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A single amino acid substitution beyond the C2H2-zinc finger in Ros derepresses virulence and T-DNA genes in *Agrobacterium tumefaciens*

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Abstract

Ros is a chromosomally-encoded repressor containing a novel C2H2 zinc finger in *Agrobacterium tumefaciens*. Ros regulates the expression of six virulence genes and an oncogene on the Ti plasmid. Constitutive expression of these genes occurs in the spontaneous mutant 4011R derived from the octopine strain Ach-5, resulting in T-DNA processing in the absence of induction, and in the biosynthesis of cytokinin. Interestingly, the mutation in 4011R is an Arg to Cys conversion at amino acid residue 125 near the C-terminus well outside the zinc finger of Ros. Yet, Ros bearing this mutation is unable to bind to the Ros-box and is unable to complement other *ros* mutants.

Keywords: Ros; Virulence gene; *Agrobacterium tumefaciens*

1. Introduction

Virulence genes on the Ti plasmid harbored in all plant tumor-inducing strains of *Agrobacterium tumefaciens* are regulated both positively and negatively. Recently, the *ipt* gene on the T-DNA of the Ti plasmid was also found to be negatively regulated despite the fact that the gene is regulated by eukaryotic transcriptional machinery in the host plant [1]. The negatively-regulated virulence genes are contained in the *virC* and *virD* operons, whose products process the T-DNA at its left and right borders for transfer from the bacterial cell to the plant host cell (reviewed recently in [2,3]). For nopaline-type Ti plasmids such as pTiC58, the T-DNA is a specific 25-kb sector of the Ti plasmid and contains the plant oncogenes *ipt*, *iaaM* and *iaaH*, whose promoters are regulated by the plant transcription system recognizing their TATA boxes [4,5] to synthesize isopentenyl transferase, tryptophan monooxygenase and indoleacetamide amidohydrolase, respectively. The *ipt* gene encodes the enzyme dimethyl-allylpyrophosphate:AMP transferase [6–8], which catalyzes the covalent linkage of dimethylallylpyrophosphate to the N^6 of AMP, yielding isopentenyladenosine-5′-monophosphate, a class of cytokinins [9]. The *iaaM* gene encodes a mono-oxygenase which converts tryptophan to indole-3-acetamide [10,11]. Indole-3-acetamide is then hydrolyzed into indole-3-acetic acid by an aminohydrolase encoded by the *iaaH* gene [12,13]. Once the T-DNA is integrated into the plant genome, the expression of *ipt*, *iaaM* and *iaaH* results in elevated levels of these growth hormones culminating in the formation of the crown gall tumor [14,15].

Negative regulation of the *virC*, *virD* [16,17] and *ipt* [1] genes is mediated by Ros, a 15.5-kDa zinc finger protein encoded by the *ros* chromosomal gene in *A. tumefaciens*. A mutation in the *ros* gene results in the derepression of *virC*, *virD* and *ipt* genes [1,16,17] resulting in the production of: (1) T-DNA intermediates in the absence of induction of the positive regulator VirG by acetosyringone [18] and (2) cytokinin by the expression of the *ipt* gene normally expressed in the plant rather than in *A. tumefaciens* [1]. The nature of the spontaneous mutation in the original *ros* mutant [16] has remained unknown. In this study, we report the identification and characterization of this mutation, which surprisingly is distal to the zinc finger in Ros. Ros is the first transcriptional regulator in prokaryotes to

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bear a C2H2 zinc finger [1]. Phylogenetic analysis revealed that Ros has close evolutionary linkages to Ros homologues in bacteria, while it is distantly related to eukaryotic zinc finger regulators [19].

2. Materials and methods

2.1. Bacterial strains, growth conditions and plasmids

The original *ros* mutant 4011R was isolated as a spontaneous mutant from octopine strain 4011 originating from strain Ach-5 [16]. Strain Ach-5 was originally isolated by Dr. Peter Ark (University of California, Berkeley, CA, USA) from a crown gall on yarrow (*Achillea ptarmica* L.) on a farm in Contra Costa County, CA, USA. *A. tumefaciens* 4011 and 4011R were grown in medium 523 [20] at 28°C. *Escherichia coli* BL21DE3 containing plasmid pUCD4401 and DH5α containing plasmid pUCD4402 were grown in Luria–Bertani medium containing ampicillin, 100 μg ml^{-1}, at 37°C. Plasmids pUCD4402 containing the *virC/D* promoter regions and pUCD4401 containing the *ros* gene were constructed and isolated as described previously [21]. The cloning vector Bluescript® was purchased from Stratagene.

2.2. Preparation of protein extracts

Proteins were partially purified from 4011 and 4011R according to D'Souza-Ault et al. [21] with minor modifications. Cells were grown until $OD_{600} = 0.6$, then harvested by centrifugation (10 000 × *g*, 20 min) at 4°C, resuspended and washed twice with TE buffer (50 mM Tris–Cl, pH 8.0, and 1 mM Na_2EDTA) containing 10 mM β-mercaptoethanol. The pellet was quickly frozen in liquid nitrogen and then thawed in crushed ice for 10–20 min. This step was repeated twice. Cells were resuspended in 1 ml extraction buffer (50 mM Tris–Cl, pH 8.0, 1 mM Na_2EDTA, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonide and 5% glycerol) containing lysozyme (2.5 mg ml^{-1}) and left in crushed ice for 30 min. Cells were sonicated for 8 pulses of 30 s each, at setting 50 with an Ultrasonic cell disrupter (Heat Systems Ultrasonics, Farmingdale, NY, USA) and the protein extract was partitioned in the supernatant after centrifugation at 10 000 × *g* for 20 min, at 4°C. The supernatant was adjusted to 30% glycerol and stored at −20°C.

2.3. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Proteins were fractionated by sodium dodecyl sulfate–PAGE in a 12% polyacrylamide gel, electrophoretically transferred onto a nitrocellulose membrane and probed with a 1:5000 diluted adsorbed polyclonal antiserum against Ros. Ros protein was visualized by the enhanced chemiluminescence system employing horseradish peroxidase-labelled antibodies (Amersham).

2.4. Radiolabelling of DNA

Plasmid pUCD4402 was isolated from *E. coli* DH5α using a Miniprep kit (Qiagen) and digested with *Sal*I to generate the 210-bp DNA fragment containing the divergent *virC/virD* promoters. The purified fragment was dephosphorylated with calf intestinal phosphatase, then labelled with γ[^{32}P]ATP using bacteriophage T4 polynucleotide kinase (Boehringer Mannheim). The probe was purified by extraction with phenol and precipitated from the aqueous phase with ice-cold 95% ethanol.

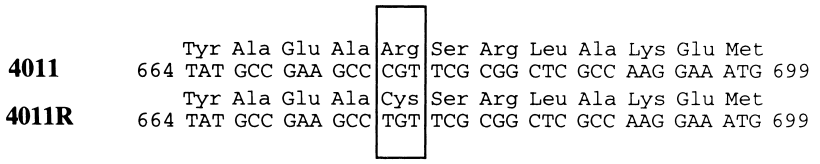
2.5. Gel mobility shift analysis

The DNA–protein binding reaction contained 20 ng labelled DNA probe, 20 μg partially purified protein, 1 μg sonicated salmon sperm DNA in binding buffer (10 mM Tris–Cl, pH 8.0, 0.5 μg ml^{-1} bovine serum albumin, 100 mM KCl and 5% glycerol) in a total volume of 20 μl. DNA–protein complexes were allowed to form at room temperature for 23 min and resolved on a 5% non-denaturing polyacrylamide gel in 1 × TAE buffer (40 mM Tris–acetate, 1 mM Na_2EDTA, pH 8.0) at 5 V cm^{-1}. The gel was dried under vacuum at 80°C and autoradiographed to visualize DNA bands.

3. Results and discussion

The chromosomal locus bearing the mutant *ros* gene in strain 4011R cloned in Bluescript as pJA4R.1 was sequenced by the method of Sanger et al. [22]. This sequence was compared to that of the wild-type *ros* gene in strain 4011. As shown in Fig. 1, the mutation is localized to a single base substitution of C into T and results in the codon change from Arg to Cys at residue 125 near the carboxyl-terminus of Ros, outside of the zinc finger motif, which is located between residue 79 and 96, as described previously [1]. This change resulted in a hydrophobic shift from normally a hydrophilic domain just below the demarcation for amphipathic properties [23] into one that is hydrophobic (Fig. 1). The mutation removes a positively-charged amino acid to an uncharged residue. The area in general is highly charged and the local high charge density may be essential for protein function. Another possibility is that the Cys residue could interfere with the DNA-binding domain through its thiol group.

The Arg to Cys substitution does not affect translation of the mutant *ros* gene. Western immunoblot analysis revealed a protein product in 4011R of comparable size to that of wild-type Ros protein and is reactive to Ros antibody (Fig. 2), indicating that the key epitopes for Ros antibody recognition remain essentially unchanged by



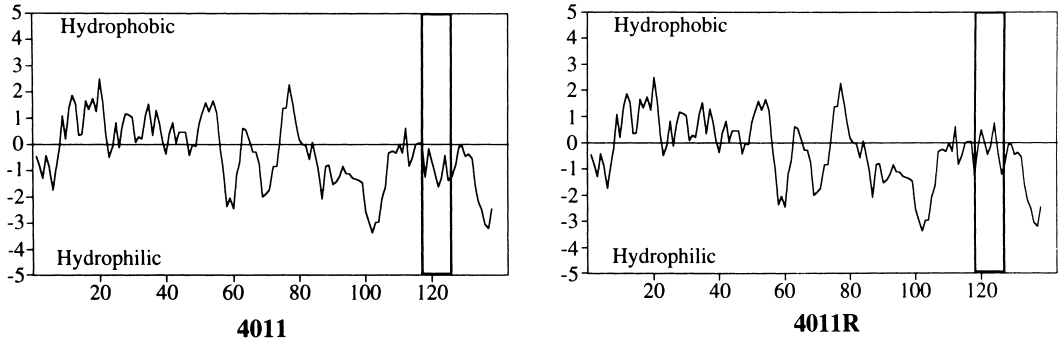


Fig. 1. Hydrophobicity plots of 4011 and 4011R as described by Kyte and Doolittle [23]. The boxed region shows the change in hydrophobicity of the Ros mutant 4011R. The amino acid and nucleotide sequences of 4011 and 4011R with the amino acid substitution indicated are shown above the plots.

the single amino acid change. Ros containing chloramphenicol acetyltransferase by the in frame fusion of the *cat* gene in either orientation in *ros* [7] was not recognized by the Ros polyclonal antibody (Fig. 2). The fusion yields a truncated protein that may be too small to detect, or the epitope may not be present in the truncated protein. As shown in Fig. 2 by the appearance of the Ros protein, these mutants were complemented by the wild-type *ros* gene. The result of this analysis indicates that the single amino acid substitution does not mask the epitopes on Ros, whereas the fusion of the *cat* gene in the *ros* gene results in a truncated product insufficient for antibody recognition.

The *vir*C/D promoter contains the Ros binding site termed the Ros box of 40 bp containing a 9-bp inverted repeat TATATTTCA/TGTAATATA [21]. The promoter region which was fused to the *cat* gene in plasmid pUCD206B [16] was used to test for repressor activity of Ros and the mutant Ros proteins. Wild-type Ros was found to repress *cat* gene expression, while *cat* expression remained unaffected in the Ros mutant. These results suggest that the mutant Ros protein from 4011R is unable to bind to the Ros box. To confirm this hypothesis, a gel mobility shift assay was performed. As shown in Fig. 3 and in contrast to the wild-type Ros repressor which binds to the Ros box (lane 2), the mutant Ros protein fails to bind to the Ros box (lane 3). As a control, another Ros mutant NT1R1, containing the *cat* insertion in *ros*, also does not bind to the Ros box (lane 4). It has been demonstrated in a previous study that mutations in the zinc finger region in Ros caused the loss of DNA binding activity as well as affinity for zinc ion [1], thereby showing this region to be essential for the repressor activity of Ros. The present study identified that the spontaneous mutation in the original Ros mutant [16] is located near the C-terminal region in Ros and not in the zinc finger itself. The mutation does not affect the overall stability of the protein. It is interesting to find that the conversion of Arg to Cys abolishes the repressor activity of Ros as assessed by gel mobility shift assay. Cysteine contributes the thio group -SH that could have an appreciable affect on the tertiary structure of Ros. Conformational studies on Ros and its mutant might reveal such structural changes. Such physical studies of this interesting repressor await its forthcoming crystallization and solution nuclear magnetic resonance analyses.

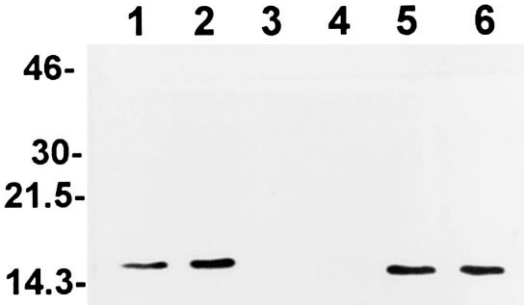


Fig. 2. Western blot analysis with polyclonal antiserum against Ros. Ten μg of protein were loaded in each lane. Lane 1: wild-type strain 4011, lane 2: mutant strain 4011R, lane 3–4: a *cat* gene insertional mutant strains 4011::39 and 4011::40, respectively, lanes 5–6: 4011::39 and 4011::40 complemented with plasmid pCR8 [17] expressing the wild-type *ros* gene.

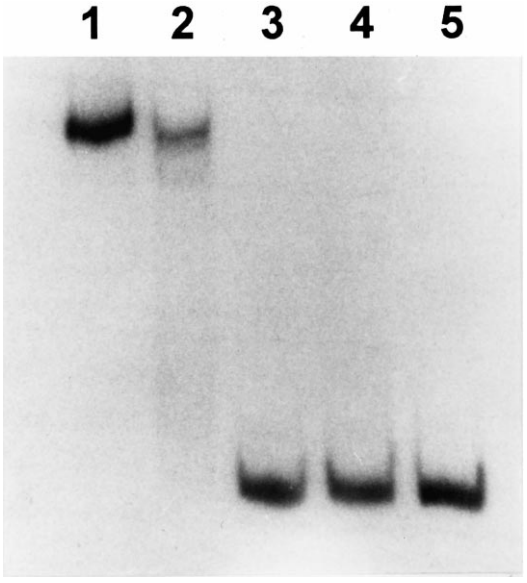


Fig. 3. Mobility shift assay using Ros and Ros mutant proteins. Lane 1: over-expressed wild-type Ros protein from BL21 DE3; lane 2: cell-free extract of wild-type Ros 4011; lane 3: mutant Ros 4011R; lane 4: Ros 4011::39 containing *cat*; lane 5: protein-free Ros box containing DNA that was used in each lane.

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