Genome-wide identification and expression analysis of glycine-rich protein genes in Chinese cabbage (Brassica rapa L. ssp. pekinensis)

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Research article

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Genome-wide identification and expression analysis of glycine-rich protein genes in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Abstract

Background

Plant Glycine-rich proteins, a superfamily with a glycine-rich domain, play an important role in various stress such as low temperature, drought, high salt, and so on. Although the research of *GRP* genes has been reported in many plants, the *GRP* gene has seldom reported in Chinese cabbage so far. Research results made a guide to further understand the function of *BrGRP* genes in Chinese cabbage.

Results

In this study, a total of 141 glycine-rich protein genes were identified in Chinese cabbage by homology comparative analysis. A further prediction of physical and chemical characteristics revealed that 58.3% of BrGRPs were alkalines, 63.1% of BrGRPs were unstable, and 73.8% were hydrophilic. Conserved domain analysis showed that 110 BrGRPs contained 18 same conserved motifs, and could be classified into five main subclasses which the evolutionary relationship and gene structure may be conserved while the other 31 BrGRPs, including *Bra014168*, *Bra040002*, etc, may gain new functions or gradually lost gene functions according to the evolution process. These identified *BrGRP* genes were also located in ten chromosomes and three different subgenomes of Chinese cabbage, and 101 pairs of orthologous GRP genes were found between Chinese cabbage and Arabidopsis. According to the opened transcriptome data, we found that 138 *BrGRP genes* showed abnormal expression at high temperature, 108 *BrGRP* genes showed abnormal expression at low temperature, and 74 at drought stress, 47 at soft rot stress, while only 3 and 7 genes at ozone and salt stress, respectively. Further promoter motif analysis found that a large number of stress-related cis-acting elements, such as DRE, MYC, MYB, and ABRE, were identified in their promoter regions, which were in correspondence with previous differential expression. In addition, some *BrGRP* genes were involved in multiple stresses suggested their broad-spectrum resistance.

Conclusion

A total of 141 *GRP* genes were identified in Chinese cabbage, which suggested their potential roles in plant stress response. But the molecular mechanisms by which *BrGRP* genes respond and resist biotic and abiotic stress remain unclear. These results may provide an important basis for the study of their function in Chinese cabbage.

Keywords

Chinese cabbage, *GRP* gene, Expression analysis, Biotic and abiotic stress response

Background

Plant glycine-rich protein (GRP) is a class of proteins consisting of glycine-rich repeat sequences. The first glycine-rich cell wall protein PtGRP1 was isolated from Petunia hybrida in 1986, and similar proteins were further found in almost all organisms [1, 2], and more and more GRPs have been isolated from plants such as Zea mays, Oryza sativa, Arabidopsis thaliana, and Nicotiana tabaccum [2].

Plant GRPs can be divided into five main subclasses based on their primary structure, the conserved domain, and the arrangement of glycine repeats [3, 4]. Class I subfamily contained a typical structural feature of GRP protein, of which the glycine-rich repeats mostly appear as (Gly)n-X, where n is generally an odd number and X can be any amino acid. At the same time, the N-terminus contains or does not contain a signal peptide sequence. For example, PtGRP1 has 67% of glycine in glycine repeat (Gly) n-X [2]. Class II of GRPs are similar to Class I at the N-terminus and contained a cysteine-rich polypeptide chain at the end of C-terminal glycine repeat region, which may play an important role in the pathogen-related process, such as AtGRP3[5]. The glycine content of class III is lower than first two classes, but the structure is abundant. For example, Oleosin-GRPs have a fat conserved area and was located in an oil-rich cell structure of tapetum, which mainly play a role in stabilizing the triglyceride and phospholipid layer in the membrane structure [6, 7]; Class IV of GRPs, also named as RNA-binding proteins (RBPs), which has no signal peptide at their N-terminus. Besides the glycine-rich sequences, these proteins also have some other structures, such as the RNA-recognition motif (RRM), cold-shock domain(CSD), and CCHC zinc finger structure, and so on. These RNA recognition domains are usually able to recognize each other or help those proteins which do not have any RNA recognition domain to interact with their target by binding [8, 9]. Class V of GRPs are similar to the members of class III, which the glycine repeats are arranged differently and presented with the mixed mode of (GGX)n and (GXGX)n [4].

The function of the plant GRP family is varied in plant growth and development. The GRPs of class I & II are almost the active components of cell walls, which play a positive regulatory role in plant cell division and organ differentiation [10]. The

AtGRPs, belong to class II, can interact with cell wall-associated receptor protein kinase AtWak1 to participate in the signal transduction, and prevent viruses from invading plants [5]. The GRPs of class III regulates pollen development and hydration while the class IV GRPs with an RNA recognition domain are involved in molecular processes such as alternative splicing or transcriptional regulation and played an important role in stomatal regulation, seed and stamen development[10, 11]. Plant *GRP* genes are often specifically expressed and plays a distinctive role in different development stage or in different tissues [10]. Compared with the wild type, the deletion mutant of *AtGR-RBP5* showed shorter roots, smaller leaves, and shorter flower axes, meanwhile overexpressed *AtGR-RBP5* promoted cell elongation and tissue growth in Arabidopsis [2].

The plant GRPs are also involved in various abiotic stress such as low temperature, drought, and high salt stress, and may play an important role in resisting the adversity stress. A notable increase of *SbGR-RNP* was also observed in S. Bicolor seedlings when subjected to NaCl treatments with 1 M and 500 mM [12]. The ryegrass *LPGRP1* gene was up-regulated under cold stress treatment [13]. Compared with elevated temperature (32℃) , the expression level of *AtGRP7* gene was higher at low temperatures [14]. *NtGRP-1a* was up-regulated under drought stress and can be maintained for 3 to 6 days [15]. On the other side, overexpression of *AtRBG2* could increase cold tolerance and showed higher germination rate under salt stress in Arabidopsis [16], Although overexpressed *AtRBG7* could also increase cold tolerance and but inhibited seed germination and plant growth under drought stress [17]. Overexpressing *AtRZ-1a*, a zinc finger-containing GRP, displayed retarded germination and seedling growth under salt or dehydration stress conditions in transgenic Arabidopsis [18], but the loss-of-function mutants of *AtRZ-1a* germinated earlier and grew faster than the wild-type plants under the same conditions[19]. Ectopic expression of *AtGRP2* and *AtGRP7* in rice could also increase crop yield under drought stress [20]. In a word, different *GRP* genes may play different functions in various tissues or stress.

Although the research of *GRP* genes has been reported in many plants [21-24], the *GRP* gene has seldom reported in Chinese cabbage so far. In previous study, we found that an Oleosin-*GRP* gene *BcGRP17* showed higher expression levels in flower buds of male fertile plant than in sterile plant but the detail function is still unknown [25]. In this study, the potential *GRP* genes in Chinese cabbage were identified based on the existing genome database BRAD (http://brassicadb.org/brad/) [26], and their phyletic evolution, module prediction, and chromosomal localization were further performed. Moreover, the expression patterns of these *BrGRP* genes were also detected in different tissues and various abiotic stresses based on the opened transcriptome databases, their functions, and evolutions in development and stress response were also discussed. These results made a guide to further understand the function of *BrGRP* genes in Chinese cabbage.

Results

Identification and characterization of BrGRP Genes in Chinese Cabbage

For identifying the *BrGRP* genes in Chinese cabbage, we first screened the *GRP* genes of *Arabidopsis* in the TAIR and NCBI database. A total of 85 *AtGRP* genes were obtained, of which 26 genes had no orthologous genes, and the remaining 59 *Arabidopsis thaliana* genes were used to identify a total of 141 *BrGRP* genes in BRAD database. Further prediction analysis revealed that protein characteristics of these *BrGRP* genes showed great variety in molecular weight, theoretical point, etc. The length of amino acids ranged from 52aa (Bra027145.1) to 2038aa (Bra028693.1), and the theoretical point ranged from 4.11 (Bra019852.1) to 12.02 (Bra009252.1), of which 58.3% BrGRPs showed a theoretical point greater over 7. More detailed information about the instability index, aliphatic index and grand average of hydropathicity were also predicted, of which 63.1% proteins showed the aliphatic index over 40 and 73.8% proteins showed a grand average of hydropathicity with negative value. All these results suggested that these BrGRPs mainly exist on the unstable alkaline hydrophilic protein (Additional file 1: Table S1).

Subcellular localization showed that most BrGRPs (102 of 141) were secreted to the extracellular, 26 BrGRPs were localized in nuclear, 7 BrGRPs were localized in mitochondria; 3 BrGRPs were localized in plasma membrane; 2 BrGRPs, Bra040170 and Bra012653, were localized in membrane-bound chloroplast; and only one BrGRP Bra005945 was localized in chloroplast (Additional file 1: Table S1). These results could provide a reference for further functional analysis of these BrGRPs in Chinese cabbage.

Analysis of phylogenetic relationships, conserved domain and gene structure of BrGRP genes

To further classified these BrGRPs, the conserved domain analysis was performed by MEME tool. A total of 110 BrGRP genes showed similar conserved domains in protein sequence, and a total of 18 motifs were identified including 4 glycine-rich motifs (motif 5, 6, 8, 15), 6 RNA recognition motifs (motif 1, 4, 7, 9, 12, 13), 2 CCHC-Zinc finger structural motifs (motif 11, 17), 1 cold shock motif (CSD) (motif 3), 1 Oleosin lipid motif (motif 2), a signal peptide motif (motif 10), and 3 unknown structures Motif (motif 14, 16, 18) (Fig. 1c, Additional file 2: Fig S1).

According to different motifs, 110 BrGRPs were employed to construct a phylogenetic tree by Nj method (Fig. 1a), and can be roughly divided into five subclasses: Class I with 8 members, which exists a cysteine-rich polypeptide chain at the end of C-terminal with glycine repeat, and similar to the Class II GRP protein defined by predecessors in Arabidopsis or others [21]. Class II with 44 members, the N-terminus of this subclasses contained a typical glycine repeat structure followed by a signal peptide sequence or not, which were like to the predecessor's Class I subfamily [4]. In addition to the

glycine-rich region, Class III with 25 members also have RNA recognition domain, cold shock domain, and CCHC-zinc finger domain structure, which like the previous Class IV, glycine-rich RNA-binding protein subfamily. The glycine content of Class IV with 17 members was lower than previous subfamily, and belong to the predecessor's Class III subfamily. the glycine content of 16 BrGRPs was the lowest, and repeated with GGX, GX (X represent any amino acid), which was coincide to the Class V subfamily (Fig. 1c). Our classifying of five subclasses in BrGRPs was highly similar to the predecessor's subfamily classification in *Arabidopsis* or others, the genes in the same subgroup shared a close phylogenetic relationship, high sequence similarity, and similar gene structures, reveals the evolutionary conservation of *GRP* gene family.

The exon-intron structure distribution of 110 GRP genomic sequences was also analyzed (Fig. 1b), and 55 *BrGRP* genes showed no intron, while the other 55 *BrGRP* genes contained 2 or more exons, of which *Bra011396* with 21 exons was the maximum. According to the motif classification, the exon numbers of Class I *BrGRP* genes subfamily were the least with 1 or 2 exons, Class II subfamily showed the maximum polymorphism from 1 to 21 exons, the others Class III, IV& V have 1-9, 1-3 and 1-14 exons, respectively.

Chromosome localization and orthologous gene analysis of BrGRPs in Chinese cabbage

To examine the chromosomal distribution of 141 *BrGRP* genes, these genes were mapped onto the chromosomes and three fractionated subgenomes of Chinese cabbage based on the B. rapa genome database (chromosome v2.5). Except 6 *BrGRP* genes (*Bra040395*, *Bra034475*, *Bra040817*, *Bra040764*, *Bra039380*, *Bra040002*) could not be assigned to any chromosome, the other 135 genes were successfully located on the 10 chromosomes, in which A03 chromosome was located most with 23 *BrGRP* genes, while A05 and A06 chromosomes were located only 7 *BrGRP* genes, each. Furthermore, 44 *BrGRP* genes were anchored on the least fractionated (LF) subgenome, 54 genes on the medium fractionated (MF1) subgenome, and 37 genes on the Most fractionated (MF2) subgenome (Fig.2; Additional file 1: Table S2). Moreover, 101 pairs of *BrGRP* genes syntenic paralogs were found on different subgenomes of Chinese cabbage (Fig.3; Additional file 1: Table S3). For example, *Bra031554* and *Bra030674* are located in MF2 and MF1 subgenomes, respectively, while both exhibited high sequence similarities with *AT1G07135*. In order to further understand the duplication of the *BrGRP* genes during the whole genome duplication in Chinese cabbage, the orthologous analysis of GRP homologous genes in Chinese cabbage and *Arabidopsis thaliana* were also compared (Fig.3 Additional file 1: Table S3). A total of 42 *AtGRP* genes have found the orthologous genes in Chinese cabbage and most *GRP* genes of Arabidopsis have 1-7 orthologous genes in Chinese cabbage. There are also 26 *AtGRP* genes that have no orthologous genes in Chinese cabbage. These results revealed the evolution of *BrGRP* genes during whole-genome duplication events in Chinese cabbage

and would provide a strong reference for discovering the functions of *BrGRP* genes in Chinese cabbage.

Expression profiling of BrGRPs in Chinese cabbage

Based on the published transcriptomic data, the expression of these *BrGRP* genes was further analyzed in Chinese cabbage under different tissues and two development stages, especially in various abiotic and biotic stress, and a set of *BrGRP* genes were identified to be abnormal expression.

Expression profiling of BrGRPs in different tissue anddevelopment stage

The expression of these *BrGRP* genes were compared on callus, root, stem, leaf, flower, silique, rosette and folding leaves, and just 5 BrGRPs, including *Bra011869*, *Bra010693*, *Bra025663*, *Bra030284*, and *Bra031210*, were detected expressing in the six tissues, their expression in flowers and siliques were lower than in other tissues but without any significant differences. Compared with other tissues, *Bra011869* has the lowest expression in flower organs, and the other four genes showed the lowest expression in silique (Fig. 4a; Additional file 3: Table S4).

Besides that, the expression of 15 *BrGRP* genes were found in rosette and folding leaves of different stages, of which 12 genes including *Bra013176*, *Bra008205*, *Bra038769*, *Bra020903*, *Bra022895*, *Bra024169*, *Bra011077*, *Bra010346*, *Bra011195*, *Bra000643*, *Bra035944*, and *Bra014000*, showed higher expression in rosette leaves than in folding leaves, while the rest 3 genes, *Bra030674*, *Bra030673*, and *Bra030284*, showed lower expressed (Fig. 4b; Additional file 3: Table S5). These found suggest that most *BrGRP* genes may not express or showed very lower during plant development and in normal growth conditions.

Expression profiling of BrGRP genes under temperature stress

The expression of *BrGRP* genes were compared under heat stress at 0, 0.5, 1, 2, 3 and 4 hours of heat stress with 45 °C in 'Chiifu' seedlings [27], a total of 138 differentially expressed *BrGRP* genes were found (Fig. 5a, Additional file 3: Table S6), which can be divided into 28 expression patterns by STEM software (Additional file 3: Table S7). Among them, six expression patterns (profile 39, 43, 44, 46, 47 and 49) and a totally of 21 *BrGRP* genes *BrGRP* genes were significantly up-regulated at 5 times under heat stress. Seven expression patterns (profile 0, 1, 8, 11, 16, 21, 35) containing *BrGRP* genes were significantly down-regulated at 5 times under heat stress. Another 15 expression patterns showed disordered fluctuating. For example, *Bra036999* of profile 9 was down-regulated at 0.5, 1h, and 2h but up-regulated at 3h and 4h. *Bra038836* of profile 22 was down-regulated at 0.5h, 1h, while up-regulated at 2h, 3h, and 4h.

Bra025622 of profile 34 was down-regulated at 1h, 4h, while up-regulated at 0.5h, 2h and 3h.

On the other side, a totally of 108 *BrGRP* genes were found to be expression in two true leaves under cold treatment based on the transcriptome data of 29-day leaves treated with 4 °C temperature [28]. Among them, the expression of 48 genes showed significant differences in low-temperature treatment than the control with 25 °C [log2fold-change(FC)>1], of which 24 genes were up-regulated, and the remain 24 genes were down-regulated after low-temperature treatment (Fig. 5b, Additional file 3: Table S8).

Expression profiling of BrGRPs under Drought Stress.

To study the expression pattern of *BrGRP* genes under drought stress, the transcriptome data of Chinese cabbage (CR2355 and ATC92037) were also analyzed [29]. CR2355 is a root transcriptome data of drought-tolerant (DT) material which can keep the required biomass for mature when suffered from transient drought stress during the reproductive phase, while ATC92037 is a root transcriptome data of drought-sensitive (DS) material which showed a significant reduction in biomass after transient drought stress. After simulant drought treatment with 2.5% PEG 6000 in seedling, 74 *BrGRP* genes showed differential expression at 4h, 8h, and 12h between DT and DS materials, and can be further divided into 13 and 15 profile, respectively (Fig. 6, Additional file 3: Table S9, S10) Among them, some genes showed common expression pattern between DS and DT materials, for example, 8 *BrGRP* genes *(Bra000643*, *Bra011869*, *Bra013176*, *Bra013176*, *Bra015926*, *Bra021807*, *Bra025663*, *Bra031210*, *Bra035944*), belong to the similar expression patterns (profile 5,6), and showed continuous down-regulated at the first 8h of drought stress, and then up-regulated at 12h, while 12 *BrGRP* genes including *Bra031554*, *Bra032933*, *Bra029489*, *Bra020903*, *Bra010730*, *Bra037035*, *Bra011396*, *Bra030284*, *Bra040170*, *Bra012653*, *Bra037697*, and *Bra022938* were up-regulated in at least one material at 4h or 8h after drought treatment, and then down-regulated or returned to a normal level. There are also some *BrGRP* genes which showed differentiated expression between DT and DS materials, such as 10 *BrGRP* genes (*Bra015350*, *Bra025674*, *Bra005798*, *Bra001972*, *Bra022895*, *Bra011869*, *Bra010693*, *Bra004190*, *Bra014000*, *Bra013997*) was down-regulated or the expression level was not significantly changed in DS material, but up-regulated in DT material after drought treatment, 2 genes, *Bra011196&Bra030325*, were up-regulated in DS plants but down-regulated or without any changed in DT plants after drought treatment. The different expression patterns of these BrGRPs in two materials under drought stress may play different roles in the response of drought tolerance in Chinese cabbage.

Expression profiling of BrGRPs under soft rot stress

We also screened the different expression *BrGRP* genes under soft rot stress based on the transcriptome data of soft rot-resistant mutant(sr) and wild control inoculated with soft rot at 0h, 6h, 12h and 24h [30], and totally 47 differentially expressed *BrGRP* genes were found (Fig. 7a, Additional file 3: Table S11), which can be divided into 20

expression patterns (Additional file 3: Table S12). The results showed that many *BrGRP* genes were down-regulated within 12 hours after inoculation and up-regulated at later 12h-24h. while 8 *BrGRP* genes were up-regulated at four times after inoculated with soft rot in mutants. These results suggested a special stress reaction of *BrGRP* genes against pathogens.

At last, a Venn diagram simply revealed that the numbers of *BrGRP* genes involved in various stress (Fig. 7b, Additional file 4: Table S13). 32 *BrGRP* genes such as *Bra033973*, *Bra011869*, *Bra035917*, showed different expression under four types of stress. 39 *BrGRP* genes, including *Bra031554*, *Bra037697*, *Bra010693*, showed different expression under heat, cold and drought stress. *BrGRP* genes showed different expression under heat and cold stress, such as *Bra013640*, *Bra027092*. 11 *BrGRP* genes showed different expression under heat, cold and soft rot stress, such as *Bra035778*, *Bra040291*, *Bra040395*, and etc. Furthermore, some *BrGRP* genes just differently expressed under single stress. For example, 33 *BrGRP* genes are only differently expressed under heat stress while just *Bra000790* showed different expression under cold stress.

At the same time, we also found that the expression pattern of *BrGRP* genes under different abiotic stresses is not completely consistent. For example, *Bra030284* is up-regulated under both high and low temperature stress, while down-regulated under drought stress, and also showed up-regulated expression in drought-stressed DS plants and down-regulation in DT plants. *Bra010693* was up-regulated under low temperature stress and in drought-stressed DT plants, while down-regulated under high temperature stress and in drought-stressed DS plants. *Bra011869* was down-regulated under low temperature, high temperature, and in drought stressed DS material, while up-regulated under drought stressed DT material and in soft rot stress. The different expression of these *BrGRP* genes under various tissues and in multiple stresses may due to the different functional dissimilation, which need further certificated.

Analysis of cis-acting elements in the promoter region of BrGRPs

In order to declare the molecular mechanism of abnormally expressed *BrGRP* genes under various adversity stresses, 141 *BrGRP* genes are classified according to the types of biotic and abiotic stresses in the Venn diagram and the stress-related cis-acting elements of their promoter regions were further analyzed (Fig 8, Additional file5: Table S14). The results indicate that lots of stress-related cis-acting elements were found in the promoter regions of 141 *BrGRP* genes, including DRE, MYB, MYC, ABRE, LTRE, W-box, TC-rich repeats, and so on. DRE has been identified as a *cis*-acting element involved in drought, high salt and low-temperature stress, MYB involved in by drought, cold and salt stress [31], MYC involved in drought and ABA [32], ABRE involved in ABA and drought stress [33], LTRE involved in low temperature stress [34]. W-box binds to WRKY transcription factor, participates in the stress response of plants such

as diseases, drought, ABA, and etc [35]. TC-rich repeats participates in plant defense and stress response[36].

The number of stress-related cis-acting elements in the promoter region of these 141 *BrGRP* genes were different, from the minimum with 4 (*Bra032933*) to the maximum with 34 (*Bra009254*), and each promoter region of *BrGRP* genes distributed 3-6 types of cis-acting elements. 97.2% (137/141) of *BrGRP* genes promoter region contained MYC element, 98.6% (139/141) contained MYB element, 84.4% (119/141) has ABRE element, 55.3% (78/141) has LTRE element, 26.2% (37/141), 51.1%(72/141) and 40.4%(57/141) contained DRE, W-box, and TC-rich repeats, respectively. These cis-acting elements distributed in the promoter region of *BrGRP* genes played an important role in the response of adversity stress which further verified the expression difference identified previously in Chinese cabbage. There are also different types of stress-related cis-acting elements in the promoter region of the *BrGRP* genes. For example, *Bra037697*, *Bra037056,* and other 15 genes only have ABRE, LTRE, MYB, and MYC. *Bra030582*, *Bra032844*, and other 9 genes only have ABRE, LTRE, MYB, MYC and TC rich repeats. Differences in the type of cis-acting elements of the *BrGRP* genes promoter region may be associated with different expression patterns of different *BrGRP* genes under biotic and abiotic stress. At the same time, the number of stress response cis-acting elements in the promoter region is not completely consistent with the expression pattern under the stress of *BrGRP* genes. For example, the ABRE element related to drought [33] is not found in the promoter region of *Bra000790* with a specific response under low-temperature stress. 11 ABRE elements were found in the promoter region of *Bra028691* who is specific response for high-temperature stress. This may be due to the loss of related functions by missing some of the cis-acting elements in promoter region. These results may provided an important reference for exploring the action mechanism of *BrGRP* genes in biotic and abiotic resistance.

Discussion

The plant *GRP* gene family is a superfamily with the domain of glycine repeat (Gly) n-X. However, due to the diversification of their protein domains, gene expression patterns, and subcellular localization, these *GRP* genes sometimes are also not considered to be a gene superfamily but a group of proteins with some repeating structural motifs [21], which may disturb the identification of *GRP* gene family. Anyway, 15, 22, 12, and 18 glycine-rich RNA-binding proteins (RBGs) have been identified in *Arabidopsis*, *Chinese cabbage*, *rice* and *maize* genomes, respectively [22, 24], furthermore, 9 and 51 GRP genes have been identified in *sweet potato* and *Curcuma longa L.*, respectively [21, 23]. In this paper, 141 *BrGRP* genes were preliminarily identified in Chinese cabbage based on the BRAD genome sequence. Except 31 genes without any conserved domain which may due to the gradual loss of functions or get the new function during evolution [37], the remaining 110 *BrGRP* genes could be roughly divided into five main subclasses according to the conserved domain, which was similar to the previous studies [3, 4]. The numbers of glycine repeat

in the glycine-rich region of BrGRPs were always as even and usually exist with the order of (Gly)6-X (motif 6), that was different from the other plants which contained the odd numbers [3, 4]. This different arrangement of BrGRP genes may lead to the unique function in Chinese cabbage which needs further study.

Among five main subfamilies, the BrGRPs of class I and class II in Chinese cabbage are similar in the term of the conserved domain, glycine repeat motif, evolutionary phylogeny and gene structure (1-3 exons), except a cysteine-rich region at the C-terminus of class II. Simultaneously, the Class IV and Class V proteins are also similar except the conserved Oleosin domain in Class IV. These results suggested that the BrGRPs of class I & class II, Class IV & Class V, each have the same ancestors which may develop additional conserved domain and get new functions during the evolution process. In addition, we also identified three Chinese cabbage Zinc Finger-Containing Glycine-Rich RNA-Binding Proteins（RZs）[38] from the Class III proteins, including *Bra032933*, *Bra025250*, *Bra005798*, all of which contained three single structural domains RRM, CSD, and CCHC, their function is still unknown.

Chinese cabbage genome was not only suffered the evolution events of three-time genome-wide replication shared with other cruciferous plants[39, 40], but also divided into three subgenomic groups, named as LF, MF1, MF2 subgenome according to the number of gene losses from small to large [37, 41]. In this paper, the syntenic analysis of *GRP* genes between Chinese cabbage and *Arabidopsis thaliana* also verified this genome-wide replication event that Chinese cabbage was originated from the hexaploid ancestor, and was rearranged to be diploid as the current cabbage species after chromosome fusion [41]. Meanwhile, it was found that 141 *BrGRP* genes of Chinese cabbage, including 44 LF genes, 54 MF1 genes, and 37 MF2 genes, were not in a three-fold relationship with 85 GRP genes of *Arabidopsis*, which may suggest that *BrGRP* genes of Chinese cabbage still evolved after the genome-wide replication event, and occurred larger-scale loss-of-function to prevent functional redundancy.

The expression of *GRP* genes in *Arabidopsis thaliana* always showed a tissue or organ-specific manner, *GRP* gene of class I&II were mainly expressed in seeds, siliques, roots, and leaves; Class III has the highest expression in shoot tips, rosettes, seeds, and flowers; Class IV is highly expressed in seeds, siliques, rosettes, and flowers; Class V is up-regulated only in inflorescence [10, 42]. Although differential expression of *BrGRP* genes were also found in different tissues in Chinese cabbage [43], the related genes were infrequent, and only part of class III genes showed different expression between various tissue. The class I and II of *GRP* genes may be acted as the active components of plant cell walls and played a crucial role in plant cell growth and organ differentiation [5]. The class III of *BrGRP* genes has strong RNA recognition and binding ability and may participate in the molecular process of plant growth and development by activating splices or regulating transcription [10]. In the study, we found that the *BrGRP* genes that differentially expressed at various developmental stages of Chinese cabbage belonged to the class I-III, which was similar to the previous studies, and may contain

similar functions in terms of growth and development. Besides that, we also found that only a small number of *BrGRP* genes showed different expression in various tissues and organs of Chinese cabbage, while most *BrGRP* genes expression can be induced by biotic and abiotic stress.

The plant *GRP* genes which can be induced by various stresses suggested another role in plant resistance. Eight glycine-rich RNA-binding protein genes (*AtGR-RBP1 - AtGR-RBP8*) have been reported in *Arabidopsis thaliana*. except *AtGR-RBP5* and *AtGR-RBP6*, another 6 *AtGR-RBP* genes were strongly induced by low-temperature stress [18, 44]. Under drought and salt stress, the expression of *AtGR-RBP1* gene was increased but the expression of *AtGR-RBP4* and *AtGR-RBP7* were gradually attenuated, while the expression of *AtGR-RBP5* and *AtGR-RBP6* did not change [44]. In tobacco, the expression of *NtGRP1* gene was induced and continuously increased at the first 24 hours of waterlogging stress, and then decreased, while kept in a low-level under whatever high or low temperature, drought, high salt and ABA stress [15, 45]. *NtRGP2* and *NtRGP3* were also induced by waterlogging, high and low-temperature stress, but were not affected by ABA treatment with 100 μmol· L-1 [15]. Among the four glycine-rich RNA-binding protein genes (*OsGR-RBP1-OsGR-RBP4*) in rice, only *OsGR-RBP4* was induced by high temperature, high salt and drought stress [46]. In this study, a total of 141 *BrGRP* genes were identified which may different expressions under high & low temperature, drought, or soft rot stress. Although the *BrGRP* genes that expressed in different biotic and abiotic stresses were different, and a total of 138, 108, 74 and 47 of *BrGRP* genes were expressed under high temperature, low temperature, drought, and soft rot treatment, respectively, the same *BrGRP* gene that differentially expressed, also showed the different expression dynamics under various stresses, suggested their multiple expression patterns and different functions under adversity stress in Chinese cabbage. *AtRZ-1* was strongly induced by low temperature and freezing stress, but had a negative regulation on seed germination and seedling growth under drought and high salt stress [18, 19, 47], Overexpression of *AtRZ-1B* and *AtRZ-1C* or loss of function mutations does not affect Arabidopsis seed germination and seedling growth under the same stress conditions [48]. *AtRZ-1B/1C* in regulating RNA splicing, gene expression, and many key aspects of plant development via interaction with proteins including SR proteins [49]. All these suggested that the *BrGRP* gene not only responds to the multiple stresses, but also resists to the various stress by different molecular mechanisms.

A large number of stress-related elements identified in the promoter region of *BrGRP* genes also proved an inextricable link and different response mechanisms between *BrGRP* genes and abiotic stress. Six important stress-related cis-acting elements of MYC, MYB, ABRE, LTRE, DRE and TC rich repeats were identified in the promoter region of *BrGRP* genes. Multiple types of cis-acting elements lied in the promoter region may be one of the reasons for the diverse expression patterns of *BrGRP* genes. Compared with the special expression patterns of *BrGRP* genes under stress, *BrGRP* genes that involved in various stresses, may play important role in broad-spectrum resistance, which should be worthy of deep research in the future.

Conclusion

In this study, 141 *BrGRP* genes were identified in the Chinese cabbage genome based on the 59 *AtGRP* genes of *Arabidopsis*. The *BrGRP* genes in Chinese cabbage are mainly composed of alkaline hydrophilic unstable protein and were secreted outside the cell membrane, only a few were found in organelles such as nuclear, mitochondria, and chloroplast. Some *BrGRP* genes may gradually lose their conserved domain during evolution, and just 110 BrGRPs shared the same conserved domain, which can be divided into five subclasses. Chromosomal localization of these *BrGRP* genes and syntenic analysis with Arabidopsis thaliana strongly confirmed that Chinese cabbage did undergo a genome-wide triple duplication event during evolution. Their specific expression under various stresses were screened, and 3-6 types of response stress cis-acting elements in the promoter region of these *BrGRP* genes were also identified, which suggested their potential roles in plant stress response. But the molecular mechanisms by which *BrGRP* genes respond and resist biotic and abiotic stress remain unclear. These results may provide an important basis for the study of their function in Chinese cabbage.

Methods

Identification of *GRP* genes in Chinese cabbage

To identify the *BrGRP* genes in Chinese cabbage, the *GRP* genes in *Arabidopsis thaliana* were isolated from TAIR database (http://www.arabidopsis.org/) [50] and used as a query to search the homeotic BrGRP genes in Chinese cabbage genome database (BRAD v1.5, http://brassicadb.org/brad/)[26]. Their nucleotide and deduced amino acid sequences were downloaded for the following characterization analysis.

Characterization analysis and subcellular localization prediction of BrGRPs

physical and chemical characteristics, including the molecular weight (MW), theoretical point (pI), instability index, aliphatic index and grand average of hydropathicity (GRAVY), of these sequences of BrGRPs were further analyzed using the ProtParam tool of ExPASy (http://web.expasy.org/protparam/). The subcellular localization of these BrGRPs were predicted by the ProtComp tool in Softberry online website (http://linux1.softberry.com/).

Multiple sequence alignment and phylogenetic tree establishment

Multiple sequence alignments of the protein sequences were performed by Clustal X with default parameters. The phylogenetic tree was constructed by Neighbor-Joining method (NJ) in software MEGA-X[51], in which the check parameter bootstrap value

was set as 1000 times, the genetic distance calculation model was set as P-distance, and the processing of vacant missing data is set as Pairwise deletion.

Analysis of the conserved domain and gene structure of *BrGRP* genes

The structures of the coding/non-coding region of *BrGRP* genes were mapped by software TBtools [52]. Next, the conservative motifs were analyzed by MEME tool (version 5.0.3; http://meme-suite.org/tools/meme) with the number of motifs set as 20 and the other parameters were default values. The LOGO of conservative motifs was listed, and the corresponding Scalable Vector Graphics (SVGs) was also exported by TBtools [52].

Identification of the orthologous *BrGRP* genes and syntenic analysis in Chinese cabbage

According to genomic and chromosome database (v2.5) of Chinese cabbage[26, 37], the identified *BrGRP* genes were located into 10 chromosomes was and three fractionated subgenomes and the chromosome positions of *BrGRP* gene was constructed by MapChart software [53]. The syntenic analysis of *BrGRP* homologs between Chinese cabbage and Arabidopsis thaliana were searched by Chinese cabbage genome database (BRAD v1.5, http://brassicadb.org/brad/) [26] and the corresponding circos were drawn out by TBtools [52].

Expression pattern analysis of *BrGRP* genes

To analyze the expression pattern of these *BrGRP* genes in Chinese cabbage, the transcriptome data of *B. rapa* 'Chiifu' [43] and the inbred line 'Fushanbaotou', a typical heading Chinese cabbage [54] were downloaded and used for gene expression profiling in eight tissues including callus, root, stem, leaf, flower, silique, rosette and folding leaves.

The expression differences of *BrGRP* genes under biotic and abiotic stress were also analyzed based on the transcriptome data of Chinese cabbage under high temperature at 45℃[27], low temperature at 4℃[28], drought [29], soft rot stress [30]. The expression levels of *BrGRP* genes were calculated with the Fragments Per kb per Million reads (FPKM) values [55] and displayed with cluster heat map drawn by TBtools [52]. Analysis of expression pattern of *BrGRP* gene under high temperature, drought and soft rot stress by STEM (Short Short Time-series Expression Miner) [56]. The Venn map was generated by TBtools [52] according to the differentially expressed data.

Analysis of the cis-acting elements in the promoter region of *BrGRP* genes

To further identified the cis-acting elements in the promoter regions of *BrGRP* genes, a 2 kb fragment in upstream of the ATG (start codon) were extracted by TBtools[52] and were further identified by PlantCare [57] (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) with the adversity

related cis-acting element including MYC (CANNTG), MYB (C/TAACNA/G) , ABRE (ABA-responsive element, ACGT), LTR (low-temperature-responsive element, CCGAAA), DRE (CCGAC), W box(TTGACC) and TC rich repeats(GTTTTCTTAC). Constructing a Venn diagram by TBtools[52] based on the type of cis-acting element.

List of abbreviations

GRP: glycine-rich protein; RBPs: RNA-binding proteins; BRAD: Brassica database; TAIR: The Arabidopsis Information Resource; BrGRP: glycine-rich proteins in Chinese cabbage; RZs: Zinc Finger-Containing Glycine-Rich RNA-Binding Proteins; LF: The least fractionatedsubgenome; MF1: The medium fractionated subgenome; MF2: The mostfractionated subgenome; FPKM: Fragments Per kb per Million reads; ABA: Abscisic acid; NJ: Neighbourhood-joining method; Scalable SVGs: Vector Graphics.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Competing interests

The authors declare that they have no competing interests

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Author information

contributions

XL and XX conceived & designed the experiments. XL and YC wrote the manuscript. XL, YC and MG were responsible for data analysis and revising the manuscript. All authors read and approved the final manuscript. ML provided helpful advice on data analysis. ML and XX revised the paper and supervised the research.

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Additional files

Additional file1 Table S1 The information on *BrGRP* genes in Chinese cabbage.

Additional file1 Table S2 The distribution of *BrGRP* genes in genome of Chinese cabbage.

Additional file1 Table S3 The orthologous gene pairs in *GRP* genes of Chinese cabbage and Arabidopsis thaliana.

Additional file 2 Figure S1 The LOGO of GRP conservative motifs in Chinese cabbage.

Additional file3 Table S4 The FPKM values of *BrGRP* genes in five different tissues of Chinese cabbage.

Additional file3 Table S5 The expression values of *BrGRP* genes in defferent development stages of Chinese cabbage.

Additional file3 Table S6 The expression values of *BrGRP* genes under 45 °C heat treatment in Chinese cabbage.

Additional file3 Table S7 The expression profiles of *BrGRP* genes under 45 °C heat treatment in Chinese cabbage.

Additional file3 Table S8 The expression values of *BrGRP* genes under low temperature treatment in Chinese cabbage.

Additional file3 Table S9 The expression values of *BrGRP* genes under drought treatment in Chinese cabbage.

Additional file3 Table S10 The expression profiles of *BrGRP* genes under drought treatment in Chinese cabbage.

Additional file3 Table S11 The expression values of *BrGRP* genes under soft rot treatment in Chinese cabbage.

Additional file3 Table S12 The expression profiles of *BrGRP* genes under soft rot treatment in Chinese cabbage.

Additional file4 Table S13 The same *BrGRP* genes expression under different biotic and abiotic stresses.

Additional file5 Table S14 Quantitative statistics of cis-acting elements related to adversity stress in the promoter region of *BrGRP* genes.

Figures

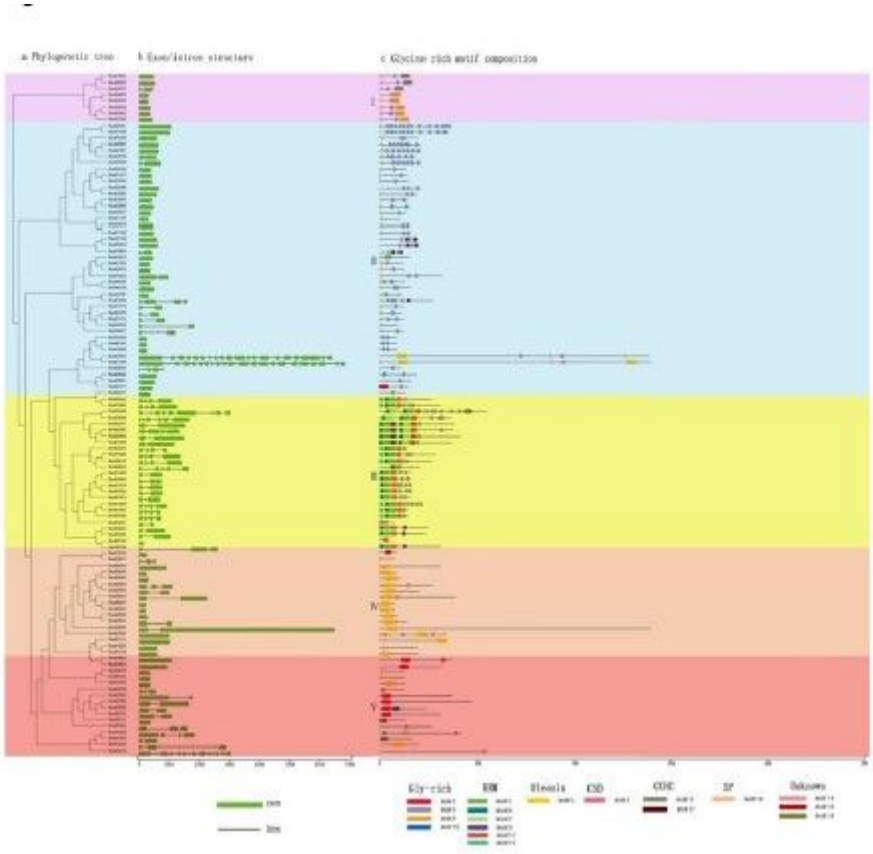

Figure 1

Phylogenetic relationship, gene structure and conserved structural composition of 110 BrGRP genes in Chinese cabbage. (a) Phylogenetic tree, by using Clustal X to align the amino acid sequence, and using MEGA X to generate a phylogenetic tree by a contiguous method with a calibration parameter of 1000. (b) the sequence structure distribution of BrGRP genes, the green box represents the exon, while the black line represents the intron, Scale indicates 1.0 kb. (c) Schematic diagram of the conserved GRP protein motif in Chinese cabbage predicted by MEME, colored boxes indicate different motifs, black lines indicate non-conservative sequences, and scale bars represent 500 aa.

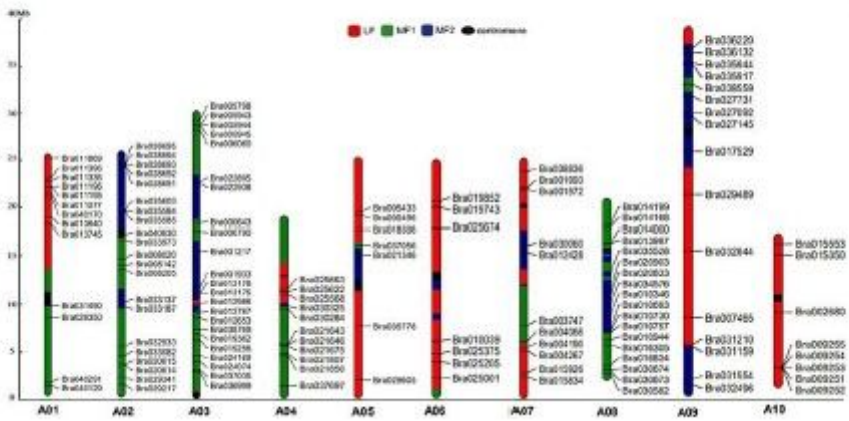

Figure 2

Chromosome distribution of BrGRP genes in Chinese cabbage. LF: the least fractionated subgenome; MF1: the medium fractionated subgenome; MF2: the most fractionated subgenome; centromere: position of chromosome centromere.

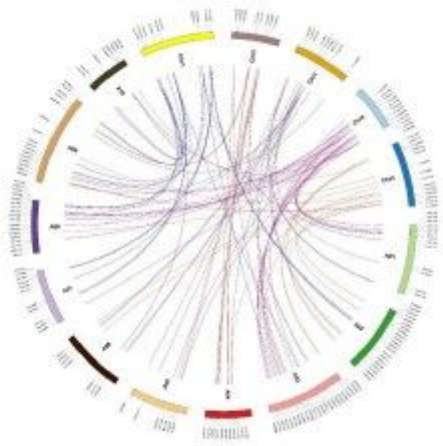

Figure 3

Syntenic analysis of GRP genes in Chinese cabbage, Arabidopsis thaliana. The orthologous and paralogous GRP genes are localized on the chromosomes of Chinese cabbage (A01-A10) and Arabidopsis thaliana (Chr1-Chr5)

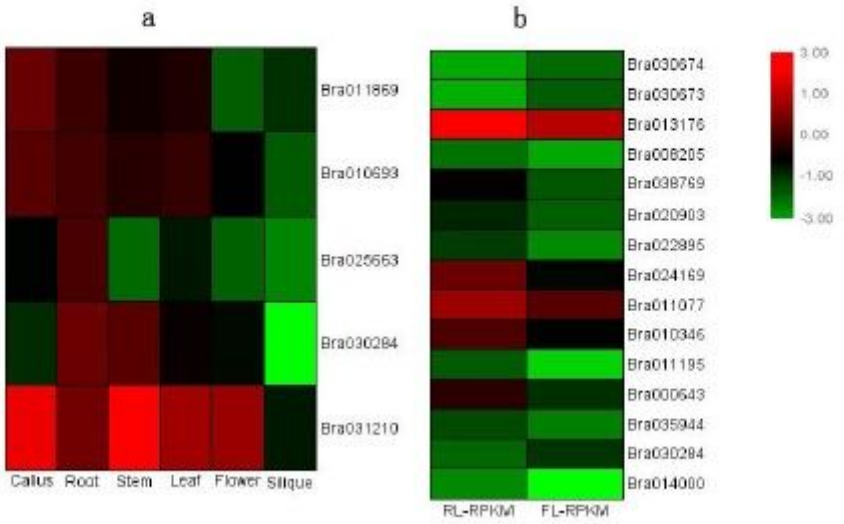

Figure 4

Expression analysis of BrGRP gene expression in Chinese cabbage. a: Differential expression pattern of BrGRP gene in different tissues of Chinese cabbage; b: Differential expression pattern of BrGRP gene of Chinese cabbage in the rosette and folding leaves.

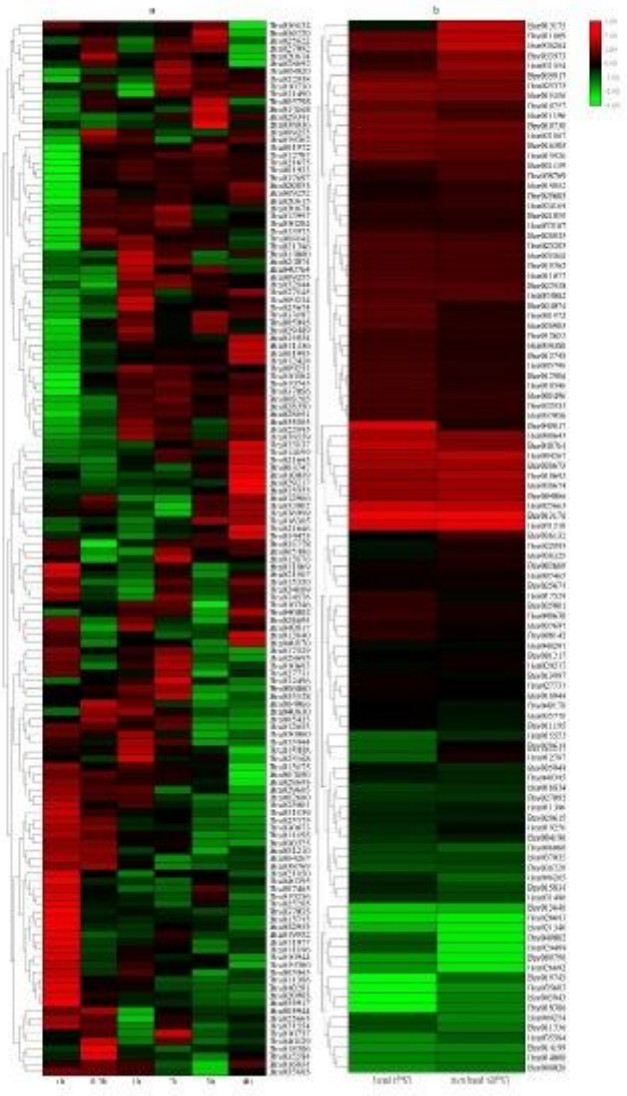

Figure 5

Expression analysis of BrGRP genes in Chinese cabbage. a: Differential expression pattern of BrGRP gene in Chinese cabbage under 45□ heat stress for 0.5h, 1h, 2h, 3h, and 4h; b: Differential expression pattern of BrGRP gene in Chinese cabbage under low-temperature stress at 25 °C and 4 °C.

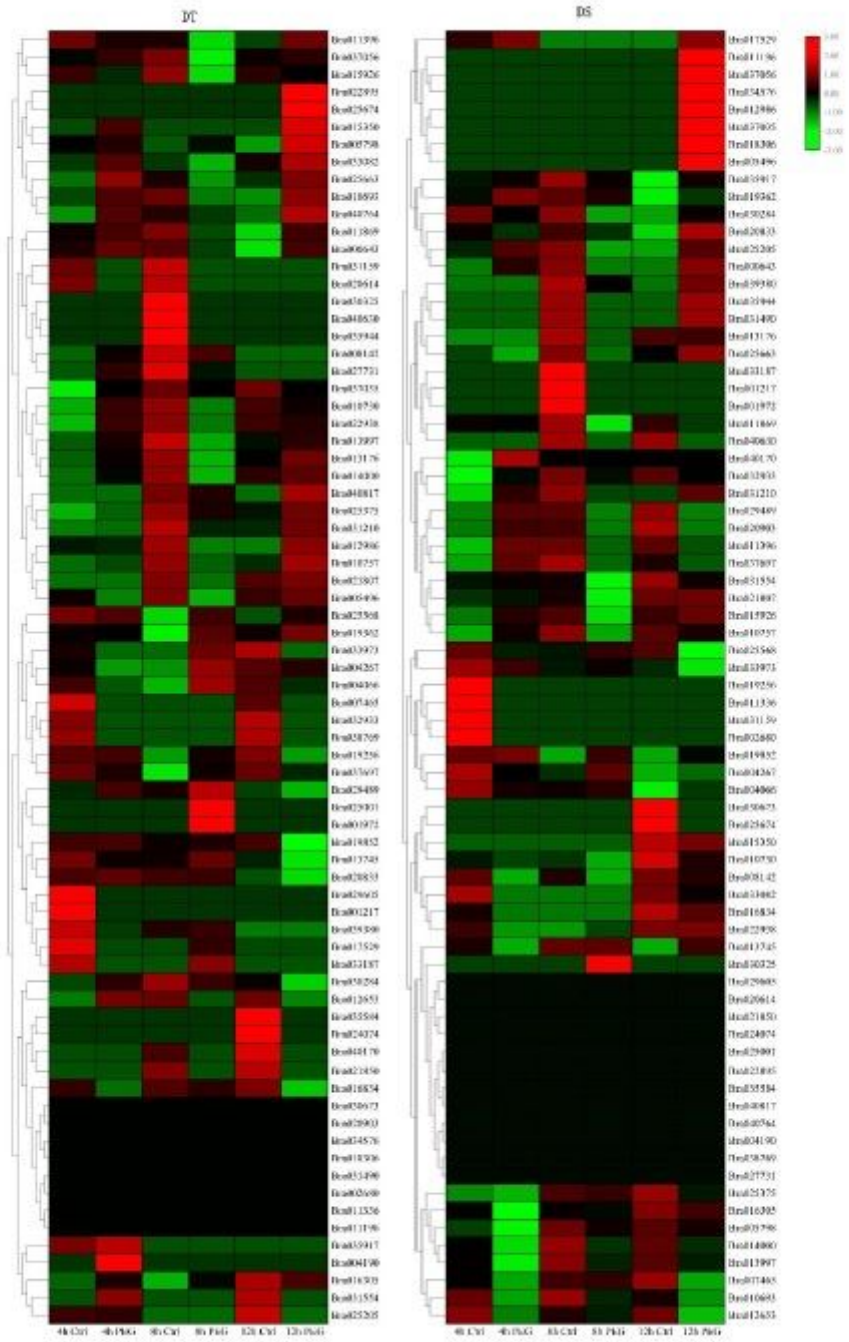

Figure 6

Differential expression profiles of BrGRP genes in drought-sensitive (DS) and drought-tolerant (DT) control and drought stress groups of Chinese cabbage at 4h, 8h and 12h.

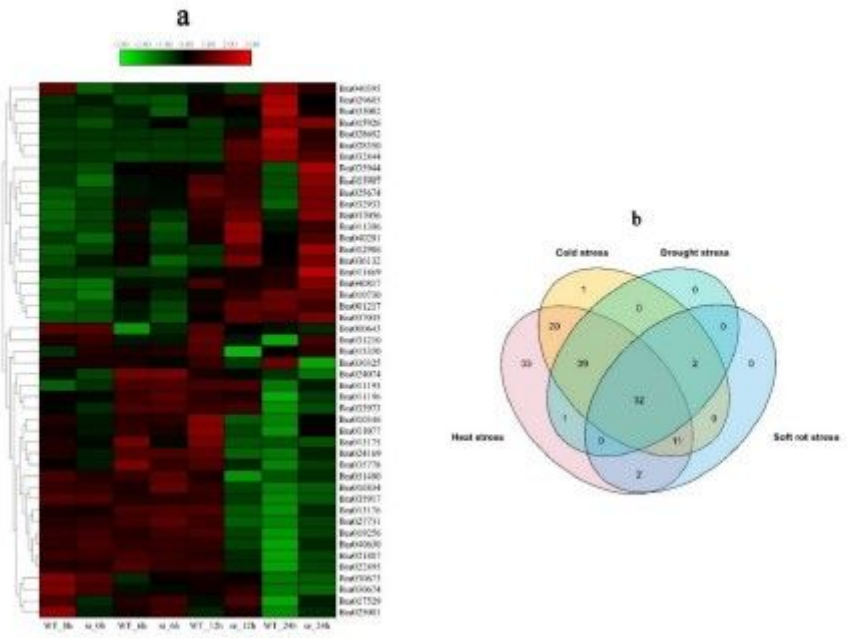

Figure 7

Expression analysis of BrGRP genes under soft rot stress (a) and the numbers of BrGRP genes involved in various stresses showed by Venn diagram (b). Fig.

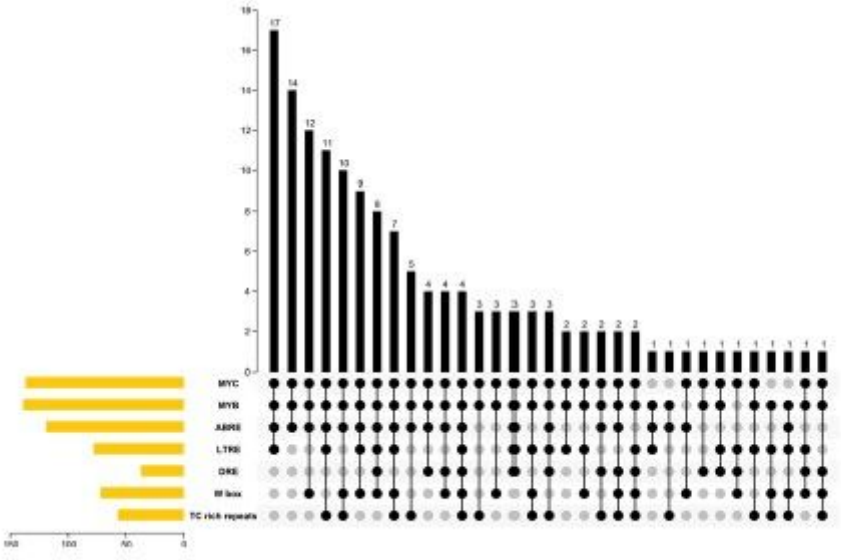

Figure 8

The numbers of the stress-related cis-acting elements in the promoter regions of BrGRP genes showed by Venn diagram. MYC (CANNTG), MYB (C/TAACNA/G), ABRE (ABA-responsive element, ACGT), LTR (low-temperature-responsive element, CCGAAA), DRE (CCGAC), W box(TTGACC), TC rich repeats(GTTTTCTTAC).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile5TableS14.xlsx
- Additionalfile2FigureS1.jpg
- Additionalfile1TableS1S3.xlsx
- Additionalfile4TableS13.xlsx
- Additionalfile3TableS4S12.xlsx