Identification of Chinese Cabbage Sentrin as a Suppressor of Bax-Induced Cell Death in Yeast

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Studies into the cell death program termed apoptosis have resulted in new information regarding how cells control and execute their own demise, including insights into the mechanism by which death-preventing factors can inhibit Bax-induced caspase activation. We investigated high temperature stress-induced cell death in Brassica rapa. Using a yeast functional screening from a Brassica rapa cDNA library, the BH5-127 EST clone encoding an apoptotic suppressor peptide was identified. However, a phylogenic tree showed that BH5-127 clusters within a clade containing SUMO-1 (Small Ubiquitin-like Modifier-1). BH5-127 was confirmed similar to have function to SUMO-1 as Fas suppression. Expression of BH5-127 showed that substantial suppression of cell death survived on SD-galactose-Leu-Ura medium. The results suggest that BrSE (Brassica rapa Sentrin EST, BH5-127) is one of the important regulatory proteins in programming cell death, especially in the seedling stage of Chinese cabbage.

Keywords: Apoptosis, Brassica rapa, sentrin, SUMO-1, yeast functional screening

Apoptosis is the process of programmed cell death (PCD) that may occur in cell organisms. The most downstream components of the cell death machinery identified to date are proteases known as caspases [11]. Although some of the interactions among known cell death components have been described, there are major gaps in the understanding of the cell death process. For example, the cellular targets of caspases that lead to cell death have not yet been fully described. Another example is the mechanism by which death-preventing members of the Bel-2 family, such as Bax Inhibitor (BI-1), can inhibit Bax-induced caspase activation [13]. Overexpression of Arabidopsis thaliana BI-1 that is a suppressor of cell death increased resistance to Bax-induced cell death in this plant [6]. Genetic engineering and modern molecular breeding methods have been used to isolate key genes involved in stress responses in plants. Functional screening of microorganisms that express heterologous cDNA libraries is a powerful tool for identifying genes with a specific function. Functional screening in yeast to identify genetic determinants capable of conferring abiotic stress tolerance in Jatropha curcas has been reported [3].

Recently, PCD in plant cells under abiotic stresses has also been investigated. Expression of BI-1 in Arabidopsis is induced under high temperature and reactive oxygen stress condition. Heat shock has been implicated in inducing abiotic stress factors including expression of BI-1 genes [5, 17]. The yield losses due to high temperature are large and are often combined with losses from other environmental stress. Chinese cabbage (Brassica rapa) is more sensitive to heat stress than other Brassica species. Although observation did not indicate the exact developmental phase at when the reproductive organs are susceptible to heat stress, pods that had passed the critical threshold developmental phase tolerated heat stress. The temperature effect on reproductive organs appeared to be responsible for the reduction in yield [1]. Expression profiling of Brassica rapa showed that heat treatment triggered significant accumulation of heat shock proteins, and some cell-wall-modifying genes related to thermotolerance were up-regulated. With respect to temperature shift, severe heat stress induces an alarming situation leading to irreversible
injury and cell death, whereas moderate heat stress triggers thermotolerance [20].

It has been demonstrated that abiotic stresses cause a significant reduction in the potential yield of crops, and this loss will only be worsened with the dramatic climate change expected in the near future [11]. Abiotic stress is widely considered a detrimental factor in crop production. In this study, we report the existence of a *Brassica rapa* EST encoding the peptide as an apoptotic suppressor otherwise known as an antiapoptotic factor under high temperature stress.

**MATERIALS AND METHODS**

**Plant Material and Stress Test**

Seeds of the *Brassica rapa* L. ssp. pekinensis inbred line “Chiifu” (289001) were received from the Brassica Resource Bank, Daejon, Korea. Seeds were germinated in Korean artificial soil and grown for 28 days in a growth chamber as previously described [10]. Three-week-old plants were subjected to a heat stress treatment, which was a growth chamber heated to 35°C (16 h day/8 h night, RH 50%) for one week. After the heat stress treatment, the leaves of the Chinese cabbage were sampled [1]. The plants that had no treatment were sampled as a control.

**RNA Isolation and cDNA Library Construction**

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions, and the RNA quality and quantity were monitored visually using RNA gel electrophoresis and spectrophotometrically using a Nanodrop instrument (Nanodrop 2000 Thermoscientific, Wilmington, DE, USA). The cDNA library was created from total RNA using a TaKaRa cDNA Library kit (TaKaRa Code 6119 and 6130, Takara Bio Inc, Japan). After digestion with EcoRI and XhoI, cDNA derived from Chinese cabbage was inserted into the EcoRI and XhoI sites of vector pYX112 (Ingenius, Wiesbaden, Germany), carrying an ARS/CEN replicon, the ratio of the cellular contents. The percentage of dead cells generated by Evans blue penetrates only dead cells and results in a blue staining of the cellular contents. The percentage of dead cells generated by Evans blue penetrates only dead cells and results in a blue staining of the cellular contents. The percentage of dead cells generated by Evans blue penetrates only dead cells and results in a blue staining of the cellular contents. The percentage of dead cells generated by Evans blue penetrates only dead cells and results in a blue staining of the cellular contents. The percentage of dead cells generated by Evans blue penetrates only dead cells and results in a blue staining of the cellular contents. The percentage of dead cells generated by Evans blue penetrates only dead cells and results in a blue staining of the cellular contents. The percentage of dead cells generated by Evans blue penetrates only dead cells and results in a blue staining of the cellular contents. The percentage of dead cells generated by each treatment was determined by scoring the cells using a microscope [19]. Functional characterization of BHS-127 in suppression of cell death was shown by transformation of S. cerevisiae and heat stressed at 35°C for 72 h. Yeast cell growth was determined by measuring the OD_{600} of a yeast suspension culture.

**Yeast Plasmid Isolation, RT-PCR, Construction of GFP, and Data Analysis**

Isolation of yeast plasmid was performed using a QIAprep Spin Miniprep kit using a microcentrifuge (Qiagen, Germany) with modifications in the membrane lyses stage. The yeast cell pellet was suspended in 250 µl of Buffer P1 containing RNase A by a Tissuemizer (Qiagen, Germany). Cells were lysed by adding 250 µl of Buffer P2 from the QIAprep kit and mixed by inverting the tube, followed by 10 min incubation at 22°C. During this period, cell lysis causes release of DNA and the mixture in the tube becomes viscous. At this stage, 350 µl of Buffer N3 was added to the lysate, and after mixing by gentle inversion, the tube was incubated on ice for 30 min. Subsequently, the protocol of the QIAprep Spin Miniprep Kit using a microcentrifuge was followed [13]. Yeast plasmids were analyzed with PCR using pYX primers: 5’-end forward primer (5’-GGAGTTTAGAAGACTTG-3’) and 3’-end complementary strand reverse primer (5’-GGATATGATCGTCG-3’) with annealing temperature of 56°C for 30 s. The PCR product profiles were monitored by agarose gel electrophoresis.

Thereafter, 500 ng of purified RNA from *Brassica rapa* was analyzed by RT-PCR using SuperScript III One-step RT-PCR with Platinum Taq DNA Polymerase (Invitrogen Co., Carlsbad, CA, USA). RT-PCR was performed using 20 pmol of specific primers for *Brassica rapa* sentrin (BSh); 5’-end forward primer (5’-GGAGTTTAGAAGACTTG-3’) and 3’-end complementary strand reverse primer (5’-TACTTATGCAGACTTG-3’). The cycling condition for PCR was 94°C for 2 min, 94°C for 15 s, 64°C for 30 s, and 72°C for 1 min. The amplification reaction was carried out for 40 cycles with a final extension at 72°C for 10 min.

The sequencing of Chinese cabbage cDNA conferring resistance high temperature stresses was performed by Solgent, Korea. We performed computational searches using BLAST sequence databases through the National Center of Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/) to understand and assign functional classes the sequence information determined in this screening. Identification of amino sequence and homology was performed using GENETYX-WIN Version.3.1. The phylogenetic tree was constructed by UPGMA using MEGA4 [7].

The indication of intracellular localization was analyzed by GFP construction. The indicated antiapoptotic gene from Chinese cabbage was constructed into vector pBin-GFP. The construction was performed by digestion with Sall and Ncol and was inserted into the pBin-GFP vector. We confirmed the intracellular distribution of GFP-tagged BHS-127 by MitoTracker, a mitochondrion-selective probe (Molecular Probes, Inc. M-7513).

**RESULTS AND DISCUSSION**

*Brassica rapa* was subjected to high temperature treatment (35°C) after the plant was 28 days old. The height, amount of leaves, and biomass were measured and compared...
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**Table 1. Characteristics of growth to 35°C in Brassica rapa, “Chiifu”.**

<table>
<thead>
<tr>
<th>Temperature treatment (°C)</th>
<th>Fresh weight (mg)</th>
<th>Dry weight (mg)</th>
<th>Leaf no. 27d</th>
<th>Plant height (cm) 27d</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>460.2±87.7</td>
<td>35.0±7.3</td>
<td>10.0±2.1</td>
<td>12.9±4.8</td>
</tr>
<tr>
<td>25</td>
<td>454.6±56.4</td>
<td>73.4±13.9</td>
<td>12.0±1.2</td>
<td>14.8±1.0</td>
</tr>
</tbody>
</table>

*a: mean±standard deviation, d: days after treatment.

The leaves of Brassica rapa after high temperature stress treatment at 35°C showed that discoloration, quantity of height, amount of leaves, and biomass were lower than Brassica rapa control, which was incubated at 25°C (Table 1). High temperature can cause considerable harvest damages, including scorching of plant leaves, branches, and stems, root growth, and reduced yield [16]. Therefore, the discoloration of leaves and the less harvest had appeared in Brassica rapa.

Yeast cells containing pYX112-cDNA were used to screen for Brassica rapa genes that can inhibit yeast growth. The QX95001 contains a galactose inducible promoter (GAL1), whereas pYX112 contains a constitutive promoter (TP1). The clones that survived on a galactose medium were isolated. The clones contain the genes that inhibit yeast growth and whose activities are suppressed [8]. Screening of 528 clones of high-temperature-induced Brassica rapa cDNA identified about 39 clones that survived following Bax expression on galactose-containing medium, and 7 colonies were recovered that exhibited substantial suppression of cell death on galactose-containing medium (Table 2). Meanwhile, screening of 324 clones of Chinese cabbage cDNA resulted in 5 clones growing on galactose-containing medium, and no colony was recovered after suppression of cell death. Yeast clones that survived on galactose-containing medium were isolated and PCR performed using the pYX-forward and pYX-reverse primers. The gel analyses of amplification of the double-stranded cDNA library displayed large sizes over 300 bp that were assumed to be the insertions of the cDNA target in the vector. Analysis of DNA sequence information from yeast transformants demonstrated that 7 sequences encoded an expressed sequence tag (EST) as apoptosis suppressor. One of the EST clones, BH5-127 (Accession No. AB609070), contained SUMO-1 (Fig. 1). Yeast clones that survived on galactose-containing medium were isolated and PCR performed using the pYX-forward and pYX-reverse primers.

**Table 2. Identification of 39 clones that survived on selection galactose medium SD-galactose-Leu'-Ura'.**

<table>
<thead>
<tr>
<th>Putative characteristic</th>
<th>Clones</th>
</tr>
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<tbody>
<tr>
<td>Prion protein</td>
<td>BH5-298</td>
</tr>
<tr>
<td>Proteinase inhibitor (serine)</td>
<td>BH5-289</td>
</tr>
<tr>
<td>Phosphoglucone isomerase PSI-1.1</td>
<td>BH5-294</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>BH5-287</td>
</tr>
<tr>
<td>Water stress inducible protein Rab21</td>
<td>BH5-127</td>
</tr>
<tr>
<td>Fe-superoxidase dismutase</td>
<td>BH5-25</td>
</tr>
<tr>
<td>SUMO (Small Ubiquitin like-Modifier)</td>
<td>BH5-127, BH5-132</td>
</tr>
<tr>
<td>Nothing significant found</td>
<td>BH5-2-1, BH5-14-2, BH5-10-3, BH5-11-5, BH5-277, BH5-362</td>
</tr>
<tr>
<td>No insert clones and small insert</td>
<td>BH5-18, BH5-275, BH5-207, BH5-12, BH5-13, BH5-32, BH5-91, BH5-94, BH5-118, BH5-216, BH5-222, BH5-223, BH5-251, BH5-268, BH5-277, BH3-274, BH3-290, BH3-299, BH3-362, BH3-244, BH3-245, BH3-246, BH3-271, BH3-272, BH3-283, BH3-285</td>
</tr>
</tbody>
</table>

Total 39 clones
plant showed that *O. sativa*, *Arabidopsis*, and *Lycopersicon esculentum* are closely related to human Sentrin/SUMO-1. Otherwise, BH5-127 clusters within a clade contained SUMO-1 of *C. nippon*, which is implicated in suppressing cell death control (Fig. 1C). We named BrSE (*Brassica rapa* Sentrin E) as Sentrin of Chinese cabbage.

A cDNA library derived from *Brassica rapa* was constructed using a yeast expression vector, which was then screened for suppression of cell death in the *S. cerevisiae* strain QX95001 obtained by transforming wild-type yeast, BF264-15Dau, containing a Leu-marked plasmid with Yep51-Bax from which the full-length mouse Bax protein is expressed under the control of a galactose-inducible yeast GAL1 promoter. Bax-induced cell death is initiated by transferring cells from glucose- to galactose-containing medium. As shown in Fig. 2A, yeast strain QX95001 transformed with the pYX112 vector, and pYX112-Bax did not grow when plated on galactose-containing medium. However, the clones AtBI-1 and BH5-127 survived and grew when plated on galactose-containing medium.

The graph in Fig. 2B shows that AtBI-1 yeast culture growth reached 0.41, which was higher (0.15) than BH5-127.
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The capability of yeast as an apoptotic suppressor was expressed by both AtBI-1 (0.41) and BH5-127 (0.15) yeast cultures. Strains transformed with the pYX112 vector or pYX112-Bax grew less than those expressing AtBI-1 or BH5-127, consistent with the fact that neither pYX112 nor pYX112-Bax contained the apoptotic suppressor.

These results were confirmed by visualizing yeast cells, where the BH5-127 clone prevented Bax-induced cell death. The number of dead cells was counted by Evans Blue staining. As shown in Fig. 2C, the cells began to die after 14 h of Bax-induced cell death expression. Visualization of strains expressing AtBI-1 or BH5-127 showed that the cells were still intact. The normal number of dead cells in yeast under these conditions was demonstrated by the pYX112 vector and pYX112-Bax samples. Bax-induced cell death of QX95001 is triggered by transferring cells from glucose- to galactose-containing medium [11]. Detection of ROS generation by Evans Blue staining was used. This reagent diffuses through cell membranes and is subsequently enzymatically deacetylated and then oxidized by ROS [19]. Expression of the SUMO-1 RNA from Brassica rapa was confirmed by RT-PCR using specific primers (BrS): 5'-end (5'-CGAGCGCGTGGTACCACGGT-3') and 3'-end complementary strand (5'-TACTTAGTCGACTCTAGACC-3'). As shown in Fig. 2D, the expression of SUMO-1 was confirmed. BH5-127 showed stronger expression than the Brassica rapa control. Expression of the proapoptotic gene Bax in yeast results in cell death with an apoptotic phenotype, which is suppressed by coexpression of anti-apoptotic genes. BH5-127 was tagged with GFP to explore the intracellular localization of BH5-127. This indicated that fusion of BH5-127–GFP was localized to mitochondria in the yeast cell (Fig. 3).

Using a similar screening system, several plant genes were isolated as suppressors of Bax-induced cell death in yeast [13, 18, 19]. Functional characterization of BH5-127 in the suppression of cell death was shown by transformation
of *S. cerevisiae* and heat stress at 35°C for 72 h. When exposed to heat stress conditions, the levels of ROS production also increased in transgenic and control yeasts, but only resulted in death of control yeast (transformed to pYX112). However, yeast expressing antiapoptotic protein was protected to a greater extent. Fig. 4 showed that transgenic yeast, including AtBI and BrSE, grew well under high temperature stress.

*S. cerevisiae* has been shown to be a useful model for apoptosis research. Features of metazoan PCD have been observed in yeast, where it has become evident that PCD occurs, exhibiting at least some of these features. Oxidative stress can cause cell death and occurs during heat-induced cell death in yeast. The use of yeast is a high throughput heterologous system for screening and identifying functionally relevant genes of interest that regulate apoptosis. Yeast may prove to be an attractive system to identify candidate plant genes with the functional ability to modulate plant cell death generated by ROS [2].

Another suppressor of Bax-induced cell death is dependent on the activation mechanism used to initiate programmed cell death. SUMO-1 is a protein that binds domains on the protein Fas but not FADD. SUMO-1 may inhibit FADD-dependent death by preventing aggregation of FADD on activated Fas and by inhibiting recruitment of caspase 8 [12]. The regulation of caspase and cell death by Bax is described in Fig. 5; Bax induces caspase activity. The most downstream components of the cell death machinery identified so far are proteins, known as caspases, a class of cysteine proteases that cleave substrates following aspartate residues. The activity of caspases can be regulated by a variety of cellular factors. Some, such as the *C. elegans* protein CED-4, the related mammalian protein Apaf-1, or the mammalian protein Fas, can activate caspases and are thus death promoters. Other proteins seem to promote cell death in part by inhibiting the activities of death-preventing proteins, such as BI-1 and SUMO-1. BI-1 blocks cell death induced by Bax, but SUMO-1 inhibits the activation of caspases [13]. In mammalian cells, tumor necrosis factor (TNF) induces programmed cell death by binding to the Fas/APO-1 receptor. The presence of a “death domain” in the cytoplasmic region of the receptor is responsible for transducing the death signal. Using the death domain as bait in two-hybrid interaction assay, SUMO-1 can be isolated. SUMO-1 shows amino acid similarity to ubiquitin, Nedd8, and *S. cerevisiae* protein Smt3. SUMO-1 provides protection against both anti-Fas/APO-1 and TNF-induced cell death [4].

We have shown that the BrSE (*Brassica rapa* Sentrin EST) is a defense factor that can suppress a cell death inducible gene like Bax. By means of transient expression of individual proteins, we identified inhibition of plant PCD (programmed cell death) as the cell death mechanism

**Fig. 3.** Fluorescence distribution of GFP and GFP-tagged BH5-127 in yeast possessing Bax. GFP and MitoTracker (left) are presented. Yeasts were cultured in glucose and galactose media until an OD$_{660}$ of ~0.5 before observation.

**Fig. 4.** Functional characterization of BH5-127 in suppression of cell death caused by high temperature. Yeast cells were transformed to express AtBI-1 (pYX112-AtBI-1), BH5-127 (pYX112-BH5-127), vector (pYX112), or Bax (pYX112-Bax), and then high temperature stressed at 35°C for 72 h.

**Fig. 5.** A model for BH5-127-suppressed FAS in Chinese cabbage. Blunt arrows indicate inhibition. Pointed arrows indicate activation.
of action of BrSE. Given the presumed importance of PCD in abiotic stress-based plant defense, it is logical that the AtBI-1 would target this process to cell death. It is possible that abiotic stress effectors that have been implicated in allowing plant growth to evade abiotic stress-based resistance also function using a similar mechanism. Kim et al. [9] reported the optimum concentration of difenoconazole for growth inhibition of Chinese cabbage for the summer production in the alpine region. This result reports that it is effective in adaptability elevation after implantation by making plug seedlings grow healthy in Chinese cabbage. Therefore, it is possible to develop a variety that can produce seedling of healthy Chinese cabbage if BrSE can be activated to control abiotic stress (high temperature) as investigated in this study.

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References