*Plant Biotechnology* 25, 365–368 (2008)

**Short Communication**

# Characterization of a dehydrogenase motif and an uORF in *Arabidopsis ANGUSTIFOLIA* gene

Kiu-Hyung Cho[1,a], Hirokazu Tsukaya[2,3], Gyung-Tae Kim[1,4,*]

[1] Department of Molecular Biotechnology, Dong-A University, Busan 604-714, Korea;[2] Graduate School, University of Tokyo, Tokyo 113-0033, Japan; [3] National Institute for Basic Biology, Okazaki 444-8585, Japan; [4] Environmental Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Korea
* E-mail: kimgt@donga.ac.kr Tel: +82-51-200-7519 Fax: +82-51-200-7524

Received March 17, 2008; accepted April 30, 2008 (Edited by Anai)

**Abstract** *ANGUSTIFOLIA* (*AN*), the first gene encoding a putative C-terminal binding protein (CtBP) identified in plants, controls leaf cell width in a polarity-dependent manner by regulating the arrangement of cortical microtubules. However, several differences in the sequence of AN have been detected in its conserved CtBP domains, which are transcriptional co-repressors in animals; thus, it is unclear whether AN is a transcriptional co-repressor in plants and how it may function. In this study, we found that AN possesses incomplete D2-HDH and GxGxxG(17x)D motifs, which confer CtBP dehydrogenase activity and NAD-binding for interaction with PxDLS motifs, respectively. In addition, full-length AN was unable to couple the reduction of pyruvate to lactic acid with the oxidation of NADH to $NAD^+$, suggesting that it might not have dehydrogenase activity. Moreover, we found that AN has an additional short open reading frame (ORF), which was identified as an upstream ORF (uORF), in its 5′-untranslated region that overlaps with the start codon of the *AN* gene. Transcriptional analysis revealed that the uORF and *AN* ORF are transcribed as a single molecule, indicating that the uORF might influence *AN* transcription during leaf development.

**Key words:** *ANGUSTIFOLIA*, CtBP, dehydrogenase activity, uORF.

Previous genetic studies of the *angustifolia* (*an*) and *rotundifolia3* (*rot3*) mutations in *Arabidopsis* revealed that genetic regulation of polar cell expansion controls two-dimensional growth of the leaf blade (Kim et al. 2002, 1999, 1998; Tsuge et al. 1996). Genetic analysis has also demonstrated that the *AN* gene regulates leaf cell width in a polarity-dependent and *ROT3*-independent manner (Kim et al. 1998). The *an* mutant has narrow leaves due to a defect in polar leaf cell elongation (Kim et al. 1998). Cytological analysis suggested that AN is involved in regulating the arrangement of cortical microtubules in leaf cells. *AN* was the first C-terminal binding protein (CtBP) homolog identified in plants, and it has since been cloned by map-based cloning (Kim et al. 1998). The microarray-based analysis of an *an* mutation suggested that AN may be capable of repressing transcription, similar to animal CtBPs (Kim et al. 1998). CtBPs, which have been identified in nematodes, arthropods, and vertebrates, are evolutionarily conserved co-repressors (Chinnadurai 2002; Nibu et al. 1998) that were originally identified as nuclear proteins based on their ability to interact with the C-terminal motif (PxDLS) of the adenovirus E1A oncoprotein. CtBP family proteins form homodimers and physically interact with a number of factors through their PxDLS motif and the recently identified RRT motif (Quinlan et al. 2006; Chinnadurai 2002); however, AN lacks both of the NAD(H)-binding motifs that are conserved in the animal CtBP family. An AN ortholog in another plant family, IAN from *Ipomoea nil* (Japanese morning glory), also lacks the above signatures (Cho et al. 2005). In general, CtBPs bear striking structural similarities to D2-hydroxyacid dehydrogenases, including an NAD-binding Rossman fold and a putative active site containing conserved histidine, glutamate, and arginine residues (Chinnadurai 2002). Moreover, low levels of dehydrogenase activity have been detected using pyruvate as a substrate (Kumer et al. 2002). It is not clear whether AN acts as a CtBP. To analyze the potential function of AN as a plant CtBP, we investigated whether AN has dehydrogenase activity, which is conserved in CtBPs.

It is uncertain whether the transcription of the plant CtBP homologs *AN* and *IAN* is regulated during leaf development (Cho et al. 2005; Kim et al. 2002). Our preliminary data suggested that AN had an additional short open reading frame (ORF) in its 5′-untranslated region (5′-UTR), which was identified as an upstream

[a] Present address: Department of Biology, Kyungpook National University, Daegu 702-701, Korea
This article can be found at http://www.jspcmb.jp/

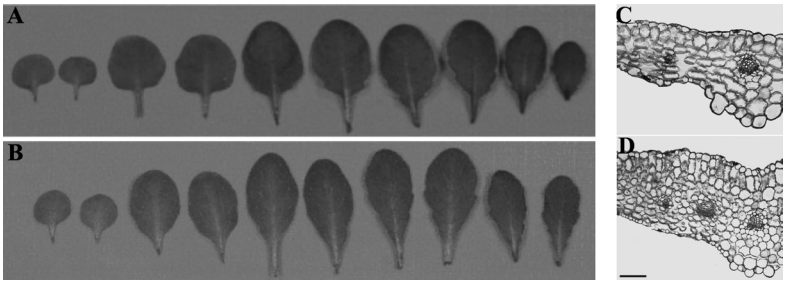


Figure 1. Leaf morphology in wild-type and *an-1* mutant plants. (A, B) Leaves of wild-type (A) and *an-1* mutant plants (B) (by row from top to bottom: aerial leaves, cotyledons, and rosette leaves). (C, D) Transverse section of the fifth rosette leaf from a wild-type (C) and *an-1* mutant plant (D). Bar, 100 $\mu$m.

ORF (uORF), that overlapped with the start codon of *AN*. In eukaryotes, uORFs play important roles in translational control in response to starvation and sucrose (Raney et al. 2000). A number of recent studies using heterologous or modified mRNAs have indicated that uORFs can influence more than translational efficiency (Imai et al. 2006; Mehta et al. 2006). In this paper, we investigated the dehydrogenase activity of AN and the possible regulation of *AN* expression by its uORF.

The *an* mutant has narrow leaves due to a defect in polar leaf cell elongation (Figure 1). Previous cytological analysis suggested that AN was involved in regulating the arrangement of cortical microtubules in leaf cells (Kim et al. 2002). AN contains not only a D2-hydroxy acid dehydrogenase (D2-HDH) motif, which is conserved among CtBPs (Kim et al. 2002), but also a putative LxCxE/D motif, a PEST motif, and a nuclear localization signal (NLS) (Cho et al. 2005; Kim et al. 2002). The observation of a D2-HDH motif in the primary sequence of AN revealed that it may be a transcriptional repressor, like animal CtBPs. To investigate whether AN is a functional dehydrogenase, we analyzed the primary sequences of the dehydrogenase motif in AN, IAN, MAN (an AN homolog in *Marchantia polymorpha*), and various CtBPs (Figure 2). Our sequence alignment revealed that AN lacks the catalytic triad necessary for dehydrogenase activity and the GxGxxG(17x)D NAD-binding motif required for interaction with PxDLS motifs. The His/Glu(Asp)/Arg triad, which has been implicated as the center for substrate binding and dehydrogenase activity, is conserved in all D2-HDHs (Figure 2); however, the structurally equivalent residues in AN, IAN, and MAN have the following substitutions: Arg266 to Ser266 and His315 to Arg315 (Figure 2). This indicates that AN may have a different motif for dehydrogenase activity that developed the ability to bind proteins having a PxDLS recognition motif, like CtBPs.

We next assayed whether AN has dehydrogenase activity by measuring the oxidation of NADH to $NAD^+$, coupled to the reduction of pyruvate to lactic acid, and vice versa, based on a loss or gain of absorbance,

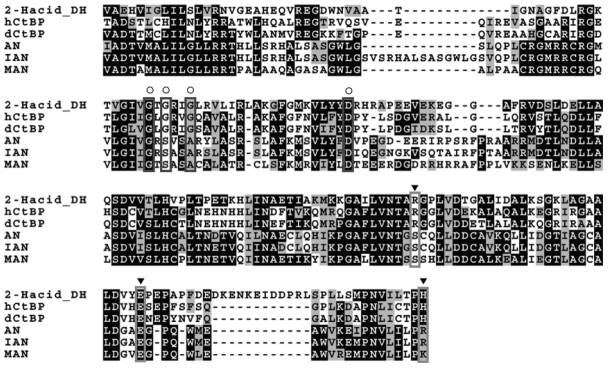


Figure 2. Primary sequence alignment of the D2-hydroxyacid dehydrogenase and D2-HDH motifs in plant ANs (IAN, *Ipomoea nil*; MAN, *Marchantia polymorpha*) and animal CtBPs (hCtBP, *Homo sapiens*; dCtBP, *Drosophila melanogaster*). Identical residues in all sequences are indicated by black boxes, while similar amino acids are indicated by gray boxes. A white circle indicates the GxGxxG(17x)D motif responsible for NAD-binding, while a black inverted triangle indicates the catalytic triad responsible for dehydrogenase activity.

respectively, at 340 nm (Adams et al. 1973). To carry out this experiment, *E. coli* XL1 cells containing pGEX-4T-3 (Amersham Biosciences Co., USA) and pGST-AN were grown overnight in 2 x YT medium containing 100 mg $l^{-1}$ ampicillin. This overnight culture was diluted 10-fold with the same medium and cultured at 37°C with shaking at 220 rpm until the $OD_{600}$ of the cultured cells became 0.3. Then IPTG was added to the medium at concentration of 1 mM and the flasks were continuously cultured for 12 h more. The cultured *E. coli* was collected by centrifugation at 6,000 *g* for 20 min and resuspended in breaking buffer (1 M $KH_2PO_4$, 0.5 M EDTA, pH 7.0), and sonicated on ice (30% duty cycle, output at 5, Sonicator TM Cell Disruptor Model W-225R; Heat System-Ultrasonics, USA). The purification of GST-AN fusion protein was carried out by Glutathione sepharose 4B (Amersham Pharmacia Biotech, Sweden) by following instructions. The purified protein was resolved by 12% SDS polyacrylamide gels, and transferred onto PVDF microporous membrane (Millipore Japan, Japan). GST-AN protein was detected in an immunoblot with anti-GST antibody developed in goat (Amersham Biosciences Co., USA) for GST-tagged

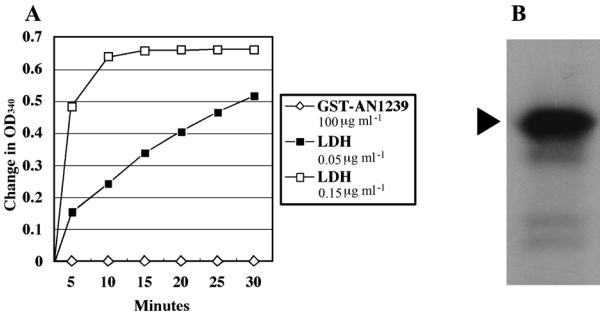


Figure 3. Dehydrogenase activity of the *AN* protein. (A) Dehydrogenase assays were performed to measure the conversion of pyruvate to lactic acid coupled to the oxidation of NADH to $NAD^+$ as described in the experimental procedures. Varying amounts of a GST-AN fusion protein purified from *E. coli* were incubated with 20 mM pyruvate, and changes in the absorbance at 340 nm were measured over time. (B) Western blot analysis was used to confirm expression of the purified GST-AN fusion protein using GST-specific antibodies.

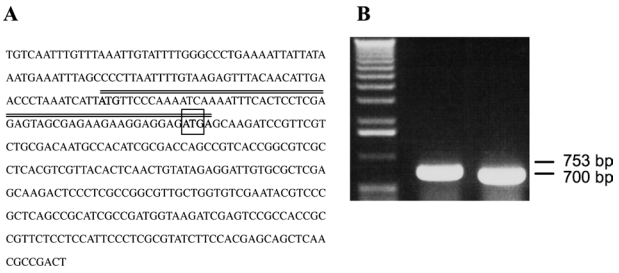


Figure 4. Preservation of a uORF in *AN*. (A) The double line indicates the putative uORF region in *AN*, while the box indicates the start codon of the *AN* ORF. (B) RT-PCR analysis using uORF- and *AN*-specific primers, respectively: 1-kb ladder (left), the uORF region (middle), and the *AN* region (right).

protein, and antibody-bound proteins was visualized by Alkaline-phosphatase-conjugated anti-goat antibody (Sigma Japan, Japan). Dehydrogenase assays were conducted in 0.2 M Tris-HCl, pH 7.3, with 20 mM NaPyruvate and 0.132 mM NADH at 25°C with the appropriate amount of AN protein. The absorbance was measured at 340 nm. Full-length AN was unable to catalyze the reaction (Figure 3A), suggesting that AN may not have dehydrogenase activity. Although the GST fusion may have altered the native protein′s dehydrogenase activity, it is more likely that the lack of activity was due to the missing arginine and histidine residues in the catalytic triad of AN (Figure 2). The well conserved catalytic triad residues of D2-HDHs in animal CtBP family lie at the confluence of the NAD- and substrate binding domains and have an important role in the interaction with both substrate and PxDLS motif (Kumar et al. 2002). Mutation analysis of catalytic triad residues (H315, E295, and R266) of human CtBP indicated that mutations disrupted in not only dehydrogenase activity but also E1A binding by conformational changes (Kumar et al. 2002). This experiment revealed that dehydrogenase motif could influence to interact with PxDLS motif and expected to play an important role in proper CtBP function as a co-repressor. Thus, these analyses supported our results that the conserved sequences in catalytic triad residues have important roles both in dehydrogenase activity and co-repressor activity of CtBP.

We recently demonstrated that AN evolved differently from other CtBPs, despite the fact that AN is a CtBP homolog (Stern et al. 2007). The lack of dehydrogenase activity and a GxGxxG(17x)D motif in AN may be the cause of the functional difference between AN and other CtBPs. Moreover, other plant homologs of AN (IAN and MAN) are also missing the dehydrogenase motif, which is conserved in animal CtBPs, supporting the idea that plant CtBPs may not have the same function as animal CtBPs. However, it is unclear whether AN is a transcriptional corepressor in plants and how it may function. A previous yeast two-hybrid analysis revealed that AN was unable to bind retinoblastoma (Stern et al. 2007); however, according to its molecular function, AN may still be a member of the CtBP/BARS/AN superfamily.

It is still unclear whether the transcription of the plant CtBP homologs *AN* and *IAN* is regulated during leaf development (Cho et al. 2005; Kim et al. 2002). Interestingly, we found that *AN* had an additional short ORF in its 5′-UTR, which was identified as a uORF, whose stop codon overlapped with the start codon of the *AN* gene (Figure 4A). Thus, we investigated whether the *AN* transcript is produced as a single molecule using RT-PCR. RT-PCR was performed by one-step RT-PCR with PLATINUM Taq Kit (Invitrogen Crop., USA). RNA was prepared with an RNeasy Mini Kit (Qiagen Inc., Japan). For detection of uORF by RT-PCR, uORF-specific primer (5UPAN, 5′-ATGTTCCCAAAATCAAAATT-TCACTCCTCGAGA-3′) was used and AN-specific primer (5AN, 5′-ATGAGCAAGATCCGTTCGTC-TGCGACAATG-3′) was used for the comparison. Antisense primer (3AN700, 5′-TTAATGCACAATGT-AGCGAAATGACATCAC-3′) was used in both reactions. Our data revealed that the uORF and ORF of *AN* are transcribed together (Figure 4B), indicating that the uORF may affect the transcription of *AN* during leaf development. In eukaryotes, uORFs have important roles in translational control in response to starvation and sucrose (Viese et al. 2005; Raney et al. 2000). In addition, a number of recent studies using heterologous or modified mRNAs have indicated that uORFs influence more than translational efficiency (Imai et al. 2006; Mehta et al. 2006). For instance, the *sac51-d* mutation, which involves a uORF in the bHLH-type transcription factor *SAC51* gene, complements the dwarf phenotype of the *Arabidopsis acl5* mutant as a result of increased *SAC51* transcription (Imai et al. 2006). Thus, we cannot exclude the possibility that *AN* regulates its own

expression level to control polar leaf expansion. Further analysis of mutation experiments of the uORF sequence in *an* mutant will elucidate the real function of uORF in regulation of expression of *AN* ORF and/or mRNA stability.

## Acknowledgements

This paper was supported by the Dong-A University Research Fund in 2005.

## References

Adams MJ, Buehner M, Chandrasekhar K, Ford GC, Hackert ML, Lijas A, Rossmann MG, Smiley IE, Allison WS, Everse J, Kaplan NO, Taylor SS (1973) Structure-function relationships in lactate dehydrogenase. *Proc Natl Acad Sci USA* 70: 1968–1972

Chinnadurai G (2002) CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol Cell* 2: 213–224

Cho KH, Takayuki S, Kim GT, Nitasaka E, Tsukaya H (2005) Characterization of a member of the AN subfamily, IAN, from *Ipomoea nil*. *Plant Cell Physiol* 46: 250–255

Imai A, Hanzawa Y, Komura M, Yamamoto KT, Komeda Y, Takahashi T (2006) The dwarf phenotype of the *Arabidopsis acl5* mutant is suppressed by a mutation in an upstream ORF of a HLH gene. *Development* 133: 3575–3585

Kim GT, Tsukaya H, Uchimiya H (1998) The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P450 family that is required for the regulated polar elongation of leaf cells. *Genes Dev* 12: 2181–2191

Kim GT, Tsukaya H, Saito Y, Uchimiya H (1999) Changes in the shapes of leaves and flowers upon overexpression of the novel cytochrome P450 in *Arabidopsis*. *Proc Natl Acad Sci USA* 96: 9433–9437

Kim GT, Shoda K, Tsuge T, Cho KH, Uchymiya H, Yokoyama R, Nishitani K, Tsukaya H (2002) The *ANGUSTIFOLIA* gene of *Arabidopsis*, a plant CtBP gene, rgulates leaf-cell expansion, the arrangement of cortical microtubules in leaf cells and expression of a gene involved in cell-wall formation. *EMBO J* 21: 1267–1279

Kumer V, Carlson JE, Ohgi KA, Edward TA, Rose DW, Escalante CR, Rosenfeld MG, Aggarwal AK (2002) Transcription corepressor CtBP is an $NAD^+$-regulated dehydrogenase. *Mol Cell* 10: 857–869

Mehta A, Trotta CR, Peltz SW (2006) Derepression of the Her-2 uORF is mediated by a novel post-transcriptional control mechanism in cancer cells. *Genes Dev* 20: 939–953

Nibu Y, Zhan H, Levine M (1998) Interaction of short-range repressors with *Drosophila* CtBP in the embryo. *Science* 280: 101–103

Quinlan KG, Nardini M, Verger A, Francescato P, Yaswen P, Dorda D, Bolognesi M, Crossley M (2006) Specific recognition of ZNF217 and other zinc finger proteins at a surface groove of C-terminal binding proteins. *Mol Cell Biol* 26: 8159–8172

Raney A, Bafon AC, Mize GJ, Morris DR (2000) In vitro translation of the upstream open reading frame in the mammalian mRNA encoding S-adenosylmethionine decarboxylase. *J Biol Chem* 275: 24444–24450

Stern MD, Aihara H, Cho KH, Kim GT, Horiguchi G, Roccaro GA, Guevara E, Sun HH, Negeri D, Tsukaya H, Nibu Y (2007) Structurally related *Arabidopsis ANGUSTIFOLIA* is functionally distinct rom the transcriptional corepressor CtBP. *Dev Genes Evol* 217: 759–769

Tsuge T, Tsukaya H, Uchimiya H (1996) Two independent and polarized processes of cell elongation regulate leaf blade expansion in *Arabidopsis thaliana* (L) Heynh. *Development* 122: 1589–1600

Viese A, Elzinga N, Wobes B, Smeekens S (2005) Sucrose-induced translational repression of plant bZIP-type transcription factors. *Biochem Soc Trans* 33: 272–275