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Studies of genetic characteristics of two heart-shaped glossy cabbage mutants

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ABSTRACT

Cabbage (*Brassica oleracea* L.var. *capitata*), an important crop of the *Brassicaceae* family, is a highly valued source of anti-cancer compounds, vitamin C and high-quality protein. Glossiness of the leaf surface is an important character that has an important influence on the quality of commercial products. In this study, two heart-shaped glossy cabbage mutants were examined to determine their characteristics and potential practical applications. Firstly, mutant-type (MT) plants showed a shiny and attractive appearance, and the total wax load in MT leaves was significantly less than that of wild-type (WT) leaves, when assessed by scanning electron micrographs. Then, the results of $\chi 2$ tests on the separated proportion proved that the glossy wax-less trait is controlled by a single recessive gene. Unexpectedly, the sulforaphane content, which has been identified as a naturally potent anticarcinogen, showed an increasing trend in MT plants than the corresponding WT plants. Lastly, our work also showed that some genes that are involved in wax biosynthesis and export pathways are down-regulated in MT leaves, which could cause the glossy phenotype of MT plants. These analyses will shed light on the benefits of incorporating the two mutants and other glossy mutants into future breeding programs to develop varieties with morphological markers linked to desirable traits.

1. Introduction

Cabbage is an important crop of the *Brassicaceae* family and originates from regions extending from the Mediterranean Sea to the North Sea coast. Cabbage is cultivated throughout the world because of its features of strong adaptability to the environment and easy cultivation. Moreover, *B. oleracea* plants are known for their abundant supply of health-promoting substances that reduce the risk of disease (Hafidh et al., 2013; Mattila and Hellstrom, 2007; Parka et al., 2014); these substances include vitamin C, vitamin E, glucosinolates, amino acids, anthocyanidins, and carotenoids. Overall, demand for cabbage is continuously increasing in both the food and pharmaceutical industries.

The leaf surfaces of cabbage are coated with an epicuticular wax layer, which is very important as the first line of defence against external factors (Kunst and Samuels, 2003). Wax widely exists in injured tissue, pollen, the seed coat, and other tissues and organs as well as in the cork layer of plants (Preuss et al., 1993). In recent years, there has been an increasing amount of research related to plant cuticular wax in *Arabidopsis thaliana* and crop species (Lee and Suh, 2015). Glossiness of the leaf surface is an important quality character that has an important influences on commercial cabbage products. The color of normal cabbage is green, gray green or dark green because of the existence of the wax on the leaves. The glossy wax-less plants are mutants whose leaves have a shiny green or glossy appearance. The glossy leaf trait is very important to plant breeding because of its specific characteristics, including glossy green leaves, high nutritional content and some resistance to the diamondback moth (Chu and Wang, 1993; Lin et al., 1984; Stoner, 1990). Furthermore, this feature can be used as a morphological marker in hybrid breeding. The inheritance of the glossy wax-less character in some crops has been studied by many researchers, and their research has shown that genetic diversity exists among different materials (Dhari and Yadava, 1993; Mo et al., 1992; Tang et al., 2015; Zhou et al., 1995). Moreover, wax-deficient genes have been isolated in a number of plant species (Aarts et al., 1995; Bourdenx et al., 2011; Chen et al., 2003; Liu et al., 2015; Sakuradani et al., 2013). However, few reports have focused on the study of cuticular wax in *Brassica* species (Tang, 2015; Zhang et al., 2013).

Heart-shaped cabbage varieties have been popular for their crispier texture and consumer appeal in regions of the Yangtze River in China (Zeng et al., 2015). However, the heart-shaped cabbage varieties have deficiencies because of the higher amounts of wax and less greenness in the leaves. Based on the trends of quality-oriented markets, leaf color

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Table 1
Primers of cabbage genes for qRT-PCR analysis.

Gene	Cabbage genome accession no.	Protein family name in Arabidopsis	Primer sequence (5′-3′)
BolFATB	Bol021081	Fatty acyl-ACP thioesterase B	ACTGGAGAAATTTTAACAAGAGCA CTCGGCAAGGATAGGGTCAG
BolLCAS1	Bol002590	Long chain acyl-CoA synthetase	AAGGGCTTCTTGAGCCAGAC TAGGCGGGGTAGTCTCTTCC
BolKCS1	Bol018447	β-Ketoacyl-coenzyme A synthase	CCGTTATACGAATCCGGCGA GGAGAAGGTCTCGAACGTCA
BolKCR1	Bol010474	β-Ketoacyl-coenzyme A reductase	AACAACGCTGGGGTTTCGTA CCAACACAGCCTGAGTAACCT
BolECR10	Bol044348	Trans-2,3-*E*noyl-Coenzyme A reductase	AGTGGCTCTGGAGGGTATCA GCGATGTTGAACCCTAGCCA
BolCER2	Bol037661	BAHD acyltransferase	AAGTCGAAGGGTTCACGGTC GGCCCAACTTAGCCCTACTG
BolCER3	Bol012187	Alkane-forming Pathway	AACAACCAAATCGAGAATGCGA TTCCCATGGACCACACGAAC

improvement is vital for heart-shaped cabbage varieties. In 2013, two spontaneous glossiness mutants from homozygous wild-type (WT) of heart-shaped cabbage materials ‘411′ and ‘429′ were found in Nanjing city, Jiangsu province of China. To determine the characteristics and potential practical applications of glossy cabbage mutants in future breeding programs to develop varieties, the two pairs of heart-shaped cabbage lines with glossy leaf traits available in China were studied. The objectives of this study were to (1) characterize the phenotypic differences and stability between the WT and the mutant-type (MT) plants, (2) explore the genetic basis of glossy wax-less characteristics, (3) study whether the glossy leaf trait influences some of the major nutritional components, and (4) analyze the ultrastructural differences between the WT and MT waxes and the expression levels of genes involved in wax synthesis.

2. Materials and methods

2.1. Plant materials

Both WT (411 and 429 wild-type) and MT (411 and 429 mutant-type) plants were grown in the greenhouse at the Liuhe Experiment Station at the Institute of Vegetable Crops, Jiangsu Academy of Agricultural Sciences, Nanjing, P. R. China and subjected to standard cultivation. The seeds were sown in mid-July 2015 and 2016. Field sampling and plant characterization were completed during August through to November. Leaves were sampled from WT and MT plants at four major developmental stages: the seedling stage, rosette stage, early heading stage and late heading stage. The two pairs of WT and MT lines were selected as parents to produce F_1, F_2 and BC generations. The individual plants were visually observed for the presence or absence of glossiness.

2.2. Scanning electron microscopy (SEM)

To examine the differences in wax load and morphology between WT and MT plants by SEM, the outer leaves at the late heading stage were collected. Leaf disks of 1.0 cm in diameter were removed by a hole punch and dried in an oven at 50 °C for 12 h. The samples were then spatter-coated with gold film and observed by SEM (EVO-LS10, Carl Zeiss, Oberkochen, Germany).

2.3. Nutrient quality analysis

The nutrient qualities of mature leaf heads and the outer leaves were analyzed separately. Nutritional quality was measured according to the national standard of analytical methods for vitamin C content (GB 6195-86), dry matter (GB 8858-88), total sugar (GB 6194-86) and protein (GB 8856-88) content. The procedure described by Li et al. (2012) was used for the determination of sulforaphane (SF). Each experiment contained five replicates, and data was analyzed by analysis of variance (ANOVA). The mean values were compared using Duncan’s multiple range test at $P \leq 0.05$.

2.4. Total RNA extraction and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the outer leaves at four developmental stages using the RNeasy Plant Mini Kit (Tiangen Co., Beijing, China) and was reverse-transcribed using Superscript II reverse transcriptase (Promega, Madison, USA) according to the manufacturer’s instructions. The transcriptional profiles of 7 genes that were related to wax biosynthesis and transport in Arabidopsis were analyzed by qRT-PCR using the SYBR Green I chemical (QIAGEN, Hilden, Germany). The wax genes were identified in the cabbage genome database (http://ocri-genomics.org/bolbase/components.htm) and validated in the National Center for Biotechnology Information (NCBI) database. The cabbage *β-actin* gene was amplified as an endogenous control along with the target gene to normalize the expression between different samples. The primers for these genes were designed with Primer Premier 5.0 software and tested to ensure the amplification of single discrete bands with no primer dimers. The sequences of the relevant primers are given in Table 1. The $2^{-\triangle\triangle CT}$ method was used to calculate relative changes in gene expression (Livak and Schmittgen, 2001).

3. Results and discussion

3.1. Phenotypic characterization of MT plants

Both ‘411WT’ and ‘429WT’ belong to parents with a heart-shaped core with excellent comprehensive properties and combining ability. In 2013, novel MT plants with wax-less character occurred spontaneously from a large population of cultivated of ‘411WT’ and ‘429WT’ plants. The MT plants have a remarkable glossy surface phenotypic trait that is easily distinguishable from the WT plants throughout their growth and development, and other phenotypes related to the MT morphology were indistinguishable from those of WT (Fig. 1).

MT plants with wax-less of balls or flat cabbage heads have been reported in the literature (Chu and Wang, 1993; Tang et al., 2015), which revealed that the growth rate of MT plants appeared slower than that of the corresponding WT plants. This resulted in shorter plants, with the MT plants did not easily producing seeds. Here, the growth rate and seed production of 411MT and 429MT were not influenced by the lack of coating wax. Thus, the inconsistency in the results of other reports may be attributed to the genetic differences controlling the trait.

3.2. Exploration of genetic inheritance of glossy wax-less characteristics

To study the inheritance of glossiness, F_1, F_2, BC_1P_1 and BC_1P_2 populations were obtained through crossing, selfing and back-crossing. The F_1s indicated that non-glossy leaves are dominant over glossy leaves. The genetic ratio of 3 non-glossy:1 glossy was obtained in F_2 generations from two crosses which were studied separately (Table 2). The back-cross generation involving a glossy parent as the recurrent parent showed a ratio of 1 non-glossy:1 glossy. The results of the $\chi 2$ test on the separated proportion proved that the inheritance of the glossy trait is controlled by a single recessive gene pair in ‘411MT’ and ‘429MT’, respectively. Furthermore, plants of the F_1 generation (crossing 411MT and 429MT) were glossy, indicating that glossiness in