	-	PI462312, D86-8286	-	-	-	-	-	-	-	-	-	-	Bonde et al. 2006
Brazil, Paraguay	2007-2010	-	-	-	-	-	-	-	-	-	PI459025	-	-							Akamatsu et al. 2013, Bonde et al. 2006, Pham et al. 2009
Argentina, Brazil, Paraguay	2007-2010	PI587855 - PI587880A PI587905	- - - -	PI200492 PI368039 PI587886	PI230970	PI417125	-	-	-	PI462312		PI459025	-	PI200526						Akamatsu et al. 2013,
South America	2001	-	-	-	-	-	-	-	-	-	-	PI459025B	-							Bonde et al. 2006, Pham et al. 2009, Kim et al. 2012
Paraguay	2001	-	-	-	PI230970	-	-													Bonde et al. 2006, Pham et al. 2009
Paraguay	2008	-	-	-	-	-	PI230970	-	-	-	-	-	-	-	-	-	-	PI567102B	-	Miles et al. 2008
Mexico	2013-2014	PI200492 PI547875	- -	- -	PI230970, PI417125	-	-	-	-	-	-		-	-	-	-	-	-	-	Pena-del-Rio et al. 2014
Uganda	2005	PI200492	-	-	PI230970	-	-	PI462312	-	-	-	-	-	-		-	-	-	-	Oolka et al. 2008, Maphosa et al. 2013
Uganda	2006	PI200492	-	-	PI230970	-	-	-	-	PI462312	-	-	-	-		-	-	-	-	Oolka et al. 2008, Maphosa et al. 2013
Uganda	2010-2011	-	-	-	-	-	-	-	PI462312	-	PI459025									Maphosa et al. 2013
Nigeria	2005	PI200492	-	PI200492	PI230970	-	-	-	-		-	-	-	-	-	-	-	-	-	Twizeyyimana et al. 2009, Twizeyyimana et al. 2011
Nigeria	2005-2006	PI594538A	PI594538A	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	Twizeyyimana et al. 2008, Twizeyyimana et al. 2009

Table 2. Continued

Origin of isolates	Year	*Rpp1* & *Rpp1-b*			*Rpp2*			*Rpp3*			*Rpp4*			*Rpp5*			*Rpp6*			References
		RB	IM	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	RB	I M	TAN	
Nigeria	2009-2011	-	-	-	-	-	-	PI462312	-	-	PI459025	-	-	-	-		-	-	-	Twizeyyimana et al. 2011
Zimbabwe	2001	-	-	PI200492	PI230970	-	-	-	-	-	PI459025B	-	-	-	-	-	PI567102B	-	-	Bonde et al. 2006, Pham et al. 2009, Ray et al. 2009, Paul et al. 2015
Zimbabwe	2006	-	-	-	-	-	PI230970	-	-	-	-		-	-	-	-	-	-	-	Bonde et al. 2006
South Africa	2001	- -	- PI594767A	PI200492 PI587886	PI230970	-	-	PI462312	-	-	-	-	-	-	-	-	PI567102B	-	-	Bonde et al. 2006, Pham et al. 2009, Ray et al. 2009, Paul et al. 2015
Hawaii	1994	PI587886 PI587905 PI594767A	PI587886 PI587905 PI594767A	- -	-	-	-	PI462312	-	-	PI459025									Pham et al. 2009, Ray et al. 2009
USA (Southern)	2006-2009	PI200492	-	PI230970	-	PI230970	-	-	-	-		-	-	-	-	-	-	-	-	Li & Young 2009, Pham et al. 2009, Ray et al. 2009, Walker et al. 2011
USA (Florida)	2011-2012	-	-	PI200492	-	-	-	-	-	-	-	-	-	PI471904	-	-	PI567102B			Paul et al. 2013, Paul et al. 2015
USA (Florida)	2006-2012	-	-	PI547875	-	PI230970	-	-	-	PI462312		PI459025B	PI459025B	PI200487	PI200487	PI200487	PI567102B	PI567102B		Walker et al. 2011, Paul et al. 2013
USA (Georgia)	2008	-	-	-	-	-	-	-	-	-	-	-	-	-	PI200456	-			PI567102B	Walker et al. 2011
USA (Georgia)	2012	PI547875	-	-	-	-	-	-	PI462312	-	-	-	-	PI200456	-	-				Walker et al. 2014
USA(Alabama & Luoisiana)	2004	-	-	-	-	-	-	PI462312	-	PI462312	PI459025B	-	-	-	-	-				Pham et al. 2009
USA(Alabama)	2007	-	-	-	-	-	-	-	-	-	-	-	-	PI200487	-	-	PI567102B	-	-	Walker et al. 2011
USA (Luoisiana)	2007-2008	-	-	-	-	-	PI417125	-	-	-	-		-	-	-	PI200487		PI567102B		Walker et al. 2011, Walker et al. 2014
USA (Mississippi) from Kudzu	2006	PI587880A	-	PI587880A PI594767A	-	-	-	-		-	PI459025	-	-	-	-		PI567102B	-	-	Li & Young 2009
USA (Central and Southern)	2007-2008	PI587880A	-	-	-	-	-	-	-	-	-	-	-	-	-	PI200526	PI567102B			Paul et al. 2015, Twizeyyimana & Hartman 2012
USA (South eastern)	2006-2012	-	-	-	PI224270,PI417125	-	-	PI462312	-	-	-	-	-	-		-	-	-	-	Walker et al. 2011, Walker et al. 2014

CHAPTER 2: MATERIALS AND METHODS

2.1 Soybean plant materials

The *Rpp* bearing NILs used in this study were developed from soybean genotypes used in the previous studies (Silva et al., 2008; Yamanaka et al., 2011; Akamatsu et al., 2013; Hossain et al., 2014). NILs were generated by crossing the donor resistant parents (bearing one of the respective *Rpp* genes) with the ASR susceptible parental variety BRS184 and following three cycles of repeated backcrossing of BRS184 as the recurrent parent. The Komata (PI 200492), PI 230970, Ankur (PI 462312), and Bing Nan (PI 459025) genotypes used as donor parents carry the resistant dominant alleles of *Rpp1*, *Rpp2*, *Rpp3*, and *Rpp4*, respectively. The recurrent parent, BRS184 was used as a susceptible control. All the soybean plants were grown in a potting mix. Four seeds per pot were planted into fifteen pots. The growth chamber environment was set by 28/20°C temperature and 14/10 hour of photoperiod (day and night respectively). All plants were kept in the same growth chamber until the second trifoliate in vegetative (V2) growth stage (Fehr et al., 1971) corresponding to about 3 weeks after planting.

2.2 Pathogen inoculation

Fresh urediniospores of the *P. pachyrhizi* isolates T1-2 (Yamaoka et al., 2014) were used. Before inoculating the soybean plants, microtubes containing frozen urediniospores were moved from the -80°C freezer and thawed in a 39°C water bath for 1 min. Then, the spores were suspended in sterile distilled water containing 0.005% (v/v) Tween 20 and adjusted to a concentration of 1×10^6 spores ml^{-1} of spore with a haemocytometer. Inoculation solution was prepared as 1×10^2 solution/leaflet and sprayed onto the abaxial surface of the leaves. The inoculated plants were incubated in a dew chamber at 21°C overnight

(approximately 16 h) and subsequently placed into a growth chamber. The whole procedure was conducted following the instructions at Japan International Research Center for Agricultural Sciences (JIRCAS) laboratory manual for studies on soybean rust resistance. https://www.jircas.go.jp/en/publication/manual_gudeline/30

2.3 Methods of phenotypic disease status evaluation

For disease status comparisons of *Rpp1-Rpp4* containing NILs, we collected samples from third trifoliate of 21 days old leaves with three replications. The disease status on leaves were evaluated and briefly presented in the figure 2. Rust severity was also measured from three replication of leaf samples using a scale based on the counted lesion density per leaflet, where 1 = no lesions; 2 = 1 to 30; 3 = 31 to 75; 4 = 76 to 150; 5 = 151 to 300; 6 = 301 to 750; 7 = 751 to 500; 8 = 1501 to 3000 and 9 = more than 3000 lesions (Miles et al., 2008). Pustule frequency was counted from three replication of leaf samples (Maphosa et al., 2012). Reaction type was also classified according to JIRCAS laboratory manual. Leaf yellowing was evaluated from three replication of leaf samples using a scale based on the leaf yellowing index per leaflet, where 1 = No yellowing (0%), 2 = Detectable (Few%), 3 = Very little (X<10%), 4 = Little (10%<X<25%), 5 = Moderate (25%<X<50%), 6 = Much (50%<X<100%), 7=Completely (100%) (Yamanaka et al., 2011).

Disease development at each of the inoculated plant leaves was visually assessed with a stereoscope (Olympus B061 of Olympus Corp., Tokyo, Japan) 21 days after inoculation. Disease symptom images were snapped with a digital camera (Nikon D5300). The number of lesions and pustules at each infection point were counted at 40X magnification. Student t-test were done for statistical analysis by office excel 2007.

2.4 Tissue collection, total RNA isolation and cDNA synthesis

Fully expanded and inoculated leaves from V2 stage plants (21 days old plant) were sampled for RNA extraction. Leaves were placed in a falcon tube wrapped with aluminium foil and dipped into liquid nitrogen, and subsequently were ground with a mortar and pestle using Invitrogen™ reagent (Thermo Fisher Scientific Inc. Carlsbad, CA 92008 USA). Total RNA was extracted for RT-qPCR from the ASR susceptible host plant BRS184 and four NILs, each of which carried one of the ASR resistance genes (*Rpp1*, *Rpp2*, *Rpp3*, or *Rpp4*). Leaves were collected at different durations after inoculation (0, 12, 24, 48, and 96 hai). Each sample was pooled from 15 plants. As a mock control plants without ASR inoculation (0 hai) were sampled. Two μg of total RNA of each sample was subsequently treated for gDNA elimination with gDNA Eraser (TaKaRa clontech), and the reverse-transcription reaction was conducted using the Prime Script™ RT reagent kit for the SYBR® Green RT-qPCR assay.

For RNA-seq, the experiment was designed based on the *P. pachyrhizi* isolate T1-2, the susceptible soybean genotype BRS184, and NIL for the *Rpp3* gene. The mock inoculation (no *P. pachyrhizi* inoculation) and *P. pachyrhizi* inoculation was conducted and subsequently RNA isolation was performed for both genotypes. Mock inoculation on BRS184 was termed as "Susceptible Mock (SM)", ASR inoculated on BRS184 was termed as "Susceptible Induced (SI)", Mock (water) inoculated on the soybean accession no PI 462312 (carrying the *Rpp3* resistance gene) was termed as "Resistance Mock (RM)", and ASR inoculated on this accession was termed as "Resistance Induced (RI)". 0 hai (SM and RM) and 24 hai (MI and RI) soybean leaf samples were collected for RNA-seq. Transcriptomic data were generated from all the combinations of SM, SI, RM, and RI which were SM vs SI, SM vs RM, SM vs RI, SI vs RM, SI vs RI, and RM vs RI. The Leaves materials for RNA-seq are shown in figure 1.

2.5 Total RNA preparation, RNA-seq library preparation, and sequencing

Total RNA was prepared from leaf tissues using RNeasy Plant Mini Kit (Qiagen, MD, USA). Leaves were completely crushed using the multi-beads shocker (YASUI KIKAI, Japan). The concentration of total RNA was measured using a NanoDrop l by dilution. The (Thermo Fisher Scientific, MA, USA) and adjusted to 100 ng/ quality was checked for integrity before performing the RNA sequencing process using the Bioanalyzer (Agilent Technologies, CA, USA). For library preparation, 500 ng of total RNA per sample was used. The library construction was carried out as previously described (Nagano et al., 2015). Sequencing was carried out on each library to generate 100 bp PE reads for transcript sequencing on an Illumina High-Seq 4000 platform by a commercial service provider (Macrogen, Kyoto, Japan). Clean reads were mapped to well-annotated 'Williams 82' soybean reference genome (assembly Glyma.Wm82.a1.0, annotation v1.1) obtained from phytozome website (Phytozome) using Tophat v2.0.13 (Trapnell et al., 2009). Raw count data were obtained by Cuffdiff embedded in Cufflinks pipeline v2.1.1 (Trapnell et al., 2012). Differential expression analysis of two samples was performed using the DESeq R package (Anders and Huber, 2010). Genes with an adjusted p-value < 0.05 by DESeq were determined to be differentially expressed genes (DEGs). For samples without biological replicates, the DEGseq R package (Wang et al., 2010) was used; q value < 0.005 and |log2 (fold change) |>1 were set as the thresholds for differential gene expression. A versatile, platform independent and easy to use Java suite for large-scale gene expression analysis was developed by genesis (Sturn et al., 2002).

2.6 Annotations

Gene ontology (GO) is a major bioinformatics initiative to unify the gene and gene product attributes/vocabulary across all species. GO enrichment analysis of the differentially expressed genes (DEGs) was performed to identify ontology terms and pathways represented by these significant genes using the soybean breeder's toolbox on the Soybase website (https://www.soybase.org/goslimgraphic_v2/dashboard.php). The enriched results were then filtered considering a 1% false discovery rate (FDR) significance threshold to obtain highly significant enriched GO terms. Homology annotations were performed using public databases including GO, KOG (Eukaryotic clusters of Orthologous Groups), and KEGG (Kyoto Encyclopedia of Genes and Genomes). Gene descriptions and functional annotation databases were downloaded from the soybean breeder's toolbox on the Soybase website (http://soybase.org/dlpages/index.php#annot), The Samuel Roberts Noble Foundation website (http://plantgrn.noble.org/LegumeIP/download.jsp), and the NCBI ftp KOG website database (ftp://ftp.ncbi.nih.gov/pub/COG/KOG/). Besides the above annotation, the Panther, Uniref, and SoyCyc database analyses for annotation were also done to cover 100% of the annotations. Based on a similarity search an in-house annotation method resulted in the quantification of 4,518 total unigenes, and 100% were quantified in at least one database (Figure 3 and Table 3).

Furthermore, the RM vs RI expressed genes were analysed, based on gene ontological databases. Generally, over-representation or under-representation was counted automatically using the calculation of meta-data coincidence by Fisher's exact test, prior to the Bonferroni-corrected pair wise technique. The online Soybase breeder's tool box website (https://soybase.org/goslimgraphic_v2/dashboard.php) was utilized for comparison.

2.7 Quantitative RT-qPCR analysis

RNA-Seq results were verified by quantitative real-time reverse transcriptase PCR (RT-qPCR) using SYBR-green (TaKaRa Biotechnology Co., Ltd., Japan). RT-qPCR was performed in a Takara biotech Real-Time Cycler using gene-specific primers (Table 4). In total, primers for 12 *P. pachyrhizi* induced genes were designed to produce amplicons of about 25 bp for each gene using the Primer3 software (http://primer3.sourceforge.net). The expression data were normalized to the soybean *Con7* and *unknown2* genes (Hirschburger et al., 2015). For each sample, the RT-qPCR reaction was repeated three times and the relative mRNA expression level was calculated as $2^{-\Delta\Delta CT}$. Correlation and significance analyses were performed (Fu et al., 2016). Statistical analysis was done by the Tukey-Kramer test.

CHAPTER 3: RESULTS

3.1 Phenotypic observation of Phakopsora pachyrhizi infection

To better understand the NIL resistance label, the ASR disease phenotypic variation with soybean NIL containing *Rpp* gene was evaluated within four soybean *Rpp* NILs with susceptible BRS184 according to categorical colour standard by JIRCAS manual and Yamanaka et al., (2010). Differences between the compatible and incompatible interactions, the interaction was categorized four classes according to the phenotypic events on NIL leaves at 21 days after *P. pachyrhizi* inoculation. The susceptible BRS184 showed abundant sporulation with no resistance response (Figure 2). *Rpp1* NIL was also showed no resistance response with abundant sporulation but leaf yellowing is higher than others. In *Rpp2* NIL, it was found moderate sporulation with urediniospore in pustules with RB lesions. *Rpp3* NIL constitutes with few pustules and RB lesions and *Rpp4* NIL showed little pustules with RB lesions.. *Rpp1* NIL had higher degrees of leaf yellowing and TAN lesion among all other NILs (Table 5).

3.2 Identification of differentially expressed genes (DEGs) from resistant and susceptible soybean genotypes infected with Phakopsora pachyrhizi

A total of 2,558 genes (56.61% of the total genes) were covered in three GO categories: BP (biological process), CC (cellular component), and MF (molecular function). Biological process, oxidation reduction process, and transcription regulation were the most represented in the BP category (Figure 4). In the case of the CC category, chloroplast, nucleus, cytoplasm, and plasma membrane ranked greater than others. The KEGG database analysis demonstrated that the large subunit ribosomal protein, Interleukin-1 receptor associated kinase 4, and phenylpropanoid biosynthesis related genes were significantly

different compared with the other genes (Figure 5). Moreover, 1,452 (32.13%) DEGs were found using the KOG functional category databases. Different ribosomal proteins and serine-threonine protein kinases represent the top two largest groups (Figure 6).

3.3 Resistance response of NILs to Phakopsora pachyrhizi by basic plant defence related DEGs

Based on the different treatments, we filtered the DEGs by making a venn diagram (Figure 7). Among 3,759 DEGs RM vs RI showed the greatest similarities with 1,742 DEGs shared. The SM vs SI, SI vs RI, and SM vs RM combinations had the fewest number of DEGs in common (three). The combinations of SM vs SI and RM vs RI represented 455 DEGs where 258 genes (56.7%) were down-regulated. Fourteen genes were more than two-fold up-regulated when RM vs RI was compared with SM vs SI, and seven genes were more than two-fold down-regulated (Table 6). According to the two-fold change baseline, there were twice as many up-regulated genes compared with down-regulated genes.

K-means clustering is one of the simplest and popular unsupervised machine learning algorithms that makes clusters in a dataset. We performed k-mean clustering to compare the DEGs common to SM vs SI, SM vs RM, and RM vs RI (Figure 8). A total of 138 genes were distributed in six different clusters. The greatest numbers of genes are in cluster 5, and are associated with chloroplast machinery, benzoxazinoids (BX), and some transcription factors. Cluster 3 was the second largest with 38 genes, and showed the opposite trend compared with cluster 5. The cluster 2 DEGs are involved mainly in primary and secondary metabolism pathways related to jasmonic acid (JA) mediated resistance mechanisms. Cluster 4 also showed a pattern similar to cluster 3, but the variation among the cluster 4 genes was great. Cluster 4 contained the greatest number of down-regulated genes. Most of them actively

contribute to changing metabolic pathways upon external agitation (Table 7). To reveal the fluctuation of the genes in the Venn diagram, this analysis found that the down regulated genes are greater in number, with the exception of in SM vs RM (Figure 9), and the greatest number of genes (1,742) were involved in the RM vs RI set. To specify the involvement of a defence system of this study discovered that 73 genes were engaged for systemic acquired resistance (SAR). Seventy-two genes were involved in carbohydrate metabolic processes. The third greatest (60 DEGs) were chitin responsive, and fifty-three DEGs were associated with JA regulation. The major set of these genes is shown in a heat map as carbohydrate metabolic process, defence response to fungus, response to chitin, MAPK cascade (Figure 10) and transmembrane transport, JA stimulus, SA stimulus, regulation of transcription by TF, signal transduction, flavonoid biosynthesis process, flavonoid metabolic process and SAR in Figure 11.

3.4 DEG mining attributes the phytoalexin biosynthesis pathway genes as prevalent candidates against Phakopsora pachyrhizi in soybean

According to our analysis the biological process GO term focused on seven distinct classes including cell growth and maintenance, energy and environmental information sensing, metabolism, oxidation, signalling, transcription related, and defence. Up- and down-regulated genes of these classes were distributed into 30 sub classes (Figure 12). Only 9 sub classes of up-regulated genes ranked higher than the down-regulated genes. Namely, they were amino acid import, amino acid transport, oxidation reduction process, response to oxidative stress, translation, ribosome biogenesis, response to UV-B, regulation of flavonoid biosynthetic process, and regulation of defence response. In the oxidation reduction process only two GO category made the highest 213 DEGs group among seven classes were involved oxidation-reduction process and response to oxidative stress. Peroxidase and cytochrome

P450 were the common genes that were up-regulated and down-regulated simultaneously. We also found the phenylpropanoid pathway genes in this category. Briefly it was presented in Table 8 of Considering the top up-regulated genes of these nine subclasses, the glycinol 4-dimethylallyltransferase (Glyma20g38930) was found in the regulation of defence response subclass. In the regulation of flavonoid biosynthetic processes under defence class, the phenylalanine ammonia-lyase (*PAL*;Glyma19g36620) was top up-regulated gene. Glyma10g43850 which encodes chalcone isomerase (*CHI*) was the top gene in the response to UV-B subclass, and for response to oxidative stress, it was Glyma11g07490, which encodes isoflavone reductase-like protein. NAD(P)H-dependent 6'-deoxychalcone synthase was identified as Glyma18g52250, which is involved in oxidation-reduction processes. With respect to these up-regulated genes, the phenylpropanoid pathway should be predominantly related to ASR resistance in soybean. In addition, 172 genes involved in oxidation reduction processes (GO:0055114) and 137 genes involved in the regulation of transcription genes were the most agitated subclasses. This agitation may have an effect on senescence as well as photosynthesis via NAC transcription factor (TF) functional network. Among the individual TF of soybean genome, the NAC gene expression showed highest percentage of expression though it ranked seven as the individual number in the genome (Table 9). In addition, we found 91 transcription factors were involved in RM vs RI for soybean defence (Table 10).

3.5 Expression profiles of genes associated with the phytoalexin biosynthesis pathway genes modulation in soybean NILs by P. pachyrhizi infection

The phenylpropanoid and isoflavonoid pathway derived phytoalexin is briefly represented in Figure 13, and the relative gene expression was performed on the genes mentioned above via RT-qPCR analysis. All resistant NILs and susceptible BRS184 plants showed the greatest *ADT6* gene expression at 12 hai (Figure 14A). In BRS184 plants, *ADT6*

gene expression was significantly different compared with the control at the 24, 48, and 96 hai time points (Figure 14A). The second greatest *ADT6* gene expression which exhibited a significant difference was found in BRS184 and the NIL of *Rpp2* at 96 and 24 hai, in comparison with 48 hai and the control time point, respectively. The other time points exhibited no significant changes (Figure 14A). Figure 14B represents *PAL*, the gateway enzyme of the phenylpropanoid pathway. Significantly great expression of the *PAL* gene at 12 hai was observed only in the NIL of *Rpp1*, even though BRS184, and the NILs for *Rpp2* and *Rpp4* showed relatively great expression at 12 hai. At 12 hai, the expression pattern of cinnamate 4-hydroxylase (*C4H*) was the greatest in the *Rpp1* soybean NIL, and slowly decreased in the *Rpp2*, *Rpp3*, and *Rpp4* NILs respectively (Figure 14C), but a significant change was found only in the *Rpp1* NIL and BRS184. Within the BRS184 and *Rpp1* NIL, the significantly different expression was found at 12 and 24 hai for BRS184. Conversely, in the *Rpp1* NIL, the *C4H* expression was significantly different at the control time point and 12 hai. In comparison, *CHS* gene expression within the *Rpp* NILs was greatest at 12 hai, except for the *Rpp4* NIL. However, no time points in any lines showed a significant difference (Figure 14D). Our RT-qPCR showed that *CHR* expression bloomed at 0 hai for all the *Rpp* lines, except *Rpp3* NIL. However, the significance level of *CHR* expression at 0 hai was found only in *Rpp4* NIL at the 96 hai time point. Significantly different expression at the 0 hai time point was only observed for the *CHR* gene among the studied gene expressions (Figure 15A). The relative expression of *CHI* and *IFS* at 12 hai was greater than the other time points, but no significant changes in expression were found in all the soybean lines, including the control (Figure 15B and 15C). *IFR* gene expression in BRS184 plants was significantly greater at 12 hai than at the 0 and 48 hai time points. With respect to the *Rpp2* NIL, the *IFR* gene expression differed significantly only at the 12 hai and 0 hai time points (Figure 15D). For *ispG*, all *Rpp* NILs showed the greatest expression at 12 hai followed by 96

hai, with an exception in the *Rpp3* NIL, but the significant expression difference was found in BRS184, and the *Rpp2* and *Rpp3* NILs. Among the BRS184, *Rpp2,* and *Rpp3* NILs *ispG* expression in *Rpp3* was significantly different at 12 and 24 hai, whereas in the BRS184 line *ispG* expression was significantly different at 0 and 12 hai. In *Rpp2 ispG* expression at 12 hai differed from that at 24 and 48 hai (Figure 16A). In the case of *G4DT*, the greatest expression was observed at 96 hai in all soybean plants, with the exception of *Rpp3* where it was seen at 12 hai. In *Rpp3 G4DT* expression was greatest at 48 hai, and significant changes were observed at 12 and 24 hai. The other significant *G4DT* expression was found in BRS184 between 96 and 12 hai (Figure 16B). Regarding *G2DT* gene expression, there is no significant change in expression between the different soybean lines. The *Rpp3* soybean line showed quite a different pattern of expression than the other NILs where *Rpp4* showed higher expression than *Rpp3* at 12 hai (Figure 16C). The relative expression of *PT3* at 12 hai was significantly greater for *Rpp1* as compared with BRS184 and *Rpp2*, while the expression of *PT3* in the *Rpp3* and *Rpp4* lines were not significantly different at the studied time points (Figure 16D).

CHAPTER 4: DISCUSSION

Study of host resistance is not only bracketed into Flor's gene for gene theory (Flor, 1946). *R* genes were found to be only one set of participants in web of interacting factors (Andersen et al., 2018)**.** van der Plank in the early 1960 mentioned plant disease resistance responses as vertical resistance and horizontal resistance. The characteristics of both resistances are not same. Vertical resistance is conditioned by one or few genes calling major genes or monogenic of oligogenic resistance. This type is not durable and often race specific or qualitative resistance. On the other, the horizontal resistance termed for nonspecific resistance governed by polygenes. It is severely known more durable than that and somewhat incomplete resistance, quantitative, partial resistance or field resistance calling by minor gene or mature/adult gene. Insights from poplar-rust interactions genomics and transcriptomics also showed that partial resistance mainly quantitative and timely regulated (Hacquard et al., 2011). In soybean, partial resistance also found in some *Rpp* gene containing line as well as pyramided line (Paul et al., 2011; Yamanaka et al., 2013, 2011, 2010). *Lr34, Lr46, Lr67* and *Lr68* confer different level of partial resistance as the adult plant resistance (APR) gene that produce stronger resistance with the combination of *Lr34* (Ellis et al., 2014). But phenotypic difference of that genes carrying plant is hard to distinguish (Ellis et al., 2014). The genes in this class mostly conform to minor gene for gene theory (Parlevliet & Zadoks, 1977). For example, PI 200492 source of *Rpp1* response against ASR isolates collected from soybean and kudzu (*Pueraria lobata*) during 1993 to 1997 in Japan (Yamaoka et al. 2002) showed susceptible reactions but showed induced resistance reactions against ASR collected during 2007 to 2009 in Japan (Yamaoka et al. 2014). Above all, the understanding of host resistance may have many dimensions.

In soybean *Rpp* genes carrying plant showed many degrees of phenotypic resistance that make complexity of individual gene mediated disease diagnostics (Yamanaka et al., 2010). In our observation of *Rpp1* NILs originated from PI 200492 into susceptible BRS 184 background showed like susceptible reaction against T1-2 indicates that genetic background have some influence on rust resistance in soybean (Garcia et al., 2011).We also found that the degree of leaf yellowing was higher in *Rpp1* NIL than other three NILs. The characteristics of leaf yellowing or preventing leaf yellowing are related to resistance to susceptible response to ASR (Yamanaka et al., 2011) and may be more linked to *Rpp1* resistance loci. In *Rpp2* NIL, we observed moderate number of pustules in *Rpp2* NIL but previously in PI230970 showed inconsistent reaction against different ASR isolates (Yamaoka et al., 2002). It is assumed that susceptible homogenous background may be able to impact on resistance responses (Maphosa et al., 2012). Before 2000, *Rpp3* (PI462312) was ineffective against Japanese races. However, it became effective in 2007 to 2009 (Yamanaka et al. 2010; Yamaoka et al. 2014). NIL from PI462312 in susceptible background also showed RB lesions with fewer uredinia production against T1-2. If *Rpp3* NIL show same RB reaction against Brazilian races, we can speculate that the resistance phenotype is related to genetic background. On the other hand, if it shows susceptible reaction it may be for the progenies or different resistance genes linked closely in this region (Hossain et al., 2015, Yamanaka et al., 2011; Akamatsu et al., 2013). PI459025, the source of *Rpp4* mediated resistance in NIL showed RB lesions with moderate number of pustule production and degree of leaf yellowing was also few against T1-2. We also observed the dark color of lesions like previous findings (Yamanaka et al., 2010). This result speculated that *Rpp4* allele from PI459025 may have linked genes. The variation accordingly between *Rpp* NIL and *Rpp* cultivar defence response also add extra mimics to understand the reason behind the complexity of individual *Rpp* gene mediated resistance mechanism.

In general, the whole genome of soybean has about 46,000 genes, however 70%-80% are duplicates. Transcriptomics is good way to avoid functional duplication to select correct genes or pathways for soybean development. This transcriptomic study found that 4518 DEGs were induced against ASR. All the genes were annotated by different databases (Table 3). KEGG functional annotation suggested the large subunit ribosomal protein, *IRAK4*, and phenylpropanoid biosynthesis related genes are greatly involved in ASR defence. Such results are similar to other studies pertaining to the regulation of defence and stress tolerance systems of soybean against ASR (Morales et al., 2013; Pandey et al., 2011; Schneider et al., 2011; van de Mortel et al., 2007). Venn diagram sorting, K-means clustering, and GO enrichment analysis specified some potential candidate genes for soybean defence to ASR.

A venn diagram (Figure 7) clearly presented the 3,759 DEGs under the SM, SI, RM, and RI interactions. 455 DEGs were found in the SM vs SI and RM vs RI combinations. These genes represent the common genes which are related basic defence during the soybean and ASR interaction. Most of the genes in this category were down-regulated. Fourteen genes were selected using the threshold of two or greater fold up-regulation in RM vs RI than SM vs SI (Table 6). These genes are mostly related to defence mechanisms of different plant species to various pathogens. For instance, *CHR* was the top up-regulated gene in the RM vs RI interaction, and exhibited three times greater expression than the *CHR* transcript observed in the SM vs SI interaction. *CHR* induced the biosynthesis of 5-deoxyisoflavonoids that suppress race-specific resistance against in *Phytophthora sojae* infection in soybean (Graham et al., 2007). The flavonoids pathway gene *CHR* also contributes to the biosynthesis of other phytoalexins, which triggers hypersensitive cell death against different pathogens (Chang et al., 2011; Graham et al., 2007). Seven genes were more than 2-fold down-regulated in the RM vs RI than SM vs SI combinations (Table 6). These genes also participate in *Rpp* gene

mediated resistance mechanisms. Specifically, *IRAK* was recruited by pattern recognition receptors (PRRs) with ligand binding which participate in the plant immune system (Couto and Zipfel, 2016). The 26S proteasome is responsible for the repression of jasmonate-zim domain protein (*JAZ*) in the JA defence mechanism (Pauwels and Goossens, 2011). However, the down-regulation of these genes may be the response of the plant to ASR attack. Cluster 2 of the K mean clustering (Table 8) analysis also showed that the *MYC2* gene which is related to the *JAZ* suppressor was also downregulated. The venn diagram redirects us to consider flavonoids and hormonal pathways for ASR defence in soybean.

K mean clustering (Figure 8) were done among 138 genes from the common DEGs from SM vs SI, SM vs RM, and RM vs RI. Six different clusters were identified which guide the gene groups during the ASR interaction. The greatest numbers of genes are in cluster 5, and the genes are associated with chloroplast machinery, *BX*, and some transcription factors. The chloroplast works as a processing unit for the regulation of plant development, metabolism, and responses to the environment via complex signalling pathways (Bobik and Burch-Smith, 2015). During ASR infection, the photosynthesis rate may be reduced and the chloroplast redox balance/state may be involved in soybean defence network (Demmig-Adams et al., 2014; Karlusich et al., 2017). The down-regulation of cluster 5 and genes in RM vs RI is expected in the ASR interaction. Cluster 3 which was the second largest with 38 genes showed the opposite trend of clusters 2 and 5. The DEGs in this cluster are involved mainly in primary and secondary metabolism pathways and have a vital role for plant defence against pathogen (Berger et al., 2007). Cluster 2 genes are mostly related to JA mediated resistance mechanisms. In JA response, the 26S-proteasome mediated polyubiquitination and jasmonate signaling is closely related to plant defence network (Nagels Durand et al., 2016). Therefore, down-regulation of these genes indicates that during

ASR defence warfare the JA signalling were inhibited. This facilitates the SA mediated defence. Generally, the SA and JA pathways are the signalling hubs that activate against biotrophic and necrotrophic pathogens, respectively, and work together antagonistically (Pieterse et al., 2012; Caarls et al., 2015). Cluster 4 showed a similar pattern to cluster 3 where the SM vs SI and RM vs RI genes were up-regulated, but the genes for SM vs RM were diverse with respect to the type of genes which were down-regulated, including the top than cluster 3. Most of the genes in this cluster actively contribute to cell wall reinforcement, secondary metabolites, and pathogenesis related genes (Table 11). Upon pathogen attack, these genes were active for host defence (Aoun Mirella, 2017). Taken together, the results of the cluster genes indicate that the resistant mechanism of *Rpp* soybean plants is dependent on perturbation of the basic plant defence system (Figure 10 and figure 11).

In the basic defence class, there were many overlapping genes involved in response to UV-B, response to wounding, regulation of flavonoid biosynthetic processes, and flavonoid metabolic processes. UV-B, response to wounding, regulation of flavonoid biosynthetic processes, and flavonoid metabolic processes often result from secondary metabolite biosynthesis (Mameda et al., 2018; Surjadinata et al., 2017). In a broader sense, the DEGs in response to secondary metabolites and phytoalexins are expressed during pathogen attacks (Pusztahelyi et al., 2015). Recent transcriptomic studies and the results of the current study suggest that the phenylpropanoid pathway is induced for soybean defence against ASR (Morales et al., 2013; Pandey et al., 2011; Schneider et al., 2011; van de Mortel et al., 2007). The RT-qPCR results optimized the relative expression of the phenylpropanoid pathway genes in this study. This results disclosed the coordination of three different set of phenylpropanoid pathway genes responsible for *Rpp* gene mediated resistance via steady state metabolite flux output (Dastmalchi et al., 2016; Lee et al., 2014, 2011; Zelezniak et

al., 2014). Firstly, the relative expression of *CHS* and *CHR* in this study (Figure 14D and 15A) was shown by their coordinate action by the division of their functional time. *CHS* worked dominantly at 12 hai in all soybean plants except the *Rpp4* line and 96 hai for *Rpp3* line. On the other hand, *CHR* functioned at 0 and 96 hai as supported by the coordinated manner with *CHS*, with the exception of the *Rpp4* line. *CHR* exhibited the second greatest expression of the *Rpp4* line at 48 hai indicates that during that period the *CHS* and *CHR* coordination for compromising some elicitation are not always same (Schneider et al., 2011). At 96 hai the expression was significantly less than that seen at 0 hai also ensure the coordination but not biphasic state (Schneider et al., 2011; van de Mortel et al., 2007). A recent study showed that the joint action of *CHR* and *CHS* has not been justified experimentally, and the intermediate substrate and channelling of *CHR* remains obscure (Dastmalchi et al., 2016). Additional studies are needed to clarify the coordination mechanism of *CHS* and *CHR* in the *Rpp4* NILs. However, this is challenging due to the fact that these genes are expressed multiple times in different gene networks. For example, *IFS* expression depends on some other co-regulating up-stream and down-stream genes. *IFS* is one of the factors affecting with very peculiar manner (Jung et al., 2000) with cross connectivity of *CHS* (Cain et al., 1997; Graham et al., 2007; Lozovaya et al., 2007). *IFS* triggers the modification of phenolic metabolism in soybean plants during ASR infection (Lozovaya et al., 2007). Our transcriptome data showed that *CHS* and *IFS* were down-regulated during the ASR interaction (Figure 14D and 15C). RT-qPCR results also showed that there were no significant changes in all the *Rpp* lines with control plants. On the other hand, the *CHR* expression was found to be very specific to *Rpp4* in this study. Considering the *CHS, CHR*, and *IFS* (Figure 14D, 15A & 15C) results suggests that *CHR* may have co-regulated effect on *Rpp* lines and with other components of co-regulation networks (Jung et al., 2000).

Secondly, *ispG* and *ADT6* coordination may play a vital role in ASR defence. In our study the *Rpp4* line showed a similar pattern of expression for both genes (Figure 14A & 16A). The rate-limiting coupling process of the isoprenoid pathway and shikimate/polyketide pathway (Sasaki et al., 2011) have great individual roles. For example, the alternative route of phenylalanine biosynthesis may hamper the main route of phenylalanine biosynthesis through arogenate (Tzin and Galili, 2010). On the other hand, *ispG* is essential for the methylerythritol phosphate pathway (MEP) pathway, but it may be inactive without a functional *ispH* or other induced stress (Chang et al., 2013; Li et al., 2017). Our results indicate that *Rpp* NILs minimize the perturbation of gene expression like a precise way of defence response. But different environmental factors and cellular compartments of either the pathogen or plants may change the picture (Lee et al., 2006). The race specific defence of the *Rpp* lines is their prime limitation. The opportunity for the asymmetrical coordination between *ispG* and *ADT6* reminds us of the limitations of the defence mechanisms of the *Rpp* lines.

Thirdly, the *G4DT* and *PT3* expression was greatest at 96 hai and 12 hai respectively, in all *Rpp* lines (Figure 16B and 14D). Lack of downstream anchoring connection of these genes (Dastmalchi et al., 2016), this result was theoretically incongruous (Akashi et al., 2009). But these genes have many possibilities of anchoring like role in the same pathway (Li et al., 2017; Zhang et al., 2013; Zhang and Liu, 2015). The *IFR* expression result indicates that the pterocarpan gene *PT3* in the *Rpp3* and *Rpp4* lines has some co-regulated genes (Figure 15D and 16D). *IFR* expression is significantly induced on the final step of downstream pterocarpan biosynthesis in *Rpp3* and *Rpp4* lines (Hua et al., 2013; López-Meyer and Paiva, 2002). Over-expression of *IFR* in soybean indicated that *IFR* induced the expression of the upstream *PAL* and *CHS* genes, and thus resistance to *Phytophthora sojae* (Cheng et al., 2015), suggesting some anchoring involvement. Another important thing was that the expression of

G2DT in *Rpp3* and *Rpp4* was greater than the other soybean lines, although the difference was not significant (Figure 16C), sharing that the pterocarpan biosynthesis is not only dependent on *IFR* (Hua et al., 2013; López-Meyer and Paiva, 2002) but also *G2DT* in *Rpp3* and *Rpp4* lines.

In summary, the transcriptome experiment revealed that the phenylpropanoid pathway genes were prevalently involved in *Rpp3* gene mediated resistance to the ASR isolate T1-2. This result was congruent with the above mentioned contemporary transcriptomic study. The relative gene expression study of select genes of that pathway was conducted by RT-qPCR using the isolates which corresponded with the *Rpp1-Rpp4* NILs. With the exception of *ispG*, *G4DT* and *CHR*, all the genes showed the greatest expression at 12 hai, but the expression patterns which occurred between 24 and 96 hai make these *Rpp* lines unique (Figure 18). This result explains the expression rate, which may limit the metabolic flux output for phytoalexin biosynthesis. New fungal races of ASR overcome plant resistance in nature (Bromfield, 1984). On the basis of our results, it is clear that the *Rpp* NILs exhibit different effects on phytoalexin biosynthesis based on their individual genetic plasticity (García-Rodríguez et al., 2017; Hartman et al., 2005; Miles et al., 2011; Pham et al., 2009) and their corresponding ASR races (Akamatsu et al., 2013; Garcia et al., 2008; Miles et al., 2011; Silva et al., 2008). For example, *CHR* showed significant variation of its expression for *Rpp4* mediated resistance response. The functional coordination of *ADT6-ispG*, *CHS-CHR*, and *G4DT-PT3* may direct us to the precise tuning of the expected metabolic flux output to overcome race specific limitations. This relative expression study among multiple *Rpp* lines discloses these comparative results for the first time. Although it is impossible to generalize these results for all *Rpp* lines against all races of ASR, these results will provide options for metabolomics and genetic engineering studies.

Breeders have too much disease pressures to settle down the durable resistance genetic factors in crops that associated with modern agricultural practices and climate change (Zhan et al., 2015). But the limited resistance resource drives them toward molecular techniques. The 21st century is continuously flourished knowledge of plant pathogen interactions. The pyramid line extended the major gene efficiency against pathogen by molecular approach but lack of functional marker it takes long to develop new breeding resources. Our study first time showed that *ADT6, ispG, G4DT, G2DT* and *PT3* have the involvement and expression variation in different *Rpp* NIL and ASR interaction. It may offer some functional marker in relation to ASR defence supposed to their linked with the defence trait (Sukumaran et al., 2018). Plant protection specialist may carry new strategy for developing plant self biopesticide like de novo glyceollin biosynthesis upon pathogen attack. Finally, our finding may able to attract different kind of researcher like secondary metabolite engineers, plant protection specialists, plant breeders and plant pathologists in obvious reason to identify the specific glyceollin type and their potential uses.

Complementary gene action or epistatic gene interaction is important for ASR resistance. Genetic background has much influence on its effectiveness in a cultivar. Experiment based on multiple genes stacking called pyramid line confirms this hypothesis. For example, pair wise gene pyramiding of *Rpp2, Rpp3* and *Rpp4* enhanced the rust resistance specially in homozygous background of *Rpp2* and *Rpp3* (Maphosa M., 2012). Among them the *Rpp3* works better in all genetic background. Pyramided line of Lr34 in wheat was showed the enhancement of resistance against leaf rust via complementary gene action (Moullet et al., 2008). The spectrum of resistance is varying in pyramided line to soybean mosaic virus in different genetic background (Maroof et al., 2009). It was suggested that the supplementation of complementary gene action may change with the change of

genetic background. But in our study, the same susceptible BRS184 background of *Rpp* NIL genetic material showed some genes coordination that was not same among them. It may suggest that coordination or complementary gene action effected by individual *Rpp* linked genes cross talking. Pyramided line confers resistance to ASR isolates that are virulent on each of the pyramided genes (Yamanaka et al., 2015). On the other hand, the capacity of inverting the rust dominancy via susceptible allele may change the temporal demand for new gene coordination (Garcia et al., 2011). In future, using the susceptible background pyramided line and other NILs may justify the gene coordination that confers resistance or susceptible via complementary gene action.

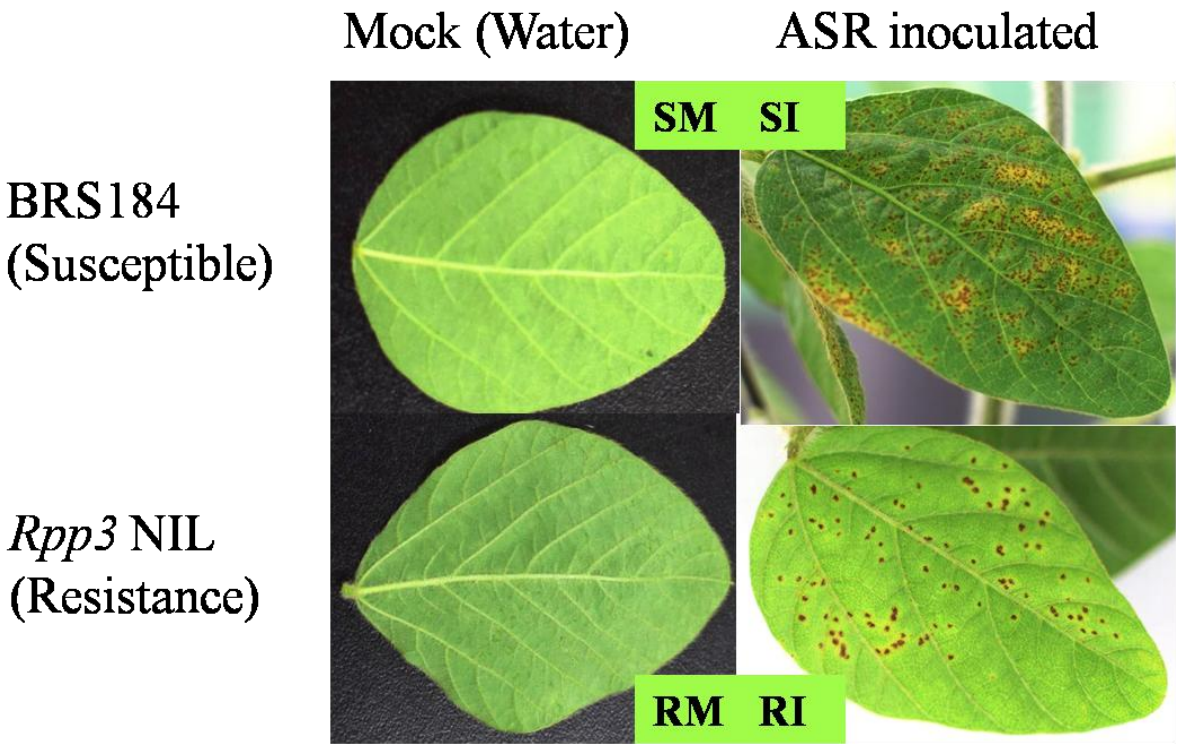

Figure 1. Soybean leave sample collection for RNA-seq : The susceptible BRS184 genotype and resistance near-isogenic line (NIL) for *Rpp3* were used for RNA-seq dataset generation. Mock (water) inoculated on BRS184 was termed as "Susceptible Mock (SM)", *P. pachyrhizi* (T1-2) inoculated on BRS184 was termed as "Susceptible Induced (SI)", Mock (water) inoculated on NIL was termed as "Resistance Mock (RM), and *P. pachyrhizi* inoculated on NIL was termed as "Resistance Induced (RI). The inoculated leaves were collected 24 hours after inoculation.

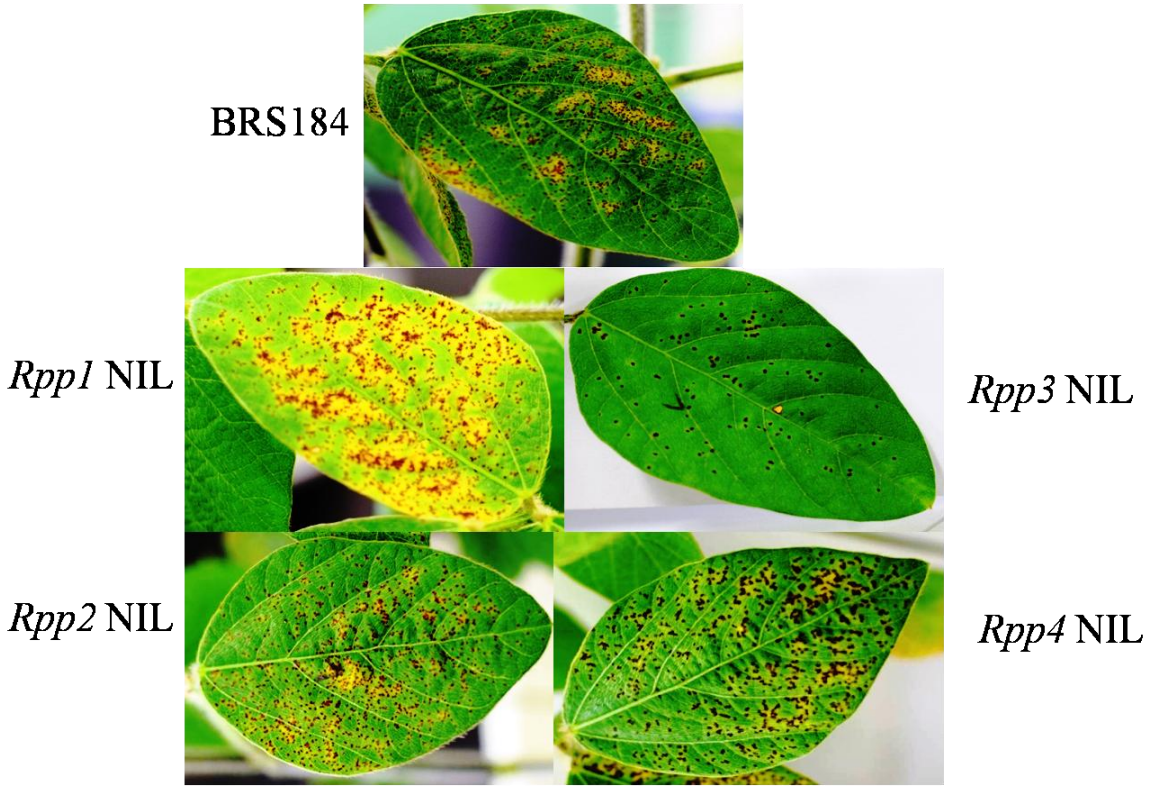

Figure 2. Differential defence response of NILs to *Phakopsora pachyrhizi*. All the samples were collected after fourteen days after ASR inoculation. The top image for BRS184 for susceptible control. The second row left side image is for *Rpp1* NIL and right side for *Rpp3* NIL. The bottom row left side for *Rpp2* NIL and right side row for *Rpp4* NIL

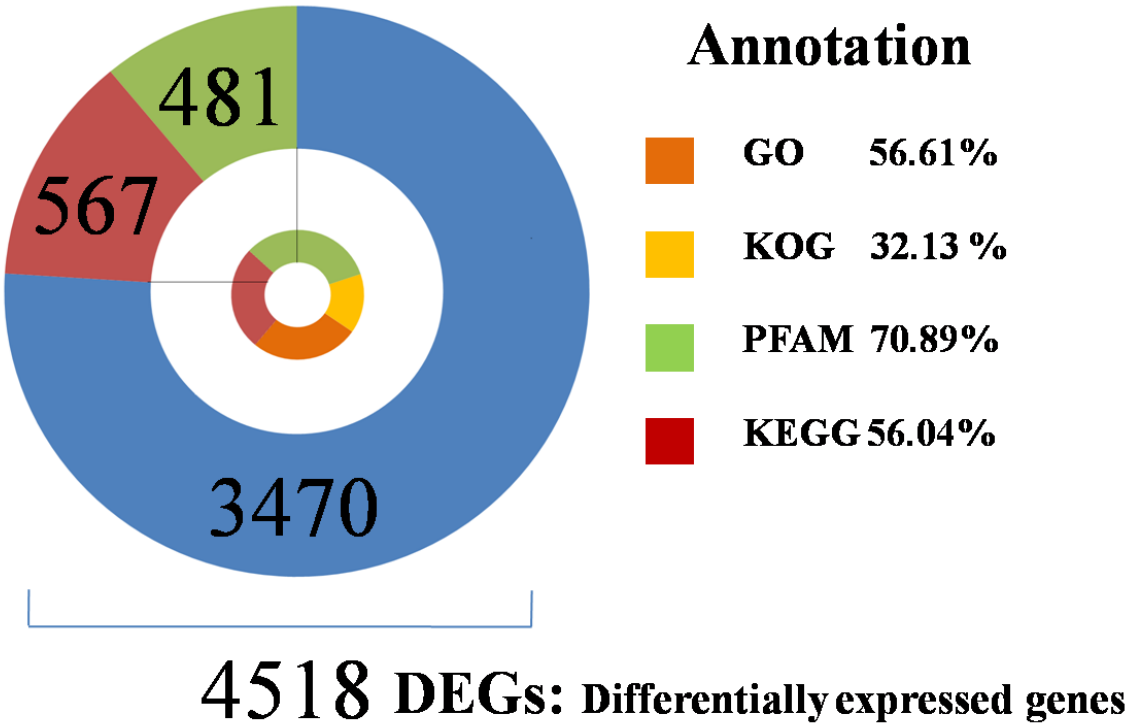

Figure 3. RNA-seq data annotation: A total of 4,518 differentially expressed genes (DEGs) were annotated using the protein family databases (PFAM), Gene Ontology (GO), Eukaryotic clusters of Orthologous Groups (KOG) and Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG) databases. In the first upper circle, blue color indicated the 3470 DEGs annotation found two or more databases, red color indicated the 567 DEGs annotation found only in KEGG databases and green color indicated the 481 DEGs annotation found only in PFAM databases. In the second inner circle shows that 70.89%, 32.13%, 56.61%, and 56.04% genes annotation were found in the PFAM, KOG, GO, and KEGG, respectively in the green, yellow, grey and red color.

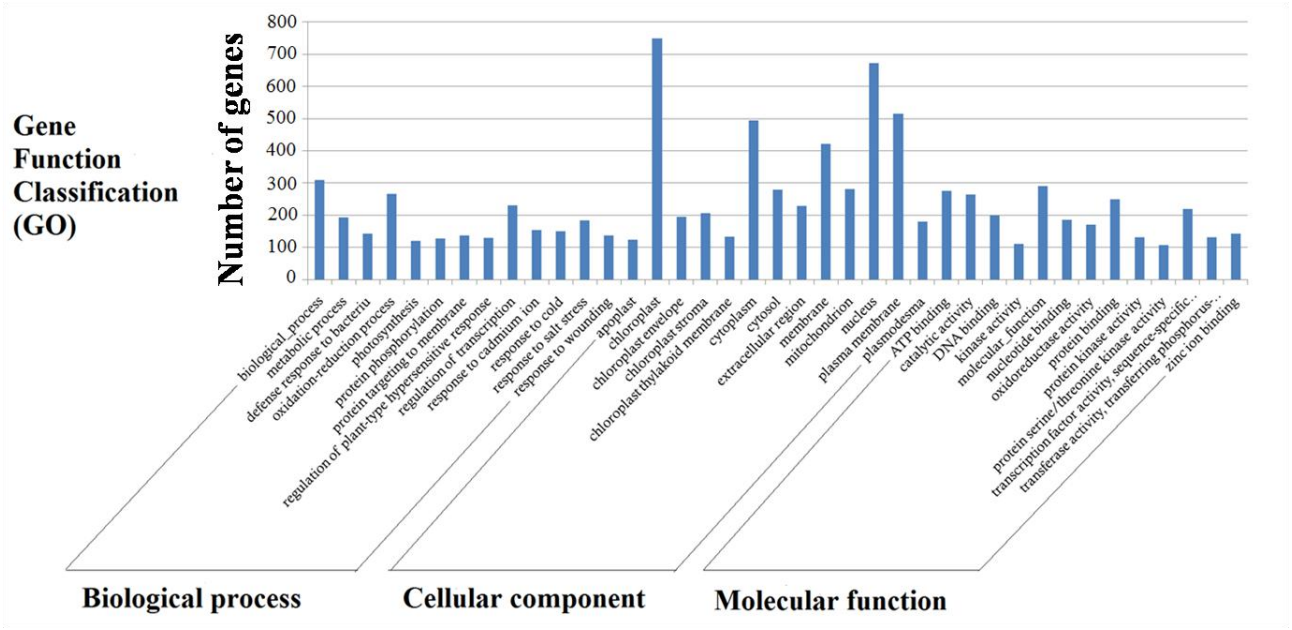

Figure 4. Gene ontological (GO) categorization of major unigenes specific to soybean resistance to *Phakopsora pachyrhizi* (*Rpp3*) NIL with the interaction of Asian soybean rust (ASR). Y axis represented the total number of genes and X axis for gene function of three major GO categories.

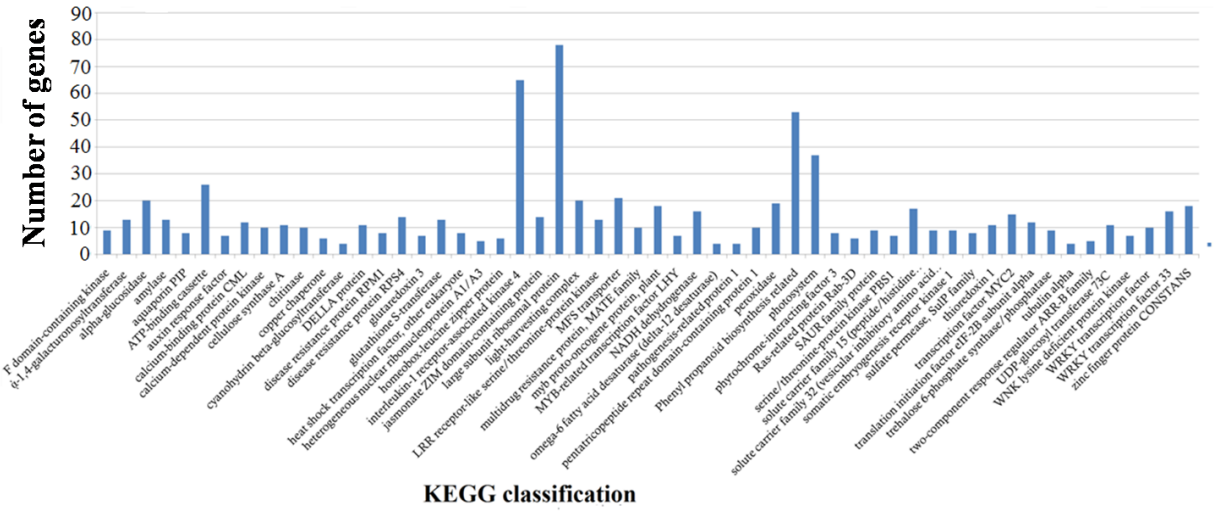

Figure 5. Functional categorization of unigenes specific to soybean resistance to *Phakopsora pachyrhizi* (*Rpp3*) NIL with the interaction of Asian soybean rust (ASR) as determined from KEGG biological process classification. Y axis represented the total number of genes and X axis for gene function..

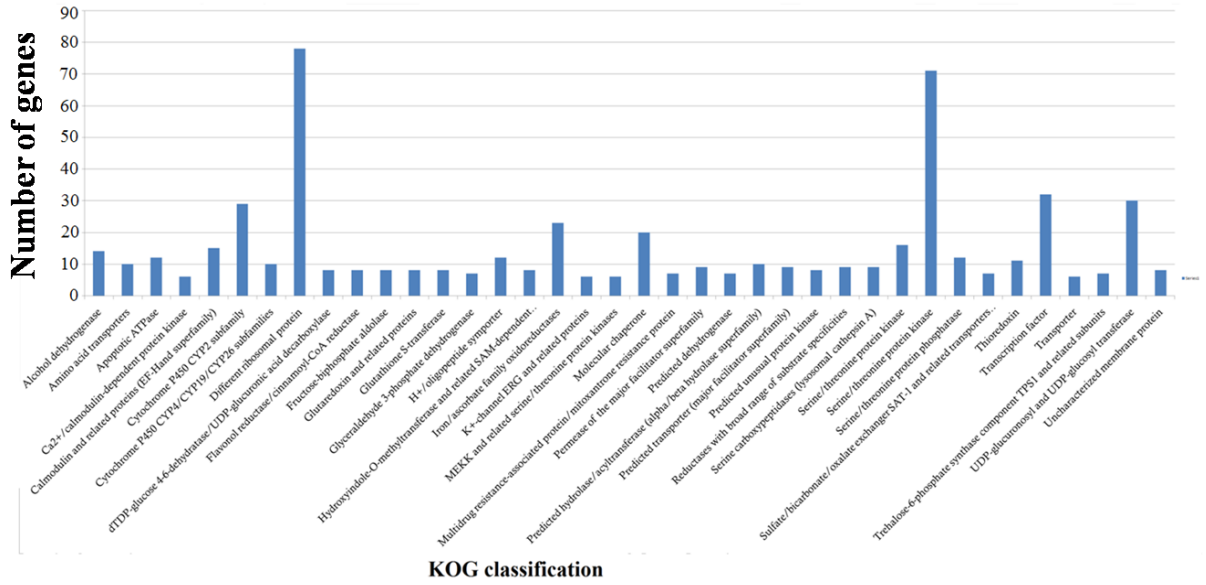

Figure 6. Functional categorization of unigenes specific to soybean resistance to *Phakopsora pachyrhizi* (*Rpp3)* NIL with the interaction of Asian soybean rust (ASR) as determined from KOG biological process classification. Y axis represented the total number of genes and X axis for gene function.

Figure 7. RNA-seq data analysis: Fifteen chamber venn diagram showing the sets of SM vs SI, SI vs RI, SM vs RM, and RM vs RI. Mock (water) inoculated on BRS184 was termed as "Susceptible Mock (SM)", *P. pachyrhizi* inoculated on BRS184 was termed as "Susceptible Induced (SI)", Mock (water) inoculated on NIL was termed as "Resistance Mock (RM)", and *P. pachyrhizi* inoculated on NIL was termed as "Resistance induced (RI)". The inoculated leaves were collected 24 hours after inoculation

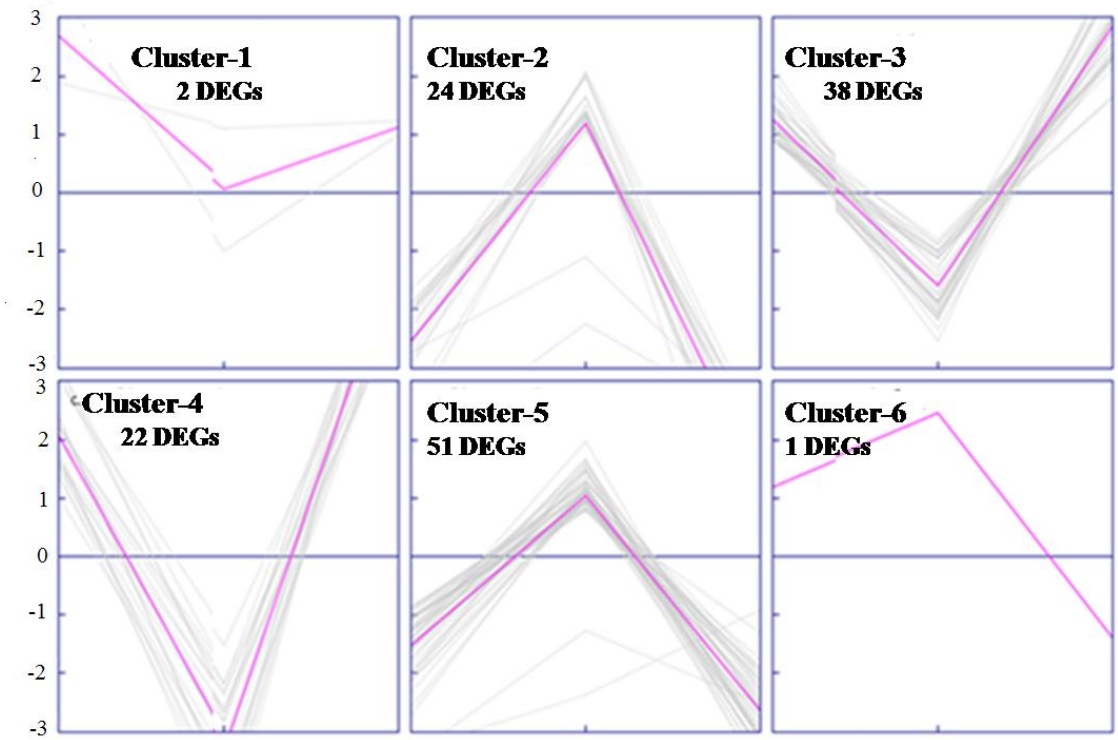

Figure 8. RNA-seq data analysis: Cluster analysis of DEGs by K mean clustering showed 138 DEGs in different clusters from the common intersection of SM vs SI, SM vs RM, and RM vs RI combinations which contained 149 genes, respectively. The remaining 11 genes have no cluster like cluster 6. The Y axis represents the level of gene expression evaluated by log10 (FPKM+1). The gene expression trend of clustered DEGs of SM vs SI, SM vs RM, and RM vs RI is represented in the X axis, respectively

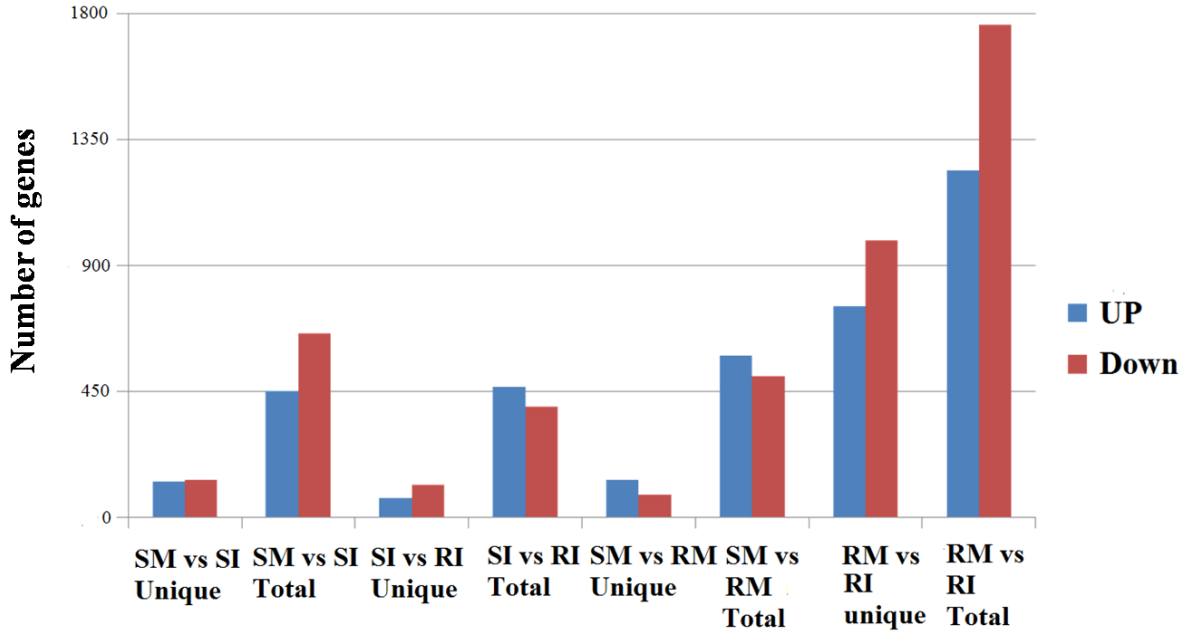

Figure 9. Number of up regulated (Blue) and down regulated (Brown) DEGs from mock and inoculated Asian soybean rust (ASR) in susceptible and resistance to *Phakopsora pachyrhizi* (*Rpp3*) NIL. Y axis indicates total number of genes and X axis indicate two types of gene set one is only single copy of respective genes termed unique and other is with multiple copy of genes termed total.

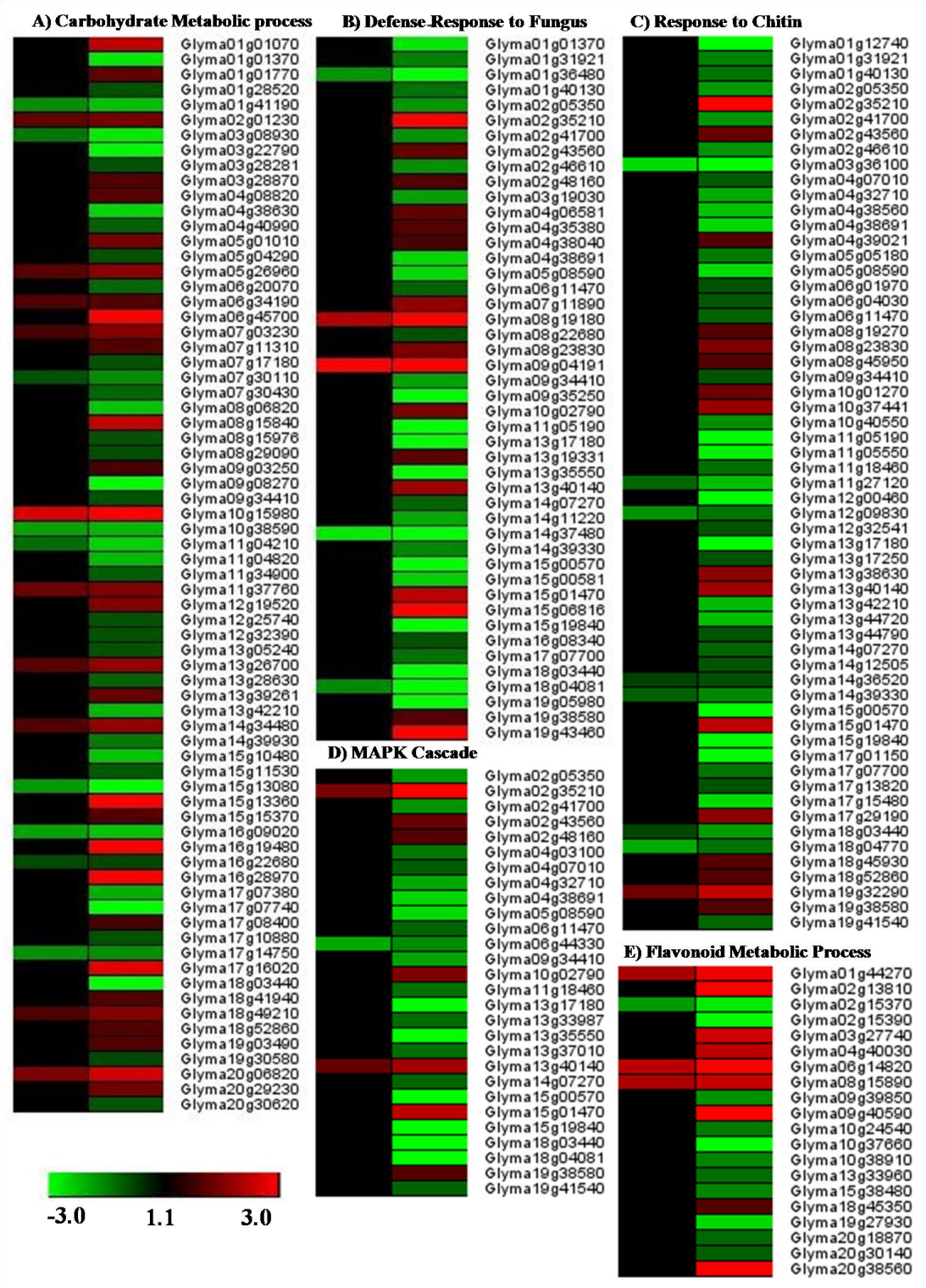

Figure 10. Heat map illustration of differential expression of genes (DEGs) of gene ontology (GO) biological process showing in various defence related mechanism in incompatible interaction Asian soybean rust (ASR) on soybean. A) Carbohydrate metabolic process B) Defence response to fungus. C) Response to chitin. D) MAPK cascade and E) Flavonoid metabolic process. Chroma color from red to green indicates log10 (FPKM+1) from high expression to less expression.

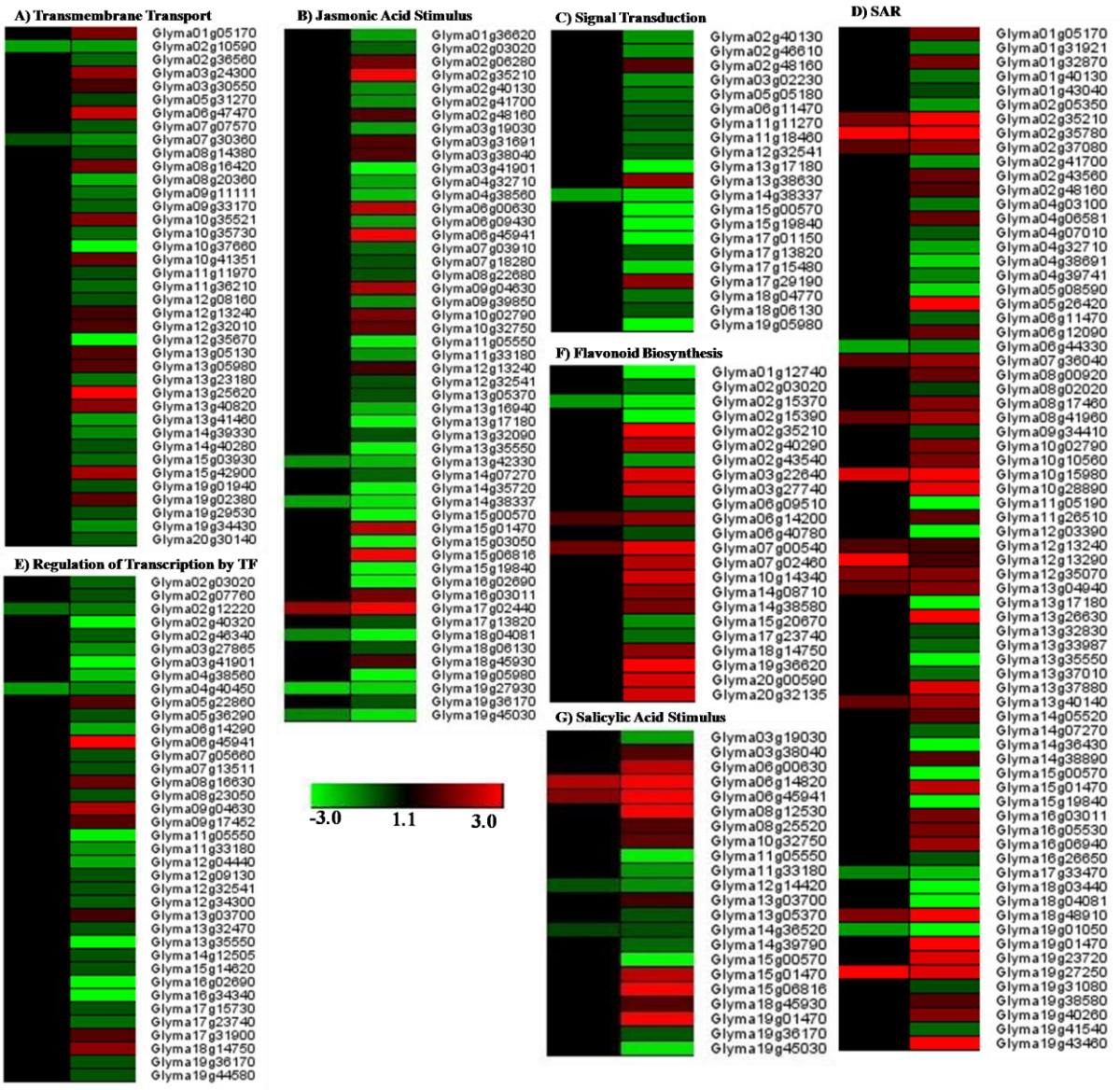

Figure 10. Heat map illustration of differential expression of genes (DEGs) of gene ontology (GO) biological process showing in various defence related mechanism in incompatible interaction Asian soybean rust (ASR) on soybean. A) Transmembrane transport. B) Jasmonic acid stimulus. C) Signal transduction. D) Systemic acquired resistance (SAR) E) Regulation of transcription by transcription factor (TF). F) Flavonoid biosynthesis and G) Salicyclic acid stimulus. Chroma color from red to green indicates log10 (FPKM+1) from high expression to less expression.

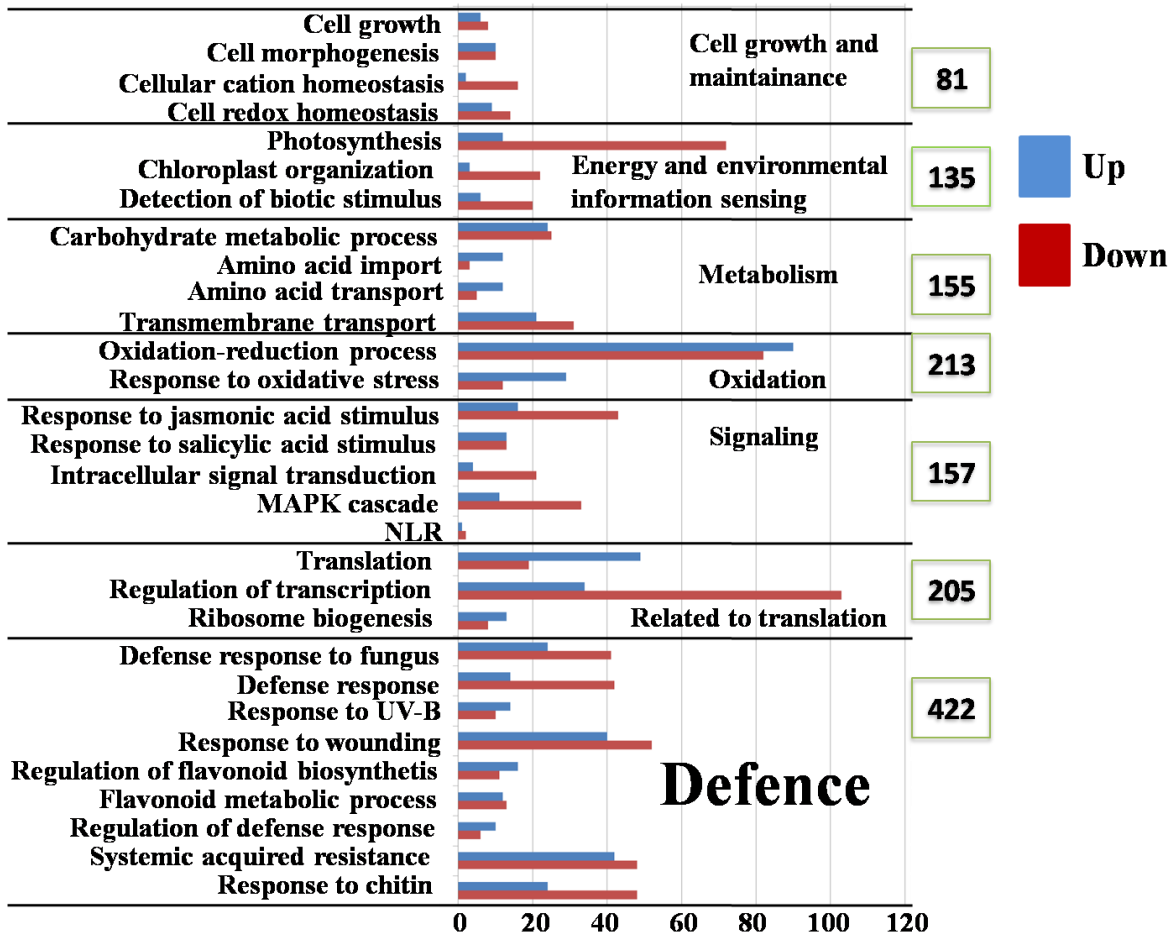

Figure 12. Over-represented gene ontology (GO) biological process classification by Fisher's exact test: RNA-seq data were used from incompatible interaction of Asian soybean rust (ASR) ie *Phakopsora pachyrhizi* on soybean plant. Up-regulated and down-regulated DEGs were represented by the blue and red color respectively. Broader functional categories also labeled by grouping of individual GO categories.

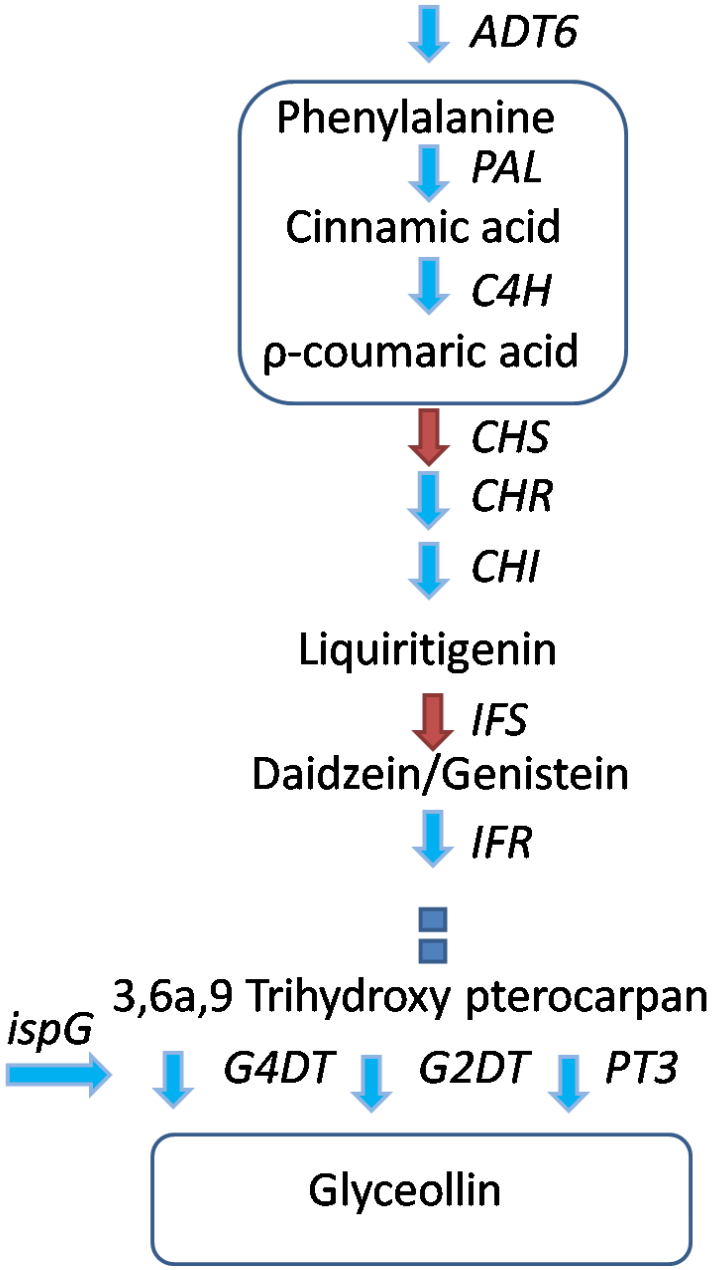

Figure 13. Major DEGs involving phytoalexin biosynthesis via phenylpropanoid pathway: *arogenate dehydratase* (*ADT6*) is the last enzyme of shikimate pathway, *phenylalanine ammonia lyase* (*PAL*) is the first enzyme in phenyl propanoid pathway, *cinnamic acid-4-hydroxylase* (*C4H*), *chalcone synthase* (*CHS*), *chalcone reductase* (*CHR*), *chalcone isomerase* (*CHI*), *isoflavone synthase* (*IFS)* and *isoflavone reductase* (*IFR*) are major enzyme to make metabolic flux, *4-hydroxy-3-methylbut-2-enyl diphosphate synthase* (*ispG*) for isoprenoid unit, *glycinol 4-dimethylallyltransferase* (*G4DT*), glycinol 2-dimethylallyltransferase (*G2DT*) and phytyltransferase 3 (*PT3*) are responsible for prenylation to glyceollin (phytoalexin) biosynthesis. Blue arrows represent the up-regulation of expression of gene and red arrows for down-regulation of expression. Double blue square block indicates the involvement of more than two genes on this pathway which weren't mentioned in this study. Up-regulation and down-regulation was mentioned by RNA-seq data.

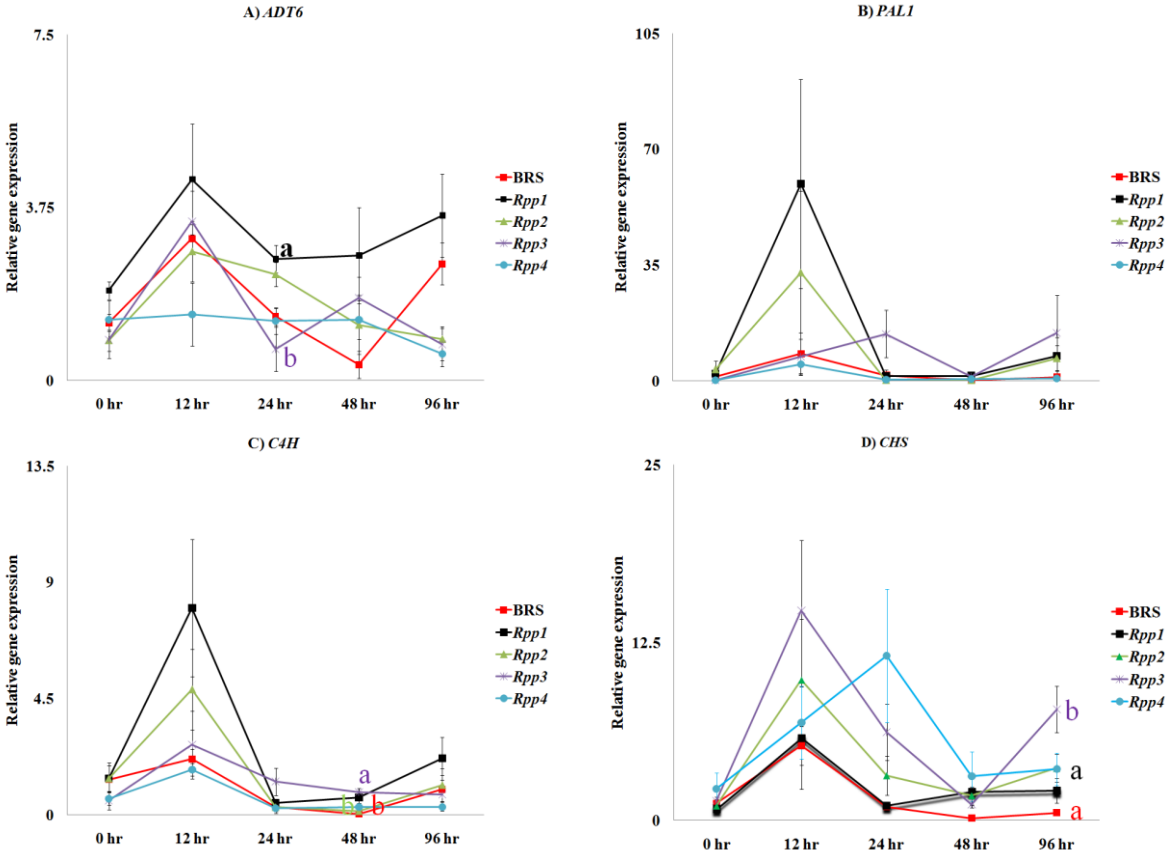

Figure 14. Relative gene expression of phytoalexin and glyceollin biosynthesis via phenylpropanoid pathway during *Phakopsora pachyrhizi* infection on different *resistance to Phakopsora pachyrhizi* (*Rpp*) lines of soybean. Twenty one-day-old plants that had been inoculated with T1-2 were utilized. The relative expression levels of A) *arogenate dehydratase 6* (*ADT6*), B) *phenylalanine ammonia-lyase* (*PAL*), C). *cinnamic acid-4-hydroxylase* (*C4H*), D) *chalcone synthase* (*CHS*), at the indicated time points (hours after inoculation (hai)) were determined by RT-qPCR using unknown2 and constans7 as an internal control. Y axis indicates the normalized mRNA expression and X axis for susceptible BRS184 (BRS) with resistance NILs for *Rpp1-Rpp4*. Bars are means ± standard deviation (SD) of 0, 12, 24, 48 and 96 hai and small letters on each bar graph showed the significant result calculated by Tukey-Kramer method in each time point, respectively.

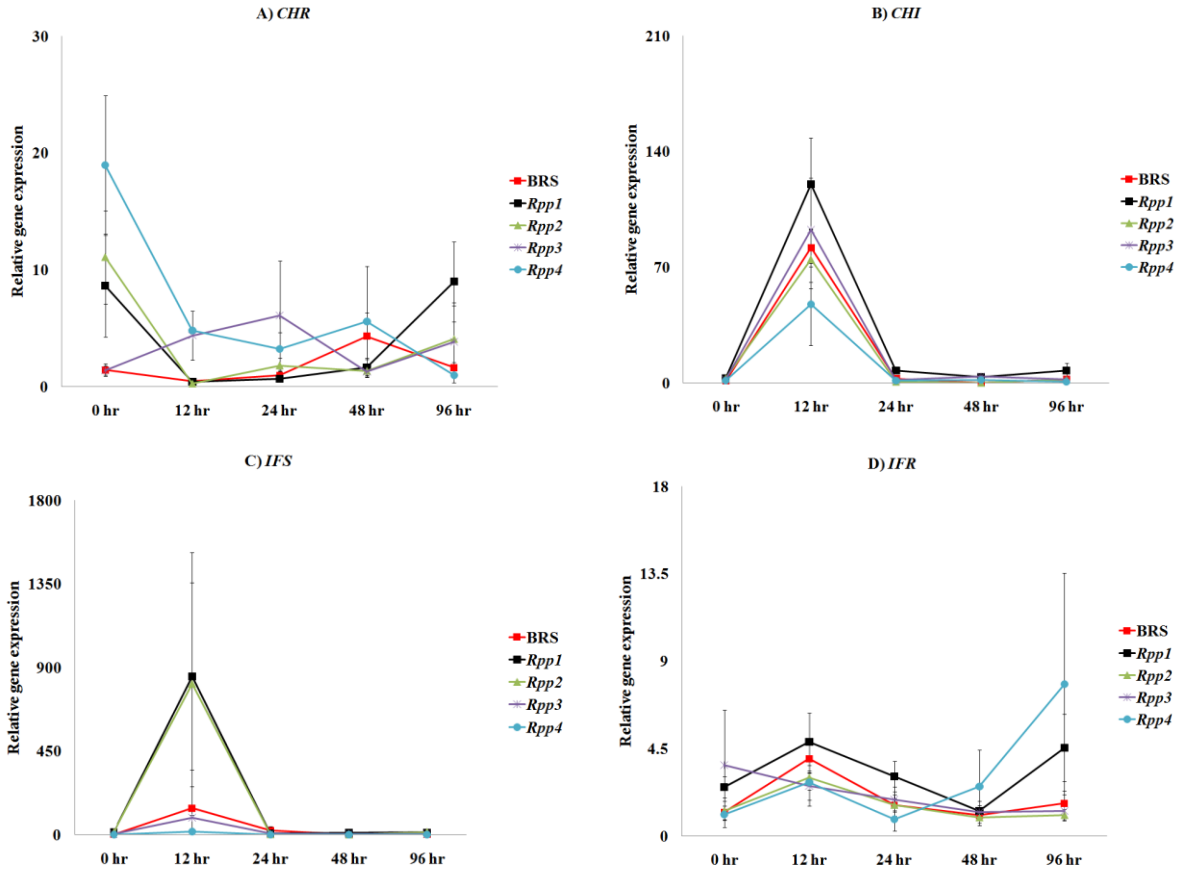

Figure 15. Relative gene expression of phytoalexin and glyceollin biosynthesis via phenylpropanoid pathway during *Phakopsora pachyrhizi* infection on different *resistance to Phakopsora pachyrhizi* (*Rpp*) lines of soybean. Twenty one-day-old plants that had been inoculated with T1-2 were utilized. The relative expression levels of A) *chalcone reductase* (*CHR*), B) *chalcone isomerase* (*CHI*), C) *isoflavone synthase* (*IFS*), D) *isoflavone reductase* (*IFR*), at the indicated time points (hours after inoculation (hai)) were determined by RT-qPCR using unknown2 and constans7 as an internal control. Y axis indicates the normalized mRNA expression and X axis for susceptible BRS184 (BRS) with resistance NILs for *Rpp1-Rpp4*. Bars are means ± standard deviation (SD) of 0, 12, 24, 48 and 96 hai and small letters on each bar graph showed the significant result calculated by Tukey-Kramer method in each time point, respectively.

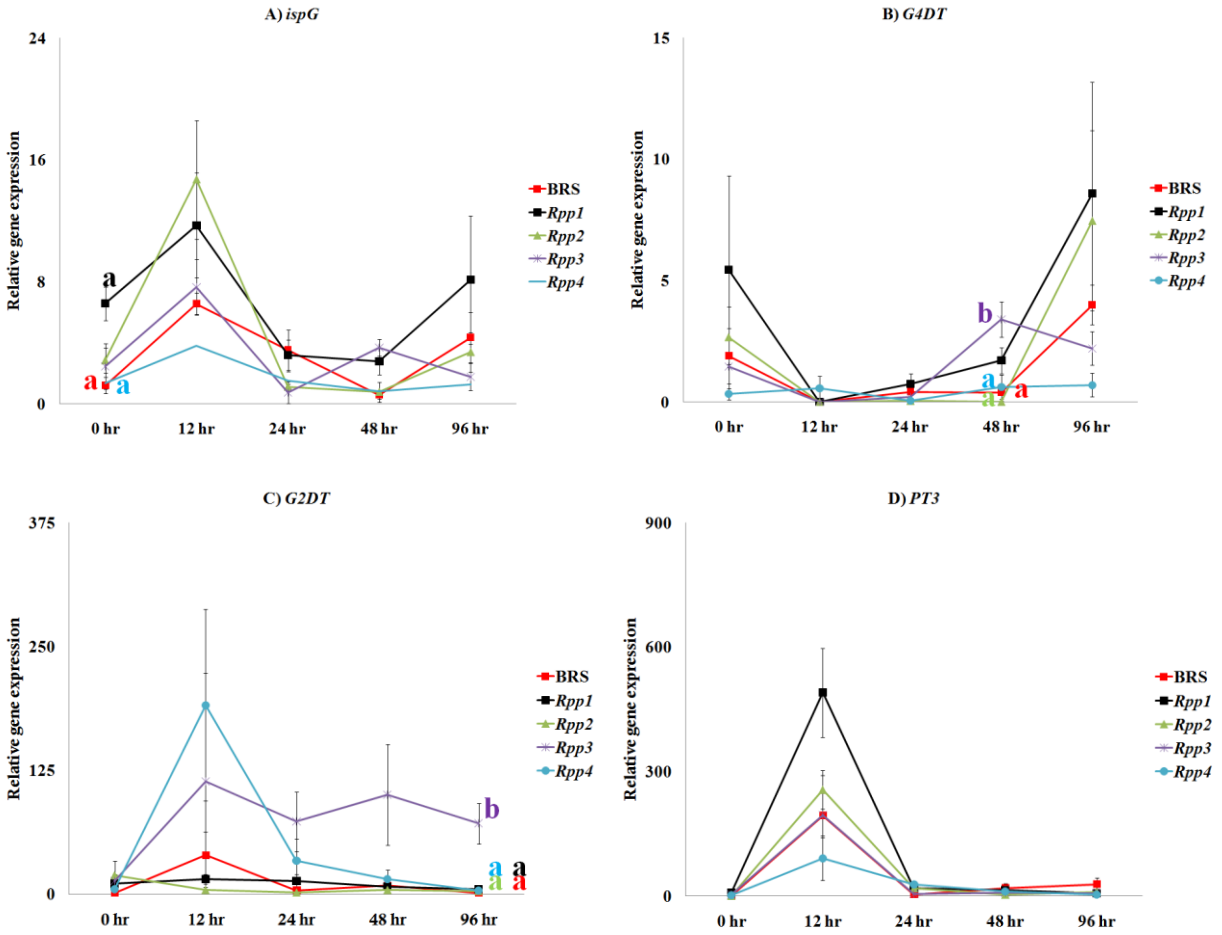

Figure 16. Relative gene expression of phytoalexin and glyceollin biosynthesis via phenylpropanoid pathway during *Phakopsora pachyrhizi* infection on different *resistance to Phakopsora pachyrhizi* (*Rpp*) lines of soybean. Twenty one-day-old plants that had been inoculated with T1-2 were utilized. The relative expression levels of A) *4-hydroxy-3-methylbut-2-enyl diphosphate synthase* (*ispG*), B) *glycinol 4-dimethylallyltransferase* (*G4DT*), C) glycinol 2-dimethylallyltransferase (*G2DT*) and D) phytyltransferase 3 (*PT3*), at the indicated time points (hours after inoculation (hai)) were determined by RT-qPCR using unknown2 and constans7 as an internal control. Y axis indicates the normalized mRNA expression and X axis for susceptible BRS184 (BRS) with resistance NILs for *Rpp1-Rpp4*. Bars are means ± standard deviation (SD) of 0, 12, 24, 48 and 96 hai and small letters on each bar graph showed the significant result calculated by Tukey-Kramer method in each time point, respectively.

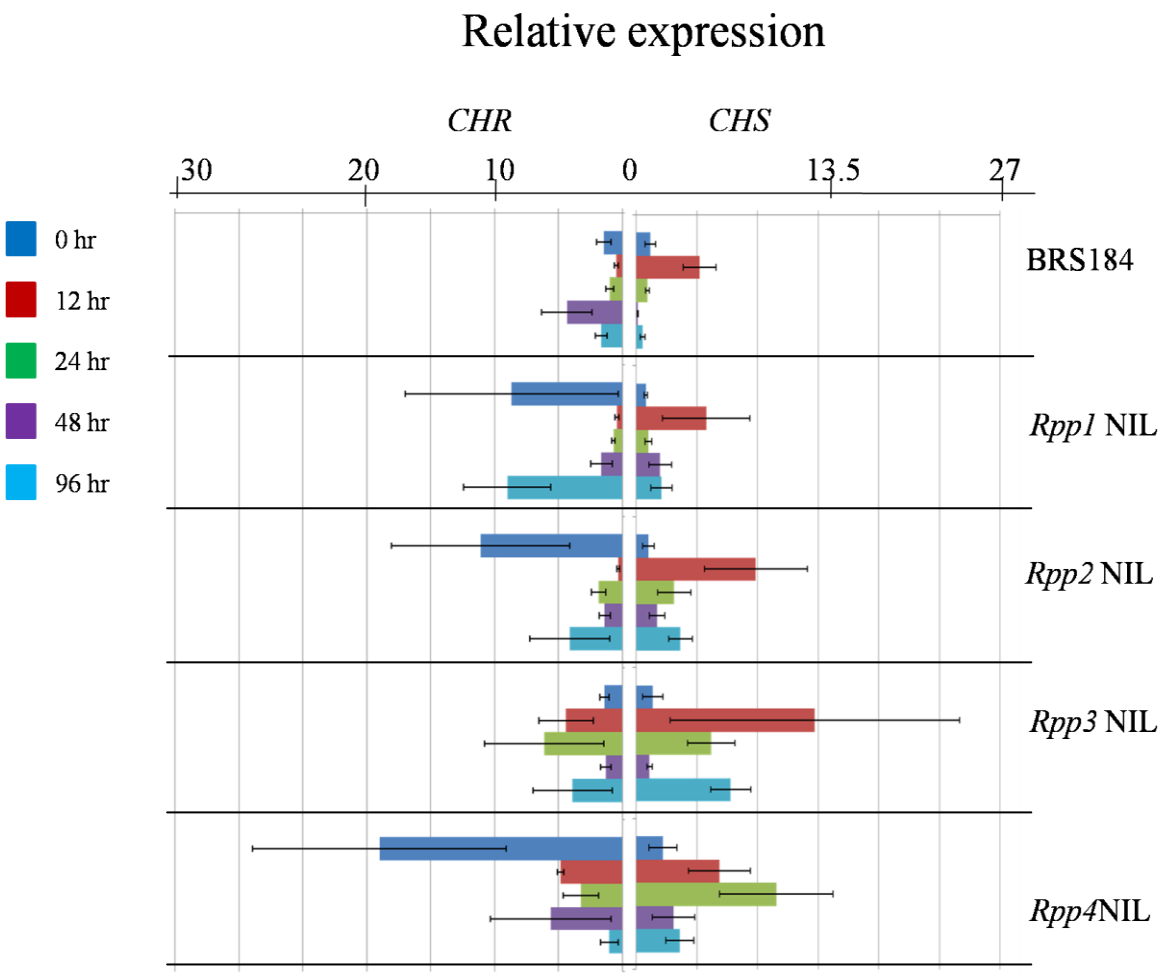

Figure 17. Relative gene expression of *chalcone synthase* (*CHS*) and *chalcone reductase* (*CHR*) showing the temporal functional reciprocal co-ordination at the indicated five time points (hours after inoculation (hai)) were determined by RT-qPCR using unknown2 and constans7 as an internal control. Top margin number indicates the normalized mRNA expression. Bars are means ± standard deviation (SD) of 0, 12, 24, 48 and 96 hai. Twenty-one-day-old plants that had been inoculated either mock- or ASR urediniospores (isolate T1-2) with 1×10^6 spores/ml (0.001% Tween 20).

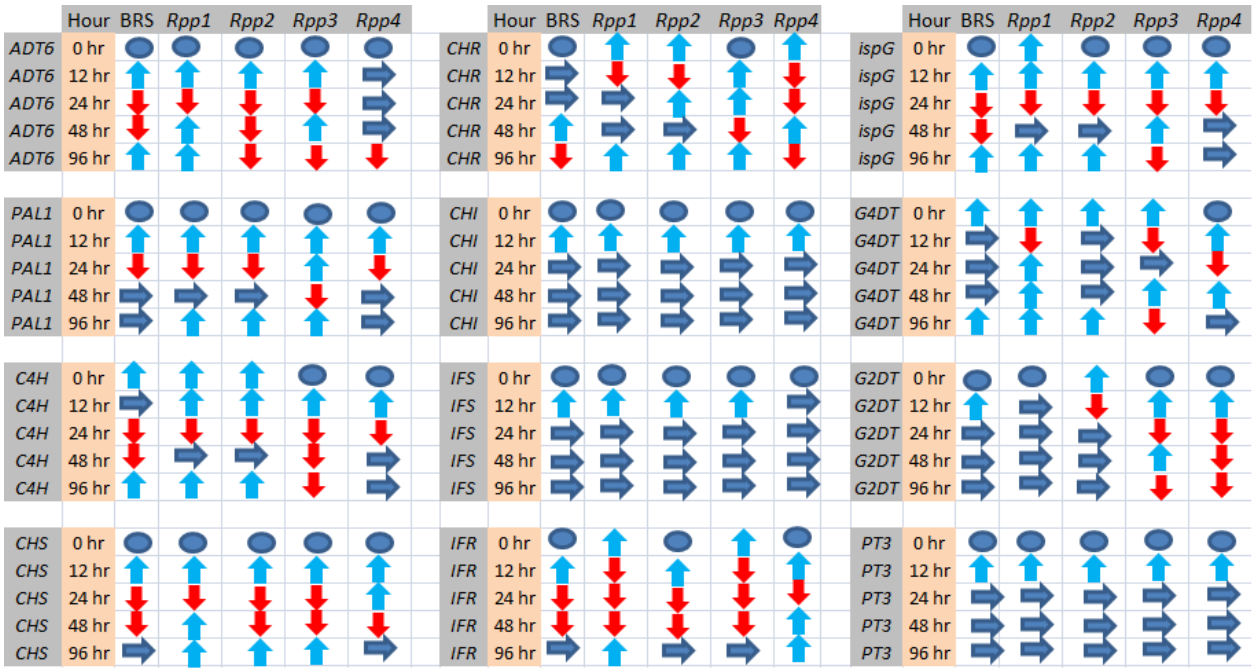

Figure 18. Arrow plot illustration of differential relative gene expression status of *arogenate dehydratase* (*ADT6*), *phenylalanine ammonia lyase* (*PAL*), *cinnamic acid-4-hydroxylase* (*C4H*), *chalcone synthase* (*CHS*), *chalcone reductase* (*CHR*), *chalcone isomerase* (*CHI*), *isoflavone synthase* (*IFS*), *isoflavone reductase* (*IFR*), *4-hydroxy-3-methylbut-2-enyl diphosphate synthase* (*ispG*), *glycinol 4-dimethylallyltransferase* (*G4DT*), *glycinol 2-dimethylallyltransferase* (*G2DT*) and *phytyltransferase* 3 (*PT*3) at 0 hr, 12 hr, 24 hr, 48 hr and 96 hr (hr=Hour) after inoculation on susceptible BRS184 and resistance to *Phakopsora pachyrhizi* near isogenic lines (NILs) of *Rpp1*, *Rpp2*, *Rpp3* and *Rpp4*. Gene expression were plotted by upward blue arrow=Up-regulated, downward red arrow=Down-regulated, right dark blue arrow =Remains same and dark blue circle=zero or close to zero.

Table 3. Statistical results of Unigenes annotation

Database type	Number of Unigenes	(Percentage (%)
PFAM	3203	70.89
Panther	1857	41.10
Uniref	2653	8.72
KEGG	2532	56.04
GO	2558	56.61
KOG	1452	32.13
SoyCyc	400	8.85
Total genes	**4518**	**100**

Table 4. Forward and reverse primers for RT-qPCR in each gene

Gene name	Glyma ID	Primer sequence (Forward and Reverse)	
ADT6	Glyma13G31900	CAGTACGGAAGGAGGACGAG	For
		CAATTTGAGCTTGGTCAGCA	Rev
PAL1	Glyma19g36620	GAACCAAACAAGGTGGTGCT	For
		TGTTGTTGAGGAGCTTGGTG	Rev
C4H	Glyma02g40290	TTGCTGAGCTTGTGAACCAC	For
		GTCGTGGAGGTTCATGTGTG	Rev
CHS	Glyma19g27930	CCCAAGCCGATTACCCTGA	For
		GATTTCCTCCACGGTCACCA	Rev
CHR	Glyma16g34570	CCAGTGAGGCTGAGACATGA	For
		ATTGACTGCAGGAGGAATGG	Rev
CHI	Glyma06g14820	TAGGCCACTTGGACCAGTTC	For
		CACCGCAGTCTCAATCTGAA	Rev
IFS2	Glyma13g24200	TCTCCACTACGCACTCATCG	For
		GCTTCCTCACGAACTTCCAG	Rev
IFR	Glyma20g02780	AGAAAGACTCGGCACTCGAA	For
		TTGACACTACCTCGCCCTCT	Rev
ispG	Glyma13g40140	TTTGCTCCCTCTGTTGCTTT	For
		CGCATTGCTCTCCCATATTT	Rev
G4DT	Glyma16g25620	GAGTGCCGTGAATTTGAACA	For
		GCCGTGAAAGAAATGAGAGG	Rev
G2DT	Glyma20g38930	ACTGCTGTCTTCATTGCTGC	For
		TGGCCTCTTCAGCACAAAAG	Rev
PT3	Glyma01g33070	CCTCTTGGGCTTTGCTGTTT	For
		ATCTGCACCCTGATGAAGCT	Rev
CONSTANS7	Glyma06g04180	ATGAATGACGGTTCCCATGTA	For
		GGCATTAAGGCAGCTCACTCT	Rev
Unknown2	AW31036	GCCTCTGGATACCTGCTCAAG	For
		ACCTCCTCCTCAAACTCCTCTG	Rev

Table 5. Disease status shown by four soybean near isogenic lines with control against T1-2 soybean rust

Genotype	Disease severity	Pustule frequency Per leaf	Reaction type	Degree of leaf yellowing
BRS184	7	705.0±10.00	TAN	4
Rpp1	6	515.70±19.96	TAN	5
Rpp2	6	494.34±25.13	RB	3
Rpp3	5	81.34±10.41	RB	3
Rpp4	6	421.00±87.37	RB	3

± Standard error of the mean, BRS=Susceptible control, RB=Reddish brown, TAN=Tan color

Table 6: Up-regulated and down-regulated DEGs in resistance response and susceptible induced during ASR pathogen infection on soybean

Glyma ID	RM vs RI	SM vs SI	Fold difference	Annotation
Glyma03g36100	-5.87536	-2.58217	-3.29319	*Interleukin-1 receptor-associated kinase 4 (IRAK4)*
Glyma12g17710	-5.66039	-2.62757	-3.03282	MADS box transcription factor
Glyma08g12440	-4.54828	-1.9953	-2.55298	Uncharacterized protein
Glyma02g03620	-4.24265	-1.83695	-2.4057	Unknown protein
Glyma02g40320	-3.83564	-1.57955	-2.25609	EREBP like factor
Glyma11g37780	-3.93722	-1.73361	-2.20361	Ring finger protein 6/12/38
Glyma18g04081	-3.78256	-1.6519	-2.13066	26S proteasome regulatory subunit T4
Glyma18g52250	6.3122	2.9179	3.3943	*Polyketide(chalcone) Reductase (CHR)*
Glyma11g36200	6.0415	2.97278	3.06872	Glutamate/aspartate-Prephenate aminotransferase
Glyma11g07490	5.98576	2.6828	3.30296	Epimerase related
Glyma15g13500	5.90954	2.8271	3.08244	Peroxidase
Glyma19g33330	5.90644	2.33861	3.56783	Histone H2A
Glyma10g31590	5.64126	2.70303	2.93823	Methionine =-gamma-lyase
Glyma02g09210	5.34852	2.58426	2.76426	2,4-dihydroxy -1,4-benzoxazin-3-one-glucoside dioxygenase
Glyma02g09220	5.21525	2.50507	2.71018	GO:0008152
Glyma18g22780	4.89944	2.03253	2.86691	Enolase
Glyma12g02250	4.82704	2.25962	2.56742	NAD(P)-Rossmann-fold superfamily protein
Glyma18g48910	4.79125	1.53804	3.25321	Mitochondrial chaperone (BCS1)
Glyma08g19180	4.66954	2.12399	2.54555	Peroxidase
Glyma05g29030	4.10446	1.72308	2.38138	Mediator of RNA Polymerase II transcription subunit 13
Glyma16g05880	4.01551	1.89608	2.11943	WRKY transcription factor 33 (WRKY33)

Table 7. Up-regulated and down-regulated genes related to oxidation during resistance response against ASR

Oxidation class	Expression type	Major family	Glyma ID	Total number in RNA-seq
Oxidation reduction process	UP-regulated	Peroxidase	Glyma15g13500,Glyma08g19180,Glyma02g40040, Glyma14g38210,Glyma15g13560	91
		Ferritin	Glyma18g43650,Glyma03g06420,Glyma07g19060, Glyma02g43040	
		Alcohol dehydrogenase	Glyma12g01780,Glyma09g29070,Glyma05g33140, Glyma18g53600	
		Malate dehydrogenase	Glyma05g35800,Glyma06g34190,Glyma12g19520, Glyma01g01180,Glyma05g01010,Glyma17g10880	
		Cytochrome P450	Glyma02g40290,Glyma14g38580,Glyma08g14900, Glyma19g32880,Glyma03g27740,Glyma16g01060, Glyma03g31691,Glyma05g27970	
	Down-regulated	Reticuline oxidase-like protein	Glyma15g14040,Glyma08g08530,Glyma15g14020, Glyma19g01160	82
		Senescence-associated nodulin 1A	Glyma02g15390,Glyma02g15370,Glyma07g33090	
		Cytochrome P450	Glyma12g09240,Glyma11g06400,Glyma08g10950, Glyma06g03850,Glyma20g01091,Glyma07g34560	
		Chlorophyllide-a oxygenase	Glyma14g23860,Glyma13g03430	
		Ferric-chelate reductase	Glyma15g13090,Glyma09g02170	
Response to oxidative stress	UP-regulated	Peroxidase	Glyma11g06180, Glyma08g19170	28
		Thioredoxin	Glyma11g20630,Glyma12g29550,Glyma13g40130	
		Phenylalanine ammonia-lyase	Glyma20g32135,Glyma19g36620	
		Isoflavone reductase-like protein	Glyma11g07490	
		Lipoxygenase	Glyma19g01470	
	Down-regulated	Peroxidase	Glyma13g01870,Glyma03g30180	13
		Chalcone synthase	Glyma19g27930	
		Galactinol synthase	Glyma19g40550,Glyma20g22700,Glyma03g38080	

Table 8. K mean clustering of 138 DEGs from SM vs SI, SM vs RM and RM vs RI combinations genes

Cluster No	Glyma ID	Major example	Number of genes	Percent
1	Glyma08g45510, Glyma14g01430	Kunitz family trypsin and protease inhibitor protein (Mcp20), Galactinol-sucrose galactosyltransferase	2	1.50
2	Glyma08g46630, Glyma13g07490, Glyma17g01150, Glyma12g09240, Glyma12g00460, Glyma18g06510, Glyma04g23110, Glyma16g02690, Glyma10g32901, Glyma13g17180, Glyma11g04130, Glyma19g01160, Glyma14g02490, Glyma13g07910, Glyma17g07400, Glyma15g40070, Glyma13g07620, Glyma07g36440, Glyma19g01050, Glyma16g15790, Glyma10g02710, Glyma08g21115, Glyma16g02320, Glyma19g44570	Allene oxide cyclase 4, Cytochrome P450 94A1, Dihydroflavonol reductase, Myc2 bHLH protein, carbonate dehydratase, Heterogeneous nuclear ribonucleoprotein R, Transcription factor MYC2	24	17.00
3	Glyma05g26420, Glyma02g13810, Glyma20g38560, Glyma03g03200, Glyma20g38930, Glyma02g03870, Glyma20g24500, Glyma13g00450, Glyma13g25620, Glyma16g20730, Glyma19g44451, Glyma03g03190, Glyma10g08540, Glyma19g36620, Glyma01g00730, Glyma03g16600, Glyma13g26630, Glyma01g31750, Glyma20g02760, Glyma17g04350, Glyma14g39160, Glyma10g28890, Glyma07g36500, Glyma20g24180, Glyma16g33840, Glyma08g00840, Glyma03g27740, Glyma16g06750, Glyma09g06250, Glyma06g47470 Glyma06g32680, Glyma08g28230, Glyma01g01180, Glyma06g08640, Glyma13g17140, Glyma07g02460, Glyma01g42420, Glyma11g04650	Chalcone isomerase, Glycinol 4-dimethylallyltransferase, Membrane protein, Caffeoyl-CoA O-methyltransferase, Calcium-dependent protein kinase SK5, Monooxygenase, H+-transporting ATPase, Hexose carrier protein HEX6, Shikimate O-hydroxycinnamoyltransferase, Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+), Coiled-coil domain-containing protein, Glutathione S-transferase GST 13	38	27.50
4	Glyma11g07510, Glyma09g40590, Glyma15g06816, Glyma05g35800, Glyma06g15411, Glyma13g42210, Glyma18g45260, Glyma03g33340, Glyma19g43460, Glyma08g14900, Glyma13g17730, Glyma08g44320, Glyma12g09810, Glyma17g04780, Glyma07g11310, Glyma08g12530, Glyma16g17680, Glyma16g05580, Glyma08g37670, Glyma11g07516 Glyma19g31580, Glyma01g26220	Beta-1,3-glucanase 1, PR1a, Endochitinase PR4, Glutathione S-transferase, Monooxygenase, Nitrate transporter, Tau class glutathione S-transferase, Acyltransferase-like protein 1-deoxy-D-xylulose-5-phosphate synthase	22	16.00
5	Glyma18g03020, Glyma10g32080, Glyma12g35670, Glyma01g40620, Glyma13g03650, Glyma01g11640, Glyma11g04530, Glyma12g03390, Glyma18g14620, Glyma06g03670, Glyma14g35720, Glyma02g45270, Glyma14g01470, Glyma04g39370, Glyma07g07150, Glyma09g34285, Glyma20g35530, Glyma06g45700, Glyma03g23870, Glyma03g22230, Glyma13g16940, Glyma15g05790, Glyma10g07090, Glyma07g00900, Glyma11g05550, Glyma18g14730, Glyma16g10880, Glyma19g32070, Glyma14g23860, Glyma12g34570 Glyma02g39460, Glyma14g07160, Glyma16g34340, Glyma03g38930, Glyma09g02170, Glyma18g52860, Glyma01g01480, Glyma01g12740, Glyma18g16060, Glyma12g00790, Glyma18g19050, Glyma08g14280, Glyma16g01870, Glyma01g36620, Glyma17g01900, Glyma13g06940, Glyma13g08390, Glyma15g24680, Glyma13g03430, Glyma04g22020, Glyma03g28281	Chlorophyll A/B binding protein, Calmodulin, Transcription factor MYC2, Photosystem I reaction center subunit N, UDP-glucosyltransferase, Ethylene-overproduction protein, Chlorophyllide-a oxygenase, Magnesium chelatase subunit, Sigma-like factor 5A, Senescence-associated protein DIN1, Glucose-1-phosphate adenylyltransferase, 2-deoxyglucose-6-phosphate phosphatase, Trehalose-phosphatase, Alcohol dehydrogenase	51	37.00
6	Glyma12g09731	Photosystem I subunit PsaO	1	1.00

Susceptible Mock=SM; Susceptible Induced=SI; Mock (water); Resistance Mock (RM); Resistance Induced =RI

Table 9. Number of transcription factors involved into the *Rpp3* soybean NIL transcriptomic response against T1-2 ASR

TFs Family	Soybean(3x + 2x + 2x)	RNAseq	Percent calculated with geneome	Percent calculated with RNAseq
bHLH	480	54	11.25	15.21
MYB	369	16	4.34	4.51
ERF	330	33	10	9.30
C2H2	267	13	4.87	3.66
bZIP	266	14	5.26	3.94
MYB_related	265	27	10.19	7.61
NAC	247	35	14.17	9.86
WRKY	233	32	13.73	9.01
G2-like	164	10	6.1	2.82
HD-ZIP	140	9	6.43	2.54
GRAS	139	17	12.23	4.79
C3H	136	8	5.88	2.25
B3	112	1	0.89	0.28
LBD	111	5	4.5	1.41
TALE	101	12	11.88	3.38
Dof	93	4	4.3	1.13
Trihelix	93	2	2.15	0.56
M-type	88	1	1.14	0.28
ARF	85	9	10.59	2.54
AP2	76	3	3.95	0.85
SBP	73	2	2.74	0.56
GATA	70	3	4.29	0.85
HSF	61	7	11.48	1.97
NF-YB	46	1	2.17	0.28
Nin-like	45	3	6.67	0.85
ARR-B	42	1	2.38	0.28
DBB	36	6	16.67	1.69
CO-like	32	9	2.813	2.54
HB-other	31	1	3.23	0.28
CAMTA	23	2	8.7	0.56
LSD	17	1	5.88	0.28
EIL	12	2	16.67	0.56
CAMTA	23	2	8.7	0.56
SBP	73	2	2.74	0.56
Trihelix	93	2	2.15	0.56
LSD	17	1	5.88	0.28
HB-other	31	1	3.23	0.28
ARR-B	42	1	2.38	0.28
NF-YB	46	1	2.17	0.28
M-type	88	1	1.14	0.28
B3	112	1	0.89	0.28

MIKC, FAR1, TCP, NF-YA, ZF-HD, NF-Yc, YABBY, SRS, WOX, GRF, E2F/DP, BBR-BPC, VOZ, BES1, CPP, Whirly, GeBP, HB-PHD, NF-X1, RAV, S1Fa-like, LFY, SAP, HRT-like, STAT, NZZ/SPL (None of these TF were in RNAseq data)

Table 10. Transcription factors DEGs involved in resistance response during *Phakopsora pachyrizi* infection on soybean.

Gene ID	Resistanse response	Gene expression p value	Transcription factor class
Glyma16g05880	RM vs RI	4.01551	WRKY
Glyma06g14720	RM vs RI	3.54822	WRKY
Glyma13g36540	RM vs RI	2.01647	WRKY
Glyma17g29190	RM vs RI	1.66867	WRKY
Glyma13g38630	RM vs RI	1.66586	WRKY
Glyma01g31921	RM vs RI	-1.53953	WRKY
Glyma05g36970	RM vs RI	-2.89904	WRKY
Glyma15g00570	RM vs RI	-3.1425	WRKY
Glyma01g43040	RM vs RI	-0.86039	TALE
Glyma09g12820	RM vs RI	-1.07394	TALE
Glyma11g02960	RM vs RI	-1.28507	TALE
Glyma12g27330	RM vs RI	-1	SBP
Glyma10g33650	RM vs RI	1	NF-YB
Glyma08g16630	RM vs RI	1.24431	NAC
Glyma02g07760	RM vs RI	-1	NAC
Glyma07g05660	RM vs RI	-1	NAC
Glyma06g14290	RM vs RI	-2.01499	NAC
Glyma01g06150	RM vs RI	-2.16484	NAC
Glyma13g35550	RM vs RI	-3.79849	NAC
Glyma06g45941	RM vs RI	3.18036	MYB_related
Glyma05g36290	RM vs RI	-1	MYB_related
Glyma19g36170	RM vs RI	-1	MYB_related
Glyma02g03020	RM vs RI	-1.2046	MYB_related
Glyma11g33180	RM vs RI	-1.82843	MYB_related
Glyma19g45030	RM vs RI	-2.71797	MYB_related
Glyma16g34340	RM vs RI	-3.4389	MYB_related
Glyma06g00630	RM vs RI	2.30422	MYB
Glyma12g32541	RM vs RI	-1	MYB
Glyma13g05370	RM vs RI	-1	MYB
Glyma15g14620	RM vs RI	-1	MYB
Glyma03g19030	RM vs RI	-1.92864	MYB
Glyma11g05550	RM vs RI	-2.84541	MYB
Glyma07g05001	RM vs RI	-1	M-type
Glyma05g23710	RM vs RI	-1.64948	LSD
Glyma01g39260	RM vs RI	-2.86723	HSF
Glyma08g21620	RM vs RI	2.03947	HD-ZIP
Glyma04g40960	RM vs RI	1.55047	HD-ZIP
Glyma07g08340	RM vs RI	1.46169	HD-ZIP
Glyma13g23890	RM vs RI	-1.72304	HD-ZIP
Glyma04g34341	RM vs RI	-1	HB-other
Glyma02g46730	RM vs RI	-1.9071	GRAS
Glyma17g01150	RM vs RI	-4.40386	GRAS
Glyma09g17452	RM vs RI	1	G2-like
Glyma13g37010	RM vs RI	-1.32994	G2-like

Table 10. Continued

Gene ID	Resistanse response	Gene expression p value	Transcription factor class
Glyma03g27890	RM vs RI	-1.61342	G2-like
Glyma09g04630	RM vs RI	2.04766	ERF
Glyma04g04355	RM vs RI	1.5749	ERF
Glyma17g31900	RM vs RI	1	ERF
Glyma05g04920	RM vs RI	-1	ERF
Glyma07g08542	RM vs RI	-1	ERF
Glyma10g07756	RM vs RI	-1	ERF
Glyma13g21570	RM vs RI	-1	ERF
Glyma14g12505	RM vs RI	-1	ERF
Glyma19g44580	RM vs RI	-1	ERF
Glyma02g46340	RM vs RI	-1.14913	ERF
Glyma05g05180	RM vs RI	-1.45522	ERF
Glyma17g15480	RM vs RI	-2.56825	ERF
Glyma02g40320	RM vs RI	-3.83564	ERF
Glyma13g03700	RM vs RI	0.784377	EIL
Glyma12g05570	RM vs RI	-0.89573	DBB
Glyma13g33987	RM vs RI	-1.2761	DBB
Glyma11g13570	RM vs RI	-1.37246	DBB
Glyma02g38870	RM vs RI	1.2623	CO-like
Glyma04g06240	RM vs RI	-1.03897	CO-like
Glyma13g01290	RM vs RI	-1.56592	CO-like
Glyma05g31190	RM vs RI	-1.07632	CAMTA
Glyma15g15350	RM vs RI	-2.07764	CAMTA
Glyma09g15600	RM vs RI	1.8329	C3H
Glyma02g39210	RM vs RI	0.911187	C3H
Glyma12g36600	RM vs RI	-0.94502	C3H
Glyma02g46610	RM vs RI	-1.73593	C3H
Glyma15g04570	RM vs RI	1.51602	C2H2
Glyma10g05190	RM vs RI	-1	C2H2
Glyma18g14750	RM vs RI	1.66599	bZIP
Glyma05g22860	RM vs RI	1	bZIP
Glyma06g47220	RM vs RI	-1	bZIP
Glyma11g06960	RM vs RI	-1.09033	bZIP
Glyma03g27865	RM vs RI	-1.73724	bZIP
Glyma05g02110	RM vs RI	1.36783	bHLH
Glyma0028s00210	RM vs RI	1.1823	bHLH
Glyma04g35380	RM vs RI	1	bHLH
Glyma07g13511	RM vs RI	-1	bHLH
Glyma13g32470	RM vs RI	-1	bHLH
Glyma12g34300	RM vs RI	-1.12671	bHLH
Glyma06g17420	RM vs RI	-1.32466	bHLH
Glyma01g12740	RM vs RI	-2.99549	bHLH
Glyma03g41901	RM vs RI	-4.21379	bHLH
Glyma16g02690	RM vs RI	-4.84037	bHLH
Glyma08g44640	RM vs RI	-1	B3
Glyma02g40650	RM vs RI	-1.40183	ARF

Table 11. Pathogenesis related (PR) DEGs involved in resistance response during *Phakopsora pachyrizi* infection on soybean.

Gene ID	Name of gene	PR group	p value
Glyma15g06790	Basic pathogenesis-related protein 1	PR1	4.2686
Glyma15g06780	Basic pathogenesis-related protein 1	PR1	5.43266
Glyma15g06816	Basic pathogenesis-related protein 1	PR1	6.21445
Glyma11g10080	Endo-1,3-beta-glucanase	PR2	4.04178
Glyma19g31580	Endo-1,3-beta-glucanase	PR2	4.22949
Glyma09g04191	Endo-1,3-beta-glucanase	PR2	1
Glyma19g43460	Chitinase	PR3	1
Glyma11g13270	Homolog of carrot EP3-3 chitinase	PR3	3.8275
Glyma13g42210	Homolog of carrot EP3-3 chitinase	PR3	5.90991
Glyma15g13500	Peroxidase	PR9	5.90954
Glyma08g19170	Peroxidase	PR9	1.3473
Glyma08g19180	Peroxidase	PR9	4.66954
Glyma09g04510	MLP-like protein 423	PR10	1
Glyma09g04530	Protein SPE-16	PR10	2.88969
Glyma15g15590	MLP-like protein 423	PR10	1

Table 12. Flavonoid metabolic process genes during resistance response upon ASR infection on soybean

Glyma ID	GO ID	P value	Annotation
Glyma10g37660	GO:0009812	-3.64614	*MATE2 transporter*
Glyma02g15390	GO:0009813	-2.98339	*Senescence-associated nodulin 1A*
Glyma02g15370	GO:0009813	-2.77657	*Senescence-associated nodulin 1A*
Glyma19g27930	GO:0009813	-2.56626	*Flavonoid biosynthesis*
Glyma09g39850	GO:0009813	-1.72813	*(+)-neomenthol dehydrogenase*
Glyma01g37370	GO:0009813	-1.72432	*Zinc finger protein CONSTANS-like protein*
Glyma15g38480	GO:0009813	-1.59624	*SRG1*
Glyma10g38910	GO:0009813	-1.57985	*Early light-induced protein*
Glyma11g05440	GO:0009813	-1.53889	*Sigma factor sigB regulation protein rsbQ*
Glyma10g24540	GO:0009813	-1.45221	*Protein ABC1*
Glyma13g33960	GO:0009813	-1.29888	*UDP-L-rhamnose synthase*
Glyma20g18870	GO:0009813	-1.24317	*Protein ABC1*
Glyma20g30140	GO:0009812	-1.14053	*MATE2 transporter*
Glyma18g45350	GO:0009813	1	*Calcium-binding protein (CML24)*
Glyma16g01060	GO:0009813	1.0035	*Monooxygenase (CYP450)*
Glyma01g26970	GO:0009813	1.41867	*CYP450*
Glyma04g40030	GO:0009813	2.19406	*Chalcone isomerase (CHI)*
Glyma08g15890	GO:0009813	2.31716	*Flavonol synthase/flavanone 3-Hydroxylase(F3H)*
Glyma03g27740	GO:0009813	2.42975	Monooxygenase
Glyma01g44270	GO:0009813	2.81319	*4-Coumarate-CoA ligase (4CL)*
Glyma06g14820	GO:0009813	3.54822	*Chalcone isomerase (CHI)*
Glyma20g38560	GO:0009813	3.87872	*Chalcone isomerase (CHI)*
Glyma02g13810	GO:0009813	3.9443	*SRG1*
Glyma09g40590	GO:0009813	6.33158	Uncharacterized protein
Glyma10g43850	GO:0009813	6.9166	*Chalcone isomerase (CHI)*

Summary

Global soybean yield projection is sharply increasing. But the potential yield of soybean has been reduced up to 90% due to Asian soybean rust (ASR) disease. ASR is a polycyclic disease caused by the obligate biotrophic foliar fungal pathogen *Phakopsora pachyrhizi*. Current ASR management is ineffective as economic and ecological perspectives. Breeding for developing resistance cultivar needs plenty of resistance resources. But very few, only eight resistance to *Phakopsora pachyrhizi* (*Rpp*) loci, have been mapped yet. Moreover, each *Rpp* line originated from different parent showed different resistance responses. In addition, the soybean defence mechanism by *Rpp* gene is also largely unknown. For this reason, this study used soybean near isogenic lines (NILs) to specify the *Rpp* gene linked candidate genes during disease response. The transcriptomes data from susceptible BRS184 and *Rpp3* NIL with *P. pachyrhizi* isolates T1-2 at 24 h after inoculation (hai) and without *P. pachyrhizi* inoculation (mock) were generated. A total 4518 differentially expressed genes (DEG) were annotated. We used similarity searching method with protein family databases (PFAM), Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG), Eukaryotic clusters of Orthologous Groups (KOG), and Gene Ontology (GO) databases. Fifty two percent phenylpropanoid pathway related genes were up-regulated according to KEGG annotated data. 1742 genes were found to *Rpp3* defence specific. Twelve genes were selected for next relative expression study to know their expression to other *Rpp* NILs. These genes are mostly related to phenylpropanoid branch isoflavonoid pathway specific phytoalexin, glyceollin biosynthesis. Prior to perform RT-qPCR of *Rpp1-4* NILs, we studied phenotypic defence status by disease severity, pustule per leaf, reaction type and degree of leaf yelloing of that NILs. This study disclosed that *Rpp3* NIL ranks better resistance status than other *Rpp* lines. The RT-qPCR results also congruent with this result that *Rpp3* NIL may use those genes efficiently for phytoalexin glyceollin synthesis. Some artistic feature we also observed that all the genes showed the greatest expression at 12 hai expression except the *glycinol 4-dimethylallyltransferase* (*G4DT*) and *chalcone reductase* (*CHR*) that were between 24 and 96 hai respectively. We also observed three different functional coordination scenario between- 1) *arogenate dehydratase 6 (ADT6)* and *4-hydroxy-3-methylbut-2-enyl diphosphate synthase* (*ispG*), 2) *chalcone synthase* (*CHS*) and *chalcone reductase* (*CHR*), and 3) *G4DT*

and *phytyltransferase 3 (PT3)*. This coordination of these resistance loci linked genes may be useful as efficient breeder's tool to develop new soybean resistance variety against ASR.

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