Genetic Relationships among 11 Nonheading Chinese Cabbage Accessions and the SCAR Marker Specific for ‘Suzhouqing’

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Abstract. ‘Suzhouqing’ is a unique landrace of nonheading Chinese cabbage [*Brassica rapa* var. *chinensis* (Linnaeus) Kitamura] with a long history of cultivation in Suzhou of Jiangsu Province, China. However, transitional and overlapped morphologic traits make it difficult to authenticate this accession from other nonheading Chinese cabbages. Genetic relationship between ‘Suzhouqing’ and the related 10 popular accessions in the Yangtze River Delta were analyzed using two well-studied single-copy nuclear genes—*ARGONAUTES 7* (*AGO7*) and *BcMF15*; the molecular identification of ‘Suzhouqing’ was determined based on the intersimple sequence repeat–sequence-characterized amplified region (ISSR-SCAR) marker. The results indicated that ‘Suzhouqing’ could be identified specifically from the other 10 accessions based on 21 specific nucleotide variations of the *AGO7* gene. Sequence variations show a strong correlation with leaf morphology, suggestive of partial causal links between the two. Genetic relationship analysis showed that five accessions with close geographic locations had a very close genetic relationship, whereas the genetic relationship of the other five accessions was related to their morphologic similarity. One exception, ‘AJH’, might undergo a special evolutionary process. Furthermore, ISSR-880 was screened as the specific primer to identify accession ‘Suzhouqing’, and a specific discrimination ISSR-SCAR marker was explored, which amplified no target band in any other accessions. The development of molecular markers for the specific identification of ‘Suzhouqing’ in 11 popular accessions in the Yangtze River Delta could provide a theoretical basis for the protective identification of other agricultural crops.

Nonheading Chinese cabbage [*Brassica rapa* var. *chinensis* (Linnaeus) Kitamura], belonging to the genus *Brassica* in Brassicaceae, is an annual or biennial herb (Wu et al., 2001). It is widely cultivated and one of the most consumed vegetable crops in Asia.

Nonheading Chinese cabbage originated in China, which has a long history of cultivation and includes numerous and complex

germplasms. As an important vegetable crop, nonheading Chinese cabbage has been treated as an independent subspecies termed *B. rapa* L. ssp. *chinensis* Makino by many agronomists (Cao and Cao, 1994; Cao and Li, 1982; Li, 1981; Lin, 1980; Tan, 1980). The identification of nonheading Chinese cabbage has long been controversial, although several authors have tried to provide a natural system to divide this subspecies into varieties (Cao and Cao, 1995; Cao and Li, 1980, 1981; Cao et al., 1994; Liu et al., 1998). The traditional identification and classification of nonheading Chinese cabbage were based mainly on the morphologic traits of shape, stem, leaf, and seedcoat of the plant. However, the morphologic features are often transitional and overlapped among varieties,

leading to difficulties and controversy in identification. For example, Li (1981) classified the nonheading Chinese cabbage into four varieties: var. *oleifera* Metzger, var. *communis* Tsen et Lee, var. *rosularis* Tsen et Lee, and var. *purpurea* Mao. Cao and Li (1982) and Lin (1980) added two varieties (var. *tai-tsai* Hort. and var. *multiceps* Hort.). Cao et al. (1994) replaced var. *tsaitai* Hort. and var. *utilis* Tsen et Lee with var. *purpurea* Mao and *parachinensis* Tsen et Lee.

Within the past two decades, several molecular markers have been developed and widely applied in genetic relationship analysis of nonheading Chinese cabbage (Han et al., 2007; Li et al., 2008; Ma et al., 2012). However, the identification results based on molecular evidence were not always consistent with those based on morphologic traits. For example, the genetic relationship revealed by the amplified fragment length among different accessions does not completely correspond to the differences in their morphologic characteristics (Guo et al., 2002; Sun et al., 2001). Furthermore, most of these studies have focused on the genetic diversity of germplasms, while markers that could discriminate one accession uniquely from the others, have not been developed yet. Only two exceptions have been reported. One is that based on amplified fragment length polymorphism molecular markers. Li et al. (2008) screened molecular markers with high efficiency in the identification of accession ‘Youdonger’ in Hangzhou. The other exception was by our research team. We developed a pair of ISSR-SCAR markers for the specific identification of Wujiang Fragrant Bok Choy (Shen et al., 2016).

Low- or single-copy nuclear genes among many species have been tested recently as effective phylogenetic markers. In *Tildenia*, a subgenus of *Peperomia* (Piperaceae), 25% of aligned characters from the *agt1* intron sequence are parsimony informative, which could be developed for species identification (Naumann et al., 2011). Eight single-copy genes contain as many as 5.8% variable sites, and primers were developed for phylogenetic studies across the Melastomataceae (Reginato and Michelangeli, 2016). In addition, Zhao et al. (2016) used 36 whole genomes and 27 transcriptomes to identify 891 orthologous genes with one or low copies, and reconstructed the rosids phylogeny. Huang et al. (2016) screened 113 low-copy orthologous nuclear genes from transcriptomes or genomes of 55 Brassicaceae species. Recent studies used transcriptome or genome data sets to reconstruct successfully phylogenies of various scales from genus to angiosperm-wide, which also shed light on research of species discrimination by low- or single-copy nuclear genes.

‘Suzhouqing’, which has a wonderful taste, is a unique landrace of nonheading Chinese cabbage with a long history of cultivation in Suzhou, Jiangsu Province. Based on the morphologic traits, it is difficult to discriminate ‘Suzhouqing’ from the other popular accessions of nonheading Chinese

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cabbage in the Yangtze River Delta. For example, there only exist slight differences in plant height, petiole length, and leaf color between ‘Suzhouqing’ and ‘Shanghaiqing’. In this study, 11 major accessions of nonheading Chinese cabbage in the Yangtze River Delta were collected. Combined with ISSR and two well-studied single-copy nuclear genes—*ARGONAUTES 7* (*AGO7*) and *BcMF15*—the genetic relationships of the major accessions of nonheading Chinese cabbage were investigated, and an authentic marker for ‘Suzhouqing’ discrimination was explored. *AGO7* has been suggested to function in leaf-shape formation, harboring high polymorphism in Chinese cabbage (Liang et al., 2016). *BcMF15* was suggested to involve in microspore development and might be male sterile related in nonheading Chinese cabbage (Tian et al., 2009). Therefore, these two genes may have the potential to be applied for identification and genetic relationship analysis of nonheading Chinese cabbage.

Materials and Methods

Plant materials. ‘Suzhouqing’ and 10 other accessions of nonheading Chinese cabbage (Table 1; the acronyms included therein are used hereafter) that mainly cultivated in the Yangtze River Delta were sampled in this study. The plant materials were provided by the Taihu base of Seed Administrative Station of Suzhou. For each cultivar, 8 to 10 healthy individuals were selected, from which tender leaves were collected and stored in silica gel until use.

Total DNA extraction. Total genomic DNA was extracted from silica-gel dried leaf tissues. One hundred-milligrams of leaves were disrupted by a tissue disrupter (Tissue-Lyser LT; Qiagen, Germany) and total DNA was isolated using the Easy Pure Plant Genomic DNA Kit (TransGen Biotechnology Co., Ltd., Beijing, China). The extracted DNA was diluted to a final concentration of 20 ng·μL⁻¹. Concentration and purity of DNA were detected using a nucleic acid and protein detector (Eppendorf; Saxony, Germany). DNA samples were stored at -20 °C for future use.

Isolation of *AGO7* and *BcMF15* genes. The sequences of *AGO7* (Gene ID: Bra003999-|PACId:22700727) and *BcMF15* (Genbank no.: EF600901) were downloaded from an

online database (<https://phytozome.jgi.doe.gov/pz/portal.html>) and Genbank, respectively, for primer designing. *AGO7* was amplified with primers *AGO7S* (5′-ATGGA-AGAAAAACCAAGAATC-3′) and *AGO7AS* (5′-TCAACAGTAAACATGAGATTC-3′). *BcMF15* was amplified with primers *LTPS* (5′-ATGAAGTTAATTTTCATTGATG-3′) and *LTPAS* (5′-TGGTGTACAAGTCCA-AAGC-3′). These primers were all designed using Primer Premier 5.0 (Premier Biosoft, CA).

Amplifications were performed using GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT). The polymerase chain reaction (PCR) mixtures contained the following components: 1.0 μL DNA template, 25.0 μL 2× Reaction Mix [Dongsheng PCR Kit, including 20 mmol·L⁻¹ Tris-HCl, 100 mmol·L⁻¹ KCl, 3 mmol·L⁻¹ MgCl₂, 400 μmol·L⁻¹ deoxyribonucleoside-5′-triphosphate (dNTPs), and bromophenol blue], 2.0 μL each of forward and reverse primers (10 μmol·L⁻¹), 1.0 μL Taq DNA polymerase (2.5 U·μL⁻¹), and double-distilled water to a total volume of 50 μL. The reaction was carried out as follows: predenaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 53 to 55 °C for 45 s, and extension at 72 °C for 1 to 3 min, then a final extension step at 72 °C for 10 min. PCR products were subjected to electrophoresis on 0.8% to 1% agarose gel containing ethidium bromide, then visualized under ultraviolet transilluminator and imaged by a gel documentation system (Bio-Rad, Hercules, CA). The amplicons with correct length were submitted to BGI Gene Technology Co., Ltd. (Shanghai, China) for sequencing, and the bidirectional results were assembled using Sequencher4.5 software (GeneCodes, Ann Arbor, MI). DNA matrices were aligned in MEGA6 (Tamura et al., 2013) and used to build a maximum likelihood tree to analyze the genetic relationship among these accessions.

ISSR analysis. DNA samples were mixed to construct DNA pools for each of the 11 accessions of nonheading Chinese cabbage. Forty-eight ISSR primers from the literature (Song et al., 2012) were synthesized by Realgene Bio-Technologies, Inc. (Nanjing, China). Each PCR had a total volume of 20 μL and contained 1.0 μL DNA template (20 ng·μL⁻¹), 10.0 μL 2× Reaction Mix (Dongsheng PCR Kit, including 20 mmol·L⁻¹ Tris-HCl, 100 mmol·L⁻¹ KCl, 3 mmol·L⁻¹ MgCl₂,

400 μmol·L⁻¹ dNTPs, and bromophenol blue), 1.5 μL of each primer (10 μmol·L⁻¹), and 0.4 μL of Taq DNA polymerase (2.5 U·μL⁻¹). PCR was performed on a BioMera T1 PCR thermocycler (Analytik Jena, Germany) as follows: initial denaturation at 94 °C for 7 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C (slightly altered for different primers) for 45 s, extension at 72 °C for 1 min, and the final extension at 72 °C for 10 min. PCR products were separated by electrophoresis in 3% agarose gel and stained by ethidium bromide. The gel was run at a constant voltage of 80 V for 1.5 h and was visualized under a gel documentation system. The 2000-base pair (bp) DNA ladder (Dongsheng Biological Technology Co., Ltd., Guangzhou, China) was used to determine the size of the amplified fragments.

The primers that can generate clear DNA bands with abundant polymorphic profiles were selected from the 48 ISSR primers set. They were used to amplify the DNA pools of the 11 accessions of nonheading Chinese cabbage. The DNA bands exhibiting divergence among different samples were picked out. Each amplification product at a specific position with the same migration rate was considered a homologous locus. The presence or absence of an allele at a particular locus was scored as 1 and 0, respectively, to generate a binary matrix for further analysis. The POPGENE 32 software (Yeh et al., 1999) were used to calculate Nei’s gene diversity index (H), the effective number of alleles (NE), Shannon’s information index (I), the genetic differentiation coefficient (GST), and gene flow (Nm). The genetic similarity coefficients and genetic distances between samples were estimated by NTSYS2.10 software (Rohlf, 2000). The obtained distance matrices were used to cluster the strains by the unweighted pair group method using arithmetic means (UPGMA) method, and a dendrogram was plotted.

Development and validation of specific ISSR-SCAR bands. A unique DNA band specific to ‘Suzhouqing’ was excised from the agarose gel and purified by the QIAquick gel extraction kit (Qiagen, Shanghai, China). The purified DNA fragments were ligated to the pMD19-T vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* DH5α competent cells by a heat-shock method. Positive clones were selected through PCR and then sequenced by Realgene Bio-Technologies (Nanjing, China). A pair of SCAR primers was designed based on the obtained sequence.

A total of 88 individual DNA samples from 11 nonheading Chinese cabbage accessions (eight individuals for each accession) were amplified with the newly designed SCAR primers. Each ISSR reaction was carried out in a final volume of 20 μL. The components and their concentration of PCR mixture were as follows: 1.0 μL DNA template (20 ng·μL⁻¹), 10.0 μL 2× Reaction Mix (Dongsheng PCR Kit, including 20 mmol·L⁻¹ Tris-HCl, 100 mmol·L⁻¹ KCl, 3 mmol·L⁻¹

Table 1. List of nonheading Chinese cabbage accessions used in this study, and their sources.

Accessions	Acronyms	Variety	Provenance	Characteristics	
				Plant type	Leaf type
Suzhouqing	SZQ	var. <i>communis</i>	Suzhou	Erect	Smooth
Zhongqibai	ZQB	var. <i>communis</i>	Changzhou	Erect	Smooth
Wuyuean	WYM	var. <i>communis</i>	Shanghai	Erect	Smooth
Aijiaohuang	AJH	var. <i>communis</i>	Nanjing	Erect	Smooth
Siyuean	SYM	var. <i>communis</i>	Shanghai	Erect	Smooth
Shanghaiqing	SHQ	var. <i>communis</i>	Shanghai	Erect	Smooth
Huangxinwu	HXW	var. <i>tai-tsai</i>	Hefei	Semiprostrate	Wrinkled
Xiaobaye	XBY	var. <i>tai-tsai</i>	Shanghai	Prostrate	Wrinkled
Huangzhongxiangqingcai	XQCH	var. <i>communis</i>	Suzhou	Semiprostrate	Wrinkled
Heizhongxiangqingcai	XQCB	var. <i>communis</i>	Suzhou	Semiprostrate	Wrinkled
Xiuhuaijin	XHJ	var. <i>communis</i>	Suzhou	Prostrate	Wrinkled

MgCl₂, 400 μmol·L⁻¹ dNTPs, and bromophenol blue), 0.8 μL of each primer (10 μmol·L⁻¹), 0.4 μL of Taq DNA polymerase (2.5 U·μL⁻¹) and 7 μL double-distilled water. The PCR program consisted of predenaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 1 min; and a final elongation at 72 °C for 8 min. The PCR products were visualized on 1% agarose gel in an electrophoresis system run at a constant voltage of 80V. After 0.5 h of electrophoresis, the PCR products were examined and imaged using a gel imaging system. A 2000-bp DNA ladder was used as a molecular weight marker.

Results

Identification of the 11 nonheading Chinese cabbage accessions based on AGO7 and BcMF15. *AGO7* and *BcMF15* were amplified successfully in 11 nonheading Chinese cabbage accessions. The length of *AGO7* was 3419 to 3433 bp, and the length of *BcMF15* was 604 to 614 bp. The aligned *AGO7* gene matrix was 3442 bp in length (Supplemental Table 1), within which 102 single nucleotide polymorphisms (SNPs) (90 located in exons, including 51 synonymous mutations and 37 nonsynonymous mutations) and seven indels (two of which were located in exons, resulting in one aa and three aa indels, respectively) were detected in the 11 accessions. All of the 11 nonheading Chinese cabbage accessions exhibited distinct and specific characters. For example, 21 specific characters were identified in accession 'SZQ', including the specific bases C, T, and T at positions 230 bp, 233 bp, and 239 bp, respectively (vs. T, C, and C, respectively, in other accessions).

The aligned *BcMF15* gene matrix was 617 bp in length (Supplemental Table 2). Seven SNPs (four nonsynonymous mutations located in exons) were detected and three indels (two located in exons, resulting in four aa indels and one aa indel, respectively). The variations in the *BcMF15* gene could divide the 11 accessions into two groups. One group contained 'SYM', 'WYM', and 'SHQ'; the other group consisted of the remaining eight accessions (Supplemental Table 2). In the first group, 'SHQ' was differentiated from other accessions based on C at position 408 bp, compared with T in other accessions. 'XBY' in the second group was differentiated by A at position 585 bp, compared with T in other accessions.

Analysis of the genetic relationship of the 11 nonheading Chinese cabbage accessions based on AGO7 and BcMF15. The results of genetic distance and the genetic similarity coefficient revealed that the genetic similarity of *AGO7* and *BcMF15* gene sequences in the 11 nonheading Chinese cabbage accessions was 97.4% to 100%, and the genetic distance was 0 to 0.016. The phylogenetic tree of the 11 nonheading Chinese cabbage accessions was based on the maximum likelihood method (Fig. 1). As shown in the Fig. 1,

the 11 nonheading Chinese cabbage accessions could be divided mainly into two clusters. Cluster I contained 'SZQ', 'XYM', 'WYM', 'SHQ', and 'ZQB', whereas the remaining six accessions were clustered in cluster II. Within cluster I, 'SYM' and 'WYM' were more closely related, as was 'SHQ' and 'ZQB', whereas 'SZQ' was obviously independent from the other four accessions. Within cluster II, 'HXW' and 'AJH' were closer together, obviously independent from the subcluster of 'XBY', 'XQCB', 'XQCH', and 'XHJ'.

Identification of the 11 nonheading Chinese cabbage accessions using ISSR molecular markers. PCR amplification of the 11 nonheading Chinese cabbage accessions was performed using 48 ISSR primers. A total of 12 primers with clear bands, high polymorphism, and good repeatability were screened. A total of 96 clear bands were obtained, with 8.0 for each primer on average. Of these bands, 55 were polymorphic bands, with 4.6 for each primer on average. The percentage of polymorphic bands was as much as 57.3%. Among the 12 primers, ISSR-807 and ISSR-834 amplified the most bands at 11 for each primer, whereas ISSR-827 amplified the fewest at five. ISSR-891 amplified the greatest percentage of polymorphic bands, which was up to 100.0%.

The genetic diversity analysis showed that the average number of effective alleles of the 11 nonheading Chinese cabbage accessions was 1.2884. *H* was 0.1773 and *I* was 0.2738, falling in between the self-pollinated plants (*H* = 0.086, *I* = 0.131) and the cross-pollinated plants (*H* = 0.178, *I* = 0.301) (Zhang and Yang, 2008). The *GST* of the 11 nonheading Chinese cabbage accessions was 1, indicating that genetic variations resided among accessions. The *Nm* was 0, implying no genetic exchange among accessions (Slatkin, 1981, 1985).

Analysis of genetic relationships among the 11 nonheading Chinese cabbage accessions based on ISSR markers. A dendrogram of the 11 accessions generated from the UPGMA procedure grouped the accessions into five major clusters at a similarity index of 0.826 (Fig. 2). Cluster I contained five accessions of var. *communis*. Cluster II consisted of two accessions differentiated morphologically by leaf color. Cluster III contained two accessions of var. *tai-tsai*. Clusters IV and V contained one accession of var. *communis* from different provenances, respectively.

Comparing the phylogenetic tree reconstructed by the *AGO7* and *BcMF15* genes with the dendrogram based on ISSR markers, the genetic relationships of 'SZQ', 'ZQB', 'SHQ', 'SYM', and 'WYM' were consistent. In the phylogenetic tree, 'XHJ' and 'XBY' clustered with 'XQCH' and 'XQCB', whereas in the dendrogram, it was represented as a single cluster ('XHJ') or clustered with 'HXW' ('XBY'). 'AJH', which clustered with 'HXW' in the phylogenetic tree, was a single cluster in the dendrogram.

Establishment and validation of specific molecular markers for 'Suzhouqing'. Among the ISSR primers, ISSR-880 was screened as the specific primer to identify accession 'SZQ'. The amplified product of this primer pair was ≈400 bp (Fig. 3), exhibiting a specific band for 'SZQ'. The ISSR-specific fragment in 'SZQ' was cloned and sequenced. The results revealed that the total length of the fragment was 424 bp, and the nucleotide sequence of the fragment is shown in Fig. 4. The sequence was searched against the *B. rapa* genome and was revealed to be an intergenic fragment.

A pair of SCAR primers was designed as SCAR-F and SCAR-R (underlined in Fig. 4), corresponding to the 133 to 153-bp and the 362 to 383-bp regions, respectively. The

Fig. 1. Maximum-likelihood phylogenetic tree of 11 nonheading Chinese cabbage accessions based on *AGO7* and *BcMF15* genes. For definitions of acronyms, refer to Table 1. Images of each accession were placed in the tree accordingly.

Fig. 2. Dendrogram of 11 nonheading Chinese cabbage accessions based on ISSR markers.

Fig. 3. Amplification profiles of 11 nonheading Chinese cabbage accessions using an intersimple sequence repeat-880 marker. Lanes 1 to 11, in order, are 'SZQ', 'ZQB', 'WYM', 'XQCH', 'AJH', 'SYM', 'HXW', 'SHQ', 'XBY', 'XQCB', and 'XHJ' (for definition of acronyms, see Table 1). M, DL2000 DNA marker; the arrow represents a specific band of 'SZQ'. bp = base pair.

designed SCAR primers were verified in 88 individuals of the 11 nonheading Chinese cabbage accessions. The results revealed that the primers amplified a clear target band of 251 bp in all eight individuals of 'SZQ', whereas the specific band was not produced in any of the other 10 accessions (Fig. 5).

Discussion

Genetic relationship among nonheading Chinese cabbage accessions. The 11 nonheading

Chinese cabbage accessions in this study are cultivated as landraces under strong artificial selection for a long time. Thus, genetic differentiation is rather high, with virtually no gene exchange. Based on horticultural classification, the 11 accessions of nonheading Chinese cabbage are divided into the var. *communis* group (which includes 'SZQ', 'SHQ', 'ZQB', 'SYM', 'WYM', and 'AJH') and the var. *tai-tsai* group (which includes 'HXW' and 'XBY'). The plants of the var. *communis* group are erect with smooth

leaves, whereas those of the var. *tai-tsai* group are prostrate or semiprostrate with wrinkled leaves. Although 'XQCH', 'XQCB', and 'XHJ' belong to the var. *communis* group, the plants display a semiprostrate and wrinkled-leaf morphology.

In this study, the genetic relationship of accessions 'SYM', 'WYM', 'SHQ', 'ZQB', and 'SZQ' were consistent based on two single-copy nuclear genes and the ISSR markers. These five accessions were cultivated in Nanjing, Suzhou, and Shanghai with close geographic locations. Our results revealed a very close genetic relationship among these accessions, which could be also inferred from sequence-related amplified polymorphism (SRAP) and simple sequence repeat markers (Han et al., 2007; Ma et al., 2012). The other accessions showed little difference in the topology between the phylogenetic tree and the dendrogram, especially for those in the var. *tai-tsai* group. Two main factors might account for this inconsistency. One is that a hybridization event occurred in some accessions, such as 'HXW', during artificial selection, leading to lineage confusion and uncertainty. The other is that, compared with the nonspecific molecular ISSR markers, the single genes—especially those highly related to agronomic traits—should have experienced directional selection during artificial cultivation. So the evolutionary rate varied between these two.

Fragrant Bok choy, which has a rich aromatic flavor, is a unique and rare variety of nonheading Chinese cabbage with a long cultivation history of more than 100 years. In this study, 'XQCH', 'XQCB', and 'XHJ' represent the three major accessions of Fragrant Bok choy. Their morphologic differences reside only in color of the leaf and vein, richness of the flavor, and amount of fiber content. The results of this study revealed that these three accessions exhibited a similar genetic background. 'XBY' and 'HXW',

both belonging to the var. *tai-tsai* group, were more closely related to the three accessions of Fragrant Bok choy of the var. *communis* group, which is consistent with similarities in

their morphologic characteristics: wrinkled leaves and prostrate plant.

Accession 'AJH' is one exception, exhibiting a close genetic relationship with 'HXW'

in the phylogenetic tree but a most distant genetic relationship with all the other accession in the dendrogram. 'AJH' is a landrace in Nanjing, Jiangsu Province. It is the shortest, almost prostrate, accession among the var. *communis* group. This accession exhibited a close genetic relationship with 'HXW', which is a semiprostrate accession in the var. *tai-tsai* group, indicating that the prostrate trait of the nonheading Chinese cabbage may be derived from the same ancestor. The analysis based on SRAP also exhibited consistent results (Han et al., 2007). Cao et al. (1997) proposed that semiprostrate var. *tai-tsai*, such as 'HXW', is the hybrid of prostrate var. *tai-tsai* and var. *communis*. Could be 'AJH' also the product of this hybridization event? This was further supported by Cao et al. (1997), who indicated that in the var. *communis* group, the green stem is an original trait whereas the white stem is an evolved one. And in our study, only 'AJH' exhibit white stems, indicating that this accession might indeed have undergone a special evolutionary process.

5'-TGGAGAGGAGAGGAGACTAATTTGATCTCTCAATCTCAATATCGGAAA
CGTGCCGGAGAAAACATCGTTTACGGCGGGACTAATCTTGCTGTCGAC
GGAGCTGGAAGCAGCTTCTTTTTCTTCTCCAGATCTCTAACCATTCTCA
CCTCCTCCTGTTTGACCGGTTCTGTTTCTCCGCCTACTTTTGGATCTCTTTC
CTCAGCTATTGGCATGATATTCCGATTACGTTGATCGTCCATTTTCTCGAA
TCCATTGTGCATTGACGACAAATCGTTGTTAAGATCGATGCCACGATAGA
TCGAAACGTCGCCGTTTCCGAATTTAATAACCTCGTCTTGACAGATCAGTA
AACATGGCTTACGATTGATAAAAAGCCTATACGTTACATTCAATCTGATT
GGATCTTCTCCTCTCCTCTCCA-3'

Fig. 4. The nucleotide sequence of a Suzhouqing-specific fragment amplified using primer intersimple sequence repeat-880. The underlined sequences were designed sequence-characterized amplified region primers.

Fig. 5. Verification of the Suzhouqing-specific intersimple sequence repeat–sequence-characterized amplified region primers by polymerase chain reaction (PCR) in eight individuals from each of the 11 nonheading Chinese cabbage accessions. (A–K) PCR results from the eight individuals of 'SZQ', 'ZQB', 'WYM', 'XQCH', 'AJH', 'SYM', 'HXW', 'SHQ', 'XBY', 'XQCB', and 'XHJ', respectively. M, DL2000 DNA marker. Lanes 1 through 8 are PCR products from eight individuals.

Molecular markers for the identification of 'Suzhouqing'. In this study, the universal sequences of *matK*, *rbcL*, *psbA-trnH*, and internal transcribed spacer (ITS) were first used to differentiate the 11 accessions, and no variations were discovered (data not shown). Then, the leaf formation-related gene *AGO7*, and the male sterile-related gene *BcMF15*, were used in the study. The results showed that 'Suzhouqing' could be identified specifically from the other 10 accessions based on 21 specific nucleotide variations of the *AGO7* gene. As one of the major targets of cultivation, the leaf shape might have undergone strong artificial selection (Liang et al., 2016). During the long-term cultivation of nonheading Chinese cabbage, the *AGO7* gene has undergone relaxed purification selection stress ($ka/ks = 0.17$ in our study; Ka/Ks value is the ratio between the number of nonsynonymous substitutions per nonsynonymous site (Ka) and the number of synonymous substitution per synonymous site (Ks)). And rich variations have accumulated in the 11 accessions of nonheading Chinese cabbage [$\pi = 0.01040$, comparable with 0.00472 in 150 accessions in Liang et al. (2016)]. Those nucleotide variations serve as reservoir and play an important role in the formation of leaf types in nonheading Chinese cabbage. The gene sequences of the two groups of nonheading Chinese cabbage with wrinkled leaves or smooth leaves were divided into two distinct groups ($Fst = 0.594^*$), indicating a significant correlation between the leaf morphology and the *AGO7* genotype. As one of the regulators in leaf adaxial/abaxial patterning, *AGO7* might serve as one of the target genes of selection during the diversification of leaf morphology shape in nonheading Chinese cabbage, which is worth paying more attention to for agronomists.

The SCAR marker developed based on ISSR markers can uniquely identify accession 'SZQ' in 11 popular accessions in the Yangtze River Delta, with an identification efficiency of 100%. Compared with the traditional morphology-based or nonspecific molecular markers, this PCR-based identification technique has the advantages of high accuracy, good reproducibility, stability, and reliability. Definitely, further studies should be carried out for the application of this specific marker in more nonheading Chinese cabbage accessions. 'Suzhouqing' is a unique landrace with a long history of cultivation in Suzhou, Jiangsu Province. The development of molecular markers for its specific identification also provides a theoretical basis for the protective identification of other agricultural crops.

Conclusions

'Suzhouqing' could be specifically identified from the other 10 popular accessions in the Yangtze River Delta based on 21 specific nucleotide variations of the *AGO7* gene. Sequence variations show a strong correlation with leaf morphology, suggestive of

partial causal links between the two. ISSR-880 was screened as the specific primer to identify accession 'Suzhouqing', and a specific discrimination ISSR-SCAR marker was explored, which amplified no target band in any other accessions. The development of molecular markers for the specific identification of 'Suzhouqing' could provide a theoretical basis for the protective identification of other important agricultural crops.

Literature Cited

- Cao, J.S. and S.C. Cao. 1994. Morphological compare and taxonomic study on the seed coats of Chinese cabbage and other groups of *Brassica*. J. Zhejiang Agr. Univ. 20: 393–399.
- Cao, J.S. and S.C. Cao. 1995. Studies on leaf characters of different growth duration of Chinese cabbage and their evolutionary courses. J. Nanjing Agr. Univ. 18:34–41.
- Cao, J.S., S.C. Cao, Y. Miao, and G. Lu. 1997. Cladistic operational analysis and study on the evolution of Chinese cabbage groups (*Brassica campestris* L.). Acta Hort. Sin. 24:35–42.
- Cao, J.S., S.C. Cao, and Y.C. Song. 1994. Studies on chromosome G-banding in 10-chromosome vegetables of *Brassica*. Acta Hort. Sin. 21:257–263.
- Cao, S.C. and S.J. Li. 1980. A preliminary study on the local variety of Chinese cabbage (*Brassica chinensis* L.): I. Morphological observation and study. J. Nanjing Agr. 2:32–38.
- Cao, S.C. and S.J. Li. 1981. A preliminary study on the local variety of Chinese cabbage (*Brassica chinensis* L.): II. A study of the biological characters. J. Nanjing Agr. 1:67–77.
- Cao, S.C. and S.J. Li. 1982. A preliminary study on the local variety of Chinese cabbage (*Brassica chinensis* L.): III. Classification of cultivars of non-heading Chinese cabbage. J. Nanjing Agr. 6:30–37.
- Guo, J.C., N.Y. Zhou, R.C. Ma, and L.Q. Cao. 2002. Genetic diversity of *Brassica rapa* revealed by AFLP molecular marker. J. Agr. Biotechnol. 10:138–143.
- Han, J.M., X.L. Hou, H.M. Xu, G.J. Shi, J.F. Geng, and X.H. Deng. 2007. Genetic differentiation of non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) germplasm based on SRAP markers. Acta Agron. Sinica 33:1862–1868.
- Huang, C.H., R.R. Sun, Y. Hu, L.P. Zeng, N. Zhang, L.M. Cai, Q. Zhang, M.A. Koch, I. Al-Shehbaz, P.P. Edger, J.C. Pires, D.Y. Tan, Y. Zhong, and H. Ma. 2016. Resolution of Brassicaceae phylogeny using nuclear genes uncovers nested radiations and supports convergent morphological evolution. Mol. Biol. Evol. 33:394–412.
- Li, J.W. 1981. The origins and variations of vegetable crops in China. Sci. Agr. Sinica 14:90–95.
- Li, H.B., S.H. Wang, and H. Wang. 2008. Application of AFLP marker technology in the identification of greengrocery variety "Youdonger". J. Anhui Agr. Sci. 36:72–73.
- Liang, J.L., B. Liu, J. Wu, F. Cheng, and X.W. Wang. 2016. Genetic variation and divergence of genes involved in leaf adaxial–abaxial polarity establishment in *Brassica rapa*. Front. Plant Sci. 7:94.
- Lin, W.S. 1980. A study on the classification of Chinese cabbages. Acta Hort. Sinica 7:21–28.
- Liu, W.X., Y.M. Shen, S.F. Ding, and S.C. Cao. 1998. A study on fuzzy cluster analysis of non-heading Chinese cabbage. J. Shandong Agr. Univ. 3:289–293.
- Ma, X.J., G.P. Li, X.J. Zheng, D.Z. Lin, and Y.J. Dong. 2012. Genetic differentiation of 20 non-heading Chinese cabbage. J. Changjiang Veg. 22:13–16.
- Naumann, J., L. Symmank, M.S. Samain, K.F. Müller, C. Neinhuis, C.W. dePamphilis, and S. Wanke. 2011. Chasing the hare - evaluating the phylogenetic utility of a nuclear single copy gene region at and below species level within the species rich group Peperomia (Piperaceae). BMC Evol. Biol. 11:357.
- Reginato, M. and F.A. Michelangeli. 2016. Primers for low-copy nuclear genes in the Melastomataceae. Appl. Plant Sci. 4:1500092.
- Rohlf, F.J. 2000. NTSYS-PC2.1. Numerical taxonomy and multivariate analysis system version 2.1. <<http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html>>.
- Shen, X.L., Y.M. Zhang, J.Y. Xue, M.M. Li, Y.B. Lin, X.Q. Sun, and Y.Y. Hang. 2016. Analysis of genetic diversity of *Brassica rapa* var. *chinensis* using ISSR markers and development of SCAR marker specific for Fragrant Bok choy, a product of geographic indication. Genet. Mol. Res. 15:1–11.
- Slatkin, M. 1981. Estimating levels of gene flow in natural populations. Genetics 99:323–335.
- Slatkin, M. 1985. Rare alleles as indicators of gene flow. Evolution 39:53–65.
- Song, Y.T., C.Y. Wu, X.L. Hou, Q.W. He, and G.J. Yin. 2012. Studies on germplasm resources of *tai-tsai* using RAPD and ISSR markers. J. Northwest A. & F. Univ. (Nat. Sci. Ed.) 40:161–174.
- Sun, D.L., Q.C. Zhao, W.Q. Song, and R.Y. Chen. 2001. Relationships analysis of Chinese cabbage species by AFLP. Acta Hort. Sinica 28:331–335.
- Tan, J.J. 1980. The origin and classification of mustard (*Brassica*) vegetables. J. Agr. Univ. Hebei 4:104–114.
- Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725–2729.
- Wu, Z.Y., P.H. Raven, and D.Y. Hong. 2001. Flora of China, p. 6–22. Science Press, Beijing, China.
- Tian, A., J. Cao, L. Huang, X. Yu, and W. Ye. 2009. Characterization of a male sterile related gene *BcMF15* from *Brassica campestris* ssp. *chinensis*. Mol. Biol. Rep. 36:307–314.
- Yeh, F.C., R. Yang, and T.J. Boyle. 1999. POPGENE 32, Microsoft windows-based freeware for population genetic analysis. Version 1.32. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Canada.
- Zhang, D.Q. and Y.P. Yang. 2008. A statistical and comparative analysis of genetic diversity detected by different molecular markers. Acta Bot. Yunnanica. 30(2):159–167.
- Zhao, L., X. Li, N. Zhang, S.D. Zhang, T.S. Yi, H. Ma, Z.H. Guo, and D.Z. Li. 2016. Phylogenomic analyses of large-scale nuclear genes provide new insights into the evolutionary relationships within the rosids. Mol. Phylogenet. Evol. 105:166–176.