African Journal of Biotechnology Vol. 11(64), pp. 12721-12727, 9 August, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1782
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Identification and characterization of longevity assurance gene related to stress resistance in *Brassica*

Nasar Uddin Ahmed[1#], Jong-In Park[1#], Hee-Jeong Jung[1], In-Ho Lee[1, 2], In-Ja Song[3], Seung-Yul Yang[1], Kwon-Kyoo Kang[4], Yong-Pyo Lim[5], Yoonkang Hur[6] and Ill-Sup Nou[1]*

[1]Department of Horticulture, Sunchon National University, 413 Jungangno, Suncheon, Jeonnam 540-742, Republic of Korea.
[2]ASIA Seed Co., 447-2, Ihwang-Ri, Janghowon, Icheon, Gyeonggi 467-906, Republic of Korea.
[3]Subtropical Horticulture Research Institute, Jeju National University, Jeju 690-756, Korea.
[4]Department of Horticulture, Hankyong National University, Ansung, Kyonggi-do 456-749, South Korea.
[5]Department of Horticulture, Chungnam National University, Daejeon, 305-764, Republic of Korea.
[6]Department of Biology, Chungnam National University, 305-764, Republic of Korea.

Accepted 23 July, 2012

Brassica **is a very important vegetable group worldwide and different stresses are a major concern for these crops. Enhancement of resistance against biotic and abiotic stresses by exploiting stress resistance related genes offers the most efficient approach to address this concern. In this study, a stress resistance related gene was identified from the full-length cDNA library of** ***Brassica rapa*** **cv. Osome, which was determined to be** ***Brassica*** **longevity assurance protein (*BrLAP*) after sequence analysis. A comparison study of this gene showed a high degree of homology with other stress resistance related longevity assurance genes and was shown to be expressed in all organs during all of the developmental growth stages. In addition, this gene significantly responded after cold, drought and ABA stress treatments in Chinese cabbage. All these data revealed that this gene may be involved in plant resistance against stresses.**

Key words: *Brassica rapa*, longevity assurance gene, gene expression, biotic and abiotic stress.

INTRODUCTION

Brassica is an important group of crops grown worldwide that belongs mainly to the species *Brassica rapa*, as well as *Brassica oleracea* and *Brassica napus* (Collinge and Slusarenko, 1987) and widely recognized for its economic importance and contribution to human nutrition (Salunkhe and Kadam, 1998). Biotic and abiotic stresses are major concern for the sustainable production of these crops. Biotic stresses include fungi, bacteria, viruses, insects and abiotic stresses include cold, salt, ABA, drought, high temperature and heavy metal pollution. Each type of stress functions through different types of molecular mechanisms that affect plants and eventually cause damage. Plants respond to biotic and abiotic stress factors by producing various defense-related proteins such as pathogenesis related (PR) proteins, hydroxyproline-rich glycoproteins (HRGPs), and several key enzymes involved in the synthesis of phenylpropanoids, phytoalexins and lignins (Bowles, 1990; Collinge and Slusarenko, 1987; Dixon and Lamb, 1990).

One promising strategy to develop resistance against biotic and abiotic stresses is based on the exploitation of the PRs and other stress responsive genes in transgenic plants. The longevity assurance gene protein (Lag1p) is located in the endoplasmic reticulum (ER), where it helps to accelerate the transport of glycosylphosphatidylinositol (GPI)-anchored proteins to the Golgi. This function of Lag1p results from its participation in ceramide synthesis. Thus, Lag1p and its homologues are likely to play a role

*Corresponding author. E-mail: nis@sunchon.ac.kr, nis@sunchon.ac.kr. Tel: +82-61-750-3249. Fax: +82-61-750-3208.

#These authors contributed equally to the work.

in ceramide signaling, which affects growth, proliferation, stress resistance, and apoptosis (Jazwinski and Conzelmann, 2002). A longevity assurance gene of tomato, *Asc-1*, which is homologous to the yeast longevity assurance gene *LAG1*, mediates resistance to *Alternaria alternate* f. sp. *lycopersici* toxins and fumonisin B1 of *Fusarium moniliforme* (Brandwagt et al., 2000).

In this study, we analyzed a stress resistance related gene that was identified from the full-length cDNA library of *B. rapa* cv. Osome and studied its homology with other stress resistance related genes. Expression of this gene was analyzed in different organs and developmental growth stages. In addition, expression was also analyzed after applying abiotic stresses in Chinese cabbage and by microarray data of biotic stress and its association with biotic and abiotic stress resistance was also discussed.

MATERIALS AND METHODS

Plant materials

Chinese cabbage (*B. rapa* 'SUN-3061') plants were grown in the Department of Horticulture, Sunchon National University, Korea. Fresh roots, stems, leaves and flower buds were harvested, frozen immediately in liquid nitrogen, and stored at –80°C for RNA isolation.

Stress treatments

Chinese cabbages seeds were aseptically grown on Murashige and Skoog (MS) agar medium in a culture room under a 16 h light photoperiod at 25°C. After 3 weeks of growth, the seedlings were transferred to fresh liquid MSH (half-strength MS medium without sucrose) medium containing 250 mM NaCl and 100 mM abscisic acid (ABA) for 24 h. To induce cold stress, the seedlings were maintained at 4°C for 24 h. Drought treatment was applied by keeping the seedling on the filter paper at 28°C for 24 h. The samples were treated with all the stresses for 0 h (wild type), 30 min, 1, 2, 4, 8 and 24 h and then frozen immediately in liquid nitrogen, and stored at –80°C for RNA isolation.

RNA extraction

Total RNA was extracted from roots, stems, leaves, flower buds and abiotic stress applied frozen samples using the Rneasy mini kit (Qiagen, USA). RNA was treated with RNase-free DNase (Promega, USA) to remove genomic DNA contaminants. The cDNA was synthesized using the Superscript® III First-Strand synthesis kit (invitrogen, USA) according to its instructions.

Sequence analysis of genes

DNA and protein sequences were processed or deduced using Software Editseq (DNAstar Lasergene, USA). ORF was obtained by ORF finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The primary structure of genes was analyzed using ProtParam (http://expasy.org/tools/protparam.html) and Augustus (http://augustus.gobics.de/submission). The alignment search was carried out using BLAST from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The program used was BLASTp and the database "nr" was selected. Typical domains were analyzed using the web tool from EMBL, (http://smart.embl.de/smart/set_mode.cgi?GENOMIC=1). Multiple protein sequences were aligned using PIR (http://pir.georgetown.edu/pirwww/search/multialn.shtml).

Expression analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed using AMV one step RT-PCR kit (Takara, Japan). The specific primers for *BrLAP,* FW- ATGTGGGTTCTTCTGATTTG and RV- CTGCAGATGTAGGAGAGGAC were used for RT-PCR. Actin primers of *Brassica*, FW-ATGGTTGGGATGGGTCAAAAA and RV-TCTTTAATGTCACGGACGATT, were used as the control. PCR was performed using 50 ng of cDNA as templates. In 0.5 ml PCR tubes, 20 *p*mol of each primer, 150 µM of each dNTP, 1.2 U of *Taq* polymerase, 1x *Taq* polymerase buffer, and double-distilled water to a total volume of 20 µl were added and mixed. The PCR procedure included pre-denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and was terminated with an additional extension for 5 min at 72°C. The microarray data on *B. rapa* subsp. *pekinensis* were obtained from the *B. rapa* Genomic Project of National Academy of Agricultural Science (Suwon, Korea) (http://brassica-rapa.org/BRGP/index.jsp).

RESULTS AND DISCUSSION

Identification and sequence analysis

We constructed a full-length cDNA library of *B. rapa cv*. Osome (Park et al., 2010) and obtained 3,429 ESTs from this library. These ESTs were then annotated and 140 biotic stress related genes were selected (unpublished). In this study, we selected one cDNA clone from these biotic stress related genes and sequenced as full-length cDNA. Full-length cDNA encoding biotic stress resistance related gene, designated as *Brassica* longevity assurance protein (*BrLAP*) was characterized. The sequence data for *BrLAP* were deposited in GenBank under accession number of EU186328.

In the sequence analysis, this gene provided complete coverage of the transcribed region and no introns were identified in the coding region. The full-length sequence of the *BrLAP* cDNA contained 1460 nucleotides and had a putative open reading frame (ORF) of 756 bp. The ORF encodes a longevity assurance protein that contained 304 amino acids, beginning at the initiation codon ATG (position 386) and ending at the stop codon TGA (position 1141) of the cDNA (Figure 1A). The deduced amino acid sequence of BrLAP was compared with other related genes from the public database. The primary structure of this gene was analyzed using bioinformatic tools and the obtained data are presented in the Table 1. Previous studies had reported that the longevity assurance protein contains multiple transmembrane domains and a highly conserved motif designated as the Lag1p motif (Jiang et al., 1998). The *BrLAP* protein contained a transmembrane domain between 20 and 40 amino acid residues. It also contained TRAM, LAG1 and CLN8 (TLC) homology domains between 69 and 283

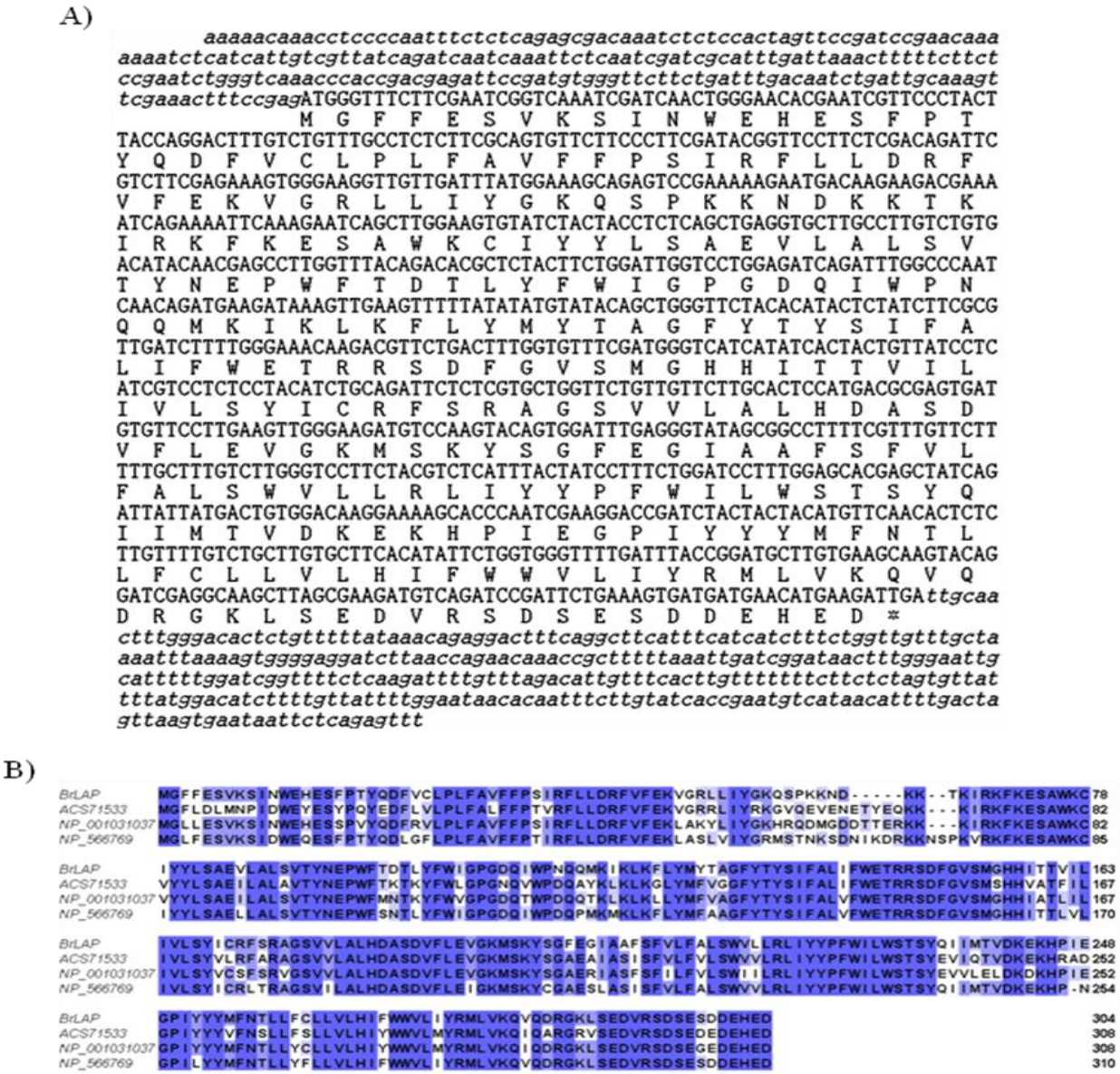

Figure 1. Deduced amino acid sequence and alignment of BrLAP. (A) The cDNA and deduced amino acid sequence, the italic letters indicate UTR region; (B) Alignment of deduced amino acid sequences of BrLAP with 3 other stress responsive genes of *Arabidopsis thaliana* (NP566769 and NP001031037) and *Orobanche cernua* var. *cumana* (ACS71533) using PIR. Numbers on the right margin indicate the positions of amino acid residues. The identical amino acids are marked in dark background, and more than 50% similarities in light.

amino acid residues, which is a protein domain with at least 5 transmembrane alpha-helices suggesting a longevity assurance protein.

Amino acid sequence comparison

The alignment search was carried out using BLAST from NCBI. The deduced protein sequence of BrLAP (ABV89617) showed high homology with the LAG1 longevity assurance-1 protein of *Arabidopsis thaliana* (NP566769 and NP_001031037) (82 and 80% identity) (Salanoubat et al., 2000) and LAG1-like protein 1 of *Orobanche cernua* var. *Cumana* (ACS71533) (74% identity) (de Zélicourt et al., 2009) (Table 2). Multiple alignment also revealed high similarity among the deduced amino acids of BrLAP and 3 other longevity assurance genes (Figure 1B), indicating its relatively conserved evolutionary relationship at the protein level. The mutated longevity assurance gene of *Orobanche cernua* var. *cumana* (ACS71533) showed an enhanced susceptibility to AAL-toxin of *Alternaria alternate*

Table 1. Primary structure analysis of *BrLAP* gene of *Brassica rapa* cv. Osome.

Name of genes	Molecular mass (kDa)	Isoelectric point (pI)	Instability index (II)	Positively charged residue (Arg + Lys)	Negatively charged residue (Asp + Glu)
BrLAP	36.05	7.65	37.26	32	31

*Analyzed using protParam, http://expasy.org/tools/protparam.html.

Table 2. Overall analysis of *BrLAP* gene of *Brassica rapa* cv. Osome.

Gene	Length (aa)	Matched clone	Matched protein	Score	Identity (%)	Top homologous species
BrLAP	304	NP566769	LAG1 longevity assurance-1	530	84	*Arabidopsis thaliana*
		NP001031037	LAG1 longevity assurance homolog 3	526	80	*Arabidopsis thaliana*
		ACS71533	Longivity assuarance LAG1-like protein 1	469	74	*Orobanche cernua var. cumana*

*Analyzed using BLAST from NCBI, http://www.ncbi.nlm.nih.gov/BLAST/.

(de Zélicourt et al., 2009).

Organ specific and growth stage expression analysis

Expression analysis was performed using specific primers with equal amounts of cDNA templates prepared from the mRNA of roots, stems, leaves and flower buds of *B. rapa* 'SUN-3061' by RT-PCR. The expression of *BrLAP* genes was observed in all the organs tested and the highest level of expression was observed in the flower buds followed by the roots, stems and leaves (Figure 2A). These organs share characteristics that make them particularly prone to pathogen attack. Thus, adequate protection of these organs against pathogen attack is critical, and the production of an antifungal molecule could be part of a local defense strategy (Hamel and Bellemare, 1995). Using microarray data, expression of this gene was analyzed at different growth stages and found to be expressed at all growth stages from mature seed to 91 day old plants (Figure 2B).

Expression analysis after applying stresses

Expression of *BrLAP* was also analyzed from the microarray database, where the *B. rapa* plant samples were infected with soft rot disease causing necrotroph bacteria, *Pectobacterium carotovorum* subsp. *carotovorum,* club root disease causing fungus, *Plasmodiophora brassicae* and TMV, which are serious enemy for most of the *Brassica* crops. But the expression of this gene was not responsive to these biotic stresses and indicating that this gene may be responsive to other pathogen infections. The results of previous studies also support the association of this gene with disease resistance, *Asc-1*, a longevity assurance gene, was able to confer resistance to infection by *A. alternata* f.sp. *lycopersici* in *Nicotiana umbratica* (Brandwagt et al., 2002).

In the past decades it has become evident that some genes respond significantly when exposed to various environmental and pathogenic stresses and have been shown to be associated with broad spectrum resistance (Lee et al., 2008). To further explore such resistance, we analyzed the expression of this gene using the specific primers after applying abiotic stress treatments including cold, salt, drought and ABA. The expression of *BrLAP* significantly increased from 0 to 4 h and decreased afterwards with cold stress treatments (Figure 3A). This gene was down-regulated during drought treatments (Figure 3C) but no responsive expression was observed after salt stress treatments (Figure 3B). After ABA stress treatments (Figure 3D), the expression of *BrLAP* gene was increased up to 2 h and decreased afterwards. This expression pattern indicates that

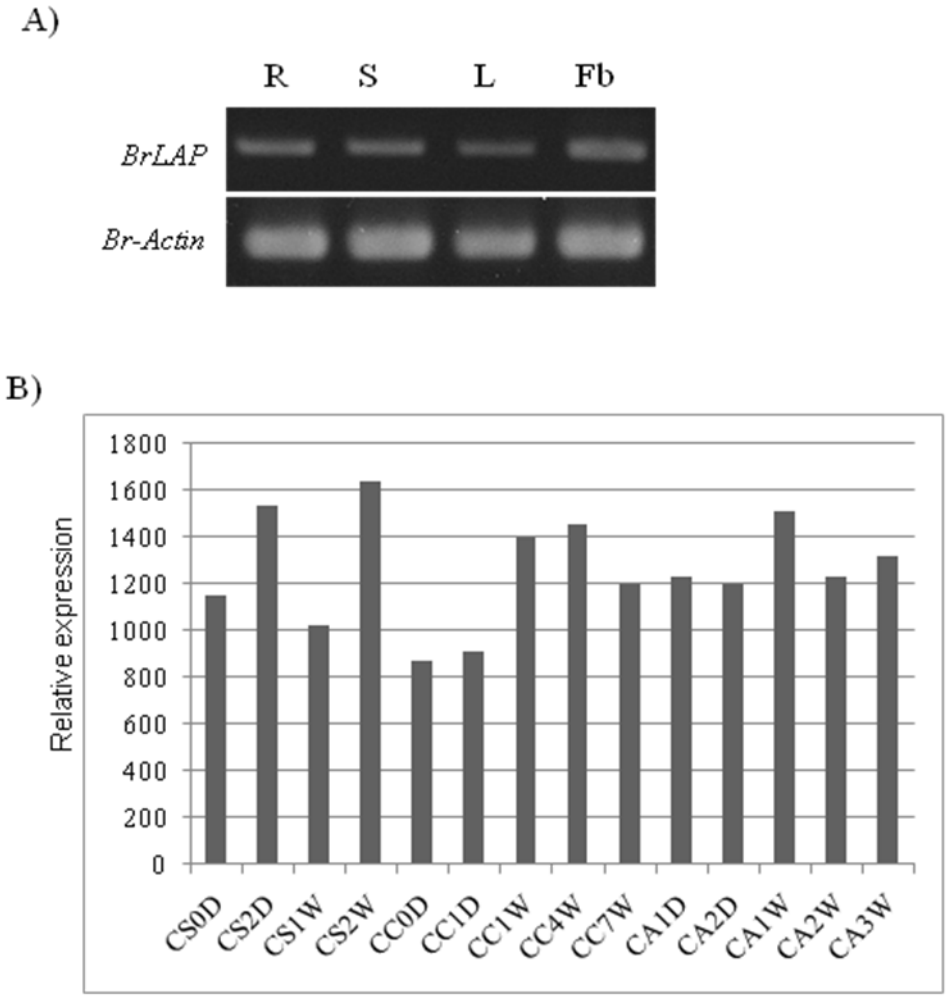

Figure 2. Expression analysis of *BrLAP* genes in healthy *B. rapa* subsp. *pekinensis*. (A) RT-PCR expression over different organs, Lanes 1–4, PCR products of roots (R), stems (S), leaves (L) and flower buds (Fb); (B) Microarray expression analysis at different growth stages, CSOD- seed, mature; CS2D- seedling, 2-days- old; CS1W- whole plant, 1- week old vegetative stage (7 days- old); CS2W- whole plant, 2- week old vegetative stage (14-days- old); CCOD- whole plant, 1 day after light-chilled at 4°C (21- days- old); CC1D- whole plant; 1 day after light- chilled at 4°C (22-days- old); CC1W- whole plant, 1 week after light chilled at 4°C (28-days- old); CC4W- whole plant, 4 weeks after light chilled at 4°C(56- days- old); CC7W- whole plant, 7 weeks after light chilled at 4°C (70- days - old); CA1D- whole plant, 1 day after green house growth (71- days- old); CA2D- whole plant, 2 days after green house growth (72- day- old); CA1W- whole plant, 1 week after green house growth (77-days- old); CA2W- whole plant, 2 weeks after green house growth (84-days- old); CA3W- whole plant, 3 weeks after green house growth (91-days- old).

these genes are most likely associated with abiotic stress resistance and we can exploit this gene for overexpression or down-regulation to enhance resistance against abiotic stresses in *Brassica*. In earlier studies, down-regulation of two genes, homologous to aspartic proteinase 2, and homolog of Jumonji class of transcription factor showed relative drought tolerant phenotypes in *Nicotiana benthamiana* (Senthil-Kumar et al., 2007).

Function of longevity assurance gene results from its participation in ceramide synthesis. The sphinganine-analog mycotoxins (SAMs) induce apoptotic cell death by inhibiting ceramide biosynthesis (Gilchrist et al., 1995; Spassieva et al., 2002; Wang et al., 1996). For example, insensitivity to the AAL-toxin (SAM) in resistant tomatoes and other plants is conferred by the *Asc1* gene, a homolog of the yeast ceramide synthase gene *Lag1* (Brandwagt et al., 2000, 2002). Other studies in *Saccharomyces cerevisae* have revealed that Lag1p and Lac1p are essential for the sphinganine N-acyl

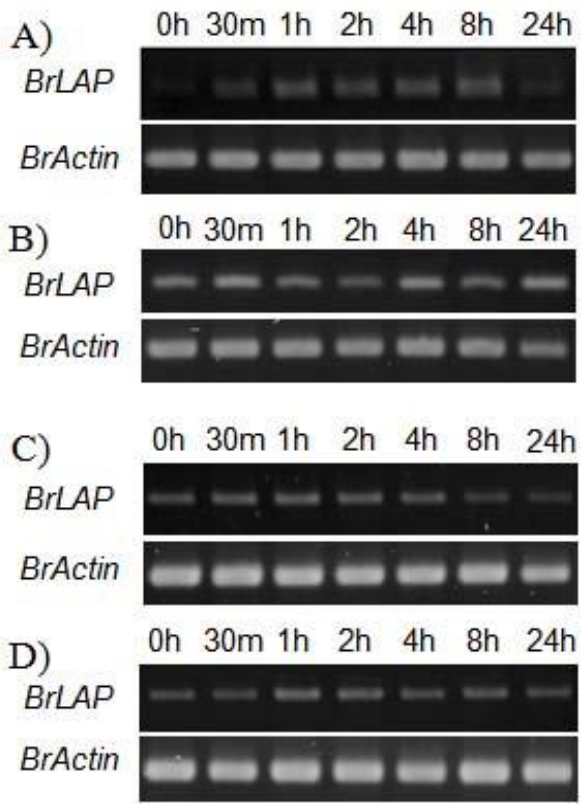

Figure 3. RT-PCR expression analysis of *BrLAP* gene after application of (A) cold, (B) salt, (C) drought and (D) ABA stresses.

transferase reaction in yeast (Guillas et al., 2001; Schorling et al., 2001). This coupled with the fact that AAL-toxin specifically inhibits sphinganine N-acyltransferase and it seems highly probable that *Asc-1* will be important for plant sphingolipid biosynthesis. To date, only circumstantial evidence exists for a role of sphingolipids in plant programmed cell death. Thus, longevity assurance and its homologues are likely to play a role in ceramide signaling, which affects growth, proliferation, stress resistance, and apoptosis (Jazwinski and Conzelmann, 2002). Taken together, it can be concluded that *BrLAP* gene may be a useful gene source for engineering transgenic plants resistant to biotic and abiotic stresses in *Brassica*.

ACKNOWLEDGEMENT

This work was supported by a grant from the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center No. PJ009085022012), Rural Development Administration, Republic of Korea.

REFERENCES

Bowles DJ (1990). Defense-related proteins in higher plants. Annu. Rev. Biochem. 59:873-907.

Brandwagt BF, Kneppers T, Nijkamp HJ, Hille J (2002). Overexpression of the tomato Asc-1 gene mediates high insensitivity to AAL toxins and fumonisin B (1) in tomato hairy roots and confers resistance to Alternaria alternata f.sp. lycopersici in Nicotiana umbratica plants. Mol. Plant–Microbe Interact. 15:35-42.

Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000). A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternate* f. sp*. lycopersici* toxins and fumonisin B1. PNAS. 97:4961-4966.

Collinge DB, Slusarenko AJ (1987). Plant gene expression in response to pathogens. Plant Mol. Biol. 9:389-410.

de Zélicourt A, Montiel G, Pouvreau JB, Thoiron S, Delgrange S, Simier P, Delavault P (2009). Susceptibility of Phelipanche and Orobanche species to AAL-toxin. Planta 230(5):1047-1055.

Dixon RA, Lamb CJ (1990). Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:339-367.

Gilchrist DG, Bostock RM, Wang H (1995). Sphingosine related mycotoxins in plant and animal disease. Can. J. Bot. 73:S459-S467.

Guillas I, Kirchman PA, Chuard R, Pfefferli M, Jiang JC, Jazwinski SM, Conzelmann A (2001). C26-CoA-dependent ceramide synthesis of *Saccharomyces cerevisiae* is operated by Lag1p and Lac1p. EMBO. J. 20:2655-2665.

Hamel F, Bellemare G (1995). Characterization of a class I chitinase gene and of wound-inducible, root and flower-specific chitinase expression in *Brassica napus*. Biochim. Biophys. Acta 1263(3):212-220.

Jazwinski SM, Conzelmann A (2002). Molecules in focus LAG1 puts the focus on ceramide signaling. Int. J. Biochem. Cell Biol. 34:1491-1495.

Jiang JC, Kirchman PA, Zagulski M, Hunt J, Jazwinski SM (1998).

Homologs of the yeast longevity gene LAG1 in *Caenorhabditis elegans* and human. Genome Res. 8:1259-1272.
Lee SC, Hwang IS, Choi HW, Hwang BK (2008). Involvement of the Pepper Antimicrobial Protein CaAMP1 Gene in Broad Spectrum Disease Resistance. Plant Physiol. 148:1004-1020.
Park JI, Kumar TS, Ahmed NU, Nou IS (2010). Construction of full-Length cDNA library and investigation of potential genes in *Brassica rapa*. J. Basic Life Res. Sci. 10:6-11.
Salanoubat M, Lemcke K, Rieger M, Ansorge W, Unseld M, Fartmann B, Valle G, Blöcker H (2000). Sequence and analysis of chromosome 3 of the plant *Arabidopsis thaliana*. Nature 408(6814):820-822.
Salunkhe DK, Kadam SS (1998). Handbook of vegetable science technology: production, composition, storage, and processing. Marcel Dekker Inc., New York.
Schorling S, Vallee B, Barz WP, Riezman H, Oesterhelt D (2001). Lag1p and Lac1p are essential for the Acyl-CoA-dependent ceramide synthase reaction in *Saccharomyces cerevisae*. Mol. Biol. Cell 12:3417-3427.
Senthil-Kumar M, Govind G, Kang L, Mysore KS, Udayakumar M (2007). Functional characterization of *Nicotiana benthamiana* homologs of peanut water deficit-induced genes by virus-induced gene silencing. Planta. 225:523-539.
Spassieva SD, Markham JE, Hille J (2002). The plant disease resistance gene *Asc-1* prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant J. 32:561-572.
Wang W, Jones C, Ciacci-Zanella J, Holt T, Gilchrist DG (1996). Fumonisins and *Alternaria alternata lycopersici* toxins: sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. Proc. Natl. Acad. Sci. USA 93:3461-3465.