Comprehensive Analysis of the Histone Deacetylase Gene Family in Chinese Cabbage (*Brassica rapa*): From Evolution and Expression Pattern to Functional Analysis of BraHDA3

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Abstract: Histone deacetylases (HDACs) are known as erasers that remove acetyl groups from lysine residues in histones. Although plant HDACs play essential roles in physiological processes, including various stress responses, our knowledge concerning HDAC gene families and their evolutionary relationship remains limited. In *Brassica rapa* genome, we identified 20 HDAC genes, which are divided into three major groups: RPD3/HDA1, HD2, and SIR2 families. In addition, seven pairs of segmental duplicated paralogs and one pair of tandem duplicated paralogs were identified in the *B. rapa* HDAC (BraHDAC) family, indicating that segmental duplication is predominant for the expansion of the BraHDAC genes. The expression patterns of paralogous gene pairs suggest a divergence in the function of BraHDACs under various stress conditions. Furthermore, we suggested that BraHDA3 (homologous of *Arabidopsis* HDA14) encodes the functional HDAC enzyme, which can be inhibited by Class I/II HDAC inhibitor SAHA. As a first step toward understanding the epigenetic responses to environmental stresses in Chinese cabbage, our results provide a solid foundation for functional analysis of the BraHDAC family.

Keywords: Chinese cabbage; duplication; epigenetics; histone deacetylase

1. Introduction

Epigenetic regulation, including DNA and histone modifications, plays an essential role in controlling chromatin structure, with the nucleosome as the basic organization unit [1]. In eukaryotes, post-transcriptional histone modifications such as acetylation, methylation, ubiquitination, and sumoylation enable the regulation of mRNA expression by recruiting histone writers, erasers, and readers [2]. Among these modifications, histone acetylation in different lysine residues of histones by histone acetyltransferases (HATs) is required for opening the chromatin structure to allow binding of RNA polymerase II and transcription factors; in contrast, histone deacetylases (HDACs) maintain the homeotic balance of histone acetylation by removing the acetyl groups from hyperacetylated histones [1,3]. The highly dynamic balance of the histone acetylation and deacetylation is mostly maintained by the physical and functional interplay between HAT and HDAC activities [3], and serves as a major epigenetic regulatory mechanism for gene transcription and in turn governs numerous physiological processes [4].

In higher plants, HDACs are generally classified into three distinct groups: the reduced potassium dependence 3/histone deacetylase 1 (RPD3/HDA1) family, silent information regulator 2 (SIR2) family, and histone deacetylase 2 (HD2) family [5]. Since 18 HDACs were identified in the *Arabidopsis thaliana* genome [6], several HDACs have been identified and isolated from rice [7], soybean [8], grape [9], litchi [10], maize [11], and cotton [12]. These indicate that genome-wide mining and comparative analysis are powerful approach to identify genes and understand the evolutionary relationships of genes. Genetic and physiological studies on *Arabidopsis* have shown that HDA6, HDA9, HDA15, HDA19, HD2C,

and AtSRT1 negatively regulate stress tolerance by regulating gene expression through histone acetylation [13]. The *Arabidopsis* HDA6 mutants showed enhanced induction of genes involved in the acetate biosynthesis pathway, which resulted in enhanced drought tolerance [14]. Similarly, the loss-of-function of HDA9 showed increased tolerance against salt and drought stresses [15]. In addition, HDA19-deficiency enhanced tolerance to high-salinity, drought, and heat stresses in *Arabidopsis* [16]. In contrast, HDA19 overexpression in *Arabidopsis* enhanced disease resistance against *Alternaria brassicicola* via increased transcription of jasmonic acid and ethylene responsive genes [17]. In rice plants, overexpression of OsHDA705, a member of the RPD3/HDA1 family, resulted in decreased salt and abscisic acid (ABA) stress tolerance during seed germination, whereas its overexpression resulted in enhanced osmotic stress resistance during the seedling stage [18]. The decrease in levels of histone H4 acetylation because of the overexpression of OsHDT701 (rice HD2 family) caused reduced resistance against rice pathogens *Magnaporthe oryzae* and *Xanthomonas oryzae* pv *oryzae* [7], whereas the overexpression of HDT701 in transgenic rice leads to increased tolerance to NaCl and polyethylene glycol stresses [19]. Furthermore, mitogen-activated protein kinase 3 phosphorylates the histone deacetylase HD2B to control the transcription of biotic stress response genes [20]. Collectively, these indicate the importance, diversity, and specificity of HDACs in response to different stimuli.

Despite the knowledge concerning HDACs, the evolutionary relationships and functional information regarding the HDAC family in *Brassica rapa*, a crop species of economic importance, remain largely unknown. In this study, we aimed at genome-wide identification of the HDAC gene family in *B. rapa* genome. Expansion and evolutionary mechanisms of this gene family were studied to explore the evolutionary relationships among *B. rapa* HDAC family (*BraHDAC*). Furthermore, we analyzed the expression pattern of *BraHDAC*s under different abiotic stress condition, providing valuable information for further study of *BraHDAC* gene functions.

2. Materials and Methods

2.1. Identification and Characterization of HDAC Family in *B. rapa*

Phytozome v12.1 (<https://phytozome.jgi.doe.gov>; *Brassica rapa* FPsc v1.3) was used to identify HDACs in *B. rapa* genome using BLASTp with *Arabidopsis* HDACs as queries. The conserved domains, molecular weight, phylogenetic, and isoelectric point (pI) analyses and subcellular localization of putative BraHDACs has been conducted by Eom and Hyun [21]. A nomenclature system for BraHDACs (generic name: BraHDA1 to 16 for the RPD3/HDA1 family, BraHDT1 and 2 for the HD2 family, and BraSRT1 and 2 for the SIR2 family) was used as described by Hollender and Liu [6].

2.2. Gene Duplication and Calculating Duplication Times of BraHDAC Genes

The Plant Genome Duplication Database (<http://chibba.agtec.uga.edu/duplication/>) was used to identify the homologous pairs among BraHDAC genes. Using PAL2NAL (<http://www.bork.embl.de/pal2nal/>), the synonymous rate (Ks), nonsynonymous rate (Ka), and evolutionary constraint (Ka/Ks) of duplicated BraHDAC genes were analyzed. The divergence time (T) was calculated using the formula $T = Ks / (2 \times 1.5 \times 10^{-8}) \times 10^{-6}$ million years ago (MYA; [22]).

2.3. Plant Growth and Treatment Conditions

Chinese cabbage (cultivar Chunkwang) seeds were grown in a growth chamber under long-day conditions at 22 °C and 60% relative humidity. After 4 weeks, Chinese cabbage plants were treated at 40 °C, and samples were harvested at the times indicated in Figure 2. Heat treatment was carried out three times, resulting in three biological replicates.

2.4. The Analysis of BraHDAC Expression

Total RNA was extracted, quantified, and qualified using nanodrop. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover. Quantitative

RT-PCR (qRT-PCR) was performed using the SYBR Green Real-time PCR Master Mix. The expression levels of each gene were normalized to the internal reference gene actin, and then was expressed relative to its value at 0 h. The specific primer pairs were designed using GenScript Real-time PCR Primer Design tool, and are listed in Table S1.

2.5. Production of Recombinant BraHDA3 in *E. coli*, and the Analysis of HDAC Activity

The PCR product of BraHDA3 was ligated into the pCR-Blunt, and subcloned into the *Escherichia coli* expression vector pPROEX-Htb vector that produces a histidine-fused translation product. To produce his tagged-BraHDA3 fusion proteins, *E. coli* strain BL21 harboring pPROEX-BraHDA3 plasmid was incubated at 37 °C. Once the optical density at 600 nm reached 0.5, fusion proteins were produced by 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG) treatment for 16 h at 30 °C. The fusion protein was purified using Ni-NTA affinity purification, and the HDAC activity was determined using HDAC Activity Colorimetric Assay Kit (BioVision, Milpitas, CA, USA). HDAC activity was calculated based on a standard curve generated with Ac-Lys-pNA (deacetylated standard).

2.6. Statistical Analyses

The experimental results are expressed as means $\pm$ SE. SPSS Statistics version 25 (IBM Microsoft, New York, NY, USA) was used to carry out statistical analyses (Duncan multiple range tests). *P* values < 0.05 were regarded as statistically significant difference.

3. Results and Discussion

3.1. Identification of Histone Deacetylases in Chinese Cabbage

Since HDACs have been introduced as erasers of epigenetic acetylation marks on lysine residues in histones, a number of plant HDAC genes have been identified from a variety of plant species [3]. From the *B. rapa* genome, we identified 20 HDAC members (Table 1). Among them, BraHDA6 was the largest protein, with 651 amino acids (AA), whereas BraHDA1 was identified as the smallest protein with 254 AA. The molecular weight of BraHDACs varied according to protein size, ranging from 28.8 KDa to 72.1 KDa, and their pI varied from 4.52 (BraHDT2) to 8.96 (BraSRT1). Subcellular locations as predicted by WoLF PSORT indicated that BraHDACs were potentially localized in the cytosol, nucleus, chloroplast, mitochondria, and peroxisome (Table 1). Similar to the *Arabidopsis* and soybean HD2 proteins [23,24], the *B. rapa* HD2 family were predicted as nuclear proteins (Table 1). In addition, BraSRT1 and BraSRT2 appeared to be localized in the mitochondria and nucleus, respectively, similar to the rice SRT (silent information regulator) family [25]. Taken together, the various localization patterns of BraHDACs indicated that they may have distinct roles.

Table 1. Histone deacetylase gene family in *Brassica rapa*.

Name	Gene ID	Location	CDS (bp)	AA	Intron Nr.	pI	kDa	Subcellular Localization
BraHDA1	Brara.A02927.1	A01:24968034..24970355	765	254	7	4.98	28.8	Cytosol
BraHDA2	Brara.A03767.1	A01:29972774..29976555	1509	502	5	5.17	56.0	Nucleus
BraHDA3	Brara.A00453.1	A01:2203688..2206445	1278	425	8	6.06	45.9	Chloroplast
BraHDA4	Brara.B03745.1	A02:30976246..30978653	1668	555	8	5.93	62.0	Cytosol
BraHDA5	Brara.C03690.1	A03:19011956..19014935	1797	598	13	6.00	65.8	Nucleus
BraHDA6	Brara.C04226.1	A03:22262637..22266032	1956	651	12	5.23	72.1	Cytosol
BraHDA7	Brara.E02343.1	A05:21170590..21174613	1638	545	16	5.60	59.8	Nucleus
BraHDA8	Brara.F01687.1	A06:10370998..10372699	1230	409	4	4.97	45.4	Cytosol
BraHDA9	Brara.F01690.1	A06:10386796..10389169	1200	399	4	5.82	44.4	Cytosol
BraHDA10	Brara.F01971.1	A06:14950658..14953055	1272	423	4	8.70	47.6	Mitochondrial
BraHDA11	Brara.F02002.1	A06:16050873..16053859	1281	426	13	5.32	48.7	Cytosol
BraHDA12	Brara.F02210.1	A06:18915988..18918237	921	306	4	4.96	34.3	Nucleus
BraHDA13	Brara.F02773.1	A06:22749037..22751166	1119	372	9	6.17	42.0	Cytosol
BraHDA14	Brara.I00647.1	A09:3621688..3625470	1959	652	12	5.32	71.5	Peroxisome
BraHDA15	Brara.I00727.1	A09:4109663..4112303	1413	470	5	4.94	52.7	Nucleus
BraHDA16	Brara.I05307.1	A09:43269577..43271821	1161	386	2	5.68	42.2	Cytosol
BraHDT1	Brara.A02332.1	A01:17662954..17671061	786	261	7	6.52	28.0	Nucleus
BraHDT2	Brara.J02801.1	A10:19273043..19274967	858	285	6	4.52	31.2	Nucleus
BraSRT1	Brara.B00303.1	A02:1417334..1419795	1047	357	8	8.96	39.7	Mitochondrial
BraSRT2	Brara.C01390.1	A03:6592244..6595515	1407	468	13	6.49	52.4	Nucleus

Plant RPD3/HDA1, HD2, and SIR2 families have been characterized by the presence of conserved domains such as the Hist_deacetyl domain (PF00850), SIR2 domain (PF02146), and ZnF_C2H2 domain (SM000355) [11,12]. As shown in Figure 1A, BraHDACs were divided into three major classes: RPD3/HDA1, HD2, and SIR2 families. The *B. rapa* genome encodes 16 proteins (Figure 1 and Figure S1) clustered in the RPD3/HDA1 family contained the conserved Hist_deacetyl domain. In addition, two SIR2 family HDACs with highly conserved SIR2 domain and two HD2 family HDACs with conserved zinc finger C2H2 type domain (ZnF_C2H2) were identified. Another type of zinc finger domain, ZnF_RTZ (SM000547), has been found in BraHDA07, indicating that BraHDT1, BraHDT1, and BraHDA07 have high binding affinity to DNA or modulate protein-protein interactions mediated by zinc finger domains [26]. The presence of domains similar to those in other plant HDACs suggested that all putative BraHDACs belong to the histone deacetylase family.

Figure 1. Phylogenetic tree, domain architectures (A) and synteny analysis (B) of the *Brassica rapa* HDAC family. Using the neighbor-joining method, phylogenetic tree was generated. The conserved domains in the putative BraHDACs were analyzed with the SMART program. Paralogous gene pairs were analyzed using plant genome duplication database.

3.2. Gene Duplication Analysis of BraHDACs

Gene duplication has long been regarded as a key evolutionary process for acquiring new genes and genetic novelty [27]. It could occur on any of these scales: whole genome duplication, segmental duplication, and single gene duplication including tandem duplica-

tion [28]. It has been shown that some HDACs expanded in the *Gossypium* and soybean genomes partly because of segmental duplication events [12,24]. In the *B. rapa* genome, 20 *BraHDACs* were asymmetrically distributed on 7 chromosomes (Table 1). Chromosome A06 contains the highest density of HDACs with six members (*BraHDA8* to 13), followed by chromosome A01 with four genes (*BraHDA1* to 3, *BraSRT1*), whereas only one *BraHDAC* was present on chromosome A05 (Table 1). A tandem duplication is characterized as two or more adjacent homologous genes located physically “close” to one another; tandem duplicated pairs are paralogous genes on the same chromosome within a 50 kb physical distance [29], indicating that only one set (*BraHDA8* and 9) was tandemly duplicated on chromosome A06 separated by 14,097 bp (Table 1) with 85.7% and 75.4% of sequence identity at the nucleotide and protein levels. To further determine whether segmental duplication events also contributed to the expansion of *BraHDACs*, we analyzed the PGDD database combined with phylogenetic analysis. As shown in Figure 1B, seven duplicated *BraHDAC* gene pairs were identified, indicating that segmental duplication is a major contributory event leading to the expansion of *BraHDAC* genes in *B. rapa* genome. The highest frequency of segmental duplication events occurred between chromosomes A06 and 09, which contained two segmentally duplicated gene pairs.

To examine the evolutionary selection process of eight duplicated gene pairs in *B. rapa* genome, we analyzed the evolutionary constraint (Ka/Ks). Ka/Ks ratios ranged from 0.0895 to 0.8713, and the average Ka/Ks value of the duplicated gene pairs was 0.404 (Table 2). This indicates that these duplicated gene pairs were subjected to negative selection (purifying selection), similar to duplicated HDAC gene pairs in *Gossypium* and soybean genomes [12,24]. The segmental duplications of HDAC genes in *B. rapa* genome occurred between 12.73 MYA and 42.04 MYA, with the average value being 29.19 MYA. The Ks of tandem duplications of *BraHDAC* genes was 0.1640, dating the duplication event at 5.47 MYA (Table 2). *Brassicaceae*, including *Arabidopsis* and *Brassica*, have been shown to have undergone three rounds of whole genome duplication, including γ triplication (135 MYA) and β (90–100 MYA) and α (24–40 MYA) duplications [30–32]. In addition, *B. rapa* exhibits this complex history, with the addition of a whole-genome triplication occurring between 13 and 17 MYA [33], indicating that the segmental duplications of *BraHDAC* genes, except *BraHDA 5/7* and *BraHDA 12/15* gene pairs, occurred before whole-genome triplication of the *Brassica* genome.

Table 2. Divergence between paralogous histone acetyltransferase gene pairs in *Brassica rapa*.

Gene 1	Gene 2	S	N	Ks	Ka	Ka/Ks	Mya
BraHDA8	BraHDA9	298.2	898.8	0.1640	0.1429	0.8713	5.47
BraHDA1	BraHDA5	176.0	574.0	1.2612	0.6244	0.4951	42.04
BraHDA1	BraHDA7	165.2	545.8	1.2066	0.4168	0.3454	40.22
BraHDA4	BraHDA6	371.3	1194.7	0.8011	0.3165	0.3951	26.70
BraHDA4	BraHDA14	399.2	1229.8	0.8870	0.3867	0.4360	29.57
BraHDA5	BraHDA7	319.5	925.5	0.4043	0.1344	0.3324	13.48
BraHDA15	BraHDA8	302.9	906.1	1.1871	0.3172	0.2672	39.57
BraHDA15	BraHDA12	215.5	693.5	0.3818	0.0342	0.0895	12.73

3.3. Effects of Heat Stress on Acetylation Status of Histone H3 in Chinese Cabbage

Heat stress induces excessive generation of reactive oxygen species, resulting in alterations in plant growth, development, photosynthesis, physiological processes, and production yield [34]. To evaluate the efficacy of heat treatment (40 °C), the physiological response of Chinese cabbage including lipid peroxidation and proline content was analyzed. As shown in Figure 2A, heat stress induced the accumulation of malondialdehyde (MDA), which is an indicator of lipid peroxidation [35]. In higher plants, the accumulation of proline, an amino acid and a compatible solute, is a common phenomenon in response to various environmental stresses [36]. We observed an increase in proline levels in response to heat stress (Figure 2B). The accumulated proline purportedly acts as a compatible osmolyte,

molecular chaperone, cytosolic pH buffer, cell redox balancer, free radical scavenger, and stabilizer for subcellular structures during various stresses, especially osmotic and salt stresses [36,37], and improves resistance to various stresses [38]. However, proline accumulation in response to heat stress leads to increased production of reactive oxygen species in the mitochondria and inhibits ABA and ethylene biosynthesis, resulting in increased sensitivity to heat stress [36]. Taken together, these physiological changes suggested that the heat stress treatment was effective, and the leaves of heat-treated plants were harvested for further analyses.

Figure 2. Physiological response to heat stress in Chinese cabbage. Changes in malondialdehyde (A) and proline (B) levels after exposure to heat stress (40 °C) were determined. (C) Expression patterns of *Brassica rapa* histone deacetylases (*BraHDACs*) in response to heat stress. Expression is indicated as a log₂ ratio of experimental treatments relative to the values at 0 h. The bars represent the mean $\pm$ standard error across three independent experiments. Different letters indicate statistically significant differences ($p < 0.05$).

To gain insight into the possible involvement of *BraHDACs* during their response to heat stress, the expression profiles of *BraHDACs* were analyzed. As shown in Figure 2C, exposure of Chinese cabbage plants to heat stress induced or repressed the expression of *BraHDACs*. After 48-h exposure to heat stress, expression levels of *BraHDA2*, *BraHDA4*, *BraHDA5*, *BraHDA6*, *BraHDA7*, *BraHDA11*, *BraHDA12*, *BraHDA15*, *BraHDA16*, *BraHDT2*, and *BraSRT2* had increased, whereas those of other *BraHDACs* decreased, remained unchanged, or were undetected. In addition, the transcript level of *BraHDA13*, *BraHDA15*, *BraHDA16*, *BraHDT2*, and *BraSRT2* was up-regulated in response to drought, salt, and wounding, whereas the decreasing level of *BraHDA2*, *BraHDA3*, *BraHDA9*, and *BraHDA14* transcripts was observed (Figure S2). Under heat-stress condition, *BraHDA4* and *BraHDA14* exhibited a different expression pattern, although *BraHDA4* and *BraHDA14* were clustered into same sub-group in RPD3/HDA1 family, indicating that the duplication of those genes resulted in divergence of their expression pattern and function. Similar with *BraHDACs*, several *Arabidopsis HDACs* and maize *HDACs* in the class II of RPD3/HDA1 family were induced by heat treatment [11,39]. In addition, *Arabidopsis*, maize and cotton *HDACs* were differentially expressed in response to hormones and stresses, suggesting the functional divergence and specific regulation of *HDACs* in response to a particular stress [11,12,39]. According to the abiotic stress induced expression pattern of *BraHDACs*, we found that *BraHDA8* was not expressed in leaves and on abiotic stresses. Based on transcriptome analysis, transcript level of *BraHDA8* has been detected only in the silique [40], indicat-

ing that *BraHDA8* should be expressed in specific tissue. In addition, the expression pattern of paralogous gene pair (*BraHDA4/14*) diverged, whereas three paralogous gene pairs (*BraHDA4/6*, *BraHDA5/7*, and *BraHDA12/15*) exhibited similar expression patterns (Figure 2C) under heat stress condition. However, these expression patterns were altered in response to different stresses including drought, salt, and wounding (Figure S2), indicating that *BraHDACs* had diversified substantially, which might be due to the different divergent fates after duplications.

3.4. Histone Deacetylase Activity of *BraHDA3*

Although many fundamental questions remain unanswered regarding the physiological function of plant HDACs in response to environmental stresses, it has been shown that RPD3/HDA1 family including Arabidopsis HDA5/14/15/18/19 positively or negatively regulated to the environmental-stress tolerances [41]. Among them, AtHDA14 controlled the activation state of RuBisCO under low-light condition, and is involved in biosynthesis of melatonin, which plays an important role in plant response to environmental stresses [42,43]. Under heat stress condition, exogenous melatonin improved the antioxidant defense systems, resulting in the suppression of heat stress-induced damage [44]. In Chinese cabbage, *BraHDA3*, homologous of AtHDA14 (Figure S1), dramatically down-regulated in response to heat stress (Figure 2C). This indicates that down-regulation of *BraHDA3* might be required for the expression of heat response genes, although the physiological function of *BraHDA3* needs to be characterized. To analyze the function of *BraHDA3*, recombinant *BraHDA3* (*BraHDA3*-His) was prepared using *E. coli* expression system. As shown in Figure 3, purified recombinant *BraHDA3* protein gave an activity of 18.1 $\mu\text{M}/100 \mu\text{g}$ of protein. When HDAC inhibitor SAHA (5 μM) was mixed with purified recombinant *BraHDA3* protein, the HDAC activity decreased to 3.8 $\mu\text{M}/100 \mu\text{g}$ of protein. This indicates that *BraHDA3* encodes the functional HDAC enzyme, which can be inhibited by Class I/II HDAC inhibitor SAHA.

Figure 3. HDAC activity of recombinant *BraHDA3*. SDS-PAGE exhibited the purified recombinant *BraHDA3* proteins. *BraHDA3* enzyme activity was analyzed using HDAC Activity Colorimetric Assay Kit. Different letters indicate statistically significant differences ($p < 0.05$).

4. Conclusions

Despite the knowledge concerning HDACs, evolutionary and functional information regarding HDACs in *B. rapa* remains relatively unknown. In this study, we identified 20 putative *BraHDAC* genes divided into three major classes: RPD3/HDA1, HD2, and SIR2 families. Most *HDAC* gene duplications in *B. rapa* appeared to have been caused by segmental duplication, which occurred between 12.73 MYA and 42.04 MYA. Several *BraHDACs* were up- or down-regulated by abiotic stress treatments, indicating that the variation of *BraHDAC* transcription levels may contribute to alteration in histone H3 acetylation levels in response to various stresses. Our results provide a solid foundation for further understanding of the underlying evolutionary mechanisms in the HDAC family and

will provide the basis for future research on functional characterization of the BraHDAC family in response to environmental stresses.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2077-0472/11/3/244/s1>, Table S1: Primer sequences for PCR analysis, Figure S1. Phylogenetic relationships of Arabidopsis, maize, and *Brassica rapa* HDACs, Figure S2: Expression pattern of BraHDAC genes in response to wounding, salt, and drought stresses.

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