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A full-length R-like basic-helix-loop-helix transcription factor is required for anthocyanin upregulation whereas the N-terminal region regulates epidermal hair formation

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ABSTRACT

Earlier studies have shown that the *Lc* gene of maize, a member of the *R* gene family that encode basic-helix-loop-helix (bHLH) transcription factors, is involved with anthocyanin production and trichome formation in *Arabidopsis*. We previously reported that the N-terminus of R protein interacts with CAPRICE (CPC), a regulatory protein, in triggering epidermal hair differentiation in *Arabidopsis*. In this study, we investigated the roles of full-length R, the N-terminal region of R (RN) and the C-terminal region of R (RC) in epidermal cell differentiation and anthocyanin production. We found that the N-terminal region was responsible for leaf trichome and root hair differentiation, whereas full-length R was required for anthocyanin upregulation. Yeast two-hybrid analysis showed that the C-terminal region was the binding site for the formation of homo- or hetero-dimers of the R-like bHLH transcription factor. To stimulate anthocyanin production, full-length R is required.

1. Introduction

The basic helix-loop-helix (bHLH) domain was first identified as a conserved region present in ten DNA-binding proteins from animals [1]. The bHLH proteins belong to a family of transcription factors (TFs) present in all three eukaryotic kingdoms [2–4]. In plants, a bHLH TF was first identified as a regulator of anthocyanin biosynthesis in maize and named R(Lc) [2]. The maize *B* gene is a homolog of the *R* gene, and their two gene products act in concert with proteins encoded by the *C1/Pl* gene family in activating most of the structural genes of the anthocyanin biosynthetic pathway [5,6]. The maize R and B proteins have been shown to induce anthocyanin pigmentation in other monocots such as wheat and barley, and in dicots such as *Arabidopsis* and petunia [7–10]. Orthologous genes of the maize *R* gene, which also regulate anthocyanin pigmentation, were identified in *Antirrhinum*, petunia and rice [11–13]. The *Arabidopsis GL3* gene was finally identified as an *R* gene ortholog [14].

The *GL3* gene regulates trichome development as a bHLH TF with significant homology to R [14]. Mutations in the *TRANSPARENT TESTA GLABRA1* (*TTG1*) locus in *Arabidopsis*, which result in several defects including a lack of trichomes and ectopic root-hair formation [15], could be rescued by overexpression of the maize *R* gene [7,8]. Therefore, *TTG1* was long considered to encode an *R*-like bHLH TF gene; however, the initial cloning of TTG1 demonstrated that it contains a WD40 motif and has no homology with bHLH TFs [16].

Molecular genetic analyses have revealed the involvement of several additional TFs in regulating the fate of epidermal cells in *Arabidopsis*. In addition to *TTG1*, *GLABRA2* (*GL2*) and *WEREWOLF* (*WER*) are involved in the formation of non-hair root cells, as observed by ectopic root-hair formation in *ttg1*, *gl2* and *wer* mutants [8,17,18]. GL2 is a homeodomain-leucine zipper (HD-Zip) protein expressed in non-hair root cell files [18–20]. *WER*, which encodes an R2R3-type MYB protein, can activate *GL2* expression [17]. Two bHLH TF genes, *GL3* and *ENHANCER OF GLABRA3* (*EGL3*), a *GL3* homologous gene, also act as negative regulators of root-hair differentiation in a redundant manner [21].

Positive regulation of *Arabidopsis* root-hair cell differentiation is controlled by *CAPRICE* (*CPC*) [22]. The *cpc* mutant was isolated based on its low number of root hairs [22]. *CPC* encodes a small R3-type MYB protein [22]. *TRIPTYCHON* (*TRY*), *ENHANCER OF TRY AND CPC1* (*ETC1*), *ENHANCER OF TRY AND CPC2* (*ETC2*) and *ENHANCER OF TRY AND CPC3* (*ETC3*)/*CPC LIKE MYB3* (*CPL3*) were isolated as *CPC* homologs and also act as positive regulators of root-hair formation

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[23–28]. For the aerial organs of *Arabidopsis*, *TTG1*, *GL2*, and *WER* family R2R3-type MYB genes [*WER*, *GLABRA1* (*GL1*) and *MYB23*] are positive regulators of trichome formation [8,16,18,20,29,30]. The bHLH TFs, *GL3* and *EGL3*, also act as positive regulators of trichome formation in a redundant manner [14,31]. R3-type MYB genes *CPC*, *TRY*, *ETC1*, *ETC2* and *ETC3/CPL3* are involved in negative regulation of trichome initiation [22–28,32–34]. As a result of these molecular genetic studies in *Arabidopsis*, it is evident that formation of root hairs and trichomes is regulated by common molecular mechanisms.

GL3 and EGL3 interact with TTG1 and WER in yeast cells, suggesting the existence of a MYB-bHLH-WD40 transcriptional complex that regulates the fate of root-hair cells [14,21,31,35]. CPC also physically interacts with both GL3 and EGL3, suggesting a competitive model for CPC and WER for GL3 or EGL3 binding sites [17,21,36]. In aerial organs of *Arabidopsis*, the MYB-bHLH-WD40 complex that includes GL1 or MYB23, GL3 or EGL3 and TTG1 activates downstream gene expression and promotes trichome formation [23,37–39]. TRY disturbs the formation of the GL1-GL3/EGL3-TTG1 complex and inhibits trichome initiation [24,35].

In this study, we designed experiments to define the precise function of R-like bHLH TF using maize R. Previously, R was reported to interact with WER, and we demonstrated that the N-terminal region of R interacts with CPC and functions in root epidermal cell differentiation in *Arabidopsis* [17,32]; however, the function of the C-terminal region of R remained unknown. In this report, we compare the functions of full-length R, the N-terminal region of R (RN) that includes an acidic region, and the C-terminal region of R (RC) that includes a bHLH region in *Arabidopsis* epidermal cell differentiation and anthocyanin pigmentation. Our data identifies the functions of the N-terminal region of R-like bHLH TF in trichome and non-hair cell differentiation. In contrast, the C-terminal region of R-like bHLH TF might be involved in anthocyanin pigmentation.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) plants were used as the wild type in this study. Seeds were surface-sterilized and sown on 1.5% agar plates as described previously [40] and propagated for observation of seedling phenotypes. Seeded plants were kept at 4 °C for 2 d and then incubated at 22 °C under constant white light 50–100 $\mu mol\, m^{-2}\, s^{-1}$. For each transgenic line, at least ten individual 5-day-old seedlings were assayed for root hairs, and at least five individual 2-week-old third leaves were assayed for trichomes. *35S::R 35S::WER*, *35S::RN 35S::WER*, *35S::RC 35S::WER* and *35S::R 35S::MYB23* plants were obtained by crossing homozygous transgenic lines, and F2 progeny were analyzed.

2.2. Gene constructs

35S::R construct was provided by Alan M. Lloyd [7]. The *35S::RN* construct was created as previously described [32]. For *35S::RC* construction, a *Xba*I-*Pst*I fragment including the 3′ UTR region was created using the following primers: TW1105, 5′-CTAGAATCGAGTTGTTGTACTCTTCGCAGATAGGCGCGTGATGCA-3′; TW1106, 5′-TCACGCGCCTATCTGCGAAGAGTACAACAACTCGATT-3′, and the *Pst*I-*Xba*I fragment of the C-terminal region of *R* was ligated into the *Xba*I site of *pBluescript SK+*. This plasmid DNA was digested with *Sac*I and *BamH*I, and subcloned into the *Sac*I and *BamH*I sites of the *pCHF3* binary vector to create *35S::RC*. The construction of *35S::WER* was described previously [17].

2.3. Transgenic plants

Plant transformation was performed by a floral dip method [41], and transformants were selected on 0.5× MS agar plates containing 50 mg/L kanamycin. Homozygous transgenic lines were selected based on kanamycin resistance. We isolated at least 20 T1 lines for each construct and selected at least 10 T2 and 4 T3 lines on the basis of their segregation ratios for kanamycin resistance.

2.4. Light microscopy

To observe trichomes, images were recorded with a VC4500 3D digital fine microscope (Omron, Kyoto, Japan) or digital microscope (VH-8000; Keyence, Osaka, Japan). At least five 2-week-old third true leaves were analyzed for trichome number for each transgenic line. Trichome number was analyzed on the ad-axial side of the leaves. Root phenotypes were observed using an Olympus Previs AX70 microscope and an Olympus SZH binocular microscope. For each transgenic line, at least ten individual 5-day-old seedlings were analyzed for root-hair number.

2.5. Extraction and analysis of anthocyanin

Wild type and transgenic plants were grown together in a growth chamber as described above. Leaves from four to six independent homozygous transgenic lines were harvested after 3 weeks and the fresh weight was determined. Total plant pigments were extracted overnight in 0.3 mL acidic methanol (1% HCl). After the addition of 0.2 mL water and an equal volume of chloroform, anthocyanins were separated from the chlorophylls by partitioning into the aqueous methanol phase and the absorption was measured at 530–657 nm in a spectrophotometer (GENios, TECAN). Anthocyanin levels were then normalized to the total fresh weight of tissue used in each sample [42,43].

2.6. Yeast two-hybrid assay

For binding domain fusions (-BD), DNA fragments corresponding to the C-terminal 237 amino acid region of *GL3* cDNA and C-terminal 299 amino acid region of *EGL3* cDNA were amplified using primers with an EcoRI site at the 5′-end and SalI at the 3′-end, and cloned into pBGT9 (Clontech) to create GL3 C-BD and EGL3 C-BD, respectively. For activation domain fusions (-AD), DNA fragments corresponding to the coding regions of the *TTG1* cDNA, *GL3* cDNA, *EGL3* cDNA, the N-terminal 405 amino acid region of *GL3* cDNA, N-terminal 367 amino acid region of *EGL3* cDNA, C-terminal 237 amino acid region of *GL3* cDNA and C-terminal 299 amino acid region of *EGL3* cDNA were amplified using primers with an EcoRI site at the 5′- and SalI at the 3′-end, and cloned into pGAD424 (Clontech) to create TTG1-AD, GL3-AD, EGL3-AD, GL3 N-AD, EGL3 N-AD, GL3 C-AD and EGL3 C-AD, respectively. The yeast two-hybrid assay was performed as described [32]. The activity was expressed as β-Gal activity units: OD_{420}/OD_{600} of assayed culture × volume assayed × time.

OD_{420} is the optical density of the product, o-nitrophenol. OD_{600} is the optical density of the culture at the time of assay. Volume is the amount of the culture used in the assay in mL. Time is in minutes.

3. Results

3.1. Evolutionary relationships among R(Lc) orthologous bHLH TF proteins

The maize *R*(*Lc*) gene encodes a TF protein containing a bHLH motif [2]. Three orthologs of *R*(*Lc*) were characterized from