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SEQUENCING AND IDENTIFICATION OF HOMOLOGOUS REGION ENCODING RUST RESISTANT-GENE IN SOYBEAN *(Glycine max L.)*

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ABSTRACT

Soybean (Glycine max L.) is an important crop plant both easy cultivation and high efficiency. Its product is commonly used such as fresh-seed,plant oil, cake, candy, milk and feed etc… for enhancing the protein requirement in daily meals and to export for providing valuable currency resource. On the other hand, it is also used in the process of land improvement, contributing to increase the yield. Soybean is not only useful in medical as an application to cure disease in human, but also utilized to provide materials for the process of animal feed with rich-protein resource, and tend to industrial application.

Based on the result of phenotypic evaluation for rust disease, we have classified into three groups: group A consists of 37 varieties with the level of resistance at scale 0 and 1, group B has 30 varieties which are identified at scale 3 and 5, and group C has 14 varieties with level of resistance at scale 7 and 9. We have designed two primers for targeting coding region of rust resistant-gene, represented by Langrisat1 and Langrisat2.

We have shown that soybean varieties in group A was capability of high rust resistance, providing further application in breeding programme. A key task for future is to determine whether rust resistant-gene can be transferred to susceptible soybean varieties. Two designed primers are needed for synthesis and testing through Polymerase Chain Reaction (PCR). To the best of our knowledge, the results presented here are the first characterization of nucleotides of rust resistant-gene in soybean. The further analysis is necessary for identifying nucleotide sequence and its functional site more exactly.

Key word: Glycine max L., Rust resistance gene, Sequencing, Soybean.

INTRODUCTION

With its significantly economic value and well-characterized morphology, Glycine max L. is one of the important crops after rice and maize. Due to high content of protein, soybean seeds and its products are used widely to feed human and cattle in the worldwide. Soybean seeds contain the high nutritional component, the content of protein approximately 38-40%, lipid range from 18-20%, rich vitamin and mineral salt. It is an only type of plant that its value evaluated both protein and lipid. On the other hand, soybean is able to fix nitrogen through symbiotic bacteria that invade the roots of leguminous plants and form root nodules in which they fix nitrogen, supplying both the bacteria and the plants. Because each nodule is considered as "tiny nitrogen fixation-manufactory", soybean cultivation reduces not only used nitrogen, but also the significant effect for the improvement and reinforcement of the land. However, its commercial value was not appreciated correctly although it can be considered such as resource for high foreign earnings and also as a potential material for human in the future. There are over 100 diseases destroy on soybean in the world, thirty-five of them affected to economic value. In Vietnam, according to the statistic of whole country in 1990, there were about 30 soybean rust diseases which caused by fungi. It is considered that the rust, caused by Phakopsora pachyrhizi H&P Syd., was one of serious disease

in soybean because of widely dispersal area and considerable reduction of the yield. Thus, plant breeders attempt to find much new methods so that they can select promising soybean varieties, resistant to rust disease constantly. However, because of the natural pressure of selection, herbicide and pesticide, they have broken environmental balance, leading to unstable rust resistance. Although during last decades, there has been progress and studies on the elucidation of the genes involved in rust resistant genes, very little is known. Blad and Baker (1972) indicated that Komata variety (PI0492) contained a dominant allele resistant to rust strain in Australia.. Sweets (2007) reported that Ankur variety (PI462312) also has a dominant allele resistant to rust disease. In this article, the aim of study is to identify nucleotide sequence and design primer for rust resistant gene, serve for further research.

MATERIALS AND METHODS

Plant material: The experiment was conducted with nine soybean varieties which come from CuuLong Delta Rice Research Institute Genebank, with NamVang and OMDN87 as two check varieties. Seventy-two soybean varieties derived from Institute of Agricultural Science for Southern Vietnam were also included for screening rust disease. Crossing technique in soybean was done the following by CLRRI protocol. Seedling leaf material was colectted at 30 days after sowing and stored in a fresh 1.5 ml tube at 4^0C.

Evaluation of phenotype: Phakopsora phakyrizhi fungi from CLRRI Genebank was cultured in M1 medium with the total of volume 1L including 10g glucose, 1g K_2HPO_4, 5g peptone, 0.5 $MgSO_4.7H_2O$, 20g agar and adjust sterile distilled water to 1L. Transfer fungal colonies from a master plate to two pre-prepared M1 medium plate. Incubate at room temperature for 36-48h. The experimental soybean varieties were designed by randomly completely block design (RBD) with three replications, two leaves of a plant from each variety per replication were infected . After 8-10 days, evaluation of affected level was recorded followed IRRI's standard protocol. Damage due to rust disease on leaves was grouped using NTSYS pc software version .Rohfl F. J. 1992

DNA isolation: Genomic DNA was isolated from seedling leaves using CTAB method. The protocol was described by Lang 2002 and modified by Dr. Nguyen Thi Lang (2000). Two to three (2-3) cm fresh leaf and placed it into mortar. Grind sample in extraction buffer (with the total volume of 1L containing 20g CTAB, 81.82g NaCl, 100ml 1M Tris pH 8.0, 40ml 0.5M EDTA pH 8.0 and sterile distilled water). Add 400µl extraction buffer, grind until green buffer appears, showing the breakage of the cells occurred, leading to release chlorophyll. Then add 400µl extraction buffer. Mix thoroughly and transfer to a clean 1.5 ml tube. Incubate in water-bath at 65^0C for 15 min. Add 400µl chloroform:isoamylalcohol (24:1). Mix vigorously and incubate in water-bath at 65^0C for 15min. Centrifuge at 4^0C, 12000rpm for 1min. Transfer supernatant to a clean 1.5ml tube. Add 800µl ethanol 100% and inverse carefully. Spin at 4^0C, 12000rpm for 5min. Remove supernatant. Rinse pellet with ethanol 70%. Dry DNA pellet at room temperature or vacuum hood for 20-30min, dissolve DNA in 50µl TE and store at 4^0C.

PCR reaction for sequencing: The PCR reaction was conducted with a final volume of 20µl using GenAmp®PCR system 9700, containing 4µl of terminator ready reaction mixture 2.5X for 384-well plates, 2µl of Bigdye ® terminator V1.1/3.1 sequencing buffer 5X, 1µl of -21M13 forward primer, 1µl of M13 reverse primer, 1µl DNA template and deionized water. The reaction conditions for PCR included a denaturing step of 96^0C for 1 min followed by 25 cycles of 10 sec. at 96^0C, 5 sec. at 50^0C and 4 min. at 60^0C, ending with a step of sample storage at 4^0C. Rapid thermal ramp to 4^0C and hold until ready to purify. After agarose gel 1% electrophoresis, PCR products were purified by using QIA quick gel Extraction Kit (QIAGen, USA).

Sequencing and sequence analysis: PCR products, pre-denatured with hidi-formamide at 95^0C for 5 min., were sequenced by using a capillary automated Applied Biosystem 3130 DNA sequencer until the whole these fragments were sequenced completely following standard sequencing process by manufacturer. Search similarity between nucleotide and deduced amino acid sequences was done by BLAST NCBI and EMBL/SwissProt available online (www.expasy.org). A

multi-sequence alignment was performed using ClustalW (www.ebi.ac.uk). Prediction of coding regions and gene structure was done by ORF finder and CLC combined workbench version 3.0.3, respectively. Signal peptide was predicted by SiganlP 3.0 server (www.cbs.dtu.dk/services). Scanprosite was used to analyze functional site of putative amino acid sequence (www.expasy.org/Prosite). Primers were designed by Primer3-BLAST available online.

PCR Amplification: PCR amplification components and conditions were done based on the methods used by Lang (2002). The PCR reaction mixture contained 20-50 nanogram (ng) template DNA, 50ng of each primers, 0.05 mM dNTP's, 1xPCR buffer (10mM Tris pH 8.4, 50mM KCl, 1.8mM $MgCl_2$ and 0.01 mg/ml gelatin) and 1 unit of Taq DNA polymerase in a total volume of 20 µl. Template DNA was initially denatured at 940C for 5 min. followed by 30 cycles of PCR amplification using the following parameters: 30 sec. denaturation at 940C, 30 sec. primer annealing at 55^0C or 60^0C and 1 min. primer extension at 72^0C. Completion of primer extension was alllowed by a final 5 min. incubation at 72^0C.

An aliquot of 10 µl of the PCR product was routinely taken for gel electrophoresis to determine if amplification was successful. When the primers detected an amplicon length polymorphism, the samples were readily scored. The remaining 10 µl of PCR product was used for restriction digestion to detect PBR polymorphism in the case of other primers. The digestion reaction normally contained 3.2 µl sterile distilled water, 1.5 µl restriction buffer (10X), 0.3 µl restriction enzyme (10 U/µl) and 10.0 µl of PCR products in a total volume of 15 µl. The digestion reaction was incubated for 4 hours to overnight at appropriate incubation temperature for the enzyme used.

The PCR products or the DNA fragments produced by restriction digestion were resolved electrophoretically on 1% agarose gel in 1 X TAE buffer.

RESULTS

Classifying based on phenotype: With the level of genetic variability 2.92%, soybean varieties were classified into three major groups: group A has 37 varieties including 27 varieties at scale 1 such as PI0830881, LS201, AGS374, GC84058, OMDN1, BR23, HL203, 9800410, SSE137559, BR24, MTD483-4, PI200429, LEIRCHART, MTD164-1, OMDN111, DT85, ALIANT, ATF15, MTD652-5, QUANGPHU, MTD652-2, AGS360, ATS16, AGS371, DT94, PI085089, PI548484 and 10 varieties at scale 0 namely GLS2111, CPAC365-76, OMDN29, DT2000, AGS376, DH4, GC990013-12-15-10, IAC100, 96033B, AGS365; group B has 30 varieties, of which 14 varieties at scale 5 as DT200, L07515, OMDN109, ATF8, 13176, PRANA, DT93, 9907A-4, MSBR22, 9005A-7, GC90013-21-23, MTD664, MANTA, MTD517-8, and 16 varieties at scale 3 such as 903551CR, 9603331-1-1-1, TL57, AGS367, HL2, GC990013-1-1-39, GC90013-12-15-6, 9804512, G85-5126, OMDN64, MSBR17, 96033B, OMDN87, PI417088, HL92, OMDN130, Dau trang DT; and group C has 14 varieties including 7 varieties at scale 9 such as 980464, PI518759, MTD176, Dau den DT, MSPR20, OMDN110, MTD514-6, and 7 varieties at scale ? 95389, 95389-1, AGS129, 5113, Nam Vang, HQ1, PI103.

Sequence analysis of PCR products: PCR reaction was successful to amplify inserted DNA sequences with a band pattern on gel approximately 110bp in size. This will be convenient for subsequent sequencing.

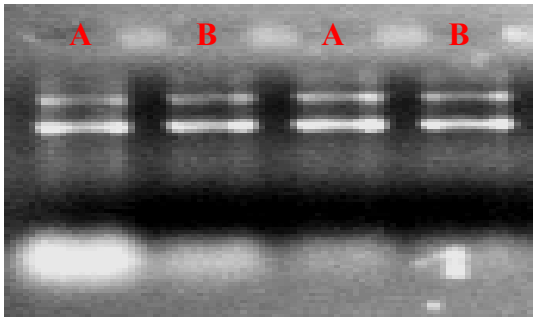

Figure 1: PCR products of OMDN1 and OMDN110 with a band pattern around 1100bp. A: OMDN1, B: OMDN110

Through analysis of ORF finder, we have identified coding region in nucleotide sequence of OMDN1 and OMDN110 with 2 exons including exon 1 (114 nucleotides start 85 position and end

in 198 position), exon 2 (156 nucleotides from 281-436 position) and 3 exons consist of exon 1 (216 nucleotides from 1-215 position), exon 2 (168 nucleotide from 48-215 position), exon 3 (210 nucleotide from 5-214 position), respectively. The nucleotide sequence of OMDN1 encoded a protein with pI/Mw (9.99 / 15339.14) and that of OMDN110 encoded a protein with pI/Mw (5.23 / 6954.88). Using BLAST search similarity, the nucleotide sequence of OMDN1 and OMDN110 were identical with nucleotide sequence of rust resistant gene in wheat with accession number S79982, S79983 in Genebank database. Two nucleotide sequence of OMDN1 and OMDN110 were also submitted to Genebank Database with accession number EU077601.2 and EU077601.1, respectively. The result of multi-sequence alignment using Clustal W showed that there was a relatively low similarity between sequences OMDN1, OMDN110, S79982 and S79983 (Figure 2).

Primer design: With two sequenced nucleotide sequences, we have designed two primers, denoted by Langrisat1: forward primer5'GGCCCCGA

GTGATTTAAGGA3', reverse primer 5'G

GTGGTGACTCACGCTGTGG 3' and

Langrisat2: forward primer 5'GTGGTGT

AGCTGAATCTGGA 3', reverse primer 5' GAGTGGTGTGAGGTGATGGT 3' for amplifying coding region of rust resistant gene. The GC ratio account for 50-55% for forward primer and 55-65% for reverse primer. These results were relatively suitable for the theory of primer design the following by Nguyen Thi Lang's standard protocol.

ANALYSIS OF THE F2 POPULATION

DNA genome from the F2 population of the cross between OMDN 29/MTD176 was assessed through PCR amplifcation using primer Langrisat L-R. The resultant PCR products were spliced out by double enzymes digestion with HinfI and AluI. Fragments were resolved on agarose gels and the banding patterns were scored with reference to those of the parents. The banding pattern of the F2's individuals could be classified into homozygote for the OMDN29 type by 250bp fragment, homozygote for MTD176 type by 210bp fragment, and heterozygotes displaying both fragments OMDN29 and MTD176 (Fig 2).

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OMDN110   AGGTTAAAGGGATAGTCGAGTTGGTTTCTGAT--AGATGATAGTAAGATTAAT-----AG 54
OMDN1     AGATAAAAAAAGTGTGTGAGTTATTTGGAGGTTTATTTATTCATAAGGTAAAT-----CA 143
S79982    TGGCAAAGAGGCCTCATCCATCGCCCTGGACGGCTCGTTTTGGTCAAATCAGTGATTGCG 599
S79983    TGGCAAAGAGGCCTCATCCATCGCCCTGGACGGCTCGTTTTGGTCAAATCAGTGATTGCG 567
           *   **              *                  *  *  *  *  * *

OMDN110   AGTGA---TGTGAATGATGG---GGTGAAAGATGTG---GTGTAGCTGAATCTGGAAAT- 104
OMDN1     GGTGA---CTTGCACAGTTGATGGGAGGAGAAGAAA---GTGGATCTGGTTGGGGGAAT- 196
S79982    GCTAAACCCATCCATCATTTCATGGTGACACATGCTCCCGTGTGGGTATTTGAAGAGATC 659
S79983    GCTAAACCCATCCATCATTTCATGGTGACACATGCTCCCGTGTGGGTATTTGAAGAGATC 627
            * *     *  *   *     ** *    *       ***    *   *   *  **

OMDN110   -----------GAA-TAGGGATGATGGATAGAAAGTGGATAAGTTGAGTGGTGTGAGGTG 152
OMDN1     -----------AAAATTTGGTCGGTGGGGGCAAAACAAAAAGAAAAAAAAATT---GTTA 242
S79982    ----------TGCGATCCTTCTTTTGGGCTGGCAAGGAAAAAATCCAACGGTGGCCAGTG 709
S79983    GAGCAGTGGATGGGATCCTTCTTTTGGGCTGGCAAGGAAAAG-TCCAACGGTGGCCAGTG 686
                    *        ***      *    * *     *    *       *

OMDN110   ATGGTTGAAATTGAGTTTGGTTGT--GCGTTGGTCGGGGTAAAG--TGGTGGGGTGAGAG 208
OMDN1     GGGGTTAAAATCCGTTCTCGCCCCCGGCCCCGAGTGATTTAAGGATAGGTTTGTTGGGGG 302
S79982    CTTGGTAACTTGGAAATCAGTCTGCAAACCCACTTCGCTTGGTGGTCTGGGAATCCGCAA 769
S79983    CTTGGTAACTTGGAAATCAGTCTGCAAACCCACTTCGCTTGGTGGTCTGGGAATCCGCAA 746
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Figure 2: The comparison of nucleotide sequence of OMDN1 and OMDN110 with others in Genebank database. Accession number of nucleotide sequence of rust resistant-gene in wheat is followed as S79982, S79983. The column conservation represents the level of homologous between sequences. Bold lines in background show homologous of two nucleotide sequence for rust resistant gene in wheat.

DISCUSSION

With phenotypic evaluation of 81 soybean varieties, tolerant levels of these varieties were grouped, allowing to screen soybean varieties which are able to stably resist to rust disease. These results open a new opportunity to utilize them as materials in plant breeding program. We assumed that morphology markers are very useful for screening directly based on phenotype. However, the plant phenotype is an interaction between genotype and environment. Thus, a practical comprehension is to need distinct approaches which evaluated on genotype to isolate stable resistance with soybean rust disease correctly.

The nucleotide sequence analysis of two soybean varieties (OMDN1 and OMDN110) indicated that they were relatively low identical with others of submitted authors in Genebank database. In additional, nucleotide data for soybean rust disease is still very restriction, this lead to be difficult to unravel and identify rust resistant nucleotide sequence. To the best of our knowledge, the results presented here are the first characterization of nucleotide of rust resistant-gene in soybean. The further analysis is necessary for identifying nucleotide sequence and its functional site more exactly. We have designed two primers, represented by Langrisat1 and Langrisat2, to amplify coding region of target gene. However, only primers Langrisat 1 detected the gene with F2 population from OMDN 1/ MTD 176 .

The accuracy of marker selection for rust resistance was verified through F2, progeny tests. The accuracy of predicting homozygous resistant genotypes based on flanking marker data was 85 % for rust using a single marker. A single marker could thus be as accurate as two flanking markers provided.

The successful PCR based marker for rust resistance gene have made it possible to use these markers in future MAS program for the transfer of rust resistance gene into elite breeding lines of soybean.

CONCLUSION

We have shown that: the soybean varieties in group A was capability of high rust resistance, provide further application in breeding programming. But at present, studies for soybean rust are still so little. We proposed that further analysis and characterization of rust resistant gene will help to identify nucleotide sequence and its functional sites. A key task for future is to determine whether rust resistant-gene can be transferred to susceptible soybean varieties. An additional task is to determine the efficiency of breeding methods that is very important for improving rust resistance in soybean varieties.

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Giải trình tự và phân lập vùng mã hóa gen kháng bệnh rỉ sắt trên bộ gen đậu nành (*Glycine max* L.)

Đậu nành (Glycine max L.) là cây trồng có vị trí quan trọng ở Việt Nam, phục vụ chủ yếu trong lĩnh vực thức ăn chăn nuôi. Bệnh rỉ sắt khá quan trọng trong sản xuất đậu nành ở Đồng Bằng Sông Cửu Long. Trên cở sở đánh giá kiểu hình tính kháng bệnh rỉ sắt đậu nành, chúng tôi đã phân ra làm 3 nhóm vật liệu: nhóm A gồm 37 mẫu giống có mức kháng cao, điểm 0 và 1, nhóm B có 30 mẫu giống ở mức điểm phản ứng là 3 và 5; nhóm C có 14 mẫu giống ở thang điểm phản ứng 7 và 9. Chúng tôi đã thiết kế 2 cặp mồi nhằm tìm kiếm vùng mã hóa gen kháng rỉ sắt theo Langrisat1 và Langrisat2.

Giống đậu nành thuộc nhóm A có khả năng kháng bệnh cao, là nguồn vật liệu đầy tiềm năng phục vụ công tác lai tạo giống. Hai primers được thiết kế dựa trên nguyên tắc chỉ thị phân tử PCR. Kết quả cho thấy tính chất của những nucleotides trong genome nơi định vị các gen kháng bệnh rỉ sắt đậu nành.