Laboratory manual for studies on soybean rust resistance

Version 24

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Japan International Research Center for Agricultural Sciences (JIRCAS)

Contents

Chapter 1: Evaluation of resistance to soybean rust

Chapter 2: Simple sequence repeat (SSR) marker analysis

(Toward marker-assisted selection to introduce *Rpp* genes)

Chapter 1

Evaluation of resistance to soybean rust

1-1. Multiplication of rust urediniospores

Before experimental use, rust spores obtained from field-grown soybeans or those preserved at −80°C for a long time should be multiplied on disease-free leaflets of susceptible soybean varieties for the preparation of highly germinative spores. Multiplication of spores by the "leaf culture method" is recommended because it enables the propagation of multiple rust isolates (populations) in a single growth chamber without the cross contamination. For the multiplication of spores, healthy soybean plants without rust infection must be used. In addition, if rust populations suspected of containing various races are to be propagated, it is desirable to use susceptible varieties carrying no resistance gene to maintain the original composition of races in the population after the multiplication step.

(Protocol)

1. Grow susceptible soybean plants (e.g., the cultivars: BRS154 and BRS184) in a growth chamber (image 1).

2. Detach mature adult leaflets from the plants.

3. Use your fingers to rub the lower surface of the leaves with

Image 1: Plants in the growth chamber

   distilled water for approximately 10 s per leaflet to spread the spore suspension well.

4. Cut the bottom (5–10 mm) of leaflets, place them in Petri dishes, and cover the cut end of the leaflets with sterilized paper moistened with distilled water (*1) (image 2).

5. Suspend [thawed (*2) or raw] spores in 0.04% Tween® 20 solution.

6. Dilute the spore suspension with 0.04% Tween® 20 solution and adjust the concentration to $5–10 \times 10^4$ spores/mL (this may change depending on the expected germination ratio) using a hemocytometer (image 3) (*3).

Image 2: Leaves for inoculation

7. Prepare the inoculation solution (approximately 100 µL per leaflet).

8. Homogenously spread the spore suspension on the lower surface of the leaves using a paintbrush.

9. Place the Petri dishes in a dark place at 21°C for 12 h (*4).

10. Use the remaining spore suspension to check the germination ratio of spores (*5).

11. Transfer the Petri dishes into a growth chamber at 21°C and culture the leaflets for approximately 13 days or more.

12. Moisten the paper in the Petri dishes with distilled water (*1) approximately twice a week and replace the sterilized paper when required.

13. Collect spores by tapping the leaflets over a paraffin paper when spores are observed in rust lesions on the leaflets (image 4).

14. Transfer collected spores into 2-mL microtubes (or 15- or 50-mL Falcon® tubes) and place the tubes with caps opened in a desiccator overnight to dry the spores.

15. Seal the microtubes and store them at −80°C (or in liquid nitrogen).

16. Repeat steps 12–15 to harvest the spores multiple times.
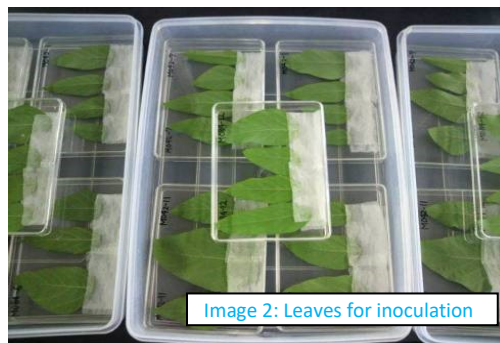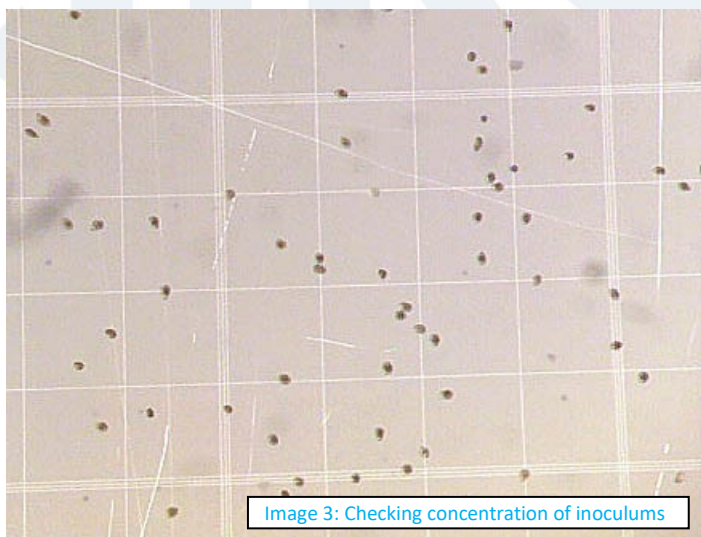

Image 3: Checking concentration of inoculums

Image 4: Spore collection

*1: Instead of distilled water, a culture solution such as a 500-fold diluted Hyponex® solution (Hyponex, Japan) or 40 ppm gibberellic acid could be used.

*2: For thawed spores, incubate the microtubes containing frozen rust spores stored at −80°C (or in liquid nitrogen) in a 39°C water bath for 1 min.

*3: For the "Fuchs–Rosenthal: Bright-Line (depth = 0.2 mm)" hemocytometer, the concentration of spores (spores/mL) is calculated as "the number of spores in 1 mm" × 0.5 × 10^4.

*4: The Petri dishes could be covered with aluminum foil before placement in the growth chamber at 21°C.

*5: See the section "1-3. Inoculation of soybean with spore suspension" for details.

(Requirements)

· Distilled water

· Culture solution [a 500-fold diluted Hyponex® solution (Hyponex, Japan) or 40 ppm gibberellic acid] (instead of distilled water, optional)

· Petri dishes (glass or plastic plates)

· Sterilized paper (Kimwipe®, etc., cut to the size of 1.5 cm × 4.0 cm, autoclave)

· **0.04% Tween® 20 solution** (10 mL of distilled water + 4 µL of polyoxyethylene sorbitan monolaurate; Tween® 20, Promega; prepare a fresh solution each time)

· Paraffin paper

· 2.0 mL-microtubes (or 15/50-mL Falcon® tubes)

· Laboratory scissors

· Paintbrush

· Counter (for counting the number of spores)

· Hemocytometer

· Growth chamber [for growing plants; 24°C, 14-h light photoperiod, more than 19,000 lux from fluorescent lamps. Biotron (Nippon Medical & Chemical Instruments Co.) is used in JIRCAS]

· Growth chamber [for leaf culture; 21°C, 12-h light photoperiod, approximately 3,000 lux from fluorescent lamps. Biotron (Nippon Medical & Chemical Instruments Co.) is used in JIRCAS]

· Freezer at −80°C (or a tank for liquid nitrogen)

· Water bath at 39°C (for thawing frozen spores)

· (Stereo) microscope

· Desiccator

1-2. Single-lesion isolation

Rust spores obtained from field-grown soybeans must be considered as a population suspected of containing various rust races. If performing a race study of the pathogen, the pathogen population must be purified into an isolate using the "Single-lesion isolation." The leaf culture method described in the "1-1. Multiplication of rust urediniospores" section should be used for single-lesion isolation.

For multiplication of spores, susceptible varieties should be used to maintain the composition of races in the rust population. For single-lesion isolation, resistant varieties could be used, and the use of resistant varieties results in rust isolates with specific virulence to resistance genes (*Rpp*: Resistance to *Phakopsora pachyrhizi*) or resistant varieties.

(Protocol)

1. Prepare the leaflets for leaf culture by following steps 1–4 in the section "1-1. Multiplication of rust urediniospores."
2. Suspend (thawed or raw) spores in 0.04% Tween® 20 solution.
3. Spread the spore suspension (Approximately 20 µL of 1×10^4 spores/mL) on the lower surface of the leaves with a small piece of filter paper using a pair of tweezers (*1).
4. Place the Petri dishes in a dark place at 21°C for overnight (*2).
5. Transfer the Petri dishes into a growth chamber at 21°C and culture the leaflets.
6. When lesions appear on the inoculated leaflets (About 7-10 days), cut the leaflet into segments (5 × 5mm ~), such that each segment has a single lesion (*3).
7. Place each leaf segment on sterilized paper (*4) moistened with distilled water (*5) in another Petri dish.
8. Incubate the Petri dish in a growth chamber until the uredinia formed on the leaf segment rupture and produce spores (About 14 - 20 days; image 5).
9. Transfer the leaf segment with the sporulating lesion to a 2.0-mL microtube (*6).
10. Add 100 µL of 0.04% Tween® 20 solution to the microtube.
11. Briefly vortex the spore suspension and drop it on the lower surface of the rubbed leaflet of susceptible variety with a micropipette (1 leaflet for 100 µL suspension).
12. Homogenously spread the spore suspension with a small piece of filter paper using a pair of tweezers.
13. Incubate the Petri dish in a growth chamber again and harvest freshly reproducing spores from the infected leaves (About 2 - 4 weeks) (*7).
14. Repeat spore multiplication process to prepare enough spores for inoculation in the subsequent experiment.

*1: Drip the drop of suspension by micropipette, then spread the inoculum by filter paper with a pair of tweezers (Figure 1).

*2: The Petri dishes could instead be covered with aluminum foil for the dark treatment.

*3: The leaflets must be cut into segments before spores are produced to prevent cross-contamination.

*4: To avoid dehydration of the small leaf segments, about 10 pieces of papers are used.

*5: Instead, 1/2,000 × Hyponex® solution or 40 ppm gibberellic acid could be used.

*6: Could be stored at −80°C.

*7: See steps 9–16 in the section "1-1. Multiplication of rust urediniospores" for details.
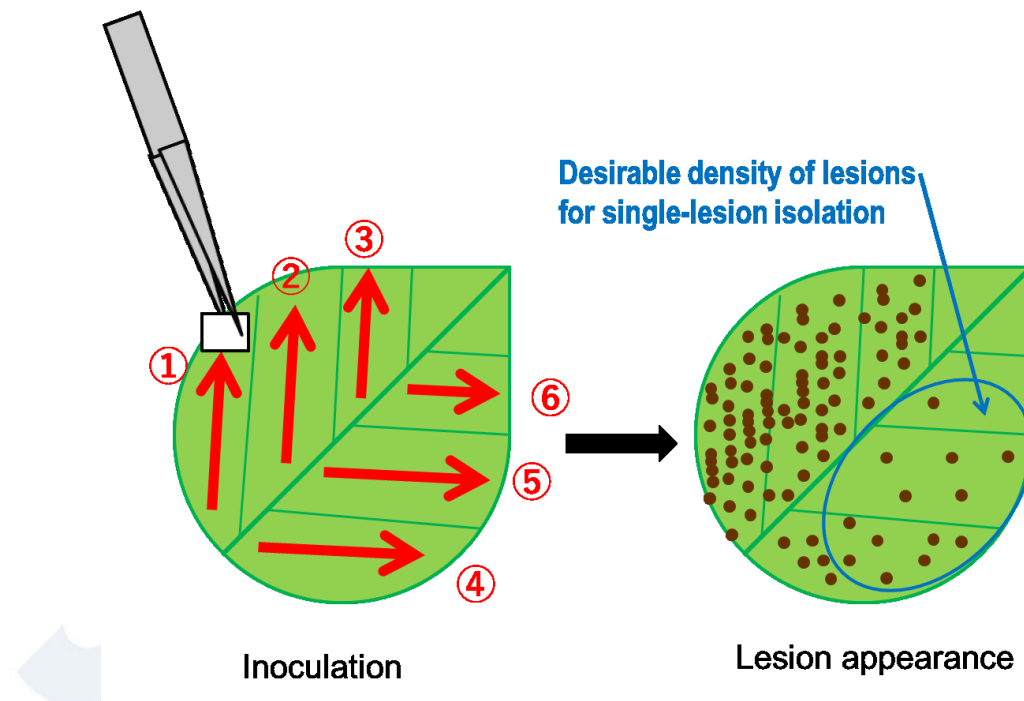
Figure 1. Method of spreading inoculum to obtain adequate density of soybean rust lesions. Red arrows and numbers indicate direction and order of single-time spreading, respectively.

Image 5: Sporulating lesion on a leaf segment

(Requirements)

· Distilled water

· Petri dishes

· Sterilized paper (Kimwipe®, etc., cut to the size of 1.5 × 4.0 cm, autoclave)

· **0.04% Tween® 20 solution** (10 mL of distilled water + 4 µL of polyoxyethylene sorbitan monolaurate; Tween 20®, Promega; prepare a fresh solution each time)

· Filter paper

· 2.0-mL microtubes

· Paintbrush

· Counter (for counting the number of spores)

· Hemocytometer

· Scalpel

· A pair of tweezers

· Growth chamber [for leaf culture; 21°C, 12-h light photoperiod, approximately 3,000 lux from fluorescent lamps. Biotron (Nippon Medical & Chemical Instruments Co.) is used in JIRCAS]

· Freezer at −80°C (or a tank for liquid nitrogen)

· (Stereo) microscope

1-3. Inoculation of soybean with spore suspension

Healthy soybean plants without any disease infection must be used for experimental inoculation. To prevent natural infection of soybean rust and other diseases, growing soybean plants in a growth chamber is recommended. Plants grown in a disease-free greenhouse could also be used. However, the plant growth in a greenhouse might be affected due to various environmental factors like photon flux density, day length, temperature etc. Both rust pathogenicity (section 1-4 "Evaluation of rust pathogenicity") and rust resistance in soybean genotypes (section 1-5 "Rust resistance of soybean genotypes") are evaluated using the same protocol.

For evaluating pathogenicity of rust isolates, we used a differential variety set (Table 1). To prevent internal variation in the resistance phenotype of the varieties, these varieties were multiplied from a single seed. We can compare the pathogenicity of rust isolates each other by using the same variety set and evaluation methodology. This variety set can be distributed for research use under the Material Transfer Agreement (MTA). Please be careful of seeds-contamination in multiplying the seeds. From 2016, our soybean rust research consortium was decided to reduce the numbers of differential varieties from 18* to 12 by excluding the varieties putatively carrying same *Rpp* gene (allele)**.

*Previous differential variety set consisted of
1: PI 200492 (Komata, *Rpp1*); **2**: PI 368039 (Tainung 4, *Rpp1*); **3**: PI 230970 (No. 3, *Rpp2*);
4: PI 417125 (Kyushu 31, *Rpp2*); **5**: PI 462312 (Ankur, *Rpp3*); **6**: PI 459025 (Bing Nan, *Rpp4*);
7: PI 200526 (Shiranui, *Rpp5*); **8**: PI 416764 (Akasaya, *Rpp3*); **9**: PI 587855 (Jia Bai Jia, *Rpp1-b*);
10: PI 587880A (Huang Dou, *Rpp1-b*); **11**: PI 587886 (Bai Dou, *Rpp1*);
12: PI 587905 (Xiao Huang Dou, *Rpp1-b*); **13**: PI 594767A (Zhao Ping Hei Dou, *Rpp1-b*);
14: BRS154 (Susceptible); **15**: TK5 (Taita Kaohsiung 5, Susceptible);
16: PI 548628 (Wayne, Susceptible); **17**: PI 567102B (MARIF 2767, *Rpp6*);
18: No6-12-1 (*Rpp2+Rpp4+Rpp5*).

** PI 587886 (RDV2) was not excluded from the new set since it may have different resistance allele of *Rpp1* from that of PI 200492 (RDV1) (Yamaoka et al. 2014). PI 416764 (RDV5) was not excluded from the new set since it may be another gene flanking to *Rpp3* of PI 462312 (RDV4) or another resistance allele of *Rpp3* (Hossain et al. 2015). Although two varieties, PI 587880A (RDV9) and PI 594767A (RDV10) may carry the same *Rpp1-b* gene, both of them are included into new set since they are important material for developing near-isogenic lines and *Rpp*-pyramided lines (Table 9).

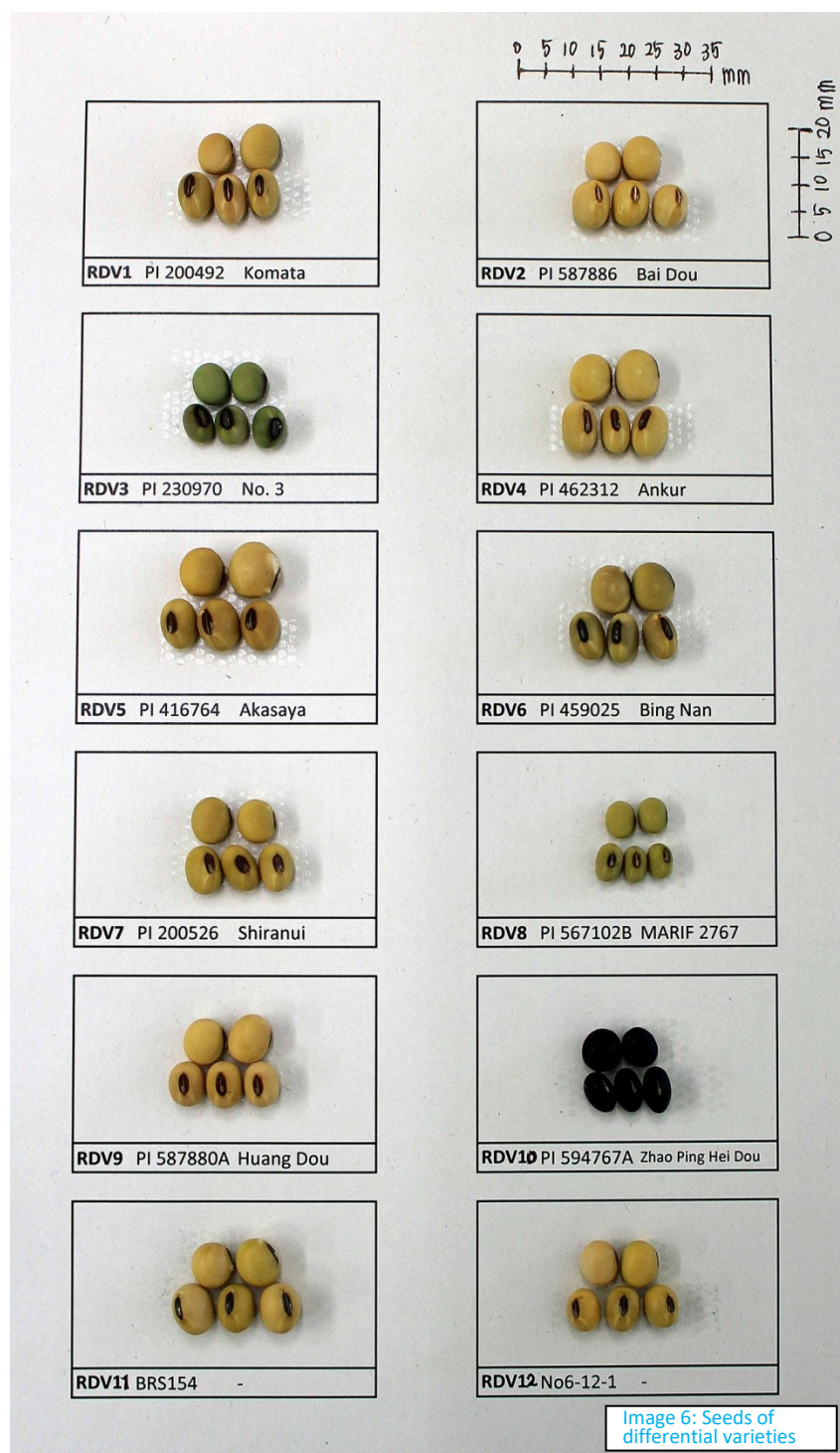**Table 1.** Soybean differential varieties*

No.	Genotype	Alternative	*Rpp*	Origin	Prev. No.	100-seeds weight**	Flower color	Pod color	Pubescence color	Hilum color**	Seed coat color**	Cotyledon color**	Reference
RDV1	PI 200492	Komata	*Rpp1*	Japan	1	23.47g	Purple***	Brown	Brown	Brown	Yellow	Yellow	Hartwig and Bromfield, 1983
RDV2	PI 587886	Bai Dou	*Rpp1*	China	11	20.99g	Purple	Light B	White	Light B	Yellow	Yellow	Ray et al., 2009
RDV3	PI 230970	No. 3	*Rpp2*	Japan	3	13.08g	Purple	Grey	White	Black	Green	Green	Hartwig and Bromfield, 1983
RDV4	PI 462312	Ankur	*Rpp3*	India	5	22.90g	White	Grey	White	Brown	Yellow	Yellow	Hartwig and Bromfield, 1983
RDV5	PI 416764	Akasaya	*Rpp3*	Japan	8	29.88g	Purple	Dark B	Dark B	Dark B	Yellow	Yellow	Hossain et al.,2015
RDV6	PI 459025	Bing Nan	*Rpp4*	China	6	24.75g	Purple	Light B	Brown	Black	Greenish Y	Yellow	Hartwig, 1986
RDV7	PI 200526	Shiranui	*Rpp5*	Japan	7	19.66g	Purple	Grey	Dark B	Brown****	Yellow	Yellow	Garcia et al., 2008
RDV8	PI 567102B	MARIF 2767	*Rpp6*	Indonesia	17	8.36g	Purple	Brown	Brown	Brown	Yellowish G	Yellow	Li et al., 2012
RDV9	PI 587880A	Huang Dou	*Rpp1-b*	China	10	21.36g	Purple	Brown	Brown	Brown	Yellow	Yellow	Ray et al., 2009
RDV10	PI 594767A	Zhao Ping Hei Dou	*Rpp1-b*	China	13	13.46g	Purple	Brown	Brown	Black	Black	Yellow	Hossain et al., 2015
RDV11	BRS154	-	(None)	Brazil	14	21.22g	White	Light B	Brown	Black	Yellow	Yellow	Akamatsu et al., 2013
RDV12	No6-12-1	-	*Rpp2, 4, 5*	Japan	18	17.10g	Purple	Brown	Brown	Dark B	Yellow	Yellow	Yamanaka et al., 2013; 2015c

*This differential variety set was renewed in 2016.

**The seeds cultivated at JIRCAS experimental field in Tsukuba, Ibaraki Japan (E140.08, N36.05) in 2014 (June - November) were used for evaluation of seed traits. Please see image 6 of seeds in the next page. The other phenotypic traits were evaluated at the same field in 2015.

***Though *Rpp1* of this differential was confirmed in BC$_3$F$_2$ populations, flower color of PI 200492 is white and different from that in USDA database (https://npgsweb.ars-grin.gov/gringlobal/search.aspx).

****Hilum color of PI 200526 is black in USDA database (https://npgsweb.ars-grin.gov/gringlobal/search.aspx).

Image 6: Seeds of differential varieties

Lowest trifoliate leaflets of soybean plants at the V3–V4 growth stage could be used for inoculation. However, the maturity of leaflets used for inoculation must be visually assessed, because the growth and the maturity rate of the plant and the leaflet depends on the soybean variety used. In this section, the protocol for inoculation using soybean plantlets is mentioned. Please see next "section: 1-3" for Inoculation protocol by leaf culture method.

(Protocol)

1. Sow the seeds in pots filled with soil (*1).
2. Grow three plants per variety in a growth chamber (24°C, 14-h light photoperiod, and more than 19,000 lux from fluorescent lamps).
3. Use soybean plants for inoculation when all plant material reaches the V3–V4 growth stage (*2).
4. Use your fingers to rub the lower surface of leaves (**the lowest trifoliate leaflets**) with distilled water for approximately 10 s per leaflet.
5. Suspend [thawed (*3) or raw] spores in 0.04% Tween® 20 solution.
6. Dilute the spore suspension with 0.04% Tween® 20 solution and adjust the concentration to 50,000 spores/mL using a hemocytometer (*4).
7. Prepare the inoculation solution (*5).
8. Homogenously spread the spore suspension on the lower surface of the leaves using a paintbrush.
9. Cover the inoculated plants with a plastic bag (to maintain high humidity) and place them in a dark place at approximately 24°C for 12 h.
10. Use the remaining spore suspension to check for the germination ratio of spores (see the following protocol).
11. Transfer the inoculated plants into a growth chamber at 24°C and grow plants for more than 13 days.
12. Use infected leaflets for evaluation 14 days after inoculation.

*1: Instead of sowing seeds, transplanting one-week-old plants grown on vermiculite (or germination chamber) could also be used.

*2: Approximately 3 weeks after sowing the seeds.

*3: For thawed spores, incubate microtubes containing frozen rust spores stored at −80°C (or in liquid nitrogen) at 39°C in a water bath for 1 min.

*4: This concentration is for the spores with moderate germinability (60–80%)

*5: Approximately 100 µL per leaflet.

(Protocol for checking spore germination)

1. Pour approximately 1.0 mL of 0.5% Agar gel (heated to melt) on a glass slide.
2. After the agar gel set, add 15–20 µL of the spore suspension on the gel.
3. Place the glass slide in a Petri dish with a piece of wet paper (image 7).
4. Place the Petri dish in the same place where the inoculated plants were placed (i.e., the growth chamber).
5. After 4–12 h, count the number of germinated and ungerminated spores (more than 300 in total) under a microscope (image 8).

6. Calculate the germination ratio of spores.

Image 7: Checking germination

Germinated spore

Ungerminated spore
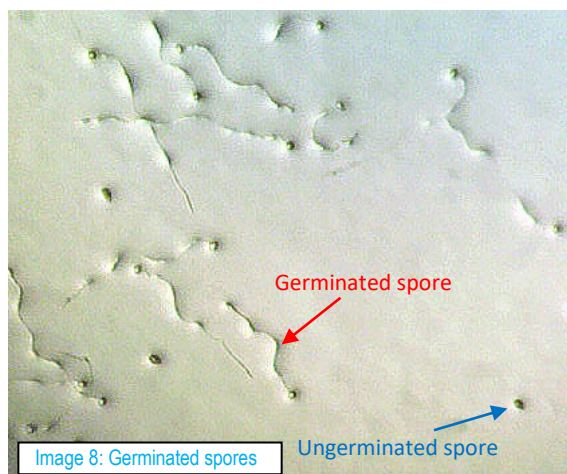
Image 8: Germinated spores

(Requirements)

· Distilled water

· **0.04% Tween® 20 solution** (10 mL of distilled water + 4 µL of polyoxyethylene sorbitan monolaurate; Tween® 20, Promega; prepare a fresh solution each time)

· **0.5% Agar gel** (100 mL of distilled water + 0.5 g of agar, autoclave)

· Growth chamber [for growing plants; 24°C, 14-h light photoperiod, more than 19,000 lux from fluorescent lamps. In JIRCAS Biotron (Nippon Medical & Chemical Instruments Co.) is used]

· Pot (size: approximately 1/10,000 a (are))

· Hemocytometer

· 50-mL Falcon® tube

· Paintbrush

· Plastic bag

· Glass slide

· Water bath at 39°C

· Petri dish (glass or plastic plates)

· (Stereo) microscope

· Counter (for counting the number of spores)

1-3'. Inoculation of soybean with spore suspension

(By leaf culture method)

As it is mentioned in the section 1-1, "leaf culture method" is useful because it enables the inoculation of multiple rust isolates (populations) in a single growth chamber. But healthy soybean plants without any disease infection are needed to obtain healthy leaves to be used for inoculation. Please be careful of difference on the conditions for growing plants and culturing leaflets.

(Protocol)

1. Carry out the inoculation by following the steps 1–10 in the section "1-1. Multiplication of rust urediniospores" using the plant materials of which you want to evaluate resistance. (*1)
2. Transfer the Petri dishes into a growth chamber at 21°C and culture the leaflets for more 13 days.
3. Moisten the paper in the Petri dishes with distilled water (*2) approximately twice a week and replace the sterilized paper when required (*3).
4. Use infected leaflets for evaluation 14 days after inoculation. For evaluation, please follow the steps mentioned in the sections: 1-4 or 1-5 (*4).

*1: Though any adult leaflets can be used for spore multiplication, the lowest mature trifoliate leaflets should be used to be inoculated for resistance evaluation.

*2: Instead of distilled water, a culture solution such as a 500-fold diluted Hyponex® solution (Hyponex, Japan) or 40 ppm gibberellic acid could be used.

*3: To avoid collapse of accumulated spores, the replacement of paper should be done carefully.

*4: Threshold values of NoU, %LU, and SL for leaf-culture experiment are different from those for plantlet experiment.

(Requirements)

· (Same as section 1-1)

1-4. Evaluation of rust pathogenicity

For evaluating the pathogenicity of rust isolates or populations (section 1-4) and resistance of soybean genotypes (section 1-5), classification of "lesion (infection) type" based on the soybean responses induced by rust inoculation is commonly used. However, some differences are observed between the methodologies of these two evaluation purposes.

Lesion color (LC) has traditionally been used to evaluate resistance to soybean rust. However, particularly under the growth chamber condition, it has been demonstrated that LC is not always indicative of resistance (Yamanaka et al., 2010). We use the following four other resistance characters: sporulation level (SL), number of uredinia per lesion (NoU), frequency of lesions with uredinia (%LU), and frequency of open uredinia (%OU). Taking into account the fact that these four characters are highly correlated with each other (Yamanaka et al., 2010, 2011), "SL" and "NoU" are mainly used for evaluating the "pathogenicity of rust isolates" (Akamatsu et al., 2013; Yamaoka et al., 2014) and for evaluating the "resistance of soybean genotypes" (Yamanaka et al., 2011).

(Protocol)

1. Carefully detach the infected leaflets from the plants 2 weeks after inoculation.
2. If the number of samples is small and the evaluation could be performed in a single day, initiate the evaluation by following "Step 6."
3. If the number of samples is too large to evaluate all in a single day, place the detached leaflets in a Petri dish with the lower surface of the leaves turned upward.
4. Ensure that the Petri dish is airtight (to protect the leaves from dehydration) and preserve it at −80°C (or −20°C) until evaluation.
5. Thaw frozen samples at room temperature and use them for evaluation.
6. Evaluate SL with the naked eye using the scale in Figure 2A.
7. Remove spores using a paintbrush.
8. Count the NoU (both open and closed uredinia) in 30 lesions under a stereomicroscope (Figure 3).
9. Determine the reactions of differential varieties (Table 1) according to the criteria in Table 2.

(A)
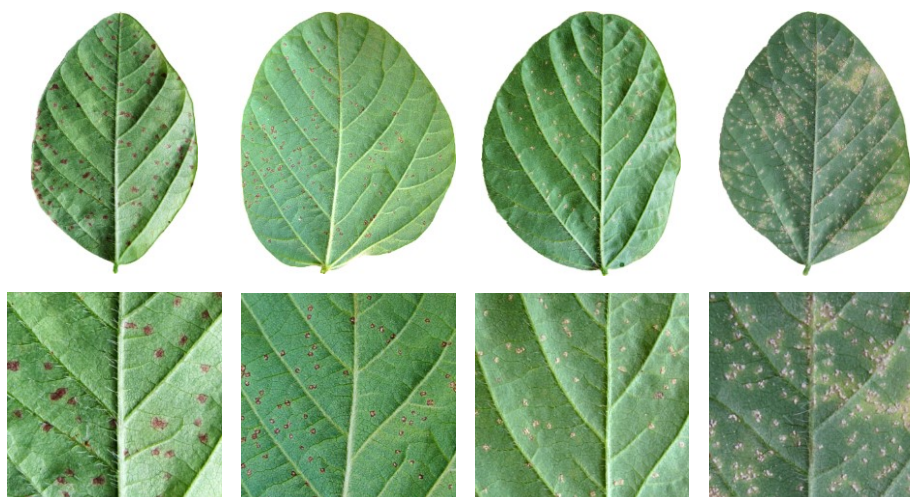
| Category | SL = 0 None | SL = 1 Little | SL = 2 Moderate | SL = 3 Abundant |

(B)

Category	Example	Characteristic
0:None		Sporulation is not observed microscopically at all. Uredinia could be observed.
1:Little		Very little sporulation is observed. Sporulation is detectable by the naked eye. Uredinia sometimes seem to be closed in this sporulation level.
2:Moderate		Moderate sporulation is defined as more spores than '1: Little' but less than '3: Abundant'. Variation of sporulation is large in this category
3:Abundant		Massive spores are observed. They sometimes collapse because of large amount of spore production.

Figure 2. The scale of SL for evaluation with the naked eye (A) and for evaluation under a microscope (B)
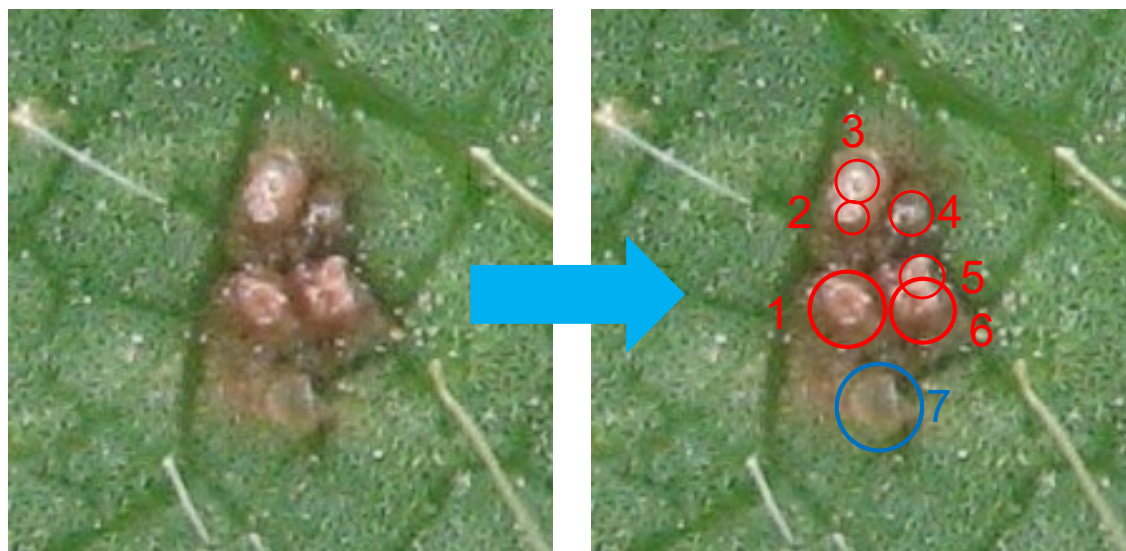
Figure 3. Example of counting the NoU. In this lesion, six open (**red**) and one closed (**blue**) uredinia are observed. The NoU and %OU were calculated as 7 and 85.7%, respectively.

Table 2. Classification criteria of rust reaction in differential varieties (Akamatsu et al., 2013). This classification was applied to evaluate the pathogenicity of rust populations and isolates. SL was evaluated with the naked eye, and the NoU was calculated from 30 lesions evaluated microscopically.

Presence of lesions	SL	NoU*	Reaction type of differentials
−	-	-	Resistant (immune)
+	0 or 1	× <1.5	Resistant
+	2 or 3	× <1.5	Intermediate
+	0 or 1	1.5≤ ×	Intermediate
+	2 or 3	1.5≤ ×	Susceptible

* For leaf-culture experiment, the criteria value of NoU = 1.2 is used instead of 1.5 (Yamanaka et al. 2013)

(Requirements)

· Freezer at −80°C (or −20°C)

· Paintbrush

· Petri dish (glass or plastic plates)

· Stereomicroscope

1-5. Rust resistance of soybean genotypes

To assess the **"degree of resistance in soybean genotypes,"** number of uredinia per lesion (NoU), frequency of lesions with uredinia (%LU), and sporulation level (SL) in the lesions of various soybean genotypes (genetic resources, mapping populations, or breeding lines) are microscopically evaluated. In addition, the NoU, %LU, and SL [as well as optional characters lesion color (LC) and frequency of open uredinia (%OU)] are all numerical vales. Thus, they could be used for quantitative trait locus (QTL) and other statistical analyses.

The two criteria described in sections 1-4 and 1-5 are similar to each other. Compared with the criteria described in section 1-4, in the classification criteria described in this section, even weaker resistance tends to be considered as "resistant," because identifying such minor resistance may also be important for genetics and breeding purposes.

(Protocol)

1. Carefully detach the infected leaflets from the plants 2 weeks after inoculation.
2. If the number of samples is small and the evaluation could be performed in a single day, initiate the evaluation by following "Step 6."
3. If the number of samples is too large to complete the evaluation in a single day, place the detached leaflets in a Petri dish with the lower surface of leaves turned upward.
4. Ensure that the Petri dish is airtight (to protect the leaves from dehydration) and preserve it at −80°C (or −20°C) until evaluation.
5. Thaw frozen samples at room temperature and use them for evaluation.
6. Evaluate the SL for each of 30 lesions at first under a stereomicroscope using the scale in Figure 2B.
7. Remove spores from the diseased leaflets using a paintbrush.
8. Count the NoU and the number of open uredinia (optional, Figure 3) and evaluate LC (optional, Figure 4) in 30 lesions under a stereomicroscope.
9. Enter the data into a datasheet (see the example of the Microsoft Office Excel® datasheet in Figure 5). %LU and %OU could be calculated using the software function.
10. Classify the phenotypic values for the NoU, %LU, and SL into "**Resistant**" or "**Susceptible**" according to the criteria in Table 3.
11. Finally determine the resistance of soybean genotypes according to the criteria in Table 4.

18

Category	Color	Example	Characteristic
1			The darkest color of lesions. Imperfect formation of lesions derived from strong resistance are also classified into here
2			Most of the lesions correspond to this category produce non or less spores
3			Both resistant and susceptible genotypes show this LC
4			Most of the lesions correspond to this category produce spores but resistant genotypes sometimes (i.e. PI459025) show this lesion color
5			Abundant spore production and large number of uredinia usually observed in these 2 categories.
6			

Figure 4. Six categories for LC in soybean rust lesions. To clearly reveal the color, images of lesions were taken after removal of urediniospores.

Soybean materials used for evaluation

Input numerical data for independent 30 lesions (LC, Total NoU, Open NoU, and SL)

Phenotype of the material caluclated from 30 lesions (LC, NoU, %LU, %OU, and SL)

No.	Genotype	LC	Total NoU	Open NoU	SL	NoL	LC	SD	NoU	SD	OU	%LU	SL	SD
1	PI200492	5 5 4 4 5 5 5 5 5 5	5 2 3 5 6 4 7 5 3 4	5 2 3 5 6 4 7 5 3 3	3 3 3 3 3 3 3 3 3 3	30	4.9	0.4	4.9	1.5	93.8%	100.0%	3.0	0.0
		5 5 5 5 5 5 5 5 4 5	5 6 7 6 5 6 6 8 3 3	5 6 7 6 5 4 5 7 3 3	3 3 3 3 3 3 3 3 3 3						137			
		4 5 5 5 5 6 5 5 5 5	3 4 3 6 6 3 5 5 7 5	3 3 3 6 5 2 5 4 7 5	3 3 3 3 3 3 3 3 3 3						146			
3	PI230970	3 3 3 3 3 3 3 3 3 3	5 3 3 5 7 1 3 4 4 2	5 3 3 5 5 1 3 3 4 2	3 3 3 3 3 3 3 3 3 3	30	3.0	0.0	3.7	1.8	94.6%	100.0%	3.0	0.0
		3 3 3 3 3 3 3 3 3 3	6 5 3 2 8 3 1 6 4 5	6 5 3 2 8 3 1 6 4 5	3 3 3 3 3 3 3 3 3 3						106			
		3 3 3 3 3 3 3 3 3 3	3 5 5 5 3 2 1 2 4 2	3 5 4 5 3 2 1 2 3 1	3 3 3 3 3 3 3 3 3 3						112			
4	PI417125	5 5 5 4 4 4 4 4 5 5	3 4 2 2 3 3 4 2 2 2	3 4 2 2 2 3 2 2 2 2	3 3 3 2 2 2 3 3 3 2	30	4.8	0.4	2.9	1.3	85.2%	100.0%	2.8	0.4
		5 5 5 5 4 4 5 5 5 5	5 3 2 3 2 3 4 7 4 3	5 2 2 3 1 3 3 7 2 3	3 3 3 3 3 3 3 3 3 3						75			
		5 5 5 5 5 5 5 5 5 5	3 3 3 5 1 1 2 2 3 2	2 1 3 5 1 1 1 2 2 2	3 3 3 3 2 3 3 3 3 3						88			
7	Shiranui	4 4 4 4 4 4 5 5 4 4	1 2 1 2 1 1 2 1 1 1	1 2 1 2 1 1 2 1 1 1	1 1 1 1 3 1 1 1 2 2	30	4.1	0.3	1.6	0.7	87.5%	100.0%	1.5	0.7
		4 4 5 4 4 4 4 4 4 4	1 1 4 1 2 1 1 1 2	0 1 3 1 1 1 2 1 1 2	1 2 1 1 1 1 1 1 3 1						42			
		4 4 4 4 4 4 4 4 4 4	1 2 2 2 2 2 2 3 2 1	1 2 2 1 2 1 2 3 1 1	2 2 2 1 1 1 2 2 2 3						48			
13	PI459025A	5 5 4 4 4 5 4 3 3 2 2	1 0 1 1 1 0 1 2 0 3	0 0 1 1 1 0 0 1 0 2	2 2 1 2 1 2 1 1 1 2	30	3.3	0.8	1.6	1.1	61.2%	83.3%	0.9	0.8
		2 4 4 3 3 3 3 3 3 3	3 1 2 3 2 0 3 2 1 3	3 1 0 1 0 0 2 0 1 3	2 2 0 0 1 1 1 1 1 1						30			
		4 3 3 3 4 3 3 3 3 3	2 1 1 2 0 2 3 3 2 3	1 1 0 1 0 1 2 3 1 3	1 0 0 0 0 0 0 0 0 0						49			

Figure 5. Example of a datasheet for evaluating lesion characters. The phenotypes of five lesion characters are shown.

Table 3. Classification criteria to assess resistant or susceptible phenotypes for the three following resistance characters of lesions: %LU, NoU, and SL. These values are calculated as the average of 30 lesions.

Resistance character	Resistant phenotype	Susceptible phenotype
%LU*	$0.0 \leq \times < 70.0$	$70.0 \leq \times \leq 100.0$
NoU*	$0.0 \leq \times < 2.0$	$2.0 \leq \times$
SL*	$0.0 \leq \times < 2.0$	$2.0 \leq \times \leq 3.0$

* For leaf-culture experiment, %LU=80.0, NoU=1.2, and SL=1.5 are used as threshold values, respectively (Yamanaka et al. 2013)

Table 4. Classification criteria of rust resistance of soybean genotypes

| Resistance category	Criteria
Immune	Having no lesions
Highly resistant	Having lesions showing the resistant phenotype for all three characters and *without uredinia*
Resistant	Having lesions showing the resistant phenotype for all three characters and *with uredinia*
Slightly resistant	Having lesions showing the resistant phenotype for any of the three characters
Susceptible	Having lesions showing the susceptible phenotypes for all three resistance characters

(Requirements)

· Freezer at −80°C (or −20°C)

· Paintbrush

· Petri dish (glass or plastic plates)

· Stereomicroscope

1-6. Rust tolerance of soybean genotypes

The resistance observed in rust lesions is commonly used to evaluate "pathogenicity of rust populations or isolates" and "resistance of soybean genotypes." However, the "degree of leaf yellowing" and "infection index (IFI)" were sometimes used as indexes to identify soybean genotypes may be tolerant to soybean rust (Yamanaka et al., 2011; 2015b). Therefore, for identifying candidate breeding materials, the evaluation of these two characters is also described in this manual.

If the resistance characters of lesions are not to be evaluated, the infection index could be evaluated less than 2 weeks after inoculation, whereas leaf yellowing should be evaluated more than 2 weeks after inoculation (i.e., approximately 3 weeks after inoculation) to clearly observe the differences among genotypes. No classification criteria for these two characters have been established yet, although some varieties clearly differ from others (image 9).

Image 9: Different LY by ASR infection in two varieties
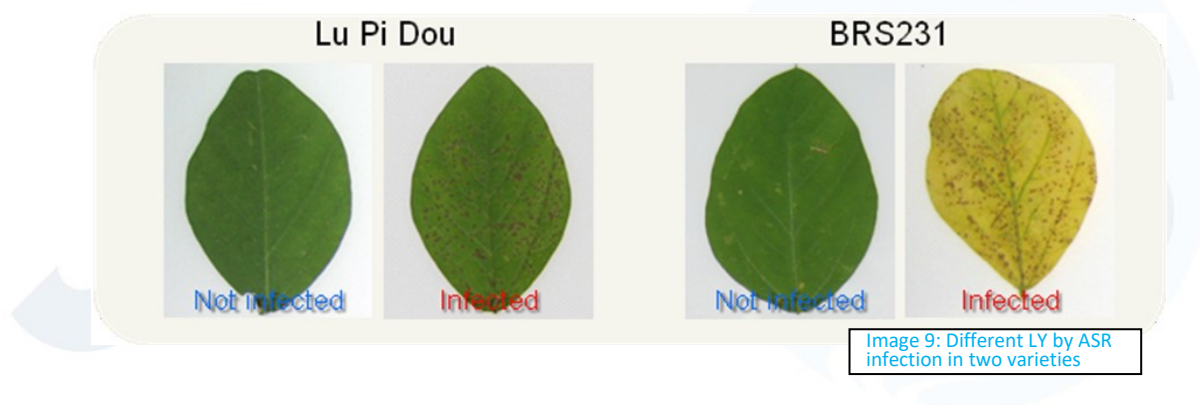
(Protocol)

1. Carefully detach the infected leaflets from the plants 1–3 weeks after inoculation (*1).
2. If the number of samples is small and the evaluation could be performed in a single day, initiate the evaluation by following "Step 6."
3. If the number of samples is too large to complete all the evaluations in a single day, place the detached leaflets in a Petri dish with the lower surface of the leaves turned upward.
4. Ensure that the Petri dish is airtight (to protect the leaves from dehydration) and preserve it at −80°C (or −20°C) until evaluation.
5. Thaw frozen samples at room temperature and use them for evaluation.
6. Evaluate the lesions using the following methodology:
7. Evaluate the degree of leaf yellowing using the scale in Figure 6.

8. Measure the area (cm^2) of infected leaflets using a scanner.
9. Cover the leaflets with a plastic sheet and count the number of lesions in the scanned leaflets using a colony counter.
10. Calculate the infection index using the following formula:

$$\text{Infection index} = \frac{10^4 \cdot \text{NoL}}{\text{LA} \cdot \text{NGS}}$$

LA: leaflet area (cm^2).

NoL: the number of lesions in a single leaflet.

NGS: the number of germinated spores in 1 mL of the spore suspension.

*1: The leaf-samples used for the evaluations in sections 1-4 and 1-5 could also be used for evaluating tolerance.
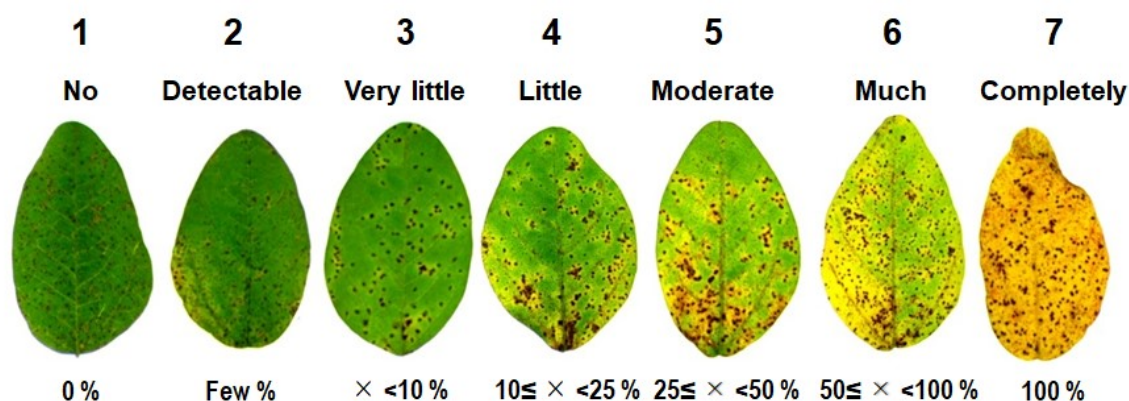

1	2	3	4	5	6	7
No	Detectable	Very little	Little	Moderate	Much	Completely
0 %	Few %	✕ <10 %	10≤ ✕ <25 %	25≤ ✕ <50 %	50≤ ✕ <100 %	100 %

Figure 6. Standards for degree of leaf yellowing. The degree of yellowing is visually categorized into seven classes from 1, no yellowing, to 7, completely yellowing. Estimated percentage of yellowed area is also shown in this figure.

(Requirements)

· Freezer at −80°C (or −20°C)

· Scanner (for infection index; Area Meter AM200, ADC BioScientific Ltd.)

· Colony counter (for infection index; CC-1, AS ONE Corporation)

· Paintbrush

· Petri dish (glass or plastic plates)

· Stereomicroscope

1-7. Data analysis

Pathogenic differences (geographical and seasonal) among rust populations can be estimated by cluster analysis using the data obtained in the sections: 1-4 or 1-5. If the differential variety set (Table 1) is used, you can detect pathogenic difference between your rust sample(s) and the samples in Latin America (Akamatsu et al., 2013; 2017; Garcia-Rodriguez et al., 2017; Silvina et al., 2019), Bangladesh (Hossain and Yamanaka, 2018) and/or Japan (Yamaoka et al., 2014). Since cluster analysis is suitable to show how different between rust samples (sample groups), Principal Coordinate or Quantification Theory III analysis are suitable for seeing the pathogenic diversity of sample groups.

Here, cluster analysis using R software (R Development Core Team, 2013) is introduced to see the pathogenic difference between rust samples. But genetic similarity between soybean genotypes based on the reaction to rust samples can also be seen similarly. Instead converting reaction categories (Resistant/Intermediate/Susceptible) into the numbers (0/1/2), numerical values such as NoU and SL can be used for analysis.

(Protocol)

1. Prepare raw data file by Excel (image 10) and save as ".csv" file. Here, "TEST.csv" (Reaction data of 12 varieties to 13 rust samples) is saved in "disc, C:" in the PC
2. Start the program "R" (image 11)(version 3.4.3 with "pvclust" and "ca" packages)
3. Read the package by input
   > **library(pvclust)**
4. Read the data file by input
   > **TEST <- read.csv("C:/TEST.csv", header=T, row.names=1)**
5. Cluster analysis by input (*1)
   > **result<-pvclust(t(TEST), method.hclust="average", method.dist="euclidian", nboot=10000)**
6. Output the dendrogram by input (image 11) (*2)
   > **plot(result)**

*1: Conditions: Method=UPGMA; Distance=Euclidian; Bootstrap resampling=10,000 times
*2: au: approximately unbiased p-value, bp: bootstrap probability value

	RDV1	RDV2	RDV3	RDV4	RDV5	RDV6	RDV7	RDV8	RDV9	RDV10	RDV11	RDV12
BRP-2	2	2	2	2	2	1	2	-	2	0	-	-
BRP-2.1	2	2	2	2	2	2	2	-	2	0	2	0
BRP-2.6	2	2	2	2	2	2	1	-	2	0	2	0
BRP-2.5	2	2	2	2	2	0	2	-	2	2	2	0
BRP-2.49	2	2	2	2	2	0	2	-	2	0	2	0
T1-2	0	2	2	0	0	0	0	-	0	0	2	0
E1-4-12	0	2	0	0	0	0	0	-	0	0	2	0
Race_1	0	2	2	0	0	2	0	-	0	-	-	-
Race_2	0	2	2	0	0	0	0	-	0	-	-	-
Race_3	0	2	1	0	0	0	0	-	0	-	-	-
Race_4	0	2	0	0	0	0	0	-	0	-	-	-
Race_5	0	0	0	0	0	0	0	-	0	-	-	-
Race_6	0	0	0	0	0	0	0	-	0	-	-	-

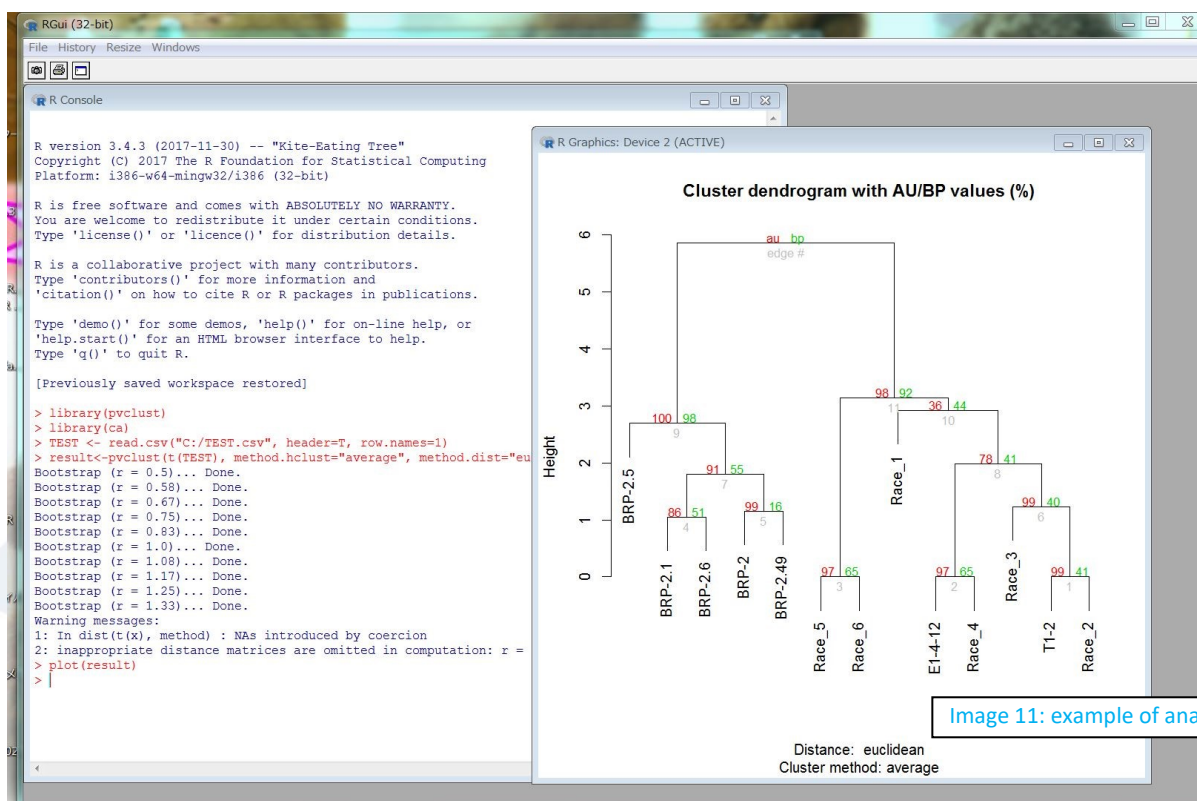Image 10: example of data file

Image 11: example of analysis

24

Chapter 2

Simple sequence repeat (SSR) marker analysis

(Toward marker-assisted selection to introduce *Rpp* genes)

2-1. Leaf sampling

Healthy young leaves without yellowing are desirable for DNA extraction. In particular, smaller developing leaves contain a larger number of cells per tissue volume. Therefore, a larger amount of DNA could be extracted from them. In fact, DNA extraction from the tissue of small leaves (one or two leaflets) at the initial growth stages of soybean provides an adequate amount of DNA for analysis using molecular markers such as simple sequence repeat (SSR). Because many backcrossed plants should be analyzed using SSR markers (Cregan et al., 1999) before flowering, leaf materials from young plants (at the V2 stage) are recommended for DNA extraction.

(Protocol)

1. Harvest healthy young leaflets (*1).
2. Place the leaflet with an area of 4–8 cm^2 into a 2-mL tube (*2).
3. Place the tubes on ice (*3) for transport to the laboratory.
4. Add three "alumina balls (*4)" to each tube.
5. Place the tubes in liquid N$_2$ (*5).
6. Vortex the tubes to convert the leaflets into powder.
7. Remove the "alumina balls" from the tubes.
8. Preserve the tubes at −80°C until DNA extraction (*6).

*1: Leaflets from plants at the V2 stage could be harvested.

*2: Large leaflets could be cut but should not be damaged a lot.

*3: Could be left for several hours.

*4: Instead of alumina balls, other kinds of balls could be used to crush leaf tissue.

*5: After this step, never melt the samples until addition of 1.5× CTAB.

*6: Instead, preservation at −20°C is also possible for a few days.

(Requirements)

· 2-mL microtubes (those from Eppendorf are recommended)

· Alumina balls (φ5 mm; Ito Seisakusho Co., Ltd.; other crusher balls could be used instead)

· Liquid nitrogen (5 L for <200 samples)

· Vortex

· Freezer at −80°C (or −20°C)

· Gloves for handling liquid nitrogen

2-2. DNA extraction

For SSR marker analysis, a high quality of DNA solution is not always necessary. Although DNA extraction according to the CTAB method (Murray and Thompson, 1980) is described in this manual, other methods including simpler extraction protocols may also be used to prepare DNA samples for analysis. If DNA is extracted using methods other than the CTAB method described in this manual, unstable amplification of PCR products may occur, owing to DNA degradation and deterioration in the quality of extracted DNA during storage.

(Protocol)

1. Heat 1.5× CTAB in a microwave oven.
2. Add 1.0 mL of 1.5× CTAB to a 2.0-mL tube containing the frozen sample stored at −80°C (*1).
3. Briefly vortex the mixture of CTAB and the leaf sample.
4. Place the samples in a 56°C incubator or water bath for 15 min and mix the solution 2–3 times.
5. Briefly vortex the mixture.
6. Centrifuge at 15,300 g for 10 min at room temperature (*2) (image 12).
7. Transfer the supernatant (800–900 μL) to new 2-mL tubes.
8. Add 1.0 mL of CIAA (*3).
9. Briefly vortex the mixture.
10. Place it in a shaker for 15 min.
11. Centrifuge at 15,300 g for 10 min at r.t. (image 13).
12. Transfer the supernatant to new 2-mL tubes using cut tips (*4).
13. Add 1,200 μL of CTAB precipitation buffer to the supernatant.
14. Mix the solution (*5).
15. Centrifuge at 15,300 g for 10 min at r.t. (*6).
16. Discard the supernatant.
17. Add 100 μL of 1 M NaCl and 0.1 μL of RNase (*7).
18. Mix, vortex, and spin.
19. Place the samples in a 37°C incubator or water bath and vortex the solution until the precipitation (PPT) is completely dissolved in the NaCl solution.
20. Spin.
21. Add 200 μL of 99.5% ethanol stored at −20°C.
22. Mix the solution (*8).
23. Centrifuge at 15,300 g for 10 min at r.t. (image 14).

24. Discard the supernatant.

25. Drain off the supernatant by turning the tubes over on paper.

26. Add 50–100 µL of 10% TE (*9).

27. Mix the solution until the PPT is completely dissolved in 10% TE.

28. Maintain the samples at 4°C for short-term storage (for a few weeks) or at −20°C for long-term storage.

Image 12: Step 6   Image 13: Step 11   Image 14: Step 23
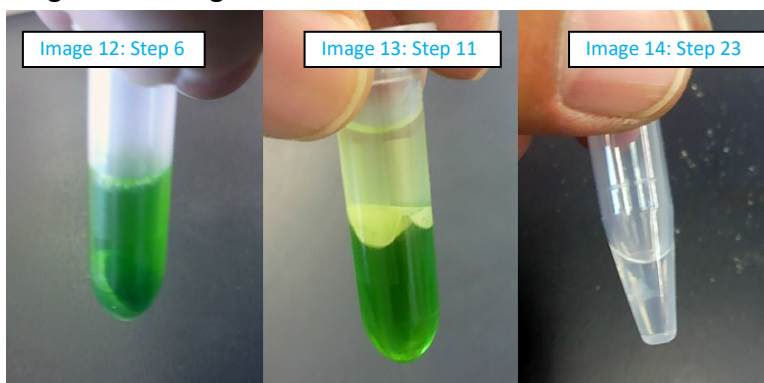
*1: Do not melt the sample before adding 1.5× CTAB.

*2: In the case of an Eppendorf rotor, F45-30-11, *15,300 g* equal to **12,000 rpm.**

*3: A chemical hood should be used for safety.

*4: Do not pipette the middle white layer.

*5: Sometimes a PPT appears. At this point, it is possible to suspend the extraction by storing the solution for 1 day at 4°C.

*6: A whitish DNA PPT will be observed; however, it is not always visible.

*7: Prepare a mixture of NaCl and RNase in advance. At this step, it is possible to suspend the DNA extraction process by storing the sample at 4°C for 1 day.

*8: A whitish DNA PPT must be observed in this step. It is possible to suspend the extraction once in this step by storing the sample at −20°C for 1 day.

*9: The volume of the TE buffer could be changed depending on the PPT size (More amount of buffer is needed for larger PPT).

(Requirements)

· **1.5× CTAB buffer** (7.5 g of CTAB + 30.7125 g of NaCl + 37.5 mL of 1 M Tris-HCl + 15 mL of 0.5 M EDTA for 500 mL, autoclave)

· **CIAA** (24:1, 500 mL of chloroform+ 20.83 mL of isoamyl alcohol)

· **CTAB precipitation buffer** (10 g of CTAB + 50 mL of 1 M Tris-HCl + 20 mL of 0.5 M EDTA for 1 L, autoclave)

· **1 M NaCl** (5.844 g of NaCl for 100 mL, autoclave)

· **RNase** (Ribonuclease A, 10 mg/mL)

· **99.5% ethanol** (store at −20°C)

· **10% TE buffer** (10 mL of TE buffer + 90 mL of dH$_2$O, autoclave)

· **1.0 M Tris-HCl (pH = 8.0)** (dissolve 60.55 g of Tris in 350 mL of dH$_2$O, adjust pH to 8.0 with HCl, make the volume to 500 mL by adding dH$_2$O, autoclave)

· **0.5 M EDTA (pH = 8.0)** (dissolve 55.83 g of EDTA in 180 mL of dH$_2$O, adjust pH to 8.0 with 10 N NaOH, make the volume to 300 mL by adding dH$_2$O, autoclave)

· **TE buffer** (5 mL of 1 M Tris-HCl + 1 mL of 0.5 M EDTA for 500 mL, autoclave)

· **HCl** (for pH adjustment, approximately 50% HCl is easiest to handle)

· **10 N NaOH** [for pH adjustment, 100 g of NaOH+ 60 mL of dH$_2$O (autoclaved); make the volume to 250 mL by adding dH$_2$O]

· Microtubes (2 mL, 1.5 mL)

· Microwave oven

· Incubator at 56°C or water bath (with shaker)

· Vortex

· Centrifuge (for 2.0- and 1.5-mL microtubes)

· Chemical fume hood (for chloroform use)

· Shaker

· Freezer at −20°C and −80°C

· Refrigerator at 4°C

2-3. Adjustment of DNA concentration

A spectrophotometer (such as NanoDrop, Thermo Fisher Scientific Inc.) is desirable for efficient and exact measurement. For SSR marker analysis, exact adjustment of DNA concentration is not always necessary. Instead, DNA concentration could be estimated using agarose gel electrophoresis. In addition, a skilled person could skip the process of diluting the extracted DNA if the amounts of extracted DNA could be estimated based on his experience. It should be kept in mind that it is undesirable to use large amounts as well as small amounts of template DNA for PCR. In addition, attention should be paid to the contamination of ethidium bromide (EtBr). If harmless stains such as "GelRed™" (Biotium, Inc.) are preferred, see "2-5. Acrylamide gel electrophoresis" for details.

(Protocol)

1. Prepare a 0.8% Agarose gel and electrophoresis buffer for Mupid® using 0.5× TBE.
2. Prepare a loading solution (3 µL of DNA solution + 1 µL of loading buffer, see Table 5; confirm that DNA PPT is completely dissolved).
3. Load the solutions into the wells of the gel.
   (Ex. Lane 1: 10 ng/µL reference; Lane 2: 20 ng/µL reference; Lane 3: 50 ng/µL reference; Lane 4: 100 ng/µL reference; Lane 5: extracted DNA samples with 20× dilution).
4. Perform electrophoresis at 100 V for 20 min.
5. Stain the gel with the EtBr solution for 10 min.
6. Visualize DNA bands under a UV transilluminator.
7. Photograph the gel (if the gel is stained too much, place gel in dH_2O for 10 min).
8. Determine approximate concentration in comparison with reference DNA.
   (If signal of sample DNA is equal to that of 50 ng/µL reference DNA, concentration of sample DNA is 50 ng/µL × 20, which is equivalent to 1,000 ng/µL; see Figure 7).
9. Prepare 100 µL of 50 ng/µL DNA solution for each sample.

Table 5. Example of preparation of DNA solution for checking.

Lane	Standards and samples	Preparation of solution for checking
1	10 ng/µL reference*	3 µL of 10 ng/µL reference DNA solution + 10× loading dye
2	20 ng/µL reference*	3 µL of 20 ng/µL reference DNA solution + 10× loading dye
3	50 ng/µL reference*	3 µL of 50 ng/µL reference DNA solution + 10× loading dye
4	100 ng/µL reference*	3 µL of 100 ng/µL reference DNA solution + 10× loading dye
5	Extracted DNA solution**	3 µL of extracted DNA solution diluted to 20× with 10% TE

*All reference DNA solutions are prepared from the 100 ng/µL solution by serial dilution.
**The concentration of extracted DNA generally ranges from 400 to 2,000 ng/µL.
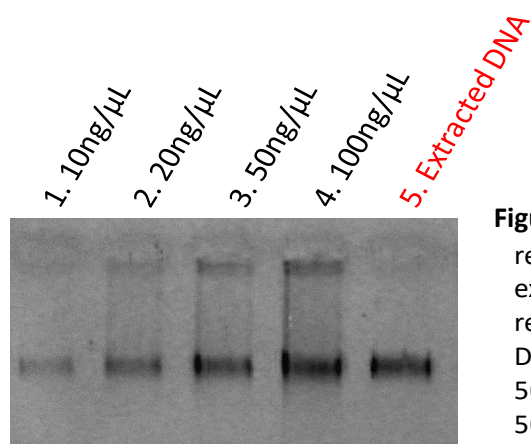
Figure7. Gel image of the standard reference DNA samples and the extracted DNA. Comparing with reference DNAs, the new extracted DNA can be similar signal strength as 3. 50ng/µL, and thus, estimated as 50ng/µL X 20 =1000ng/µL.

(Requirements)

· **0.5× TBE buffer** (20× dilution of 10× TBE using dH$_2$O)

· **Agarose** (for electrophoresis)

· **EtBr solution** (15 µL of 10 mg/mL EtBr solution in 300 mL of autoclaved dH$_2$O; instead of EtBr, the use of less harmful chemicals such as GelRed^{TM} is recommended)

· **Loading dye (10×)** [0.25% bromophenol blue + 0.25% xylene cyanol + 40% (w/v) sucrose, autoclave]

· **Reference DNA solution (10, 20, 50, and 100 ng/µL)** (λ DNA/soybean genomic DNA whose concentration has been exactly adjusted in advance may be used)

· **10× TBE** (54 g of Tris + 55 g of boric acid + 20 mL of 0.5 M EDTA for 500 mL, autoclave)

· Mupid® system (Advance Co. Ltd.)

· UV transilluminator

· Camera (with a filter for UV)

2-4. PCR (for SSR markers)

Instead of "Quick Taq® HS DyeMix" (Toyobo Co., Ltd.) used in this manual, various *Taq* polymerases could be used for SSR amplification. However, in case other *Taq* polymerases are used, the conditions (composition of PCR reagents, thermal condition of PCR, etc.) will also need to be changed to avoid unclear PCR bands due to nonspecific amplification or low efficiency of specific amplification. Sequences of all soybean SSR primers in this manual are available at SoyBase (Grant et al., 2010).

(Protocol)

1. Prepare the following mixture on ice for the PCR reaction (Table 6) for each SSR marker.

Table 6. Composition of reagents for PCR

PCR reagents	For one reaction	For 17 reactions
dH$_2$O	3 µL	51 µL
2× Quick Taq® HS DyeMix	5 µL	85 µL
5 µM primers (forward + reverse)	1 µL	17 µL
50 ng/µL DNA	1 µL	-
Total	10 µL	9 µL × 17*

*If 14 BC$_1$F$_1$ plants need to be checked, a master mixture for a total of 17 reactions (14 BC$_1$F$_1$ + two parents + one addition for reserve) should be prepared.

2. Add 9 µL of the mixture to 0.25-mL microtubes, 96-well plate, or 384-well plate for PCR.
3. Add 1 µL of 50 ng/µL DNA solution.
4. Seal the microtubes.
5. Mix the solution and spin.
6. Place them on a thermal cycler and initiate the reaction using the following in Table 7.
7. Take the PCR samples out of the thermal cycler.
8. Spin, add 1 µL of the 10× loading dye, mix well, and spin again. (*1)
9. Use PCR products for electrophoresis or preserve at −20°C for a few days until electrophoresis.

*1: Though "Quick Taq® HS DyeMix" solution contains the loading dye, additional 10× loading dye to PCR products will aid in the loading of the PCR samples into the wells of the gel more easily.

Table 7. Thermal conditions for PCR

Steps	Temperature	Time
1. Preheating, denaturation	96°C	2 min
2. Denaturation	94°C	1 min
3. Annealing	48°C	1 min
4. Extension	68°C	1 min
5. Repeat: 32 times of steps 2–4		
6. Preservation	10°C/4°C	-*

*Till taking the products out from the machine.

(Requirements)

· **Autoclaved dH$_2$O** (for PCR use only)

· **2× Quick Taq® HS DyeMix** (Toyobo Co., Ltd., preserved at −20°C)

· **Primers** (working solution containing 5 µM forward and 5 µM reverse primers, each is prepared from 100 µM forward and 100 µM reverse primer stock solutions and TE buffer)

· **50 ng/µL DNA solution** [test samples, parents, and a heterozygous (F$_1$) plant]

· **I.5-mL microtubes** (for mixture)

· **0.25-mL microtubes/96- (384) well plates** (for PCR reaction, together with caps or seals)

· **Loading dye (10×)** [0.25% bromophenol blue + 0.25% xylene cyanol + 40% (w/v) sucrose, autoclave]

· 96-well thermal cycler/384-well thermal cycler

2-5. Acrylamide gel electrophoresis

Here, we describe a slab gel electrophoresis unit for 16-cm^2 acrylamide gel plates (Nihon Eido, Co.). In JIRCAS, two electrophoresis units with four gels (NA-1114) and six gels (NA-1116) are used (Yamanaka et al., 2006, 2007a, 2007b) (image 15). These units are also used in Embrapa-Soja (Brazil), Nikkei-Cetapar (Paraguay), IPTA (Paraguay), INTA (Argentina), INIA (Uruguay) and BSMRAU (Bangladesh).

Instead of ethidium bromide (EtBr), the use of less harmful chemicals such as GelRed^{TM} (Biotium, Inc.) is recommended for gel staining, although EtBr is one of the most cost-effective chemicals for this purpose. This manual provides protocols not only for EtBr but also for GelRed^{TM} used in JIRCAS. The detection system (to visualize the bands of PCR products) used for GelRed^{TM} is the same as that used for EtBr. If staining chemicals other than EtBr/GelRed^{TM} are used, you must check whether your detection system is appropriate.

For visualization of amplified products, various gel scanners could be used. Such gel scanners are useful for photographing multiple gels (up to 9 gels) with high image quality. In JIRCAS, "Typhoon 9410 (GE Healthcare)" and "Pharos FX^{TM} (Bio-Rad)" are used.

Image 15: NA-1116 (Left) and NA-1114(Right)

(Protocol)

1. Wipe the glass plates and combs with 99.5% ethanol.
2. Assemble the glass plates with silicon packing and clips.
3. Prepare the "running gel" solution using a conical flask with a stirrer (see Table 8).
4. Pour the running gel solution (approximately 16 mL per unit) between a pair of glass plates.
5. Pour dH$_2$O over the running gel solution to cover it to initiate gel polymerization.
6. After completion of polymerization, completely remove water using filter papers.
7. Prepare the "stacking gel" solution using a 50-mL (or 15-mL) Falcon® tube (see Table 8).
8. Pour the stacking gel solution (approximately 3 mL per unit) between a pair of glass plates.
9. Insert the combs between the glass plates.
10. After completion of polymerization, take out the comb from the gel in between the glass plates under flowing water (*1).

11. Wash the wells with water using a hypodermic syringe to remove any liquid stacking gel solution (*2).

12. Remove the silicon packing and clips.

13. Set up the electrophoresis unit and pour Tris-glycine buffer into the unit (*3).

14. Load PCR products and DNA size marker into the wells (*4).

15. Initiate electrophoresis (*5).

16. Stop electrophoresis 0–30 min after the Xylene cyanol band has run off the gel.

17. Take the gels out from the glass plates (*6).

18. Stain the gel with the EtBr solution (10 µL/200 mL dH$_2$O) for 10 min (*7).

19. Visualize DNA bands under a UV transilluminator.

20. Photograph the gel.

21. Assign genotypes to soybean plants.

Table 8. Running and stacking gel composition.

Running gel	For 1 gel	2	3	4	5	6
30% Acrylamide stock solution	10.0 mL	20.0 mL	30.0 mL	40.1 mL	50.1 mL	60.1 mL
dH$_2$O	3.7 mL	7.4 mL	11.1 mL	14.9 mL	18.6 mL	22.3 mL
1.5 M Tris-HCl (pH 8.8)	4.7 mL	9.5 mL	14.2 mL	18.9 mL	23.6 mL	28.4 mL
10% APS	71.8 µL	143.6 µL	215.3 µL	287.1 µL	358.9 µL	430.7 µL
TEMED	17.8 µL	35.6 µL	53.3 µL	71.1 µL	88.9 µL	106.7 µL
Total	18.5 mL	37.1 mL	55.6 mL	74.2 mL	92.7 mL	111.2 mL

Stacking gel	For 1 gel	2	3	4	5	6
30% Acrylamide stock solution	0.7 mL	1.4 mL	2.0 mL	2.7 mL	3.4 mL	4.1 mL
dH$_2$O	2.3 mL	4.6 mL	6.9 mL	9.2 mL	11.5 mL	13.8 mL
0.5 M Tris-HCl (pH 6.8)	1.1 mL	2.2 mL	3.2 mL	4.3 mL	5.4 mL	6.5 mL
10% APS	33.8 µL	67.5 µL	101.3 µL	135.0 µL	168.8 µL	202.5 µL
TEMED	4.5 µL	9.0 µL	13.5 µL	18.0 µL	22.5 µL	27.0 µL
Total	4.1 mL	8.2 mL	12.3 mL	16.4 mL	20.4 mL	24.5 mL

*1: Remove the combs as soon as detection of well formation.

*2: Completely remove any liquid stacking gel solution.

*3: Running buffer could be used repeatedly (generally once or twice); however, multiple uses prolong the duration of electrophoresis.

*4: DNA size markers are used as references to calculate the sizes of polymorphic bands for marker-assisted selection and to identify any problems in electrophoresis in case clear amplified bands are not observed.

*5: Perform electrophoresis at 300 V for approximately 5–6 h. Overnight electrophoresis at a lower voltage (50–60 V) is also possible. For overnight electrophoresis, the voltage could be set to 300 V for 15 min at first, then changed to 50–60 V in the night and changed again to 300 V in the morning to complete electrophoresis.

*6: Confirm the direction (the front and the back, and right and left) of gel

*7: For the "GelRed" system, see the following additional protocol:

(Protocol for the GelRed^{TM} staining system)

*Preparation of the "GelRed^{TM}" staining solution*
1. Mix 67.5 µL of GelRed^{TM} in 450 mL of dH$_2$O.
2. Use this solution approximately seven times for three to six gels (for Pharos FX^{TM} scanner).
3. If the signal well could not be seen, increase the GelRed^{TM} concentration or decrease the number of times the solution is used.

Protocol
1. Transfer gels to the GelRed^{TM} staining solution.
2. Shake it gently on a shaker for 1 h at room temperature.
3. Transfer gels to dH$_2$O for scanning.
4. Scan the gels using a scanner (Typhoon or Pharos FX^{TM}) or photograph under a UV transilluminator.

(Requirements)

· **1.5 M Tris-HCl (pH = 8.8)** (dissolve 90.825 g of Tris in 400 mL of dH$_2$O, adjust pH to 8.8 with HCl, make the volume to 500 mL by adding dH$_2$O, autoclave)

· **0.5 M Tris-HCl (pH = 6.8)** (dissolve 30.275 g of Tris in 400 mL of dH$_2$O, adjust pH to 6.8 with HCl, make the volume to 500 mL by adding dH$_2$O, autoclave)

· **30% acrylamide stock solution** (dissolve 150 g of acrylamide monomer + 4 g of N,N'-methylenebisacrylamide in 500 mL of dH$_2$O, filter through a 0.4–0.8-µm filter, preserve at 4°C)

· **Autoclaved dH$_2$O** (for dilution, 500 mL, autoclave)

· **10% APS** (ammonium peroxodisulfate, dissolve 100 mg in 1 mL of autoclaved dH$_2$O, preserve at −20°C after preparation)

· **TEMED** (N,N,N,N-tetramethyl ethylenediamine, 4°C)

· **Tris-glycine buffer** (prepare by diluting 10× Tris-glycine with d-H$_2$O)

· **DNA size marker** (for SSR markers, a 20-bp ladder marker/PhiX-*Hae*III digest marker is used) (see image for checking its banding pattern and size in acrylamide gel)

Image: Bands of DNA size markers
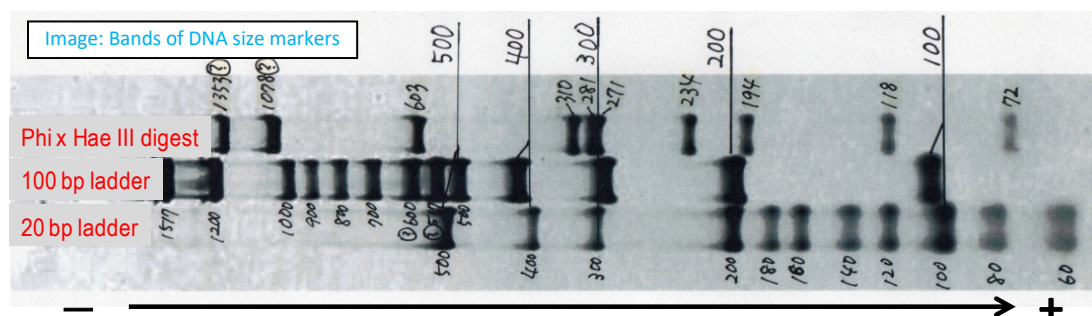
· **EtBr solution** (15 μL/300 mL of autoclaved dH$_2$O, 4°C)/**GelRed^{TM} solution** (at room temperature)

· **99.5% ethanol** (for cleaning the glass plates)

· **10× Tris-glycine** (30.3 g of Tris + 144.1 g of glycine for 1 L, autoclave)

· Acrylamide gel electrophoresis unit (Nihon Eido Co., NA-1114 or NA-1116)

· Magnetic stirrer

· Measuring pipette/graduated cylinder (to measure the gel solution)

· Conical flask

· Filter paper (cut into small pieces)

· Hypodermic syringe

· Electric power supply

· Spatula (for weighing small amounts of chemical powder and for separating the gel from the glass plates)

· UV transilluminator/gel scanner

· Camera (with a filter for UV)

2-6. Data analysis

Considering the recombination between SSR markers and *Rpp*, two polymorphic markers that closely locate and sandwich *Rpp* should be chosen to determine whether backcrossed plants carry the targeted resistance alleles for *Rpp*. When a recurrent variety is crossed with F_1 (or backcrossed plants), a ratio of 1:1 indicates that the progeny is heterozygous or homozygous for the recurrent parent allele.

Figure 8 shows an example of marker-assisted selection (MAS) in BC_1F_1 plants. If BC_1F_1 plants that are heterozygous for one SSR marker at the one flanking genomic location of *Rpp4* are also heterozygous for the other marker at the opposite flanking genomic location of the same gene (Silva et al., 2008; Garcia et al., 2008; Yamanaka et al., 2008; Lemos et al., 2011)*,* the progeny could be considered as carrying the resistance allele of the resistance gene in a heterozygous state and could be used for the next backcross. Currently available resistant soybean materials for MAS include *Rpp*-pyramided lines developed in JIRCAS (Table 9) and soybean varieties carrying each of *Rpp1–6* (Table 10).

As reference, genetic linkage maps around *Rpp* regions are shown in Figures 9 a-d. The maps will aid in selecting markers for your breeding material. Table 11 is the list of markers our group is using for MAS. These markers were selected based on the maps (Figures 9 a-d).
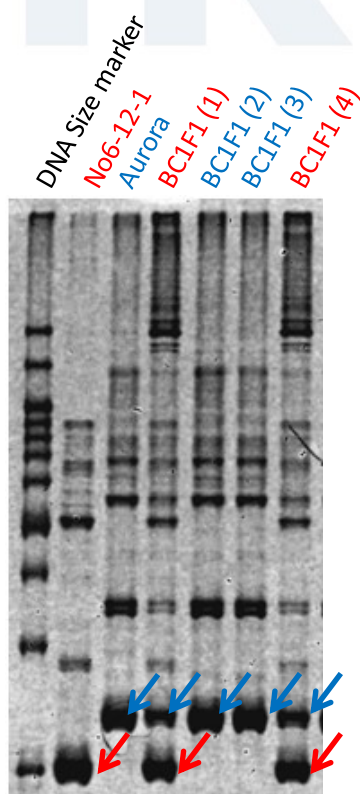
Figure 8. Gel image of PCR products by SSR marker, Satt288 which is linked to *Rpp4*. Donor parent, 'No6-12-1' carries resistant allele of *Rpp4* but recurrent parent, 'Aurora' doesn't have it. Among 4 BC_1F_1 plants, BC_1F_1 (1) and (4) are heterozygous for Satt288 locus but (2) and (3) are homozygous to recurrent parent.

PCR band derived from 'Aurora'

PCR band derived from 'No6-12-1'

Table 9. *Rpp*-pyramided lines developed by JIRCAS till 2019[a]. All lines listed here are considered to be pure line since they have reached to the generation of F_8 by SSD.

Name	*Rpp*	Ovule parent A	Pollen parent
Two genes			
Mo84-6	*Rpp1 + Rpp2*	PI 200492	An76-1
Py4-4-12-3	*Rpp1 + Rpp3*	Mo84-6	Hyuuga
Mo83-7	*Rpp1 + Rpp4*	PI 200492	An76-1
Py7-1-82	*Rpp1-b + Rpp2*	PI587880A	No12-1-A
Py5-2-24-13	*Rpp1-b + Rpp4*	No6-12-B	PI587880A
Py7-1-47	*Rpp1-b + Rpp5*	PI587880A	No12-1-A
Oy7-5-9	*Rpp2 + Rpp3*	An76-1	Hyuuga
An76-1	*Rpp2 + Rpp4*	BRS 184 × PI 230970	BRS 184 × PI 459025
No12-1-A	*Rpp2 + Rpp5*	An76-1	Kinoshita
Oy15-3	*Rpp3 + Rpp4*	An76-1	Hyuuga
Py8-1-15-11	*Rpp3 + Rpp5*	No12-1-A	Hyuuga
No6-12-B	*Rpp4 + Rpp5*	An76-1	Kinoshita
Three genes			
Py4-4-5	*Rpp1 + Rpp2 + Rpp3*	Mo84-6	Hyuuga
Mo42-1	*Rpp1 + Rpp2 + Rpp4*	PI 200492	An76-1
Py7-1-8-5	*Rpp1-b + Rpp2 + Rpp5*	PI587880A	No12-1-A
Py5-2-31-3	*Rpp1-b + Rpp4 + Rpp5*	No6-12-B	PI587880A
Oy49-4	*Rpp2 + Rpp3 + Rpp4*	An76-1	Hyuuga
Py8-1-15-6	*Rpp2 + Rpp3 + Rpp5*	No12-1-A	Hyuuga
No6-12-1	*Rpp2 + Rpp4 + Rpp5*	An76-1	Kinoshita
Py6-1-17	*Rpp3+ Rpp4 + Rpp5*	Hyuuga	No6-12-B
Four genes			
Py1-1-45	*Rpp1 + Rpp2 + Rpp3 + Rpp4*	Mo42-1	Oy49-4
Py2-3-39	*Rpp1 + Rpp2 + Rpp4 + Rpp5*	No6-12-1	Mo42-1
Py3-1-31	*Rpp2 + Rpp3 + Rpp4 + Rpp5*	Oy49-4	No6-12-1

[a] These *Rpp*-pyramided lines can be distributed for the research use by concluding the Material Transfer Agreement (MTA) like the differential variety set shown in Table 1.

Table 10. Resistant varieties to ASR.

Name of line	PI number	*Rpp* [a]	Origin	Dif. No. [b]	Reference
Komata	PI 200492 [c]	*Rpp1*	Japan	RDV1	Hyten et al. (2007)
Bai Dou	PI 587886	*Rpp1*	China	RDV2	Ray et al. (2009)
Xiao Jin Huang	(Not assigned)	*Rpp1*	China	-	Yamanaka et al. (2015a)
Himeshirazu	PI 594177	*Rpp1*	Japan	-	Yamanaka et al. (2015a)
Jia Bai Jia	PI 587855	*Rpp1-b*	China	-	Yamanaka et al. (2016)
Huang Dou	PI 587880A	*Rpp1-b*	China	RDV9	Ray et al. (2009)
Xiao Huang Dou	PI 587905	*Rpp1-b*	China	-	Hossain et al. (2015)
Zhao Ping Hei Dou	PI 594767A	*Rpp1-b*	China	RDV10	Hossain et al. (2015)
Min Hou Bai Sha Wan Dou	PI 594538A	*Rpp1-b*	China	-	Chakraborty et al. (2009)
Gou Jiao Huang Dou	PI 594760B	*Rpp1, rpp1*	China	-	Garcia et al. (2011)
Jin Yun Dou	PI 561356	*Rpp1*	China	-	Kim et al. (2012)
No. 3	PI 230970 [d]	*Rpp2*	Japan	RDV3	Silva et al. (2008)
Hougyoku	PI 224270	*rpp2*	Japan	-	Garcia et al. (2008)
Iyodaizu B	(Not assigned) [e]	*Rpp2*	Japan	-	Yamanaka et al. (2015a)
Ankur	PI 462312	*Rpp3*	India	RDV4	Hyten et al. (2009)
Akasaya	PI 416764	*Rpp3*	Japan	RDV5	Hossain et al. (2015)
Hyuuga [f]	PI 506764	*Rpp3* [g]	Japan	-	Monteiros et al. (2007)
FT-2 [h]	PI 628932	*Rpp3*	Brazil	-	Brogin et al. (2005)
MARIF 2740	PI 567099A	*Rpp3* [i]	Indonesia	-	Ray et al. (2011)
Bing Nan	PI 459025	*Rpp4*	China	RDV6	Silva et al. (2008)
Takema	PI 423972	*Rpp4-b*	Japan	-	King et al. (2017)
Shiranui	PI 200526	*Rpp5*	Japan	RDV7	Garcia et al. (2008)
Awashima Zairai	PI 200456	*rpp5*	Japan	-	Garcia et al. (2008)
Kinoshita	PI 200487	*Rpp5*	Japan	-	Garcia et al. (2008)
Orba [j]	PI 471904	*Rpp5*	Indonesia	-	Garcia et al. (2008)
MARIF 2767	PI 567102B	*Rpp6* [k]	Indonesia	RDV8	Li et al. (2012)
-	PI 605823	*Rpp7*	Vietnam	-	Childs et al. (2018)

Since their resistance genes are mapped with molecular markers, we can use them for marker-assisted breeding for ASR. Some information was obtained from the websites of United States Department of Agriculture (USDA) in USA and National Institute of Agrobiological Sciences (NIAS) in Japan.

[a] Resistance genes are represented as *Rpp* (resistant allele is dominant) or *rpp* (resistant allele is recessive), respectively

[b] Identification number of differential varieties (Table 1)

[c] PI 368039 (Tainung 4) is derived from PI 200492 and may share the same *Rpp1* allele (Boerma et al. 2011)

[d] PI 197182, PI 230971, and PI 417125 are reported to carry the same *Rpp2* as PI 230970 has (Laperuta et al. 2008)

[e] Several PIs are present for 'Iyodaizu'

[f] PI 506764 (Hyuuga) is reported to carry *Rpp5*, too (Kendrick et al. 2011)

[g] 'Hyuuga' is derived from 'Asomusume' × 'Akasaya' and may share the same *Rpp3* allele as 'Akasaya'

[h] ASR-resistant variety, 'BRSMS-Bacuri' is derived from 'FT-2' and may share the same resistant allele, *Rpp3*

[i] PI 567099A is reported to carry *Rpp3* or another gene close to *Rpp3* (Ray et al. 2011)

[j] ASR-resistant Taiwanese variety, 'TK 5 (Taita Kaohsiung 5) was developed from 'Orba'

[k] The other Indonesian variety, PI 567104B is reported to carry *Rpp6* or another gene close to it (Liu et al. 2016)

USDA website: https://npgsweb.ars-grin.gov/gringlobal/search.aspx
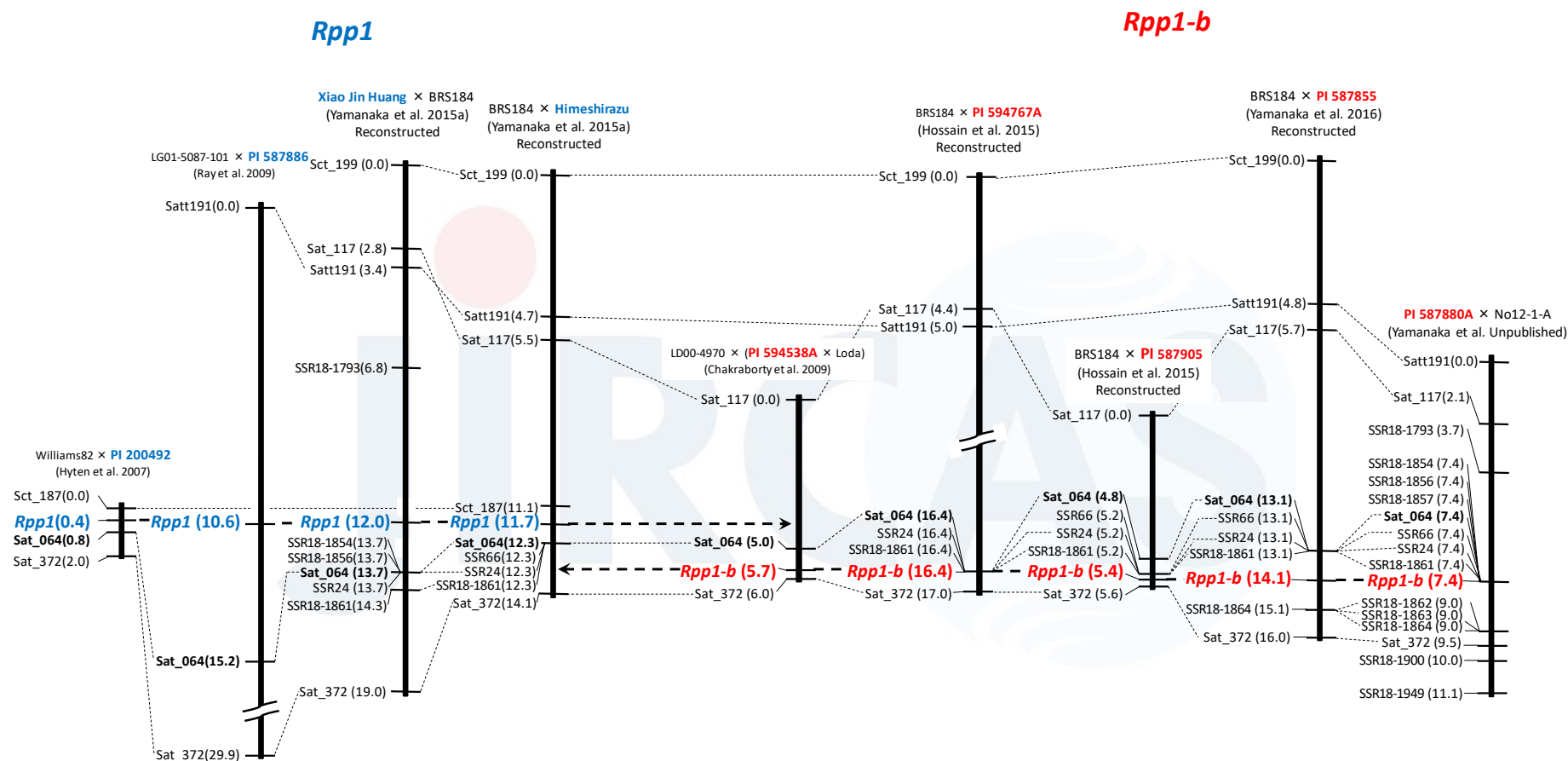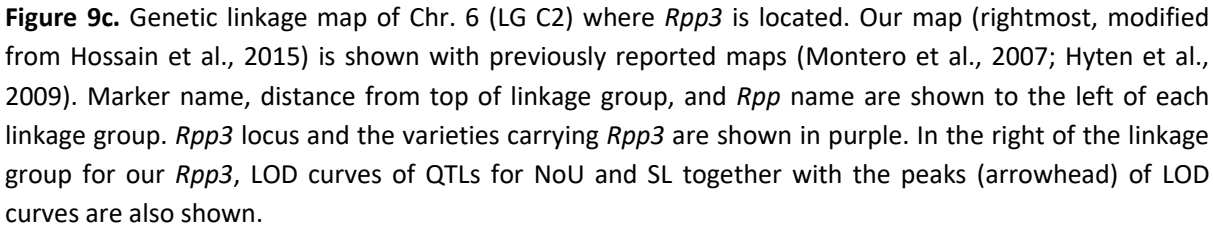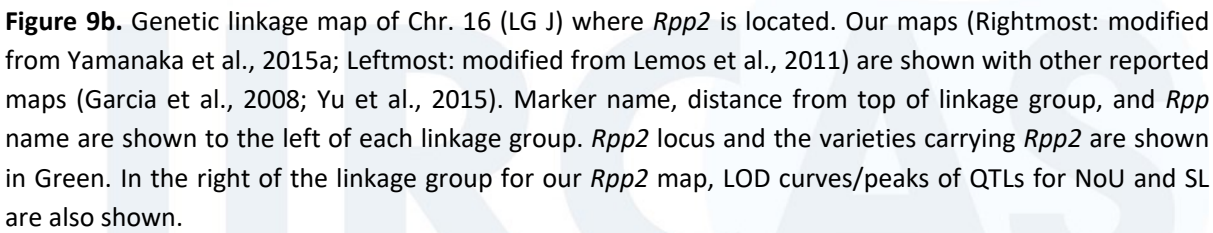NIAS website: http://www.gene.affrc.go.jp/index_en.php

Chromosome 18 (LG G)

Figure 9a. Genetic linkage map of chromosomes (Chr.) 18 (linkage group: LG G) where *Rpp1* and *Rpp1-b* are located. Our linkage maps (Hossain et al., 2015; Yamanaka et al., 2015a; 2016; unpublished) are shown with modifications together with previously reported maps (Chakraborty et al., 2009; Hyten et al., 2007; Ray et al., 2009). Marker name, distance from top of linkage group, and *Rpp* name are shown to the left of each linkage group. *Rpp1* locus and the varieties carrying *Rpp1* are shown in blue, and *Rpp1-b* locus and the varieties carrying *Rpp1-b* are shown in red, respectively.

Chromosome 16 (LG J)

Figure 9b. Genetic linkage map of Chr. 16 (LG J) where *Rpp2* is located. Our maps (Rightmost: modified from Yamanaka et al., 2015a; Leftmost: modified from Lemos et al., 2011) are shown with other reported maps (Garcia et al., 2008; Yu et al., 2015). Marker name, distance from top of linkage group, and *Rpp* name are shown to the left of each linkage group. *Rpp2* locus and the varieties carrying *Rpp2* are shown in Green. In the right of the linkage group for our *Rpp2* map, LOD curves/peaks of QTLs for NoU and SL are also shown.

Chromosome 6 (LG C2)

Figure 9c. Genetic linkage map of Chr. 6 (LG C2) where *Rpp3* is located. Our map (rightmost, modified from Hossain et al., 2015) is shown with previously reported maps (Montero et al., 2007; Hyten et al., 2009). Marker name, distance from top of linkage group, and *Rpp* name are shown to the left of each linkage group. *Rpp3* locus and the varieties carrying *Rpp3* are shown in purple. In the right of the linkage group for our *Rpp3*, LOD curves of QTLs for NoU and SL together with the peaks (arrowhead) of LOD curves are also shown.
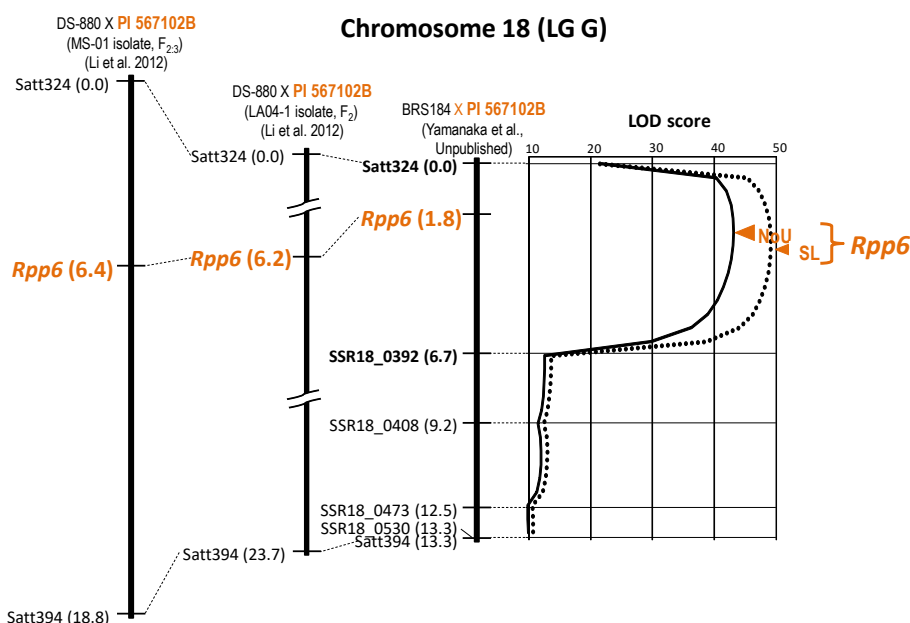
Chromosome 18 (LG G)

Figure 9d. Genetic linkage map of Chr. 18 (LG G) where *Rpp4* is located. Our map (right, Yamanaka et al. unpublished) is shown with a previously reported map (Meyer et al., 2009). Marker name, distance from top of linkage group, and *Rpp* name are shown to the left of each linkage group. *Rpp4* locus and the varieties carrying *Rpp4* are shown in brown. In the right of the linkage group for our *Rpp4*, LOD curves of QTLs for NoU and SL together with the peaks (arrowhead) of LOD curves are also shown.

Chromosome 3 (LG N)
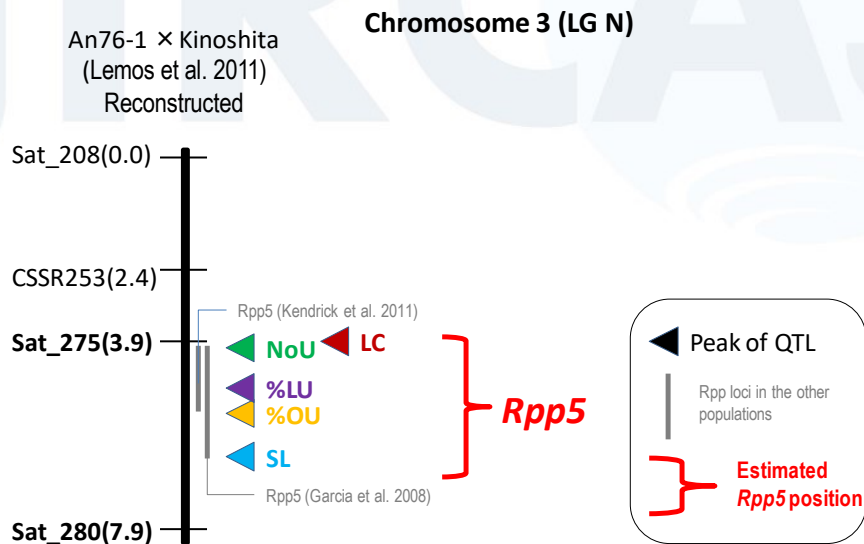
Figure 9e. Genetic linkage map of Chr. 3 (LG N), where *Rpp5* is located. This linkage map was constructed using an F_2 population (141 plants) derived from the cross between the An76-1 (*Rpp2* and *Rpp4*) cultivar and Kinoshita (*Rpp5*). This map was updated from our previous study (Lemos et al., 2011). Marker name and distance from top of the linkage group are shown to the left of linkage group. To the right of ~~each~~ linkage group, peaks of QTLs for five resistance characters by composite interval mapping and candidate *Rpp5* locus estimated using this F_2 population and in other studies are shown.

Figure 9f. Genetic linkage map of Chr. 18 (LG G) where *Rpp6* is located. Our map (rightmost, unpublished) is shown with previously reported maps (Li et al., 2012). Marker name, distance from top of linkage group, and *Rpp* name are shown to the left of each linkage group. *Rpp6* locus and the variety carrying *Rpp6* is shown in orange. In the right of the linkage group for our *Rpp6*, LOD curves of QTLs for NoU and SL together with the peaks (arrowhead) of LOD curves are also shown.

Table 11. SSR markers sandwiching *Rpp*s. Each 1 marker for upper (U) and lower (L) sides is used for MAS. In case 2 marker candidates are listed for one side, Marker_1 overrides Marker_2.

		Marker	Forward primer (5'–3')	Reverse primer (5'–3')
Rpp1	U_marker	**Sct_187**	CATGCTCCCATTCTCT	AACATTGGCTTTTTACTTAG
	L_marker_1	**Sat_064**	TAGCTTTATAATGAGTGTGATAGAT	GTATGCAAGGGATTAATTAAG
	L_marker_2	**SSR66**	AGATTGGGTGAGAACATAAG	GGAGAGCGTAAAAGAAATTC
Rpp1-b	U_marker_1	**SSR66**	AGATTGGGTGAGAACATAAG	GGAGAGCGTAAAAGAAATTC
	U_marker_2	**Sat_064**	TAGCTTTATAATGAGTGTGATAGAT	GTATGCAAGGGATTAATTAAG
	L_marker_1	**SSR18-1864**	TGAATGATATATGTTTTGCGAAGA	CAATAGAGCCGGATGGATGT
	L_marker_2	**Sat_372**	GCGTCTCGAGGTAATTATCTATTTATCTTTT	GCGAGTTTGGTAACATCGAGTATTGAT
Rpp2	U_marker_1	**Satt620**	GCGGGACCGATTAAATCAATGAAGTCA	GCGCATTTAATAAGGTTTACAAATTAGT
	U_marker_2	**SSR16_0895**	CATTCCCCAAGGCACATATT	TGGTTCTTTCTATGGCAGGG
	L_marker_1	**SSR16_0908**	TGCCCTACGATCTCTACGAAA	CGGACAAATGATCAGACCAA
	L_marker_2	**SSR16_0912**	TCGGGTGCTGCTAAAAATTC	AGTCAAAGGGGCTCTTGCTT
Rpp3	U_marker_1	**Sat_238**	GCGCTGAGACGTTTGTGATTGTTTGTAA	GCGGTGCATGCAATTCTAGTTAGATATT
	U_marker_2	**Satt460**	GCGCGATGGGCTGTTGGTTTTTAT	GCGCATACGATTTGGCATTTTTCTATTG
	L_marker_1	**SSR06_1521**	CAACCTCCCACCACTAAACTG	CCCTTTAGCCTTTTCTTTGC
	L_marker_2	**SSR06_1530**	CTTTTGAGTTTTGCCTCCCA	AGTCAAAGGGGCTCTTGCTT
Rpp4	U_marker_1	**Satt288**	GCGGGGGTGATTTAGTGTTTGACACCT	GCGCTTATAATTAAGAGCAAAAGAAG
	U_marker_2	**Sc21_2922**	GACTCAACTCATCCGTGC	GAACATATACAAAGGAAGCAT
	L_marker_1	**SSR18_1576**	TTTTGCACTCCCTGATGGAT	AACAGGGAATACATGCCAAA
	L_marker_2	**SSR18_1580**	TGCCAAGAAACTATAGGCCG	TGAGTTTCCCTGGTAGTGTGG
Rpp5	U_marker	**Sat_275**	GCGCGCTGGCAATTATTCAAAACTTAACGAT	GCGAAGGCTACGGTGAATAGAAAGGAC
	L_marker	**Sat_280**	GGCGGTGGATATGAAACTTCAATAACTACAA	GGCGGGCTTCAAATAATTACTATAAAACTACGG
Rpp6	U_marker	**Satt324**	GTTCCCAGGTCCCACCATCTATG	GCGTTTCTTTTATACCTTCAAG
	L_marker_1	**SSR18_0392**	AAAATTTTCACTCCGGTCATT	ACTGCAGGAGCAAGAACCTC
	L_marker_2	**SSR18_0408**	GCAACAGGATTTGGAGCTGT	TGTGGTCTCGGTAGCCTTCT

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Providing materials: Soybean rust pathogens described in this manual were derived from University of Tsukuba and Embrapa-Soja. The differential varieties except for the *Rpp*-pyramided line developed by JIRCAS were provided by Embrapa-Soja.

Abbreviations of institutes- **JIRCAS**: Japan International Research Center for Agricultural Sciences; **Embrapa**: Empresa Brasileira de Pesquisa Agropecuária;   **INTA**: Instituto Nacional de Tecnología Agropecuaria;   **IPTA-CICM**: Instituto Paraguayo de Tecnología Agraria- Centro de Investigación Capitán Miranda;   **INIA**: Instituto Nacional de Investigación Agropecuaria;   **BSMRAU**: Bangabandhu Sheikh Mujibur Rahman Agricultural University;   **UNNOBA**: Universidad Nacional del Noroeste de Buenos Aires,   **INIFAP**: Institute Nacional de Investigaciones Forestales, Agricolas y Pecuarias

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