Wheat Rust Diseases

Methods and Protocols

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Methods in Molecular Biology

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Wheat Rust Diseases

Methods and Protocols

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Rust disease, caused by strains of the fungus *Puccinia*, is a major threat to global wheat production and food security. For instance, the Ug99 stem rust that emerged in Eastern parts of Africa overcame the majority of genetic resistance present in commercial bread wheat. Along with the ability to evolve additional virulence, the pathogen spread rapidly to neighboring regions, and to date there are more than eight Ug99 lineage races spread across 13 countries. The emergence of highly virulent and temperature adaptable isolates of wheat stripe rust in parts of Europe, USA, Southeast Asia, Africa, and Australia has become an additional threat to worldwide wheat cultivation. To combat the threat posed by these rapidly evolving fungi, the global wheat rust research community came together through programs such as the Borlaug Global Rust Initiative and identified state-of-the-art techniques and tools for monitoring and preventing the spread of wheat rust diseases. Therefore, this Springer Protocol Series on “Wheat Rust Diseases” is a valuable collection of advanced tools that are currently used for characterization of rust, the host plant wheat, and the interactions between the two. Parts I and II of this volume consist of routinely used and advanced tools for characterizing rust pathogen where protocols for rust surveillance, genotyping, and molecular pathogenicity studies are discussed. Part III describes tools for genetic analysis of rust resistance while the subsequent Part IV covers new methods on rust resistance gene cloning which were based on next-generation sequencing and assembly tools. It also covers molecular assays for the functional analysis of cloned resistance genes. The last part (V) of the volume has a chapter on the isolation and screening of bacterial endophytes as biocontrol agents for rust disease management.

In summary, this volume covers a wide range of topics right from the rust pathogen to the genetics of the host plant wheat. Techniques covered in this volume are of value to both established and new generations of wheat rust researchers and to some extent to the whole of plant science and the microbial research community.

*Canberra, ACT, Australia*  
*Sambasivam Periyannan*
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Chapter 12

Genetic Analysis of Resistance to Wheat Rurs

Caixia Lan, Mandeep Singh Randhawa, Julio Huerta-Espino, and Ravi P. Singh

Abstract

Leaf rust, stripe rust, and stem rust pose a significant threat to global wheat production. Growing rust resistant cultivars is the most efficient and environment friendly method to reduce yield losses. Genetic analysis is undertaken to identify genes and study their roles in conferring rust resistance in a given wheat background. This chapter summarizes the protocol for genetic analysis of rust resistance at both seedling and adult plant stages. Additionally, it examines statistical analysis and related software to characterize quantitative trait loci (QTL) linked with rust resistance.

Key words Wheat, Leaf rust, Stem rust, Stripe Rust, Resistance, Identification, Mapping

1 Introduction

Rusts are the most important diseases of wheat which cause significant losses in the absence of proper chemical or genetic control measures. Leaf (brown) rust, stripe (yellow) rust, and stem (black) rust, which are caused by *Puccinia triticina* (*Pt*), *P. striiformis* f. sp. *tritici* (*Pst*), and *P. graminis* f. sp. *tritici* (*Pgt*), respectively, are the three important rust diseases of wheat. If any of these rusts reach an epidemic level, devastating yield losses can occur and wipe out as much as 100% of the crop in an individual field with susceptible varieties. Leaf rust has caused serious epidemics throughout wheat growing regions in Europe, North America, Oceania, Southern Africa, South America, and South Asia [1]. Recently, its incidence increased in China due to warmer temperatures favoring disease development and the widespread cultivation of susceptible varieties. Stripe rust is generally found in northern latitudes, highlands, and wheat-growing regions with cooler temperatures during early growth stages. However, recent large-scale epidemics are in warmer wheat-growing areas with the emergence of two closely related *Pst* strains with increased aggressiveness and tolerance to warm temperatures [2]. Highly virulent stem rust is also moving into new
areas with the emergency of a new \textit{Pgt} race, Ug99 (designated as TTKSK using the North American differentials set), which was detected in Uganda in 1998 and has virulence to most of the widely deployed race-specific resistance genes. It was recognized as a significant threat to global food security \cite{3}. There are many ways to manage these diseases; however, development and cultivation of resistant varieties are the most efficient control methods particularly for low-income smallholder farmers of developing countries.

Two types of rust resistance genes are often defined in wheat. Race-specific, or major, resistance genes which usually confer protection throughout the growth cycle are also referred to as “all-stage resistance” \cite{4}. These resistance genes cause hypersensitive reactions in the host when infected with rust isolates carrying corresponding avirulence alleles \cite{5}. In contrast, race nonspecific or minor genes confer adult plant resistance (APR) and are normally present together with other similar effect genes and are therefore associated with quantitative inheritance \cite{6,7}. Most cultivars with multiple genes for APR are susceptible at the seedling stage but later display resistance to a number of races, as its name (APR) indicates \cite{8}.

2 Materials

2.1 Phenotyping at Seedling Stage

1. Greenhouse: Light and temperature controlled according to the rust type.

2. Trays: Plastic trays filled with soil (a standard size which will allow us to evaluate 24 entries for yellow and stem rust or 48 entries in the case of leaf rust).

3. Soil: Steam a mixture of raw soil and peat moss (3:1) at 95 \degree C for 8 h, add fertilizer (for example, for 100 kg soil, add 22 g of Urea, 11 g of Scat, 5 g of KCl, 10 g of Magnesium sulfate, and 0.3 g of Ultra mix) after 24 h to the cooled down soil. We mix the prepared soil with peat moss (1:1) again before planting.

4. Inoculum: Dried urediniospores are usually kept at \(-70 \degree C\). Before inoculation, urediniospores of leaf rust and stem rust have to be placed in a water bath (heat shock) at 40 \degree C for 4 min, then placed in a humid chamber (40 \degree C, at least 4 h) to gain moisture, whereas stripe rust urediniospores can be put in the humid chamber (40 \degree C, at least 4 h) directly.

5. Mineral oil: Soltrol 170. Specialized inoculators have been designed to use compressed air to spray spore suspensions in mineral oil with fine droplet sizes \cite{9}.

6. Chamber: High humidity is generated by humidity chambers, which can be either permanent or temporary in design; the temperature can be controlled.
7. Several gelatin capsules or small glass bottles used to keep urediniospores of purified rust pathotypes in ultrafreezer.
8. Test material: Seeds of wheat varieties, advanced breeding lines, recombinant inbred lines, and a set of differentials carrying known resistance genes.

2.2 Phenotyping at Adult Plant Stage
1. Seeds: Wheat varieties, advanced breeding lines, recombinant inbred lines, and a set of differentials carrying known resistance genes.
2. Field: The experiment area should be in the “hot spot” of disease.
3. Spreaders: Mixture of susceptible varieties or lines with specific known resistance genes for specific disease.
4. Inoculum: Urediniospores can be mixed with carrier talcum powder or paraffinic mineral oil such as Soltrol. Urediniospores are very hydrophobic, so they do not mix readily with water; however, water-based urediniospore suspensions can be injected into elongating wheat stems to infect plants in the field without the need for exogenous moisture. Suspensions of urediniospores in mineral oil can be efficiently applied to plants using sprayers of various kinds. Typically, handheld sprayers are used [10, 11].

2.3 Genotyping
1. Genomic DNA of each line.
2. SSR markers and KASP assays.
3. SNP and GBS genotype platforms.

2.4 Softwares for Analysis
2. Kyazma B.V. software from Wageningen University [13].

3 Methods

3.1 Phenotyping at Seedling Stage

3.1.1 Sowing
For phenotyping of wheat varieties, about 8–10 seeds of advanced breeding lines or recombinant inbred lines are sown as hills in a tray or pot. Spacing of hills varies depending on the expected growth of the seedlings and number of days required before notes to be taken.
If the objective is to screen segregating populations, then the number of plants per population or line is higher and depends on the number of expected resistance genes. Simultaneously, a set of differentials carrying known resistance genes is also sown to determine the race use in the study and the expression of resistance genes. Differential sets are available as near-isogenic lines, single resistance genes carrying lines, or varieties with known or temporarily designated genes (see Note 1). The trays are placed in a greenhouse room or growth chamber with good light and optimum temperatures (15–25 °C), allowing good seedling growth and vigor. Seedlings are watered once every 2 days using a handheld water sprinkler. A single dose (4 g/L) of Nitrogen fertilizer (Ultra-sol) is applied to 7-day-old seedlings. About 9- to 12-day-old seedlings with their second leaf one-third to halfway expanded is the most common stage for carrying out inoculation.

3.1.2 Inoculation

Nine- to twelve-day-old seedlings (two-leaf stage) are inoculated by using an atomizer to spray urediniospores of purified rust pathotypes suspended in light-weight mineral oil, e.g., Soltrol 170, at concentrations of 2–5 mg spores/mL or by observing the light brown or yellow color of the suspension. For stripe rust, the trays/pots carrying inoculated seedlings are subsequently placed in a mist chamber in trays filled with water, covered with plastic hoods and incubated at 7–9 °C for 24 h. However, for leaf and stem rust, trays/pots are placed in chambers at 18–22 °C and subjected to continuous mist produced by ultrasonic or other devices (see Note 2). The next day, seedlings are moved into a greenhouse maintained at 18–25 °C for leaf rust, 10–18 °C for stripe rust, and 18–28 °C for stem rust phenotyping. Disease scoring is conducted 10–14 days after inoculation; leaf rust about 10 days after inoculation, whereas for the other two rusts it is conducted for about 14 days.

3.1.3 Scoring

Leaf rust and stem rust infection types on wheat seedlings are recorded after 10 and 14 days post-inoculation, respectively, and are usually based on a 0–4 scale as described in Roelfs et al. [11], where “0” = no visible symptoms; “;” = only necrotic/chlorotic flecks without any uredinia; “1” = small uredinia surrounded by necrosis; “2” = small to medium uredinia surrounded by chlorosis or necrosis; “3” = medium-sized uredinia without chlorosis or necrosis; “4” = large-sized uredinia without chlorosis or necrosis; “X” = random distribution of variable-sized uredinia; and “+” and “−” were used when uredinia were somewhat larger or smaller than normal for the infection types (ITs). Seedling ITs of 0, ;, 1, 2, and X are generally considered as resistant, whereas 3 and 4 are susceptible.

For stripe rust the infection types are recorded about 14 days post-inoculation using a 0–9 scale [17], where “0” = no visible infection; “1” = necrotic/chlorotic flecks without sporulation;
“2” = necrotic/chlorotic stripes without sporulation; “3” = necrotic/chlorotic stripes with trace sporulation; “4” = necrotic/chlorotic stripes with light sporulation; “5” = necrotic/chlorotic stripes with intermediate sporulation; “6” = chlorotic stripes with moderate sporulation; “7” = stripes without chlorosis or necrosis and with moderate sporulation; “8” = stripes without chlorosis or necrosis and with sufficient sporulation; and “9” = stripes without chlorosis or necrosis and abundant sporulation. ITs of 0–5, 6, and 7–9 are categorized as resistant, intermediate, and susceptible, respectively.

### 3.2 Phenotyping at Adult Plant Stage

Although phenotyping of plants at post-seedling growth stages can be conducted in the greenhouse using the methods described for seedlings, it is more common to conduct adult plant studies in field trials where plants are exposed to multicycling disease progression under natural conditions. These tests are important to determine the effectiveness of race-specific as well as adult-plant resistance genes in reducing the disease severity and crop losses.

#### 3.2.1 Field Trials Layout

For phenotyping varieties, advanced breeding lines and recombinant inbred lines, the size of field plots can vary from hills to short rows depending on the resources and objective. At CIMMYT we use paired rows plots, i.e., two short rows of 0.7 m length, 30 cm apart sown on top of 80 cm wide raised beds. This allows 50 cm spacing between the rows of two different lines. If planting is done on flat land, furrows are opened 30 cm apart; two furrows are used for planting, and a furrow is left unplanted between each plot. We also leave a 0.3 m alleyway and sow a hill plot using 8–10 seeds of susceptible spreader on one side of the plot in the alleyway. This allows each plot to have a spreader adjacent to it for a uniform disease buildup and spread. About 60–80 seeds (about 3–5 g) of each line are planted. If hill plots are sown, then using 8–10 seeds of each line is recommended. The experimental field should also be surrounded with two rows of spreader plots. The spreader plots are often a mixture of susceptible varieties depending on the objective, spreaders with differential susceptibility can also be used, e.g., varieties susceptible to leaf rust, but resistant to stripe rust and vice versa, or varieties susceptible to a specific pathotype but resistant to the other. For the screening of breeding materials, a mixture of susceptible varieties allowing the establishment and multiplication of the most important and relevant pathotypes is recommended for the field nurseries.

#### 3.2.2 Field Inoculation

The most efficient method of inoculation is spraying 4–8-week-old spreaders with rust urediniospores suspended in Soltrol 170, similar to inoculations in the greenhouse. Stripe rust inoculations are carried out earlier in the season and should coincide with cool night temperatures with good dew formation or wet conditions.
Leaf rust and stem rust inoculations can be done at later growth stages but should be completed by heading stage. Dew formation, or availability of free moisture, on leaves and stems following the inoculation is critical for establishing infections, however, temperature can also play role, especially for stripe rust. If Soltrol or another similar lightweight mineral oil is not available, urediniospores can be suspended in water with a few drops of Tween 20 to break the surface tension, and can be used for spraying or injecting in plants through a syringe as described in Roelfs et al. [11]. Syringe inoculation can be done throughout the day, and is the safest method to establish disease. However, new suspensions are recommended every 3–4 h. The spore–water–Tween 20 suspension should be made fresh and sprayed late in the evening so that water does not evaporate and the process coincides with dew formation. Another way to inoculate the nursery is to mix urediniospores with talcum powder and dust the spreaders using a handheld duster or a cloth bag. Finally, pots of spreader seedling inoculated in greenhouse can also be placed at regular intervals in the field to establish rust diseases on field spreaders. Depending on the objective, urediniospores of different pathotypes of the same rust fungi or different rust fungi can be mixed (see Note 3).

3.2.3 Disease Evaluation

Disease evaluation in the field involves two components namely, disease severity visually estimated by percentage using the modified Cobb’s Scale [18], and host response to infection as described in Roelfs et al. [11]. Host responses commonly used are: R (resistant)—small uredinia (or stripes) with chlorosis or necrosis and little sporulation; MR (moderately resistant)—medium sized uredinia (or stripes) with chlorosis or necrosis and some sporulation; MS (moderately susceptible)—medium to large sized uredinia (or stripes) with slight or no chlorosis and without necrosis and with moderate sporulation; and S (susceptible)—large sized uredinia (or stripes) without chlorosis or necrosis and with profuse sporulation. Disease severity and reaction can be recorded at the first appearance of the disease, but it is common to wait until the susceptible checks, parents or some lines in mapping population have close to a 60–80% disease severity. Repeated disease data can be recorded at weekly to 10-day intervals until plants reach close to physiological maturity. For breeding materials it is common to record one disease data at an appropriate stage of disease development when data is more relevant for the selection of resistant materials.

3.3 Methods for Genotyping

With the advancement of genotyping technologies over the past two decades, molecular markers have been widely used in the mapping and identification of resistance genes to wheat rusts. Several molecular technologies have been used, including restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR), diversity arrays technology (DArT),
3.4 Single Gene Analysis

3.4.1 Mendelian Analysis of Segregation Ratios in Different Generations

Many genetic analyses have been conducted historically using the segregation ratios in different segregating generations. Using Mendelian genetics, the observed segregation ratio is tested with the expected segregation ratio to determine the number of segregating genes and establish independence or linkage if more than one gene involved. Monosomic analysis was then done to identify chromosome location, followed by telocentric mapping to establish chromosome arm location. Reliable phenotyping in the seedling or adult plant stage played a crucial role in these studies.

When inheritance of resistance is complex, i.e., based on minor genes with additive effects, also known as quantitative trait loci (QTL), inheritance studies are more reliable when conducted using mapping populations of recombinant inbred lines in F5 or F6 generations, or double-haploid populations. Phenotyping is more accurate in these populations due to high homozygosity. As a result, most of the remaining genetic effects are additive. The number of rust resistance genes in the RIL population can be estimated using Mendelian segregation analysis [28, 29], where
the observed frequencies for each phenotypic category is tested against the expected frequencies (Table 1) for different numbers of additive genes using Chi-squared ($\chi^2$) analysis.

Moreover, the minimum number of resistance genes can also be estimated using the quantitative approach described by Wright [30] as $n = (GR)^2/4.57(\sigma^2_g)$, where $GR$ (genotypic range) = phenotype range $\times b^2$ (narrow-sense heritability), $\sigma^2_g$ = genetic variance of the F5 RILs in the present population, $b^2 = \sigma^2_g/\sigma^2_g + \sigma^2_e$. The analysis of variance (ANOVA) is carried out using SAS 9.2 (SAS Institute, Cary, NC) with the final disease severity (FDS) in each environment.

### Table 1

<table>
<thead>
<tr>
<th>Number of genes</th>
<th>Generation</th>
<th>Lines (%)</th>
<th>HTPR$^a$</th>
<th>HTPS$^b$</th>
<th>Other$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F5</td>
<td>43.75</td>
<td>43.75</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F6</td>
<td>46.875</td>
<td>46.875</td>
<td>6.25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F5</td>
<td>19.1</td>
<td>19.1</td>
<td>61.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F6</td>
<td>22.0</td>
<td>22.0</td>
<td>56.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F5</td>
<td>8.4</td>
<td>8.4</td>
<td>83.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F6</td>
<td>10.3</td>
<td>10.3</td>
<td>79.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>F5</td>
<td>3.7</td>
<td>3.7</td>
<td>92.6</td>
<td></td>
</tr>
<tr>
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<td>F6</td>
<td>4.8</td>
<td>4.8</td>
<td>90.4</td>
<td></td>
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<td>97.4</td>
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<td>5</td>
<td>F6</td>
<td>2.3</td>
<td>2.3</td>
<td>95.4</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ HTPR = Homozygous parental type resistant  
$^b$ HTPS = Homozygous parental type susceptible  
$^c$ Other = Lines with intermediate levels of disease severity

3.4.2 Single Gene Mapping Using Molecular Methods

The prerequisite for any molecular mapping is consideration of both phenotypic and genotypic data. Any relevant genotyping platforms can be used and the cost and accessibility often dictates the selection. It has become common to outsource genotyping as it is less expensive than any alternative. Chi-square ($\chi^2$) tests are performed to evaluate the goodness of the fit of observed segregation with expected genetic ratios and to detect marker–trait linkages. Recombination fractions are calculated using the software MapManager QTXb20 [12] or Joinmap 4.0 [13] and converted to centiMorgens using the Kosambi mapping function [31]. Logarithm of odds (LOD) scores of 3–4 is usually used to determine significance of genetic linkages. Linkage maps were graphically visualized with MapChart [14]. The workflows of MapManager QTXb20, Joinmap 4.0, and MapChart are listed in Figs. 1, 2, and 3, respectively.
3.5 Methods for QTL Analysis

Quantitative trait locus (QTL) mapping of biparental population is conducted on the basis of available linkage maps with whole wheat genome molecular markers and phenotypic data from multiple environments/locations. The linkage maps can be constructed by JoinMap software as mentioned above. QTL mapping using disease severity from each experiment can be carried out using inclusive composite interval mapping (ICIM) [15] and QTL Cartographer software [16]. The workflows of IciMapping 4.1 and QTL Cartographer are listed in Figs. 4 and 5, respectively.

4 Notes

1. The number of lines in differential sets varies between labs and rust diseases but often have about 50 lines for leaf rust, 30 lines for stripe rust, and 50 lines for stem rust.

2. For stem rust, it is also important to add light about 12 h after misting while seedlings are still wet and to let them dry slowly.
Fig. 2 Workflow of Joinmap 4.1 to construct linkage maps

Purchase and install Joinmap 4.1

Prepare the input file as described in the Manual

Open the software and assign a name to the ‘New Project’

‘Load Data’ and Click ‘Grouping (tree)’

Click ‘Options’ and select the ‘Calculation Options’

Click ‘Calculate’, Select the targeted group, click ‘Population’ and select ‘Create Groups using Grouping Tree’

Select the ‘Group’ on left side and make the linkage group with button ☐

Linkage maps of whole wheat genome

Fig. 3 Workflow of Mapchart to draw maps

Download and install MapChart

Prepare MCT file with linkage map and QTL information in a Text file

Open the software and ‘Open’ the file

Click ‘Chart’ to see the Fig and ‘Copy page’ to a PowerPoint file

Save the Fig as ‘.JPEG’ format
Download and install IciMapping 4.1

Prepare the input `.bip` file that includes genotype and phenotype of the biparental population

Open the software and give it a new ‘Project’ name

Open the prepared `.bip` file and load the data into software

Select the parameters and ‘Start’. The logarithm of odds (LOD) threshold to declare a QTL significant at $P = 0.05$ for each trait was determined based on 1,000 permutation tests. Stepwise regression is used to detect the percentages of phenotypic variance explained (PVE) by individual QTL and additive effects at the LOD peaks

The significant QTL result is present in the `.qic` file

---

Fig. 4 Workflow of Icimapping 4.1 to do QTL analysis

Download and install QTL Cartographer

Open the file and ‘Import’ data source from ‘QTL cartographer INP format (*.inp, *.imp)’, click ‘Next’ and select ‘Map File’ and ‘Cross data’ from Mapmanager software as mentioned in 3.3.2, then click ‘Finish’ or ‘Open’ data source of `.mcd` file from IcIMapping software as mentioned above

Select ‘Composite Interval Mapping’ in the Analysis section

Click ‘Go’, Set the Threshold value either LR=11.5 (= LOD 2.5) or by 1,000 permutations time at 0.05 significance level. Then Click ‘Start’

QTL result is listed in ‘EQTL’ file

---

Fig. 5 Workflow of QTL Cartographer to do QTL analysis
3. It is strongly recommended that single or a few pathotypes of known virulences of the same fungi should be used for genetic studies.

4. However, limited D genome markers, too many repeat markers at the same position, and confusing chromosome information from SNP markers in 90K SNP assays may affect widespread use in genetic analysis (Per. Comm. Jizeng Jia).

Acknowledgments

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References


5. Flor HH (1942) Inheritance of pathogenicity in Melampsora lini. Phytopathology 32:653–669


