Stable inheritance of RF2a transgene integration in transgenic IR64 rice plants and evaluation of their tolerance against Rice tungro virus

A Estiati*, D Astuti1, D Widyajayantie1 and S Nugroho1

1 Genomics and Crop Improvement Laboratory, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI) Jln. Raya Bogor Km.46 Cibinong 16911, Jawa Barat, Indonesia Phone: 62-21-8754587; Fax: 62-21-8754588

*Email: nugroho_amy@yahoo.com

Abstract. Rice tungro disease (RTD) is one of the devastating diseases in Asia, caused by simultaneous infection of Rice tungro bacilliform virus (RTBV) and Rice tungro spherical virus (RTSV). The spread of these viruses is difficult to control especially in areas with intensive rice planting. RF2a is a rice transcription vector, regulate the expression of RTBV promoter and is also important for rice development. The overexpression of RF2a in transgenic rice plants has been reported can reduce virus accumulation and leads to tolerance against tungro virus. Transformation of RF2a transgene into a commercial susceptible cultivar, IR64 had been conducted. Four trangenic rice lines at T1 and T2 generations have been obtained. Stable inheritance and expression of RF2a transgene in successive generations are an indication of the successful development of tungro-tolerant cultivars. Therefore, PCR analysis for RF2a transgene and bioassay on transgenic plants at T2 and T3 generations against tungro virus had been done. PCR analysis showed that the RF2a transgene which is indicated by the presence of hpt was stably inherited and several transgenic rice plants harboring the overexpressed RF2a are gaining tolerance to an Indonesian tungro Purwakarta isolate compared to the IR64 wild type under greenhouse conditions.

1. Introduction

Rice (Oryza sativa L.) as a staple food for people in Asian countries is threatened by a variety of factors including insects, fungi, bacteria and viruses. Among rice virus diseases, rice tungro disease (RTD) is the most devastating viral disease of rice in South and Southeast Asia [1, 2]. When rice tungro virus infects plants at early seedling stage, no yield will be obtained [3, 4]. The disease causes yield loss with approximately 1.5 billion US dollars worldwide each yr [3, 5]. In Indonesia, in 2012, the areas affected by RTD reached 6,441 ha and 151 ha of them were puso [6]. Tungro virus-infected rice plants exhibit symptoms such as stunting, yellowing or yellow-orange leaf discoloration and produce few tillers [7, 8].

RTD is caused by 2 viruses namely Rice tungro bacilliform virus (RTBV) and Rice tungro spherical virus (RTSV), both are transmitted from plant to plant by green leafhoppers (GLH; Nephotettix virescens Distant) as a vector of infection [5, 9, 10]. The endemic areas of tungro virus are small comparing to the total areas, however, the endemic areas become the sources of virus to spread the disease to another areas and develop into large-scale outbreaks [11].
Breeding of tungro-tolerant cultivars is a good approach to control RTD [12, 13]. RF2a is a rice transcription factor and plays an important role in rice development and virus replication as well. The overexpression of RF2a in infected transgenic rice lines cv.TP309 has proven to improve plant tolerance by reducing tungro virus accumulation [3].

Agrobacterium-mediated transformation of RF2a into a commercial susceptible cultivar, IR64 to enhance tolerance against tungro virus, had been conducted in our laboratory. The T-DNA of plasmid recombinant, pCAMBIA1305.1 contains RF2a and hygromycin phosphotransferase (hpt) genes and each gene driven by CaMV35S promoter [14]. Since hpt is closely linked to the RF2a, the existence of RF2a transgene in transgenic rice plants can be determined by the presence of hpt gene. Four transgenic rice lines with single copy of hpt insertion at sec (T1) and third (T2) generations have been developed. However, these 4 lines were still categorized as heterozygous lines for hpt [15].

Stable inheritance and expression of RF2a transgene in subsequent generations are an indication of the successful development of tungro-tolerant plants. Therefore, PCR analysis and bioassay against tungro virus on 4 transgenic rice lines at third (T3) and fourth (T4) generations had been carried out. In this article, we report the presence of the RF2a transgene which is indicated by the presence of hpt was stably inherited and the overexpression of RF2a in transgenic rice plants confer tolerance against an Indonesian tungro Purwakarta isolate compared to the original IR64.

2. Methods

2.1. Plant and tungro virus materials
IR64 transgenic rice lines at third (T3) and fourth (T4) generations i.e T3 IR64.2.2-12-1-1 (IRT1), T3 IR64.2-9-1 (IRT2), T3 IR64.6.2-26-4-1 (IRT3), T3 IR64.8.2-13-4-1 (IRT4) and IR64 cultivar as a wild type and susceptible check as well, were used in this experiment. Taichung Native1 (TN1) and Tukad Petanu (TP) were also included in bioassay study as control of susceptible and tolerant cultivars to tungro virus, respectively. Indonesian tungro Purwakarta isolate was used in bioassay studies was obtained from Indonesian Center for Rice Research (ICRR) -Sukamandi, West Java, Indonesia.

2.2. Selection of homozygous transgenic rice lines
The presence of RF2a transgene in transformed plants was identified by using the linked hpt marker gene as the target for amplification. Thirty plants from each transgenic rice line were subjected to PCR. The pCAMBIA1305.1 binary vector was used as a positive control. PCR analysis was performed with a pair of primers specific to hpt gene (hpt forward: 5'-GATTTGTGTACGCCCGACAGT-3’ and hpt reverse: 5’- CGCTGTTCTACAACCCGTCG -3’). These primers will amplify the hpt fragment with the expected amplification product of 600 bp [15].

Total genomic DNA was extracted from fresh leaf of transformed and non-transformed plants [16]. The PCR reactions contained 1 µL of 100 ng DNA, nuclease-free water, DreamTaq™ Green PCR Master Mix (2x) (Thermo Scientific), and 0.4 µM for each primer. PCR conditions for hpt gene was 3 min pre-denaturation at 95 °C, 1 min denaturation at 95 °C, 1 min annealing at 60 °C, 1 min extension at 72°C for 35 cycles, 7 min extension at 72 °C [15]. PCR was conducted using Biometra Thermocycler (AnalytikJena). The expected fragment size of PCR product for hpt gene was 600 bp and the amplicon was detected by loading the PCR product on a 1.2% agarose gel and visualized under UV light upon Ethidium Bromide (EtBr) staining. Chi-square test was performed for goodness-of-fit analysis using the expected Mendelian 3:1 or 1:1 ratios.

2.3. Bioassay study on homozygous transgenic IR64 rice lines harboring RF2a overexpression against rice tungro virus
Plant inoculation was conducted in a greenhouse of Indonesian Center for Rice Research (ICRR) in Sukamandi, West Java, Indonesia. Only homozygous transgenic rice lines were used in bioassay study. Non-transgenic IR64 and TN1 cultivars were used as susceptible checks, meanwhile TP was used as tolerant check. Prior to bioassay, to ensure that the rice plants to be subjected to virus inoculation were
transgenic plants, PCR analysis was performed using the same primers specific to hpt as described earlier.

Tungro virus inoculation method was conducted as described previously [15]. Seven days old healthy transgenic rice plants that had been verified by PCR for hpt and control rice plants were allowed to grow in plastic box and kept in gauze mesh cage in the glasshouse. Viruliferous GLH (vector that contain virus) were introduced into gauze mesh cage containing healthy transgenic and control rice plants for 24 hr. Observation was conducted 2 wk after infestation. Symptoms of each individual plant was scored visually according to Standard Evaluation System for rice [17] as follows: 1= no symptoms observed; 3= 1-10% height reduction, no distinct yellow to yellow orange leaf discoloration; 5= 11-30% height reduction, no distinct yellow to yellow orange leaf discoloration; 7= 31-50% height reduction, with distinct yellow to yellow orange leaf discoloration; 9= more than 50% height reduction with distinct yellow to yellow orange leaf discoloration.

3. Results and discussions

3.1. Selection of homozygous transgenic rice lines
Eleven is the minimum number of plants to show at least 1 unexpected phenotype appeared under 95% probability for a character controlled by a recessive allele. If all of the 11 plants tested showed phenotype from expected dominant allele, it is indicated that the population was originated from homozygous parent for the target gene [18]. Thus, in order to select homozygous transgenic rice lines, we used 30 plants from each transgenic rice line for PCR analysis.

![Figure 1](image_url)

**Figure 1.** Detection of hpt gene using primers specific to hpt in 4 transgenic rice lines at T2 and T3 generations. Each line consisted of 30 plants. The presence of RF2a transgene in transformed plants was identified by using the linked hpt marker gene as the target for amplification. M: 200 bp ladder marker; P: pCAMBIA 1305.1 harboring hpt gene; K+: positive control plant; W: dH2O as a replacement for DNA; 1-30: transgenic rice plants. (a) IRT1; (b) IRT2; (c) IRT3 and (d) IRT4 lines.
Table 1. Segregation analysis of hpt gene in four transgenic rice lines.

| Transgenic rice lines | Total plants tested | Segregation analysis | Df:1; α: 0.05 $\chi^2$:3.84 | Segregation ratio |
|-----------------------|---------------------|----------------------|----------------------------|------------------|
| IRT1                  | 30                  | 30+ hpt gene 0- hpt gene | Homozygot                  |                  |
| IRT2                  | 30                  | 30+ hpt gene 5- hpt gene | Heterozygout 3:1           |                  |
| IRT3                  | 30                  | 30+ hpt gene 0- hpt gene | Homozygot                  |                  |
| IRT4                  | 30                  | 30+ hpt gene 0- hpt gene | Homozygot                  |                  |

The presence of RF2a transgene in transformed plants was identified by using the linked hpt marker gene as a target for amplification with the expected amplification product of 600 bp. PCR analysis on 4 transgenic rice lines (IRT1, IRT2, IRT3 and IRT4) showed that all plants tested from each of 3 transgenic rice lines i.e. IRT1, IRT3, IRT4, were able to amplify hpt with the same size as a fragment that can be amplified by pCAMBIA 1305.1 as a positive control. Meanwhile, 5 plants out of 30 plants tested from IRT2 rice line were unable to amplify hpt gene. These results indicated that 3 transgenic rice lines i.e. IRT1, IRT3 and IRT4 are homozygous, meanwhile 1 transgenic rice line namely IRT2 is still heterozygous for hpt gene which following Mendelian segregation ratio of 3:1 (Fig. 1 and Table 1). Thus, these 3 homozygous transgenic rice lines were then selected for bioassay study.

3.2. Bioassay study on homozygous transgenic IR64 rice lines harboring RF2a overexpression against rice tungro virus

Prior to bioassay, PCR analysis was performed to ensure that the rice plants to be subjected to virus inoculation were transgenic plants. PCR analysis was conducted using specific primers for hpt gene. The sequences of the primers were same as mentioned previously. Ten plants from each of 3 transgenic rice lines (IRT1, IRT3 and IRT4) were chosen and PCR analysis was conducted. The results showed that all plants tested were found harboring hpt (Fig. 2). Thus, these plants were chosen to be subjected to virus inoculation.

Figure 2. Detection of hpt gene using primers specific to hpt in 3 homozygous transgenic rice lines, namely IRT1 (a), IRT3 (b) and IRT4 (c) prior to bioassay study. All transgenic rice plants were able to amplify hpt gene. Thus, these plants were chosen to be subjected to virus inoculation. P: pCAMBIA 1305.1 harboring hpt gene; K: negative control plant; W: dH2O as a replacement for DNA; 1-10: transgenic rice plants. (a) IRT1; (b) IRT3 and (c) IRT4 lines.
Figure 3. The appearance of transgenic rice lines (IRT1, IRT3 and IRT4) and control rice plants following inoculation with tungro virus at 78 d after inoculation. (a) Plants with score of 1; (b) plants with score of 9. TP as resistant check cultivar had score of 1; non-transgenic IR64 and TN1 as susceptible cultivars showed severe RTD symptoms with score of 9.

Ten plants from each homozygous transgenic rice line at T₃ generation and control plants were allowed to grow in plastic box and kept in gauze mesh cage in the greenhouse. Viruliferous GLH were introduced into gauze mesh cage containing healthy transgenic rice and control plants for 24 hr. The observation was conducted at 2 WAI. Using the scoring system provided by IRRI [13], most transgenic rice plants were clustered under the score of 7 and 9, followed by a score of 1. Susceptible cultivars (IR64 and TN1) had score of 9 and tolerant check cultivar (TP) had score of 1.

Transgenic rice plants and control of susceptible cultivars with score of 9 showed severe RTD symptoms, i.e. stunted growth and yellow-orange colored leaves and even died. Meanwhile, transgenic
rice plants with score of 1 showed no RTD symptoms, similar to the level of tolerant cultivar, Tukad Petanu (Fig. 3).

The appearance of transgenic rice plants harboring RF2a overexpression following challenge inoculation with tungro virus still varied. This presumably due to the different levels of RF2a in transgenic plants. Transgenic plants with sufficient levels of RF2a may gain tolerance to RTD since the availability of RF2a can support plant development. In contrast, transgenic plants with insufficient levels of RF2a may exhibited severe RTD symptoms and higher levels of virus accumulation [3, 8]. To determine the levels of RTBV and RF2a transcripts in inoculated transgenic rice plants, qRT-PCR should be conducted [3, 15]. Furthermore, the transgenic plants that showed tolerance against the tungro virus with a score of 1 were allowed to grow to produce seeds for further studies.

4. Conclusion

Transgenic IR64 rice plants harboring RF2a overexpression have been developed. Successful application in the development of tungro virus-tolerant cultivars depend on the stable inheritance and expression of target gene in successive generations. By using the linked hpt marker gene as the target for amplification, PCR analysis on 3 homozygous transgenic rice lines at T3 generation exhibited that the RF2a transgene were stably inherited. Viral inoculation on 3 homozygous transgenic rice lines showed that several transgenic rice plants gained tolerance against RTD, similar to the control of tolerant cultivar, Tukad Petanu. On the contrary, the remaining transgenic plants exhibited RTD symptoms similar to susceptible check cultivars (IR64 and TN1) performance. These results are similar to the previous studies [15].

The different expression of transgenic rice plants against tungro virus although they were transformed with the same T-DNA construct was suggested due to the availability of RF2a in plants. The levels of RF2a play an important role in determining the level of tolerance [3, 15]. The levels of RF2a are inversely proportional to the accumulation of RTB. The higher availability of RF2a, reduced the RTBV accumulation which leads to tolerance [3]. Thus, it could be assumed that transgenic rice plants with a score of 1 have sufficient accumulation of RF2a to support plant development rather than virus replication, nevertheless, to estimate the levels of RF2a in tolerant plants, qRT-PCR should be conducted. To ensure that the RF2a transgene and the levels of RF2a are stable inherited in selected transgenis rice lines through generations, the inheritance and expression studies will be conducted at least until T3 generation of transgenic rice plants.

Acknowledgements

We thank Dr. Suprihanto, Celvia Roza and Oco Rumasa from Indonesian Center for Rice Research (ICRR)- Sukamandi, West Java, Indonesia for assisting in the bioassay against tungro virus in the greenhouse.

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