GENE IDENTIFICATION AT THE INSERTION SITE OF A HIGH SALINITY TOLERANT INSERTIONAL MUTANT RICE LINE

Identifikasi Gen pada Situs Insersi Galur Padi Mutasi Insersi Toleran Salinitas Tinggi

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ABSTRAK

Salinitas tinggi adalah salah satu cekaman abiotik utama yang mempengaruhi produksi padi, terutama di daerah pesisir. Pada penelitian sebelumnya telah diidentifikasi galur-galur padi mutan insersi transposon *Ds* cv Nipponbare yang menunjukkan peningkatan toleransi terhadap cekaman salinitas. Penelitian ini bertujuan untuk mengetahui situs penyisipan transposon *Ds* pada DNA kromosomal dari salah satu galur mutan insersi (170-10) dan mengidentifikasi gen di sekitarnya. *Amplicon* sekitar 450 pb dari padi mutan 170-10 berhasil diisolasi dengan teknik TAIL-PCR menggunakan *degenerate primer* dan primer spesifik Ds. Analisis berbasis bioinformatika mendapatkan insersi terletak pada ekson kedua di suatu *coding sequence* (CDS) yang merupakan gen putatif *Os11g0686500* pada kromosom 11. Analisis bioinformatika juga mengidentifikasi dua CDS di sekitar lokasi insersi, yaitu daerah *downstream* dan *upstream*.

Kata kunci: padi (*Oryza sativa* L.), salinitas, mutasi insersi

ABSTRACT

High salinity is one of the major abiotic stresses affecting rice production. Previously, we have identified transposon Ds insertional mutant rice lines cv Nipponbare showing improved tolerant under salinity stress The objective of this research was to determine the Ds insertion site in chromosomal DNA from one of the salt tolerant mutant line (170-10) and identify the genes in the proximity. A specific amplicon around 450bp from the mutant rice line 170-10 was successfully isolated with TAIL PCR technique using the degenerate and a specific Ds primers. Bioinformatics analyses found that the insertion was located in a putative CDS designated as Os11g0686500 in chromosome 11 at the 2nd exon. Two putative CDS's within the proximity of the Ds insertion site downstream and upstream the insertion sites were also identified.

Key words: *rice (Oryza sativa L.), salinity, insertion mutation*

INTRODUCTION

Rice production in Indonesia is facing many challenges, related to biotic and abiotic stresses. Salinity is one of the major problems in rice production. In Asia, 12 million hectares of cultivated land are exposed to high salinity (Lafitte *et al*., 2004). Salinity affects the growth of roots, stems and leaf area, due to metabolic imbalance caused by ionic poisoning, osmotic stress and nutrient deficiency (Munns, 2002). Rice plants are very sensitive to salinity stress, especially during the germination stage. The salinity stress on rice can result in decreased in yield (Lafitte *et al*., 2004).

Other problem that is faced in rice farming is the lack of environmental stress tolerant varieties, such as high salinity stress tolerance Discovery of genes with a specific trait to deal with environmental stresses, such as salinity stress, can be done with the availability of complete genome information that allows the exploration of the properties of molecules, and regulation of gene expression.

Insertion mutation is one of the strategies to discover gene function. The *Ac/Ds* transposon found in maize is the most common system that is used in the insertion mutations in plants, such as Arabidopsis (Wiegel *et al*., 2000), carrot (Van Sluys *et al*., 1987), tomato (Meissner et al., 2000), lettuce (Yang *et al*., 1993), tobacco (Fitzmaurice *et al*., 1999), and potato (Knapp *et al*., 1988). Previous studies shown that transposon *Ac/Ds* can also be used on rice as a potential insertional mutagen (Izawa *et al*., 1997). Activation - tagging system with Ds element carrying four copy enhancers are now used in rice to maximize finding important genes (Upadhyaya *et al.*, 2002). Insertion mutation strategy is expected to be able to assist in revealing the potential of rice as a source of genes, or the factors and elements that control the expression of related abiotic stresses tolerant genes, such as salinity stress. In the future, insertion mutation can be used in the breeding of rice or other important crop.

Previously, we reported 10 Nipponbare rice insertional mutant lines harboring activation tag that showed increased tolerance to salinity stress at germination stage (Zannati *et al*., 2015). Among 10 potential mutants, mutant 170-10 had the highest vigour index under salinity stress condition (Zannati *et al.*, 2015). The growth performances under salinity stress of those mutant lines have been reported previously (Windiastri *et al*., 2017). In this paper the determination of the insertion sites of line 170-10 and the identification of the genes in the proximity of the insertion which may play roles in the improved salinity tolerant of the insertional mutant rice lines are explored.

MATERIALS AND METHODS

Rice Material

Rice insertional mutant lines 170-10 Cv Nipponbare harboring activation tag which showed the best performance among salinity tolerant mutants identified was chosen for these experiments.

Insertion Site Determination

Analysis of flanking sequences of the insertion region in the rice genome was performed using TAIL-PCR (Thermal Asymmetric Interlaced PCR) (Liu *et al*., 1995) with Dream Taq DNA Polymerase (Sigma). TAIL-PCR was performed by using three nested primers specific to the transposon *Ds* were Ds5'-1a (ACGGTCGGGAAACTAGCTCTAC), Ds5'-2a (TCCGTTCCGTTTTCGTTTTTTAC), Ds5'-3a (TATACGATAACGGTCGGTAC), and degenerate primers were AD1(NTC GA(G/C) T(A/T)T (G/C)G (A/T) GTT), AD2(NGT CGA (G/C)(A/T)G ANA (A/T) GA A), AD3 (A/T)GT GNA G(A/T)A NCA NAG A). The TAIL-PCR was performed essentially according to the method described by Liu *et al.* (1995), with minor modifications in the time length. The PCR products were analyzed on a 0.8% agarose gel (w/v) electrophoresis, and purified using the DNA Extraction Kit (fermentas). A total of 35 ng of DNA sample in a 30 mL nuclease free H_2O (fermentas) were sent for sequencing (PT. Genetika Science Indonesia).

In-silico Analysis of Insertion Site

Identification of the insertion site was done through in-silico analysis using several databases that are publicly available, such as www.ncbi.nlm.nih.gov for the identification of the insertion site, www.gramene.org for obtaining information associated with chromosome 11, www.softberry.com, STRING (string-db.org) to determine the predicted genes and proteins in non-coding area, www.geneontology.org to determine the gene ontology information and the surrounding genes, and www.expasy.org to predict protein interactions. The length of the analyzed region on chromosome 11 were around 60 Kbp, from nucleotide number 29.600.000 to 29.661.000.

RESULTS AND DISCUSSION

Flanking Sequences Analysis

Flanking sequence analysis was done on rice mutant 170-10, which performed salinity tolerance in 200 mM

NaCl treatment. Amplification analysis of flanking sequence of insertion Ds transposons conducted by TAIL-PCR, using the protocol described by Liu et al. (1995). TAIL-PCR results showed the specific band generated from specific Ds3 primer amplification and arbitrary primer AD2 (Figure 1).

Insertion Area Prediction

Identification of insertion site was conducted through *in-silico* analyses performed using several publicly available databases blast analyses of the flanking sequence identify using TAIL-PCR of mutant lines 170-10 showed that the insertion occurred in chromosome 11, at the exon 2nd at 29,639,691 nucleotide (nt) of the *Os11g0686500*. The gene is 4,044 kb in size, and consists of two exons and one intron (Figure 2), therefore it is predicted that the insertion interrupted the gene's function (Ramachandran and Sundaresan, 2001). *Os11g0686500* (4,044 bp), located from 29,636,486 to 29,640,529 nt on chromosome 11. Blast analysis result showed that *Os11g0686500* has 90% similarity with *pikm2-TS* and -*pikm1TS* genes, which are responsible for blast disease resistance in rice (Ashikawa *et al*., 2008).

Stress researches have mainly focused on single stress aspects, for simplicity. However, in their natural environment, plants have to adapt to numerous environmental stresses at the same time and different stresses can occur at different stages of the plant's life cycle. The recognition and signaling pathways regulating the responses to abiotic stresses (*e.g.* drought, salinity, cold and heat) could be similar to those used for responding to biotic stresses (Tippmann *et al*., 2006).

The adaptation to one stress condition can therefore affect tolerance to other non-related stresses, a phenomenon referred to as cross-tolerance (Tippmann *et al*., 2006). Tolerance to one environmental stress or stress

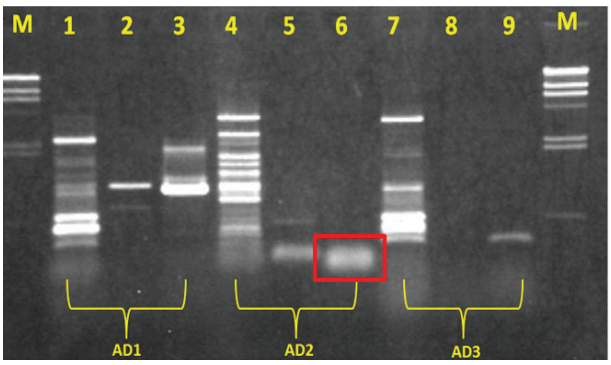

Figure 1. Tail PCR results of rice mutant line 170-10 dengan nested primer Ds3 dan Degenerate Primer AD1, AD2 and AD3. M = Marker Lamda HindIII, 1 = 1st Round of AD1 primer, 2 = 2nd Round of AD1 primer, 3 = 3rd Round of AD1 Primer, 4 = 1st Round of AD2 primer, 5 = 2nd Round of AD2 primer, 6 = 3rd Round of AD2 Primer, 7 = 1st Round of AD3 primer, 8 = 2nd Round of AD3 primer, 9 = 3rd Round of AD3 Primer. Red box indicated amplicon purified for sequencing (± 450bp)

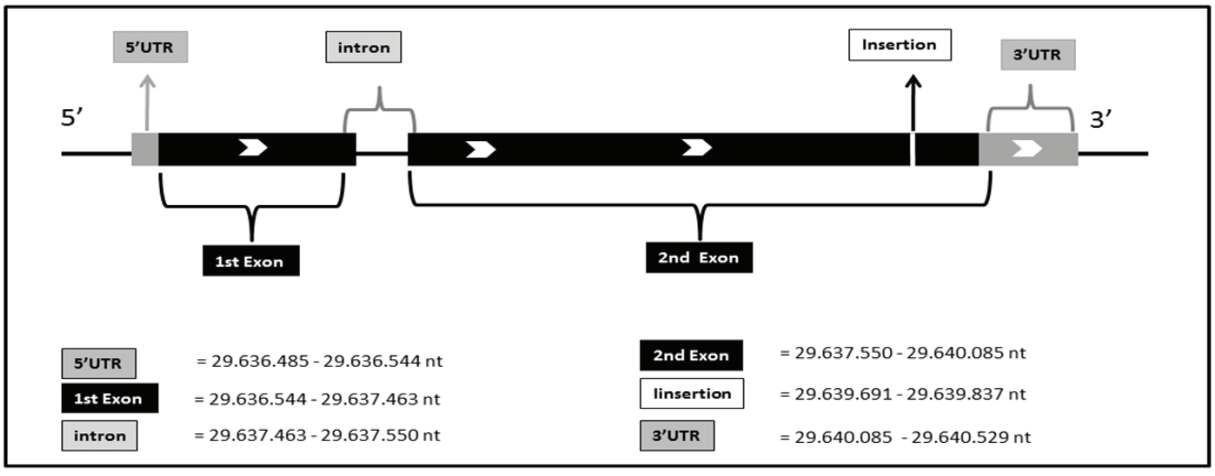

Figure 2. Scheme of *Os11g0686500*. The gene with exon, intron, untranslated region (UTR), and the location of insertion. (www.ncbi.nlm.nih.gov).

condition often affects the tolerance to another stress or condition. For example, exposure of plants to sublethal abiotic stress leads to enhanced biotic stress tolerance in tobacco (Yalpani et al., 1993) and Arabidobsis (Prasch and Sonnewald, 2013)

In this study, *Os11g0686500* which insertional mutation predicted to confer improved salinity tolerant phenotype of rice mutant line 170-10, has sequence similarities with a blast resistance *pikm2-TS* and – *pikm1TS* and is thought to be an example of a crosstalk - tolerance phenomenon between biotic and abiotic stresses. In the case of Arabidopsis, the tolerance to salinity and resistance to fungi was reported, the gene were MYB4 (Vanini *et al*., 2004).

The adaptation to various stresses has led to the development of common stress transduction pathways and includes, among others, the increased synthesis of secondary metabolites, Ca2+ fluxes, an oxidative burst and an overlapping set of stress response genes (Cheong *et al*., 2002, Roberts *et al*., 2002). In many species, genes have been identified, which respond to pathogen infection, pathogen defense signal components or elicitors and non-biotic stresses, such as salt or oxidative stress (Lee *et al*., 2004; Hong and Hwang, 2005).

Prediction of Upstream and Downstream Insertion Area

The activation-tagged contained four copy of enhancers, which allows the activation of transcription of genes in the vicinity (Suzuki *et al*., 2001). Enhancers can function in both directions, different orientations, up-stream and down-stream region to a certain distance (thousands of nucleotides) (Ramachandran and Sundaresan, 2001). To determine the relationship between the genes, we need information about the area surrounding genes up-stream and down-stream. Through the prediction of protein interaction databases (STRING) of www.expasy.org, the proteins marked with uncharacterized, unknown interaction.

NCBI reference sequence analysis showed that *Os11g0686100* gene positioned at up-stream of *Os11g0686500*, with distance were 32.169kb. *Os11g0686100* gene have 3,446 bp size, located from 29,600,872 to 29,604,317 nt on chromosome 11. According to the predictions of www.geneontology.org and databases on www.gramene.org, *Os11g0686100* on japonica rice, produce C79D7 protein, which belong to the cell wall-like protein. While at the down-stream area there is *Os11g0686900* gene with 9,335 bp size, lies from 29,651,805 to 29.661.139 nt (Figure 3). The distance from *Os11g0686500* gene were 47.276 kb. *Os11g0686900* belongs to disease resistance proteins group.

Analyses of Non-Coding Areas

We have applied approach from open source analysis tools to examine the proteins in the data base, the protein are listed as having unknown function. The approach identification with FGENESH of www.softberry.com of the non-coding region between *Os11g0686500* gene and *Os11g0686100* gene (non-coding area A) predicted area A1, which may contain one coding sequence consisted of 14 exon located at 29606121-29615120 nt of chromosome 11. The protein was predicted to be expressed on the membrane of chloroplasts. There was also another predicted coding region in area A2 located at 29615121-29624120 nt, which contained one exon. The predicted protein is predicted to be expressed in the mitochondria. Another predicted coding region in area A3 (Figure 3) located from 29,624,121 to 29,633,120 nt contained five exon. Analyses of regions between *Os11g0686500* and *Os11g0686900*, which are located from 29,642,121 to 29,651,120 nt (non-coding area B), predicted one coding region with three exons.

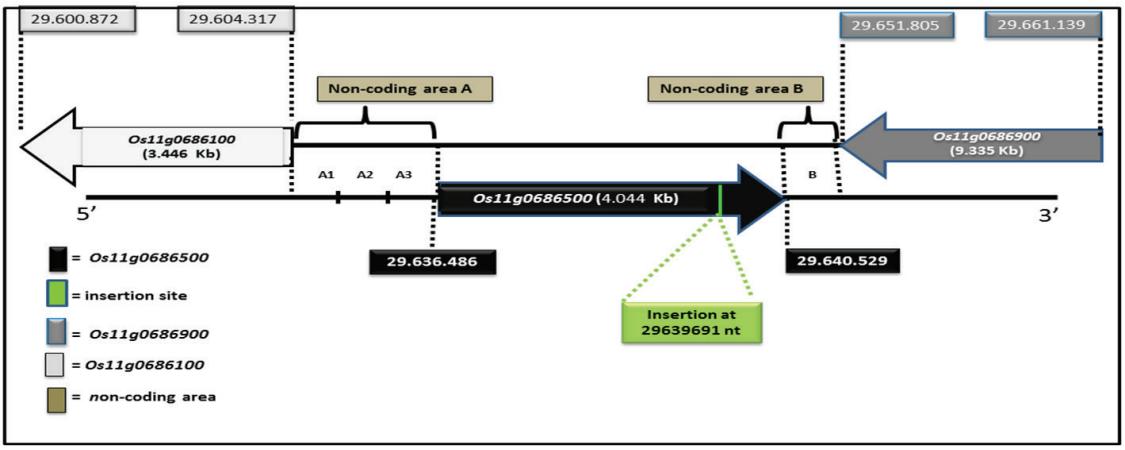

Figure 3. Insertion site and the position of *Os11g0686500* gene in chromosome 11 along with nearby genes, *Os11g0686100* and *Os11g0686900* (www.ncbi.nlm.nih.gov)

CONCLUSION

The analyses of the salinity stress tolerant mutant rice line 170-10 indicated that there was an insertion at the 2nd exon of the predicted coding sequence *Os11g0686500*. Since the insertional fragment contained activation tag, analyses of the flanking regions of the insertion site was important to obtain better picture of the genes might involve in the development of the increase salinity tolerant phenotypes.

Analyses of the flanking regions of the insertion site identified other predicted coding sequences. The roles of those genes identified in the improved salinity tolerance phenotypes might need to be analyzed.

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