The role of *AUX1* gene and auxin content to the branching phenotype of Kenaf (*Hibiscus cannabinus* L.)

E.L. Arumingtyas¹, R. Mastuti² and S. Indriyani³

¹Laboratory of Molecular Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang, Indonesia

²Laboratory of Plant Physiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang, Indonesia

³Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang, Indonesia

ABSTRACT

The objectives of this research were to identify auxin gene, *AUX1*, and to determine the plant auxin content and their role in conferring branching on Kenaf. PCR analysis using *AUX1* primer capable to amplify the DNA of non branching (KR11) and branching kenaf mutant, resulting in 800 bp PCR product. The sequence of the PCR product showed high degree of homology with the sequence of *AUX1* gene of other plants in the NCBI GenBank database, confirming kenaf possession of the gene *AUX1*. However, some variation on the DNA sequence was found between branching and non branching phenotype indicated allele differences of the same gene which were responsible for the variation in the type of branching. Identification of auxin content in the roots, apical shoot, and axillary branches using spectrophotometry method showed that the branching plant has higher auxin content in the apical shoot compared to the content in the branches. This indicate that *AUX1* controls the formation of branches by controlling either the content of auxin in the apical shoot and branches, or the ratio of auxin content in the shoot and branches. [*Physiol. Mol. Biol. Plants* 2010; 16(1) : 93-98] E-mail : larasbio@gmail.com

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INTRODUCTION

Branch formation in plants has been known to be related to the phenomenon of apical dominance which was related to the presence of auxin. Genes which convert branching phenotype and are related to auxin signalling have been identified on *Arabidopsis thaliana*, among them are auxin influx carrier (*AUX1*)/like-*AUX1* (*LAX*) and *AXR1* genes. The *AUX1/LAX* gene produce auxin influx carrier protein 1 (*AUX1*)/like-*AUX1* (*LAX*) proteins which involved in active auxin transport across cell membrane (Bennett *et al.* 1996; Parry *et al.*, 2004). Auxin resistance1 (*AXR1*) gene involved in the degradation of negative regulator in the signaling auxin process (Leyser *et al.*, 1993; Paciorek and Friml, 2006). Other than these roles, it is also known that *AUX1* and

AXR1 dominant allele cause non branching appearance of plant, on the other hand the recessive allele (*aux1* and *axr1*) causes branching appearance (Stirnberg *et al.*, 1999) which is caused by the loss-of-function of the genes (Nordström *et al.*, 2004).

Although there are many genes that have been known to influence the auxin transport and signalling. However, the mechanism of the hormone in influencing the apical dominance remains unclear. Radiolabelled auxin applied apically does not enter the bud in any quantity (Booker *et al.*, 2003), and the levels of auxin in buds often increase at the time of bud formation (Gocal *et al.*, 1991). Additionally, direct auxin application onto the bud does not inhibit outgrowth (Brown *et al.*, 1979).

Further studies show evidence that auxin does not directly affect branching (Romano *et al.*, 1993, 1995; Tamas, 1995; Cline, 1991, 1994; Nordström *et al.*, 2004; Leyser, 2006; Quint and Gray, 2006; Teale *et al.*, 2006).

Correspondence and reprint requests : Estri L. Arumingtyas
Phone: 62-0341-575841; Fax: 62-0341-554403

There was an indication of synergic role of auxin and cytokinin in the branching emergence (Cline, 1991; 1994). Application of auxin rapidly reduces flux through the isopentenyladenosine-5'-monophosphate-independent cytokinin biosynthetic pathway (Nordström *et al.*, 2004). However, until now, the control mechanism of branching by auxin and cytokinin remains uncovered yet. Some experiments show the evidence of a signal or the presence of substance that connects the level of auxin with the number of branching, however identity of the substance is not yet reported. Due to the lack of information about the identity of the substance and the evidence of existence of auxin along with branching formation in kenaf. This experiment was conducted to identify the gene involved in the synthesis of auxin. In the previous experiment mutation was induced using chemical mutagen *ethyl methanesulfonate* (EMS), giving rise to branching phenotype of mutant line (Arumingtyas *et al.*, 2005).

MATERIALS AND METHODS

Plant material and DNA isolation

Plant material used was non branching kenaf line KR11 and the branching mutant arose from EMS mutation of KR11. The seed of original non branching kenaf line KR11 was supplied by Indonesian Tobacco and Fibre Crops Research Institute, Malang, Indonesia. Plant was grown in polybags at green house, one plant per polybag. The auxin and the genomic DNA were extracted from 3 months old plants, when the plants entered the end of vegetative growth phase. DNA isolation was conducted using CTAB Doyle and Doyle (1987).

Polymerase Chain Reaction (PCR)

PCR was done using forward and reverse primers designed based on the sequence of *AUX1* gene of *Arabidopsis thaliana* with the sequence of : Forward: 5'-CAAGTGGCACAAGTGCTGTT-3' and Reverse : 5'-GGTGGCTCCGGTAAAGTACA-3'. Reaction mixture (25 µl) consist of 10x buffer Ex Taq Polymerase, 2 mM MgCl₂, 200 µM dNTP, 25 pmol primer, 1U Taq Polymerase (Takara, Japan) and aquadest. Program was set on 93 °C for 1 minutes preheating , continued with 35 cycles consist of 93 °C for 1 minute denaturation, 56 °C of 30 seconds annealing, and 72 °C for 1 minute extension, and finally 72 °C for 10 minutes for the last extension and 5 minutes cooling at 4 °C. The PCR product was visualized on 1.5 % agarose gel.

Sequencing

Sequencing for AUX 1 PCR product was conducted using ABI 3130 Genetic Analyzer machine at Eijkmann Institute, Jakarta, Indonesia.

Auxin extraction

Extraction of auxin indol-3- acetic acid (IAA) was done using the method of Unyayar *et al.* (1996). The samples were collected from 5 parts of the plant including the root tip, the bud at the 10th node, the bud at the 15th node, the bud at the 20th node and the shoot tip, about 3 cm from the tip of each part, 1 gram each. The experiment was laid out in Completely Randomized Design using three replication. Statistical analysis of the result were done in an SPSS 10.0 for Windows and the comparison of auxin content were computed as per Least Significant Difference (Steel and Torrie, 1984).

RESULTS

PCR and Sequencing Analysis of AUX1 gene

PCR reaction using DNA template which was isolated from kenaf leaves. Primers that was used in the PCR reaction capable to amplify the gene with the fragment size of 800 bp (Figure 1).

The fragment was detected, both on the control and mutant line. To know, whether the alleles of the gene are different in the control and the mutant line, sequencing was conducted. The sequencing result showed in the Figure 2a,b. Analysis of the sequence using Basic Local Alignments Search Tool (BLAST) of

Fig. 1. Ampification of AUX1 gene on kenaf. M = Marker, 1 = Control nonbranching (KR11), 2-5 = Branching Mutants.

Table 1. The changing of nitrogen base, codon, and amino acid sequence in the control (KR11) to the mutant line

Mutation site	Bases Changing	Codon Changing	Amino Acid Changing
1	Tranversion	GAG→CAG	Glu→Gln
2	Tranversion	TGG→TGT	Trp→Cys
3, 4	Deletion	TGG→—G	Trp→-
5	Transition	ATA→GTA	Ile→Val
6	Transition	CGG→CAG	Arg→Gln
12	Tranversion	GCC→TCC	Ala→Ser
13	Transition	CCC→TCC	Pro→Ser
15	Tranversion	TAA→TAC	Stop→Tyr
16	Tranversion	AAG→AAC	Lys→Asn
21, 22	Tranversion	CTA→ATC	Leu→Ile
25	Tranversion	CCT→GCT	Pro→Ala
28	Tranversion	GCT→TCT	Ala→Ser
29	Tranversion	GAG→GAT	Glu→Asp
34	Tranversion	TTA→ATA	Leu→Ile
35	Tranversion	TAG→GAG	Stop→Glu
36	Tranversion	AGG→TGG	Arg→Trp
37	Tranversion	GGG→GTG	Gly→Val
38	Tranversion	CTG→GTG	Leu→Val
39	Tranversion	CTA→ATA	Leu→Ile
40	Tranversion	TGT→AGT	Cys→Ser
41	Tranversion	GGA→CGA	Gly→Arg
42	Transition	CTT→CTC	Leu→Leu
43	Transition	GCT→GTT	Ala→Val
44	Transition	TTC→TCC	Phe→Ser
45	Tranversion	TGT→TCT	Cys→Ser
46	Transition	ACA→GCA	Thr→Ala
47	Transition	TCG→TTG	Ser→Leu
48	Tranversion	TTC→TAC	Phe→Tyr
49	Tranversion	TAT→GAT	Tyr→Asp
51	Transition	ATC→ACC	Ile→Thr
53	Tranversion	ATA→TTA	Ile→Leu
55, 56	Tranversion	AAG→CGG	Lys→Arg
57	Transition	ATC→GTC	Ile→Val
58	Tranversion	TCC→TAC	Ser→Tyr

Table 1. Continued

Mutation site	Bases Changing	Codon Changing	Amino Acid Changing
59, 60	Tranversion	ATT→AGC	Ile→Ser
61	Insertion	TG→TGT	-→Cys
62	Tranversion	TTA→TTT	Leu→Phe
63	Transition	TCT→TTT	Ser→Phe
64	Tranversion	ACA→ACT	Thr→Thr
65	Tranversion	TGA→TTA	Stop→Leu
66	Tranversion	GAA→GAC	Glu→Asp
67, 68	Tranversion	TGA→GGT	Stop→Gly
69, 70	Tranversion	ACC→TGC	Thr→Cys
71, 72, 73	Tranversion	GAC→TTT	Asp→Phe
74	Tranversion	CTT→GTT	Leu→Val
75	Tranversion	TAT→TAA	Tyr→Stop
76	Tranversion	TAA→GAA	Stop→Glu
77	Transition	CAA→TAA	Gln→Stop
78	Transition	TAT→CAT	Tyr→His

Table 2. Auxin (IAA) level (μg/g of tissue) at root tip, shoot tip, and branch tip of branching mutant and non branching control plants

Plant Line	Plant Part	Auxin concentration (μg/g of tissue)
KR11	Root tip	0.217 ± 0.033a
	Node 10	0.295 ± 0.034ab
	Node 15	0.365 ± 0.046b
	Node 20	0.409 ± 0.033b
	Shoot tip	0.649 ± 0.057c
Mutant	Root tip	0.214 ± 0.018a
	Node 10	0.269 ± 0.017b
	Node 15	0.327 ± 0.012b
	Node 20	0.346 ± 0.049b
	Shoot tip	0.515 ± 0.017c

Note: different characters following the value of IAA concentration show significant differences based on LSD 0.05

GACTGCTTATCTCATGAGTGGTGGCTCTACATAGAGTACAGAAGCAGGAAAGAGAAAGAGAACGTCAGCT
 TCAAGAACCATGTCATCCGGGTTGAGTCCCTGCCCCCGAGTTAAAAGACTTTGGGAAATCAGCTAAAA
 CTACCTGCTGCTGAGGGATTTTGTGGGGTCTTATAGAGGGATGGGCTGTGGTGGCTATATTGTAGGGAAA
 AAGGACTTGCTTTCAACTGTACATTCCCCTCGTTCGGATATGCCATCCAACCTTATAGCTTGTGCAAAGGG
 ACCATCTATCTCACTTATCCATTCCATTCTCTGTTATCTCAAACTGGACAACGTGAAATTAAGAACTTC
 CTTGAACCGACCTTTATTAACAATAATATTTATTACTACACAACAGACAAAGAGAGAAGACAAGGACCTC
 CTTTTTTTTGGAGGATGCTGCCCATTATTCTTCATCTTCCCCTTTCTCCACTACCCTTTTCTTCTTTTC
 TTCTTGGTATTATGACCTCTACCGCGAGCTCAGCTACACGGCCGCTTCTACTAGTCGTCACCGGCTAATC
 ACCCACTTCATTCTGAACTCTTTGTTTTTCACCTATACCAAGATTGTATTTATTTTCAGGTGGACGGACTCC
 GATCCAGGGGACGGGGTGACTCGAGCTGTCCTCAAGTAGGCGGCAACACTAACCGACCCCCAAA

(a)

ATTTTCTATGGACTTGTGGGAAGCTGGACTGCTTATCTCATCAGTGTGCTCTACGTAGAGTACAGAAGCA
 GGAAAGAGAAAGAGAACGTCAGCTTCAAGAACCATGTCATCCAGGTTTCTTTTTCCTTCCCTCCCCAGTTA
 CAACACTTTACCAAATCAGCTGACCATCGCTCCCTCTGATGGATTTTTCGGTGTAAAGAGTGGGATGTG
 GTGTGGTGGATATATAGTAGGGAAAAACGACTCGTTTCCAACCTCTGCATTCCCCTTGTACGGAGATGCGA
 CCCAACTATTAGCTTGTGCGCGGGGACCATCTGTCTCACTTATCCATTACAGCCTCTGTTTTTTTTCAAAA
 CTGGACTACGTTAAATTAAGACCTTCCTGGTTGCTTTGTTTAAGAATAATAACATTTATTACTACATCAA
 CAATGAAGACATGAAGAAATGGAGGACCTTTTTGTTGGAGGATGCTGCTCCACTACCTTCTTTATCCCCT
 CCTTTCTCAACTACATCAGTTCTTCTTTTCTTCTTGGTATGATGACCACCTACGCCGCCTGCTGGTTTAT
 GGCCGCTGCTACTATTGCCACGGGCAGCTCAAACAAACCACATAACTGAATGCTTTGGCTGTATCTATA
 ACTAAGAGTGAATTTAGCTCAGGCGGACCGAGTACGATCAGGCAGACGGGGTGACACACAGCTGTACATC
 ACCGCTGCCGGCAGCACCTACCGGAGCCACCA

(b)

Fig. 2. (a) Sequence of AUX1 gene fragment of control KR11, (b) Sequence of AUX1 gene fragment of mutant line.

NCBI, show high homology between the amplified sequence of non branching KR11 and the branching mutant line with *AUX1* gene or *AUX1*-like gene of *Pisum sativum*, *Prunus avium*, *Casuarina glauca*, *Populus tomentosa*, *Cucumis sativus*, *Dianthus caryophyllus* and *Arabidopsis thaliana*. Comparison of the sequence of control KR11 and the mutant line using BLAST for two sequence show some mutation site as shown at Figure 3.

Auxin (IAA) content of plant

Auxin (IAA) were detected in the root tip, shoot tip and branching tip (node 10, 15 and 20) of both non branching KR11 and branching mutant plants. Measuring IAA content in those parts of plant expected to

uncovered its relation with the formation of branches. Table 2 showed the auxin level in the five parts of plant.

The IAA level of both non branching KR11 and branching mutant plants showed significant differences at among it was in the root tip, branch tip and the shoot tip. The level decreased from the shoot tip down to the root tip. Similar pattern of IAA level was found in the non branching KR11 and branching mutant plants.

DISCUSSION

Homology analysis using BLAST showed that the high sequence homology of the PCR product with AUX1

Fig. 3. Alignment analysis between the sequence of AUX1 gene of control KR11 and the mutant line. Circles shows nitrogen base differences

gene or AUX1-like sequence indicate that kenaf possesses a similar gene. Seventy-eight mutation sites were found in the fragment of AUX1 gene. The difference in some nitrogen bases may be the cause of difference in the morphology of kenaf plant from non-branching to branching phenotype. Further, analysis in the mutation of nitrogen bases was shown in Table 1. Some types of mutation seen were transition, transversion, insertion, and deletion. Insertion and deletion of N base are capable of inducing frame shift mutation. This, in turn, will cause large changes in the protein translated from the codon. From all mutations detected, 78.8 % were missense and nonsense mutations, whereas 21.2 % of them were silent mutations which were caused by the change of purine. This phenomenon was in accordance with Friedberg *et al.* (1995) that an addition of alkyl group to DNA bases happened non-randomly. Ethyl Methane sulfonate (EMS) has been known to add alkyl group to purine base adenine or guanine and never to pyrimidine bases, cytosine and thymine. A row of guanines will increase its negative electrostatic potential and make it easier to an alkyl group which is electrophilic. Some potential sites of mutation including 4 nitrogen

base: N¹, N³, N⁶ and N⁷ of adenine, N¹, N², N³, N⁷, O⁶ of guanine, N³, N⁴, O² of cytosine, and N³, O², O⁴ of thymine (Friedberg *et al.*, 1995; Russel, 1994; Suzuki, 1989; Segal, 1984).

Concerning the IAA content of plant, it was observed that the control non-branching plants showed similar levels of IAA content in every part of the plants with the branching mutant plants. Both the non-branching and branching plant showed a decreased level of IAA from the shoot tip to the root tip. This phenomenon is in accordance with Ljung *et al.*, (2001) that the Indole-3-acetic acid (IAA), the most abundant type of auxin in plants, has been shown to be abundantly synthesized in the shoot apex and young expanding leaves. The auxin is transported basipetally down the shoot in a polar manner by active transport in the polar transport stream in the vascular parenchyma (Blakeslee *et al.*, 2005).

Although the DNA analysis showed differences in the sequence of K11 compared to the mutant, there was no evidence that the differences affected the level of IAA in the plants. This is suggested that the IAA level in the plant was not only controlled by AUX1 gene but also by other genes as reported by Leyser, 2006; Quint and Gray, 2006; Teale *et al.*, 2006. In turn, the branching phenotype was not only manifested by the existence of auxin but also other hormones, since there were similar patterns of IAA levels in the branching and non-branching plants. There is evidence that auxin can regulate cytokinin biosynthesis. Application of auxin rapidly reduces flux through the isopentenyladenosine-5'-monophosphate-independent cytokinin biosynthetic pathway (Nordström *et al.*, 2004). On the other hand, according to nutrition theory of apical dominance, cytokinin transported from root to the branch will cause the formation of sink of nutrition in the branch, which in turn will lead to the rapid growth of branch (Cline, 1994). The existence of cytokinin in the branch will support the enhancement of the growth of branches. However, further confirmation is needed.

For the conclusion, AUX1 gene affected the branching appearance on kenaf; however, the level of IAA in the plants was not fully controlled by one gene. Furthermore, the branching phenotype was the result of a cross talk between auxin and other hormone(s) yet to be discovered.

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