Silencing of *NbECR* encoding a putative enoyl-CoA reductase results in disorganized membrane structures and epidermal cell ablation in *Nicotiana benthamiana*☆

Jong-A Park[a], Tae-Wuk Kim[b,1], Seong-Ki Kim[b], Woo Taek Kim[a], Hyun-Sook Pai[c,*]

[a] *Department of Biology, Yonsei University 134, Seoul 120-749, Korea*
[b] *Department of Life Science, Chung-Ang University, Seoul 156-756, Korea*
[c] *Division of Bioscience and Bioinformatics, Myongji University, Yongin, Kyonggi-do 449-728, Korea*

Received 23 May 2005; revised 8 July 2005; accepted 10 July 2005

Available online 26 July 2005

Edited by Michael Sussman

Abstract The very long chain fatty acids (VLCFAs) are synthesized by the microsomal fatty acid elongation system in plants. We investigated cellular function of *NbECR* putatively encoding enoyl-CoA reductase that catalyzes the last step of VLCFA elongation in *Nicotiana benthamiana*. Virus-induced gene silencing of *NbECR* produced necrotic lesions with typical cell death symptoms in leaves. In the affected tissues, ablation of the epidermal cell layer preceded disintegration of the whole leaf cell layers, and disorganized cellular membrane structure was evident. The amount of VLCFAs was reduced in the NbECR VIGS lines, suggesting NbECR function in elongation of VLCFAs. The results demonstrate that *NbECR* encodes a putative enoyl-CoA reductase and that the NbECR activity is essential for membrane biogenesis in *N. benthamiana*.

Keywords: Cell death; ER-localization; Fatty acid elongation; Lipid analysis; Very long chain fatty acid

1. Introduction

The very long chain fatty acids (VLCFAs), i.e. fatty acids with more than 18 carbon atoms, are widely distributed in the plant kingdom and have multiple functions depending on chain length [1]. The major site of VLCFA synthesis is the epidermal cells where they are utilized for the production of waxes. VLCFAs are also critical components of seed triacylglycerol and sphingolipids in plants [2–4].

VLCFAs are formed by the microsomal fatty acid elongation (FAE) system in plants. FAE involves sequential additions of C2 moieties from malonyl coenyme A (CoA) to preexisting C16 or C18 fatty acids derived from the de novo FAS pathway of the plastid. Each cycle of FAE is accomplished by a series of four enzymatic reactions: (1) condensation of malonyl-CoA with a long-chain acyl-CoA; (2) reduction to β-hydroxyacyl-CoA; (3) dehydration to an enoyl-CoA; and (4) reduction of the enoyl-CoA, resulting in the elongated acyl-CoA [5]. Together, these four activities are termed the elongase [6]. Multiple condensing enzyme genes have been characterized so far, and the mutation of those genes inhibited wax formation or accumulation of seed triacylglycerol [7–9]. Very recently, the function of *ECR* encoding enoyl-CoA reductase catalyzing the last enzymatic step of FAE was analyzed in *Arabidopsis* [10]. T-DNA knockout mutants of *ECR* (*cer10*) exhibited morphological abnormalities and reduced size of aerial organs. Furthermore, the loss of ECR activity resulted in a reduction of cuticular wax load and affected VLCFA composition of seed triacylglycerols and sphingolipids, indicating that ECR is involved in all VLCFA elongation reactions in *Arabidopsis*.

In this study, we investigated the function of *NbECR* encoding a putative enoyl-CoA reductase in *N. benthamiana* by using virus-induced gene silencing (VIGS). Depletion of NbECR resulted in cell death, particularly in the epidermal layer, and highly disorganized cellular membrane structure. Composition of the VLCFAs was altered in the effected tissues. These results indicate that enoyl-CoA reductase in the VLCFA elongation system is essential for cell viability in *N. benthamiana*.

2. Materials and methods

2.1. VIGS

The *NbECR* cDNA fragments were PCR-amplified and cloned using *Xcm*I sites into the pTV00 vector that contains a part of the TRV genome. VIGS was carried out as described [11–13].

2.2. Semiquantitative reverse transcription (RT)-PCR

Semiquantitative RT-PCR was performed with 5 μg total RNA isolated from the 4th leaf above the infiltrated leaf as described [13]. To detect the *NbECR* transcripts, the NbECR-N (5′-CTCTTTCGTAGATCCC-3′ and 5′-GTAAGCAATGAAAGCTCC-3′) and NbECR-C (5′-GGGTTAGTTTGTCAAGTTG-3′ and 5′-ATACAAGAGAACCAAGGG-3′) primers were used. Sequences of the primers for various defense genes were previously described [14].

2.3. Evans blue staining, callose staining, and ion leakage measurement

Evans blue staining, callose staining, and ion leakage measurement were carried out as described [14].

☆ GenBank accession number: DQ000300

*Corresponding author. Fax: +82 31 335 8249.
E-mail address: hspai@mju.ac.kr (H.-S. Pai).

[1] Present address: Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA 94305, USA.

0014-5793/$30.00
doi:10.1016/j.febslet.2005.07.013

2.4. Subcellular localization of NbECR

The *NbECR* cDNA corresponding to the entire coding region were cloned into the 326-GFP plasmid [15] using *Bam*HI sites to generate the NbECR:GFP fusion protein. The BiP cDNA corresponding to the N-terminal 44 amino acids were cloned into the 326-RFP plasmid using *Bam*HI sites to generate the BiP:RFP fusion protein [16]. Transformation of the two fusion constructs into *N. benthamiana* protoplasts and microscopic observation of expression of the introduced genes were carried out as described [17].

2.5. Histochemical analysis

Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light microscopy were carried out as described [13], using the 4th leaf above the infiltrated leaf from the TRV and TRV:ECR lines.

2.6. Lipid analysis

Lipid extraction was performed as described [18]. Following addition of 60 μg of 17:0 free fatty acid (Sigma, St. Louis) as an internal standard, fatty acid methyl esters were prepared by acidic methanolysis [19]. Gas chromatography/mass spectrometry (GC–MS) analyses were carried out as described [20].

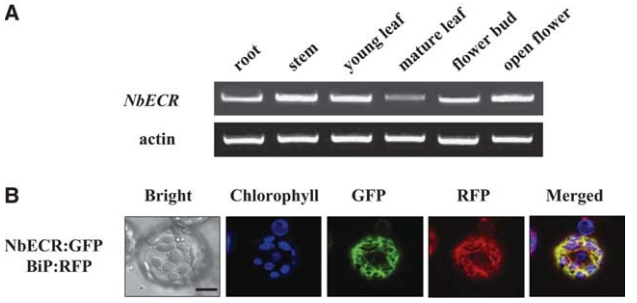

Fig. 1. Tissue-specific expression of *NbECR* and localization of the NbECR protein in the endoplasmic reticulum (ER). (A) Semiquantitative RT-PCR analyses were carried out to examine the *NbECR* mRNA level in different organs of *N. benthamiana*. (B) Subcellular localization of the *NbECR*-encoded protein was investigated by GFP (green fluorescent protein) fusion. *N. benthamiana* protoplasts were transformed with the *NbECR-GFP* fusion construct as well as with the *BiP:RFP* (red fluorescent protein) fusion construct to mark the ER, and the localization of the fluorescent signals was examined at 24 h after transformation under a confocal laser scanning microscope. The false color (blue) was used for chlorophyll autofluorescence to distinguish it from the red fluorescence of RFP.

3. Results

3.1. VIGS of NbECR caused formation of necrotic lesions on leaves

Functional genomics has been carried out in *Nicotiana benthamiana* using TRV-based VIGS. Through the screening we found that gene silencing of *NbECR* encoding a putative enoyl-CoA reductase causes spontaneous formation of necrotic lesions and abnormal leaf morphology. The full-length *NbECR* cDNA encodes a polypeptide of 310 amino acids that correspond to a molecular mass of 36272.2 Da. Alignment of the NbECR protein sequence with the related sequences, including *Arabidopsis ECR* and yeast *TSC13*, indicates that this group of the proteins is evolutionarily conserved (Supplementary Fig. 1). The high similarity in sequence and protein structure suggests that *NbECR* is a *N. benthamiana* orthologue of the *Arabidopsis ECR* and yeast *TSC13* genes.

3.2. Expression of NbECR and subcellular localization of its encoded protein

NbECR was expressed in all of the tissues tested, such as the roots, stems, young and mature leaves, flower buds, and open flowers (Fig. 1A). If NbECR is a component of ER-localized FAE complex, NbECR is likely to be localized in the ER. The subcellular localization of NbECR was examined by expressing a fusion protein between NbECR and green fluorescent protein (GFP). The *NbECR:GFP* construct under the control of the CaMV35S promoter was transformed into protoplasts isolated from *N. benthamiana* leaves (Fig. 1B). To track the ER, a DNA construct encoding BiP:RFP, a fusion protein between the ER-localized BiP (chaperonine binding protein) [16] and red fluorescence protein (RFP) was cotransformed into the protoplasts. After 24 h incubation, expression of the introduced gene was examined under a confocal laser scanning microscope. Green fluorescent signals perfectly overlapped with red fluorescent signals, demonstrating that the NbECR protein was targeted to the ER (Fig. 1B).

3.3. Suppression of endogenous NbECR expression

To confirm gene silencing of *NbECR*, we cloned three different *NbECR* cDNA fragments into the TRV-based VIGS vector pTV00 [11] and infiltrated *N. benthamiana* plants with *Agrobacterium* containing each plasmid (Fig. 2A). TRV:ECR(N) contained a 0.5 kb fragment covering a part of the 5′-UTR and the N-terminal coding region, and TRV:ECR(C) contained a 0.33 kb fragment covering a part of the 3′-UTR and the C-terminal coding region. TRV:ECR(F) contained the full-length coding region of the cDNA. VIGS with these constructs all resulted in the formation of large necrotic lesions on young leaves and abnormal leaf development (Fig. 2B). Some of the newly emerged leaves of the ECR VIGS lines were wrinkled and irregular in shape. The brown/transparent lesions were usually formed in the middle to lower part of the young leaves at about 14 days after infiltration. The cell death sometimes progressed to complete collapse and disappearance of the leaf tissues. Interestingly, lesions were found only in leaves. This cell death phenotype has been observed reproducibly in all of the >300 *N. benthamiana* plants that have been subjected to *NbECR* VIGS to date.

The effect of VIGS on endogenous *NbECR* mRNA levels was examined by semi-quantitative RT-PCR (Fig. 2C). RT-PCR using the *NbECR-N* primer (marked in Fig. 2A) showed significantly reduced PCR product levels in the TRV:ECR(C) and TRV:ECR(F) lines relative to the control TRV, indicating that the endogenous level of the *NbECR* mRNA was greatly reduced in those plants. The same primers detected high levels of the viral genomic transcripts containing the N-terminal region of the *NbECR* cDNA in the TRV:ECR(N) lines. RT-PCR with the *NbECR-C* primer (marked in Fig. 2A) revealed suppression of *NbECR* expression in the TRV:ECR(N) and TRV:ECR(F) plants, and detected the viral genomic transcripts in the TRV:ECR(C) plants. The actin transcript levels, which serve as a control, remained constant. These results demonstrate that the endogenous *NbECR* expression was significantly reduced in the VIGS lines and the cell death pheno-