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Differentiated cuticular wax content and expression patterns of cuticular wax biosynthetic genes in bloomed and bloomless broccoli (*Brassica oleracea* var. italica)

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ABSTRACT

The aerial surfaces of plants are covered with a wax layer that serves the essential functions of limiting non-stomatal water loss and acting as protective barrier against environmental stresses. We selected two broccoli lines, bloomed (MC91) and bloomless (MC117), and analyzed their phenotypes related to cuticular wax accumulation. The total wax amount was 1.93-fold higher in MC91 leaves compared to MC117 leaves. All of the studied cuticular wax compounds were 1.07–3.79-fold higher in MC91 plants compared to MC117 plants except for the C_{31} alkane. The wax compositions did not essentially different between the two broccoli lines, but some compounds were found at significantly higher levels in MC91 plants compared to MC117 plants, mainly reflecting differences in C_{29} alkanes, C_{29} secondary alcohols and C_{29} ketones. To investigate gene regulation by bloom phenotype, we analyzed the mRNA expression patterns of various cuticular wax biosynthetic genes. Our results revealed that *LACS1*, *KCS1*, *KCR1*, *ECR*, *CER3* and *MAH1* were expressed more in MC91 plants compared to MC117 plants at both 3 and 10 weeks. The expression levels of the studied cuticular wax biosynthetic genes were significantly induced by drought stress, which is known to induce cuticular wax deposition. Together, these results show that the cuticular wax accumulation of broccoli is regulated by cuticular wax biosynthetic gene expression and can be affected by environmental signals.

1. Introduction

The plant cuticle is a non-cellular outermost hydrophobic layer that protects plants against biotic and abiotic stresses, including drought, UV radiation, insect attack and pathogen infection [1–4]. Cuticular wax biosynthesis is regulated in response to drought or the need to reduce water loss, which affects plant growth and productivity and reduces crop yield [5,6].

This cuticle layer is synthesized by epidermal cells and is largely comprised of cutin and cuticular wax [7,8]. Cuticular wax is produced from very long-chain fatty acids (VLCFA; C_{20} to C_{34}), which are generated and then modified into primary alcohols and wax esters (via acyl-reduction pathways), or aldehydes, alkanes, secondary alcohols and ketones (via decarbonylation pathways) [9–11]. Fatty acid elongation involves four sequential reactions: condensation of two-carbon units to acyl-CoA by 3-ketoacyl-CoA synthetase (*KCS*); reduction of 3-ketoacyl-CoA by 3-ketoacyl-CoA reductase (*KCR*); dehydration of 3-hydroxyacyl-CoA by 3-hydroxyacyl-CoA dehydratase; and reduction of trans-2,3-enoyl-CoA by trans-2-enoyl-CoA reductase [12–14]. For the generation of a very long-chain (VLC) alkane, the multiprotein enzyme complex, *CER3*/*WAX2*/*YRE*/*FLP1*, catalyzes the conversion of VLC acyl-CoA to the VLC alkane [8]. A primary alcohol is generated by fatty acyl-CoA reductase (*FAR3*/*CER4*), and may then be condensed into wax esters by *WSD1*. Midchain alkane hydroxylase 1 (*MAH1*) catalyzes an alkane into a secondary alcohol and subsequently oxidizes the

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secondary alcohol to a ketone [8]. Cuticular wax components are generated in the ER, transported to the plasma membrane, and then exported to the apoplast via an ATP-binding cassette (*ABC*) transporter [15]. Lipid transfer proteins (*LTP*) have been suggested to be involved in transporting cuticular wax components through the hydrophilic cell wall. For example, GPI-anchored lipid transfer protein 1 (*LTPG/LTPG1*) and *LTPG2* have been shown to be directly or indirectly involved in the export or accumulation of cuticular waxes [16,17].

Although the quality and quantity of plant cuticular waxes reportedly differ by species and organ [18], relatively few reports have studied cuticular wax in *Brassica* (broccoli) species. Some studies in broccoli have reported the identification of a lipid transfer protein [19] and the expression patterns of various wax-associated proteins [20]. The bloomed phenotype is often used as a morphological marker in breeding and genetic studies on broccoli, and the epicuticular wax composition is believed to influence insect resistance, reproduction, and leaf color in *Brassica oleracea* [21–23]. Leaf color, which reflects the wax composition, is a main target trait of breeding, and breeders therefore need molecular markers that will enable them to select this trait in *Brassica* species. However, the wax components of broccoli have not yet been studied in detail. Furthermore, the relationship between cuticular wax composition and wax biosynthesis gene expression has not yet been fully elucidated in this plant.

In the present study, we quantified and compared cuticular wax components in two broccoli lines (*B. oleracea* var. *italica*) with different cuticular wax phenotypes. We also analyzed the expression patterns of 10 genes involved in cuticular wax biosynthesis, and assessed correlations between metabolic pathways and gene transcription.

2. Materials and methods

2.1. Plant materials

Seeds of broccoli (*B. oleracea* var. italica) strains MC117 and MC91 were obtained from Koregon Seed Co., Ltd. (Ansung, Korea) and grown in soil-based compost under standard greenhouse conditions (Daejeon, Korea). Leaves were sampled from 3- or 10-week-old plants, immediately frozen in liquid nitrogen, and stored at −80 °C for RNA isolation. Fresh 10-week-old plants were subjected to gas chromatography–mass spectrometry (GC–MS) and gas chromatography-flame ionization detector (GC-FID) analysis.

2.2. Analysis of cuticular wax composition

Fresh leaves (10 g) were obtained from 10-week-old plants and cuticular wax was extracted in methylene chloride for 30 s at room temperature. Palmitic acid, stearic acid, pentacosane, behenic acid, heptacosane, lignoceryl alcohol, lignoceric acid, nonacosane, 1-hexacosanol, hexacosanoic acid, hentriacontane, 1-octacosanol, *n*-tritriacotane, 1-triacontanol and melissic acid were added to the extracted methylene chloride solvents as standards. The solvent was then evaporated under a gentle stream of nitrogen, and the residue was dissolved in a mixture of 100 μl of pyridine and 100 μl of bis-*N*,*N*-(trimethylsilyl)trifluoroacetamide. Each sample was then heated at 80 °C for 30 min to convert the waxes into trimethylsilyl derivatives. The wax composition was determined by capillary gas chromatography (GC) (6890N; Agilent) and a mass spectrometric detector (5973N; Agilent). The utilized temperature profile was as follows: 50 °C for 2 min, increased at 40 °C/min to 200 °C, held for 2 min at 200 °C, increased at 3 °C/min to 320 °C, and held for 30 min at 320 °C. Qualitative and quantitative compositional analyses were performed as previously described [24].

Table 1
List of primer sequences used for RT-PCR of the cuticular wax biosynthesis genes.

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)
LACS1	GATTAGTCCACCTAAACCATTT	ATCCGGTATACACATAGCAG
KCS1	TCTGACGTTCGAGACCTTCTCC	TGAATCCAAAGCACCGAACA
KCR1	TCCATCCTCAGGTTCACCTT	CGCTTCCTCTTCAGCATATTCAC
ECR	GAGTACTGTGATGGAAACACAG	TTCGCCGTAGCCCAGGTATT
CER3	CCGAACCAAGAGGGATGGCA	CCACAAGACTGGCTGAAGAT
MAH1	AGAGAGGAGTTAAGTCGCGG	GAGGATGCATAGAGAGAAGCCAG
LTP2	TCAGCTAGCGTGTCAGAACAAATGG	CCGACCCTAATCCTTCGGGTGA
FAR3	GAAATGTGAAAACGCTAAGATCT	CGTAACGCTTTTATCCAACAATT
WSD1	AACAACACCGGAGACACGTCC	TGGACGGACTGAGCTCCACT
ABCG11	CCTCGGTCGGGGGAGGGAAC	AGCACAGTCCTGAAGACCCA
ACT2	ATCACACTTTCTACAATGAGC	TCGTAGATTGGCACAGTGTGAG

Molecular identities were determined using an Agilent 6890 GC and an Agilent 5973 mass spectrometric detector. Cuticular waxes were quantified in three biological replicates for each sample.

2.3. Drought-stress treatment

Leaves were collected from 10-week-old plants, placed between sheets of 3 MM What-man chromatography paper, and incubated in the dark in a growth room at 22 °C. After 24 h, the drought-stressed leaves were collected. Leaves sampled at the same time as those subjected to drought stress (0 h) were used as control samples.

2.4. RNA extraction and RT-PCR analysis

Total RNA was isolated using the TRIzol reagent (Gibco-BRL), and first-strand cDNA synthesis was performed using ReverTra Ace (TOYOBO) according to the manufacturer's instructions. Orthologs of the wax biosynthesis genes were identified from our internal *B. oleracea* transcriptome database (unpublished data) using the *Arabidopsis* gene sequences of *LACS1* (NM_130292), *KCS1* (NM_099994), *KCR1* (NM_105441), *ECR* (NM_115394), *CER3* (NM_125164), *MAH1* (NM_001124037), *LTP2* (NM_129410), *FAR3* (NM_119537), *WSD1* (NM_123089) and *ABCG11* (NM_101647). To determine the expression patterns of the wax biosynthetic genes, RT-PCR was performed using gene-specific primers and actin 2 (*ACT2*) [25], which were designed using Primer 3 (http://frodo.wi.mit.edu/primer3) and are listed in Table 1. RT-PCR runs had 28 cycles, each of which consisted of template denaturation at 94 °C for 30 s; primer annealing at 55 °C for 30 s; and elongation at 72 °C for 1 min. PCR products were separated by 1.2% agarose gel electrophoresis.

3. Results and discussion

3.1. Morphological characteristics of bloomed and bloomless broccoli

Two broccoli lines were visually selected from our in-house double haploid collection based on their bloomed (MC91) and bloomless (MC117) phenotypes. Both lines were examined for bloom deposition on the plant surface from 3 to 10 weeks after sowing. Bloom deposition was detected in the shoot systems (e.g., the leaves, petioles, stems, internodes and terminal buds) of 3-week-old MC91 plants; in contrast, MC117 plants showed no visible bloom deposition at this time point (Fig. 1). Both lines showed visible bloom deposition at 10 weeks after sowing, but there were prominent differences in the degree of blooming on the leaf surface. As shown in Fig. 1, MC91 plants had a distinctive glaucous or gray appearance with more bloom accumulation compared to the glossy phenotype of MC117 plants. There has been no report of any mutant that is completely deficient in cuticular wax, reflecting