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# BnNHL18A shows a localization change by stress-inducing chemical treatments

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**Abstract**

The two genes, named *BnNHL18A* and *BnNHL18B*, showing sequence homology with *Arabidopsis NDR1/HIN1-like* (*NHL*) genes, were isolated from cDNA library prepared with oilseed rape (*Brassica napus*) seedlings treated with NaCl. The transcript level of *BnNHL18A* was increased by sodium chloride, ethephon, hydrogen peroxide, methyl jasmonate, or salicylic acid treatment. The coding regions of *BnNHL18A* and *BnNHL18B* contain a sarcolipin (SLN)-like sequence. Analysis of the localization of smGFP fusion proteins showed that BnNHL18A is mainly localized to endoplasmic reticulum (ER). This result suggests that the SLN-like sequence plays a role in retaining proteins in ER membrane in plants. In response to NaCl, hydrogen peroxide, ethephon, and salicylic acid treatments, the protein localization of BnNHL18A was changed. Our findings suggest a common function of BnNHL18A in biotic and abiotic stresses, and demonstrate the presence of the shared mechanism of protein translocalization between the responses to plant pathogen and to osmotic stress.


*Keywords:* *Brassica napus*; *HIN1*; Hypersensitive response; *NDR1*; Osmotic stress; Sarcolipin

Higher plant has an efficient surveillance system based on disease resistance (*R*) gene to recognize avirulence (Avr) factors displayed by pathogens, and the pathogen recognition results in activation of multi-faceted defense response, including the so-called hypersensitive response (HR) [1]. A defense-associated gene in tobacco, *harpin-induced gene 1* (*HIN1*), was isolated and shown to be induced by harpins and bacteria (e.g., *Pseudomonas syringae*) that elicit hypersensitive response [2]. The *Arabidopsis* gene, *nonrace-specific disease resistance gene 1* (*NDR1*), shows sequence similarity to *HIN1* and is involved in some *R*-gene-mediated resistance to both the bacterial pathogen *P. syringae* pv. *tomato* and the fungal pathogen *Peronospora parastica* [3].

The *Arabidopsis* genome contains 45 genes that show sequence similarity to *NDR1* and *HIN1* [4]. These genes have been designated as *NHL* (*NDR1/HIN1-like*) 1–45 and grouped into several subclasses on the basis of sequence similarities [4,5]. NHL proteins share three conserved sequence motif of unknown function [5], which are unique to this family of proteins [4]. Among the three conserved motifs, two motifs are highly conserved (motif 2, NPNKRIGIYYD; motif 3, PFYQGHKN) and the other is less conserved (motif 1, LILWLILRPXKPKFXVQDATV) [5]. Analysis of amino acid sequence reveals NHL proteins have one or two putative transmembrane-spanning regions [5]. Subcellular localization studies showed that NHL3 and NDR1 are localized to plasma membrane and are glycosylated [6,7]. Both proteins are predicted to have an uncleavable N-terminal signal anchor sequence [6,7].

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There are several papers reporting the roles of some members of *NHL* gene family in plant–pathogen interaction. Mutation of *NDR1* gene causes susceptibility to both bacterial and fungal pathogen [3]. Overexpression of *NHL2* in transgenic *Arabidopsis* plants results in elevated levels of *PR-1* expression and light-dependent 'speck disease-like' symptoms in the leaves [5]. *NHL10* is up-regulated in the hypersensitive response to *Cucumber mosaic virus* infection and is specifically induced in an incompatible plant–bacterial pathogen interaction [4]. *NHL25* and *NHL3* transcripts also accumulate specifically during infection with avirulent bacterial pathogen strains [7]. *NHL3* overexpressing plants show increased resistance to *P. syringae* pv. *tomato* DC3000 [7].

Newly synthesized membrane proteins enter the endoplasmic reticulum (ER) in unfolded state, where they undergo modifications such as glycosylation, disulfide bond formation, and assembly into oligomers [8]. They are then transported through a series of membrane-bound compartments which include the various cisternae of the Golgi complex, where further carbohydration modification occurs [8]. As a default pathway, membrane proteins move from ER to the plasma membrane or to the vacuole membrane [9]. For resident proteins in the ER, specific signals and mechanisms are necessary to retain the proteins within the ER [8,9]. For the integral membrane proteins, a cytosolic di-lysine motif, KKxx or xKxK, located near the C-terminus has been identified as ER retention signal for the type 1 membrane proteins (N-terminus in the lumen), whereas the di-arginine motif xRRx, located near the N-terminus, is involved in ER retention for the type II membrane proteins (C-terminus in the lumen) [9,10].

Sarcolipin (SLN) is a 31 amino acid transmembrane protein that regulates $Ca^{2+}$-ATPase of fast-twitch skeletal muscle (SERCA1a) function [10–12]. The SLN can be divided into three domains: a hydrophilic N-terminal domain of 7 amino acids exposed to cytosol, a hydrophobic transmembrane helical domain of 19 amino acids, and a hydrophilic C-terminal lumenal domain consisting of 5 amino acids [10,13]. SLN is localized to ER, although it does not contain the previously characterized ER localization signals. Gramolini et al. [10] reported that the SLN retention in ER depends on it C-terminal RSYQY sequence and its interaction with endoplasmic SERCAs. In the absence of SERCA, retention of SLN in the ER is mediated through its association with other components, such as ER luminal chaperone proteins [10].

In this study, we described the characterization of oilseed rape (*Brassica napus*) *NHL* genes, named *BnNHL18A* and *BnNHL18B*. The proteins encoded by *BnNHL18s* contain an SLN-like sequence. We showed that BnNHL18A::GFP fusion protein was localized to ER, but osmotic stress and hydrogen peroxide, and the hormones related to plant defense signaling pathways changed the protein localization from ER. Our findings suggest BnNHL18A functions in a shared mechanism between a response to plant pathogen and a response to osmotic stress.

## Materials and methods

*Plant materials and chemical treatments.* Oilseed rape (*B. napus*) seeds were purchased at the local market. The seeds were surface sterilized in 70% (v/v) ethanol for 2 min, soaked in 15% (v/v) bleach for 15 min, and then rinsed four to five times in sterile water. Shaking culture growth medium (40 ml; one-strength Murashige and Skoog basal salt mixture salts [Sigma, Australia], 1% [w/v] sucrose, and 1 mM Mes [pH 5.7]) was inoculated with approximately 30 sterile seeds. Cultures were grown for 5 days, with 18 h of light/6 h of dark at 23 °C, with shaking at 80 rpm.

For chemical treatments, 5-day-old seedlings were harvested and hydroponically treated with 200 mM NaCl, 100 μM abscisic acid, 1 mM ethephon ([2-chloroethyl]phosphonic acid), 1 mM hydrogen peroxide, 50 μM methyl jasmonate, or 1 mM salicylic acid for various time points and then frozen in liquid nitrogen for further analyses.

*cDNA library screening.* A cDNA library was constructed from RNA extracted from oilseed rape seedlings treated with 200 mM NaCl for 1 and 3 h. Approximately $1 \times 10^5$ clones were screened from the cDNA library by using a $^{32}$P-labeled partial cDNA fragment obtained previously from differential display [14]. Hybridization was performed according to a published protocol [15]. After hybridization, the membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at room temperature for 10 min twice, with 0.1× SSC containing 0.1% SDS at room temperature for 10 min, and with 0.1× SSC containing 0.1% SDS at 65 °C for 5 min. The membranes were exposed to X-ray film with an intensifying screen at −70 °C for 48 h. Positive clones were excised with the helper phage and recircularized to generate a subclone in the pBluescript SK-phagemid vector (Stratagene, La Jolla, CA).

*RNA gel blot analysis.* Total RNA was extracted from 5-day-old seedlings with TRI REAGENT as described in the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Ten micrograms of total RNA was electrophoresed on a 1.5% agarose gel containing 6% formaldehyde in 3-(*N*-morpholino)-propanesulfonic acid buffer, pH 7.0 and transferred to a Nytran Plus membrane (Amersham Life Science, Buckinghamshire, England). Hybridization was performed with a $^{32}$P-labeled DNA probe according to a published protocol [16], and the membranes were washed with 2× SSC at room temperature for 10 min twice, with 0.1× SSC containing 0.1% SDS at room temperature for 10 min, and with 0.1× SSC containing 0.1% SDS at 65 °C for 5 min. The membranes were visualized directly with the BAS-2500 Phosphorimager (Fuji Photo Film, Tokyo, Japan).

The probes used in this study were prepared from an *Acc*I digestion fragment (247 bp) of full-length *BnNHL18A* cDNA (GenBank Accession No. DQ205338) harbored in pBluescript.

*Vector construction for expression of GFP and RFP fusion proteins and imaging the fusion proteins.* The termination codon of the *BnNHL18A* cDNA was removed after PCR using specific oligonucleotide primers, 5′-GGGATCCATGTCAAAAGATTGCGGCAACC ACGGT-3′ and 5′-TGGATCCCAACGTTCACACTGCATTTAGTAAC-3′. The resulting fragment was fused in-frame to the coding region of *smGFP* [16]. A *BiP::RFP* construct was kindly provided by Dr. I. Hwang [17].

*Arabidopsis* protoplasts were transfected by a method described by Jin et al. [18] with minor modification. Briefly, leaf tissues (5 g) of 3-week-old *Arabidopsis* seedlings grown on agar plate were cut into small squares (5–10 mm$^2$) with a sharp razor blade and incubated with 25 ml enzyme solution [0.25% macerozyme (Yakult Honsha, Tokyo, Japan) R-10, 1.0% cellulase (Yakult Honsha) R-10, 400 mM mannitol, 8 mM $CaCl_2$, and 5 mM Mes–KOH, pH 5.6] at 23 °C for 5 h with gentle agitation (50–75 rpm). After incubation, the protoplast suspension was filtered through 100 μm mesh and protoplasts were collected by centrifugation at 46*g* for 5 min. The pelleted protoplasts were resuspended in 5–10 ml W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes–KOH, pH 5.6), overlaid on top of 20 ml of 21% sucrose, and centrifuged for 10 min at 78*g*. The intact protoplasts at the interface were transferred to a new Falcon tube containing 20 ml W5 solution. The protoplasts were pelleted again by centrifugation at 55*g* for 5 min and

resuspended in 20 ml W5 solution. The protoplasts were then incubated on ice for 30 min.

To transform DNA into protoplasts, protoplasts were pelleted again at 46*g* for 5 min and resuspended in MaMg solution (400 mM mannitol, 15 mM $MgCl_2$, and 5 mM Mes–KOH, pH 5.6) at a density of $5 \times 10^6$ protoplasts/ml. Plasmid DNA (20–50 μg total at a concentration of 2 mg/ml) was added to 300 μl of protoplast suspension followed by 325 μl PEG solution (400 mM mannitol, 100 mM $Ca(NO_3)_2$, and 40% polyethylene glycol 4000). The mixture was incubated for 30 min at room temperature. After incubation, the mixture was diluted with 10 ml W5 solution. Protoplasts were recovered by centrifugation at 50*g* for 5 min, resuspended in 3 ml W5 solution, and incubated at 22 °C in the dark. Expression of protein was monitored at various time points after transformation, and images were captured with a cooled charge-coupled device camera using a Zeiss Axioplan fluorescence microscope. The filter sets used were XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23), XF33/E (exciter, 535DF35; dichroic, 570DRLP; emitter, 605DF50), and XF137 (exciter, 540AF30; dichroic, 570DRLP; emitter, 585ALP) (Omega, Brattleboro, VT) for GFP, RFP, and autofluorescence of chlorophyll, respectively. Data were then processed using Adobe Photoshop software (Mountain View, CA), and the images were rendered in pseudocolor.

## Results

### *Isolation and sequence analysis of the BnNHL18 cDNA clones*

We isolated several salt-stress induced cDNA fragments from oilseed rape by mRNA differential display [14]. One of the fragments showed the strongest homology with *NHL18* (loci number, At3g52470), a member of *NHL* gene family in *Arabidopsis*. Using this differential display-derived fragment as a probe, we obtained two cDNA clones from oilseed rape cDNA library. One cDNA (1054 bp), designated *BnNHL18A* (GenBank Accession No. DQ205338), has additional 3′ 258 bp UTR region compared with the second cDNA (796 bp), named *BnNHL18B* (GenBank Accession No. DQ205339). Translation of *BnNHL18A* cDNA sequence predicts a protein with a molecular mass of 23 kDa. At deduced amino acid sequence level, BnNHL18A was found to be identical with BnNHL18B, except for two amino acid substitutions.

In *Arabidopsis*, 45 *NHL* genes can be categorized into four classes (the class 1, 2, and 3, and a class that encodes proteins showing weak homology with each other) and the class 1 is further divided into two subclasses [4]. The deduced protein sequences of *BnNHL18A* and *BnNHL18B* are clustered with the NHL proteins which belong to a subclass in the class 1 (Fig. 1A). Fig. 1B shows an alignment of BnNHL18A and BnNHL18B with the previously characterized *Arabidopsis* NHL proteins and tobacco HIN1 (NtHIN1). The three conserved motifs among *Arabidopsis* NHL proteins are also presented in BnNHL18 proteins (Fig. 1B). Since BnNHL18B has near identical sequence to BnNHL18A, further study was done with BnNHL18A.

Similar to NDR1 and NHL3, BnNHL18A was predicted to be a membrane protein. The computational analysis using transmembrane prediction programs, DAS-TMfilter (http://mendel.imp.univie.ac.at/sat/DAS/DAS.html) [19] and THUMBUP (http://www.smbs.buffalo.edu/phys_bio/service.htm) [20] indicated a single putative transmembrane helix domain (residues 20–41) (*E* value of 2.076e−11 for DAS-TMfilter; 100% probability for THUMBUP) (Fig. 1B). This domain was also predicted as an uncleavable signal anchor by SignalP-3.0 (www.cbs.dtu.dk/services/SignalP) [21]. According to a prediction from THUMBUP, BnNHL18A was suggested as a type II membrane protein having an N-terminal cytoplasmic domain (residues 1–19).

Interestingly, database searches revealed that the near N-terminal region of BnNHL18A (residues 16–46) contains a sarcolipin (SLN)-like sequence. This putative amino acid stretch is 32% identical to sarcolipin from human and partially overlapped with the motif 1 (Fig. 1B). In addition to the SLN-like sequence in the near N-terminus, the remaining parts (residues 47–208) of BnNHL18A can be aligned with *Arabidopsis* syntaxin-related proteins, SYP24 (GenBank Accession No. AAG601780) and AtPLP/SYP23 (GenBank Accession No. O04378), although their primary structures revealed only limited overall homology (data not shown).

### *Expression patterns of the BnNHL18A under various stress conditions*

RNA blot analysis was used to determine the steady-state levels of *BnNHL18A* transcripts in *B. napus* seedlings at various time points following treatments with 200 mM NaCl, 100 μM abscisic acid (ABA), 1 mM ethephon (an ethylene releasing compound), 1 mM $H_2O_2$, 50 μM methyl jasmonate (MeJA) or 1 mM salicylic acid (SA) (Fig. 2). The blots were hybridized with radio-labeled probe for 3′ UTR region of *BnNHL18A*.

Upon NaCl treatment, *BnNHL18A* expression was rapidly induced within 30 min by twofold. However, treatment with ABA did not induce elevated level of the *BnNHL18A* transcript. ethephon, $H_2O_2$, MeJa or SA treatment also caused a *BnNHL18A* gene expression; maximum induction was 3.4-fold at 1 h for ethephon, 3.3-fold at 2 h for $H_2O_2$, 2.3-fold at 2 h for MeJa, and 3.4-fold at 24 h for SA treatment. *BnNHL18A* mRNA began to accumulate at 1 h after ethephon treatment and the transcripts gradually declined. For the methyl jasmonate and $H_2O_2$ treatments, expression pattern of *BnNHL18A* gene was similar to that of ethephon treatment. However, the maximal accumulations of *BnNHL18* mRNA were observed at the later time point of 2 h. Upon SA treatments, accumulation of *BnNHL18* transcript was continuously increased throughout examined time points. The differences in the kinetics of *BnNHL18A* induction suggest that *BnNHL18A* gene expression is differentially regulated by the individual stress signal.

### *Subcellular localization of BnNHL18A*

The amino acid sequence of BnNHL18A was shown to contain a membrane-spanning region in the N-terminus of

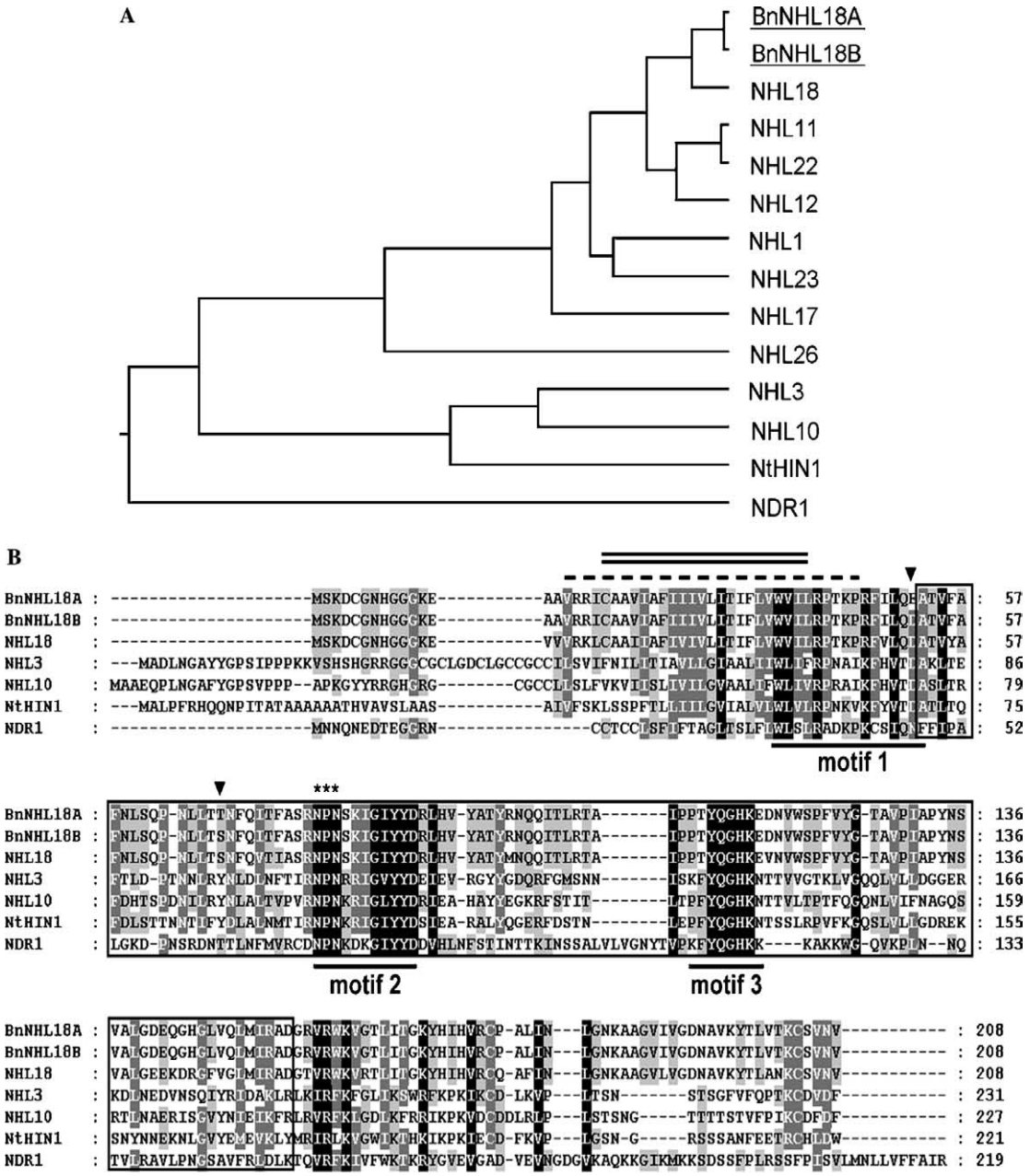


Fig. 1. (A) Phylogenetic relationship of NHL proteins in *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*) HIN1 with the BnNHL18A and BnNHL18B proteins. BnNHL18A and BnNHL18B are underlined. The following NHL gene products were subjected to analysis: NHL1 (At3g11660), NHL3 (At5g06320), NHL10 (At2g35980), NHL11 (At2g35970), NHL12 (At2g35960), NHL17 (At3g44220), NHL18 (At3g52470), NHL22 (At4g09590), NHL23 (At5g06330), NHL26 (At5g53730), NDR1 (At3g20600), and NtHIN1 (CAA68848). (B) Alignment of the amino acid sequences of BnNHL18A and other members of NHL protein family. The WHy domains are boxed and an invariable NPN motif at their N-terminus is marked by asterisks. The three conserved sequence motifs among NHL proteins are indicated by bold lines. A sarcolipin-like sequence is indicated by a dashed line above the sequence of BnNHL18A. The hydrophobic anchor sequence is indicated by double underline. Arrowheads indicate the amino acid residues showing differences between BnNHL18A and BnNHL18B. The sequence alignment was generated using the MegAlign program (DNASTAR, Madison, WI).

this protein. To determine the subcellular localization of BnNHL18A, the coding region of *BnNHL18A* was introduced into 5′-terminus of *smGFP* used as fluorescent marker under the control of the CaMV 35S promoter. The resulting construct was introduced into *Arabidopsis* protoplasts following the polyethylene glycol (PEG)-mediated transformation method. Also we used an smGFP fusion protein with *Arabidopsis* BiP as a marker for the ER localization. The transient expression of BnNHL18A::GFP showed that the fusion protein was enriched in ER (Figs. 3A, i–iii) with the same pattern of fluorescence signal as BiP::GFP (Figs. 3A, iv–vi), which was distributed homogeneously throughout the ER network with a higher concentration in the cytosolic region where ER network densely packed. Whereas smGFP control protein was found throughout the entire cytoplasm (Figs. 3A, vii–ix). In order

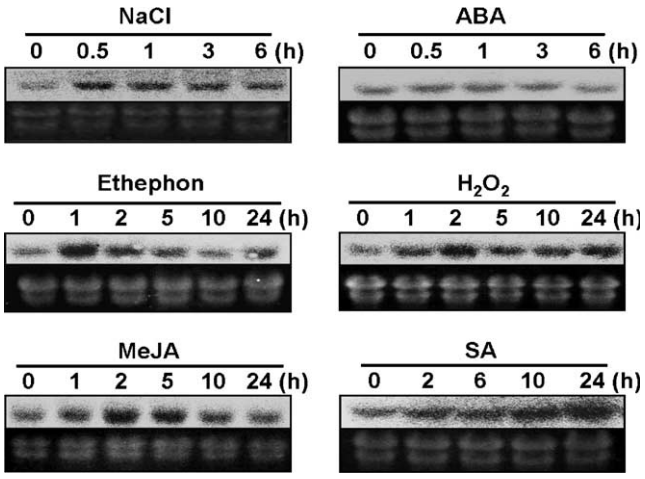


Fig. 2. Induction pattern of *BnNHL18A* gene upon treatment of NaCl, abscisic acid (ABA), $H_2O_2$, ethephon, methyl jasmonic acid (MeJA), or salicylic acid (SA). *B. napus* seedlings were treated with 200 mM NaCl, 100 μM ABA, 1 mM ethephon, 1 mM $H_2O_2$, 50 μM MeJA, and 1 mM SA, and harvested at the designated time points for total RNA extraction. For RNA gel blot analysis, 10 μg of total RNA was applied to each lane. Equal loading was verified by ethidium bromide-stained gel.

to compare the localization of BnNHL18 with that of BiP at this time, we co-expressed the red fluorescence protein (RFP) fused BiP fusion protein with BnNHL18A::smGFP in *Arabidopsis* protoplasts. Figs. 3A (x–xiii) show the GFP signals of the BnNHL18A::smGFP were colocalized with RFP signals of BiP::RFP, confirming that the BnNHL18A::smGFP protein localized to the ER. These in vivo localization results indicate the BnNHL18A are present primarily in the ER, suggesting that function of this protein is associated with ER.

Since BnNHL18A does not contain ER retention signals, BnNHL18A had been suspected to be localized to the plasma membrane without being confined in ER, as shown in NDR1 and NHL3 [6,7]. In this respect, it was of great interest that database searches revealed that the near N-terminal region of BnNHL18A (residues 16–46) contains a SLN-like sequence (Figs. 1B and 3B). Especially, the membrane-spanning domain region of BnNHL18A is 47% identical to that of human SLN, for which sequence is conserved among SLNs from other vertebrates. However, NHL3 and NDR1 which are localized in plasma

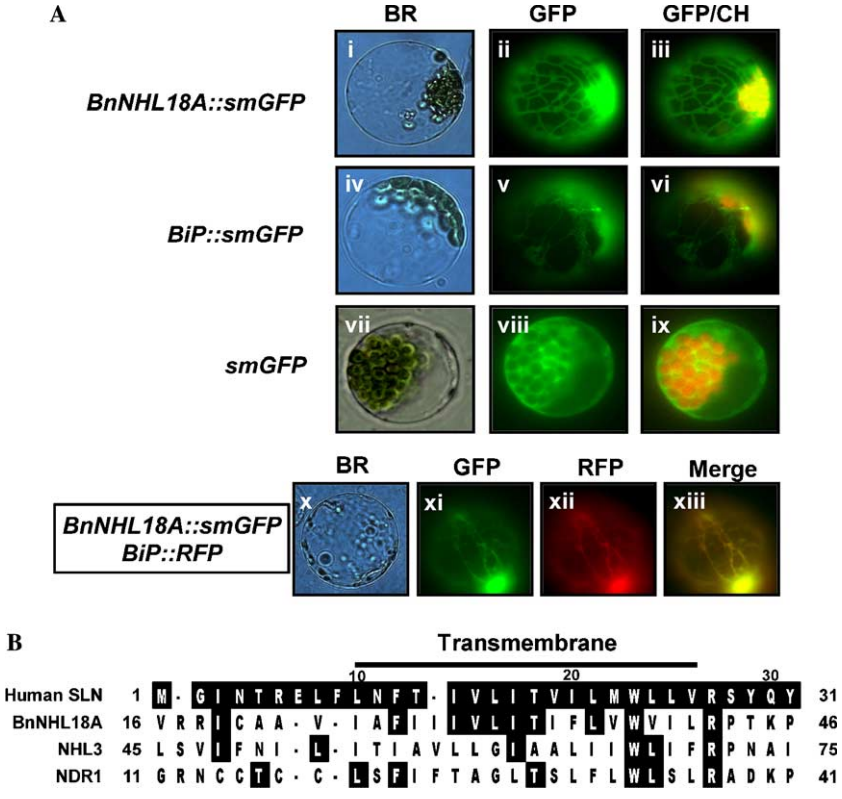


Fig. 3. (A) Localization of BnNHL18A::smGFP to the ER. *Arabidopsis* protoplasts were independently transformed with the plasmids containing *BnNHL18A::smGFP* (i–iii), *BiP::smGFP* (iv–vi), or *smGFP* (vii–ix) and co-transformed with the plasmid harboring *BnNHL18A::smGFP* and *BiP::RFP* (x–xiii). (ii, v, viii, and xi) smGFP signal (GFP) in the transformed protoplasts. (iii, vi, and ix) The overlaps of signals from smGFP and chlorophyll (GFP/CH). (i, iv, vii, and x) Bright-field images (BR). xii shows an RFP signal (RFP) of Bip::RFP. (xiii) A merged image of xi and xii (Merge). These are representative data of protoplasts that expressed these fusion proteins 16 h after transformation. (B) Alignment of SLN sequence in human (GenBank Accession No. NP003054) with SLN-like amino acid sequence in BnNHL18A and membrane-spanning region of NHL3 and NDR1. The bar indicates a transmembrane domain of human SLN. Note a conservation of a short sequence (Ile-Val-Leu-Ile-Thr; IVLIT) and a Leu at positions 14–18 and 21, respectively, for human SLN, and those at positions 29–33 and 36, respectively, for BnNHL18A.

membrane show 18% and 29% identity to this domain, respectively (Fig. 3B). Therefore, we suppose that ER retention of BnNHL18A might be mediated by the SLN-like sequence.

*Changes in localization of BnNHL18A in the response to stress-related chemicals*

Since *BnNHL18A* expression was increased by the stress-related treatments (Fig. 2) and BnNHL18A showed sequence homology with the syntaxins which are involved in protein trafficking, we attempted to test whether the stress-related treatments could change the ER localization of BnNHL18. Time-lapse changes in the localization of BnNHL18A::smGFP signals were monitored with *Arabidopsis* protoplasts in culture media containing 50 mM NaCl or 100 μM $H_2O_2$. Figs. 4A and B show the multi-channel time-lapse imaging of the *Arabidopsis* protoplasts. Treatment with 50 mM NaCl or 100 μM $H_2O_2$ caused the fusion protein to translocate to the cytosolic region, showing depletion of signals from peripherals of ER network (30–120 min in Figs. 4A and B).

Before the treatments (0 min in Fig. 4), the part of the GFP signals of BnNHL18A and BiP fusion proteins were shown as net- or knotted mesh-like structures uniformly distributed in the region overlapped with the vacuolar space, which were observed typically spherical, fully transparent, and colorless in the bright-field light (indicated with V in Fig. 4A). That showed the location of the BnNHL18A proteins in the ER network embosoming vacuole. However, after the onset of stresses the net-like structures became fainter and the pores of 'mesh' were growing larger (30–120 min in Figs. 4A and B). After all, majority of the GFP signals in the vacuolar space disappeared (120 min in Figs. 4A and B). Whereas, the remaining portion of GFP signals of BnNHL18A fusion protein in the cytosolic region where ER is densely packed with chloroplasts emitting red auto-fluorescence remained unchanged, suggesting that the disappearance of signal was not due to the protein degradation.

It was noticeable that the fluorescence micrograph time-lapse series also showed small discrete patches of GFP signals (arrows in Fig. 4B), likely to be protein aggregates, moved into cytosolic region upon treatments. On the contrary, the protoplast transformed with BiP::GFP revealed that BiP located invariably in ER network during the stress-inducing treatment, indicating the integrity of ER structure remained intact and excluding a nonspecific effect of the treatments (Fig. 4C). Taken together, these results indicate that the localization of BnNHL18A was changed in the response to NaCl and $H_2O_2$ treatments. However, even though BnNHL18A::smGFP fusion protein was translocated outside of ER, we could not assign the final destined location of BnNHL18A to cytosol or other membranous compartments based on the present experiments. Further studies also should be done to find out whether this protein indeed contains a syntaxin-like activity.

To gain further insights into the mechanism(s) underlying the BnNHL18 translocation from ER, we investigated the effect of other stress-related hormones. The protein translocation in *Arabidopsis* protoplast with BnNHL18::smGFP was observed after treatments of following chemicals:

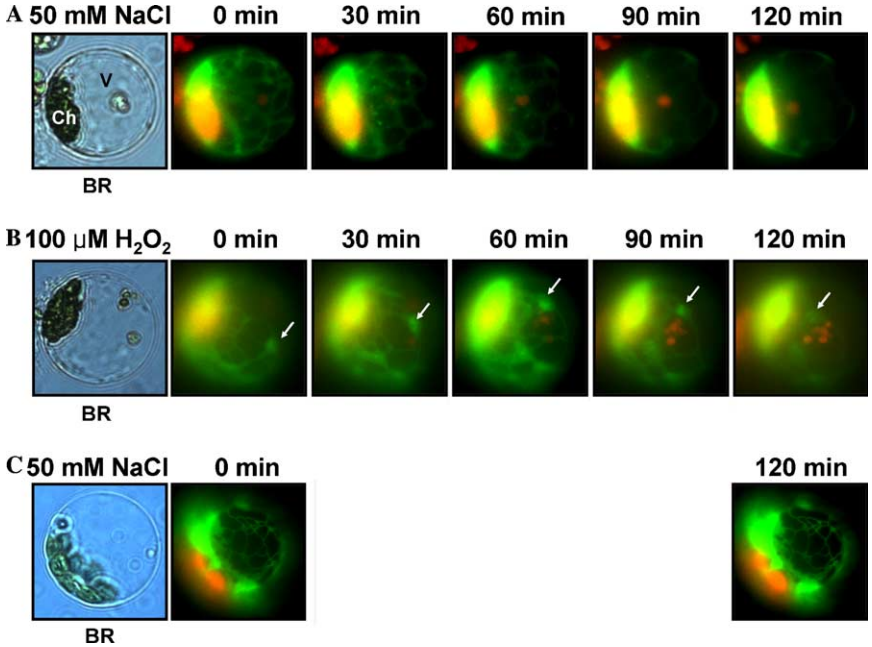


Fig. 4. The effect of NaCl (A) and $H_2O_2$ (B) on the subcellular localization change of BnNHL18A::smGFP. Protoplasts were treated with 50 mM NaCl or 100 μM $H_2O_2$ at 16 h after transfection. The time-lapse changes in the localization of the fusion protein were examined at indicated time points. Fluorescent patterns of BiP::GFP (C) were also examined for comparison. Arrows indicate the discrete GFP signals suggesting the BnNHL18A::smGFP translocalization from ER. V, vacuole; Ch, chloroplast; BR, bright-field image.

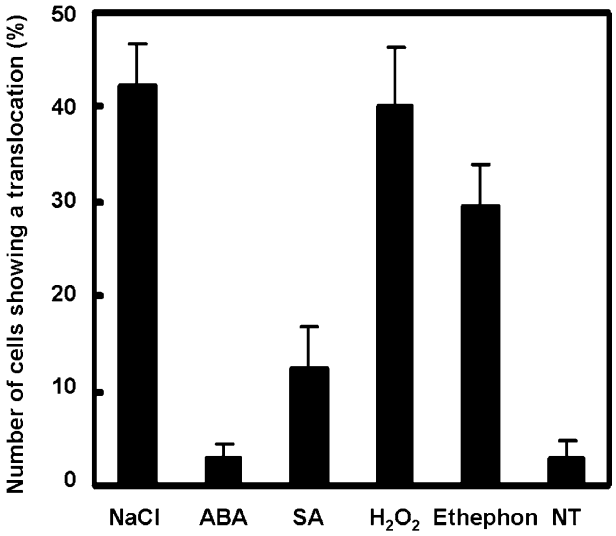


Fig. 5. The number of protoplasts showing the translocalization of the BnNHL18A::GFP upon various chemical treatments. Protoplasts transfected with plasmid harboring the *BnNHL18A::GFP* were subjected to treatments with the indicated chemicals (50 mM NaCl [NaCl], 100 μM ABA [ABA], 100 μM SA [SA], 100 μM $H_2O_2$ [$H_2O_2$], 100 μM ethephon [Ethephon], or no treatment [NT]) and counted after 2 h post-treatments (18 h after transfection). At least 50 cells were used for each measurement. Experiments were performed in triplicate. The error bars represent standard deviations.

ABA, SA, $H_2O_2$ or ethephon in a concentration of 100 μM each, or 50 mM NaCl. As a quantitative assay, we counted cells in which the net-like GFP signals were completely dissipated after 2 h post-treatments. As shown in Fig. 5, more than 30% of transfected cells showed the translocation in the response to ethephon as well as osmotic and oxidative stresses. SA also affected the BnNHL18A translocation to a lesser extent but significantly. ABA did not have influence on the translocation.

## Discussion

In this study, we isolated two *B. napus NHL* genes, named *BnNHL18A* and *BnNHL18B*, which contain SLN-like sequence. Using the GFP fusion protein, we showed that BnNHL18A was localized to ER. The results infer that the SLN-like sequence may play a role in retaining BnNHL18A in ER. We also showed that BnNHL18A expression was induced by various treatments including osmotic stress and chemicals that are closely related to plant defense responses. Moreover, osmotic stress treatments change the localization of the fusion protein. The localization change of BnNHL18A was also mediated by biotic stress-related chemicals.

These results suggest a common function of BnNHL18A in biotic and abiotic stresses, and demonstrate the presence of the shared mechanism of protein translocalization between the responses to plant pathogen and to osmotic stress. This is novel, since the known functions of NHL proteins are limited to plant–pathogen interaction and senescence. Recently, Ciccarelli and Bork [22] identified a protein module, named WHy domain (for water stress and hypersensitive response domain), that links NHL proteins to the plant family LEA-14, which is expressed under water stress conditions and during late embryogenesis. The detection of shared domain in two families of proteins suggested a common molecular mechanism between the plant response to pathogen, HR, and a response to water stress [22].

To our knowledge, there has been no report which shows that a protein containing SLN-like sequence localized to the ER in plants. Gramolini et al. [10] reported that SLN retention in ER is dependent on its C-terminal RSYQY sequence (residues 27–31). In the immuno-microscopic study with the serial deletion constructs of SLN, they showed that the shortest sequence of SLN for the localization into ER is only N-terminal 27 amino acids. Moreover, in the case of the SLN constructs without the Arg-27, very little immuno-staining was produced in ER; the signal was enriched at plasma membrane and was diffused in cytosol [10]. The SLN-like sequence in BnNHL18A does not contain the conserved sequence corresponding to SLN (for BnNHL18A, RPTKP, residues 41–46). However, it is noteworthy that both proteins do share an Arg (residue 41 for BnNHL18A and residue 27 for SLN) at the identical position related to the transmembrane domain (Fig. 3B). Besides, in the presence of SERCA2a, the SLN mutants without RSYQY sequence were able to be localized in ER [10], suggesting the importance of transmembrane domain sequence for ER retention. The transmembrane domain is conserved among SLNs from vertebrates. The transmembrane domain region of BnNHL18A is 47% identical to that of human SLN. However, NHL3 and NDR1 which are localized in plasma membrane show 18% and 29% identity to this domain. It was suggested that the different NHL family members are not located in the same organelles [4]. Therefore, it will be interesting to study whether the motif 1, the less conserved sequence shared among NHL proteins, and its N-terminal flanking sequence contribute to the heterogeneity in NHL protein localization.

The observation of BnNHL18A translocation from ER after various stress-inducing treatments is reminiscent of the ATF6 translocation upon ER stress in animal system. The bZIP transcription factor ATF6 is a major mediator of transcriptional induction by ER stress [23]. An unusual property of the transcription factor ATF6 is that it exists as an ER membrane-tethered precursor regulated by intramembrane proteolysis [24–26]. ATF6 is a type II ER transmembrane protein with its C-terminus located in the ER lumen and its N-terminal DNA binding domain facing the cytosol [24,25]. In the response to ER stress, ATF6 translocates from ER to the Golgi where it is processed to its active form [26,27]. The trafficking is controlled by the ER chaperone BiP/GRP78 which binds to the lumenal domain of ATF6 [24]. BiP expression has been shown to respond to variety of abiotic and biotic stress conditions, such as water stress, fungus infestation, insect attack,

nutritional stress, cold acclimation, and elicitors of the plant pathogenesis response [28]. Interestingly, Gramolini et al. [10] revealed BiP/GRP78 interact with SLN. Therefore, there is a close analogy between translocation mechanism of BnNHL18A and ATF6. We suppose that BnNHL18A translocation might be mediated by stress-responsive chaperons which associated with SLN-like sequence and/or other sequence(s) in BnNHL18A. Knowledge of BnNHL18A localization and its movements at the subcellular level will provide insight into the elucidating the molecular functions of NHL proteins.

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