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Identification of Low Temperature Inducible Genes of *Lactuca sativa* by Using Suppression Subtractive Hybridization Method

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Key message Genes encoding vacuolar processing enzymes leading to program cell death were induced by low temperature in lettuce.

Lettuce (*Lactuca sativa)* is one of important vegetables taken as raw state. It is expected to be kept as high quality under low temperature conditions, especially freezing state. However, there are few research regarding to its molecular response to abiotic stress of lettuce. As we reported previously, lettuce plants acquire low levels of freeze tolerance. In the present paper, we have isolated 192 cDNA clones corresponding to cold induced genes of lettuce plant by using a PCR–based suppression subtractive hybridization method. Most clones were categorized into 62 distinct known genes based on homology search. Out of the corresponding genes, 45 genes were confirmed to be low–temperature–inducible with reverse transcription-qPCR. Some of the genes encoded stress–related proteins, such as late embryogenesis abundant proteins including dehydrin, which were expected to be involved in enhancement of freezing tolerance. On the other hand, some of proteins encoded by genes were suspected to be involved in suppressing the enhancement of freezing tolerance, such as vacuolar processing enzyme (VPE), adagio protein, and gigantea–like protein. In particular, VPE have been reported to be associated with program cell death, suggesting that it is negatively involved in freezing tolerance of lettuce.

Key words: cDNA subtractive subtraction, low–temperature–inducible gene, freezing tolerance, lettuce

INTRODUCTION

Environmental stress, such as drought, cold, and heat, to plants have threatened the stable production and supply of agricultural produce such as crops and vegetables. Although a representative leafy vegetable, lettuce, is grown under relatively cool conditions, there are not many reports concerning its response to environmental changes such as abiotic stress. Several reports regarding response of lettuce to drought or salt stress are found (Garrido *et al*., 2013; Leyva *et al*., 2011; Porcel *et al*., 2006), however there are few reports concerning responses of lettuce to cold stress.

Recently, we reported that low temperature treatment of lettuce led to acquisition of the tolerance (Honjoh *et al*., 2018). Expression of C–repeat/dehydration–responsive–element binding factor (CBF) and galactinol synthase (GolS) genes were induced in lettuce during low temperature treatment. These genes are well known as low–temperature–inducible genes in plants which acquire freezing tolerance (Gilmour *et al*., 1998; Taji *et al*., 2002). As described above, there is little information of low–temperature–inducible genes in lettuce. Generally, since lettuce plants are well known as a freezing sensitive vegetable, identification of low–temperature–inducible genes in lettuce might lead to identification of factors that suppress the development of freezing tolerance, as well as factors that enhance cold tolerance.

In the present paper, in order to identify genes that affect the strength of freezing tolerance, an expressed sequence tag (EST) library of genes specifically expressed in lettuce during low temperature treatment was generated using a PCR–based suppression subtractive hybridization (SSH) method. The isolated EST clones were sequenced and searched for homologous genes in databases. The expression patterns of functionally identified genes corresponding to ESTs were analyzed by RT–qPCR and the involvement of the genes in freezing tolerance was discussed.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *Lactuca sativa* L. (Papa lettuce) were obtained from Nakahara Seeds Co. Ltd. (Fukuoka, Japan). The seeds were disinfected with 70% ethanol for 10 – 15 s, and then dipped in 0.2% sodium hypochlorite solution for 10 min. The seeds were then rinsed three times with sterile water. The seeds were sown in pots (7.5 cm diameter) with soil and lettuce plants were cultivated in a growth chamber (model MLR–350;

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SANYO, Co. Ltd., Tokyo, Japan) at 22°C under a photosynthetic photon flux density of about 70 μmol/m^{2}s with a 16 h photoperiod. Sterilized tap water was poured to the soil 2–3 days intervals.

Low temperature treatment

Lettuce plants grown for 2 weeks were incubated in a refrigerator (model SPR–T1281; SANYO) at 2°C for indicated period under a photosynthetic photon flux density of about 70 μmol/m^{2}s with a 16 h photoperiod. After this treatment, lettuce plants were used for following freezing treatment and a several leaves were harvested for preparation of total RNA. Harvested leaves were immediately frozen in liquid nitrogen and stored at –80°C until preparation of total RNA. Low temperature treatments and sampling for performing qPCR experiments were independently done three times.

Preparation of poly (A)$^+$RNA

The frozen leaves were grounded in liquid nitrogen using mortar and pestle until the leaves became powder. Total RNA was prepared from this grounded powder using RNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions.

Poly (A)$^+$RNA was purified from total RNA using Oligotex™–dT30 <Super> mRNA Purification Kit (Takara, Kyoto, Japan), according to the manufacturer's instruction. The purified poly (A)$^+$RNA was dissolved in RNase–free water and the concentration of RNA solution was adjusted to 0.5 μg/μl.

cDNA subtraction

In order to obtain ESTs corresponding to low temperature inducible genes from lettuce leaves, a PCR–based cDNA subtraction method was done by the use of BD Clontech PCR–Select™ cDNA Subtraction Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the instruction manual. Tester cDNA was prepared from 2 μg of poly (A)$^+$RNA derived from 72–h low temperature treated lettuce leaves. Driver cDNA was made from non–treated lettuce leaves. At the end of the protocol, the PCR products were subcloned into a pGEM®–T easy vector (Promega, Madison, WI, USA).

DNA sequencing and homology analysis

Determination of nucleotide sequences of the subcloned cDNA was directly performed by a contractor (Macrogen Japan, Tokyo, Japan). DNA sequences were analyzed in both directions. The obtained nucleotide sequences were identified by comparing with those of nr and dBEST databases using the BlastX or the BlastN programs (Altschul *et al*., 1997) on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST) at the threshold e–value of 10^{-5} or better. The ESTs were grouped into functional categories either manually according to the classification method of Bevan *et al*. (1998).

Primer design for qPCR and identity of the PCR-products

Primer pairs for reverse transcription–quantitative PCR (RT–qPCR) were designed using Primer 3 Software (https://bioinfo.ut.ee/primer3–0.4.0/). By using the primers, RT–qPCR was performed with *Ex–Taq* DNA polymerase (TaKaRa). Gene–specific primers were designed so that the resulting PCR product had the size of 70–300 bp (Table 1). The quality of PCR products was visually inspected by agarose gel electrophoresis, the generation of only one band of the expected size was taken as a criterion for specificity. The identity of the PCR products was confirmed by subcloning each product into a pGEM®–T easy vector (Promega), followed by sequencing of the inserts.

RT-qPCR

In order to ensure the transcriptional up-regulation, RT–qPCR analyses were performed. Poly (A)$^+$RNAs were prepared from 0, 6, 12, 24, and 72 h low temperature treated lettuce leaves according to the method described above. Two micrograms of each poly(A)$^+$RNA were reverse–transcribed using Rever Tra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. The synthesized cDNA was used as a template for RT–qPCR. Quantification of gene–specific cDNA was performed on Mx3000P™ Real–Time PCR System (Agilent Technologies Japan, Ltd., Hachioji, Tokyo, Japan) with THUNDERBIRD® SYBR® qPCR Mix (TOYOBO) according to the manufacturer's protocol. Each reaction was performed on 2 μl of 1:10 (v/v) dilution of the first cDNA strands with 0.3 μM of each primer in a total reaction of 20 μl. The qPCR was performed as follows: 40 cycles of 15 s at 95°C, 15 s at 55°C, and 60 s at 72°C. The specificity of the PCR amplification procedures was checked with a heat dissociation protocol (from 55°C to 95°C) after the final cycle of the PCR. Poly (A)$^+$RNA solutions were used in place of the RT reaction mixture in order to confirm the absence of genomic DNA in the RNA solution. Each reaction was done in duplicate for determination of corresponding threshold cycle (C_T) values. The relative levels of transcription were calculated by normalization of expression level of *actin* gene from lettuce (Accession number as mRNA, AY260165) as internal standards. All qPCR experiments were done using three independent biological samples and results are expressed as the mean values ± standard deviations. Genes, which showed relative expression levels over 2 at any low temperature treatment times, were defined as low temperature inducible.

Statistical analysis

For comparison between control and low–temperature–treated samples, all the data for expression levels of the genes were normalized by logarithmic transformation and then analyzed as using one–way analysis of variance (ANOVA), followed by the Dunnett multiple comparison post hoc test. Effects of low temperature treatment time on the expression levels of the genes were evaluated with Jonckheere–Terpstra Test. Statistical analysis was performed using EZR, which is a graphical user interface for R (The R Foundation for Statistical

Computing, Vienna, Austria, version 4.2.0) (Kanda, 2013).

RESULTS AND DISCUSSION

Isolation of EST clones for low temperature inducible genes and their categorization based on nucleotide sequence

An SSH–EST library, which was enriched with cDNAs corresponding to low temperature inducible genes, was constructed. Two hundred sixty clones were obtained as EST clones. All clones were sequenced and searched for homologous genes. Out of them, 192 clones showed similarity to the genes registered in databases and were categorized into 62 distinct groups based on the functions of the proteins, which were encoded by the clones, according to the classification method of Bevan *et al*. (1998).

In order to study the involvement of 62 genes corresponding to the categorized EST clones in freezing tolerance of lettuce, the expression levels of the genes during low temperature treatment were investigated with RT–qPCR. Out of them, 45 genes were confirmed to be low temperature inducible genes, although their expression patterns were diverse (Fig. 1). On the other hand, relative expression levels of other 17 genes were not enhanced (data not shown), so the possibilities of their low–temperature–inducibilities and their involvement in

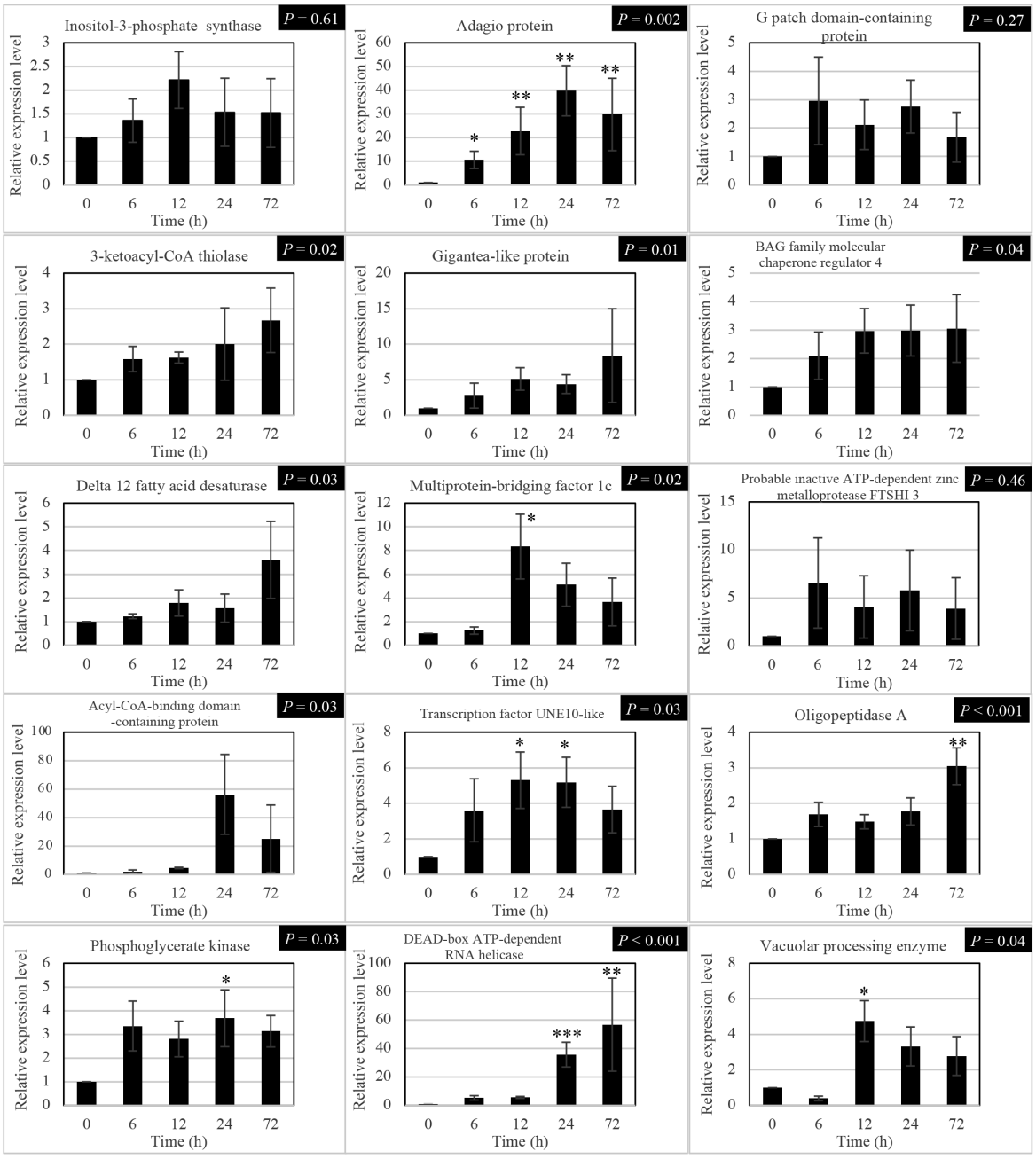
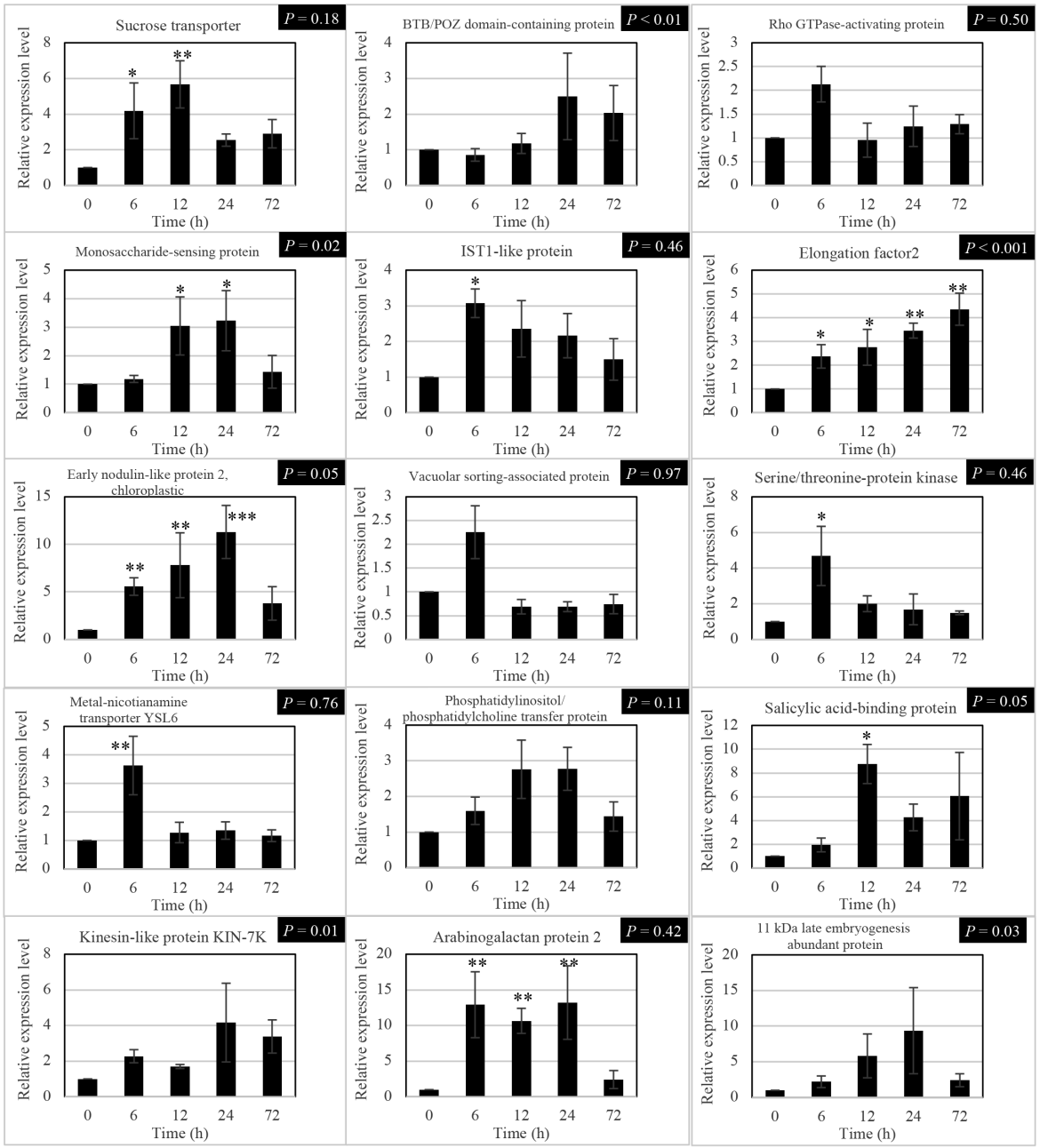


Fig. 1. Expression patterns of low–temperature–inducible genes in lettuce during low temperature treatment. Data values are the means ± SD of three independent biological samples. The asterisks indicate significant differences vs control (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). Effects of low temperature treatment time on the expression levels of the genes were evaluated with Jonckheere–Terpstra test and the results were shown with P values.

Fig. 1. Continued.



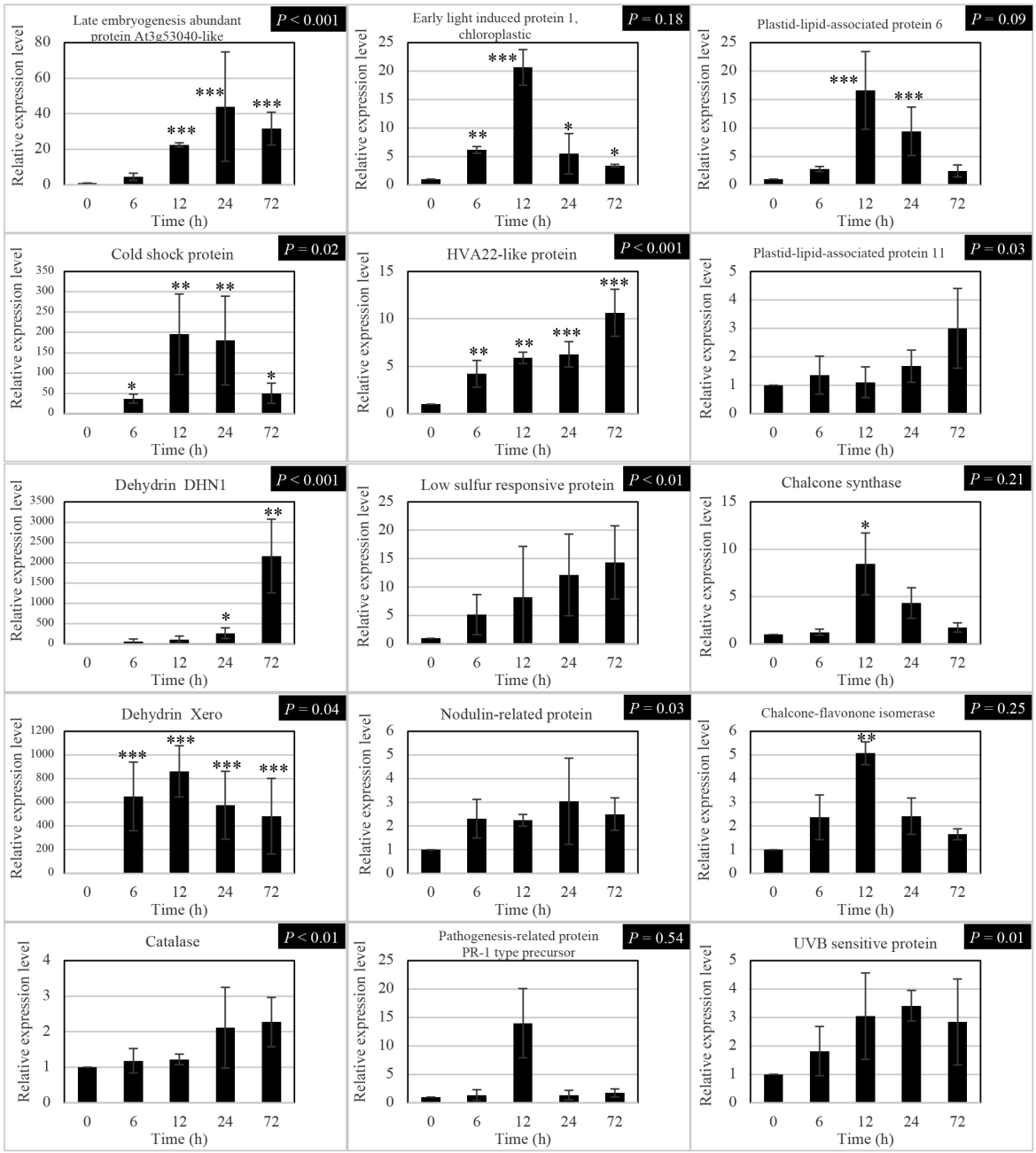
the freezing tolerance of lettuce plants were considered low. The nucleotide sequences of the ESTs corresponding to genes that were confirmed to be low temperature inducible were deposited to DDBJ with accession numbers shown in Table 1.

The clones, which were confirmed to be low temperature inducible, were categorized into 13 intracellular functions as follows: metabolism, energy, cell growth/division, transcription, protein destination and storage, transporters, intracellular traffic, cell structure, signal transduction, disease/defense, unclear classification, secondary metabolism based on their putative functions (Bevan *et al.*, 1998). In particular, the "disease/defense" category contained 13 distinct deduced proteins. The second most abundant category was "signal transduction" and "transporters" which respectively contained five distinct deduced proteins. Other categories were as follows: "protein destination and storage" (four distinct deduced proteins), "transcription" (four distinct deduced proteins), "intracellular traffic" (four distinct deduced proteins), "metabolism" (three distinct deduced proteins), "secondary metabolism" (two distinct deduced proteins), "cell growth/division" (two distinct deduced proteins), "energy" (one deduced protein), "cell structure" (one deduced protein), "unclear classification" (one deduced protein), and "unknown". The involvement of freezing tolerance of the categorized genes were discussed as follows.

Effects of duration of low temperature treatment on expression levels of the genes

As shown in Fig. 1, expression patterns of genes were diverse. By using a statistical method with Jonckheere–Terpstra Tests, effects of duration of low temperature

Fig. 1. Continued.



treatment on expression levels of the corresponding genes were investigated. Some genes were transiently induced during low temperature treatment ($P > 0.05$) and the other most genes were continuously along with duration of the treatment ($P < 0.05$). Expression levels of some genes, encoding such as 3–ketoacyl CoA thiolase, gigantea–like protein, delta 12 fatty acid desaturase etc. in treated samples gradually increased but did not show any significant differences to those in the non-treated sample.

Involvement of "metabolism"–categorized genes in freezing tolerance

In the category of "metabolism", six ESTs individually encoded a part of three proteins, whose amino acid sequences were respectively similar to amino acid sequence of inositol–3–phosphate synthase (*myo*–inositol phosphate synthase; 7 clones), 3–ketoacyl–CoA thiolase (four clones), acyl–CoA–binding domain–containing protein (one clone), or delta 12 fatty acid desaturase (FAD2; one clone).

Inositol–3–phosphate synthase (*myo*–inositol phosphate synthase: MIPS) is the key enzyme of *myo*–inositol synthesis (Tan *et al*., 2013b). *Myo*–inositol may function as a compatible solute for protection against abiotic stress and can also be converted to other compatible solutes (Tan *et al*., 2013b). Furthermore, *myo*-inositol induces the expression of galactinol synthase gene of *Medicago falcata* (Zhuo *et al*., 2013). As we previously showed, low temperature enhanced the expression levels of two *GolS* genes (Honjoh *et al*., 2018). Thus, MIPS would play an important role for enhancement of freezing tolerance in lettuce.

3–ketoacyl–CoA thiolase (KAT) is an important

Table 1. Summary of EST clones corresponding to low–temperature–inducible genes in lettuce and primers used for RT–qPCR. Data values are the means ± SD of three independent biological samples. The asterisks indicate significant differences (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Category	Accession number	Isolated clone numbers	Homologous gene (Accession number in GenBank)	Formward primer	Reverse primer	Amplicon (bp)
Metabolism	HX999310	7	Inositol-3-phosphate synthase (XM_023911192)	5'-AACATTGCCCTTTGTTTTGC-3'	5'-CTTTTGGCTGCTCCAATCAT-3'	197
	HX999313	4	3-ketoacyl-CoA thiolase 2 (XM_023880635)	5'-CGTTGTGTTGCGACTCTGTT-3'	5'-GCCACCCTTGCACACTTATT-3'	245
	HX999319	1	Delta 12 fatty acid desaturase (XM_023904690)	5'-TTCCCGCGTCGGAGATAAAG-3'	5'-ACTCCAACACCGGGTCAATC-3'	261
	HX999323	1	Acyl-CoA-binding domain-containing protein (XM_023880835)	5'-ACAGAGCTCAGGTGGGGTAT-3'	5'-AGCGTTATTTGGTTTTTGGGCA-3'	73
Energy	HX999315	1	Phosphoglycerate kinase (XM_023881287)	5'-GATGGCTGGATGGGATTAGA-3'	5'-CACTAAGCTCCGCCAGTTTC-3'	166
Cell growth/division	HX999300	1	Adagio protein (XM_023914869)	5'-ACGGAACTCCATTGGTCAAC-3'	5'-GATTGGACGTCGTTGTTGTG-3'	163
	HX999325	3	Gigantea-like protein (XM_023887441)	5'-CGGCAACCGACACTTGAGAA-3'	5'-GCTGGAATGGGGAGAATCCG-3'	70
Transcription	HX999298	5	Multiprotein-bridging factor 1c (XM_023880219)	5'-CGCCGTCGAATTTCTTCACC-3'	5'-GCTCCACAAATCCAGACCCA-3'	107
	HX999320	1	Transcription factor UNE-10 (XM_023899894)	5'-GACCCATTCTCCGGTCGAAA-3'	5'-ACGAGCAATCTGCTCAGGTC-3'	117
	HX999330	1	DEAD-box ATP-dependent RNA helicase (XM_023875246)	5'-GATACCGTAGGCTGCTGCTC-3'	5'-ACATGAGACAAAATTGGAGGGA-3'	136
	HX999335	1	G-patch domain containing protein (XM_023885233)	5'-GAGAGAAATGCACGGAAGCAG-3'	5'-TCTCTTTTTCCTGGCGCAAC-3'	71
Protein destination and storage	HX999295	1	BAG family molecular chaperone regulator 4-like (XM_023902522)	5'-ACCGGCAACGAAGTTGAACA-3'	5'-AGTGGATTGGGGAAGGACGA-3'	99
	HX999296	1	Probable inactive ATP-dependent zinc metallo-protease FTSHI 3, chloroplastic (XM_023892841)	5'-CTTGACTGCCCAAGCAATCG-3'	5'-AGCGGTTGAAAGGGCAAAAC-3'	114
	HX999307	1	Oligopeptidase A (XM_023896127)	5'-CGCTTGAGGAAGACAGGTTC-3'	5'-GTCTCTCTCACCGCCTTGTC-3'	160
	HX999331	22	Vacuolar processing enzyme (XM_023901824)	5'-CGCATGCTTTTGTTTCAAGA-3'	5'-CAAAGGTGGAAGTGGAAAGG-3'	154
Transporter	HX999303	1	Sucrose transporter (XM_023907593)	5'-TTTAGTGTCCCTTGCGCTCT-3'	5'-TAAATTGCCACCACCAAACA-3'	159
	HX999306	2	Monosaccharide-sensing protein 2-like (XM_023906419)	5'-ATCGCGTGGGTGTTTGTGTT-3'	5'-AATCAGTTACCCTTTGCGGC-3'	119
	HX999329	2	Early nodulin-like protein 2 (XM_023894333)	5'-CTCCAACAGGTAATCCGTCTG-3'	5'-GAGTCAGTGAGCGAGGAAGC-3'	150
	HX999332	1	Probable metal-nicotianamine transporter (XM_023894681)	5'-GCCTTTTCATCATCGCGTCA-3'	5'-GAACATCGATTTCGCGGACG-3'	158
	HX999338	2	Kinesin-like protein KIN-7K (XM_023912350)	5'-AGGACGAAAGAACAGGTGGA-3'	5'-ACCCACATATTCGCAAGCTC-3'	176
Intracellular traffic	HX999301	2	BTB/POZ domain-containing protein (XM_023916806)	5'-ATAAGCAAGCCTGGGCAGTT-3'	5'-TGATATCGTCCGTTGTGGGG-3'	109
	HX999318	1	IST1-like protein (XM_023905074)	5'-GTTTTCCGGCGACAACCATC-3'	5'-AGATTGTTCACCAGGGCTCC-3'	223
	HX999328	1	Vacuolar sorting-associated protein 32 homolog 2 (XM_023886650)	5'-GAAGGCTTCTGCGGAGGTAG-3'	5'-TTCGTTGCTTTCTGCATGGC-3'	240
	HX999337	1	Phosphatidylinositol/phosphatidylcholine transfer protein (XM_023887226)	5'-AGGATGCCAAAAGGACCGAG-3'	5'-GCGACAAACAGCAGCATTCA-3'	215
Cell structure	HX999309	2	Arabinogalactan protein 2 (XM_023879790)	5'-AGTGCAGTCCCAGCGATAAC-3'	5'-CTGATCTTCCACCTAGCGGC-3'	70
Signal transduction	HX999297	1	Probable Rho GTPase-activating protein (XM_023872885)	5'-ACCAGGACTTGAATGCACCA-3'	5'-TAATGGCTGAGGAAGGCTCG-3'	95
	HX999299	2	Elongation factor 2 (XM_023900272)	5'-TGCCCGACGTGTGATCTATG-3'	5'-GCTCCGGTGCTTGAATTTCC-3'	89
	HX999304	4	Serine/threonine-protein kinase (XM_023909656)	5'-TACCTTCTCGACTGGGCGTA-3'	5'-TGATGACATTGGTGGGCGAA-3'	162
	HX999336	1	Salicylic acid-binding protein (XM_023884658)	5'-ACACCGCTAGAAGCTTGGTT-3'	5'-TTTTGCCGTGGCTAGGTCTT-3'	195
Disease/defense	HX999321	2	11 kDa late embryogenesis abundant protein-like (XM_023888546)	5'-GTAGAGCGTGACCAAAACGC-3'	5'-TACCAATCGGGTGAGACCCA-3'	187
	HX999314	3	Late embryogenesis abundant protein At3g53040-like (XM_023902984)	5'-AGAAACCGCTGACGTTGCTA-3'	5'-TCGCTCTTGCCAATTGTTGC-3'	297
	HX999312	1	Cold shock protein (XM_023885330)	5'-GGAGGCCACCATGGAGTTAG-3'	5'-GGGGGACGCTTTGATGCT-3'	130
	HX999308	2	Dehydrin DHN1-like (XM_023909772)	5'-TGGTGGTCATAACACGGACG-3'	5'-TGGGTTTCCCCTTCCCCATA-3'	81
	HX999334	10	Dehydrin Xero 2 (XM_023885323)	5'-CCCACTTCACTCTAGCACCC-3'	5'-CCCATAACCACCGCCACTAA-3'	208
	HX999302	2	Catalase (XM_023874935)	5'-CACCCATGAGATCCGCACAA-3'	5'-TGGCTTCACATTCAAGCGAG-3'	94
	HX999317	5	Early light inducible protein 1, chloroplastic (XM_023878313)	5'-GCAACTCCCATCACACTCCT-3'	5'-TAAACGCCAACACGTCAGAG-3'	190
	HX999316	1	HVA22-like protein (XM_023891438)	5'-ACGATCAGCAGTGGCTTTCT-3'	5'-GACGACGACGATGATTTGTG-3'	244
	HX999322	1	Low sulfur responsive protein (XM_023901232)	5'-ATCCGTCGGTGAAAGACCAC-3'	5'-CCCCTAGCTGCGAACAAAGA-3'	198
	HX999327	4	Nodulin-related protein (XM_023876698)	5'-CCAAGGAGTGGGGCAGTATC-3'	5'-GTTGTAGCACTAGCACCGGA-3'	78
	HX999294	1	Pathogenesis-related protein PR-1 type-like (XM_023883232)	5'-ACTCCCAAAACCGTCCCTAT-3'	5'-ACCGAATTGCTCCAAACAAC-3'	178
	HX999333	1	Plastid-lipid-associated protein 6 (XM_023882531)	5'-GGCCATCGACCAATAGAGGA-3'	5'-GTTCACCCCTATCACCTCTTGT-3'	87
	HX999326	1	Plastid-lipid-associated protein 11 (XM_023899888)	5'-GAGTCGATGGCGTCAGTGAT-3'	5'-AAACCCACCGAACCCTAACC-3'	157
Secondary metabolism	HX999324	9	Chalcone synthase (XM_023879789)	5'-GTAGGCCGAGGAGCTTAGTG-3'	5'-GTCCCCAAGCTCGGTAAAGA-3'	148
	HX999305	1	Chalcone-flavonone isomerase (XM_023915225)	5'-AGACAAGAAGATCACAGCCCG-3'	5'-GCGTACTCTCCTTCGACCATT-3'	147
Unclear classification	HX999311	1	Root UVB sensitive protein 5 (XM_023873941)	5'-AACGTGCCCGGATATTGGTT-3'	5'-CACTTCGAAGCACCGAGAGA-3'	300
Control for RT-qPCR		–	Actin (AY260165)	5'-TTGTGAGCAACTGGGATGAC-3'	5'-GAAAGCACAGCCTGGATAGC-3'	199

enzyme involved in fatty acid degradation and is positively involved in abscisic acid (ABA) synthesis via β–oxidation of fatty acids (Jiang *et al*., 2011). ABA is well known to play an important role in plant development and stress adaptation as a plant hormone. In the present study, the *KAT* gene was up–regulated by low temperature treatment, suggesting that ABA–regulated genes would be induced in lettuce.

Overexpression of one type of acyl–CoA–binding protein (ACBP6) was reported to enhance freezing tolerance of *Arabidopsis* (Chen *et al*., 2008; Liao *et al*., 2014). ACBP6–mediated freezing tolerance was accompanied by increased phospholipase D (*PLDd*) gene expression, decreased phosphatidyl choline (PC) content, and increased phosphatidic acid (PA) content (Chen *et al*., 2008). Qiao *et al*. (2018) suggest that PLDd convert PC into phosphatidyl glycerol (PG), phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE), all of which stabilize the cell membrane and the membrane skeleton, conferring tolerance to various abiotic stress.

FAD2 is delta 12 fatty acid desaturase localized in endoplasmic reticulum and was also shown to be induced by low temperature in several plants including *Chlorella* (Suga *et al*., 2002) and cotton (Kargiotidou *et al*., 2008). As generally well known, desaturation of membrane lead to increase in fluidity of membrane at low temperature, and to cold acclimation of plant.

Involvement of "energy"–categorized genes in freezing tolerance

In the category of "energy", one EST encoded parts of a protein, whose amino acid sequence was similar to amino acid sequences of phosphoglycerate kinase.

Phosphoglycerate kinase (PGK) was shown to be induced by low temperature in *Arabidopsis* (Bae *et al*., 2003). Overexpression of phosphoglycerate kinase–2 in tobacco plants improved salinity stress tolerance by higher chlorophyll retention and enhanced proline accumulation, besides maintaining better ion homeostasis (Joshi *et al*., 2016). There are several isotype genes encoding phosphoglycerate kinase in plants, so chilling tolerant plant might have an isotype *PGK* gene, which are induced during low temperature treatment. In the present study, this enzyme would be at least involved in chilling tolerance of lettuce plants.

Involvement of "cell growth/division"–categorized genes in freezing tolerance

In the category of "cell growth/division", four ESTs individually encoded a part of two proteins, whose amino acid sequences were, respectively, similar to amino acid sequence of adagio protein (1 clone) or that of gigantea–like protein (3 clones).

According to UniProt database (https://www.uniprot.org), adagio protein has alternative name, ZTL protein, and a component of E3 ubiquitin ligase complex involved in the regulation of circadian clock–dependent processes including the transition of flowering time, hypocotyl elongation, cotyledons and leaf movement rhythms (https://www.uniprot.org/uniprot/Q94BT6). Furthermore, Norén *et al*. (2016) proposed a model that low levels of ZTL protein would result in increased protein levels of two transcriptional factors, long hypocotyl5 (HY5) and pseudo–response regulator5 (PRR5), and in a repression of *C–repeat binding factors* (*CBFs*), which are important transcriptional factors for cold tolerance. HY5 and PRR5 act in concert to repress *CBF3*, and PPR5 represses *CBF1* and *CBF2*. Thus, cold–induction of ZTL (adagio) protein might negatively affect the expression of *CBF* genes and other cold–responsive genes.

Gigantea (GI) protein is known as flowering time regulator which connects networks involved in developmental stage transitions and environmental stress responses, furthermore repression of one GI protein led to enhancement of salt stress tolerance in poplar (Ke *et al*., 2017). Fornara *et al*. (2015) and Xie *et al*. (2015) respectively reported that loss of GI function led to increase in freezing tolerance of *Arabidopsis* and *Brassica rapa*. Furthermore, GI and ZTL are likely to be closely related for adaptation to environmental stress (Gil and Park, 2019; Kim *et al*., 2007). In the present paper, EST clones encoding GI and ZTL homologous proteins were identified at the same time. Therefore, considering the negative involvement of the two proteins in stress tolerance in previous reports described above and the fact that they were induced at low temperature in the present study, it is considered that they might have a negative effect on the low–temperature tolerance of lettuce.

Involvement of "transcription"-categorized genes in freezing tolerance

In the category of "transcription", eight ESTs individually encoded a part of four corresponding proteins, whose amino acid sequences were respectively similar to amino acid sequences of multiprotein–bridging factor 1c (MBF1c; 5 clones), transcription factor UNE–10 (1 clone), DEAD–box ATP–dependent RNA helicase (1 clone), and G–patch domain containing protein (1 clone).

Overexpression of *MBF1c* gene from antarctic moss, *Polytrichastrum alpinum*, in *Arabidopsis* seems to enhance tolerances against salt, osmotic, cold, and heat stress (Alavilli *et al*., 2017). Furthermore, the overexpression of the gene up–regulated the expression of 10 salt–stress inducible genes without salt treatment in *Arabidopsis*. On the other hand, overexpression of *MBF1c* gene from *Capsicum annuum* in *Arabidopsis* reduced abiotic stress tolerance, accompanying reduced expression levels of stress tolerant genes compared to those in wild type *Arabidopsis* (Guo *et al*., 2014). *P. alpinum* is antarctic moss, which lives under severe cold condition, so it is strong against cold. On the other hand, *C. annuum* is likely to be weak against cold. Thus, even both the genes encode same MBF1c proteins, the protein encoded by a gene from non–cold tolerant plant might not function to enhance the cold tolerance. In the present paper, the *MBF1c* gene was up–regulated by low temperature treatment in lettuce and encoded a transcriptional regulator protein. It would be necessary

for the protein to investigate whether this protein induces other stress responsive genes and functions for acquisition of freezing tolerance in lettuce as a transcriptional factor.

Although several transcriptional factors are reported to be involved in response to cold stress in plants (Yamaguchi–Shinozaki and Shinozaki, 2006), there are little information regarding to transcription factor UNE10–like protein except for one report regarding to response to light (Jaspers *et al*., 2009). Thus, it was difficult to discuss the involvement of UNE10–like protein in cold stress tolerance.

DEAD–box ATP–dependent RNA helicase has been reported to be involved in chilling and freezing tolerance of *Arabidopsis* as a regulator of CBF genes (Gong *et al*., 2002). In cyanobacteria, a cold–induced DEAD–box RNA helicase was suggested to unwind cold–stabilized secondary structure in the 5'–untranslated region of RNA during cold stress (Yu and Owttrim, 2000). Lu *et al*. (2020) showed that DEAD–box RNA helicase 42 plays a critical role in pre–mRNA splicing for adaptation to cold stress in rice. Furthermore, as Liu *et al*. (2016) shows, this enzyme would be an important role in growth and development of plant under low temperature. So, the enzyme coded by this clone would be necessary for cold acclimation of lettuce.

Information of proteins with G–patch (glycine–rich motif) domains, whose roles are predicted as RNA binding or RNA processing (Aravind and Koonin, 1999), are very rare in plant kingdoms. Zhang *et al*. (2005) suggested that MOS2, a protein containing G–patch and KOW (Kyprides, Ouzounis, Woese) motifs, is essential for innate immunity in *Arabidopsis thaliana*. The KOW motif seems to be found in a variety of ribosomal proteins. By searching Pfam database, the G–patch domain containing protein encoded by a gene, to which the isolated EST clone in the present paper showed similarity, does not contain a KOW motif, but it contains RNA binding and zing–finger motifs, suggesting this G–patch domain containing protein might regulate translation of transcripts of some genes for stress response in lettuce.

Involvement of "protein destination and storage"–categorized genes in freezing tolerance

In the category of "protein destination and storage", 25 ESTs individually encoded a part of four proteins, whose amino acid sequences were respectively similar to amino acid sequence of BAG family molecular chaperone regulator 4 (BAG4; 1 clone), probable inactive ATP–dependent zinc metalloprotease FTSHI 3 (1 clone), oligopeptidase A (1 clone), or vacuolar processing enzyme (22 clones).

BAG (Bcl–2–associated athanogene) protein is a ubiquitous family of chaperone regulators of apotosis by interacting with Hsp70 and Hsc70 and stress tolerance of transgenic tobacco plants with different expression levels of *BAG4* gene from *Arabidopsis* were investigated (Doukhanina *et al*., 2006). Tobacco plant or *Arabidopsis* plant with low expression level of *AtBAG4* reduced chlorosis by abiotic stress such as UV–, oxidative–, drought–, salt– and cold stresses and showed enhancement of stress tolerance compared to other transgenic plants and wild–type plants. Thus, BAG4 protein at low expression level was considered to play an important role for regulating apotosis, which is induced by stress, in plants. Thus, in the present paper, the expression level of this *bag4* gene in lettuce would be probably important for cold tolerance of lettuce.

Probably inactive ATP–dependent zinc metalloprotease FTSHI 3 gene was likely to be induced by low temperature in lettuce. FTSH means filamentation–temperature–sensitive protein H and additional "I" means protease–inactive (Mishra *et al*., 2019). Five kinds of *Arabidopsis* FTSHIs are localized in the chloroplast envelope and, out of them, *FtsHi1* is reported to be involved in biogenesis and division of chloroplast (Kadirjan–Kalbach *et al*., 2012). It is difficult to discuss the role of this gene in lettuce, however, this gene was highly induced by low temperature.

There are few information regarding to low–temperature–inducible oligopeptidase. However, overexpression of a gene encoding prolyl oligopeptidase from rice was reported to confer abiotic stress tolerance to *Escherichia coli* (Tan *et al*., 2013a), suggesting that oligopeptidase is likely contribute to enhancement of stress tolerance of organisms including plants.

Vacuolar processing enzyme (VPE) is a vacuole-localized cysteine proteinase responsible for the maturation and activation of vacuolar proteins, which are synthesized on the endoplasmic reticulum (ER) as a proprotein precursor and are then transported to vacuoles (Hatsugai *et al*., 2015). The enzyme has a caspase–1–like activity which is related with program cell death (PCD) (Hara–Nishimura and Hatsugai 2011). Under heat stress condition, gVPE deficiency suppressed vacuolar disruption and led to enhancement of heat tolerance in *Arabidopsis* (Li *et al*., 2012). Koukalová *et al*. (1997) reported that cold stress induced PCD of plant cells. Qiao *et al*. (2002) reported that overproduction of animal cell death suppressors, Bcl–xL and Ced–9, in tobacco cells enhanced resistance to salt, cold, and wound stress. Furthermore, Bcl–2 protein was suggested to suppress hydrogen peroxide-induced PCD by suppressing the expression of *OsVPE2* and *OsVPE3* in rice (Deng *et al*., 2011). Thus, the induction of VPE gene was considered to lead to promotion of PCD and play a negative role in acquiring cold tolerance.

In the present papers, several types of proteases or peptidases were identified as low–temperature–inducible. These proteases might quickly and properly degrade denatured proteins for utilization as parts of newly synthesized protein. It is necessary to make clear the involvement in response to low temperature by investigating individual target protein or localization of them in future.

Involvement of "transporters"–categorized genes in freezing tolerance

In the category of "transporters", eight ESTs individually encoded five proteins, whose amino acid sequences

were respectively similar to amino acid sequence of sucrose transporter (1 clone), monosaccharide-sensing protein 2–like (2 clones), early nodulin–like protein (2 clones), metal–nicotianamine transporter (1 clone), or kinesin–like protein KIN–7K (2 clones).

Involvement of sucrose transporter in cold tolerance of plants was reported by several researchers (Jia *et al*., 2015; Yue *et al*., 2015). By searching WoLF PSORT database, the identified protein in the present paper was predicted to localize in plastid (data not shown). Furthermore, as Patzke *et al*. (2019) reported, plastidic sucrose transporter of *Arabidopsis* is likely to be involved in freezing tolerance. Then, sucrose transporter is also likely to function for development of cold tolerance in lettuce.

There are few papers regarding to monosaccharide-sensing protein like–2. However, deduced amino acid sequence of monosaccharide–sensing protein 2–like in the present paper is likely to be similar to that of tonoplast monosaccharide transporter 2. According to Klemens *et al*. (2014), sugar accumulation in vacuole seems important for freezing tolerance. Induction of tonoplast monosaccharide transporter by low temperature treatment might be important for enhancement of freezing tolerance in lettuce.

An early nodulin–like protein is reported as a cold–responsive protein in cypress (Pedron *et al*., 2009). However, as original function of this protein remains unclear, it is difficult to discuss its role in lettuce.

Nicotianamine forms a complex with a metal ion and metal–nicotianamine transporter YSL6 is involved in transportation of metal movement within the plant and functions for homeostasis in plants (Conte *et al*., 2013). AtYSL6 are likely to function in the influx from the acidic vacuole to the cytoplasm in *Arabidopsis*. *OsYSL6* was constitutively expressed in rice plants (Koike *et al*., 2004). Although it is difficult to discuss why this gene was induced by low temperature in lettuce, this protein would be involved in cold acclimation.

Kinesin superfamily proteins are important microtubule–based motor proteins with a kinesin motor domain that is conserved among all eukaryotic organisms (Li *et al*., 2012). Gao *et al*. (2017b) showed that kinesin–like protein KCA2 was phosphorylated by cold stress in cold tolerant banana cultivar not in cold sensitive cultivar, suggesting that KCA2 plays important role in development of cold tolerance of banana. The protein was also reported to play critical roles in mitosis, morphogenesis, signal transduction, and regulating gibberellin biosynthesis and cell growth by transcriptionally activation (Li *et al*., 2012). Thus, this protein might play a role in cold tolerance of lettuce.

Involvement of "intracellular traffic"–categorized genes in freezing tolerance

In the category of "intracellular traffic", five ESTs individually encoded four proteins, whose amino acid sequences were respectively similar to amino acid sequence of BTB/POZ domain–containing protein (2 clones), IST1–like protein (1 clone), phosphatidylinositol/phosphatidylcholine transfer protein (1 clone), or vacuolar sorting–associated protein 32 homolog 2 (1 clone).

BTB/POZ domain–containing protein contains a Broad–complex Tramtrack and Bric–a–brac (BTB) or POX virus and Zinc finger (POZ) domain. This protein was reported to participate in plant responses to biotic and abiotic stress (He *et al*., 2019). The protein in pepper plant was cold inducible and suggested to interact with DREB2A, an important transcription factor in abiotic stress, resulting in regulation of plant stress response (He *et al*., 2019). However, reason of the expression of the protein in response to cold stress is unclear.

Endosomal Sorting Complex Required for Transport (ESCRT) pathway is composed of increased salt tolerance 1 (IST1) protein, LIP5, charged multivesicular body protein (CHMP) 1, and SKD1 (Buono *et al*., 2016). ATPase activity of SKD1 and endosomal trafficking by ESCRT system are regulated by LIP5 and IST1. In *Arabidopsis*, 12 types of IST1–like (ISTL1) proteins were identified (Buono *et al*., 2016). Because interaction of one of ISTL1 proteins with LIP5 is likely to be essential for normal plant growth and repression of spontaneous cell death by regulating an important ATP enzyme, SKD1, for endosomal recruitment (Buono *et al*., 2016), induction of *ISTL1* is also probably important for repression of cell death in lettuce plants under low temperature.

Vacuolar sorting–associated protein 32 (VPS32) homolog 2 is also recognized as CHMP2–1 or sucrose non–fermenting (SNF) 7.1 (https://www.uniprot.org/uniprot/Q9SZE4). This protein is also one of core components of ESCRT Ⅲ (Ibl *et al*., 2012) and is considered isotype of CHMP1 described paragraph of IST1–like protein. It is also reported to play a role of MVB biogenesis, endosomal sorting or viral replication (Gao *et al*., 2017a). To our knowledge, induction of VPS32 or IST1–like protein by low temperature has not been reported. In the present paper, identified homologs to VPS32 or IST1 would be different from normal components of ESCRT Ⅲ.

Phosphatidylinositol/phosphatidylcholine transfer protein was reported to be homologous to Sec14 protein of yeast (Mo *et al*., 2007). Wang *et al*. (2016) reported that *ZmSEC14p*, Sec14 protein from maize was a cold responsive protein and overexpression of the protein in transgenic *Arabidopsis* conferred tolerance to cold stress. Thus, clarification of the function of the protein is expected in future.

Involvement of "cell structure"–categorized genes in freezing tolerance

In the category of "cell structure", two ESTs individually encoded one protein, whose amino acid sequence was similar to amino acid sequence of arabinogalactan protein 2 (2 clones).

Arabinogalactan protein is grouped into a superfamily of highly glycosylated hydroxyproline–rich proteins (Gong *et al*., 2012). Gong *et al*. (2012) reported the

involvement of an arabinogalactan protein, GhAGP31, in cold stress tolerance of cotton and the enhancement of freezing tolerance of yeast and transgenic *Arabidopsis* by overexpression of its encoding gene. They also suggested that GhAGP31 might interact with pectin to form protein–carbonhydrate linkage within cell walls, which contribute to the stability of the cell wall. In lettuce plants, induced arabinogalactan protein might contribute to enhancement of freezing tolerance.

Involvement of "signal transduction"-categorized genes in freezing tolerance

In the category of "signal transduction", 8 ESTs individually encoded five proteins, whose amino acid sequences were respectively similar to amino acid sequence of Rho GTPase–activating protein (1 clone), elongation factor 2 (2 clones), serine/threonine-protein kinase (4 clones), or salicylic acid-binding protein (1 clone).

Rho GTPase–activating protein was reported to be involved in the regulation of tolerance to dehydration stress in barley (Suprunova *et al*., 2007). Although very little information is available on this protein in plants, Rho GTPase–activating protein in yeast has been reported to be an important regulator of multiple biological process including stress resistance and so on (Rahim *et al*., 2017). This protein might play an important role for response to low temperature stress in lettuce.

Elongation factor 2 (EF–2) protein is also well reported as cold responsive proteins for cold tolerance and plays an important role in protein synthesis (Shi *et al*., 2019). Shi *et al*. (2019) reported that overexpression of EF–2 gene enhanced freezing tolerance in tobacco plants by regulating hundreds of protein synthesis under low temperature conditions. In the present paper, this protein is thought to play an important function.

Some of serine/threonine–protein (STP) kinase is also known as cold inducible in *Arabidopsis* (Mizoguchi *et al*., 1995). A serine/threonine–protein kinase, OST1, acts upstream of *CBF* genes to positively regulate freezing tolerance (Ding *et al*., 2015). Identified STP kinase in the present paper seems to play an important role in signal transduction for cold response in lettuce.

One type of salicylic acid-binding protein (SABP2) possesses methyl salicylate (MeSA) esterase activity, thus it catalyzes the conversion of MeSA to salicylic acid (SA) (Li *et al*., 2019). Exogenic SA seems to induce freezing tolerance in wheat via hydrogen peroxide and abscisic acid (Wang *et al*., 2018a). Furthermore, endogenous SA is also likely to be important for chilling tolerance in maize seedlings (Wang *et al*., 2018b). Although chilling tolerance is different from freezing tolerance, SA seems to influence on tolerance to low temperature stress.

Involvement of "disease/defense"–categorized genes

In the category of "disease/defense", 34 ESTs individually encoded 13 proteins, whose amino acid sequences were respectively similar to amino acid sequence of 11 kDa late embryogenesis abundant (LEA; 2 clones), LEA (3 clones), cold shock protein (1 clone), dehydrin Xero (10 clones), dehydrin DHN1 (2 clones), catalase (2 clones), early light inducible protein (5 clones), HVA22–like protein (1 clone), low sulfur responsive protein (1 clone), nodulin–related protein (4 clones), pathogenesis–related protein (1 clone), plastid–lipid–associated protein 11 (1 clone), or plastid–lipid–associated protein 6 (1 clone).

LEA proteins are one of probably most well–known stress responsive proteins in plants. In the present paper, 4 kinds of LEA (11–kDa LEA, LEA, dehydrin Xero, dehydrin DHN1) proteins were identified. For classification of LEA proteins, family domain search database, Pfam (https://pfam.xfam.org), is well used (Battaglia *et al*., 2008; Hundertmark and Hincha, 2008). By using the Pfam database, 11–kDa LEA and another LEA was grouped into LEA group1 and group 4, respectively (data not shown). Cold shock protein was also categorized into dehydrin by using Pham database (data not shown). These proteins would play important roles in freezing tolerance of lettuce.

Catalase is also well known as one of antioxidative enzymes for development of stress tolerance of plants (Saker and Oba, 2018). Feki *et al*. (2015) suggested that *TdCAT1*, a catalase gene from durum wheat, is a promising candidate gene for the development of crops with multiple stress tolerances.

Early light–inducible protein is also known as cold inducible under light condition (Adamska and Kloppstech, 1994; Hayami *et al*., 2015). This protein is likely to play a role in the protection of photosynthetic apparatus from excess light under cold condition (Arora and Rowland, 2011). Thus, the protein would probably play the same role in lettuce.

HVA22–like protein is likely to be localized in ER and Golgi membranes (Guo and Ho, 2008). In particular, HVA22 proteins were estimated to be involved in vesicular traffic in stressed cells of citrus and one of them is likely to be involved in dehydration tolerance and oxidative stress reduction (Ferreira *et al*., 2019). The identified HVA22–like protein would also function vesicular traffic in lettuce plant, leading to low temperature stress.

Although information regarding to low sulfur responsive protein is not so much, a plant–specific low–sulfur responsive gene is reported to be responsive to a several stresses including cold stress (Tombuloğlu *et al*., 2016). Investigation of role of this protein in cold acclimation would be necessary.

Information of regarding to nodulin-related protein is not found. However, the deduced protein assigned to nodulin–related protein also showed sequence-similarity to the drought–induced unknown protein of sunflower (Ouvrard *et al*., 1996).

According to Van Loon and Van Strien (1999), there are several type proteins known as pathogenesis–related proteins, including chitinase, endoproteinase, peroxidase, defensin, and so on. This gene whose part was identified in the present paper as an EST clone was cate-

gorized into genes encoding cysteine–rich secretory protein family by using Pfam database (https://pfam.xfam.org).

Two kinds of plastid–lipid–associated proteins contained fibrillin domains according to BLAST search on UniProt database (https://www.uniprot.org). Twelve sub–families of fibrillin were reported and some fibrillins categorized in group 1 were likely to be induced by cold (Singh and McNellis, 2011). Furthermore, Arabidopsis knock out mutant of fibrillin 5 was sensitive to cold stress (Kim *et al*., 2015). Thus, some of fibrillins are likely to be involved in cold tolerance.

Involvement of "secondary metabolism" and "unclear classification"–categorized genes in freezing tolerance

In the category of "secondary metabolism", 10 ESTs individually encoded two proteins, whose amino acid sequences were respectively similar to amino acid sequence of chalcone synthase (CHS; 9 clones) or chalcone–flavone isomerase (CHI; 1 clone).

CHS is one of the key and rate–limiting enzymes of anthocyanine pathway and converts *p*–coumaroyl CoA and malonyl CoA to naringenin chalcone (Christie *et al*., 1994). Then, CHI converts naringenin chalcone to naringenin, which is one of polyphenols during flavonoids or anthocyanin biosynthesis (Christie *et al*., 1994). In Arabidopsis, mRNA of CHS is induced by cold stress under light (Leyva *et al*., 1995). Leyva *et al*. (1995) concluded that CHS induction is light–dependent and Arabidopsis acquire freezing tolerance with exposure to low temperature without light.

An EST clone which encodes root UVB sensitive (RUS) protein 5 is categorized in unclear classification and this encoded protein is one of RUS family. There are very few regarding the functions of RUS proteins. Recently, *Rus5* knockout mutant of *Arabidopsis* was reported to show no visible phenotypic difference to wild type under normal growth conditions (Perry *et al*., 2021). At present, function of lettuce RUS5 is also not estimated under low temperature. Further research using knockout *rus5* plant will be expected under low temperature condition.

AUTHOR CONTRIBUTIONS

K. Honjoh wrote the manuscript, Y. Masuda, T. Miyamoto participated in the discussion of this manuscript, H. Okano, M. Sasaki, M. Kurokawa cloned EST, and T. Kimura, K. Shibata performed qPCR. All authors have read and agreed to publish this version of the manuscript.

REFERENCES

Adamska, I, and Kloppstech, K. 1994 Low temperature increases the abundance of early light–inducible transcript under light stress conditions. *J. Biol. Chem*., **269**: 30221–30226

Alavilli, H., Lee, H., Park, M., and Lee, B. 2017 Antarctic moss multiprotein bridging factor 1c overexpression in Arabidopsis resulted in enhanced tolerance to salt stress. *Front. Plant Sci*., **8**: 1206

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J. 1997 Gapped BLAST and PSI–BLAST: a new generation of protein database search programs. *Nucleic Acids Res*., **25**: 3389–3402

Aravind, L. and Koonin, E. V. 1999 G–patch: a new conserved domain in eukaryotic RNA–processing proteins and type D retroviral polyproteins. *Trends Biochem. Sci*., **24**: 342–344

Arora, R. and Rowland, L. J. 2011 Physiological research on winter–hardiness: deacclimation resistance, reacclimation ability, photoprotection strategies, and a cold acclimation protocol design. *Hortscience*, **46**: 1070–1078

Bae, M. S., Cho, E. J., Choi, E.–Y. and Park, O. K. 2003 Analysis of the *Arabidopsis* nuclear proteome and its response to cold stress. *Plant J*., **36**: 652–663

Battaglia, M., Olvera–Carrillo, Y., Garciarrubio, A., Campos, F. and Covarrubias, A. A. 2008 The enigmatic LEA proteins and other hydrophilins. *Plant Physiol*., **148**: 6–24

Bevan, M. and Bancroft, I. *et al*. 1998 Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature*, **391**: 485–488

Buono, R. A., Paez–Valencia, J., Miller, N. D., Goodman, K., Spitzer, C., Spalding, E. P. and Otegui, M. S. 2016 Role of SKD1 regulators LIP5 and IST1–like1 in endosomal sorting and plant development. *Plant Physiol*., **171**: 251–264

Chen, Q.–F., Xiao, S. and Chye, M.–L. 2008 Overexpression of the Arabidopsis 10–kilodalton acyl–coenzyme A binding protein ACBP6 enhances freezing tolerance. *Plant Physiol*., **148**: 304–315

Christie, P. J., Alfenito, M. R. and Walbot, V. 1994 Impact of low–temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*, **194**: 541–549

Conte, S. S., Chu, H. H., Chan–Rodriguez, D., Punshon, T., Vasques, K. A., Salt, D. E. and Walker, E. L. 2013 *Arabidopsis thaliana* Yellow Stripe1–Like4 and Yellow Stripe1–Like6 localize to internal cellular membranes and are involved in metal ion homeostasis. *Front. Plant Sci*., **4**: 283

Deng, M., Bian, H., Xie, Y., Kim, Y., Wang, W., Lin, E., Zeng, Z., Guo, F., Han, N., Wang, J., Qian, Q. and Zhu, M. 2011 Bcl–2 suppresses hydrogen peroxide–induced programmed cell death via *OsVPE2* and *OsVPE3*, but not via *OsVPE1* and *OsVPE4*, in rice. *FEBS J*., **278**: 4797–4810

Ding, Y., Li, H., Zhang, X., Xie, Q., Gong, Z. and Yang, S. 2015 OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in *Arabidopsis*. *Dev. Cell*, **32**: 278–289

Doukhanina, E. V., Chen, S., van der Zalm, E., Godzik, A., Reed, J. and Dickman, M. B. 2006 Identification and functional characterization of the BAG protein family in *Arabidopsis thaliana*. *J. Biol. Chem*., **281**: 18793–18801

Feki, K., Kamoun, Y., Mahmoud, R. B., Farhat-Khemakhem, A., Gargouri, A. and Brini, F. 2015 Multiple abiotic stress tolerance of the transformants yeast cells and the transgenic *Arabidopsis* plants expressing a novel durum wheat catalase. *Plant Physiol. Biochem*., **97**: 420–431

Ferreira, M. D. G., Castro, J. A., Silva, R. J. S. and Micheli, F. 2019 HVA22 from citrus: a small gene family whose some members are involved in plant response to abiotic stress. *Plant Physiol. Biochem*., **142**: 395–404

Fornara, F., de Montaigu, A., Sánchez–Villarreal, A., Takahashi, Y., van Themaat, E. V. L., Huettel, B., Davis, S. J. and Coupland, G. 2015 The GI–CDF module of Arabidopsis affects freezing tolerance and growth as well as flowering. *Plant J*., **82**: 695–706

Gao, C., Zhuang, X., Shen, J. and Jiang, L. 2017a Plant ESCRT complexes: moving beyond endosomal sorting. *Trends Plant Sci*., **22**: 986–998

Gao, J., Zhang, S., He, W.–D., Shao, X.–H., Li, C.–Y., Wei, Y.–R., Deng, G.–M., Kuang, R.–B., Hu, C.–H., Yi, G.–J. and Yang, Q.–S. 2017b Comparative phosphoproteomics reveals an important role of MKK2 in banana (*Musa spp.*) cold signal network. *Sci. Rep*., **7**: 40852

Garrido, Y., Tudela, J. A., Marín, A., Mestre, T., Martínez, V. and Gil,

M. I. 2013 Physiological, phytochemical and structural changes of multi–leaf lettuce caused by salt stress. *J. Sci. Food Agric.*, **94**: 1592–1599

Gil, K. and Park, C. 2019 Thermal adaptation and plasticity of the plant circadian clock. *New Phytol.*, **221**: 1215–1229

Gilmour, S. J., Zarka, D. G., Stockinger, E. J., Salazar, M. P., Houghton, J. M. and Thomashow, M. F. 1998 Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold–induced COR gene expression. *Plant J.*, **16**: 433–442

Gong, S.–Y., Huang, G.–Q., Sun, X., Li, P., Zhao, L.–L., Zhang, D.–J. and Li, X.–B. 2012 GhAGP31, a cotton non–classical arabinogalactan protein, is involved in response to cold stress during early seedling development. *Plant Biol.*, **14**: 447–457

Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B. and Zhu, J.–K. 2002 RNA helicase–like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc. Natl. Acad. Sci. USA*, **99**: 11507–11512

Guo, W.–L., Chen, R.–G., Du, X.–H., Zhang, Z., Yin, Y.–X., Gong, Z.–H. and Wang, G.–Y. (2014) Reduced tolerance to abiotic stress in transgenic *Arabidopsis* overexpressing a *Capsicum annuum* multiprotein bridging factor 1. *BMC Plant Biol.*, **14**: 138

Guo, W.–J. and Ho, T.–H. D. 2008 An abscisic acid-induced protein, HVA22, inhibits gibberellin–mediated programmed cell death in cereal aleurone cells. *Plant Physiol.*, **147**: 1710–1722

Hara–Nishimura, I. and Hatsugai, N. 2011 The role of vacuole in plant cell death. *Cell Death Differ.*, **18**: 1298–1304

Hatsugai, N., Yamada, K., Goto–Yamada, S. and Hara–Nishimura, I. 2015 Vacuolar processing enzyme in plant programmed cell death. *Front. Plant Sci.*, **6**: 234

Hayami, N., Sakai, Y., Kimura, M., Saito, T., Tokizawa, M., Iuchi, S., Kurihara, Y., Matsui, M., Nomoto, M., Tada, Y. and Yamamoto, Y. Y. 2015 The responses of Arabidopsis *early light–induced protein2* to ultraviolet B, high light, and cold stress are regulated by a transcriptional regulatory unit composed of two elements. *Plant Physiol.*, **169**: 840–855

He, Y.–M., Liu, K.–K., Zhang, H.–X., Cheng, G.–X., Ali, M., Haq, S. U., Wei, A.–M. and Gong, Z.–H. 2019 Contribution of *CaBPM4*, a BTB domain–containing gene, to the response of pepper to *Phytophthora capsica* infection and abiotic stresses. *Agronomy*, **9**: 417

Honjoh, K., Okano, H., Kawabata, A., Kurokawa, M., Kimura, T., Machida, T., Masuda, Y. and Miyamoto, T. 2018 Freezing tolerance of *Lactuca sativa* and induction of CBF and *GolS* genes during cold treatment. *J. Fac. Agr., Kyushu Univ.*, **63**: 249–257

Hundertmark, M. and Hincha, D. K. 2008 LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. *BMC Genomics*, **9**: 118

Ibl, V., Csaszar, E., Schlager, N., Neubert, S., Spitzer, C. and Hauser, M. 2012 Interactome of the plant-specific ESCRT–Ⅲ component AtVPS2.2 in *Arabidopsis thaliana*. *J. Proteome Res.*, **11**: 397–411

Jaspers, P., Blomster, T., Brosché, M., Salojärvi, J., Ahlfors, R., Vainonen, J. P., Reddy, R. A., Immink, R., Angenent, G., Turck, F., Overmyer, K. and Kangasjärvi, J. 2009 Unequally redundant RCD1 and SRO1 mediate stress and developmental responses and interact with transcription factors. *Plant J.*, **60**: 268–279

Jia, W., Zhang, L., Wu, D., Liu, S., Gong, X., Cui, Z., Cui, N., Cao, H., Rao, L. and Wang, C. 2015 Sucrose transporter AtSUC9 mediated by a low sucrose level is involved in Arabidopsis abiotic stress resistance by regulating sucrose distribution and ABA accumulation. *Plant Cell Physiol.*, **56**: 1574–1587

Jiang, T., Zhang, X.–F., Wang, X.–F. and Zhang, D.–P. 2011 Arabidopsis 3–ketoacyl–CoA thiolase–2 (KAT2), an enzyme of fatty acid β–oxidation, is involved in ABA signal transduction. *Plant Cell Physiol.*, **52**: 528–538

Joshi, R., Karan, R., Singla–Pareek, S. L. and Pareek, A. 2016 Ectopic expression of Pokkali phosphoglycerate kinase–2 (OsPGK2–P) improves yield in tobacco plants under salinity stress. *Plant Cell Rep.*, **35**: 27–41

Kadirjan–Kalbach, D. K., Yoder, D. W., Ruckle, M. E., Larkin, R. M. and Osteryoung, K. W. 2012 *FtsHi1/ARC1* is an essential gene in Arabidopsis that links chloroplast biogenesis and division. *Plant J.*, **72**: 856–867

Kanda, Y. 2013 Investigation of the freely available easy–to–use software "EZR" for medical statistics. *Bone Marrow Transplant.*, **48**: 452–458

Kargiotidou, A., Deli, D., Galanopoulou, D., Tsaftaris, A. and Farmaki, T. 2008 Low temperature and light regulate *delta 12* fatty acid desaturase (FAD2) at a transcriptional level in cotton (*Gossypium hirsutum*). *J. Exp. Bot.*, **59**: 2043–2056

Ke, Q., Kim, H. S., Wang, Z., Ji, C. Y., Jeong, J. C., Lee, H., Choi, Y.–I., Xu, B., Deng, X., Yun, D.–J. and Kwak, S.–S. 2017 Down–regulation of *GIGANTEA-like* genes increases plant growth and salt stress tolerance in poplar. *Plant Biotechnol. J.*, **15**: 331–343

Kim, E.–H., Lee, Y. and Kim, H. U. 2015 Fibrillin 5 is essential for plastoquinone–9 biosynthesis by binding to solanesyl diphosphate synthases in Arabidopsis. *Plant Cell*, **27**: 2956–2971

Kim, W., Fujiwara, S., Suh, S., Kim, J., Kim, Y., Han, L., David, K., Putterill, J., Nam, H. G. and Somers, D. E. 2007 ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. *Nature*, **449**: 356–360

Klemens, P. A. W., Patzke, K., Trentmann, O., Poschet, G., Büttner, M., Schulz, A., Marten, I., Hedrich, R. and Neuhaus, E. 2014 Overexpression of a proton–coupled vacuolar glucose exporter impairs freezing tolerance and seed germination. *New Phytol.*, **202**: 188–197

Koike, S., Inoue, H., Mizuno, D., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N. K. 2004 OyYSL2 is a rice metal–nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant J.*, **39**: 415–424

Koukalová, B., Kovařík, A., Fajkus, J. and Široký, J. 1997 Chromatin fragmentation associated with apoptotic changes in tobacco cells exposed to cold stress. *FEBS Lett.*, **414**: 289–292

Leyva, A., Jarillo, J.A., Salinas, J. and Martinez–Zapater, J.M. 1995 Low temperature induces the accumulation of *phenylalanine ammonia–lyase* and *chalcone synthase* mRNAs of *Arabidopsis thaliana* in a light–dependent manner. *Plant Physiol.*, **108**: 39–46

Leyva, R., Sánchez–Rodríguez, E., Ríos, J. J., Rubio–Wilhelmi, M. M., Romero, L., Ruiz, J. M. and Blasco, B. 2011 Beneficial effects of exogenous iodine in lettuce plants subjected to salinity stress. *Plant Sci.*, **181**: 195–202

Li, J., Xu, Y. and Chong, K. 2012 The novel functions of kinesin motor proteins in plants. *Protoplasma*, **249**: 95–100

Li, Q., Wang, G., Guan, C., Yang, D., Wang, Y., Zhang, Y., Ji, J., Jin, C. and An, T. 2019 Overexpression of *LcSABP*, an orthologous gene for salicylic acid binding protein 2, enhances drought stress tolerance in transgenic tobacco. *Front. Plant Sci.*, **10**: 200

Li, Z., Yue, H. and Xing, D. 2012 MAP Kinase 6–mediated activation of vacuolar processing enzyme modulates heat shock-induced programmed cell death in Arabidopsis. *New Phytol.*, **195**: 85–96

Liao, P., Chen, Q.–F. and Chye, M.–L. 2014 Transgenic Arabidopsis overexpressing acyl–CoA–binding protein ACBP6 are freezing tolerant. *Plant Cell Physiol.*, **55**: 1055–1071

Liu, Y., Tabata, D. and Imai, R. 2016 A cold–inducible DEAD–box RNA helicase from *Arabidopsis thaliana* regulates plant growth and development under low temperature. *PLoS One*, **11**: e0154040

Lu, C.–A., Huang, C.–K., Huang, W.–S., Huang, T.–S., Liu, H.–Y. and Chen, Y.–F. 2020 DEAD–box RNA helicase 42 plays a critical role in pre–mRNA splicing under cold stress. *Plant Physiol.*, **182**: 255–271

Mishra, L. S., Mielke, K., Wagner, R. and Funk, C. 2019 Reduced expression of the proteolytically inactive FtsH members has impacts on the Darwinian fitness of *Arabidopsis thaliana*. *J. Exp. Bot.*, **70**: 2173–2184

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H.

and Shinozaki, K. 1995 Two genes that encode ribosomal–protein S6 kinase homologs are induced by cold or salinity stress in *Arabidopsis thaliana*. *FEBS Lett.*, **358**: 199–204

Mo, P., Zhu, Y., Liu, X., Zhang, A., Yan, C. and Wang, D. 2007 Identification of two phosphatidylinositol/phosphatidylcholine transfer protein genes that are predominately transcribed in the flowers of *Arabidopsis thaliana*. *J. Plant Physiol.*, **164**: 478–486

Norén, L., Kindgren, P., Stachula, P., Rühl, M., Eriksson, M. E., Hurry, V. and Strand, Å. 2016 Circadian and plastid signaling pathways are integrated to ensure correct expression of the CBF and COR genes during photoperiodic growth. *Plant Physiol.*, **171**: 1392–1406

Ouvrard, O., Cellier, F., Ferrare, K., Tousch, D., Lamaze, T., Dupuis, J. and Casse-Delbart, F. 1996 Identification and expression of water stress– and abscisic acid–regulated genes in a drought-tolerant sunflower genotype. *Plant Mol. Biol.*, **31**: 819–829

Patzke, K., Prananingrum, P., Klemens, P. A. W., Trentmann, O., Rodrigues, C. M., Keller, I., Fernie, A. R., Geigenberger, P., Bölter, B., Lehmann, M., Schmitz–Esser, S., Pommerrenig, B., Haferkamp, I. and Neuhaus, H. E. 2019 The plastidic sugar transporter pSuT influences flowering and affects cold responses. *Plant Physiol.*, **179**: 569–587

Pedron, L., Baldi, P., Hietala, A. M. and Porta, N. L. 2009 Genotype–specific regulation of cold–responsive genes in cypress (*Cupressus sempervirens* L.). *Gene*, **437**: 45–53

Perry, N., Leasure, C. D., Tong, H., Duarte, E. M. and He, Z. 2021 RUS6, a DUF647–containing protein, is essential for early embryonic development in *Arabidopsis thaliana*. *BMC Plant Biol.*, **21**: 232

Porcel, R., Aroca, R., Azcón, R. and Ruiz–Lozano, J. M. 2006 PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. *Plant Mol. Biol.*, **60**: 389–404

Qiao, J., Mitsuhara, I., Yazaki, Y., Sakano, K., Gotoh, Y., Miura, M. and Ohashi, Y. 2002 Enhanced resistance to salt, cold and wound stresses by overproduction of animal cell death suppressors Bcl–xL and Ced–9 in tobacco cells – Their possible contribution through improved function of organella. *Plant Cell Physiol.*, **43**: 992–1005

Qiao, K., Wang, M., Takano, T. and Liu, S. 2018 Overexpresson of *acyl–CoA–binding protein 1* (*ChACBP1*) from saline–alkali–tolerant *Chlorella* sp. enhances stress tolerance in *Arabidopsis*. *Front. Plant Sci.*, **9**: 1772

Rahim, K., Huo, L., Li, C., Zhang, P., Basit, A., Xiang, B., Ting, B., Hao, X. and Zhu, X. 2017 Identification of a basidiomycete-specific Vilse–like GTPase activating proteins (GAPs) and its roles in the production of virulence factors in *Cryptococcus neoformans*. *FEMS Yeast Res.*, **17**: fox089

Sarker, U. and Oba, S. 2018 Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. *Sci. Rep.*, **8**: 16496

Shi, H., He, S., He, X., Lu, S. and Guo, Z. 2019 An eukaryotic elongation factor 2 from *Medicago falcata* (*MfEF2*) confers cold tolerance. *BMC Plant Biol.*, **19**: 218

Singh, D. K. and McNellis, T. W. 2011 Fibrillin protein function: the tip of the iceberg? *Trends Plant Sci.*, **16**: 432–441

Suga, K., Honjoh, K., Furuya, N., Shimizu, H., Nishi, K., Shinohara, F., Hirabaru, Y., Maruyama, I., Miyamoto, T., Hatano, S. and Iio, M. 2002 Two low–temperature-inducible *Chlorella* genes for Δ12 and ω–3 fatty acid desaturase (FAD): isolation of *Δ12* and *ω–3 fad* cDNA clones, expression of *Δ12 fad* in *Saccharomyces cerevisiae*, and expression of *ω–3 fad* in *Nicotiana tabacum*. *Biosci. Biotechnol. Biochem.*, **66**: 1314–1327

Suprunova, T., Krugman, T., Distelfeld, A., Fahima, T., Nevo, E. and Korol, A. 2007 Identification of a novel gene (*Hsdr4*) involved in water–stress tolerance in wild barley. *Plant Mol. Biol.*, **64**: 17–34

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi–Shinozaki, K. and Shinozaki, K. 2002 Important roles of drought– and cold–inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J.*, **29**: 417–426

Tan, C.–M., Chen, R.–J., Zhang, J.–H., Gao, X.–L., Li, L.–H., Wang, P.–R., Deng, X.–J. and Xu, Z.–J. 2013a *OsPOP5*, a prolyl oligopeptidase family gene from rice confers abiotic stress tolerance in *Escherichia coli*. *Int. J. Mol. Sci.*, **14**: 20204–20219

Tan, J., Wang, C., Xiang, B., Han, R. and Guo, Z. 2013b Hydrogen peroxide and nitric oxide mediated cold– and dehydration-induced *myo*–inositol phosphate synthase that confers multiple resistances to abiotic stresses. *Plant Cell Environ.*, **36**: 288–299

Tombuloğlu, H., Ablazov, A. and Filiz, E. 2016 Genome–wide analysis of response to low sulfur (LSU) genes in grass species and expression profiling of model grass species *Brachypodium distachyon under* S deficiency. *Turk. J. Biol.*, **40**: 934–943

Van Loon, L. C. and Van Strien, E. A. 1999 The families of pathogenesis–related proteins, their activities, and comparative analysis of PR–1 type proteins. *Physiol. Mol. Plant Pathol.*, **55**: 85–97

Wang, W., Wang, X., Huang, M., Cai, J., Zhou, Q., Dai, T., Cao, W. and Jiang, D. 2018a Hydrogen peroxide and abscisic acid mediate salicylic acid–induced freezing tolerance in wheat. *Front. Plant Sci.*, **9**: 1137

Wang, X., Shan, X., Xue, C., Wu, Y., Su, S., Li, S., Liu, H., Jiang, Y., Zhang, Y. and Yuan, Y. 2016 Isolation and functional characterization of a cold responsive phosphatidylinositol transfer-associated protein, *ZmSEC14p*, form maize (*Zea may* L.). *Plant Cell Rep.*, **35**: 1671–1686

Wang, Y., We, T., Huang, Y., Guan, Y. and Hu, J. 2018 Salicylic acid biosynthesis inhibitors increase chilling injury to maize (*Zea mays* L.) seedlings. *Plant Growth Reg.*, **85**: 11–21

Xie, Q., Lou, P., Hermand, V., Aman, R., Park, H. J., Yun, D., Kim, W. Y., Salmela, M. J., Ewers, B. E., Weinig, C., Khan, S. L., Schaible, D. L. P. and McClung, C. R. 2015 Allelic polymorphism of *GIGANTEA* is responsible for naturally occurring variation in circadian period in *Brassica rapa*. *Proc. Natl. Acad. Sci. USA*, **112**: 3829–3831

Yamaguchi–Shinozaki, K. and Shinozaki, K. 2006 Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Ann. Rev. Plant Biol.*, **57**: 781–803

Yu, E. and Owttrim, G. W. 2000 Characterization of the cold stress–induced cyanobacterial DEAD–box protein CrhC as an RNA helicase. *Nucleic Acids Res.*, **28**: 3926–3934

Yue, C., Cao, H.–L., Wang, L., Zhou, Y.–H., Huang, Y.–T., Hao, X.–Y., Wang, Y.–C., Wang, B., Yang, Y.–J. and Wang, X.–C. 2015 Effects of cold acclimation on sugar metabolism and sugar-related gene expression in tea plant during the winter season. *Plant Mol. Biol.*, **88**: 591–608

Zhang, Y., Cheng, Y. T., Bi, D., Palma, K. and Li, X. 2005 MOS2, a protein containing G–patch and KOW motifs, is essential for innate immunity in *Arabidopsis thaliana*. *Curr. Biol.*, **15**: 1936–1942

Zhuo, C., Wang, T., Lu, S., Zhao, Y., Li, X. and Guo, Z. 2013 A cold responsive galactinol synthase gene from *Medicago falcata* (*MfGolS1*) is induced by *myo*–inositol and confers multiple tolerances to abiotic stressed. *Physiol. Plant.*, **149**: 67–78