Genome-wide identification and expression analysis of the cytokinin gene family in Brassica oleracea L. reveals its role in clubroot resistance

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Brassica oleracea, genome-wide, cytokinin family genes, clubroot
Abstract

Background: cytokinins have important functions in regulating plant growth and response to abiotic stress. Cytokinin family genes have been described in several plant species, but a comprehensive analysis of the cytokinin family genes in Brassica oleracea has not been reported to date, especially their roles in dealing with the invasion of P. brassicae. Results: Cytokinins are a class of phytohormones that promote cell division and differentiation and are thought to affect plant immunity to multiple pathogens. To reveal the mechanisms of the Brassica oleracea cytokinin family genes in response to clubroot disease, a total of 36 cytokinin genes were identified using a genome-wide search method. Phylogenetic analysis classified these genes into three groups. They were distributed unevenly across nine chromosomes in B. oleracea, and 15 of them did not contain introns. The results of colinear analysis showed that each cytokinin gene in the B. oleracea genome had at least one homologous gene in the Arabidopsis genome. A cis-element analysis indicated that these genes possessed several stress response cis-elements. The heatmap of the cytokinin gene family showed that these genes were expressed in various tissues and organs. Five and eight genes were up- and downregulated, respectively, in the susceptible material after inoculation. In addition, two and one genes were up- and downregulated, respectively, in resistant material. This may indicate that these cytokinin genes play important roles in the host plant response to clubroot disease. In addition, the results provide insights for better understanding the role of cytokinin in the B. oleracea–P. brassicae interaction. Conclusions: Our results are helpful to elucidate the role of cytokinin family genes in cabbage response to infection by P. brassicae, and lay a
foundation for further study on the function of these genes. Keywords: *Brassica oleracea*, genome-wide, cytokinin family genes, clubroot

**Background**

Cytokinin is a plant hormone associated with cell division and differentiation, consisting of a glandular peptide derivative with a substituted N\(^6\)-isoprenoid side chain (Miller et al. 1956; Shaw et al. 1994). Cytokinin plays important roles in the growth and development of plants. Cytokinin can promote cell proliferation in buds and increase the activity of meristematic tissues by regulating the PIN protein in shoots (Kieber et al. 2018; Waldie et al. 2018). In the roots, cytokinin not only acts as a signaling molecule regulating root growth (Zhao et al. 2015) but also determines the size of the roots by controlling cell differentiation (Ioio et al. 2007).

Cabbage (*Brassica oleracea var. capitata* L.) is a very important vegetable crop that is widely grown around the world, with great economic value. As a member of the U’s triangle (Nagaharu 1935), cabbage is also of great theoretical significance for comparative and evolutionary analysis. Clubroot, caused by *Plasmodiophora brassicae* Woronin, which mainly invades cruciferous crops including cabbage, is a worldwide soil-borne disease (Yu et al. 2017; Rocherieux et al. 2004). The life cycle of *P. brassicae* includes two stages: the root-hair infection stage and the cortex infection stage. Resting spores germinate in a suitable soil environment to form primary zoospores, which then infect the root hairs and form primary plasmodia. The primary plasmodia can form secondary resting zoospores through a series of complex processes. The secondary resting zoospores in turn infect cortical cells and form secondary plasmodia, which are the key cause of swollen roots (Rolfe et al. 2016). The clubroot hinders the absorption and transport of water and nutrients in
plants (Pang et al. 2018), causing crop yield reduction. Multiple signaling pathways are activated when plants are infected by *P. brassicae*. The formation of resting spores is restricted within cortex cells in resistant cabbage (Ning et al. 2018). The expression of cytokinins can significantly reduce clubroot symptoms and the content of the pathogen in susceptible lines (Chen et al. 2018). Chu et al. (2014) reported that the signaling and metabolic activity of ethylene (ET) and jasmonic acid (JA) were significantly upregulated in resistant lines compared to susceptible lines at 15 days after inoculation (dai). Schuller et al. (2014) found that brassinosteroid (BR) synthesis and signal perception were involved in clubroot development. Manoharan et al. (2016) reported that methyl salicylate (SA) plays an essential role in the response to a chronic pathogen during clubroot development in *B. oleracea*. In addition, the accumulation of sugar at the infected site can help plants reduce the risk of clubroot disease (Walerowski et al. 2018).

The formation of galls is the most typical symptom of plants infected by *P. brassicae* (Lisha et al. 2018). Microscopic observation of root infection showed that the galls are caused by cell elongation and division (Kobelt et al. 2000). The galls can be regarded as a powerful metabolic sink (Rausch et al. 1981) where auxins and cytokinins are synthesized in large amounts (Dekhuijzen and Overeem 1971; Ando et al. 2005). Similar to clubroot disease, leguminous plants form tumors in the roots when they are infected by rhizobia (Miri et al. 2015). As a key endogenous plant signal, the role of cytokinin in this process is to induce the formation of a mass. Many phenomena indicate that cytokinin must play an essential role in the plant response to the invasion of *P. brassicae*. Cytokinin and auxin synergistically induce hypertrophy and hyperplasia by increasing the cell division rate and cell enlargement during the formation of a mass in the roots (Ludwig-Müller and Schuller 2005).
The detection and analysis of five cytokinin synthase genes from *Brassica rapa* indicated that the expression of cytokinin synthase genes was increased during the primary developmental stage of clubroot disease (Ando et al. 2005; Schuller et al. 2014). Similarly, throughout the process of gall formation, cytokinin biosynthesis is upregulated, with downregulation of adenosine kinase (Ludwig-Müller et al. 2009). RNA-seq is considered a powerful approach for detecting differentially expressed genes and is widely used to study the interactions between host plants and *P. brassicae*. Siemens et al. (2006) identified more than 1000 DEGs in infected individuals compared with the normal roots of *Arabidopsis*. A transcriptome study of *Brassica oleracea var. italica* and *Brassica macrocarpa Guss.* in different infection periods of *P. brassicae* showed that genes associated with glucosinolate biosynthesis, cell wall biosynthesis, and plant hormone signal transduction were activated much earlier in resistant lines (Zhang et al. 2016). In addition, comparative transcriptome analysis between susceptible and resistant Chinese cabbage varieties revealed that genes associated with pathogen-associated molecular patterns (PAMPs), pathogenesis-related (PR), calcium ion influx, hormone signaling, transcription factors, and cell-wall modification played important roles in the interactions between *B. rapa* and *P. brassicae* during the primary infection stages (Chen et al. 2016).

Although the function of cytokinin has been analyzed in various plant cultivars, such as *Arabidopsis* (Riou-Khamlichi et al. 1999), *Spirodea polyrhiza* (Kurepa et al. 2018), *Phaseolus lunatus* (Martin et al. 1999), rice (Ashikari et al. 2005), tomato (Chen et al. 2016), apple (Feng et al. 2017), potato (Lomin et al. 2018) and maize (Schmülling et al. 2003; Chettoor et al. 2017), the roles of the cytokinin gene family in cabbage with clubroot disease are currently unknown. In this study, we identified
36 cytokinin genes in *B. oleracea*. The phylogenetic relationships, chromosome locations, colinearity relationships, and gene structures of all detected genes as well as the *cis*-acting regulatory elements in promoters were then systematically analyzed. The transcription and expression patterns of cytokinin genes during different periods following inoculation with *P. brassicae* were also investigated. Our results will promote the understanding of the molecular mechanism of the cytokinin response to the development of clubroot disease in *B. oleracea*.

**Results**

**Genome-wide identification and phylogenetic analysis of cytokinin genes in *B. oleracea***

A total of 36 cytokinin genes were identified, and the detailed information for each cytokinin gene is shown in Table 1. The proteins encoded by the cytokinin genes varied from 355 to 594 amino acids (aa) in length. Among these proteins, the *Bol024927* protein sequence was the longest, with 594 amino acids, and the *Bol006929* protein sequence was the shortest, with 355 amino acids. Among all of the proteins, 18 members shared similar localization to cytoplasm, three to the extracellular region, four to the vacuole, and eleven members were located in more than one compartment.

To study the evolutionary relationships of the cytokinin genes in *B. oleracea*, *Arabidopsis*, and *B. rapa*, 135 cytokinin genes were analyzed to construct an unrooted phylogenetic tree. The cytokinin genes were classified into three groups (Fig. 1), which contained 34 (class I), 32 (class II) and 56 (class III) members. The remaining 10 members could not be clustered into any group. The numbers of cytokinins from *B. oleracea*, *A. thaliana* and *B. rapa* in each class are represented in
Chromosomal distribution and differential retention of cytokinin genes in *B. oleracea*

The 36 cytokinin genes were assigned to nine chromosomes of *B. oleracea* (Fig. 2). The distribution of the cytokinin genes on each chromosome was uneven. Chromosome C07 contained the largest number (8) of cytokinin genes, followed by chromosomes C01, which contained six genes. Both chromosome C03 and C04 contained five genes. Three genes were located on C05. Chromosome C02, C06 and C08 each contained only two genes.

To obtain a better understanding of the evolutionary history of the cytokinin gene family in *B. oleracea* and *Arabidopsis*, duplications of putative cytokinin genes in the *B. oleracea* and *Arabidopsis* genomes were analyzed. Every cytokinin gene in the *B. oleracea* genome has at least one homologous gene in the *Arabidopsis* genome. In addition, segmentally duplicated gene pairs were observed among the 36 identified cytokinin genes in *B. oleracea* (Fig. 3; Additional file 2: Table S3). The *B. oleracea* genome contained 1–7 copies of each cytokinin gene found in *Arabidopsis*, while 13 of the *A. thaliana* cytokinin genes had no homologs in *B. oleracea* (Fig. 3; Additional file 3: Table S4). For example, AT3G47930.1 contained only one homologous gene (*Bol024927*) in *B. oleracea*, while AT2G41510.2 contained up to seven homologous genes (*Bol036074, Bol020547, Bol006929, Bol005172, Bol027725, Bol045724, and Bol035751*) in *B. oleracea*.

Structure and conserved motif analysis of cytokinin genes

The structures and phases of introns/exons were determined to further study the cytokinin genes. In general, genes with closer phylogenetic relationships exhibited similar genetic structures, such as *Bol006383* and *Bol028361* or *Bol035751* and
Nearly half (16, 44.4%) of all the genes contained two or more introns, while 5 genes contained only 1 intron. The remaining 15 genes without introns were almost the same length. The length of each intron in different genes showed great divergence. For example, Bol43933 contained 17 short introns, whereas Bol006389 contained an extremely long intron (Fig. 4). The differences in the gene structure of Bolcy in B. oleracea might indicate the diversity of the potential biological functions of these genes.

The intron pattern is also related to the phylogenetic classification (Fig. 1; Fig. 4). Fifteen intron-free genes were classified into two subclasses. Bol018140, Bol031036 and Bol010168 all contained only one intron and showed a very close relationship. The same phenomenon occurred between Bol035751 and Bol036047. Taken together, the distribution patterns of the introns in cytokinin genes with similar relationships were roughly the same.

To further reveal the functional diversification of cytokinin genes during their evolution, 10 conserved motifs were detected in the 36 cytokinin genes (Fig. 5). Among the 36 cytokinin proteins, motifs 1 and 3 existed in almost all of them, except for Bol024927, which exhibited no motifs. Bol005172 contained motifs 4, 1, 3 and 6. Bol022730 contained motifs 8, 1 and 3. Bol043933 contained motifs 4, 1 and 3. Bol006929, Bol020547 and Bol045724 all contained motifs 1, 3, and 6. Bol009917, Bol010168, Bol014258, Bol018140, Bol027725, Bol031036, Bol033608, Bol035751, Bol036074 and Bol037999 only contained motifs 3 and 6. Bol027388 contained the same motifs as Bol024952 except for motif 9. Motifs 4, 8, 1, 3, 9, 5, 10, 7, 6, and 2 were present in half of the family members, which may indicate similar functions of these proteins.

**Cis-elements in the promoters of B. oleracea cytokinin genes**
To further elucidate the regulatory mechanisms of *B. oleracea* cytokinin genes in response to clubroot disease, the promoter sequences were submitted to the PlantCARE database to identify *cis*-elements. Seventeen types of *cis*-acting regulatory elements were detected (Fig. 6). All 36 cytokinin genes contained 2-16 *cis*-elements related to light-responsiveness. Twelve cytokinin genes contained defense and stress-response-related *cis*-elements. MeJA-responsiveness *cis*-elements were detected in 28 chitinase genes, while *cis*-elements related to endosperm expression and anoxia-specific inducibility were only detected in five and one cytokinin genes, respectively. Anaerobic induction *cis*-elements was detected in 28 cytokinin genes, as were drought induction *cis*-elements. The numbers of *cis*-elements related to circadian control, flavonoid biosynthesis and seed-specific regulation were relatively small, being detected in 2, 2 and 3 cytokinin genes, respectively.

**Expression patterns of cytokinin genes in different organs and treatments**

The RNA-Seq dataset (GSE42891) was examined to determine the expression levels of cytokinin genes in the leaves, stem, flowers, siliques, buds, calli and roots of *B. oleracea*. Most of the cytokinin genes exhibited different expression patterns (Fig. 7a). Fifteen were expressed in all organs, while the expression of two was not detected. Some genes were expressed in only one or two organs. For example, *Bol024949, Bol024952, Bol028359* and *Bol028360* were only expressed in siliques, whereas *Bol022730* and *Bol036628* were expressed in the flowers and buds. The various expression patterns suggested a broad range of biological functions of the cytokinin genes during the growth and development of *B. oleracea*.

To explore the relationship between cytokinin and clubroot disease, we tried to inoculate two cabbage materials, Xiangan 336 (resistant) and Jingfeng No. 1
and collected 8 different kinds of samples (Fig. 7b) for RNA-seq. The expression patterns of most cytokinin genes under the eight different treatments were quite different (Fig. 7b). The expression levels of several genes (Bol024952, Bol024949, Bol009917, Bol028359, Bol036628 and Bol036948) were almost the same in all treatments. Five (Bol014258, Bol027725, Bol022730, Bol036074 and Bol006388) and six (Bol020547, Bol027390, Bol018140, Bol006929, Bol024927 and Bol028392) genes were significantly up- and downregulated, respectively, in J7I compared with J7C. Similarly, three (Bol020547, Bol006383 and Bol005172) and two (Bol022730 and Bol043933) genes were significantly up- and downregulated, respectively, in X7I compared with X7C. Moreover, three (Bol014258, Bol024330 and Bol028363) and four (Bol018140, Bol010168, Bol037842 and Bol045724) genes were significantly up- and downregulated, respectively, in J28I compared to J28C, whereas the number of up- and downregulated genes in X28I compared to X28C were five (Bol014258, Bol028392, Bol028361, Bol028363 and Bol045724) and five (Bol027725, Bol018140, Bol027388, Bol028360 and Bol024927), respectively. In addition, some genes showed different expression levels in different inoculation periods in the same material. For example, Bol027725, Bol022730 and Bol036074 exhibited high expression levels in J7I but low levels in J28I. In contrast, Bol028363, Bol011927 and Bol006385 exhibited low expression levels in X7I but high levels in X28I.

Overall, after inoculation, the expression of Bol027725, Bol036074 and Bol006388 in the susceptible material were upregulated, and the expression of these genes in the resistant material was maintained at a low level. However, the expression patterns of Bol006383 and Bol005172 were exactly the opposite. The expression of Bol027390, Bol006929, Bol024927 and Bol028392 in the susceptible material was
downregulated after inoculation, whereas the expression levels of these genes in the resistant material remained unchanged. In contrast, Bol043933 showed an opposite expression pattern to the above two genes. In addition, the expression patterns of Bol020547 and Bol045724 were completely opposite that of Bol022730, which was upregulated in susceptible material after inoculation but downregulated in resistant material. Generally, the expression patterns of cytokinin genes between Jingfeng No.1 and Xiangan 336 were quite different under the same treatment, possibly because of the difference in resistance between them.

To further examine these findings, quantitative reverse-transcription PCR (qRT-PCR) was performed to analyze the expression patterns of eight cytokinin genes under different treatments. The results showed that the expression levels of the eight genes were basically consistent with those in the heat map (Fig. 7b; Fig. 8). Generally, the expression levels of Bol028363 and Bol006383 were relatively low in each treatment. Bol011927 and Bol005172 showed similar expression patterns because of their higher expression levels in X28C and X28I and low expression levels in J28C and J28I. Overall, the expression levels of the 8 genes in X7I were relatively low.

Discussion

Cytokinins are closely related to various physiological activities in plants such as cell division, nutrient transfer and leaf senescence (Rioukhamlichi et al. 1999; Matsumotokitano et al. 2008; Amzallag et al. 1992; Merewitz et al. 2010). Additionally, they play a very important role in the plant responses to different types of adversity, including drought stress, heat stress, pathogen infection and pest infestation (Rivero et al. 2007; Liu et al. 2002; Babosha et al. 2009; Smigocki
et al. 1995). However, the expression patterns cytokinin genes in response to *P. brassicae* have remained uncertain until now. In this study, we mainly analyzed the functions of the cytokinin gene family in the development of clubroot disease in *B. oleracea*. The members of cytokinin gene family detected in this study adopt an alpha+beta sandwich structure, with an antiparallel beta-sheet, in a ferredoxin-like fold. These structures are predominantly found in plant cytokinin dehydrogenase 1 proteins and are capable of binding both FAD and cytokinin substrates (Malito et al. 2004). To further understand this gene family, 36 genes were detected, and we then analyzed their colinearity, structures, chromosomal locations, *cis*-elements and expression patterns under different treatments. This research provides comprehensive information for a better understanding of the cytokinin gene family in *B. oleracea*.

Genes with few or no introns are thought to be expressed quickly in plants (Jeffares et al. 2008). Long introns can prolong the gene transcription time, so that genes with fewer introns will react more rapidly to biotic and abiotic stresses as well as the invasion of pathogens. Some stress-related gene families such as the *Hsp20* family (Peng et al. 2018), the leucine-rich repeat family (Zhou et al. 2016) and the GRF family (Sang et al. 2018) also contain few introns. Among the genes examined in this study, 15 genes were intronless, and 5 genes contained only 1 intron, potentially supporting the above hypothesis. In other words, these genes will be able to respond faster when plants are invaded by *P. brassicae*.

Many plants increase their resistance to pathogens by activating the signaling pathways of SA and JA (Cyclic et al. 2015; Xie et al. 2011). Furthermore, cytokinins can interact with SA and JA to strengthen plant defense responses (Sano et al. 1994; Choi et al. 2010; Choi et al. 2011;). Exogenous application of JA or SA can
significantly contribute to resistance to the biotrophic clubroot agent \textit{Plasmodiophora brassicae} in \textit{Arabidopsis}, broccoli and pak-choi (Séverine et al. 2015; Lovelock et al. 2013; Zhu et al. 2017). In this study, MeJA-responsive \textit{cis}-elements were detected in 24 genes, while salicylic acid-responsive \textit{cis}-elements were detected in 10 genes, which may indicate their different effects in the response to clubroot. Promoters control gene activity by binding to transcription factors. In this study, many types of \textit{cis}-elements, such as stress-, hormone- and metabolism-related \textit{cis}-acting regulatory elements were detected in the promoters of \textit{B. oleracea} cytokinin genes (Fig. 6), possibly reflecting an interconnected induction mechanism involving transcription factors.

Gene duplication is a potential driver of biological evolution (Epstein et al. 1971). One of the characteristics of the eukaryotic genome is the existence of multiple gene families, which are groups of genes produced via the duplication and mutation of an ancestral gene. Interchromosomal replication is the main origin of gene duplication in eukaryotic genomes (Friedman et al. 2001). Three whole-genome duplications that have occurred in Arabidopsis over the past 350 million years have provided an opportunity for the large-scale expansion of certain classes of genes (Maere et al. 2005; Wang et al. 2015). In this study, we found that each homologous gene in Arabidopsis presented several copies in the \textit{B. oleracea} genome; for example, AT1G26380.1 presented 3 copies, and AT5G44400.1 presented 5 copies (Fig. 3; Additional file 2: Table S3). This may suggest that the cytokinin genes were replicated during the evolution of \textit{B. oleracea}, resulting in more diverse functions. Conserved genes are more likely to give rise to functional and persistent duplicates, thus contributing more to their long-term survival ability in eukaryotic genomes. Moreover, genes with many \textit{cis}-regulatory regions, which are expressed in numerous
tissues or encode multidomain proteins, will be preferentially preserved (Davis et al. 2004). The promoter region of each cytokinin gene examined in this study contained a large number of cis-elements, which may indicate that they have more diverse functions during gene evolution and thus have a greater chance to be retained.

According to the RNA-seq data of *B. oleracea*, a certain number of cytokinin genes, such as Bol037842, Bol045724 and Bol036074 exhibited incongruous expression patterns in various tissues, indicating that different cytokinin proteins may have diverse functions. Three genes, Bol028363, Bol031036 and Bol018140, were relatively highly expressed in all of the investigated tissues under normal conditions, showing the expression characteristics of housekeeping genes (Lopes et al. 2013). Various expression patterns were observed under the different treatments in the two cultivars (Jinfeng No.1 and Xiangan 336). The invasion of *P. brassicae* can promote the division of root cells in susceptible plants and eventually lead to swelling of the roots (Siemens et al. 2006). Thus, the cytokinin-related genes may be activated by the invasion of *P. brassicae*. Bol1027725, Bol022730 and Bol036074 may be play an important role in the process of cell division after inoculation because they were upregulated in J7I compared to J7C. Bol014258 and Bol024330 showed the same expression characteristics between J28I and J28C; however, the opposite expression pattern was found for Bol018140, Bol027388, Bol033608, and Bol006389, which may indicate the different roles of these genes. Additionally, Bol024952, Bol1009917 and Bol1036948 were considered to have no effect on the response to pathogen invasion because they exhibited the same expression pattern under each treatment.

*P. brassicae* infects the root hairs of *oleracea* at 7 dai and the cortex cells at 28 dai
(Ning et al. 2018). Therefore, genes with significant differential expression between Jinfeng No.1 and Xiangan 336 after inoculation may be crucial genes for the plant response to invasion by *P. brassicae*. *Bol022730* was upregulated in Jinfeng No.1 after inoculation but downregulated in Xiangan 336, possibly because its expression is inhibited by *P. brassicae* in resistant materials. In contrast, *Bol020547* and *Bol045724* were downregulated in Jinfeng No.1 after inoculation but upregulated in Xiangan 336. This may mean that these two genes play a key role in responding to infection by *P. brassicae*. Further analysis showed that the promoter regions of the two genes contained MeJA-responsiveness-related *cis*-elements, and *Bol020547* also contained a salicylic acid responsiveness-related *cis*-element. Therefore, *Bol020547* and *Bol045724* may be key genes in the *B. oleracea* response to clubroot disease, and their specific functions need to be further studied.

**Conclusions**

Here, a genome-wide analysis of *B. oleracea* cytokinin gene family was performed, and 36 cytokinin genes were confirmed. Subsequently, analyses of cytokinin genes on gene structures, phylogeny, chromosomal location, gene duplication and gene expression patterns were conducted based on bioinformatics and qRT-PCR methods. Five and eight genes were up- and downregulated, respectively, in the susceptible material after inoculation. In addition, two and one genes were up- and downregulated, respectively, in resistant material. This may indicating that cytokinin genes play important roles in the interaction between *B. oleracea* and *P. brassicae*. The study provides comprehensive information on the cytokinin gene family in *B. oleracea* and will aid in determining the cytokinin gene functions.
Methods

Identification of cytokinin family genes in *Brassica oleracea*

The cabbage whole-genome protein sequences were downloaded from the *Brassica oleracea* Genomics Database (www.ocri-genomics.org/bolbase/blast/blast.html). The HMM profile of the *Cytokin-bind* domain was first downloaded from the Pfam (http://www.sanger.ac.uk/Software/Pfam/) database (Pfam: PF09265). The CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.xfam.org/) databases were used to analyze the conserved domains.

Phylogenetic analyses of cytokinin genes

The amino acid sequences of cytokinin proteins derived from *B. oleracea*, *Arabidopsis*, and *B. rapa* were used for phylogenetic analysis. An unrooted neighbor-joining phylogenetic tree was constructed using MEGA 6.0 software (Tamura et al. 2013) with 1000 bootstrap test replicates.

Chromosomal location of cytokinin genes

All identified cytokinin genes were mapped to *B. oleracea* chromosomes using Mapdraw V2.1 (Liu and Meng 2003) based on the information available from the *B. oleracea* genome database (http://plants.ensembl.org/Brassica_oleracea/Info/Index).

Collinearity analysis of cytokinin genes

The microsyntenic relationships of the cytokinin genes in *B. oleracea* and *Arabidopsis thaliana* were detected through BLAST searches of these genes against the whole genomes of these crops. The physical locations of the cytokinin genes on each chromosome were obtained from the respective databases. Then, TBtools was used to visualize the relationships between the two crop cultivars.
Gene structure and conserved motif analyses of cytokinin genes

The exon-intron structures of *B. oleracea* cytokinin family genes were analyzed at the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/index.php) (Guo et al. 2007). The NCBI-CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchlerbauer et al. 2015) and the MEME program (http://meme-suite.org/index.html) (Machanick et al. 2011) were used to identify protein sequences and the conserved motifs, respectively.

Analysis of cis-acting elements in cytokinin genes

The upstream sequences (1.5 kb) of the initiation codons (ATG) of each cytokinin gene were submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Rombauts et al. 1999) to study cis-acting elements in the promoters of the cytokinin genes.

Plant materials and treatments

The *P. brassicae* used in this study was collected from Changyang, Hubei Province, China, and was identified as belonging to race 4 (Ning et al. 2018) based on the differential sets of Williams (1966). A resting spore inoculum of $2 \times 10^8$ spores/mL was prepared before inoculation. Two commercial cabbage cultivars, Xiangan 336 and Jinfeng No. 1, which were resistant and susceptible to *Plasmodiophora brassicae*, respectively, were bought from the market. Then they were sown in plastic cups (25 cm × 10 cm) containing sterile nursery medium. *P. brassicae* inoculation was carried out when the seedlings had produced two real leaves by injecting 2 mL of the resting spore suspension at the roots using a transferpettotor. Two kinds of treatment were performed for each cultivars. A treatment without inoculation was set as the control. Every treatment included 24 seedlings, with 8 seedlings consisting of one biological replicate. Then, the plants were transferred to
a culture room with a 16 h photoperiod and 19-25°C temperature. For transcriptional analysis of the cytokinin genes after *P. brassicae* inoculation, the roots from plants subjected to 8 treatments (J7C, J7I, X7C, X7I, J28C, J28I, X28C, X28I) were collected (24 individuals per treatment, 8 individuals per replicate). All of the samples were quickly frozen in liquid nitrogen and stored at −80°C until RNA extraction.

**Total RNA extraction, cDNA synthesis, and qRT-PCR analysis**

Total RNA was extracted from cabbage samples using TRIZol following the supplier's instructions (Transgen, Beijing, China). Then, the RNA quality was assessed by using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and 1% formaldehyde gel electrophoresis. The cDNA was reverse transcribed with the HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). The specific primers for cytokinin genes were designed with Premier 3.0, (Additional file 1: Table S2). qRT-PCR was carried out using ChamQ Universal SYBR qPCR Master Mix (2×) (Vazyme, Nanjing, China) in a Bio-Rad CFX96 Real Time PCR System. Each amplification reaction was conducted in a 20-μl reaction volume containing 10 μl KAPA SYBR, 0.4 μl of a 10 μM solution of each primer, 2 μl diluted cDNA and 7.2 μl ddH₂O. The PCR program was set as follows: 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. Melting curve analysis was performed from 65°C to 95°C with increments of 0.5°C every 5 s. Three independent biological and technical replicates were carried out for each reaction. The housekeeping gene actin was used as the internal reference gene.

**Expression analysis of cytokinin genes using RNA-seq data**

To assay cytokinin gene expression profiles, Illumina RNA-seq data from the 8 different treatments as well as various tissues, including the leaves, stems, flowers,
siliques, bud, calluses and roots, were downloaded from the NCBI (GSM1052958-964). FPKM values were used to calculate gene expression levels, and the positively expressed genes were evaluated according to a default empirical abundance threshold of 1 FPKM. The fragments per kb of exon per million mapped reads (FPKM) algorithm was used in this study to normalize the gene expression values. Heat maps of hierarchical clustering were constructed using MeV4.9 and TBtools (https://github.com/CJ-Chen/TBtools) software.

Abbreviations

qRT-PCR: Quantitative real-time polymerase chain reaction; dai: Days after inoculation; ET: Ethylene; JA: Jasmonic acid; SA: Salicylate; BR: Brassinosteroid; aa: amino acids; DEGs: Differentially expressed genes; PAMPs: Pathogen-associated molecular patterns; PR: Pathogenesis-related; HMM: Hidden markov model; CDD: Conserved domain database; FPKM: Fragments per kilobase of transcript per million mapped reads; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information

Declarations

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Availability of data and materials

Data of this study have been included in the article or as additional file.
Authors’ contributions

MZZ and YN collected the public dataset, perform bioinformatics analysis and also drafted the manuscript. LXY and WXC contributed to bioinformatics analysis and the making of all the figures and tables. CCK conceived this study and reviewed the manuscript. MZ, YYZ, HHL, ZYF, LMY, YW and XLH reviewed the manuscript. All of the authors read and approved the final manuscript.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Information on B. oleracea cytokinin genes
| Gene ID   | Chr | Genomic Location      | Gene Length (bp) | Protein Length (aa) | Predicted Localization         |
|----------|-----|-----------------------|------------------|---------------------|-------------------------------|
| Bol028359| C01 | 7442998-7444581       | 1584             | 527                 | Cytoplasm                     |
| Bol028360| C01 | 7449780-7451360       | 1581             | 526                 | Cytoplasm                     |
| Bol028361| C01 | 7453035-7454639       | 1605             | 534                 | Cytoplasm                     |
| Bol028363| C01 | 7482109-7483644       | 1536             | 511                 | Cytoplasm                     |
| Bol028392| C01 | 7729897-7731465       | 1569             | 522                 | Cytoplasm                     |
| Bol031036| C01 | 30380413-30382523     | 2111             | 548                 | Chloroplast/Cytoplasm         |
| Bol014258| C02 | 9886003-9888966       | 2964             | 518                 | Endoplasmic reticulum/Vacuole |
| Bol036074| C02 | 6024676-6027814       | 3139             | 517                 | Extracellular                 |
| Bol011927| C03 | 50037656-50039251     | 1596             | 531                 | Cytoplasm                     |
| Bol020547| C03 | 12074968-12077007     | 2040             | 545                 | Vacuole                       |
| Bol024927| C03 | 39976312-39978905     | 2594             | 594                 | Chloroplast/Vacuole           |
| Bol024949| C03 | 40218413-40219993     | 1581             | 526                 | Cytoplasm                     |
| Bol024952| C03 | 40274541-40276136     | 1596             | 531                 | Cytoplasm                     |
| Bol005172| C04 | 39333424-39335907     | 2484             | 412                 | Vacuole                       |
| Bol027388| C04 | 21332565-21335127     | 2563             | 505                 | Cytoplasm                     |
| Bol027390| C04 | 21363848-21365476     | 1629             | 542                 | Cytoplasm                     |
| Bol037842| C04 | 35866576-35869681     | 3106             | 532                 | Cytoplasm                     |
| Bol018140| C05 | 22809900-22811690     | 1791             | 561                 | Chloroplast/Cytoplasm         |
| Bol036628| C05 | 25011639-25013231     | 1593             | 530                 | Cytoplasm                     |
| Bol037999| C05 | 4599782-4602880       | 3099             | 465                 | Endoplasmic reticulum/Vacuole |
| Bol010168| C06 | 25330129-25332003     | 1875             | 561                 | Chloroplast/Cytoplasm         |
| Bol027725| C06 | 2274860-2277663       | 2804             | 531                 | Extracellular                 |
| Bol006383| C07 | 27111633-27113243     | 1611             | 536                 | Cytoplasm                     |
| Bol006385| C07 | 27055734-27058578     | 2845             | 537                 | Cell membrane/Cytoplasm       |
| Bol006388| C07 | 27010262-27011857     | 1596             | 531                 | Cytoplasm                     |
| Bol006389| C07 | 27003157-27008056     | 4900             | 543                 | Cytoplasm                     |
| Bol022730| C07 | 7797363-7801639       | 4277             | 505                 | Endoplasmic reticulum         |
| Bol024330| C07 | 41865890-41867509     | 1620             | 539                 | Cytoplasm                     |
| Bol033608| C07 | 45245680-45249406     | 3727             | 524                 | Endoplasmic reticulum         |
| Bol036948| C07 | 40966319-40967965     | 1647             | 548                 | Cell membrane/Cytoplasm       |
| Bol031324| C08 | 38898546-38900162     | 1617             | 538                 | Cytoplasm                     |
| Bol045724| C08 | 33615188-33616880     | 1693             | 361                 | Vacuole                       |
| Bol009917| C09 | 24618802-24620803     | 2002             | 587                 | Cytoplasm                     |
| Bol035751| C09 | 29462698-29465742     | 3045             | 520                 | Extracellular                 |
| Bol043933| C09 | 38523554-38527278     | 3725             | 570                 | Chloroplast/Cytoplasm/Vacuole |
Figure 1

Phylogenetic tree of cytokinin genes from B. oleracea, A. thaliana and B. rapa. Th
Figure 2

Distribution of cytokinin genes on B. oleracea. chromosomes. The chromosome n.
Figure 3

Syntenic relationships of B. oleracea and A. thaliana cytokinin genes in chromoso
Figure 4

Gene structures of 36 cytokinin genes identified in B. oleracea. Exons are represented by green boxes, while introns are represented by gray lines.

Figure 5

Distribution of conserved motifs in cytokinin genes. Ten putative motifs are indicated in different colored boxes. The lengths of the motifs in each protein are shown proportionally.
Figure 6

Predicted cis-acting elements in the promoter regions of cytokinin genes.

Figure 7

Expression patterns of cytokinin genes analyzed by RNA-Seq. a Heatmap showing
Figure 8

Expression levels of 8 cytokinin genes analyzed by qRT-PCR.

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