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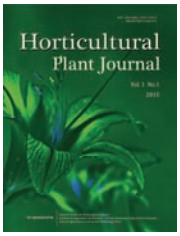

Preliminary Study of the Characteristics of Several Glossy Cabbage (*Brassica oleracea* var. *capitata* L.) Mutants

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Abstract

To determine the characteristics and potential practical applications of glossy cabbage (*Brassica oleracea* var. *capitata* L.) mutants, five different glossy mutants were studied. The amount of epicuticular wax covering the mutant leaves was only approximately 30% that of the wild-type (WT) leaves. The wax crystals of WT plants were columnar and linear, while they were granular and rod-shaped in the mutants. Additionally, in WT cabbage, the primary wax components were alkanes, alcohols, fatty acids, ketones, and aldehydes. There was a significant decrease in the abundance of alkanes and ketones in the wax of the mutants. The glossy-green trait of the mutants may be the result of an inhibited alkane-forming pathway. Higher rates of chlorophyll leaching and water loss demonstrate that the mutant leaves were more permeable and sensitive to drought stress than the WT leaves. Growth curve results indicated that the growth rate of *mutant-1* and *mutant-3* was slower than that of the corresponding WT cabbage, resulting in shorter plants. However, the growth rate of *mutant-2* was not influenced by the lack of coating wax. An investigation of the agronomic traits and heterosis of the glossy cabbage mutants indicated that all five mutants had glossy-green leaves, which was a favorable characteristic. The F_1 plants derived from crosses involving *mutant-2* exhibited obvious heterosis, suggesting the observed glossy-green trait is controlled by a dominant gene. Therefore, *mutant-2* may be useful as a source of genetic material for future cabbage breeding experiments.

Keywords: cabbage; glossy mutant; agronomic trait; microstructure; wax component

1. Introduction

The outer cabbage leaves are coated with an epicuticular wax layer, which is very important as the first line of defense against externa1 factors. Color of wild-type (WT) cabbage leaves are green, gray green, or blue green, but wax-deficient mutants appear glossy-green because of the lack of wax. The glossy cabbage mutant has some favorable characteristics over WT cabbage such as a crispier texture and a greater abundance of sugar, vitamin C, and dry matter (Chu and Wang, 1993; Li et al., 2012; Liu et al., 2014).

Plant cuticular wax production involves a series of complex processes, including the biosynthesis of various wax components, transportation of products, and regulation of the signal involved in wax biosynthesis. Cuticular wax is composed mainly of very long chain fatty acids (VLCFAs) and

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their derivatives, including alkanes, primary and secondary alcohols, aldehydes, ketones, and alkyl esters (Samuels et al., 2008; Buschhaus & Jetter, 2011). The steps involved in cuticular wax biosynthesis are as follows: (1) synthesis of C16/C18 fatty acids in plastids, (2) extension of the C16/C18 fatty acids to form VLCFAs, (3) and differentiation of the VLCFAs into wax components via the alcohol- and alkane-forming pathways. Primary alcohols and alkyl esters are produced in the alcohol forming pathway and aldehydes, alkanes, secondary alcohols, and ketones are generated in the alkane-forming pathway (Bernard & Joubès, 2013).

Previous studies regarding the glossy cabbage mutant have usually focused on only one mutant. In this study, several glossy cabbage mutants collected from different regions and at different growth stages were analyzed. Differences in wax structure and content between the mutants and WT cabbage were investigated using scanning electron microscopy (SEM) and gas chromatography mass spectrometry (GC-MS). Examining cuticle permeability, growth, and agronomic characteristics enabled the characterization of the effects of wax deficiency on cabbage physiology. The observed heterosis associated with one of the mutants (i.e., *mutant-2*), whose wax-less trait was controlled by a dominant gene, may have practical implications for future attempts at breeding glossy cabbage cultivars.

2. Materials and methods

2.1. Material

This study was completed between July 2013 and December 2014 on the Beijing Nankou Farm at the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences. Experimental materials included five glossy cabbage (*Brassica oleracea* var. *capitata* L.) mutants and three WT cabbage cultivars (Table 1, Fig. 1). Comparisons of heterosis were completed with the following crosses: *mutant-2* × 13Q-201, *mutant-2* × 13Q-208, *mutant-2* × 13Q-352, and *mutant-2* × 13Q-508. The waxy parents, 13Q-201, 13Q-208, 13Q-352, and 13Q-508, were inbred lines.

Table 1 Glossy cabbage mutants and wild-type cabbage

Material No.	Material name	Inheritance of glossy trait
mutant-1	21-3you	Single recessive
WT-1	21-3	
mutant-2	10Q-385you	Single dominant
WT-2	10Q-385	
mutant-3	10Q-961	Single recessive
WT-3	10Q-962	
mutant-4	11Q-Ld1	Single recessive
mutant-5	10Q-960	Double recessive

Fig. 1 Five glossy cabbage mutants, three wild-type cabbage plants, a hybrid parent (13Q-208), and an F_1 plant

The five glossy cabbage mutants were sown in July 2013 and then transplanted to the greenhouse after maturing. Plants were artificially pollinated between March and May 2014. Seeds were harvested in June and sown in July. Plants were transferred to the field in August. Field sampling and plant characterization were completed between September and December 2014.

2.2. Scanning electron microscopy and gas chromatography mass spectrometry analysis of cuticular wax

Scanning electron microscopy was used to study leaf cuticular wax. The outer leaves were collected, cut into 5 mm × 10 mm samples, and fumigated with 2% osmic acid for 24 h. The next procedures were performed as follows: leaves were immersed in hexane for 30 s to extract cuticular wax. As an internal standard, 20 μg n-tetracosane (C24; Supelco, Sigma) was added to each sample, which were then dried using nitrogen. The extracted monomers were treated with 100 μL bis-N,N-(trimethylsilyl) trifluoroacetamide at 90 ℃ for 30 min to transform hydroxyl-containing compounds into their corresponding trimethylsilyl derivatives (Mu et al., 2013). After drying samples with nitrogen, 1 mL hexane was added to dissolve samples, which were then filtered and transferred to new chromatographic bottles (Jenks et al., 1995). The composition of the samples was analyzed by GC-MS using the GCMS-QP2010 system (Shimadzu, Japan). The auto-retrieved sub-mass spectral data were compared with a reference standard chart and data from published studies to determine the composition of samples. Contents were measured using an area normalization method (Mao et al., 2012).

2.3. Chlorophyll-leaching and water loss rates

To assess chlorophyll efflux, 2 g leaves were immersed in 30 mL 80% ethanol. We collected 3 mL aliquots after 0, 0.5, 1, 2, 4, 8, 10, 12, and 24 h. The aliquots were subjected to spectrophotometric analysis (absorption measured at 647 and 664 nm) to quantify the amount of leached chlorophyll. Experiments were repeated three times. We calculated the total chlorophyll concentration of fresh leaf tissue using the following equation: total chlorophyll (micromoles) = 7.93 (absorption at 664 nm) + 19.53 (absorption at 647 nm). Chlorophyll efflux during each interval was expressed as a ratio (chlorophyll at that interval: total chlorophyll extracted after 24 h) (Lolle et al., 1998). The leaf loss rate (%) was calculated as follows: (original quality - quality after water loss) / (original quality - final dry mass) × 100 (Chen et al., 2003).

2.4. Growth curve

After planting, we investigated the growth rates of the mutants and their corresponding WT plants every 7 days until plants reached maturity. We assessed maximum leaf length, maximum leaf width, and plant height. Experiments were repeated three times, with each replicate consisting of seven samples.

2.5. Comparison of agronomic characteristics and heterosis

The following agronomic characteristics were analyzed in mature plants: single ball quality, ball diameter, length of center column, shape of cabbage head, and the days needed from planting to maturity. The F_1 population from crosses involving *mutant-2* was used in investigations of heterosis, which involved an examination of plant size, maximum outer leaf width, and single ball quality. Heterosis over mid-parent were calculated as follows: $[F_1 - (P_1 + P_2) / 2] / [(P_1 + P_2) / 2]$. Super affinity was calculated as follows: $(F_1 - Ph) / Ph$ (Ph represented the highest affinity).

2.6. Nutrient quality

The nutrient qualities of mature balls and outer leaves were analyzed separately. We measured dry matter, total sugar, protein, vitamin C, and crude fiber at the safety and quality detection center of Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences. Experiments were repeated three times, with each replicate consisting of three leaf balls or outer leaves from three plants.

3. Results

3.1. Leaf surface microstructure

According to SEM analysis, there was considerably more wax on the surface of WT cabbage leaves than on mutant leaves. The main wax crystal structures on WT leaves were columnar (Fig. 2, WT-1 and WT-2) or linear (Fig. 2, WT-3). The wax on the surface of mutant leaves was sparsely distributed and its crystal structures were mainly granular (Fig. 2) and rod-shaped (Fig. 2, abaxial side of *mutant-3*).

We compared the leaf ultrastructure among the five glossy cabbage mutants. The wax crystal structures of *mutant-1*–*mutant-4* were granular, while those of *mutant-5* were flaky. There was significantly more wax on the adaxial leaf surface than on the abaxial surface for all five mutants by visual. *mutant-5* contained the most wax, followed by *mutant-1*–*mutant-4* in that order. Additionally, a visual assessment of the plants using the naked eye revealed that the bright green color of the five mutants (Fig. 1) was related to the amount of wax on the leaf surface. Lower amounts of leaf wax resulted in brighter and greener plant leaves (Fig. 2).

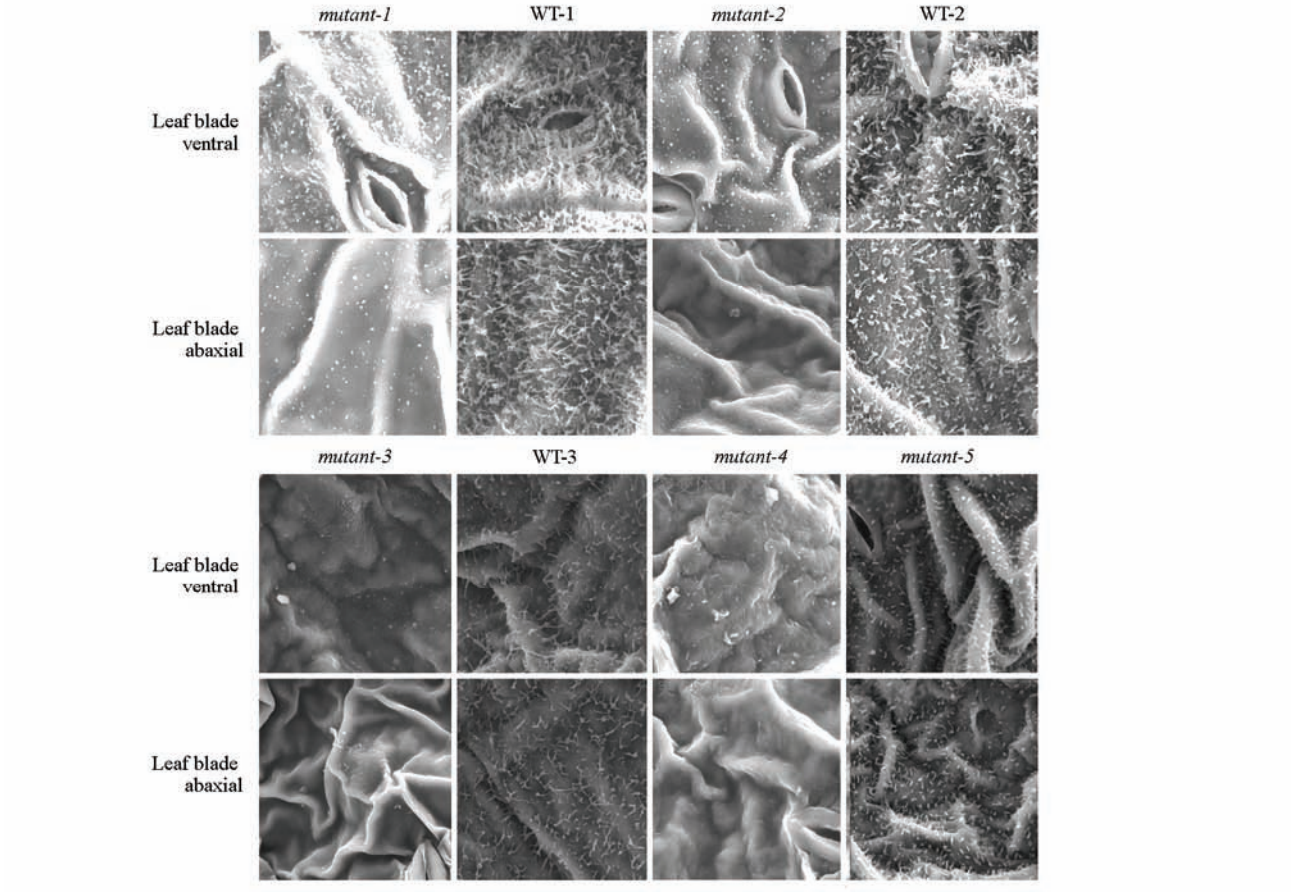

Fig. 2 Microstructural features of the adaxial (upper panels) and abaxial (lower panels) leaf surfaces

3.2. Wax composition

The wax composition of mutant and WT cabbage plants was analyzed by GC-MS. As shown in Fig. 3, the main wax components of WT cabbage plants were alkanes, fatty acids, alcohols, aldehydes, and ketones. The alkanes were the most abundant among the wax components. The abundance of wax contents was considerably decreased on the mutant leaves, with *mutant-1*, *mutant-2*, and *mutant-3* containing only 26.73%, 29.71%, and 30.57% of the total wax content of WT cabbage respectively. Overall, the mutant leaves had approximately 70% less wax than the WT cabbage leaves. The wax components lacking in the three glossy cabbage mutants were mainly alkanes, ketones, and alcohols. The loss of alkanes and ketones was the most serious in terms of wax production as these two components are the main products of the alkane-forming pathway. The inhibited activity of the alkane-forming pathway in the glossy cabbage mutants resulted in a dramatic decrease in leaf surface wax abundance.

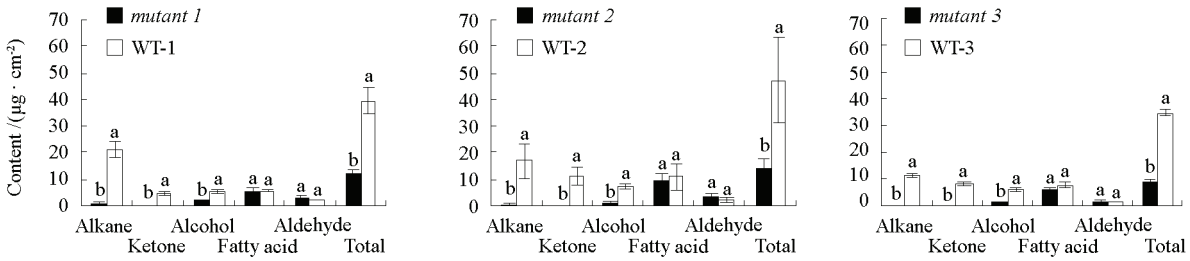

Fig. 3 Wax composition on cabbage leaves

3.3. Leaf cuticle permeability

Rates of chlorophyll efflux and leaf water loss were measured to determine the permeability of mutant leaf cuticles. Chlorophyll efflux and water loss in the three mutants were significantly higher than those of the WT cabbage (Fig. 4). Because the rates of chlorophyll efflux and leaf water loss are the main indicators of leaf cuticle permeability, the results indicate that the leaf cuticles of the mutants were more permeable than those of WT cabbage leaves.

3.4. Growth curve and agronomic traits

Growth indices of the mutant and WT cabbage plants were examined to identify any differences in growth (Fig. 5). *mutant-1* was shorter than the corresponding WT cabbage 14 days after planting in the field. Additionally, the biggest leaf of *mutant-1* was smaller (i.e., length and width) than that of the WT cabbage 21 days after planting. However, significant differences were not observed between *mutant-2* and the corresponding WT cabbage in terms of the largest leaf length

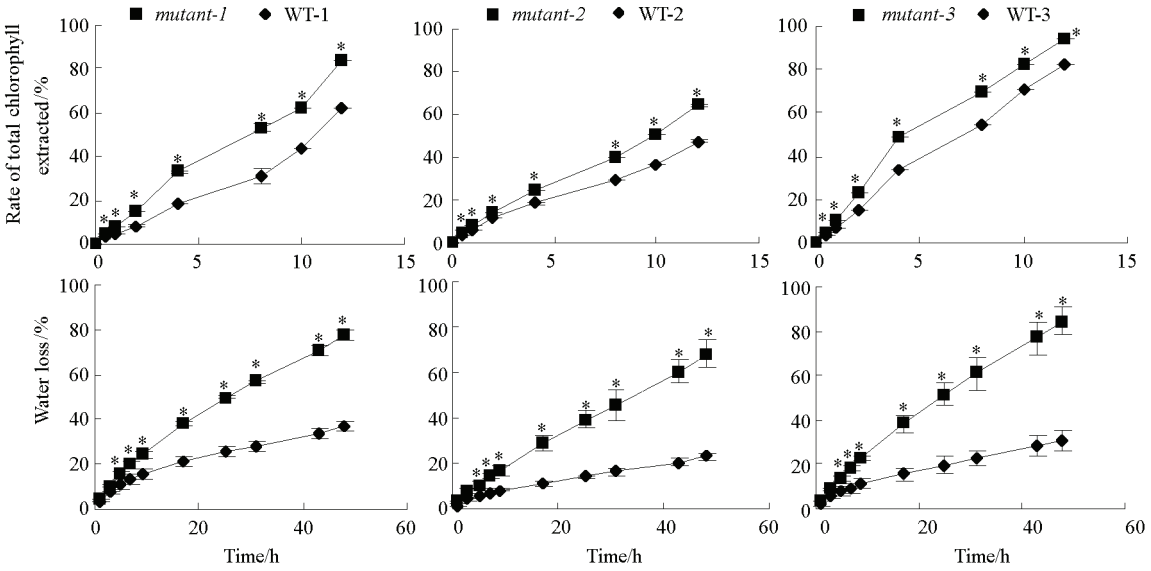

Fig. 4 Chlorophyll efflux and leaf water loss rates

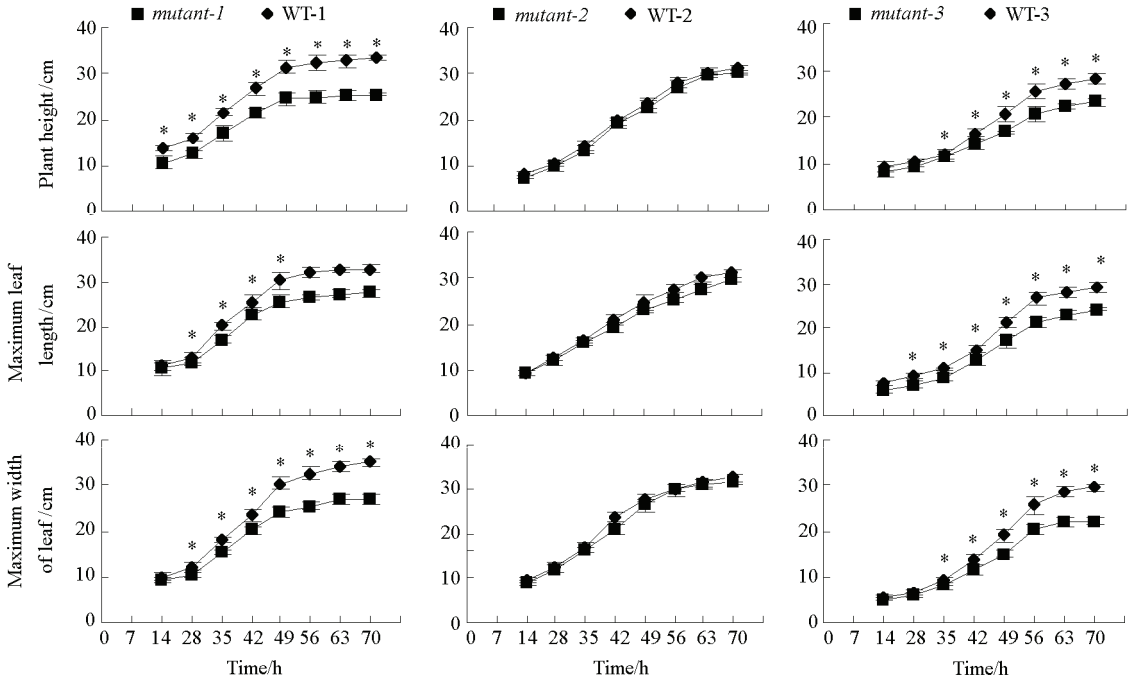

Fig. 5 Growth curves of glossy cabbage mutant and wild-type cabbage plants

and width and plant height. The length of the largest *mutant-3* leaf was shorter than that of the largest WT leaf 21 days after planting. *mutant-3* was shorter than the corresponding WT cabbage, and had a smaller largest leaf width 28 days after planting.

The five mutants were deficient in terms of wax, but they exhibited more favorable characteristics than the WT cabbage plants (Table 2, Fig. 1). There were no significant differences between the mutant and WT plants regarding shape of the cabbage head (i.e., spherical), maturation, and ratio of the lengths of the central column and the ball. *mutant-1* was smaller than the corresponding WT cabbage in terms of plant height, plant expansion, ball weight, and head size.

Except for leaf color, there were no significant differences in agronomic characteristics between *mutant-1* and the corresponding WT cabbage. *mutant-2* exhibited good head shape and glossy-green leaves, which were desirable characteristics. *mutant-3* was smaller than the corresponding WT cabbage regarding plant height, plant expansion, head weight, and head size.

Because the corresponding WT plants for *mutant-4* and *mutant-5* were unavailable, we could only investigate the agronomic traits of the mutants. *mutant-4* cabbage plants had a round head and short central column, and exhibited a medium maturity rate and growth vigor. *mutant-5* was a late-maturing cabbage with a round head and a medium maturity rate.

The growth of *mutant-1* and *mutant-3* lagged behind that of the corresponding WT cabbage, resulting in smaller plants. However, the growth rate of *mutant-2* was not affected by wax abundance, indicating that the effects of wax deficiency on plant growth are diverse and depend on the plant.

3.5. Nutrient components

There were no significant differences in the leaf ball nutrient contents among *mutant-1* and *mutant-3* and the corresponding WT cabbage. This suggests that wax deficiency did not affect the head leaf nutrient contents in these two mutants. Because wax is present mainly on the outer leaves, we investigated the nutrient contents of these leaves. There was more protein and vitamin C in *mutant-1* than in the corresponding WT, but less crude fiber. There were no other differences in nutrient content. Additionally, there were no significant differences in outer leaf nutrient content between *mutant-2* and the corresponding WT cabbage. We observed higher dry matter and crude fiber contents in *mutant-3* than in the corresponding WT (Table 3).

3.6. Heterosis

We used 12 *mutant-2* lines to hybridize with WT cabbage. There were four inbred progenies and the F_1 populations exhibited a wax-less characteristic, indicating that the glossy trait of *mutant-2* was conferred by a dominant gene. This is the first report of the dominant inheritance of the wax-less trait in cabbage. We investigated the main agronomic traits of four wax-less F_1 plants. We observed that the F_1 plants were significantly bigger than those of *mutant-2*, except for the *mutant-2* × 13Q-352 cross. All other crosses exhibited heterobeltiosis and mid-parent heterosis. In terms of leaf width, all crosses showed strong heterobeltiosis and mid-parent heterosis.

Table 2 Agronomic characteristics of glossy cabbage mutant and wild-type cabbage plants

Material	Plant height/ cm	Plant breadth/ cm	Head shape	Leaf color	Head weight/ kg	Head length/ cm	Head width/cm	Core length/ Head length
mutant-1	23.30 ± 0.53 f	55.90 ± 0.90 bc	Flat	Blight green	1.21 ± 0.11 b	10.55 ± 0.45 e	20.50 ± 0.44 b	0.48 ± 0.05 b
WT-1	25.83 ± 1.16 e	60.03 ± 1.31 ab	Flat	Grey	1.53 ± 0.13 a	10.98 ± 0.04 de	22.93 ± 0.71 a	0.46 ± 0.02 b
mutant-2	34.08 ± 1.65 c	50.25 ± 2.77 d	Flat	Blight green	0.71 ± 0.03 de	11.81 ± 0.74 cd	14.13 ± 0.64 d	0.45 ± 0.02 b
WT-2	35.22 ± 0.20 c	56.56 ± 2.07 bc	Flat	Grey	0.82 ± 0.06 cd	12.15 ± 0.38 bc	14.85 ± 0.41 d	0.48 ± 0.02 b
mutant-3	40.22 ± 1.35 b	41.50 ± 1.45 e	Round	Blight green	0.78 ± 0.21 d	10.17 ± 0.17 e	14.78 ± 0.79 d	0.56 ± 0.08 a
WT-3	47.89 ± 1.50 a	48.61 ± 0.92 d	Round	Grey green	1.10 ± 0.04 bc	13.00 ± 0.60 ab	16.78 ± 0.35 c	0.62 ± 0.04 a
mutant-4	39.33 ± 1.16 b	63.33 ± 5.20 a	Round	Blight green	0.44 ± 0.06 e	12.33 ± 1.04 bc	11.50 ± 0.87 e	0.31 ± 0.05 c
mutant-5	30.33 ± 0.58 d	52.50 ± 2.60 cd	Round	Dark blight green	1.19 ± 0.34 b	13.67 ± 0.76 a	16.33 ± 1.16 c	0.56 ± 0.02 a

Table 3 Nutrient analysis of cabbage outer leaves

Leaf	Material	Dry matter/%	Total sugar/%	Protein/%	VC/(mg · g^{-1})	Crude fiber/%
Head leaf	*mutant-1*	8.02 ± 0.16 d	3.76 ± 0.05 b	1.40 ± 0.09 e	0.49 ± 0.011 e	0.56 ± 0.02 d
	WT-1	8.11 ± 0.41 d	4.01 ± 0.10 b	1.27 ± 0.07 e	0.46 ± 0.032 e	0.57 ± 0.02 d
	mutant-3	6.43 ± 0.39 e	2.80 ± 0.05 cd	1.27 ± 0.10 e	0.43 ± 0.034 e	0.58 ± 0.01 d
	WT-3	6.85 ± 0.32 e	3.01 ± 0.43 c	1.46 ± 0.09 e	0.44 ± 0.025 e	0.58 ± 0.02 d
Outer leaf	*mutant-1*	10.50 ± 0.36 b	2.48 ± 0.18 d	2.24 ± 0.22 c	0.69 ± 0.040 c	0.84 ± 0.01 c
	WT-1	10.13 ± 0.15 b	2.52 ± 0.18 d	1.88 ± 0.08 d	0.61 ± 0.016 d	0.91 ± 0.04 b
	mutant-2	15.43 ± 0.46 a	4.60 ± 0.36 a	2.82 ± 0.15 a	1.10 ± 0.051 a	1.13 ± 0.04 a
	WT-2	15.53 ± 0.40 a	4.54 ± 0.42 a	2.89 ± 0.31 a	1.00 ± 0.072 a	1.13 ± 0.01 a
	mutant-3	9.34 ± 0.42 c	1.18 ± 0.13 e	2.54 ± 0.19 b	0.84 ± 0.043 b	0.89 ± 0.03 b
	WT-3	8.57 ± 0.38 d	0.89 ± 0.02 e	2.32 ± 0.12 bc	0.67 ± 0.030 cd	0.83 ± 0.03 c

Note: Different letters within each column indicate significant difference (P = 0.05)

Table 4 Heterosis analysis of *mutant-2*

Material	Plant breadth			Max leaf width		
	Plant breadth/cm	Over median/%	Over parents/%	Max leaf width/cm	Over median/%	Over parents/%
mutant-2 × 13Q-208	60.50 ± 1.80 ab	9.72	0.78	36.33 ± 3.21 b	19.94	5.52
mutant-2 × 13Q-352	53.17 ± 4.54 cd	− 1.46	− 7.80	33.67 ± 0.58 bc	17.82	8.60
mutant-2 × 13Q-201	61.67 ± 2.89 a	18.35	14.29	42.00 ± 1.73 a	38.89	22.33
mutant-2 × 13Q-508	62.17 ± 4.54 a	17.22	11.38	31.67 ± 2.89 cd	14.14	7.95
mutant-2	50.25 ± 2.77 d			26.15 ± 1.50 e		
13Q-208	60.03 ± 1.31 ab			34.43 ± 1.71 bc		
13Q-352	57.67 ± 1.53 abc			31.00 ± 3.61 cd		
13Q-201	53.96 ± 2.15 cd			34.33 ± 1.53 bc		
13Q-508	55.81 ± 1.84 bc			29.33 ± 2.08 de		

Material	Weight			Head shape	Leaf colour
	Weight/kg	Over median /%	Over parents /%		
mutant-2 × 13Q-208	1.40 ± 0.32 bc	24.98	− 8.33	Flat	Bright green
mutant-2 × 13Q-352	1.63 ± 0.16 ab	78.00	45.82	Flat	Bright green
mutant-2 × 13Q-201	1.75 ± 0.27 a	98.33	66.61	Flat	Bright green
mutant-2 × 13Q-508	1.33 ± 0.08 bcd	100.55	86.30	Flat	Bright green
mutant-2	0.71 ± 0.03 e			Flat	Bright green
13Q-208	1.53 ± 0.13 ab			Flat	Grey
13Q-352	1.12 ± 0.17 cd			Flat	Grey
13Q-201	1.05 ± 0.07 d			Flat	Grey
13Q-508	0.61 ± 0.12 e			Flat	Grey

Head weight is one of the main traits that influence the economic value of cabbage cultivars. The heads of four F_1 plants were significantly heavier than those of *mutant-2*. Furthermore, except for the *mutant-2* × 13Q-208 cross, the F_1 populations produced heads that were significantly heavier than those of the parents. All crosses had obvious mid-parent heterosis. For example, the mid-parent heterosis of the *mutant-2* × 13Q-508 cross was 100.55%. Additionally, all the four crosses except for the *mutant-2* × 13Q-208 showed strong heterobeltiosis (Table 4).

Among the four crosses, the *mutant-2* × 13Q-208 cross showed the strongest heterosis. Additionally, the head and leaves of *mutant-2* were bright green with a favorable appearance (Fig. 1). Therefore, *mutant-2* may be useful for breeding of cabbage with improved traits.

4. Discussion

Differences in wax content and crystal structure were observed between the mutants and WT plants. Results demonstrated that all mutant plants were coated with wax, but there were differences in abundance and crystal structure among all mutants. For example, there was more wax in *mutant-5* than in the other four mutants, but less than in the WT cabbage. Additionally, the shape of the wax crystal structures in *mutant-5* was different from that of the other mutants.

The mutants contained only 30% of the wax present in the WT plants, and alkanes and ketones were the main wax components missing in the mutants. Alkanes and ketones are the main products of the alkane-forming pathway.

A number of wax metabolism-related genes have been cloned from *Arabidopsis thaliana* (Bernard and Joubès, 2013), rice (Qin et al., 2011; Mao et al., 2012; Zhou et al., 2013), corn (Dietrich et al., 2005; Monica Sturaro et al., 2005), barley (Richardson et al., 2007), wheat (Muria and Taira, 1999), and *Thellungiella halophila* (Zhao, 2007). The wax metabolic pathway has been preliminarily characterized, but further research is necessary to clarify specific details. Future research on the wax-less mutants will likely increase our knowledge of the wax metabolic pathway in cabbage.

The development of green leaves is an important and desirable agronomic trait in cabbage. The results described in this study regarding the glossy-green mutants should help increase our understanding of the wax-less cabbage mutant. Furthermore, because *mutant-2* exhibits strong heterosis, which is controlled by a single dominant gene, it may be useful for breeding cabbage with ideal agronomic characteristics.

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