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Research article

Response of NBS encoding resistance genes linked to both heat and fungal stress in *Brassica oleracea*

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ABSTRACT

Environmental stresses, including both abiotic and biotic stresses, cause considerable yield loss in crops and can significantly affect their development. Under field conditions, crops are exposed to a variety of concurrent stresses. Among abiotic and biotic stresses, heat and *Fusarium oxysporum*, are the most important factors affecting development and yield productivity of *Brassica oleracea*. Genes encoding the nucleotide-binding site (NBS) motif are known to be related to responses to abiotic and biotic stresses in many plants. Hence, this study was conducted to characterize the NBS encoding genes obtained from transcriptome profiles of two cabbage genotypes with contrasting responses to heat stress, and to test expression levels of selected NBS- leucine reich repeat (LRR) genes in *F. oxysporum* infected plants. We selected 80 up-regulated genes from a total of 264 loci, among which 17 were confirmed to be complete and incomplete members of the TIR-NBS-LRR (TNL) class families, and another identified as an NFYA-HAP2 family member. Expression analysis using qRT-PCR revealed that eight genes showed significant responses to heat shock treatment and *F. oxysporum* infection. Additionally, in the commercial *B. oleracea* cultivars with resistance to *F. oxysporum*, the *Bol007132*, *Bol016084*, and *Bol030522* genes showed dramatically higher expression in the *F. oxysporum* resistant line than in the intermediate and susceptible lines. The results of this study will facilitate the identification and the development of molecular markers based on multiple stress resistance genes related to heat and fungal stress under field conditions in *B. oleracea*.

1. Introduction

The adaptability and productivity of crops are limited by major abiotic stresses including heat, drought, chilling, waterlogging, and salinity. Although the type and severity of stress depends on the specific location, abiotic stresses can result in crop damage at levels as high as those caused by biotic stresses. Furthermore, crops suffering abiotic stress are usually more susceptible to bacteria, fungi and viral diseases (Dita et al., 2006). Many studies have identified responses of plants to combinations of stresses such as drought, salt, high temperature, heavy metals, high light, and biotic stresses (Mittler, 2006; Ahmed et al., 2013; Atkinson and Urwin, 2012; Narusaka et al., 2004). Among these, Angadi et al. (2000) suggested that high temperature stress has a direct effect on the reproductive development and yield of *Brassica* species (Angadi et al., 2000). In addition, (Björkman and Pearson, 1997) reported that high temperature causes unevenly-sized flower buds on broccoli (*Brassica oleracea* var. *italica* L.) inflorescences (Björkman and Pearson, 1997). Young et al. (2004) confirmed that high temperature stress has effects on fruit and seed production and induces flower abortion as well as parthenocarpic silique production in *Brassica napus* (Young et al., 2004). Furthermore, in many plants, the damage caused by high temperature stress, is mainly revealed by secondary stress such as severe infection and disease. High temperature conditions affect plant disease development by attenuating plant disease resistance while promoting pathogen growth. The phenotypes of several transgenic mutants of *Arabidopsis thaliana*, with misregulated R genes are suppressed by environmental cues (Fujita et al., 2006), suggesting the existence of crosstalk between R-gene mediated disease resistance responses and abiotic stress responses. In addition, the abundance of the *Hordeum vulgare* (barley) R proteins *MLA1* and *MLA6* decrease dramatically within several hours of a temperature shift from 18 °C to 37 °C, without reduction in *MLA1* and *MLA6* abundance (Bieri

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et al., 2004). Among biotic stresses, *Fusarium oxysporum* is a major constraint to growth of cabbage in most regions, with one of the most serious threats being *Fusarium wilt* disease related to high temperature (Caroline et al., 2009; Sharma et al., 2011). *F. oxysporum* is a common soil-borne fungal pathogen that causes damping-off and wilting of many economically important plants, including *Brassica* species, cotton, banana, tomato, melons, legumes, and commercial flowers, resulting in severe yield reductions (Caroline et al., 2009). The relationship between temperature and *F. oxysporum* has made it a useful model for investigations of host defense strategies against abiotic and biotic stress (Correll et al., 1994; Pu et al., 2012). As defense mechanisms against biotic stress, many plant species contain alleles of disease resistance R loci that encode proteins that undergo specific interaction with proteins encoded by the corresponding pathogen avirulence (avr) gene loci. The largest class of known R proteins includes those that contain nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains (Sharma et al., 2011; Dangl and Jones, 2001; Correll et al., 1994). Plant NBS-LRR proteins can be separated into two subgroups; TIR-NBS-LRR (TNL) proteins that contain a Toll-like domain, and the CC-NBS-LRR (CNL) proteins, which are characterized by a coiled-coil domain. Some other genes encode so-called partial or incomplete R proteins, classified as TIR-NBS (TN) and TIR-X (TX) (Gururania et al., 2012; Marone et al., 2013). TN proteins contain the TIR and NBS domains, but lack LRRs, whereas TX proteins lack both NBS and LRR domains, but contains the TIR domain and often have a small and variable C-terminal domain (Gururania et al., 2012). Despite lacking some domains, these partial R proteins appear to be active as defense proteins, as evidence by the fact that Arabidopis transgenic lines overexpressingTX and TN resistance to the necrotrophic fungal pathogen *F. oxysporum* (Nandety, 2011). Consequently, identification and localization of the NBS-encoding genes in plants is important to step in efforts to improve tolerance and resistance to abiotic and biotic stresses. However, although many studies have identified numerous NBS-encoding transcription factors activated in response to abiotic and biotic stress in plant species (Pu et al., 2012), no investigations of the effects of combinations of fungal infection and high temperature on *B. oleracea* have been conducted to date. Cabbage (*B. oleracea*) is one of most widely cultivated vegetable crops in the world, and the availability of genome data make it useful for comparative genomic studies to predict gene functions. Here, we report (1) the identification and domain analysis of NBS-encoding genes in *B. oleracea* RNA-seq data under heat shock treatment, (2) assignment of linkage groups to the nine physical chromosomes of *B. oleracea*, (3) the NBS encoding gene expression level patterns of multiple genes in response to heat stress and *F. oxysporum* based on RT-qPCR analysis and (4) the ability to distinguish *F. oxysporum* resistant, intermediate, and susceptible lines in commercial *B. oleracea* cultivars using RT-qPCR.

2. Materials and methods

2.1. RNA sequencing

Two inbred lines of *B. oleracea* 'ASC621', Bo1 (high temperature resistant, HR) and Bo2 (high temperature susceptible, HS), were subjected to heat shock treatment at 45 °C for 0, 0.5, 1, 2, 3, or 4 h, after which total RNA was extracted from the 3rd and 4th leaf of Bo1 and the 2nd and 3rd leaf of Bo2. Briefly, leaf samples were separately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was then isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quality of the RNA was determined with a 2100 Bioanalyzer RNA nanochip (Agilent Technologies, Santa Clara, CA, USA). To create unigene sequence of cabbage, short read sequencing data were obtained using the Illumina HiSeq 2000 platform (101 bp paired end reads; Seeders, Republic of Korea). Raw reads of more than 20 bp ($Q \geq 20$) were subjected to quality control and trimming using the Solexa QA package. All of the sequencing reads from the different tissue samples were used to optimize the *de novo* assembly using the software tools Velvet (v1.2.07) and Oases (v.0.2.08) based on the de Bruijn graph algorithm. Several hash lengths were taken into consideration to select the best *de novo* assembly. Transcripts of unigenes assembled from the total reads of the mRNA samples of each tissue were validated by direct comparison with gene sequences in the Phytozome15 (http://www.phytozome.net/) using BLASTx against the non-redundant (nr) database (e-value cutoff of 1e-10). Reads for each sequence tag were mapped to the assembled unigenes using the mapping software tool Bowtie (version 0.12.7; http://bowtie-bio.source-forge.net/index.html). Moreover, the number of mapped clean reads for each unigene was counted and normalized using the DESeq package in R (Anders and Huber, 2010). Only those loci with tag counts of 50 or above in all experimental samples were retained for further analysis. Fold change and *t*-Test analyses were used to identify differentially expressed genes between each sample. The FDR (false discovery rate) was applied to identify the threshold of the *p*-value in multiple tests and analyses, and this value was calculated *via* DESeq. All correlation analyses and hierarchical clustering were performed using the AMAP library in R. Functional enrichment analysis was carried out *via* DAVID, which is a web-accessible set programs providing comprehensive functional annotation tools for investigators to understand biological meaning behind a large list of genes (Dennis et al., 2003). The gene lists by annotated TAIR ID of transcripts of up- and down-regulated DEG were analyzed with default criteria (counts ≥ 2 and EASE score ≤ 0.1) for each GO term and KEGG pathway. The contigs were subsequently annotated based on *Arabidopsis* protein (TAIR10), *Brassica rapa* (http://brassicadb.org/brad/), and *B. oleracea* (http://www.ocri-genomics.org/bolbase/) data (Yu et al., 2013; Cheng et al., 2011). All RNA Seq raw data from this study were deposited in the Omics database of NABIC (National Agricultural Biotechnology Information Center) (http://nabic.rda.go.kr).

2.2. Annotation of B. oleracea putative NBS-encoding genes

A total of 264 loci were annotated as putative TNL family genes. The lists of annotated TAIR IDs of transcripts of up and down-regulated differentially expressed genes were analyzed using the default criteria. A search of the *B. oleracea* genome database was conducted to identify all members of the TNL family. The genomic DNA database for *B. rapa* subsp. *pekinensis* was downloaded from the *Brassica* database website (http://brassicadb.org/brad/) (Cheng et al., 2011, 2012; Wanget al, 2011). The sequences of all NBS-encoding genes in the genomes of the other species assessed were downloaded from the plant TFDB database (http://planttfdb.cbi.edu.cn/) (Zhang et al., 2011) and the amino acid sequences of one or the most representative members (i.e. the maximum number of different conserved motifs of each group) for each group defined by Nakano et al. (2006) were used as queries to search the Bolbase database (Nakano et al., 2006). Every sequence identified was subsequently checked against the *Arabidopsis* and *B. rapa* databases to confirm that they belonged to the TNL family. As a final quality check, the presence of the TNL domain was confirmed in every *B. oleracea* candidate TNL gene using SMART (Letunic et al., 2012). Subsequently, the protein domains were analyzed using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq) and myHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Each subfamily motif was identified using the MEME program (Bailey