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***Journal of Scientific and Engineering Research*, 2017, 4(12):92-98**

Research Article

ISSN: 2394-2630
CODEN(USA): JSERBR

Pyramiding the Candidate Genes of Rice Bacterial Leaf Blight Resistance *xa5, Xa7* and *xa13* into the Elite Rice Variety

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Abstract Bacterial leaf blight disease caused by the gram negative bacterium *Xanthomonas oryzae pv.oryzae* and is ranked as the most important widespread disease of the major rice producing countries in the world. The objectives of this study were to identify the bacterial leaf blight tolerance the candidate genes *xa5* and *xa13* and design the markers namely *xa5add35* and *xa13add4* based on the databases of 36 genome sequencing of Vietnamese native rice cultivars and pyramided them into an elite Vietnamese variety. We have used P3 marker to detect *Xa7* among the 36 genome sequenced of rice lines. Based on the screening results of *xa5, Xa7* and *xa13,* Hom rau variety was used as the donor plant to introgress the resistant genes into elite cultivar, An dan 11. The individual plants of BC_1F_1, BC_2F_1 and BC_3F_1 have been examined to identify the individual plants carrying the *xa5, Xa7* and *xa13.* The results have found that 06 plants of BC_3F_1 carried the resistant genes *xa5, Xa7* and *xa13* in heterzygote. They have been selfed to develop BC_3F_2 population to select the elite lines with potential bacterial leaf blight resistance and being released to the farmers.

Keywords Bacterial leaf blight, resistance, marker design, candidate gene, genome

1. Introduction

Bacterial leaf blight (BB) disease caused by *Xanthomonas oryzae pv.oryzae (Xoo)* and is ranked as the most important widespread disease of rice producing countries in the world. In Asia, consecutive rice cultivation has resulted in high BLB affection that caused yield reductions of 50% in certain environments [1] and up to 100% yield loss with extreme severity and caused poor folder [2]. In rice, all stages of rice are susceptible to BLB and yield losses due to the disease range between 20 and 30% and in the case of severe infection can be reduced by up to 80%, depending on the crop stages, degree of susceptibility and environmental condition where it occurs [3-4].

Rice (*Oryza sativa L.*) is most important crops for over half of worldwide populations and supplies 20% of daily calories. In Vietnam, rice is a stable food and plays a key role in the economy of the country. Currently, Vietnam is the second biggest rice exporter in the word, and accounts for 50% of worldwide rice trade [4]. Vietnam, with 3260 km of coastline where there are two biggest low-lying rice deltas including Red River and Mekong deltas, are being significantly influenced by BLB infestation, caused rice yield severe reduction due to the adverse effects from climate change such as in temperature rise which leads to higher susceptibility of rice crop to *Xoo* and further provide favorable conditions for development of the pathogen [5]. Total rice growing areas in Red River and Cuu Long Deltas were affected by BLB in 2016 were approximately 88.021 ha, increased by 68.796 ha to compare with in 2015, in which 10.703 ha was caused by extreme severity of BLB [6]. To combat this problem, the attempts of worldwide scientists have been paid to generate BLB resistant rice varieties is one of the most feasible ways, economical and environmentally safe option to manage BLB disease. To date, 42 different resistance (R) genes designated from *Xa1* to *Xa42* have been identified to confer BLB

resistance, among them 9 genes have been isolated and clones [7-8]. A number of these QTLs/genes have been tagged by tightly linked molecular markers [9, 10, 7].

Recently, the basis of molecular assisted backcrossing (MABC) strategy is to transfer a specific allele at the target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genome [11]. The use of molecular markers, which permit the genetic dissection of the progeny at each generation, increase the speed of the selection process, thus enhancing genetic gain per unit time [12].

Pyramiding multiple resistant QTLs/genes into a single elite rice variety is proposed as a strategy to prevent or delay the breakdown of resistance. Owing to availability of the tightly linked molecular markers together with the complete genome sequences of two different rice subspecies japonica and indicia and three different races of BLB pathogen have made the possibility to identify plant with multiple resistance genes. Genomics-based strategies for gene discovery, couple with advanced powerful molecular tools, included the complete genome sequence of rice was initially launched in 2005 [13] are available which have accelerated the identification of a functional profile of candidate genes tolerance to abiotic and biotic stresses.

In this study, based on the genome sequence databases of 36 Vietnamese rice landraces, the work was to focus on identifying the bacterial leaf blight tolerance of candidate genes and design the markers, then pyramided them into an elite variety by applying molecular breeding.

2. Materials and Methods

2.1. Materials

The database genome sequence of 36 Vietnamese landraces were used in this study. The donor rice plant was Hom rau, the local rice landrace carrying the candidate genes tolerant to bacterial leaf blight disease was used. While, An dan 11 was developed from the cross between Q5, Khang dan 18 and Soc trang rice varieties. An dan 11 landrace showed to be desirable phenotypes with good tillering ability, seed compact with high spikelet capacity, low unfilled seeds, high yield with stability [6].

2.2. Methods

The nucleotide sequences were analyzed and compared by using the ClustalW2 software ((http://www.ebi.ac.uk/Tools/msa/clustalw2/). The specific primers were designed by applying Primer 3.0 software (http://primer3plus.com/web_3.0.0/primer3web_input.htm) and MEGA 6.0 Windows (http://www.megasoftware.net/mega.php). All the software products are opening sources and available to use as the above links.

2.3. Plant Crossing Scheme

The scheme for crossing the plant materials used in this study is presented in Figure 1. Hom rau was used as the donor plant of BLB resistance, where, An dan 11 was the elite rice variety were used as the recipient plant. For marker assisted selection (MAS) scheme An dan 11 was crossed with Hom rau to develop F_1 seeds (Figure 1). The F1s were backcrossed with An dan 11 to obtain a large number BC_1F_1 seeds. In the BC_1F_1 generation, the individual plants that were heterozygotes at the candidate genes of *xa5*, *Xa7* and *xa21*, were identified reducing the population size. The second and third BC generations were applied the same above strategy to develop the next generation. The selected BC_3F_{1s} was self-pollinated for further analyses.

2.4. Method to Test the Candidate Genes Resistance

Leaf samples of each selected plant were collected and extracted DNA following the CTAB methods with some modification. PCR reactions were performed by Veriti 96-well Thermal cycler. Total volume was 15 µl, included: 5µl DNA; 0.15µM primer; 0.2 mM dNTPs; 1X Buffer PCR; 2.5mM MgCl2 and 0.25 Taq polymerase. PCR products were performed on electrophoresis on 6% gel polyacrylamide for further analysis.The gels were stained in 0.5 mg/ml ethidium bromide and were documented using Alpha Imager 1220 (Alpha Innotech, CA, USA).

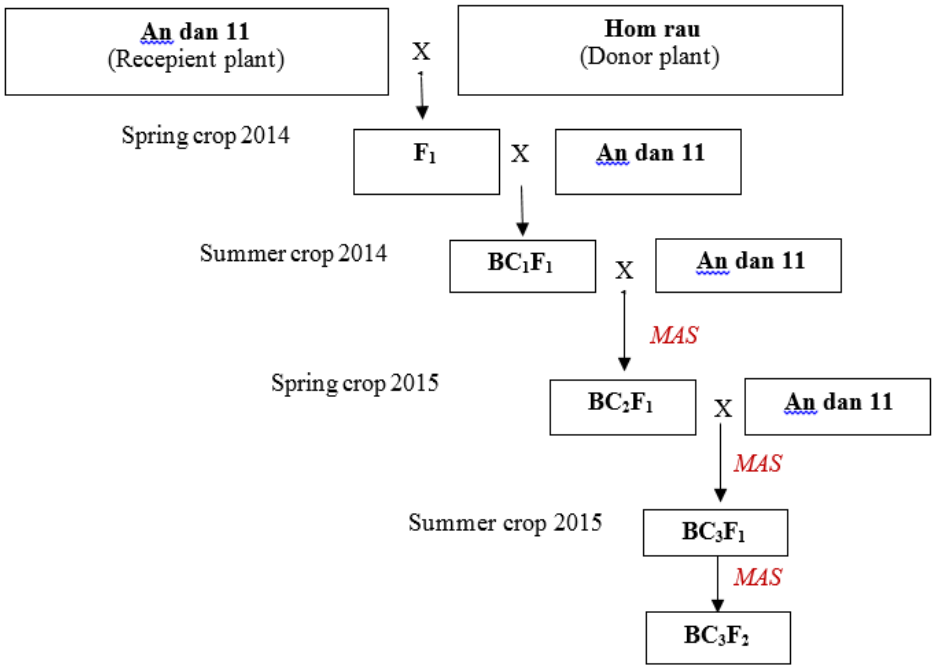

Figure 1: The scheme of applying MAS to improve bacterial leaf blight resistance in An dan 11 variety

2.5. Data Analysis

The analysed data by the bioinformatic software included ClustalW2, MEGA 6.0 Windows were further analyzed by Excel version 2010.

3. Results and Discussion

3.1. Identification of candidate genes and markers designing involving in BLB resistance from the genome sequencing of rice landraces

3.1.1.Screening and designing markers to identify candidate gene xa5 based on the genome sequence of rice landraces

The locus gene *xa5* with the code LOC_Os05g01710 (*Xanthomonas oryzae* PV. Oryzae Resistance 5) (Rice Genome Annotation Project release 5.6; http://rice.plantbiology.msu.edu/cgi-bin/sequence_display.cgi?orf=LOC_Os05g01710.1 has been identified based on 6261 nucleotides, CDS region with 321 nucleotides and 106 amino acids coding. It has found that 36 out of 33 rice landraces attain candidate gene *xa5* carrying the similar number of amino acids and nucleotides as the published CDS region *xa5* coded LOC_Os05g01710. However, the number of nucleotide gen xa 5 indicated that there are 18 sequence analogues (candidate gene *xa5*) in 18 rice landraces which showed mostly similar nucleotides of *xa5* (the published code LOC_Os05g01710) with less than 4 nucleotides. There are difference in T, C, A, G and number of nucleotides (less than 12 to 36 nucleotides) in other 15 rice landraces (Figure 2)

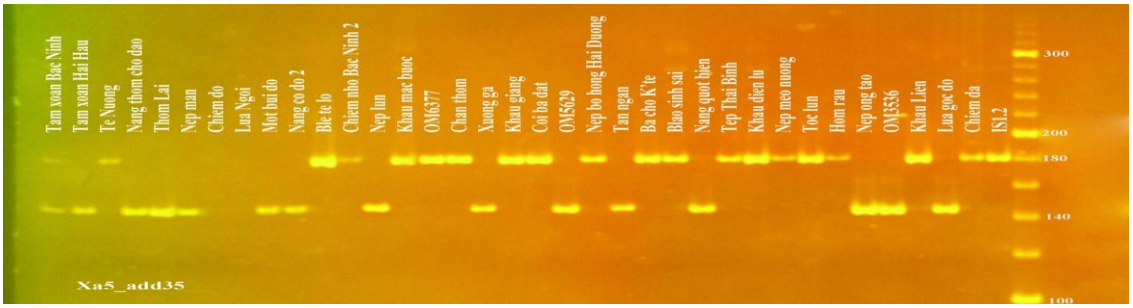

Figure 2: PCR and Electrophothesis to examine primers xa5add35F/xa5add35R to identify candidate gene xa5 from the Vietnamese rice landraces.

Based on the result analyses by the software, the comparison was made between *xa5* locus of 36 native rice landraces and *LOC_Os05g01710* bacterial leaf blight resistant gene, it showed rather high the frequent appearance of single nucleotide polymorphism (SNPs) and the Indels among the landraces. Particularly, the sequence of 35 nucleotides missing was found to be in 14 other native rice landraces which are similar with the published gene of *xa5*. Based on this specific difference, the primer namely *xa5add35* with the sequence: *xa5add35R*: taggagaaactagccgtcca and *xa5*add35F: tagtggcatgggaaatatgg) was designed by using the Primer 3.0 software. The amplified primer *xa5*add35 was estimated 179bp which disclosed in the landraces which carried the candidate gene of bacterial leaf blight resistance. Contrarily, the 144bp size was found in the landraces which showed different *xa5* sequence. PCR results of 36 genome sequences showed that 19 landraces pinpointed the 179 bp including: Te Nuong, Ble te lo, Chiem Nho Bac Ninh 2, Khau mac buoc, OM6377, Chan thom, Khau giang, Coi ba dat, Nep bo hong Hai Duong, Ba cho K'te, Blao sinh sai, Tep Thai Binh, Khau dien lu, Nep meo nuong, Toc lun, Hom rau, Khau Lien, Chiem da and IS1.2 landraces. It implied that the landraces carrying the *xa5* candidate gene in form of homozygote, any only Tam Xoan Bac Ninh was heterzygoted form which found to carry both band size with 179 bp and 144 bp.

3.1.2. Screening and designing markers to identify candidate gene *xa13* based on the genome sequence of rice landraces

Similar analyses based on the coding DNA sequence and the amino acids of database of 36 rice genome sequence as well as comparing the *xa13* with the code *LOC_Os08g42350* (*Xanthomonas oryzae* PV. *Oryzae* Resistance 13) (Rice Genome Annotation Project release 5.6; http://rice.plantbiology.msu.edu/cgi-bin/ORF_infopage.cgi?orf=LOC_Os08g42350), it showed 8 landraces carrying nucleotides in CDS region and amino acids which were similar with the published resistant gene *xa13 LOC Os08g42350* (924 nucleotides and coding 307 amino acids). Especially, two landraces Hom rau and Chan Thom revealed full similarity of nucleotides and amino acids of the published resistant gene *xa13 LOC_Os08g42350.*

We have compared *xa13* locus sequence of 36 genome sequence of native rice landraces with the resistant gene *LOC_Os08g42350* , It found that the difference occurred in two sequence segments in gene, specifically, 6 nucleotides and 4 nucleotides missing to compare with the published *xa13*, which were found in some rice landraces. Based on this difference, the functional marker namely *xa13add4* (the sequence: *xa13add4R*: cattagcagctagttaacttac and *xa13add4F*: tcactcactcactcactcaa) were designed and amplified at 97 bp (in candidate gene resistant) and 93bp (in the different sequence with the published *xa13*). By PCR confirmation of 36 genome sequence with the primer *xa13add4*, it showed that 8 landraces found to carry the lane 97 bp included: Tam xoan Bac Ninh, Tam xoan Hai Hau, Coi ba dat, Nep bo hong Hai Duong, Tan ngan, Khau dien lu, Hom rau and Khau lien).

3.2. Identification of *Xa7* in the landraces

For the *Xa7* gene, it is frequently used the P3, M5, RM5509 to identify this gene [14-15]. In the current study, the P3 was used *Xa7* to examine the possibly carried gene in the landraces. The electrophoresis results showed that some landraces including Lua ngoi, Ble te lo, Khau mac buoc, Chan Thom, Coi ba dat, Ba cho K' te, Blao sinh sai, Khau dien lu, Nep meo nuong and Hom rau carried the band 300 bp which was similar with the possitive control (IRBB7) (Figure 3; Figure 4).

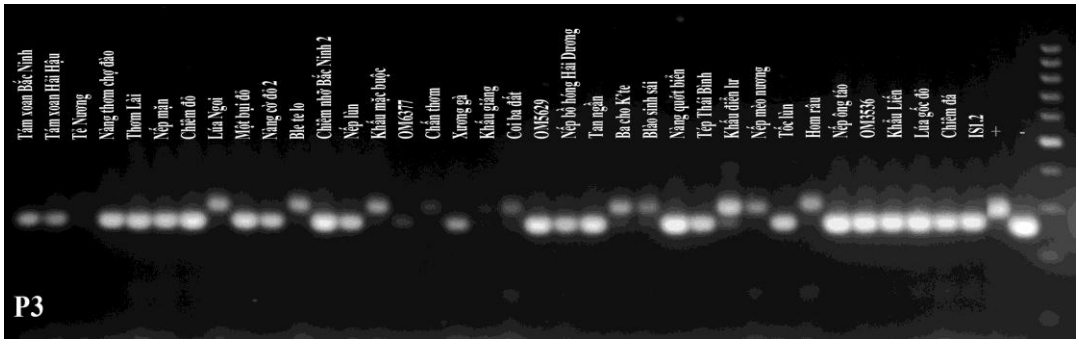

Figure 3: PCR identification of Xa7 by use the primer P3 among the rice landraces

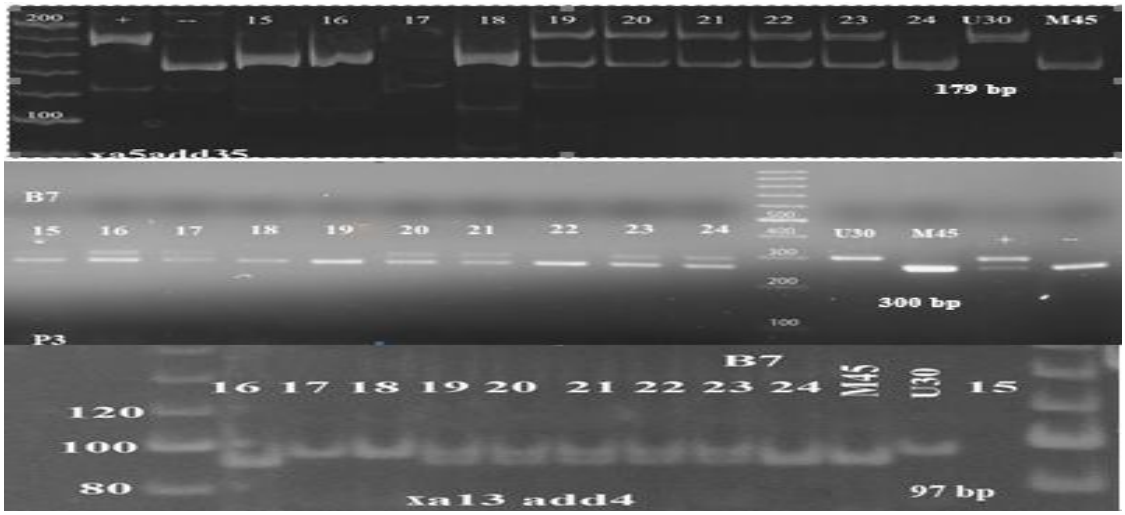

Figure 4: PCR checked by use the primer xa5add35, P3 and xa13add4 to identify xa5, Xa7 and *xa13 gene in the individual plants of BC1F1* of the *An dan 11 x Hom rau*

M45: An dan 11; U30: Hom rau; 15-24: individual plants BC1F1; +: Possitive control (IRBB5, IRBB7, IRBB13); - : Neggative control (IR24)

The analyses of the results, we have found that the Hom rau landrace has carried the candidate genes *xa5*, xa13 and *Xa7*. Therefore, this landrace was used as the donor plant, while the An Dan 11 was the recipient plant. In BC_1F_1 population, the individual plants were examined for the target genes *xa5*, *Xa7* and *xa13*, In heterzygote, it showed that 5 plants carried the *xa5*, 7 plants were *Xa7* and 5 plants were *xa13*, respectively. Therefore, we have selected the individual plants of BC_1F_1 number 20, 21 and 23 which carried 3 genes *xa5*, *Xa7* and *xa13* to develop BC_2F_1.

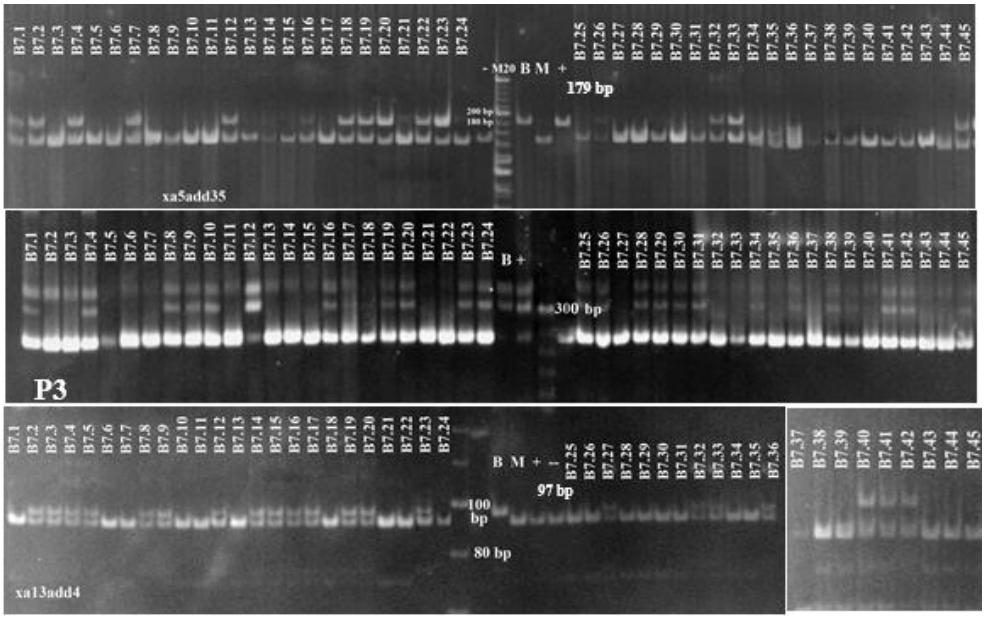

Figure 5: PCR checked by use of the primers xa5add35, P3 and xa13add4 to identify xa5, Xa7 and xa13 genes in the individual plants of BC_2F_1 population in the An dan 11 x Hom rau; M: An dan 11; B: Hom rau; B7.1-B7.45: the individual plants of BC_2F_1; +: Positive control (IRBB5, IRBB7, IRBB13); - : Negative control (IR24)

In summer season crop of 2015, we have examined the individual plants which carried the target genes. The results showed that 18 individuals of BC_2F_1 carrying *xa5* gene, while 22 individual plants carried the *Xa7* and 21 plants were found to harbor *xa13*, respectively. All the carried target genes of the plants were in form of heterozygotes. Based on the attained results, the individual plants number B7.4, B7.16, B7.19 and B7.20 carried

the *xa5, Xa7* and *xa13* were used to develop the BC_3F_1 the summer season crop in 2015. We have continued examining the individual plants of BC_3F_1 population as shown in Figure 6. The plants number 29, 30, 32, 43, 45 and 47 were carried 3 genes including *xa5, Xa7* and *xa13*. Similarly, 6 individual plants number 13, 19, 20, 37 and 38 were found to carry *xa5* and *Xa7*, respectively. While, 13 other plants number 25, 27, 28, 31,33, 34, 35, 36, 39, 40, 41, 42 and 44 possessed *xa5* and *xa13*.

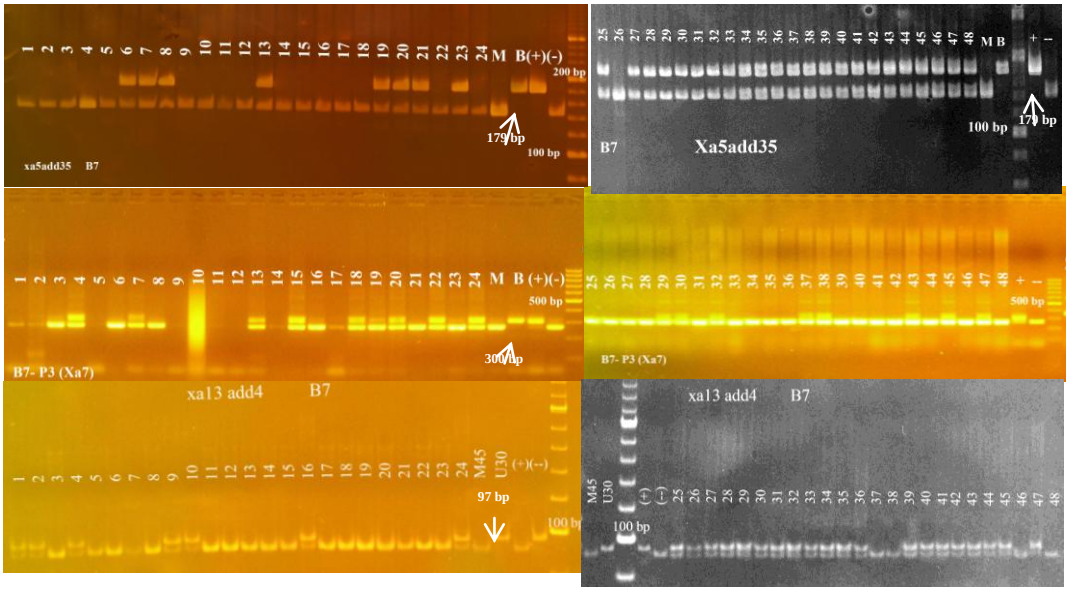

Figure 6: PCR examination by use the primer xa5add35, P3 and xa13add4 to identify the xa5, Xa7 and xa13 of the individual plants of BC_3F_1 of the An dan 11 x Hom rau
M: An dan 11; B: Hom rau; the number 1-48: the individual plants of BC_3F_1; +: positive control (IRBB5, IRBB7, IRBB13); - : Negative control (IR24)

The plants number 4 and 24 carried *Xa7* and xa13, respectively. In general, the selected individual plants of BC3F1 carrying the target gene were used to develop BC3F2 by selfing in the spring season crops, 2016.

4. Conclusions

We have successfully identified the candidate genes of *xa5*, *Xa7* and xa13 of some Vietnamese native rice landraces. Three candidate genes *xa5, Xa7* and *xa13* of the Hom rau landrace were introgressed into An dan 11 variety. The results showed 6 individual plants of BC_3F_1 carried 3 candidate gene *xa5*, *Xa7* and xa13 ; 6 plants were possessed *xa5* and *Xa7*, and 12 individual plants carried *xa5* and xa13 were found. The further works are ongoing to develop BC_3F_3 and BC_3F_4 by selfing and examine the target genes of interest. Simultaneously, the selected plants will be artificially examined for bacterial leaf blight resistance.

Acknowledgement
The authors would like to send the thankfulness to Ministry of Agriculture and Rural Development (MARD) and Ministry of Science and Technology (MOST) to support the projects code: 164/HĐ-KHCN/CNSH dated February 27th, 2014 and 26/2014/HĐ-NĐT, June 20th, 2014.

References

[1]. Mew, T.W., Alvarez, A.M., Leach, J.E, Swings, J. (1993). Focus on Bacterial Blight of Rice. *Plant Diseases*, 77:5–12.
[2]. Agrawal, G.K, Rakwal, R., Jwa, N.S, Agrawal, V.P. (2005). Signaling molecules and blast pathogen attack activates rice *OsPR1a* and *OsPR1b* genes: a model illustrating components participating during defense stress response. *Plant Physiology and Biochemistry*, 39:1095-1103.

[3]. Khan, M.A., Naeem, M., Iqbal, M. (2014). Breeding approaches for bacterial leaf blight resistance tin rice (*Oryza sativa* L.), current status and future direction. *European Journal of Plant Pathology*, 139:27.

[4]. Linh, L.H., Linh, T.H., Xuan, T.D., Ham L.H., Ismail, A.M., Khanh, T.D. (2012). Molecular breeding to improve salt tolerance of rice (*Oryza sativa* L.) in the Red River Delta of Vietnam. *International Journal of Plant Genomics*, Article ID 949038, 9 pages.

[5]. Anh, L. H., Hue, H. T., Quoc, N. K. Nghia, L. T. Trung, K. H., Trung, T.N. Trang, D.H., Xuan, T. D. Khanh, T. D. (2016). Effect of Salt on Growth of Rice Landraces in Vietnam, *International Letters of Natural Sciences*, 59:72-81.

[6]. MARD- Nong nghiep Vietnam (2016). http://nongnghiep.vn/giong-lua-an-dan-11-post94876.html|NongNghiep.vn) (in Vietnamese).

[7]. Bhatia, D., Vikal, Y. (2017). Genetics and Genomics of bacterial blight resistance in rice. In Advances in International Rice Research. Intech Publisher, 175-213.

[8]. Kim, S.M., Suh, J.P., Qin, Y., Noh, T.H., Reinke, R.F., Jena, K.K.(2015) Identification and fine-mapping of a new resistance gene, Xa40, conferring resistance to bacterial blight races in rice (*Oryza sativa L.*). *Theoretical and Applied Genetics*, 128: 1933–43.

[9]. Rao, K.K., Lakshminarasu, M., Jena, K.K. (2002) DNA markers and marker-assisted breeding for durable resistance to bacterial blight of rice. *Biotechnological Advances*, 20:33–47

[10]. Gu, K., Sangha, J.S., Li, Y., Yin, Z. (2008) High resolution genetic mapping of bacterial blight resistance gene *Xa10*. *Theoretical and Applied Genetics*, 116:155–163.

[11]. Neeraja, C. N., Rodriguez, M., Pamplona, R. A. *et al*. (2007) A marker-assisted backcross approach for developing submergence-tolerant rice cultivars. *Theoretical and Applied Genetics*, 115:767–776.

[12]. Hospital, F. (2003) Marker-assisted breeding in Plant Molecular Breeding, H. J. Newbury, Ed., pp. 30–59, Blackwell Publishing, Oxford, UK.

[13]. Goff, S.A.et al. (2005). A draft sequence of the rice genome (*Oryza sativa* L. ssp. Japonica), Science 296: 92-100.

[14]. Taura S, Sugita Y, Kawahara D, et al. 2004. Gene distribution resistance to bacterial blight in Northern Vietnam rice varieties. *Abstracts of the 1st international Conference on Bacterial Blight of rice.* March17-19, 2004, Tsukuba, Japan, p. 42.

[15]. Hoa, L.V., Hai, T.V., Thu, T.M., Rui, L.Y. (2010). Investigation of rice germplasm to evaluate gene resistance of bacterial leaf blight by molecular marker. Journal of Science and Development: 8(1): 9-10 (*in Vietnamese*).