ISOLATION OF UPSTREAM AND DOWNSTREAM FRAGMENT OF ACRYLTRANSFERASE (AT3) GENE FROM Capsicum frutescens L. CV. CAKRAHIJAU

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ABSTRACT
Capsicum frutescens cv. CakraHijau is a local cultivar that has been widely cultivated in Indonesia due to its several advantages, including its pungency. Pungent taste of Capsicum is generated by capsaicin compound encoded by AT3 gene. Recently, 404 bp fragment of AT3 gene had been isolated. This research aimed to isolate upstream and downstream fragments of AT3 gene. PCR method used two pairs of primers: F2/R2 (F2 5’-TCT CCA TGC TGA CAA CAA CA-3’, and R2 5’-CGA TGA AAG ATA GCT TGT G-3’) and F3/R3 (F3 5’-GCA TCT CTT GCA GAG AGC ATA G-3’, and R3 5’-TGT ACG CAC TCG TTG AGA CT-3’). F2/R2 primers amplified 326 bp upstream fragments, while F3/R3 primer amplified 261 bp downstream fragments. The alignment of those two fragments with one previously obtained produces a 675 bp partial sequence with 230 bp located upstream of presumed start codon. ClustalX analysis reveals that this fragment is located upper half compare to C. frutescens cv. Shuanla AT3 gene. Further primer design is necessary to obtain downstream of AT3 gene.

Key words: Capsicum frutescens cv. CakraHijau, Capsaicin, AT3 gene

INTRODUCTION
Capsicum frutescens L. is a member of Solanaceae’s family which is widely cultivated in Indonesia (Djarwaningsih, 2005). One local cultivar of the C. frutescens L. is CakraHijau. This cultivar has a high economic value for its high level of pungency and resistancy against disease (Rukmana, 2002).

The pungency of Capsicum frutescens L. comes from a secondary metabolite named capsaicin (Stewart et al., 2007). Capsaicin is syntheses in leaf and accumulated in placenta of fruit (Stewart et al., 2005). The biosynthesis of capsaicin involves two secondary metabolic pathways; there are phenylpropanoid and fatty acid pathway (Stewart et al., 2005). In capsaicin biosynthesis there is an enzyme which combine phenillylamine and 8-methyl-6-nonenoic acid forming Capsaicin. This enzyme is Capsaicin Synthase (CS) (Stewart et al., 2005), encoded by Acyltransferase gene (AT3) (Kim et al., 2001).

AT3 gene had been reported for three species i.e Capsicum annum, Capsicum chinense, and Capsicum frutescens (Stewart et al., 2005). For Capsicum frutescens’s AT3 had been reported from Capsicum frutescens cv. BG 2814-6 and Capsicum frutescens cv. Shuanla and partial segment of Capsicum frutescens cv. CakraHijau (Habibi et al., 2013).

Previous research on Capsicum frutescens cv. CakraHijau reported 404 bp length middle fragment of AT3 compared to Capsicum frutescens cv. BG 2814-6 and Capsicum frutescens cv. Shuanla (Habibi et al., 2013). This research was aimed to isolate an upstream and a...
downstream fragment of AT3 based on sequent of the fragment already revealed.

MATERIALS AND METHODS
Total DNA of Capsicum frutescens cv. CakraHijau was isolated according to the procedure of Nucleospin® II plant DNA isolation kit (Macherey-Nagel) with slight modification. AT3 fragments isolation was performed by PCR method. The primers designed based on AT3 sequences of Capsicum frutescens cv. CakraHijau previously reported (Habibi et al., 2013). The primers used are F2 5’-TGC TGA TCT CCA CAA CAA CA-3’, R2 5’-GCT ATA AAG TGA CGA TGT G-3’, F3 5’-GCA TCT CTT GCA AGC ATA GAG G-3’, and R3 5’-TGT TCG TTG ACG CAC AGA CT-3’. PCR cycles for F2/R2 is as follow: denaturation 94°C for 1 min, annealing 52°C for 1 min, and extension 72°C for 1 min. PCR cycles for F3/R3 is as follow: denaturation 94°C for 1 min, annealing 55°C for 1 min, and extension 72°C for 1 min. PCR products were checked using 1.5% agarose gel electrophoresis and sequenced by the Big Dye Transluminator through ABI 3130 Genetic analyzer machine at Eijkman Institute for Molecular Biology Jakarta. The sequencing results was analyzed using DNA Baser, BLAST, Clustal X software, and SIXFRAME.

RESULTS AND DISCUSSION
In this research, F2/R2 primers pair obtained 326 bp upstream (Figure 1A), meanwhile F3/R3 primers pairs obtained 261 bp downstream fragments relative to AT3 gene fragment had been previously isolated (Figure 1B).

![Figure 1. AT3 gene consensus sequence isolated from C. frutescens cv. CakraHijau using pair of primers: (A) F2/R2; (B) F3/R3](image)

BLAST analysis results shows that the query coverage of F2/R2 fragment is 8% and 5% of the total AT3 sequence of C. frutescens cv. BG2814-6 and C. frutescens cv. Shuanla, respectively; and sharing 99% and 98% similarity with AT3 of C. frutescens cv. BG2814-6 and C. frutescens cv. Shuanla, respectively for (Figure 2).

BLAST analysis results of F3/R3 fragments shows query coverage of 7% and 17% from total sequence of C. frutescens cv. BG2814-6 and C. frutescens cv. Shuanla AT3 gene, respectively; and sharing 96% and 95% similarity compared to C. frutescens cv. BG2814-6 and C. frutescens cv. Shuanla AT3 gene, respectively (Figure 3).
The result of merging the $F_2/R_2$ and $F_3/R_3$ fragments with previously obtained fragments ($F_1/R_1$) of $AT_3$ from $C. frutescens$ cv. CakraHijau resulted on 905 bp length fragment which arranged as follow: nucleotide 1 to 326 is $F_2/R_2$ fragment, nucleotide 327 to 646 is $F_1/R_1$ fragment, and nucleotide 646 to 905 is $F_3/R_3$ fragment. Open reading frame analysis reveal that ORF #3 is the most possible reading frame of the target gene since it is contained no stop codon in the middle of the fragment (Figure 4).

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The result of BLAST analysis of merged $F_1/R_1-F_2/R_2-F_3/R_3$ sequence compared to $AT3$ gene of *C. frutescens*cv. Shuanla and *C. frutescens*cv. BG 2814-6 shows that the fragment spends 675 bp and 905 bp of total $AT3$ length from both references, respectively. Moreover, BLAST result also shows that the query coverage of that target genes compared to $AT3$ complete sequence of *C. frutescens* cv. BG 2814-6 and *C. frutescens* cv. Shuanla are 24% and 40%, respectively, with 99% similarity level to both references sequence (Figure 5). These results give more confident that the successfully isolated fragments are correctly parts of $AT3$ gene.

![Figure 5. The results of the BLAST sequence AT3 C. frutescens cv. CakraHijau with gene comparison: (A) C. frutescens cv. BG2814-6, (B) C. frutescens cv. Shuanla. 1: F2R2 fragment; 2: F1/R1 fragment; 3: F3R3 fragment.](image)

ClustalX alignment analysis of merged $F_1/R_1-F_2/R_2-F_3/R_3$ fragment amino acid sequence revealed that $F_1/R_1-F_2/R_2-F_3/R_3$ fragment compiled the 1$^{st}$ up to 225$^{th}$ amino acid sequence of *C. frutescens* cv. Shuanla (Figure 6). Taking a consideration of $F_1/R_1-F_2/R_2-F_3/R_3$ position toward $AT3$ gene of *Capsicum frutescens* cv BG2814-6 revealed by BLAST analysis, we suggest that upstream sequence of *C. frutescens* cv. BG2814-6 $AT3$ gene relatives to both $AT3$ of *C. frutescens* cv. CakraHijau and *Capsicum frutescens* cv Shuanla is a promoter region of the respective gene.

Further analysis shows that $F_1/R_1-F_2/R_2-F_3/R_3$ fragment is highly conserved compared to the respective $AT3$ sequence from both *C. frutescens* cv. Shuanla and *C. frutescens* cv. BG2814-6. There are only three amino acids which are exclusively belongs to *C. frutescens* cv. CakraHijau, i.e. amino acid #14, 191, 211; with amino acid #191 is an inserted amino acid compared to both *C. frutescens* cv. Shuanla and *C. frutescens* cv. BG2814-6.

Examining the position of $F_1/R_1-F_2/R_2-F_3/R_3$ fragment relative to both *C. frutescens* cv. Shuanla and *C. frutescens* cv. BG2814-6, possible open reading frame, and taking an account on the amino acid alignment result with those two references, which gives a highly shared amino acid sequences and it is suggested that there is about 900 bp fragment left which has not yet been isolated. There are more works need to be done to retrieve a complete sequence of this gene.
This research combined with previously obtained fragment has successfully isolated as long as 905 bp \textit{AT3} gene fragment which is 675 bp length of the 3’ fragment is suggested to encode a functional part of \textit{C. frustescens cv. CakraHijau} \textit{AT3} gene. Further research is ongoing in order to retrieve as long as 972 bp of the 3’ last fragment to obtain the complete sequence of \textit{AT3} gene from \textit{Capsicum frustescens cv. CakraHijau}. A careful primers designing is necessary.

CONCLUSION

This research combined with previously obtained fragment has successfully isolated as long as 905 bp \textit{AT3} gene fragment which is 675 bp length of the 3’ fragment is suggested to encode a functional part of \textit{C. frustescens cv. CakraHijau} \textit{AT3} gene. Further research is ongoing in order to retrieve as long as 972 bp of the 3’ last fragment to obtain the complete sequence of \textit{AT3} gene from \textit{Capsicum frustescens cv. CakraHijau}. A careful primers designing is necessary.
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