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# Critical Role of Alanine-161 in Delila Protein Involved in Regulation of Anthocyanin Pigmentation for Transcriptional Activation in Yeast

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**Abstract**

Delila protein is a member of MYC-like transcriptional activator family, which regulates the expression of structural genes in the biosynthesis of anthocyanin in snapdragon. We have mutated several amino acid of Delila to elucidate their function using transcriptional activation by yeast one-hybrid system. Among several site-directed mutations, the replacement of alanine-161 with 18 different amino acids resulted in severe decrease in the transcriptional activation of the yeast *GAL1* promoter Delila fused to the yeast GAL4 DNA-binding domain. This lack of induction indicates the importance of alanine-161 for transcriptional activation by Delila protein. Although the mutational change at residue 161 from alanine to aspartic acid exhibited no activity, the deletion of N-terminus (1-136 amino acids) of this mutant resulted in the recovery of activity. These results suggest that alanine-161 located outside the activation domain in Delila plays a critical role for its transcriptional activation in yeast.

## 1. Introduction

Delila protein is a member of MYC-like transcriptional activator family containing a characteristic basic helix-loop-helix (bHLH) binding domain. Delila is involved in the regulation of gene expression for anthocyanin biosynthesis in the corolla tube and a variety of other tissues in snapdragon (Goodrich *et al.*, 1992). Several other functional homologues of Delila have been described in maize (Consonni *et al.*, 1993; Ludwig and Wessler, 1990), petunia (Quanttrocchino *et al.*, 1998) and *Perilla frutescens* (Gong *et al.*, 1999). Functional analysis of the transcriptional activation of maize B protein, a maize Delila homologue, indicated that an interaction domain exists in the amino-terminal sequence of B protein (Goff *et al.*, 1992). B protein functions as a transcriptional activator in anthocyanin biosynthesis of maize by interacting with C1, a MYB-like protein from maize (Goff *et al.*, 1992). Delila and MYC-RP, a Delila homologue from perilla, when fused with the DNA-binding domain of yeast GAL4 transcriptional factor, could transactivate the promoter of *GAL1* gene in yeast (Gong *et al.*, 1999). Analysis by deletion clones of Delila and MYC-RP suggested the presence of a transactivation domain between the interaction domain and bHLH domain (**Fig. 1**) (Gong *et al.*, 1999). Delila and MYC-RP could also transactivate the promoter of gene for dihydroflavonol-4-reductase (DFR) from perilla in yeast (Gong *et al.*, 1999).

The primary structure of the transcriptional activator proteins have been elucidated: in many transcriptional activators, a polypeptide stretch rich with acidic amino acids is found in the domain

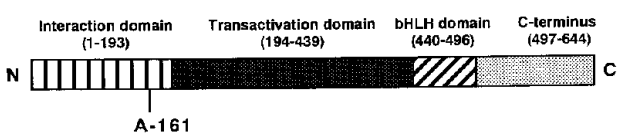


**Fig. 1.** Schematic representation of the functional domains of Delila protein (Goodrich *et al.*, 1992). Delila protein presumably contains three distinct functional domains; an interaction domain (1-193 amino acids) (Goff *et al.*, 1992), a transactivation domain (194-439 amino acids) (Gong *et al.*, 1999), and a DNA-binding domain (bHLH domain, 440-496 amino acids) (Goodrich *et al.*, 1992). The function of C-terminus region (497-644 amino acids) is unknown.

responsible for transactivation, and the activity of transcriptional activation was correlated with the net negative charge of this domain (Gill and Ptashne, 1987; Gill *et al.*, 1990; Ptashne, 1988). Change of single hydrophobic amino acid in transactivation domain was sufficient to significantly reduce the activity of transactivation in herpes simplex virus VP16 protein (Cress and Triezenberg, 1991; Regier *et al.*, 1993) and maize C1 protein (Sainz *et al.*, 1997).

In our previous study on MYC-RP, we found that the truncated protein only with transactivation domain (193-420 amino acids) exhibited stronger activity of transactivation than the entire protein, when they were fused with GAL4 DNA-binding domain (Gong *et al.*, 1999). Similar examples, in which shortened polypeptides exhibited stronger transactivation activity, have been reported in a rice homeobox protein (Tamaoki *et al.*, 1995) and an Arabidopsis CIP7 nuclear protein (Yamamoto *et al.*, 1998). These results suggest that the structure of peptide outside the activation domain plays an important role in the activity of transactivation.

In the present study, we show that alanine-161 placed in the interaction domain outside the activation domain of Delila is critical for transcriptional activation in yeast. Although the entire mutated protein at alanine-161 exhibited no activity of transcriptional activation, deletion of N-terminus in an alanine-161 mutant resulted in recovery of the activity.

## 2. Materials and Methods

### *2.1. Site-directed and deletion mutagenesis*

The *Xho*I-*Bam*HI fragment of *Delila* obtained from pJAM173 (Mooney *et al.*, 1995) was subcloned into pBluescriptSK(+) vector. Using the QuickChange kit from Stratagene, point mutations were introduced into the SK(+) *Delila* plasmid. The primer used for generation of point mutations at alanine-161 was 5'-GCTTGCAAAGAGTNNNT-CAATTCAGACAG-3' (N is a mixture of G, A, T, C). The mutations were verified by sequencing using the dideoxy-chain termination method with Thermo Sequenase (Amersham) using a DNA sequencer (model DSQ 1000; Shimadzu, Japan). The plasmids with mutated Delila were digested with *Nco*I and *Bam*HI. The released insert was translationally ligated to GAL4 DNA-binding domain (1-147 amino acids) of pAS2-1 vector (Clontech) to yield an effector construct. Mutations at other different amino acid residues were generated by polymerase-chain-reaction (PCR) method. The PCR products digested with *Bam*HI and *Pst*I were ligated to GAL4 DNA-binding domain of pAS2-1 vector, and mutations were verified by sequencing. The deletion clone (deleted 1-136 amino acids) of the mutant changed at alanine-161 to aspartic acid (A161D) was constructed by PCR using a forward primer 5'-CGGGATCCAAGCAGTATGGCTAT-GCAACGCTCATCGTGC-3' with a *Bam*HI site (underlined) and a reverse primer 5'-AAACTG-CAGACTTCATAGTAACTTTCTGAAGAGCTTG-TTTG-3' with *Pst*I site (underlined). The PCR product digested with *Bam*HI and *Pst*I were ligated to GAL4 DNA-binding domain in pAS2-1 vector.

### *2.2. Analyses of mutated Delila expressed in yeast*

The effector plasmids carrying GAL4 DNA-binding domain (1-147 amino acids) translationally fused with different Delila mutations were introduced into a yeast strain Y187, which contained the reporter construct of the upstream activating sequence (UAS) of *GAL1* promoter for controlling *Lac*Z reporter gene (Harper *et al.*, 1993). The effector plasmids were also introduced into a strain YM4271 (Wilson *et al.*, 1991) (Clontech), which contained the perilla *DFR* promoter (1.3 kb) linked to a minimal promoter of the yeast iso-1-cytochrome C gene (*CYC1*) for controlling the expression of *Lac*Z reporter gene as described previously (Gong *et al.*, 1999). The assay of reporter $\beta$-galactosidase ($\beta$-gal) was carried out by a paper filter method using X-gal as a substrate and by a liquid method using O-nitrophenyl $\beta$-D-galactoside as substrate as reported previously (Gong *et al.*, 1999).

For western blot analysis, proteins extracted from transformed yeast strain Y187 were subjected to SDS-polyacrylamide gel (12%) electrophoresis and electrotransferred to polyvinylidene difluoride (PVDP) membranes (Millipore). The membranes were probed with a mouse monoclonal antibody against GAL4 DNA-binding domain (Clontech).

## 3. Results

### *3.1. Probing critical amino acids of Delila on transcriptional activation*

To find which amino acid residues of Delila are critical for transcriptional activation, we introduced several point mutations into the wild-type Delila. We chose six mutations, N52A (arginine-52 to alanine, *etc.*), A134S, A161S, L255S, E266G, and N428A, because all these mutations result in alteration of secondary structure of Delila protein predicted by the Chou and Fasman's program (1978). These site-directed mutants were fused with the DNA-binding domain of a yeast transcriptional

activator GAL4. Transactivation analysis of the resulting fusion proteins (effector) on the expression of *GAL1-LacZ* fusion gene (reporter) indicated that the activity of A161S mutant was severely decreased compared with that of wild-type Delila; however, the rest of five mutants exhibited no significant changes in transactivation activity (data not shown). These results suggested that alanine-161 is critical for transactivation of Delila protein among the amino acids examined.

### *3.2. Analyses of transcriptional activation of Delila alanine-161 mutants on GAL1 promoter*

To further address the importance of Delila alanine-161 for transcriptional activation, we made extensive substitutions of this alanine-161 with different amino acids. The fusion effector constructs of alanine-161 mutants linked with GAL4 DNA-binding domain were analyzed for their transactivation activity on the *GAL1* promoter. As shown in **Table 1**, the activities of all mutants except for a silent substitution were dramatically decreased. Three different groups could be categorized according to the degrees of activity. One group contains ten mutants which exhibited no transcriptional activity; this includes A161D, A161E, A161H, A161K, A161N, A161P, A161Q, A161R, A161W and A161Y (sometimes, A161Y showed activity in paper filter method), most of which are mutated to charged amino acids. The second group showed weak but substantial activity, which includes A161F, A161I, A161L, A161T and A161V. The third group, including A161C, A161G and A161S, showed relatively strong activity. These mutated

**Table 1.** Transcriptional activation by fusion construct of Delila alanine-161 mutant with GAL4 on the yeast *GAL1* promoter

| Clone No. | Codon | Mutant | Activity of fusion of GAL4-DB with Delila mutant | |
|---|---|---|---|---|
| | | | Paper assay | Liquid assay (%) |
| 10* | GCT* | A161A* | +++ | 100 |
| 8 | TGT | A161C | ++ | 17 |
| 32 | GAT | A161D | − | nd |
| 2 | GAG | A161E | − | nd |
| 1 | TTC | A161F | ± | 0.3 |
| 26 | GGG | A161G | ++ | 12.4 |
| 11 | CAC | A161H | − | nd |
| 22 | ATC | A161I | + | 0.2 |
| 39 | AAG | A161K | − | nd |
| 7 | TTG | A161L | ++ | 0.6 |
| 12 | AAT | A161N | − | nd |
| 27 | CCA | A161P | − | nd |
| 3 | CAA | A161Q | − | nd |
| 13 | CGG | A161R | − | nd |
| 16 | TCG | A161S | ++ | 8.7 |
| 4 | ACA | A161T | + | 0.3 |
| 23 | GTG | A161V | ++ | 1.6 |
| 6 | TGG | A161W | − | nd |
| 35 | TAC | A161Y | − | nd |
| Control | Empty vector | | − | nd |

The yeast strain Y187, containing the reporter construct of *GAL1* UAS fused with *LacZ*, was transformed with the effector fusion construct of GAL4 DNA-binding (DB) domain with each alanine-161 mutant. Six independent clones were picked up and cultured on SD plates for two days. The harvested cells were applied to the paper filter assay for $\beta$-gal or further cultured in SD liquid medium for the liquid assay. Yeast cells transformed with empty vector pAS2-1 were used as a negative control. For the paper filter assay, the activity was measured by the relative intensity of blue spots : +++, strong ; ++ medium ; + weak ; ±, faint ; −, no blue color. For the liquid assay, the relative activity was calculated by comparison with that of a silent mutation (clone No. 10). nd, activity was not detected.

*A silent mutation. GCG is the codon of alanine-161 in wild-type Delila. No difference in the activities of wild-type Delila (GCG) and a silent mutant clone 10 (GCT) could be determined.

amino acids are of small hydrophilic side chain.

Protein extracts from yeast cells transformed with each mutant were analyzed by SDS-polyacrylamide gel electrophoresis and immunostaining using a monoclonal antibody against GAL4 DNA-binding domain (data not shown). These indicated that the mutant proteins were translated at almost equal quantity in yeast. Thus, we concluded that the reduction of transactivation activity of the mutants is due to the impaired function of the mutated proteins.

### *3.3. Analyses of transcriptional activation of Delila alanine-161 mutants on DFR promoter*

To determine if these alanine-161 mutants would affect the ability of Delila to activate the promoter of an anthocyanin biosynthetic gene, *DFR*, encoding dihydroflavonol-4-reductase, we introduced these mutated effectors into yeast strain YM4271 (Wilson *et al.*, 1991), which carries the promoter of perilla *DFR* gene linked with a minimal promoter from the yeast gene for control the expression of the *LacZ* reporter gene (Gong *et al.*, 1999). In this experiment, we used the CL plasmid, which contains the whole *GAL4* gene as a control, showing that GAL4 protein itself poorly activated the *DFR* promoter. The results shown in **Table 2** are similar to those for *GAL1* promoter activation shown in **Table 1**. Only mutants A161C, A161G and A161S showed apparent transcriptional activities; however, the rest of mutants exhibited almost no or faint activities.

### *3.4. Deletion analysis of Delila mutant A161D*

We selected one of the Delila mutants, A161D, which showed no transactivation activity, as a tester to determine if the deletion of a partial peptide could recover its transactivation ability. The fusion

**Table 2.** Transcriptional activation by fusion construct of Delila alanine-161 mutant on *DRF* promoter

| Clone No. | Codon | Mutant | Activity of fusion of GAL4-DB with Delila mutant | |
|---|---|---|---|---|
| | | | Paper assay | Liquid assay (%) |
| 10* | GCT* | A161A* | +++ | 100 |
| 8 | TGT | A161C | ++ | 2.25 |
| 32 | GAT | A161D | − | nd |
| 2 | GAG | A161E | − | nd |
| 1 | TTC | A161F | + | 0.13 |
| 26 | GGG | A161G | ++ | 2.18 |
| 11 | CAC | A161H | − | nd |
| 22 | ATC | A161I | − | nd |
| 39 | AAG | A161K | − | nd |
| 7 | TTG | A161L | + | 0.12 |
| 12 | AAT | A161N | − | nd |
| 27 | CCA | A161P | − | nd |
| 3 | CAA | A161Q | − | nd |
| 13 | CGG | A161R | − | nd |
| 16 | TCG | A161S | ++ | 1.52 |
| 4 | ACA | A161T | ± | 0.05 |
| 23 | GTG | A161V | ± | 0.05 |
| 6 | TGG | A161W | − | nd |
| 35 | TAC | A161Y | ± | 0.05 |
| Control | CL | | ± | 0.3 |
| Control | Empty vector | | − | nd |

The yeast strain YM4271, carrying the reporter gene of perilla *DFR* promoter fused with *LacZ*, was transformed with the effector plasmids and analyzed as described in Table 1. Yeast cells transformed with CL containing rhe full-length GAL4 gene or empty vector pAS2-1 were used as controls. For the paper filter assay, the activity was measured by the relative intensity of blue spots : +++, strong ; ++ medium ; + weak ; ± , faint ; − , no blue color. For the liquid assay, the relative activity was calculated by comparison with that of a silent mutation (clone No.10). nd, activity was not detected.

*A silent mutation. GCG is the codon of alanine-161 in wild-type Delila. No difference in the activities of wild-type Delila (GCG) and a silent mutant clone 10 (GCT) could be determined.

construct of the truncated A161D (deletion for 1-136 amino acids) with GAL4 DNA-binding domain exhibited the same transactivation activity as that by the identically truncated wild-type Delila. The relative transactivation activity of these truncated Delila proteins were about 10% of that of full-length wild Delila. These results indicated that the mutation of alanine-161 impaired the transactivation activity only when the N-terminal region (1-136 amino acids) was present in Delila protein.

## 4. Discussion

In our previous study, we isolated two cDNAs of Delila homologues, MYC-RP and MYC-GP, from red and green varieties of *P. frutescens*, respectively (Gong *et al.*, 1999). There was only one amino acid substitution between the deduced amino acid sequences of MYC-RP and MYC-GP: alanine-132 in MYC-RP was substituted by serine in MYC-GP (Gong *et al.*, 1999). The predicted secondary structures of MYC-RP and MYC-GP are different. However, comparison of transactivation activities of these two proteins was difficult because of their low activities in yeast. We thought that, if similar mutation is introduced into a homologous snapdragon Delila exhibiting stronger transactivation activity, the transactivation could be comparably measured between wild-type Delila and its mutants. Thus, we firstly constructed a Delila mutant, A134S, in which alanine-134 corresponds to alanine-132 in MYC-RP. This alanine is conserved in most MYC-like proteins involved in regulation of anthocyanin biosynthesis. However, no apparent change in transactivation activity was found in this A134S mutant Delila. We constructed a mutant at another conserved serine, A161S, and found the severe decrease of transactivation activity. Alanine-161 is also conserved in all MYC-like proteins involved in the regulation of anthocyanin biosynthesis. The mutations at other amino acids that cause the changes in predicted secondary structures did not affect the transactivation activity.

The extensive mutation analyses showed that alanine-161 of Delila is critical for its transcriptional activation of the *GAL1*. The transactivation of each mutant correlates to the side chain properties of the substituted amino acid as described in **Results**. The analyses of alanine-161 mutants on *DFR* promoter suggest that relative activity of mutants were essentially the same on the *GAL1* promoter.

Single amino acid substitution, located at the catalytic or interaction residue, severely affects the protein function as described in dihydrofolate reductase (Howell *et al.*, 1986), VP16 (Regier *et al.*, 1993), and hGH (Cunningham *et al.*, 1989). Because alanine-161 in Delila is outside the activation domain (about 194-439 amino acids) (**Fig. 1**), the substitutions at this alanine residue were not expected to severely perturb the structure of activation domain itself. However, the mutation at alanine-161 resulted in severe decrease of transcriptional activation of the whole Delila protein. One possible explanation for this severe decrease of activity in this mutation would be that alanine-161 mutants probably changed the structure in the interaction domain of Delila as to mask the activation domain, which interacts with the general transcriptional machinery (Cress and Triezenberg, 1991). The findings that the truncated mutant A161D recovered partial activity, although the full-length mutant exhibited no activity, would support this masking hypothesis. As shown in **Fig. 2**, the predicted secondary structure of Delila protein is changed by mutation at alanine-161 to aspartic acid. This change in secondary structure around alanine-161 may affect the transactivation activity of Delila, although the correlation between alteration of secondary structure and reduction transactivation activity of all mutants was not perfectly observed.

Most of previous studies on transcriptional activators have focused on the functional analysis of activation domains, such as VP16 (Cress and Triezenberg 1991; Regier *et al.*, 1993), C1 (Sainz *et al.*, 1997), GAL4 (Gill and Ptashne, 1987; Gill *et al.*, 1990), GCN4 (Drysdale *et al.*, 1995) and Sp1 (Gill *et al.*, 1994). Some mutations occurring in the activation domains affected on transcriptional activation. However, the activities of some transcriptional activators are also affected by structures outside activation domain such as CIP7. In CIP7, the whole protein (1-1058 amino acids) exhibited no transcriptional activation when fused with GAL4 DNA-binding domain. However, the activation

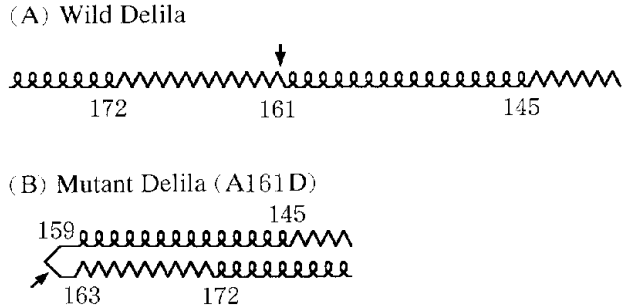


**Fig. 2.** Predicted secondary structures of (A) wild Delila and (B) the mutant A161D. The prediction was performed as described by Chou and Fasman (1978). *Arrowhead* indicates the position of the 161 residue.

domain (387-1058 amino acids) alone activated transcription in both yeast and plant (Yamamoto *et al.*, 1998). The evidence obtained in our present study on Delila mutants indicated that alanine-161 outside the activation domain is important for its transcriptional activation probably by keeping the structural integrity of Delila protein.

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