KnowledgeE Publishing Services
KnE Life Sciences
ISSN 2413-0877 Volume 2 (2015) 21-26
The 3rd International Conference on Biological Science 2013
(The 3rd ICBS-2013)

ISOLATION OF UPSTREAM AND DOWNSTREAM FRAGMENT OF *ACYLTRANSFERASE* (*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

Selection and Peer-review under responsibility of the 3rd ICBS-2013
Doi http://dx.doi.org/10.18502/kls.v2i1.111

downstreamfragment 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).

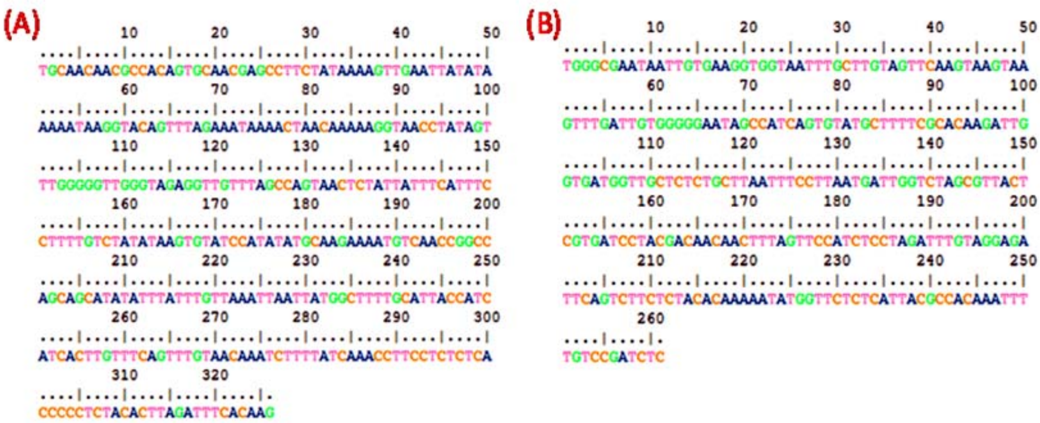

Figure 1. *AT3* gene consensus sequence isolated from *C. frutescens* cv. CakraHijau using pair of primers: (A) F2/R2; (B) F3R3

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, respectivelty; 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).

Figure 2. BLAST analysis result of C. frutescens cv. CakraHijau *AT3* gene obtained by F2/R2 primers compared to *AT3* of : A. *C. frutescens* cv. BG2814-6; B. *C. frutescens* cv. Shuanla

Figure 3. BLAST analysis result of C. frutescens cv. CakraHijau *AT3* gene obtained by F3/R3 primers compared to *AT3* of : A. *C. frutescens* cv. BG2814-6; B. *C. frutescens* cv. Shuanla

The result of merging the *F2/R2* and *F3/R3* fragments with previously obtained fragments (*F1/R1*) of *AT3* from *C. frutescens* cv. CakraHijau resulted on 905 bp length fragment which arranged as follow: nucleotide 1 to 326 is *F2/R2* fragment, nucleotide 327 to 646 is *F1/R1* fragment, and nucleotide 646 to 905 is *F3/R3* 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).

```
     Q  Q  R  H  S  A  T  S  L  L* K  L  N  Y  I  K  I  R  Y
3    caacaacgccacagtgcaacgagccttctataaaagttgaattatataaaaataaggtac  62
     S  L  E  I  K  L  T  K  R  *  P  I  V  W  G  L  G  R  G  C
63   agtttagaaataaaactaacaaaaaggtaacctatagtttggggggttgggtagaggttgt  122
     L  A  S  N  S  I  I  S  F  P  F  V  Y  I  S  V  S  I  Y  A
123  ttagccagtaactctattatttcatttccttttgtctatataagtgtatccatatatgca  182
     R  K  C  Q  P  A  S  S  I  Y  L  F  V  K  L  I  M  A  F  A
183  agaaaatgtcaaccggccagcagcatatatttatttgttaaattaattatggcttttgca  242
     L  P  S  S  L  V  S  V  C  N  K  S  F  I  K  P  S  S  L  T
243  ttaccatcatcacttgtttcagtttgtaacaaatcttttatcaaaccttcctctctcacc  302
     P  S  T  L  R  F  H  K  L  S  F  I  D  Q  S  L  S  N  M  Y
303  ccctctacacttagatttcacaagctatctttcatcgatcaatctttaagtaatatgtat  362
     I  P  C  A  F  F  Y  P  K  V  Q  Q  R  L  E  D  S  K  N  S
363  atcccttgtgcatttttttaccctaaagtacaacaaagactagaagactccaaaaattct  422
     D  E  L  S  H  I  A  H  L  L  Q  T  S  L  S  Q  T  L  V  S
423  gatgagctttcccatatagcccacttgctacaaacatctctatcacaaactctagtctct  482
     Y  Y  P  Y  A  G  K  L  K  D  N  A  T  V  D  C  N  D  M  G
483  tactatccttatgctggaaagttgaaggacaatgctactgttgactgtaacgatatggga  542
     A  E  F  L  S  V  R  I  K  C  S  M  S  E  I  L  D  H  P  H
543  gctgagttcttgagtgttcgaataaaatgttccatgtctgaaattcttgatcatcctcat  602
     A  S  L  A  E  S  I  V  L  P  K  D  L  P  W  A  N  N  C  E
603  gcatctcttgcagagagcatagttttgcccaaggatttgccttgggcgaataattgtgaa  662
     G  G  N  L  L  V  V  Q  V  S  K  F  D  C  G  G  I  A  I  S
663  ggtggtaatttgcttgtagttcaagtaagtaagtttgattgtggggaatagccatcagt  722
     V  C  F  S  H  K  I  G  D  G  C  S  L  L  N  F  L  N  D  W
723  gtatgcttttcgcacaagattggtgatggttgctctctgcttaatttccttaatgattgg  782
     S  S  V  T  R  D  P  T  TTT L  V  P  S  P  R  F  V  G
783  tctagcgttactcgtgatcctacgacaacaactttagttccatctcctagatttgtagga  842
     D  S  V  F  S  T  Q  K  Y  G  S  L  I  T  P  Q  I  L  S  D
843  gattcagtcttctctacacaaaaatatggttctctcattacgccacaaattttgtccgat  902
     L
903  ctc   905
```

Figure 4. The third ORF of complete fragment of merged *F2/R2* (Blue font) and *F3/R3* (Pink font) into the previously obtained *F1/R1* fragments (Black font) of *C. frutescens* cv. CakraHijau *AT3*. Red asterisk depict STOP codon; M: Methionin or START codon.

The result of BLAST analysis of merged *F1/R1-F2/R2-F3/R3* 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.

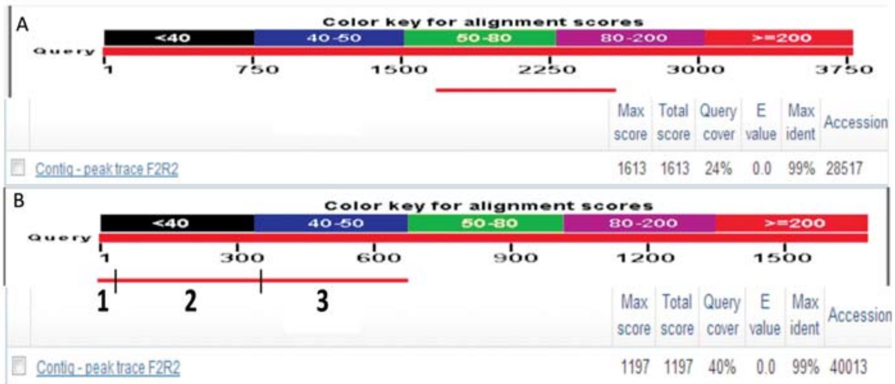

Figure 5. The results of the BLAST sequence AT3 C. frutescens cv. CakraHijau with gene comparison: (A) C. frutescens cv. BG2814-6, (B) C. frutescens cv. Shuanla. 1: F2R2 fragment; 2: F1/R1 fragment; 3: F3R3 fragment.

ClustalX alignment analysis of merged *F1/R1-F2/R2-F3/R3* 3rd ORFamino acid sequence revealed that *F1/R1-F2/R2-F3/R3* fragment compiled the 1st up to 225th amino acid sequence of *C. frutescens* cv. Shuanla (Figure 6). Taking a consideration of *F1/R1-F2/R2-F3/R3* position toward *AT3* gene of *Capsicum frustescens* cv BG2814-6 revealed by BLAST analysis, we suggest that upstream sequence of *C. frustescens* 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 *F1/R1-F2/R2-F3/R3* fragment is highly conserved compared to the respective *AT3* sequence from both *C. frutescens* cv. Shuanla and *C. frustescens* 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. frustescens* cv. BG2814-6.

Examining the position of *F1/R1-F2/R2-F3/R3* fragment relative to both *C. frutescens* cv. Shuanla and *C. frustescens* 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.

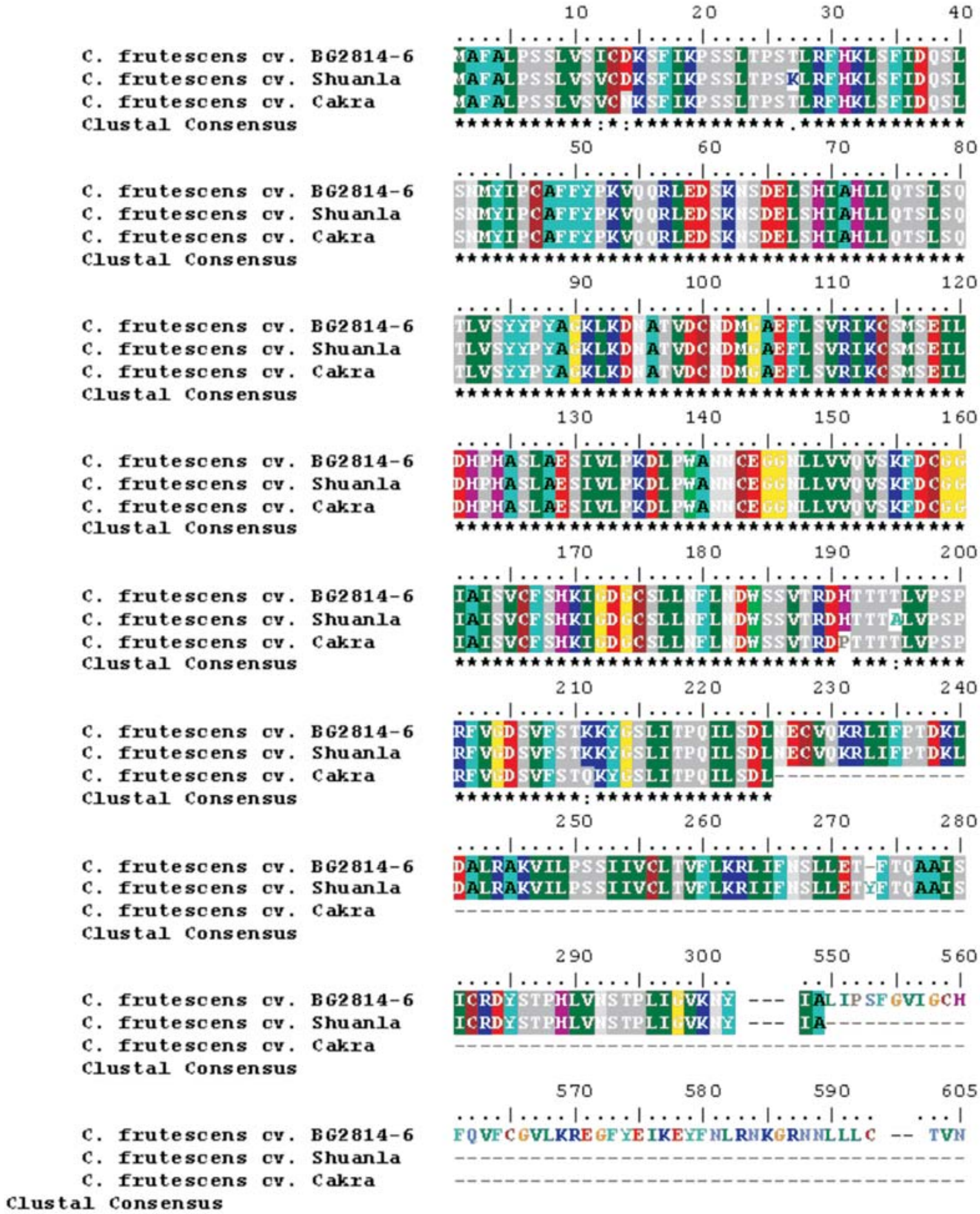

Figure 6. Alignment of amino acid sequences of *C. frutescens* cv. CakraHijau*AT3* with *C. frutescens* cv. BG2814-6 and *C. frutescens* cv. Shuanla; Asterisks depict conserved amino acids.

CONCLUSION

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

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