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SHORT COMMUNICATION

Cloning and Characterization of Partial Chlorophyll a Oxygenase (*CAO*) Gene Involved in Shade Tolerance Mechanism in Soybean

Nurul Khumaida*[A)], Kisman[B)], Didy Sopandie[A)]

[A)] Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University (IPB). Jl. Meranti, Kampus IPB Darmaga, Bogor 16680 Indonesia.
[B)] Department of Agronomy, Faculty of Agriculture, Mataram University. Jl. Pendidikan 37, Mataram, West Nusa Tenggara, Indonesia

*Corresponding author; email: nkhumaida@yahoo.com

Abstract

Plant tolerance mechanism under shade stress can be indicated by high chlorophyll-b content, which is synthesized from chlorophyll a by chlorophyll a-oxygenase (CAO) gene. This study reported the CAO gene involvement in shade tolerance mechanism in soybean. Total RNA was isolated from the second trifoliate leaves of soybean plants, and the first strand cDNA was prepared using Reverse Transcriptase Moloney Murine Leukemia Virus (RT-M-MLV) (RNase H^-). Northern blot hybridization was used to analyze expression of partial CAO gene. The results showed that one partial gene, CAO 3-4 (1,052 bp), comprised of 292 adenine (a), 241 cytosine (c), 276 guanine (g) and 242 thymine (t), therefore the number of amino acid deduction was 338. The expression of CAO 3-4 partial gene was higher in shade tolerant genotypes than in shade sensitive genotypes under 50% shade or dark condition. These results demonstrated that the CAO gene is involved in shade tolerance mechanism of soybean.

Keywords: low light intensity, shading, chlorophyll b, Glycine max

Introduction

High nutrient content in soybean seeds is one of the reasons for categorizing soybean as one of the important food crops in Indonesia. National demand of soybean seed increases every year (ca 1.2 million ton) along with the people's increasing awareness on the importance of soybean-based food products. However, Indonesian national soybean production has been stagnant at about 730,000 ton per year. A potential strategy to increase national soybean production is by using lands under canopies of estate crops such as rubber and palm oil, agroforestry, or by intercropped with other food crops. These systems, however, have a main limiting factor, i.e. is low light intensity due to tree canopy shading.

Soybeans grown under simulated shaded condition using 50% shade showed changes in agronomical, morpho-anatomical, physiological, and molecular characters. These changes are forms of soybean adaptation mechanism to low light stress through avoidance and tolerance mechanisms. One important indication of shade tolerance mechanism is the increase of chlorophyll b formation from chlorophyll a.

Chlorophyll (chl) b pigment is essential for efficient photosynthesis in plants; clorophyll b is required for assembly of light-harvesting complexes (LHCs) (Hoober and Eggink, 2001). Previous studies have revealed that chlorophyll b in leaves of upland rice and soybean increased under shade stress, which resulted in decreases of chlorophyll a/b ratio (Sopandie et al., 2006). It is also known that an increase in leaf chlorophyll b increased antenna size as an implication of decreased chlorophyll a/b ratio (Yamasato et al., 2005). Expression of LHCP gene on soybean under different light intensity indicated positive correlation between LHCP gene expression and chlorophyll b content, whereas it was negatively correlated to chlorophyll a/b ratio (Khumaida, 2002). Low light intensity decreased chlorophyll a/b ratio as an effect of the increased chlorophyll b, and correlated with the increased of chlorophyll a/b

protein at LHCII (Hidema et al., 1992). Antenna enlargement for the photosystem II enhanced light capture efficiency (LCE); and even though chlorophyll content increases, the level of leaf chlorophyll decreases due to thinner leaves under shade (Nilsen and Orcutt, 1996). Plants under low light intensity have larger chlorophyll antenna because the photosystems (PS) contain a relatively high chlorophyll b and light harvesting complex of chlorophyll a-b (LHC), and a lower chlorophyll a/b ratio (Masuda et al., 2003). On the other hand, plants under the high light intensity have smaller antennas due to a lower chlorophyll c contained in the photosystems, smaller LHC antennas and a higher chlorophyll a/b ratio. The ability of plants to enlarge or reduce the antenna size allows plants to adapt under variable levels of light intensity.

Chlorophyll b, a photosynthetic antenna pigment, is synthesized from chlorophyll a via two steps of oxygenation reaction by chlorophyllide a oxygenase (*CAO*) gene (Nagata et al., 2004; Lee et al., 2005). In soybean, however, the characterization and expression of chlorophyll a oxygenase (*CAO*) gene still not well understood.

This study employed the standard gene cloning technique, followed by characterization using Northern blot hybridization were used to identify the chlorophyll a oxygenase (*CAO*) gene in three soybean genotypes, i.e. a shade tolerant "Ceneng" (G1), shade sensitive "Godek" (G2), and high yielding elite line "CG-30-10" (G3). All genotypes were planted in pots under light exposure treatments: full sunlight condition or no shading condition (C0), three hours of 50% shading (C1), two days 50% shading (C2), three hours of dark (C3), and two days of dark conditions (C4). The third trifoliate fully developed leaves were harvested and subjected to RNA total extraction. The specific primers were designed manually based on NCBI database related to *CAO* gene (Table 1).

Table 1. Gene specific primer of *CAO* characteristics

Sequence ('5 – 3')	Gc[1)] (%)	T[2)] (ºC)	No. of Accession	Target size (bp)
1-F: GGATTGGCGTGCTCGTCAAG	60	64	Ab021316	750
1-R: GTAAACCGTGTTCCACCGGG	60	64	*Arabidopsis t*	
3-F: CCCACGCGTCCGGCGTCCCG	85	74	AB021310	420
3-R: GGATAGTCGACTTCGGTGGG	60	64	*Oryza s*	
4-F: CGTGAGTACAAGTCCATCGG	55	62	AB021316	500
4-R: CGGGTTTCCCGTCTTCACCC	65	64	*Arabidopsis t*	
5-F: CCTTCTCTGCTGCCTCCTTC	60	65.4	AB021310	600
5-R: GCAACCTGTCTACTCCCCTC	60	62.6	*Oryza s*	
6-F: CGGAATACATGTGCGCATAG	50	63.9	AB021316	650
6-R: GTTCAGCGAAATGTCTCCAC	50	62.3	*Arabidopsis t*	

Note: [1)] GC : guanine-cytosine content; [2)] T: temperature

Fragment sequence of cDNA CAO was characterized using bioinformatics tools in terms of amino acid deduction (www.expasy.ch/tool/dna.html), open reading frame (ORF) or coding sequence (CDS) (www.ncbi.nlm.nih.gov/gorf.html) and (http://genes.mit.edu/GENSCANS.html), homology based on the nucleotide and amino acid sequences (www.ncbi.nlm.nih.gov/BLAST2) and (www.ebi.ac.uk/clustalW/index.html).

Results Summary

One of the cloned *CAO* cDNA using six GSPs that fits as a shade tolerant candidate is *CAO 3-4*. The characteristics of the *CAO 3-4* partial gene that was isolated with pT7Blue vector were amplified using gene specific primer. Fragment sequence of *CAO* cDNA resulted from cloning indicated that *CAO 3-4* partial gene (1,052 bp) comprised of 292 adenine (a), 241 cytosine (c), 276 guanine (g) and 242 thimine (t). The nucleotide fragment was then translated into amino acid deduction, and the number of amino acid deduction on *CAO 3-4* partial gene was 338. GenScan analysis of the fragment

sequence of CAO partial gene showed that of CAO partial gene contained partial coding sequence (CDS) as internal exon (274 bp) 568-841 did not have poly-Adenin (polyA), and the partial CDS consisted of 92 amino acids

Our previous studies on shade tolerance related genes had screened positive genes encoding Cytochrome and psaD (sub unit of PSI) from shade tolerant soybean genotypes using differential display methods (Khumaida et al., 2007). The full length gene of psaD was submitted to GenBank data base, i.e. Glycine max photosystem I sub unit PsaD (psaD) mRNA, complete cds/ Accession: EF628505 (Khumaida et al., 2007). However, whether or not CAO3-4 partial gene sequence fragment was related to psaD gene (sub unit of PSI) still need to be elucidated.

In the current study, we used six combinations of gene specific primers (GSP) to clone CAO cDNAs from shade tolerant soybean genotype, and found 10 fragments of cDNA candidates. Three gene specific primers including CAO-1, CAO-2, and CAO-5 could not separate the amplified products successfully. However, CAO-3, CAO-4, and CAO-6 gene specific primers generated 10 fragment cDNAs of CAO partial gene candidates. The nucleotide sequences of one CAO 3-4 fragment showed high sequence identity to a gene in *A. thaliana* chromosome 3.

Expression of CAO 3-4 partial Gene in Soybean

CAO gene is located in thylakoid membrane of chloroplast (Eggink et al., 2004). The shade tolerant soybean varieties have relatively darker green leaves compared to those of shade sensitive varieties, and this might be due to conversion of chlorophyl a to chlorophyl b by CAO gene. The role and function of CAO gene in the greening of leaves, in both shade tolerant and sensitive soybean genotypes, warrants further study. We hypothesise that persisting and sustaining green leaf area for a longer period of time could be used as a soybean improvement strategy by using the potential CAO gene.

To understand the molecular mechanism of changes in Chl b content and Chl a/b ratio, one soybean genotype from each of light tolerant and light sensitive were grown under different light intensities, and the CAO 3-4 partial gene (putative) expression was analyzed. The intensity of CAO 3-4 partial gene expression on the sensitive genotype “Godek” decreased up to 40-50% compared to the tolerant genotype “Ceneng” when grown under 50% shading or totally dark condition. On the other hand, the high yielding genotype “CG-30-10” grown under 50% shading or totally dark had the expression intensity decreased only by 10-15% compared to the tolerant genotype “Ceneng”. Therefore the “CG-30-10” might be a promising shade tolerant genotype.

Our previous study (Khumaida et al., 2007) showed that the expression level of CAO 3-4 partial gene was positively correlated with chlorophyll b of the shade tolerant genotypes grown under 50% shade. The increase in leaf Chl b was accompanied by an enhanced CAO expression that was highly pronounced in low light. Furthermore, expression level of CAO gene on light sensitive “Godek” correlated negatively with chlorophyll b content. We hypothesised that CAO gene might be involved in the soybean shade tolerance mechanism. Further studies are ongoing to determine the soybean shade tolerant genotypes and their shade tolerance mechanisms.

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