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Development and Application of a PCR-Based Molecular Marker for the Identification of High Temperature Tolerant Cabbage (*Brassica oleracea* var. *capitata*) Genotypes

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Abstract: Global warming accelerates the development of high temperature (HT)- and high humidity (HH)-tolerant varieties. This is further facilitated by the identification of HTHH-tolerant genes and the development of molecular markers based on these genes. To identify genes involved in HTHH tolerance in cabbage (*Brassica oleracea* var. *capitata*), we performed RNA-seq analysis of two inbred lines, BN1 (HTHH-tolerant) and BN2 (HTHH-susceptible), and selected trehalose 6- phosphate phosphatase I-2 (*BoTPPI-2*) as one of the HTHH-tolerant-associated genes. We also developed a segregating F_2 population from a cross between BN1 and BN2. RNA-seq results showed that *BoTPPI-2* transcript levels were high in the HTHH-tolerant inbred line BN1, but not detectable in the HTHH-susceptible inbred line BN2. The expression pattern of *BoTPPI-2* was not related to the expression of heat shock-related genes. Soft rot resistance, used as an indicator of HTHH tolerance, was higher in BN1 than in BN2. F_2 individuals similar to BN1 with respect to phenotype appeared to be HTHH-tolerant, whereas BN2-types were susceptible to HTHH. Analysis of the genomic DNA revealed the presence of a long terminal repeat (LTR; ca. 4.6 kb) in the ninth intron of the *BoTPPI-2_BN2* allele, thereby suppressing its transcription and exhibiting HTHH phenotype. Except for the LTR insertion, the sequence of *BoTPPI-2_BN2* was almost identical to that of *BoTPPI-2_BN1*. On the basis of the LTR and *BoTPPI-2* sequences, we developed a molecular marker to identify HTHH-tolerant genotypes and validated its efficiency using F_2 individuals, inbred lines, and cultivars from diverse sources. The marker explained the genetic basis of HTHH tolerance in at least 80%, but not 100%, of the cabbage genotypes. Thus, additional markers associated with HTHH tolerance are needed for perfect selection.

Keywords: heat tolerance; trehalose-6-phosphate phosphatase *I (TPPI)*; molecular marker; *Brassica oleracea* var. *capitata*; long terminal repeat (LTR)

1. Introduction

Global warming is one of the most detrimental environmental factors affecting agriculture, as it affects plant growth and reproduction, leading to significant losses in crop productivity [1].

Exposure to heat stress, including transitory or constant high temperature (HT), causes changes in plant morphology, physiology, and biochemistry, involving a re-organization of cell structure and metabolism, and alterations in the accumulation of several proteins and primary and secondary metabolites [2–6]. Because HT stress induces the production of reactive oxygen species (ROS), which cause extensive damage in plants, HT tolerant plants have evolved ROS detoxification systems that include enzymatic and non-enzymatic antioxidants [4,7,8]. To cope with heat stress, plants rapidly (within 10 min) induce the expression of heat stress-responsive genes (*HSRs*) through various signaling pathways, such as the phytochrome B- and phytochrome-interacting factor 4 (PIF4)-dependent pathway [9,10], cyclic nucleotide-gated channels (CNGC)-calcium-calmodulin pathway [11–13], proteolytic cleavage-activated transcriptional activation pathway [14,15], ROS-induced pathway [4,16], and H2A.Z-related pathway [17]. Two different groups of transcription factors, including HEAT SHOCK FACTOR A1s (HSFA1s) and circadian clock proteins REVEILLE4/8 (RVE4/8), mediate early *HSR* expression through HSF-dependent and -independent pathways, respectively [18].

In the open field, biotic and abiotic stresses are unpredictable, and several stresses may occur at the same time. HT is usually accompanied by high humidity (HH). HT stress causes abnormal plant phenotypes including sterility, and these effects are intensified by HH [19,20]. Compared with the thermosensitive species *Arabidopsis thaliana*, the thermotolerant plant species *Portulaca oleracea* produces antioxidant proteins under HT and HH (HTHH) conditions, thereby reducing the accumulation of ROS [21]. Soybean (*Glycine max*) matrix metalloproteinase (Gm1-MMP) confers HTHH stress tolerance by lowering ROS level in transgenic *Arabidopsis* plants [22]. In pepper (*Capsicum annuum*), HTHH induces the expression of *HSFB2a*, which directly regulates *CaWRKY40* expression, thus imparting tolerance to HTHH conditions [23]. The pepper *HAESA-LIKE 1* (*CaHSL1*) gene, which encodes an HSL receptor-like protein kinase (RLK), is activated by CaWRKY40 or other HSFs, thus conferring thermotolerance under the HH condition by inducing the expression of genes encoding heat shock proteins (HSPs) [24]. In *Brassica oleracea*, an important vegetable crop, plant growth and development is negatively impacted by both HT and low temperature (LT) compared with 20 °C [25,26]. A number of genes responsive to short-term (2–4 h) HT conditions have been identified in cabbage (*B. oleracea* var. *capitata*), and several genes including *BoHSP70* have been selected as candidates for the selection of HT-tolerant cabbage lines [27]. Alternative splicing of heat shock-related genes, such as *HsfA2* and *HsfB2a*, is important for heat stress adaptation in cabbage [28]. The HSP/chaperone network is also suggested as a major component of multiple stress responses [29]. To develop cabbage varieties resilient to global climate change and to expand their cultivation area into tropical and/or subtropical regions, identification of genes and development of molecular markers associated with HTHH tolerance is critical.

Trehalose (TRE) is a non-reducing disaccharide present in a wide variety of organisms, including bacteria, fungi, invertebrates, and plants. In these organisms, TRE serves as an energy source as well as an osmolyte or a protein/membrane protectant against various stresses [30,31]. TRE and its intermediate also act as signaling molecules in metabolic pathways important for growth [30,32]. Because TRE is resistant to acid hydrolysis and is stable in solution at HT, it may confer membrane and molecule stabilization and dehydration resistance [33]. TRE effectively stabilizes dehydrated enzymes, proteins, and lipid membranes, resulting in the promotion of cellular integrity and protection of biological structures against a variety of environmental stresses, such as desiccation, heat, and cold, among other factors [33–35]. TRE also acts as a potential signaling metabolite for mediating interactions of plants with pathogenic or symbiotic microorganisms and herbivorous insects. In plants, TRE is involved in tolerance to abiotic stresses, such as high salinity [36], HT [37], nitrogen deficiency [38], and drought [39,40], and biotic stresses such as microorganisms [41] (reviewed in Fernandez et al., 2010 [33]).

TRE biosynthesis in plants involves a two-step reaction: first, trehalose-6-phosphate (T6P) is synthesized from uridine diphosphate glucose (UDP-glucose) and glucose-6-phosphate by T6P synthase (TPS), and then T6P is dephosphorylated to produce the disaccharide TRE by T6P phosphatase

(TPP) [42]. Genomes of *Arabidopsis*, rice (*Oryza sativa*), and other plant species harbor multiple *TPS* and *TPP* genes [33,43,44], which are differentially regulated in response to a variety of abiotic stresses [32]. In *Arabidopsis*, 11 genes encode proteins with both TPS- and TPP-like domains, but only one of these (AtTPS1) appears to be active. On the other hand, 10 *TPP* genes (*AtTPPA–J*) regulate the level of T6P for plant growth and development [45]. *AtTPPI* is largely expressed in leaf primordia, meristem zone, and roots, but not in flowers [45]. In plants, transcript levels of *TPP* genes are relatively low but are transiently increased by abiotic stresses and abscisic acid (ABA) treatment [46,47], thus enhancing the tolerance to salt and cold stresses. An increase in TRE levels by overexpression of a bifunctional *TPS-TPP Escherichia coli* gene confers drought, cold, salt, and HT tolerance in rice [48] and *Arabidopsis* [49]. TRE accumulates to high levels in microorganisms at HT and functions in both osmo- and thermoprotection [50]. *AtTPPI* expression is strongly induced by cold and wounding, weakly induced by salt and drought, and repressed by heat and ultraviolet B (UV-B) [32]. Increase in TRE levels in transgenic plants expressing microbial genes enhances their drought, salt, and cold tolerance [48,49,51,52]. The activity of TPP enzymes in *Candida utilis* is enhanced by methylation, leading to HT resistance [53]. However, no experimental evidence supporting the role of TRE in abiotic stress tolerance is available in *Brassica* species.

Retrotransposons are widely distributed in the plant kingdom and constitute a large portion of plant genomes [54]. For example, the *B. oleracea* genome contains 60–570 copies of copia-like retrotransposons [55]. Retrotransposons alter gene expression by inserting themselves within or near transcriptionally active regions [56,57]. Depending on the location of retrotransposon insertion (gene coding sequence or promoter region), the event causes gain- or loss-of-function mutation. For example, in grape (*Vitis vinifera*), insertion of the grapevine retrotransposon 1 (*Gret1*) in the upstream region of *VvMYBa1* results in white-skinned grapes [58]. In rice, insertion of Renovator in the promoter region of the blast resistance gene *Pit* leads to its activation [59]. In cauliflower (*B. oleracea* var. *botrytis*), insertion of a copia-like LTR retrotransposon in exon 3 of the *Orange* (*Or*) gene results in three alternatively spliced *or* transcripts, thereby causing the accumulation of β-carotene to high levels (production of orange curd) [60]. Insertion of LTR elements frequently exhibits *cis*-regulatory activities [61] because transcription start sites are usually found in LTR regions [57,62] and non-LTR regions [63].

From the point of view of cabbage breeders, HTHH-tolerant cabbage lines must exhibit normal phenotypic traits, such as normal head formation and soft rot resistance under HTHH conditions. Contrary to the HTHH-tolerant lines, the HTHH-susceptible lines exhibit early maturity, soft tissues, no head formation, and soft rot susceptibility under HTHH conditions. In this study, we carried out RNA-seq analyses of two cabbage inbred lines with contrasting characteristics, BN1 (HTHH-tolerant) and BN2 (HTHH-susceptible). Because previous studies showed that plant heat shock tolerance is largely due to constitutive expression of many genes prior to stress treatment [3,64], we selected the trehalose -6- phosphate phosphatase I-2 (*BoTPPI-2*) gene as a candidate to develop HTHH-tolerant marker because its basal level of expression was higher in BN1 than in BN2. Genomic DNA sequence analysis showed that the *BoTPPI-2* sequence in BN2 contains a 4.6 kb LTR retrotransposon inserted in its ninth intron. This insertion might repress the expression of the *BoTPPI-2_BN2* allele. On the basis of this difference in the genomic DNA sequence of *BoTPPI-2* between the two inbred lines, we developed a molecular marker to discriminate between HTHH-susceptible and tolerant lines, and validated this marker using the F_2 progeny of BN1 × BN2 cross and a large number of inbred lines and cultivars.

2. Materials and Methods

2.1. Plant Materials

Seeds of HTHH-tolerant and -susceptible inbred lines of cabbage (*Brassica oleracea* L. var. *capitata*), BN1 and BN2, respectively, and F_1 and F_2 progeny of BN1 × BN2 cross were produced at the Biotechnology and Breeding Institute of Asia Seed Co., Icheon-si, Gyeonggi-do, Republic of Korea. Seeds of other cultivars or inbred lines were either provided by the Golden Seed Project (GSP)

Horticultural Center, Suncheon National University, or purchased from Korean breeding companies including Joen Seeds Co., Goesan-gun, Chungbuk, Korea. Leaf samples of some cabbage lines were supplied by Korean seed companies, including Joen Seed Co. Seeds, and were germinated and grown in a growth chamber at 22 °C under a 16 h light/8 h dark photoperiod and 140 μmol m^{-2} s^{-1} photon flux density. Three-week-old cabbage seedlings were treated with short-term heat shock treatment (45 °C for 1 h). To conduct the HTHH treatment, seedlings were grown in a greenhouse under conditions indicated in Figure 1. Leaf samples were harvested, ground in liquid nitrogen to a fine powder, and used for DNA or RNA extraction. Cabbage genotypes used in this study are summarized in Table S1.

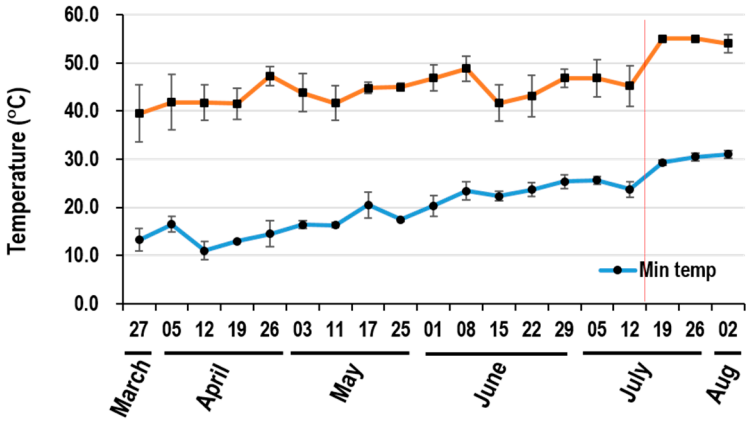


Figure 1. Temperature fluctuations in the glass greenhouse during the cabbage growth period. One-month-old seedlings were grown in a culture room at 22 °C, and then transplanted in pots (20 cm height × 20 cm diameter) on 27 March 2018 and maintained in the greenhouse until August 2, with frequent watering to maintain high humidity. Min temp, weekly average minimum temperature; max temp, weekly average maximum temperature.

To analyze the genetic basis of HTHH tolerance, seeds of parental lines (BN1 and BN2), F_1 hybrids, and segregating F_2 progeny were sown in a tray containing 50 holes (4.5 × 4.5 cm) on 27 March 2018. Seedlings were grown in a glass greenhouse until the end of April 2018. Then, each of the 10 BN1, 10 BN2, 10 F_1, and 116 F_2 seedlings was transplanted into a pot (20 cm height × 20 cm diameter) and grown until 2 August 2018 with frequent watering to maintain HH conditions. HYPONEX (N/P/K = 20:20:20) fertilizer (Hyponex, Osaka, Japan) was directly applied to the soil once a week. Temperature during the growing period is summarized in Figure 1.

2.2. RNA-Seq Analysis

Two inbred lines of cabbages, BN1 and BN, treated with short-term heat shock were used in RNA sequencing. The total RNA was extracted from cabbage leaves using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Total RNA (20 μg) from each sample, BN1_22 °C and BN2_22 °C (control) and BN1_45 °C and BN2_45 °C (treated), were used for Illumina sequencing (33 G 101 bp paired-end reads; Seeders, Daejeon, Korea). Transcripts of unigenes assembled from the total reads were validated by direct comparison with gene sequences in the Phytozome 15 (https://phytozome.jgi.doe.gov/pz/portal.html) using BLASTx (threshold e-value ≤ 1e $^{-10}$). The number of mapped clean reads for each unigene was counted and normalized using the DESeq package in R on two independent biological replicates. The reads per kilobase million (RPKM) were used to represent the expression level of unigenes.

2.3. Genomic DNA Extraction and Cloning and Sequence Analysis of BoTPPI Genes

To identify cabbage homologs of the *AtTPPI* gene (AT5G10100), two *B. oleracea* databases were searched: BRAD (http://brassicadb.org/brad/) for *B. oleracea* var. *capitata* and Ensembl Plants (http://plants.ensembl.org/Brassica_oleracea/Info/Index) for *B. oleracea* var. *oleracea*. Initially, no

B. oleracea homolog of *AtTPPI* was detected in BRAD; however, using the sequences identified in Ensembl Plants, two *B. oleracea* genes were identified in BRAD: Bol008761 (Bo3g005430) and Bol043695 (Bo9g173440). These were renamed as *BoTPPI-1* and *BoTPPI-2*, respectively, according to their sequence similarity with *AtTPPI*.

Genomic DNA was isolated from leaf samples of BN1 and BN2 using the DNeasy Plant Mini Kit (QIAGEN GmbH, Germany). To amplify the *BoTPPI-1* and *BoTPPI-2* genes, primers were designed on the basis of their sequences in *B. oleracea* and other *Brassica* species, including *B. rapa* and *B. napus*. The *BoTPPI-1* gene was amplified using BoTPPI-1-F (5′-TGCGAGCTTCTTCTCCTACTCATCC-3′) and BoTPPI-1-R (5′-CCTCTTCACACWCYTGGCTGCA-3′), whereas *BoTPPI-2* was amplified using BoTPPI-2-F (5′-TCCTCATCTTCATCATCCACTCCATCTC-3′) and BoTPPI-2-R (5′-TCTACAACGTT CCATGAAAGGTTAGCCA-3′). PCR was performed using the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of amplification (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2–5 min), and a final extension at 72 °C for 7 min. PCR products were purified using the LaboPass Gel Extraction Kit (Cosmogenetech, Seoul, Korea) and cloned into the TA vector using the T&A Cloning Kit (RBC Bioscience Co., Taiwan). The resulting plasmids were transformed into *Escherichia coli* (DH5α) cells. Plasmid DNA was purified using DNA-Spin (Intron Biotech. Inc., Seongnam, Korea) and sequenced. Because cabbage plants may contain multiple *BoTPPI-1* or *-2* alleles, at least 10 clones of each cabbage line were sequenced and analyzed. Any possible PCR and/or sequencing errors were eliminated by aligning independent sequences.

2.4. Cloning BoTPPI-2 from BN2 by Inverse PCR (iPCR)

The *BoTPPI-1* gene (2015 bp) was cloned from both BN1 and BN2. However, primers listed in Table S2 could amplify the *BoTPPI-2* gene (2509 bp) only from BN1, not from BN2. To clone the *BoTPPI-2* gene from BN2, iPCR was performed on the basis of *BoTPPI-2* (Bo3g005430) gene sequence using Universal GenomeWalker 2.0 (Takara Bio USA, Mountain View, California, USA). The sequence for iPCR was composed of the 3′ region of the 1769 bp fragment, amplified from both BN1 and BN2 using the primer pair BoTPPI-2-F and BoTPPI-2-R3 (5′-TGAGCACTCACCAAGTGACTC-3′), and the 5′ region of the adjacent gene *Bo9g173430* (*Glutathione S-transferase DHAR3*) on chromosome 9 (http://plants.ensembl.org/Brassica_oleracea/Info/Index). On the basis of the sequence of the 1769 bp fragment, two forward primers specific to *BoTPPI-2* were designed: iPCR-F1 (5′-CGACAAATATACTTGTCGTGCTCTTCA-3′) and iPCR-F2 (5′-GTATGACATGACGTTCTCTCATTTGAC-3′).

The DNA extracted from line BN2 and control DNA from the kit were digested with *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I for 16–18 h at 37 °C. The digested DNAs were purified using the NucleoSpin Gel Kit and PCR Clean-Up Kit (Macherey-Nagel GmbH & Co., Düren, North Rhine-Westphalia, Germany), and then ligated to the GenomeWalker adaptor. Then, a primary PCR was performed using a gene-specific primer iPCR-F1 and adaptor primer AP1. PCR products amplified from *Eco*RV and *Pvu*II libraries were subjected to a secondary PCR using a gene-specific primer iPCR-F2 and adaptor primer AP2. The 1175 bp fragment amplified from the *Eco*RV library after the second PCR was sequenced and analyzed. To obtain further sequence information, PCR was carried out using the *Pvu*II library with a gene-specific primer iPCR-F3 (5′-TAGAAGTTCTTGTAACCAGATGGCTTG-3′, designed against the 3′ region of the 1175 bp fragment) and adaptor primer AP2, and the resulting 2222 bp DNA fragment was sequenced. Using the 3′ region of this 2222 bp sequence, a gene-specific primer iPCR-F4 (5′-CTAAACTTATCAAACGCTTCTCCCTTC-3′) was designed. A final PCR was performed using BN2 DNA (template) and primers iPCR-F4 and Bo9g-430-R (5′-ACCGCTTGATAAATCCGACGTG-3′, designed against the 5′ region of Bo9g173430) to amplify a 3205 bp fragment. The PCR products were electrophoresed on 1% agarose gels, and DNA bands were excised, purified, and cloned using the T&A Cloning Kit (RBC Bioscience Co., Banqiao, Taiwan). To minimize sequencing errors, at least five clones from each library were sequenced and analyzed.

To validate the sequence of the *BoTPPI-2_BN2* allele, PCR was performed using the forward primer Bo9g440-F and reverse primer Bo9g440-R or Bo9g440-R1. The expected PCR products were 2.4 kb for BN1 and 7.7 kb for BN2.

2.5. RNA Extraction and Gene Expression Analysis

Total RNA was extracted from plant samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Promega, Madison, WI, USA) to remove genomic DNA contamination. Then, reverse transcription PCR (RT-PCR) was performed using the Avian Myeloblastosis Virus (AMV) One-step RT-PCR Kit (Takara, Kyoto, Japan) and gene-specific primers (Table S3). Primer sequences were designed on the basis of sequences in the BRAD database (http://brassicadb.org/brad/).

2.6. Marker Development and Validation

DNA was extracted from leaf samples of various cabbage genotypes using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Twenty ng genomic DNA was used as the template and PCR was performed using sequence-specific primer sets (Table S2). To identify HTHH-tolerant genotypes (BN1-type), PCR was conducted at a volume of 20 μL, containing 10 pmol each of forward and reverse primers (BoTPPI-2-RF and BoTPPI-2-RR, respectively) and 4 μL 5X PCR Master Mix (Elpisbiotech, Daejeon, Korea), using the following conditions: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. To identify HTHH-susceptible (BN2-type) genotypes, PCR was performed as described above but with different primers (BoTPPI-2-SE and BoTPPI-2-SR). To verify the presence of genomic DNA, *BoActin 2* was simultaneously amplified. The PCR products were separated by electrophoresis on 1% agarose gels.

3. Results

3.1. Genetic Analysis of HTHH Tolerance in Cabbage

To examine the genetic basis of HTHH tolerance in cabbage, 10 plants of each parent inbred line (BN1 and BN2), 10 F_1 plants, and 116 segregating F_2 plants were phenotypically scored from 15 June (head forming stage) to 3 August (the head of the HTHH-susceptible line BN2 was completely rotten) (Figure 1). During the examination period, LT and HT were >20 °C and >40 °C, respectively (Figure 1; LT > 30 °C and HT > 50 °C during the last 2 weeks (19 July to 2 August)), and humidity was >90% (data not shown). During this period, all plants produced a head; therefore, we checked soft rot resistance as an indicator of HTHH tolerance. The experiments were carried out in summer 2017 and 2018, but the data shown in this paper are from summer 2018.

Leaves of the HTHH-tolerant inbred line BN1 were hard and bluish green in color, whereas those of the HTHH-susceptible inbred line BN2 were soft and green (or deep green) in color (Figure 2A). With respect to the HTHH tolerance, on 29 June, BN2 plants were completely infected (100%) by *Pectobacterium carotovorum* (bacterial pathogen causing soft rot), whereas only 2 out of 10 (20%) BN1 and F_1 plants were infected with this pathogen (Figure 2B). On 19 July, 50% of BN1 and 70% of F_1 hybrids showed infection symptoms. Complete (100%) resistance to soft rot was not observed, indicating that soft rot resistance, in relation to HTHH tolerance, might be not due to a single gene. On the basis of leaf color, we classified the segregating F_2 population into four groups (A–D). The leaf color of plants in groups A and B was similar to that of BN1, although plants in group A looked more similar to BN1 than those in group B. By contrast, plants in groups C and D were similar to BN2 (Figure 2C). Additionally, the incidence of soft rot from 15 June to 3 August was the lowest in group A and highest in groups C and D (Figure 2C,D). Together, these data indicated that BN1-type progeny possess the HTHH tolerance trait.

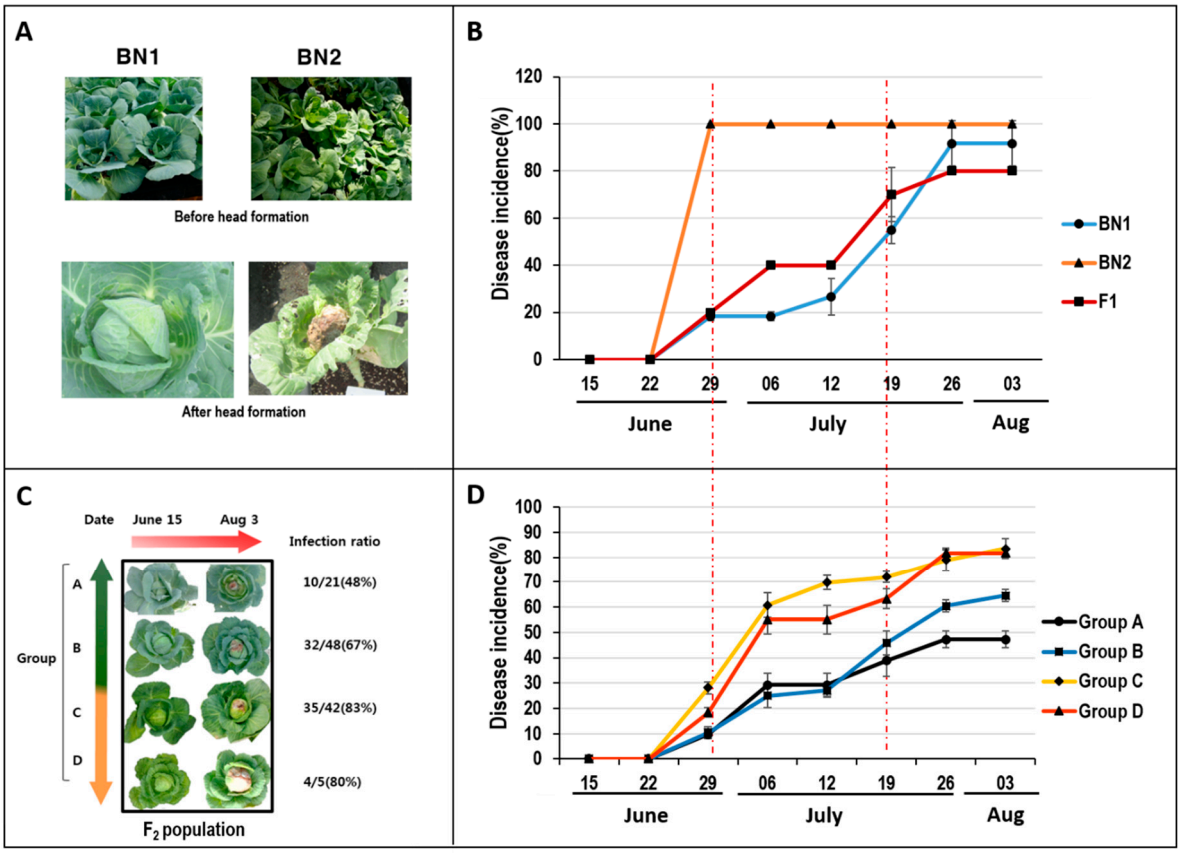


Figure 2. Morphology of soft rot incidence in plant materials used in this study. (**A**) Morphology of parental inbred lines before and after head formation, showing soft rot symptoms and leaf color (bluish green and deep green). (**B**) Soft rot incidence in parental lines and F_1 plants during the cultivation period. (**C**) Morphology of F_2 plants on the basis of leaf color and soft rot incidence. (**D**) Soft rot incidence in F_2 plants during the cultivation period. Vertical dotted lines between (B, D) indicate the period exhibiting the remarkable difference in soft rot incidence.

3.2. Selection and Genomic DNA Analysis of BoTPPI-2

To identify HT(HH) tolerance-related genes in cabbage, we carried out RNA-seq analysis of BN1 and BN2 plants grown under normal condition (22 °C) and heat shock condition (45 °C for 1 h) (Tables S4 and S5). More than 19,000,000 short reads were obtained for each sample of cabbage, and the average length of each read was 101 bp, resulting in sequencing raw data of about 1.9 Gb in total length and covering 56,000 unigenes. Because the content of TRE in plants is correlated to abiotic stress resistance, we analyzed the expression patterns of all *BoTPP* genes identified by RNA-seq (Figure 3). No *B. oleracea* counterpart for *AtPTTC* and *AtTPPF* was detected in this study. Relative transcript levels of *BoTPPI-2* in the HTHH-tolerant line BN1 were significantly higher than those in the HTHH-susceptible line BN2 under both conditions (11- to 12-fold) (Figure 3). Transcript levels of another paralog, *BoTTPI-1*, were negligible (<4 RPKM (reads per kilobase of transcript per million mapped reads)). Previous studies suggest that heat shock tolerance is largely due to constitutive expression of many genes prior to stress treatment [3,64]. In other words, heat shock-tolerant plants exhibit higher basal expression levels of heat shock-responsive genes than susceptible lines, which change moderately upon heat shock. Therefore, it is reasonable to study the *BoTPPI-2* gene in relation to heat tolerance.

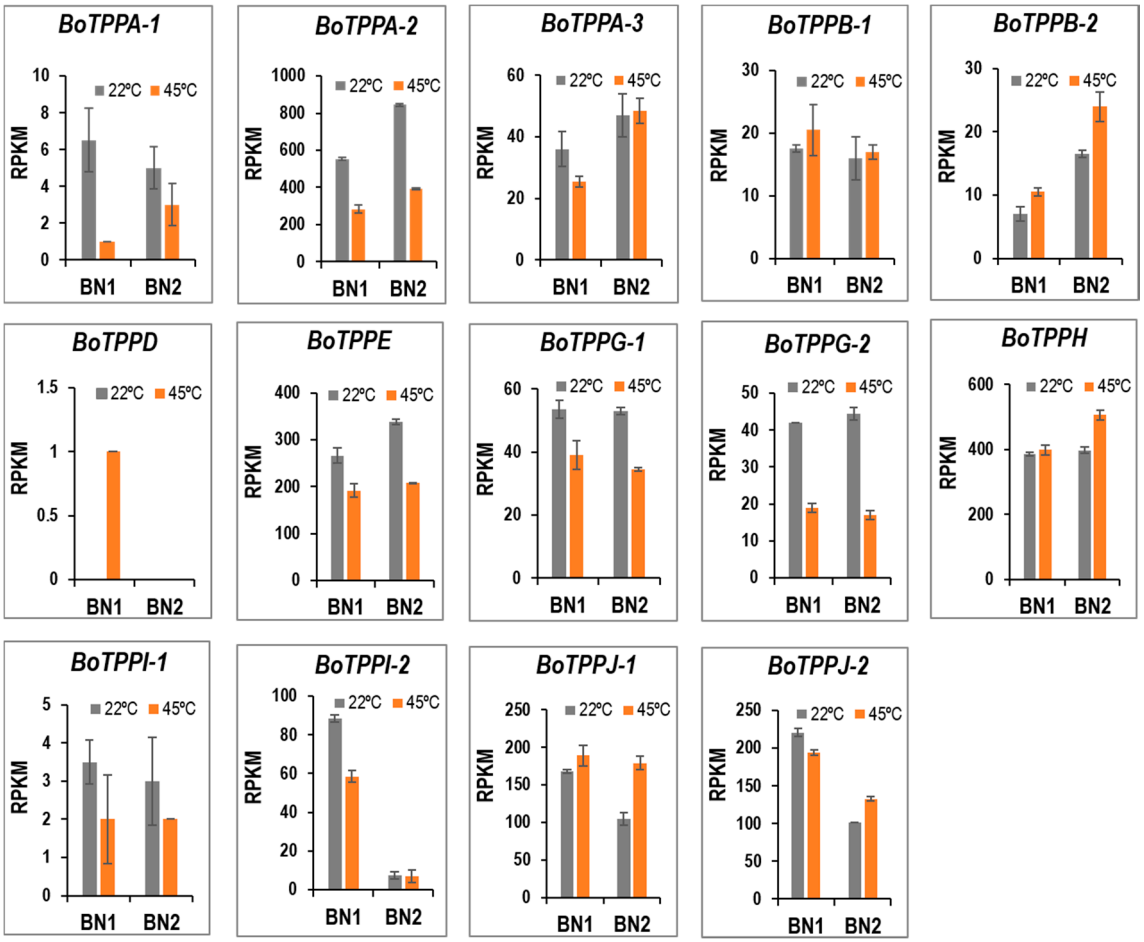


Figure 3. Expression of trehalose-6-phosphate phosphatase *(BoTPP)* genes under normal conditions (22 °C) and after heat shock (45 °C for 1 h). Expression levels were obtained from RNA-seq data as reads per kilobase of transcript per million mapped reads (RPKM).

Two loci corresponding to *Arabidopsis TPPI* (AT5G10100) were identified in the genome sequence of *B. oleracea* var. *oleracea* (http://plants.ensembl.org/Brassica_oleracea/Info/Index): Bo3g005430 and Bo9g173440. The Bo3g005430 locus showed higher identity to *AtTPPI* than Bo9g173440; therefore, we designated Bo3g005430 and Bo9g173440 as *BoTPPI-1* and *BoTPPI-2*, respectively. The expression of only *BoTPPI-2* was associated with the heat shock treatment (Figure 3). Because nucleotide sequences of *BoTPPI-1* and *BoTPPI-2* in *B. oleracea* var. *oleracea* showed relatively high sequence identity (>80%), we decided to clone both these genes from *B. oleracea* var. *capitata* inbred lines BN1 and BN2. The resulting sequence information was used for designing allele-specific primers. Using sequence information available from above website, we designed primers to clone the genomic DNAs of *BoTPPI* genes from BN1 and BN2 (Table S1, Figure 4).

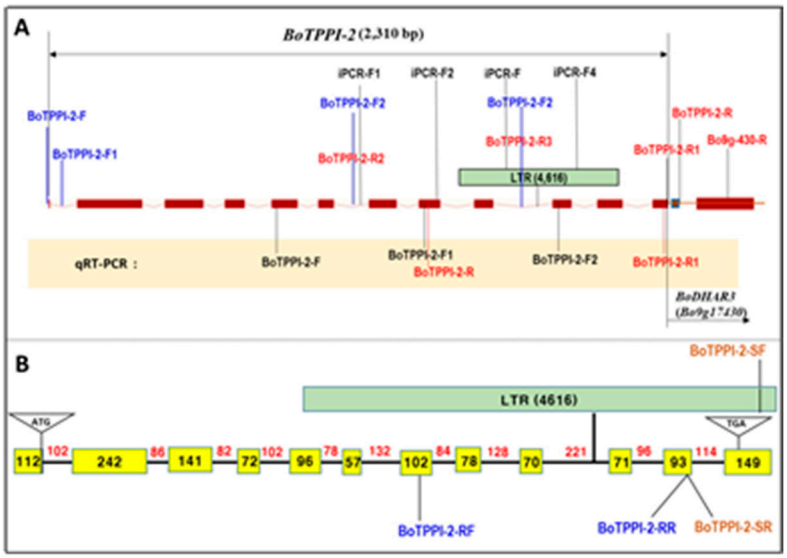


Figure 4. Schematic representation of the *BoTPPI-2* gene and adjacent regions along with the names and binding sites of primers used for cloning, RT-PCR, and marker development. (**A**) Genomic structure of the *BoTPPI-2* gene and the 5′ region of *BoDHAR3* (Bo9g17430). The binding sites of primers used for cloning, inverse PCR (iPCR), and qRT-PCR are shown. (**B**) Names and binding sites of primers used for marker development. Long terminal repeat (LTR; 4616 bp) was inserted into the ninth intron of *BoTPPI-2_BN2* in the reverse orientation.

Sequence analysis revealed that the *BoTPPI-1* gene was 2110 bp in size and contained nine exons and eight introns. Several sequence variations were detected between *BoTPPI-1_BN1* (GenBank accession no.: MN442122) and *BoTPPI-1_BN2* (GenBank accession no.: MN442123); eight single nucleotide polymorphisms (SNPs) were detected in exons (four of which caused amino acid substitutions) and six SNPs and two simple sequence repeats (SSRs) in introns (Figure S1). The *BoTPPI-2* gene could be cloned only from BN1 using the designed primer set. Genomic DNA of *BoTPPI-2_BN1* (GenBank accession no.: MN477455) was 2511 bp in size and contained 12 exons and 11 introns (Figure S1). *BoTPPI-1_BN1* and *BoTPPI-2_BN1* sequences showed 81% identity over the whole gene and 91% identity in exons (936 bp for *BoTPPI-1_BN1*; 1087 bp for *BoTPPI-2_BN2*), indicating relatively high sequence similarity.

The full-length *BoTPPI-2* gene could not be amplified from the BN2 inbred line using any of the designed primer sets; only a partial sequence representing exon 9 could be amplified (Figure S2), implying a structural or sequence variation in the 3′ region of the *BoTPPI-2_BN2* allele. To test this possibility, we designed a reverse primer (Bo9g-430-R) located in the next gene (Bo9g17430; *BoDHAR3*) and used it in combination with the forward primer BoTPPI-2-F to perform a long PCR. We obtained a 3179 bp fragment from BN1 but a 7795 bp fragment from BN2 (Figure 5), indicating that a long DNA fragment was inserted in the *BoTPPI-2_BN2* allele. Using iPCR (see Materials and Methods), we obtained the full-length sequence of *BoTPPI-2* from BN2. Sequence analysis revealed that *BoTPPI-2_BN2* contained an LTR retrotransposon (GenBank accession no.: MN477456); except for this insertion, *BoTPPI-2_BN1* and *BoTPPI-2_BN2* sequences were highly identical (Figure S3). The LTR was inserted in the reverse orientation in the ninth intron of *BoTPPI-2_BN2* and encoded a 1380 amino acid (aa) (or 1333 aa) polyprotein with 63% identity with the *Arabidopsis* putative retroelement Pol polyprotein (AAD32898; 1307 aa) and 59% identity with *Brassica oleracea* var. *botrytis* putative Pol polyprotein (ABH0740; DQ482460, an LTR sequence within the *OR* gene) [60]. The same LTR was identified in two additional locations in the *B. oleracea* var. *capitata* genome: chromosome 6 (C06:20217457–20222073 bp; 98% identity [4577/4617]) and chromosome 5 (C05: 13853467–13858082 bp; 97% identity [4522/4616]) (http://brassicadb.org/brad/).

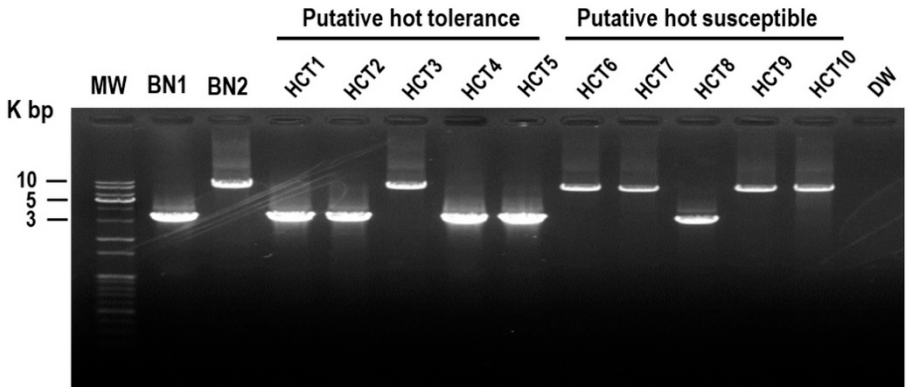


Figure 5. Validation of two *BoTPPI-2* alleles (BN1-type and BN2-type) in cabbage inbred lines by PCR using the BoTTI-2-F/-R primer pair. Putative high temperature (HT)-tolerant lines (HCT1–5) and HT-susceptible lines (HCT6–10) were provided by Joen Seed Co., Korea.

3.3. Sequence and Phylogenetic Analyses of BoTPPI-2

To validate the insertion of LTR in *BoTPPI-2* gene, we performed genomic PCR using DNA of BN1, BN2, and 10 inbred lines provided by Joen Seed Co. (Figure 5). In addition to BN2, four HTHH-susceptible lines and one HTHH-tolerant line provided by Joen Seed Co. appeared to carry the LTR sequence in the *BoTPPI-2* gene. These results indicate that the *BoTPPI-2* gene in BN2-type plants (HTHH-susceptible genotypes) contains the LTR insertion.

Next, we examined the phylogenetic relationship among *BoTPPI* genes using coding sequences (CDSs) (Figure S4). Interestingly, the *BoTPPI-2* CDS showed 100% identity with Bo9g173440 (only two SNPs); however, one-third of the 3′ region of *BoTPPI-2* was completely different from that of Bol043695 (*B. oleracea* var. *capitata*). Therefore, the *BoTPPI-2* sequence of *B. oleracea* var. *capitata* (http://brassicadb.org/brad/) should be replaced with our results. No other BN2-type was identified among other *B. oleracea* species and *B. rapa* and *B. napus* (Figure S5). On the other hand, *BoTPPI-1* was correctly annotated in both cabbage websites or both cabbage plants.

3.4. Effect of LTR Insertion on BoTPPI-2_BN2 Expression

To examine *BoTPPI* expression and its association with the expression of heat shock-related genes in BN1 and BN2, three-week-old seedlings were treated with various temperatures (Figure 6A), and RT-PCR was carried out using various primer sets (Figure 6B, Table S3). *BoTPPI-1* transcripts were almost undetectable in both lines using the BoTPPI-1-F/-R primer pair. *BoTPPI-2* transcripts were amplified from BN1 using all primer combinations, but no primer pair could detect *BoTPPI-2* transcripts in BN2. No detection of transcripts in BN2 using Bo9g-F/-R (= BoTPPI-2-F/-R) primer pair implies that either the *BoTPPI-2* gene was not transcribed or *BoTPPI-2* transcripts were quickly degraded by short interfering RNAs (siRNAs), as described in Lisch (2013) [65]. Interestingly, the level of *BoTPPI-2_BN1* transcripts was slightly lowered by heat shock treatment (consistent with RNA-seq data) but recovered by warming and heat shock after warming. This expression pattern implies that *BoTPPI-2* does not have a rapid response upon exposure to HT. Expression of heat shock-related genes was heat-inducible and not dependent on *BoTPPI-2* expression (Figure 6C). This implies that the role of BoTPPI-2 in HT tolerance might not be related to the heat stress pathway.

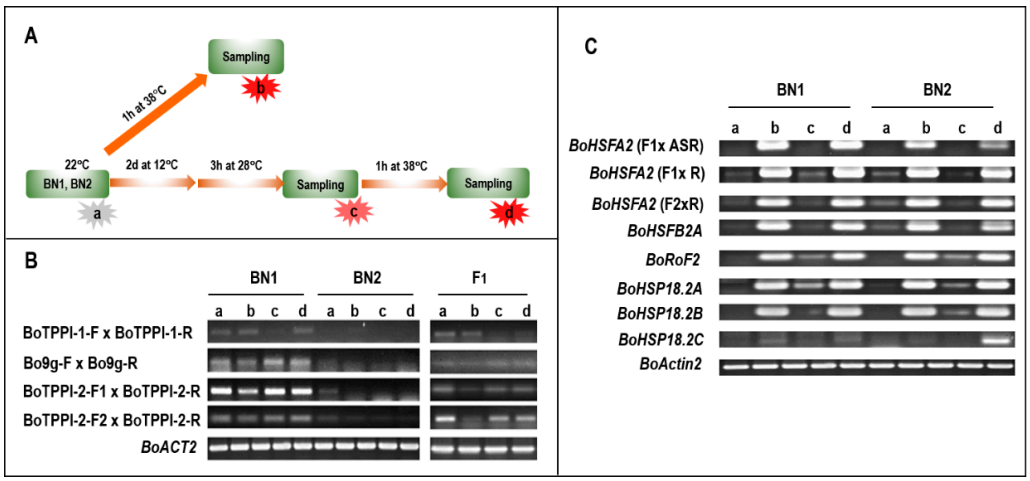


Figure 6. Expression of *BoTPPI* genes and selected heat shock-related genes in cabbage genotypes. (**A**) Temperature treatments such as heat shock (b), warming (c), and heat shock after warming (d). (**B**) Expression of *BoTPPI-1* and *BoTPPI-2* in BN1 and BN2 inbred lines and their F_1 hybrid. (**C**) Expression of heat shock (HS)-related genes in BN1 and BN2 inbred lines.

3.5. Development of Molecular Marker for Genotyping HTHH Tolerance in Cabbage

To distinguish between HTHH-tolerant (BN1-type) and -susceptible (BN2-type) genotypes, we designed a primer set based on the genomic DNA sequence of *BoTPPI-2*. To identify BN1-type genotypes, the forward primer (BoTPPI-2-RF) was located in exon 7, and the reverse primer (BoTPPI-2-RR) was located in exon 11. To identify BN2-types, forward primer (BoTPPI-2-SF) was located in the LTR, and reverse primer (BoTPPI-2-SR) was located in exon 11 (Table S2, Figure 4B). Primer sets were confirmed using BN1 and BN2 DNAs, showing specific amplification (Figure 7A). Then, we validated the specificity of these primers with samples used in Figure 5 (Figure 7B). The banding pattern using these primers was identical to that shown in Figure 5, thus confirming the sequence-specificity of these primers.

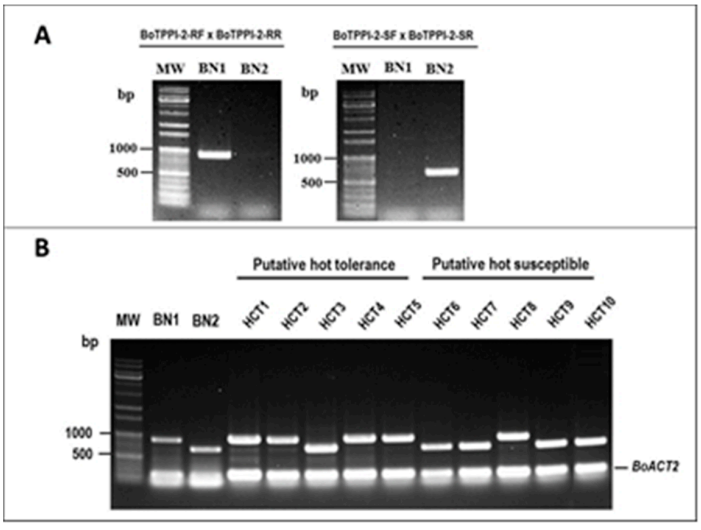


Figure 7. Development and validation of the PCR-based marker used for the identification of HT-tolerant and -susceptible cabbage genotypes. (**A**) Marker development using two different primer sets: BoTPPI-2-RF/-RR for the HT-tolerant (BN1-type) allele and BoTPPI-2-SF/-SR for the HT-susceptible (BN2-type) allele. (**B**) Marker validation using cabbage inbred lines obtained from Choen Seed Co. The same primer sets were used as in A, and PCR products were separated on 1% agarose gel. *Brassica oleracea Actin2* (*BoACT2*) was used as a control.

Next, these primers were tested on F_2 progeny of BN1 × BN2 cross (Figure 2). First, we selected five representative F_2 plants from each of the four groups (A–D), on the basis of leaf color (Figure 8). All five plants in group A and group D were BN1-type and BN2-type, respectively. In group B, four plants showed the BN1-type banding pattern, and one plant showed the BN2-type banding pattern. However, in group C, four out of five plants showed the BN2-type banding pattern, and three of the four plants also contained the BN1-type band. Generally, marker bands reflected the HTHH tolerance phenotypes, as shown in Figure 2. Deviation from our expectation was probably because of the incorrect grouping of plants by leaf color or the involvement of several genes in HTHH tolerance. Thus, leaf color and soft rot resistance did not appear to be controlled by a single gene, and *BoTPPI-2* was found to not be directly related to leaf color.

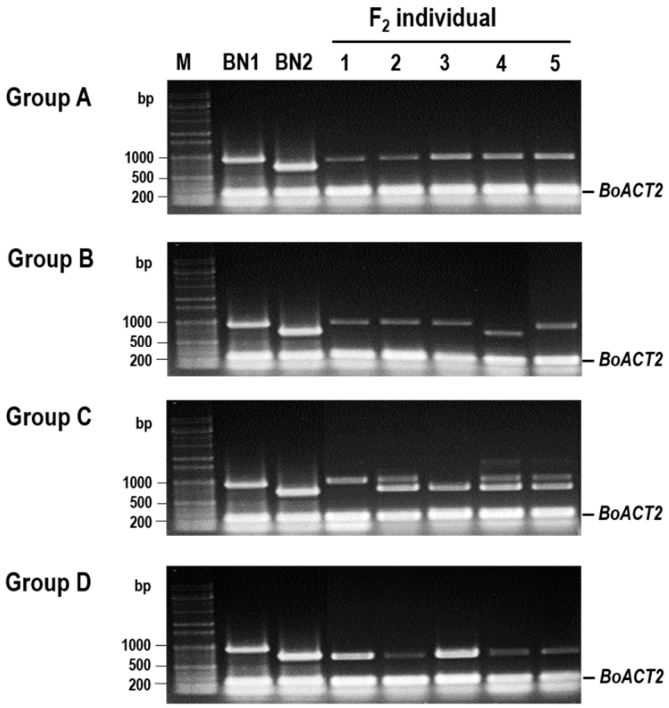


Figure 8. Marker validation using five individuals selected from the segregating F_2 population derived from the BN1 × BN2 cross. Groups A to D were the same as those shown in Figure 2C. Five individuals in each group were selected on the basis of leaf color.

3.6. Application of the Molecular Marker

First, we tested the molecular marker on cabbage samples provided by the Biotechnology and Breeding Institute of Asia Seed Co. These samples included inbred lines and cultivars produced by the company and cultivars purchased from other companies in order to use for comparison or breeding. Most cabbage genotypes were BN1-type, several were heterozygous, and a few were BN2-type (Figure S6). Figure S6B showed contrasting cabbage inbred lines used by Park et al. (2013) [27], including three HT-tolerant and two HT-susceptible lines. All HT-tolerant lines were BN1-type, but of the two HT-susceptible lines, one was BN2-type and the other was BN1-type, suggesting that the *BoTPPI-2*-based marker does not account for 100% of the HT tolerance phenotype, or that HTHH tolerance might be different from HT tolerance.

Although the genetic material used in this study was bred at Asia Seed Co., another Korean seed company, Joen Seed Co., has recently been very active in the development of cabbage cultivars suitable for tropical regions. Samples provided by Joen Seed Co. showed a good match between breeders' selection and the molecular marker developed in this study (Figures 7B and 9). HCT-01–05 represent five inbred lines used as breeding materials for the production of HTHH-tolerant cultivars, characterized by flat head, soft rot resistance, and head formation under high temperature; however,

bolting was easily induced in these lines upon exposure to slightly low temperature (Figure 7B). Except for HCT-03, all inbred lines produced a BN1-type banding pattern (HTHH tolerance marker). On the other hand, HCT-06–10 represent five inbred lines used as breeding materials for the production of cultivars with a round head and early maturity in China and Europe. These inbred lines had a soft texture with bolting resistance during spring cultivation but were HTHH-susceptible. Except for HCT-08, all four lines showed a BN2-type banding pattern (HTHH-susceptible) (Figure 7B). Together, these results show that the *BoTPPI-2*- and the inserted LTR-based molecular marker could identify 80% of the HTHH-tolerant or -susceptible *B. oleracea* lines. However, eight inbred lines provided by Joen Seed Co. showed HTHH-tolerant genotypes (Figure 9A).

Cabbage genotypes shown in Figure 9B included six cultivars grown in South-East Asia, exhibiting HT tolerance and a flat head, and JS107 F_1 hybrid derived from a cross between HCT-07 and HCT-08 (Figure 7B). Figure 9C shows the F_1 hybrid of HCT-03 (Figure 7B) and HCT-199 (HT-tolerant line). Three cultivars in Figure 9D represented leading varieties of Joen Seed Co. JS21 (HomeRun) was an F_1 hybrid of HCT-08 and one of the HT-susceptible inbred lines exhibiting very early maturity and soft tissue; the F_1 hybrid was heterozygous for the *BoTPPI-2* marker. These results support the reliability of the marker.

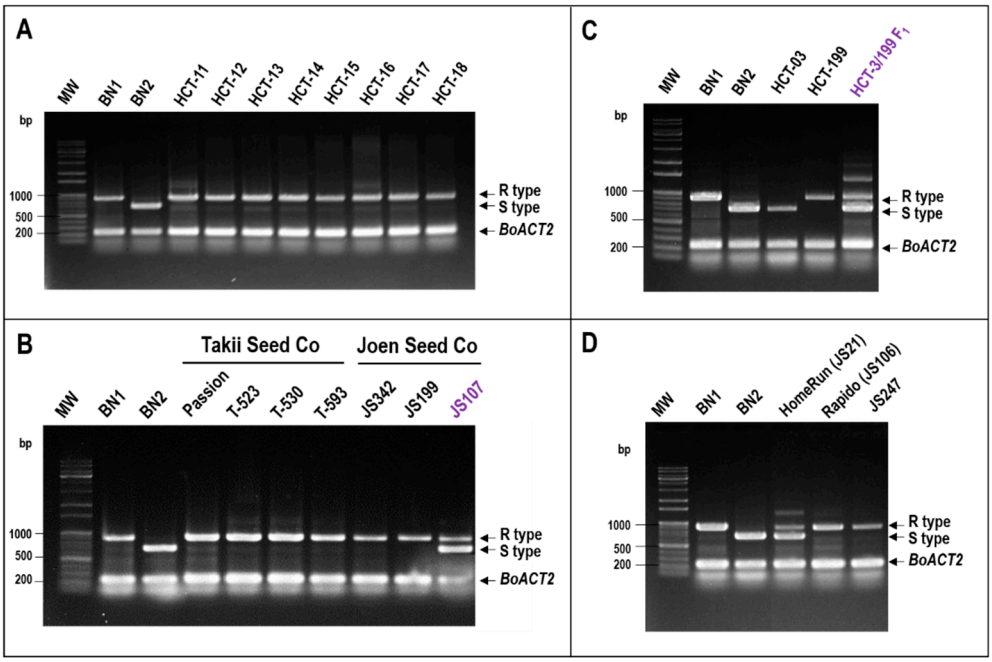


Figure 9. Validation of hot tolerance of cabbages provided by Joen Seed Co. (**A**) Inbred lines that have been used for hot/cold tolerance source. (**B**) Cabbages that have been used for heat-tolerant and flat head sources. JS107 was a heat-susceptible line used as a reference, which was hybrid of HCT-07 and HCT-08 in Figure 7B. (**C**) Breeding material for hot tolerance selection. (**D**) Three representative cultivars for selling.

We obtained seeds of 65 Japanese cultivars from the Center of Horticultural Seed Development of the GSP, Suncheon National University, Suncheon, Korea. All 65 cultivars showed the BN1-type (HTHH-tolerant) banding pattern (Figure S7). The Chinese cultivars usually were BN2-type *BoTPPI-2* (Figure S7), thus supporting the Chinese markets that have a high demand for green leaf cabbage, as well as breeding programs in China that focus on early maturing cultivars with a round head. Two cultivars developed by Korean Seed Co. Dongbu Farmhanong for export to China also showed the BN2-type banding pattern (Figure S8B). However, most cultivars from Alaska, USA, contained a *BoTPPI-2* that was type BN1 (Figure S8C).

Taken together, our data show that most inbred lines and cultivars from Korea and Japan carry the *BoTPPI-2_BN1* (HTHH-tolerant) allele, whereas a large proportion of Chinese cultivars harbor the *BoTPPI-2_BN2* (HTHH-susceptible) allele but with green leaf color. Thus, the *BoTPPI-2*-based molecular marker was reliable, but not perfect, for genotyping cabbage lines for the HTHH tolerance trait.

4. Discussion

Tolerance to HT and HH conditions (HTHH tolerance) is indispensable for the cultivation of cabbage in the tropics and subtropics, as well as during the summer in Korea. However, no molecular marker was available for the selection of HTHH-tolerant cabbage lines to date, which would accelerate the pace of cultivar development in breeding programs. In this study, we report the identification and application of a molecular marker on the basis of the insertion of an LTR retrotransposon in the *BoTPPI-2* gene of HTHH-susceptible cabbage genotypes. This marker is associated with lower accumulation of *BoTPPI-2* transcripts and loss of HTHH tolerance.

4.1. Analysis of HTHH-Tolerant and -Susceptible Cabbage Genotypes

Breeding materials for the production of HTHH-tolerant cabbage cultivars are usually characterized by a flat head, soft rot resistance, bolting under HTHH, and easily induced bolting under slightly low temperature. On the other hand, breeding materials for production of spring cabbage cultivars in China and Europe are characterized by a round head, early maturity, soft texture, bolting resistance, and HTHH susceptibility. In this study, we used the HTHH-tolerant inbred line BN1 with bluish leaf color and a flat head, and the HTHH-susceptible inbred line BN2 with green leaf color and a round head. These two genotypes were crossed to produce a segregating F_2 population. Because small heads were formed on 15 June, we examined the incidence of soft rot, as one of the HTHH tolerance indexes, until 3 August when the head of the HTHH-susceptible line BN2 was completely rotten (Figure 2). Classification of cabbage genotypes by their leaf color and soft rot incidence showed strong correlation with the HTHH tolerance trait (Figure 2).

4.2. Utility of the BoTPPI-2 Gene as a HTHH Tolerance Marker

On the basis of RNA-seq analysis, we selected *BoTPPI-2* as a putative candidate marker for the examination of HTHH tolerance in cabbage (Table S4, Table S5). Transcript levels of *BoTPPI-2* were high in the HTHH-tolerant inbred line BN1 but negligible in the HTHH-susceptible inbred line BN2 (Figures 3 and 6). Genomic DNA sequence analysis revealed the insertion of LTR, a putative retroelement Pol polyprotein, in intron 9 of the *BoTPPI-2* gene in the HTHH-susceptible inbred line BN2 (Figure 4). We speculated that the absence of *BoTPPI-2* transcripts in BN2 was either due to promoter activity or silencing of the LTR-containing *BoTPPI-2* transcript by siRNAs, as described by Lisch (2013) [65]. Because of differences in promoter activity in different plant organs, the expression of *AtTPPI* is high in root and hypocotyl-root junction [45]. Because of the lack of function or physiological role of TPPI, we could not explain the function of BoTPPI-2 in HTHH tolerance. TRE protects heat-induced membrane damage [37], but we hypothesized that the loss of *BoTPPI-2* expression reduces TRE content in BN2-type lines because of the presence of multiple *TPP* genes in cabbage. Our results showed that the expression of major heat stress-responsive genes showed no difference between BN1 and BN2 under heat shock and heat acclimation conditions (Figure 6). These results suggest that *BoTPPI-2* is not directly associated with the heat stress pathway required for conferring HT tolerance.

4.3. Effect of LTR Insertion

Insertion of transposable elements (TEs) can give rise to deleterious mutations or overall genetic instability; however, TEs also have a positive impact on gene regulation and adaptation [65]. Retrotransposons in plants are usually repressed by DNA methylation, but to avoid this silencing, LTRs employ various strategies. One of these strategies is the recruitment of a major plant heat shock

transcription factor such as HSFA2 under heat stress condition [66]. We did not detect the expression of LTR in BN-2 type genotypes (data not shown), so that it was hard to explain LTR function in cabbages.

LTR insertion usually alters plant physiology including gene expression. This change results from the insertion of the LTR within or near transcriptionally active regions of genes [56,57,67]. The *or* gene mutation in cauliflower results from the insertion of a copia-like LTR retrotransposon in exon 3, which leads to the production of three alternatively spliced *or* transcripts associated with the accumulation of high levels of β-carotene, leading to the production of orange curd [60]; alternative splicing is caused by the translocation of the LTR. In pepper, insertion of the 3′ untranslated region (UTR) region of a non-LTR retrotransposon in the promoter region of *CaAn2* encoding a **my**elo**b**lastosis (MYB) transcription factor activates *CaAn2* expression, leading to increased anthocyanin biosynthesis [63]. Because LTR regions possess transcription start sites, LTR insertion usually activates gene transcription [57,61,62]. However, in this study, we showed that insertion of the LTR in *BoTPPI-2* suppressed its transcription and was associated with loss of HTHH tolerance. To extend our knowledge on LTR insertion, it is important to study the effect of LTR insertion at two other regions in the genome (chromosomes 5 and 6) on gene expression, and the effect of LTR insertion in *BoTPPI-2* on the expression of a nearby gene, *BoDHAR3* (chromosome 9). In *Arabidopsis*, dehydroascobate reductase 1 (*DHAR3*) is associated with high light intensity stress and ROS removal [68,69]. It is possible that the insertion of LTR in *BoTPPI-2_BN2* allele changes the expression of *BoDHAR3*, thus decreasing HTHH tolerance.

4.4. Marker Validation

The molecular marker developed in this study on the basis of the difference between *BoTPPI-2_BN1* and *_BN2* sequences explained the HTHH tolerance trait of almost 80% of the tested inbred lines and segregating F_2 plants (Figures 7–9, Figures S6–S8). Most Korean and Japanese cultivars possessed the HTHH-tolerant *BoTPPI-2_BN1* allele. Some breeding materials used by seed companies in Korea and most China cultivars possessed the HTHH-susceptible *BoTPPI-2_BN2* allele. We predicted that the insertion of LTR in *BoTPPI-2_BN2* allele would have an evolutionary advantage. It is possible that this LTR insertion is associated with insensitivity to vernalization for bolting resistance during spring cultivation or the maintenance of soft texture and green color of leaves. However, this needs further investigation.

It is impossible to obtain perfect (100%) association between a marker and the trait if the trait is not controlled by a single dominant gene. According to previous studies, traits such as flowering time, which are controlled by multiple genes, could be exploited by several molecular markers in combination. In a segregating F_2 population derived from a cross between late-flowering (*BoFLC2*) and early-flowering (*boflc2*) lines of cauliflower, the *BoFLC2* gene behaves in a dosage-dependent manner and accounts for up to 65% of the variation in flowering time [70]. In *B. oleracea* var. *capitata*, insertions/deletions (InDels) present in introns of the *peroxidase* (*BolPrx.2*) gene [71] and *BoFLC1.C9* gene [72] account for approximately 80% of the variation in flowering time among F_2 individuals and commercial lines. Therefore, this is the first report of a gene-specific marker for genotyping the HTHH tolerance trait in cabbage, which accounted for over 80% of variation. This marker could be used for molecular breeding in Brassicaceae. Development of additional similar markers and their combined use can help increase the pace of breeding HTHH-tolerant cultivars to tackle the ongoing global warming.

5. Conclusions

The LTR insertion into *BoTPPI-2* gene from HTHH-susceptible genotypes, such as BN2, led to the inhibition of its transcription, thereby losing HTHH tolerance. Although positive roles of LTR insertion have not been identified in cabbages, useful molecular markers to discriminate HTHH tolerance or susceptibility could be developed using *BoTPPI-2* and the LTR sequences.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/1/116/s1. Figure S1. Schematic representation of the *BoTPPI-1* gene in inbred lines BN1 and BN2, and that of *BoTPPI-2* in

BN1. Figure S2. Primer sets used to amplify DNA from BN1 and BN2. Figure S3. Comparison of *BoTPPI-2_BN1* and *BoTPPI-2_BN2* sequences after removal of the LTR sequence from *BoTPPI-2_BN2*. Figure S4. Phylogenetic analysis of *BoTPPI* genes on the basis of the coding sequence (CDS). Figure S5. Marker validation using diverse *B. oleracea* genotypes. Figure S6. Marker validation using cabbage samples provided by Asia Seed Co. Figure S7. Marker validation using Japanese cultivars supplied by the Golden Seed Project (GSP) Center. Figure S8. Marker validation using cabbage genotypes obtained from various sources. Table S1. *B. oleracea* samples used in marker development and validation for hot tolerance and susceptible. R, heat-resistant, S, heat-susceptible. Table S2. List of primers used in gene cloning and marker development. Table S3. List of primers used in RT-PCR analysis. Table S4. Summary of RNA sequencing and assembly. Table S5. Summary of RNA-seq data expressed as reads per kilobase of transcript per million mapped read (RPKM).

Author Contributions: Data curation, H.S., B.-H.H., and J.-I.P.; formal analysis, J.-I.P. and C.-T.H.; investigation, H.S., M.L., and J.-I.P.; project administration, Y.H.; resources, B.-H.H. and J.-I.P.; supervision, Y.H.; validation, H.S. and Y.H.; writing—original draft, H.S.; writing—review and editing, C-T.H. and Y.H. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare that they have no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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