Deletion of C-terminus of ETC2 confers partial protein stability in Arabidopsis root epidermal cells

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ABSTRACT

Root hair is a specialized epidermal cell in plants, which is important for water absorption and nutrient uptake from the soil. The ENHANCER of TRY and CPC2 (ETC2) encodes an R3-type MYB transcription factor that belongs to the CAPRICE (CPC) gene family in Arabidopsis thaliana. Although, the CPC family genes have a common function to promote root hair cell differentiation, ETC2 hardly promotes root hair formation. Previously, we showed that ETC2 had a longer C terminus than that of the other CPC family proteins and that the deletion of the extended C terminus of ETC2 conferred root hair forming function to ETC2. We hypothesized that the C-terminal region contributed to the rapid degradation of ETC2 protein. In this study, we tried to compare the accumulation of the ETC2 protein and the C termini-truncated ETC2 protein (ETC2∆C) using immunoblot analysis. A faint band corresponding to the ETC2ΔC-2×GFP protein was detected, but the ETC2-2×GFP protein was not detected. Our results confirmed that the deletion of C terminus of ETC2 somehow contributed to the stability of ETC2. Moreover, ETC2 was revealed to be unstable protein even when the C terminus was removed. Our findings demonstrate intrinsic differences in the regulatory mechanism of CPC family proteins that mediate root hair formation.

Keywords: ETC2, MYB, root hair, Arabidopsis thaliana.

INTRODUCTION

Root hairs are specialized epidermal cells in plants and are important organs for water absorption and nutrient uptake from the soil. Epidermal cells differentiate into two types of cells, namely root hair cells and non-hair cells in Arabidopsis thaliana (Dolan et al., 1994, 1993). The transcription factor complexes, including WEREWOLF (WER) (Lee and Schiefelbein, 1999), GLABRA3/ENHANCER of GL3 (GL3/EGL3) (Bernhardt et al., 2003; Payne et al., 2000) and TRANSPARENT TESTA GLABRA (TTG1) (Walker et al., 1999), promote the transcription of GLABRA2 (GL2) (Koshino-Kimura et al., 2005) and induce non-hair cell formation (Bernhardt et al., 2005; Tominaga-Wada et al., 2011). ENHANCER OF TRY AND CPC2 (ETC2), which encodes an R3-type MYB protein was identified as a homolog of the CAPRICE (CPC) family genes (Kirik et al., 2004b). The CPC family includes the Tryptichon(TRY), ETC1, ETC2, ETC3/CPL3, Trichomeless1 (TCL1) and Trichomeless2 (TCL2) genes (Esch et al., 2004; Gan et al., 2011; Kirik et al., 2004a, b; Schellmann et al., 2002; Tominaga et al., 2008; Tominaga-Wada and Nukumizu, 2012; Wada et al., 1997; Wang et al., 2007).

The CPC family genes are supposed to act as positive regulators of root hair cell differentiation (Tominaga-Wada et al., 2011). Although the CPC family genes have mainly overlapping functions in root hair differentiation, their precise functions are different (Wang et al., 2008). The CPC mutant showed a dramatically reduced root hair number phenotype (Wada et al., 1997). On the other hand, an obvious reduction in the root hair number was not observed in any of the other CPC family mutants (Kirik et al., 2004a, b;
To understand the functional differences in the CPC family genes in root hair formation, we previously analyzed the transgenic plants expressing the CPC, TRY, ETC1, ETC2 and CPL3 genes under the control of CPC promoter (Tominaga-Wada and Wada, 2017). Among them, only TRY and ETC2 showed less effect on the root hair formation and were unstable in the root epidermis. Truncation of the extended C terminus of TRY and ETC2 restored the function of these proteins in the root hair formation (Tominaga-Wada and Wada, 2017). In the case of TRY, we clearly demonstrated by immunoblot analysis that the deletion of C terminus of TRY enhanced the protein stability.

In this study, we examined the effect of the C terminal region of ETC2 on protein stability by immunoblot analysis. We observed the fluorescence of GFP-fusion proteins in several independent CPCp:ETC2-2xGFP and CPCp:ETC2ΔC-2xGFP transgenic lines. In addition, we checked the effects of proteasomal inhibitors on ETC2 accumulation.

MATERIALS AND METHODS

Plant materials and growth conditions

The A. thaliana wild type Col-0, CPCp: ETC2-2xGFP and CPCp:ETC2ΔC-2xGFP transgenic plants used in this study were all previously described (Tominaga-Wada and Wada, 2017). The seeds were sown on 1.5% agar plates, as described by Okada and Shimura (1990).

Western blotting

Arabidopsis roots of 7-day-old seedlings were ground using Tissue Lyser (Qiagen, CA, USA). Proteins were extracted from whole-cell extracts using the P-PER Plant Protein Extraction Kit (Thermo Scientific) with Halt™Protease Inhibitor Single-Use Cocktail (Thermo Scientific), according to the manufacturer’s instructions. The protein content of each fraction was measured using the Pierce BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific) and bovine serum albumin as the standard. The proteins were separated by SDS-PAGE on a 10% Mini-PROTEAN EGX Precast Gel (Bio-Rad, CA, USA). The gels were electrophoresed at 200 V for 50 min and then the proteins were transferred to PVDF membrane (Bio-Rad 162-0174) (Bio-Rad, CA, USA) by wet electroblotting system for 30 min. We used mouse anti-GFP antibody (1:10000; Living Colors A.x Monoclonal Antibody)(Clontech,CA,USA) and sheep anti-GFP antibody (1:10000; Amersham ECL Anti-Mouse IgG HRP-linked species specific whole antibody from sheep) (GE Healhtcare) for immunoblotting. The immunoblotted proteins were detected with ImmunoSter LD system (Wako, Osaka, Japan) and Ez-Capture MG imaging system (ATTO, Tokyo, Japan).

RESULTS AND DISCUSSION

C-terminal region of ETC2 is somehow involved in the stability of ETC2 protein

The ETC2 gene encodes an R3-type MYB protein and extended C-terminal region of 18 amino acids compared to the other CPC family proteins (Tominaga-Wada and Wada, 2017) (Figure 1A). Previously, we showed that the deletion of the extended C-terminal region of TRY and ETC2 enhanced their root hair forming function (Tominaga-Wada and Wada, 2017) (Figure 1B). We demonstrated that TRY protein was able to degrade because of the properties of the C-terminal region, using immunoblot analysis (Tominaga-Wada and Wada, 2017). On the other hand, ETC2 was not clearly proven to possess the unstable protein property because of the C-terminal domain. In this study, to investigate whether the low levels of ETC2 protein in the transgenic roots were restored by C-terminus truncation, we performed immunoblot analysis. The subtle differences in the accumulation levels of ETC2-2xGFP and ETC2ΔC-2xGFP fusion proteins were determined in the proteins extracted from the root tissue of wild type (Col-0), CPCp:ETC2-2xGFP and CPCp:ETC2ΔC-2xGFP transgenic plants using an anti-GFP antibody (Figure 1C). Unfortunately, a distinct band, as previously observed in the protein extract from CPCp: TRYΔC-2xGFP (Tominaga-Wada and Wada, 2017) was not detected either in the CPCp: ETC2-2xGFP or CPCp: ETC2ΔC-2xGFP transgenic plants (Figure 1C). However, faint bands of the predicted molecular mass (65 kDa) corresponding to the ETC2ΔC-
A ETC2 MDNTNLRGGPSLRQTKFTRSRYSDEEVSSI EWEFI SMEQEDDSL SRMYRLVG NRWDLI AGRVVGRKANE ERYW MRNSDFSHKRRRLNNSPFFSTSPNLQENLKL

B

<table>
<thead>
<tr>
<th>Col-0</th>
<th>CPCp:ETC2 ΔC-2xGFP(kDa)</th>
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<tbody>
<tr>
<td>20</td>
<td>15</td>
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<tr>
<td>65 kDa</td>
<td>50 kDa</td>
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C

2xGFP protein were detected in the CPCp: ETC2ΔC-2xGFP transgenic plants (Figure 1C). The bands corresponding to 2xGFP (50 kDa) appeared relatively stronger in the CPCp: ETC2-2xGFP plants compared to that in the CPCp: ETC2ΔC-2xGFP transgenic plants (Figure 1C). The bands corresponding to ETC2-GFP or ETC2ΔC-GFP (38 kDa) appeared relatively stronger in the CPCp: ETC2ΔC-2xGFP transgenic plants than in the CPCp: ETC2-2xGFP transgenic plants (Figure 1C). These results suggest that ETC2-2xGFP and ETC2-GFP proteins degraded faster than the ETC2ΔC-2xGFP and ETC2ΔC-GFP proteins in the transgenic plants. Although, truncation of the C terminus of ETC2 enhanced the protein accumulation, the ETC2ΔC-2xGFP protein appeared to be unstable in the CPCp: ETC2ΔC-2xGFP transgenic plants.

We also observed GFP fluorescence more precisely than in the previous study by using several independent transgenic plants (Tominaga-Wada and Wada, 2017) (Figure 2). In agreement with the previous report, the roots of CPCp: ETC2ΔC-2xGFP transgenic plants showed stronger GFP fluorescence than those of the CPCp: ETC2-2xGFP plants (Figure 2). This result confirmed that the truncation of C terminus of ETC2 does not favor the degradation of the ETC2 protein. The slight GFP fluorescence observed in CPCp: ETC2-2xGFP#1 and #2 (Figure 2) might correspond to the slight bands of 2xGFP (approximately 50 kDa) (Figure 1C).
Figure 2: Distribution of ETC2-2xGFP and ETC2ΔC-2xGFP fluorescence. GFP fluorescence (green) and propidium iodine (PI) fluorescence were observed in A) CPCp:ETC2-2xGFP (#1, #2, and #3), B) CPCp:ETC2ΔC-2xGFP (#1, #2, and #3), and C) CPCp:ETC1-2xGFP and CPCp:TRYΔC-2xGFP transgenic plants. Scale bar: 200 µm.

(lanes 3 to 6). The GFP fluorescence in the roots of CPCp:ETC2ΔC-2xGFP#1 and #2 transgenic plants (Figure 2) might correspond to the bands of ETC2ΔC-2xGFP, 2xGFP and ETC2ΔC-GFP (approximately 65, 50 and 38 kDa, respectively) (Figure 1C) (lanes 7 to 10). However, the GFP fluorescence in the CPCp:ETC2ΔC-2xGFP(#1 and #2) transgenic plants was still much weaker than that in the CPCp:ETC1-2xGFP or CPCp:TRYΔC-2xGFP transgenic plants (Figure 2, bottom panel). Our results suggest that the degradation of ETC2 protein was not completely stopped by C terminal truncation. In this aspect, ETC2 is distinctly different from TRY in which the degradation was clearly stopped by C terminal truncation (Tominaga-Wada and Wada, 2017). We hypothesized that there was another ETC2 protein specific degradation mechanism, different from that of the TRY protein. In addition, we observed relatively strong GFP fluorescence in the stele of CPCp:ETC2-2xGFP #3 and CPCp:ETC2ΔC-2xGFP #3 transgenic plants (Figure 2). The accumulation of GFP fusion proteins in the stele, with or without C terminus of ETC2 proved that the ETC2 protein degradation was an epidermal cell-specific function.

Effect of inhibitors on the localization of ETC2 protein

To further clarify the degradation mechanism of the ETC2 protein, we tested the effects of inhibitors on ETC2-2xGFP
protein accumulation (Figure 3). Plants selectively degrade proteins through the ubiquitin (Ub)/26S proteasome proteolytic pathway to achieve an additional layer of regulatory control (Hershko and Ciechanover, 1998; Pickart, 2001). However, four proteasome inhibitors, MG132, MG115, epoxomicin and lactacystin did not promote the accumulation of ETC2-2xGFP fusion protein (Figure 3). These results suggest that the Ub/26S proteasome pathway might not be the main mechanism for controlling the ETC2 activity. The addition of protease inhibitor E-64d, which stops the constitutive autophagy in Arabidopsis root tip cells (Inoue et al., 2006) did not promote accumulation of ETC2-2xGFP (Figure 3). To examine whether the classical secretion process is involved or not, we used brefeldin A (BFA), which blocks the secretory pathway from the endoplasmic reticulum (ER) to the plasma membrane by inhibiting the vesicle formation at the Golgi apparatus (Geldner et al., 2003; Ritzenthaler et al., 2002). The BFA treatment did not also promote the accumulation of ETC2-2xGFP (Figure 3). These results suggest that neither the protease-mediated degradation nor the vesicle secretion controlled the accumulation of ETC2.

Conclusions
In this study, we used immunoblot analysis to demonstrate that the ETC2 protein was degraded partially depending on
the property of the C-terminal domain (Figure 1C). However, the fluorescence of ETC2ΔC-2xGFP was weaker than that of ETC1-2xGFP or TRY2ΔC-2xGFP, indicating that ETC2ΔC protein was unstable (Figure 2). Our results suggest that the degradation of ETC2 protein depends not only on the C terminus but also on another amino acid sequence. The inhibitors including four proteasome inhibitors, protease inhibitor E-64d, and BFA did not affect the ETC2-2xGFP protein accumulation (Figure 3). Further investigation is necessary to elucidate the mechanism and biological function of the ETC2 protein degradation.

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