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A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation☆

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Abstract

A novel cDNA clone, *Tad1*, was isolated from crown tissue of winter wheat after differential screening of cold acclimation-induced genes. The *Tad1* cDNA encoded a 23 kDa polypeptide with a potential N-terminal signal sequence. The putative mature sequence showed striking similarity to plant defensins or γ-thionins, representing low molecular size antipathogenic polypeptides. High levels of *Tad1* mRNA accumulation occurred within one day of cold acclimation in crown tissue and the level was maintained throughout 14 days of cold acclimation. Similar rapid induction was observed in young seedlings treated with low temperature but not with exogenous abscisic acid. In contrast to defensins from other plant species, neither salicylic acid nor methyl jasmonate induced expression of *Tad1*. The recombinant mature form of TAD1 polypeptide inhibited the growth of the phytopathogenic bacteria, *Pseudomonas cichorii*; however, no antifreeze activity was detected. Collectively, these data suggested that *Tad1* is induced in cold-acclimated winter wheat independent of major defense signaling(s) and is involved in low temperature-induced resistance to pathogens during winter hardening.

Keywords: Cold acclimation; Plant defensin; Thionin; Antimicrobial protein; Antifreeze protein

Overwintering plants are capable of exhibiting high levels of cold tolerance, which is acquired through the cold acclimation process. Freezing tolerance of such plants increases substantially after a period of exposure to low but non-freezing temperature [1–3]. Cold acclimation is accompanied by the activation of a large number of cold-responsive genes [4,5]. Cold-responsive genes encoding molecular chaperones [6,7], lipid [8], and sugar [9,10] metabolism are suggested to be involved in cold adaptation. Reports have shown that some proteins that accumulate in cold-acclimated plant tissues such as antifreezing proteins [11,12] and Lea-like proteins [13,14] function to increase freezing tolerance. In addition to freezing tolerance, resistance to pathogens is an important trait for winter hardiness. It was reported that cold acclimation enhances resistance to pathogens in winter cereals [15]. Recent studies have shown that a class of pathogenesis-related (PR) proteins such as endo-chitinase and β-1,3-glucanase accumulated in cold-acclimated rye apoplast [11,16]. Cold-induced accumulation of this class of PR proteins has been correlated with enhanced resistance to pathogens [11,16]. Interestingly, cold-induced PR proteins from rye showed antifreeze activity and are thought to be bifunctional in cold acclimation. These findings suggested that activation of genes involved in the freezing tolerance and disease resistance are both required for winter survival. However, the mechanisms that bring disease resistance during cold acclimation are still mostly unknown.

Thionins are plant antimicrobial proteins that contain four conserved disulfide bonds. A divergent subgroup of thionin is γ-thionin, which has a small molecular size (5 kDa) and has three-dimensional structure similar to insect defensins, and therefore is referred to as plant defensin. Plant defensins are highly

☆ *Abbreviations:* GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-galactoside; ABA, abscisic acid.

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inducible by pathogen infection and jasmonate treatment [17–20], suggesting that they are involved in a defense mechanism against pathogen attack.

In this study, we report the isolation of a cDNA clone, *Tad1*, which encodes a novel plant defensin from winter wheat. We show that *Tad1* is induced by low temperature treatment independent of major defense signals and is possibly involved in enhanced tolerance against pathogens during cold acclimation.

Materials and methods

Plant growth conditions. Seeds of winter wheat (*Triticum aestivum* L.) cv. Chihoku were surface-sterilized with 70% (v/v) ethanol and 1% (v/v) sodium hypochlorite and subsequently planted on commercial soil mixture. Plants were grown at 22/18 °C in a controlled-growth chamber (16 h light/8 h dark) for 14 days. Control non-acclimated (NA) plants were harvested at the end of this period and the basal part of the shoot (5–7 mm from the bottom) containing crown was collected. Cold acclimation (CA) was performed by subjecting the plants to 6/2 °C (8 h light/16 h dark) cycles for additional 14 days. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until used for subsequent RNA extraction.

Low temperature treatments of seedlings. Germinated seeds were planted on a plastic mesh grid (15 × 20 cm) placed on a plastic container (1000 ml content) and grown hydroponically with tap water in a growth chamber at 23 °C under continuous illumination. The tap water for growing of seedlings was replaced daily. Five-day-old seedlings were transferred into a growth chamber at 4 °C under continuous light. At each designated time period, shoots were collected, frozen in liquid nitrogen, and stored at −80 °C for further use.

Chemical application to seedlings. Five-day-old seedlings were prepared as described above under continuous light. Seedlings were transferred to water containing 50 μM ABA (Sigma). Salicylic acid (SA) and methyl jasmonate (MeJA) were supplied as hydroponic solution and at the same time by spraying the shoot part. For an independent alternative treatment, detached shoots were floated on SA (5 mM) or MeJA (100 μM) solution, respectively. Whole shoots from each treatment were harvested and stored at −80 °C for further use.

cDNA cloning of Tad1. Isolation of the cDNA clone *Tad1* was carried out by differential screening of macroarrayed cDNA clones. Briefly, plasmid clones representing a cDNA library constructed from cold-acclimated crown tissue of wheat cv. Chihoku were heat-denatured and slot-blotted onto Hybond N^+-nylon membrane with a vacuum immunoblotter (ATTO, Tokyo Japan). Double-stranded cDNA, synthesized from mRNA isolated from both cold-acclimated and non-acclimated crown tissues described above, was random-primed with [α-^{32}P]dCTP and used for hybridization. Hybridization and washing of membrane was carried out according to the standard protocol [21]. Detection and quantification of the signals was performed by a BAS1000 image analyzer (Fuji Film, Tokyo, Japan). A cold-up-regulated clone was picked and sequenced by the dideoxy method using the Thermo Sequence v2.0 Kit (Amersham Pharmacia Biotech, Piscataway, NJ) with a DNA sequencer model 373A (Applied Biosystems, San Jose, CA). Sequence data analysis was carried out using DNASIS software (Hitachi Software Engineering, Yokohama, Japan). The sequence alignment was performed by CLUSTAL X [22]. The phylogenetic tree was calculated by Neighbor-Joining method and displayed using TreeView software [23].

Northern blot analysis. Total RNA was isolated from wheat crown and shoot tissue of wheat using TRIzol reagent (Invitrogen). Twenty μg total RNA was denatured in formamide and formaldehyde, separated on 1.2% agarose gels containing formaldehyde and transferred onto Hybond-N^+ membrane (Amersham Pharmacia Biotech) according to the standard method [21]. Equal loading of the samples was confirmed by ethidium bromide staining. RNA blots were hybridized in Rapid-Hyb buffer (Amersham Pharmacia Biotech) containing 10 μg/ml salmon testis DNA (Sigma) and a [α-^{32}P]dCTP-labeled specific *Tad1* probe at 65 °C overnight. RNA blots were subsequently washed with 1× SSC and 0.1% SDS for 15 min and twice with 0.1× SSC and 0.1% SDS for 20 min at 65 °C. After washing, RNA blots were exposed to X-ray film at −80 °C.

Production and purification of a recombinant TAD1 protein. A PCR fragment corresponding to the mature polypeptide was amplified from the *Tad1* cDNA clone with two primers, 5′-GGCGAATTCCCG GACGTGCCTGTCGC-3′ and 5′-CGCGTCGACTGGTGTTGCTT AGCA-3′, which incorporated *Eco*RI and *Sal*I sites, respectively. The amplified PCR product was cut with *Eco*RI and *Sal*I, and ligated into *Eco*RI/*Sal*I site of pGEX 6P-3 vector (Amersham Pharmacia Biotech). The resulting construct was transformed into *Escherichia coli* BL21(DE3) strain (Novagen) and used for the induction of recombinant fusion protein (GST–mTAD1). Induction of the recombinant protein was carried out by adding IPTG (0.5 mM) to the culture (OD_{595}, 0.6) and culturing further for 5 h at 30 °C. Subsequently, cells were harvested and disrupted by sonication after freeze/thawing in 1× PBS. The total soluble fraction was collected by centrifugation and subjected to a glutathione–Sepharose 4B column (Amersham Pharmacia Biotech) for affinity purification. Bound GST–mTAD1 was digested with PreScission Protease (Amersham Pharmacia Biotech) at 4 °C for 16 h. The eluted recombinant mature TAD1 (rmTAD1) was further purified by filtration with Centricon YM-30 spincolumn (Amicon). The filtrate was concentrated and substituted with 5 volumes of 1× PBS using Microcon YM-3 spincolumn (Amicon). Protein concentration was determined with the Bio-Rad Protein Assay Kit (Bio-Rad) using bovine IgG as a standard. Protein samples were separated with tricine–SDS–PAGE [24].

Antibacterial assay. Inhibitory activity of rmTAD1 on the growth of the phytopathogenic bacterium, *Pseudomonas cichorii* SPC900, was determined with a Benchmark microplate reader (Bio-Rad) as previously described [25]. A fresh colony of *P. cichorii* was cultured in YP medium (1% bactopeptone, 0.5% yeast extract) at 30 °C until an OD_{595} of 0.1–0.2 was reached. Bacterial suspensions (800 μl) were added to 200 μl of purified and filter-sterilized rmTAD1 (0–100 μg/ml in final concentrations) in 1× PBS in a flat-bottomed 48-well titer plate. The aliquot (200 μl) of each working mixture was dispensed in a 96-well titer plate. The titer plate was placed in an incubator at 30 °C without shaking and growth was monitored. Relative growth was expressed as OD_{595} with designated concentrations of rmTAD1 per OD_{595} without rmTAD1 at 12 h of incubation time. Antibacterial activity (IC_{50}) was defined as the concentration of rmTAD1 (μg/ml) that gave 50% growth inhibition of the bacterium.

Measurement of antifreeze activity. Antifreeze activity was determined by observation of the ice crystal morphology using a Leica DMLB 100 photomicroscope equipped with a Linkam LK600 temperature controller. Two microliters of rmTAD1 solution (12.5 mg/ml) or RD3, a type III AFP from antarctic eelpout (*Rhigophila dearborni*) [26] (3 mg/ml), was momentarily frozen (∼−22 °C) and warmed to 0 °C on the sample stage of the photomicroscope to create several ice crystal seeds in the solution. This solution was then cooled to ∼−1 to −5 °C and growth of ice crystal seeds was monitored.

Results

Isolation of a cDNA clone encoding wheat gene induced by cold acclimation

A 644 bp long cDNA clone, *Tad1* (*T. aestivum* defensin 1), was isolated after screening for CA-induced

genes of a crown cDNA library using differential hybridization of macroarray membranes. The cDNA clone contained a 246 bp open reading frame that encodes a 8.97 kDa basic polypeptide of 82 amino acid residues (Fig. 1). A BLAST search in the GenBank protein database suggested that the predicted amino acid sequence has considerable homology to plant defensins or γ-thionins that are representative plant antifungal/-bacterial polypeptides [27]. As found in other proteins in this class, the predicted protein sequence contained a putative signal sequence at N-terminal and eight cysteine residues (Fig. 1). Fig. 2A shows an alignment of the deduced mature polypeptide sequences of TAD1 (mTAD1) and plant defensins from several species. Although the mTAD1 sequence was rather divergent than other monocot defensins (Fig. 2B), eight cysteines and Ser^{7}, Glu^{27}, and Gly^{32} are conserved in all plant defensins. The length of mTAD1 polypeptide (45 residues) matched other plant defensins, except for tomato flower-specific γ-thionin [28] and *Capsicum chinense* γ-thionin (AF128239), which contain an additional C-terminal domain. These characteristics suggested that *Tad1* encodes a plant defensin polypeptide.

Expression of Tad1 is cold responsive in winter wheat

Northern blot analysis was performed to investigate *Tad1* expression during cold acclimation. As shown in Fig. 3A, *Tad1* transcripts was at a low level in non-acclimated crown tissue. The transcript level was highly induced at one day of cold acclimation and the levels were constantly maintained during 14 days of cold acclimation (Fig. 3A). To refine the critical time period for *Tad1* induction in response to low temperature, hydroponically grown seedlings were exposed to short period of cold treatment. In the shoot tissue of the seedlings, *Tad1* mRNA induction was first detectable after 24 h of cold treatment (Fig. 3B). Since ABA is known to be involved in the regulation of some cold-responsive genes, *Tad1* responsiveness to exogenous ABA was determined. Although minimum modulation of *Tad1* mRNA levels may occur during ABA treatment, it was clear that cold-induced expression of *Tad1* is not associated with ABA levels, which typically increase during cold treatments (Fig. 3C).

Since many plant defense proteins including defensins and PR proteins have been shown to be induced by SA [29,30] or jasmonic acid [31–33], it was determined whether *Tad1,* a CA-regulated type of defense protein, is inducible by application of SA or MeJA. Northern blots revealed that there was no induction in Tad1 mRNA levels during MeJA treatments (Fig. 4A). Similarly, treatments of young seedlings with SA did not induce *Tad1* expression (Fig. 4B). These data suggested that *Tad1* expression is not regulated by MeJA or SA and is independent of major defense signaling.

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                9          18          27          36          45          54
5' CCA CGT AGA CGC AGC AGC AGC AGC GAG CTC TGT CGG TTC TCT ATC TAG CAT

               63          72          81          90          99         108
   CTC CGG TGT GAA GCG AGC AAG CAG AGA GAG AGA GAG ATG GCG TCC ACT CGT CGC

                                                   Met Ala Ser Thr Arg Arg

              117         126         135         144         153         162
   ATG GCT GCC GCG CCG GCC GTC CTC CTT CTC CTC CTC CTG CTC GTC GCC ACA GAG

   Met Ala Ala Ala Pro Ala Val Leu Leu Leu Leu Leu Leu Val Ala Thr Glu

              171         180         189         198         207         216
   ATG GGG ACG ATG AAG ACG GCG GAG GCC CGG ACG TGC CTG TCG CAG AGC CAC AAG

   Met Gly Thr Met Lys Thr Ala Glu Ala Arg Thr Cys Leu Ser Gln Ser His Lys

              225         234         243         252         261         270
   TTC AAG GGC ACC TGC CTC AGC AAC AGC AAC TGC GCC GCC GTG TGC CGC ACC GAG

   Phe Lys Gly Thr Cys Leu Ser Asn Ser Asn Cys Ala Ala Val Cys Arg Thr Glu

              279         288         297         306         315         324
   AAC TTC CCC GAC GGC GAG TGC AAC ACG CAC CTC GTC GAG CGC AAG TGC TAC TGC

   Asn Phe Pro Asp Gly Glu Cys Asn Thr His Leu Val Glu Arg Lys Cys Tyr Cys

              333         342         351         360         369         378
   AAG CGG ACC TGC TAA GCA ACA CCA GTC CGC GCG ACG TCC TCC GAT GGA TGC TTG

   Lys Arg Thr Cys  *

              387         396         405         414         423         432
   CTA CTG GGC TAG AGC ATC CAT GCC GTC CCT TAG ATC TGT TCG TCC CTA CTC CGT

              441         450         459         468         477         486
   CTA TTT CCG TTT CCT TTA CGT TGC TCC TAG GGA TGA CTG AAA ATA AAG TAG CTA

              495         504         513         522         531         540
   CCT GCA CCC TCG GCA TTG GCT GTT CGT CTG CAT GTA TGC TGT TTA AGT GTT CGT

              549         558         567         576         585         594
   GGC TTT AGT TTG TGC TGT TGA TGT AAT AAC GAT GCC ACT GAC AAT TTG GCT TCT

              603         612         621         630         639
   ATG TCT TGT GTT GGA ACT TTT GAA TCA GCA GGT TTG TTG GCT TGT CTG TT 3'
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Fig. 1. Nucleotide and deduced amino acid sequences of *Tad1*. The deduced amino acid sequence is shown below the nucleotide sequence (DDBJ/GenBank Accession No. AB089942). The stop codon is indicated by an asterisk. Underlined amino acid sequence indicates putative signal peptides. Eight cysteine residues in the putative mature polypeptide are given in bold.

Recombinant mature TAD1 polypeptide has antibacterial activity but lacks antifreeze activity

Since *Tad1* was found to be homologous to plant defensins (Figs. 1 and 2), we tried to express and purify the recombinant mature TAD1 polypeptide to determine a defense activity against phytopathogens. Analysis by tricine–SDS–PAGE revealed that the prominent protein band whose apparent molecular size is 31.5 kDa was strongly induced in total soluble fraction from IPTG-induced cells (Fig. 5, lanes N and S). This 31.5 kDa protein was bound to a glutathione–Sepharose 4B column (Fig. 5, lane T) and the mature polypeptide (rmTAD1) was eluted after digestion with PreScission Protease. Consistently, 200–300 μg of rmTAD1 was obtained from 800 ml *E. coli* culture.

Antibacterial assay of purified rmTAD1 was performed against the growth of *P. cichorii*. As shown in Fig. 6, the growth of *P. cichorii* was inhibited by addition of purified rmTAD1 in a dose-dependent manner. GST purified from *E. coli* cells carrying the control pGEX6P-3 plasmid (Fig. 5, lane G) showed no growth inhibition at the 100 μg/ml concentration (data not shown). An IC_{50} value was estimated from the dose–response curve and found to be approximately 25 μg/ml. Since the IC_{50} value was comparable to those with other

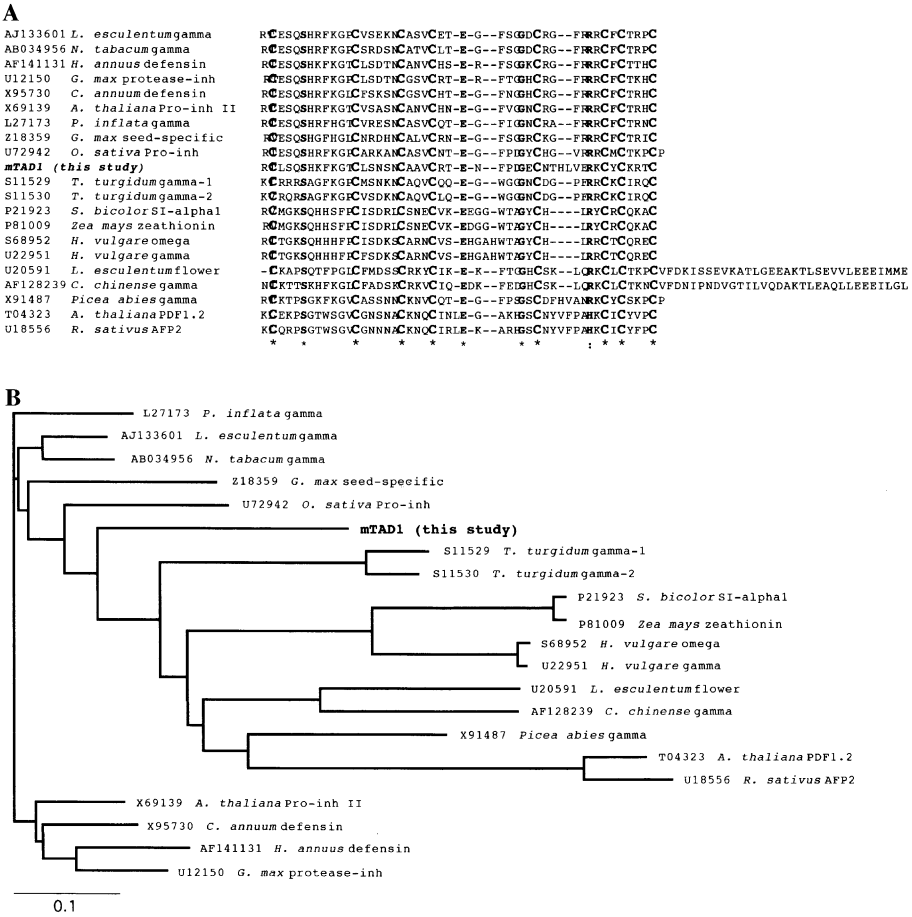

Fig. 2. Comparison of the putative mature sequence of TAD1 and that of other plant defensins (γ-thionins). (A) Alignment of plant defensin sequences available on GenBank (Accession Nos.): *Lycopersicon esculentum* γ-thionin (AJ133601); *Nicotiana tabacum* thionin-like protein (AB034956); *Helianthus annuus* defensin (AF141131); *Glycine max* protease inhibitor (U12150); *Capsicum annuum* defensin (X95730); *Arabidopsis thaliana* proteinase inhibitor II (X69139); *Petunia inflata* γ-thionin (L27173); *G. max* seed-specific sulfur-rich protein (Z18359); *Oryza sativa* proteinase inhibitor (U72942); *Triticum aestivum* mTad1 (AB089942); *Triticum turgidum* γ-purothionin 1 (S11529); *Triticum turgidum* γ-purothionin 2 (S11530); *Sorgham bicolor* α-amylase inhibitor (P21923); *Zea mays* γ-zeathionin 2 (P81009); *Hordeum vulgare* ω-hordothionin (S68952) *Hordeum vulgare* γ-thionin (U22951); *Lycopersicon esculentum* flower-specific γ-thionin (U20591); *Capsicum chinense* γ-thionin (AF128239); *Picea abies* γ-thionin (X91487); *Arabidopsis thaliana* PDF1.2 (T04323); and *Raphanus sativus* antifungal protein 2 (U18556). Asterisks indicate conserved amino acid residues. The sequence alignment was performed with CLUSTAL X software [43]. (B) The phylogenetic tree of the amino acid sequences that are aligned in (A). The tree was calculated by Neighbor-Joining method and displayed using TreeView software [23]. The bar represents evolutionary distance, expressed in the number of substitutions per amino acid.

plant defensins [34], it was concluded that rmTAD1 polypeptide has antibacterial activity.

Measurement of antifreeze activity of rmTAD1 was determined by ice crystal morphology. When a type III antifreeze protein from antarctic eelpout (*R. dearborni*) [26] was cooled at a concentration of 3 mg/ml, ice crystals grew into hexagonal bipyramids, indicative of antifreeze activity. In contrast, when rmTAD1 protein (12.5 mg/ml) was cooled similarly, no formation of hexagonal bipyramids was observed (Fig. 7A).

Discussion

In this study, we have utilized differential screening of macroarrayed cDNA library to identify a novel cold-regulated gene, *Tad1* from winter wheat. Sequence analysis revealed that *Tad1* encoded a polypeptide that is homologous to plant defensins which belongs to a subfamily of thionins (Figs. 1 and 2A). Thionins inhibit growth of a great variety of bacteria and fungi, and are the representative antipathogenic low molecular weight polypeptides in plants [35,36]. There is no considerable homology at amino acid sequence levels between α, β-thionin [36] and γ-thionin/defensin families [27]; however, mature polypeptides from both subfamilies commonly contain eight cysteine residues [37]. Those eight cysteine residues are thought to form characteristic four disulfide bonds that provide functional three-dimensional conformation [38,39]. Although the amino acid sequence of mTad1 was rather divergent, the number and position of the cysteine residues in mTAD1

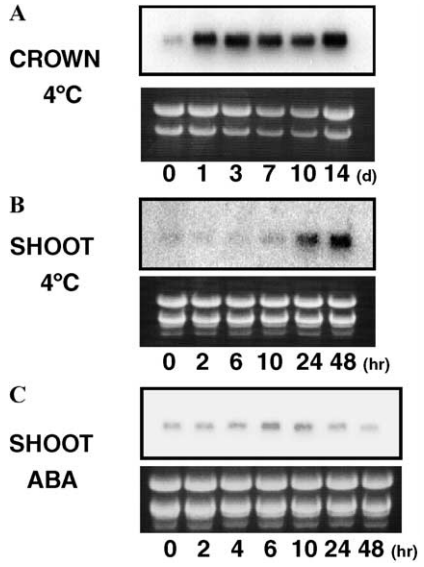

Fig. 3. (A) Expression of *Tad1* in crown tissue of winter wheat during cold acclimation. Fourteen-day-old plants were subjected to cold acclimation for designated time periods and total RNA (20 μg) from the crown tissue was analyzed by Northern blotting analysis. Ethidium bromide-stained gel indicates equal loading of the RNA samples. (B) Expression of *Tad1* in shoot of winter wheat in response to 4 °C. Hydroponically grown five-day-old seedlings were exposed to 4 °C for 0–48 h. Total RNA was isolated from shoot tissue and analyzed by Northern blotting analysis. (C) Expression of Tadl in shoot of winter wheat in response to 50 μM ABA. Hydroponically grown 5-day-old seedlings were transferred to water containing 50 μM ABA. At each time point (0–48 h), total RNA was isolated from shoot tissue and analyzed by Northern blot analysis.

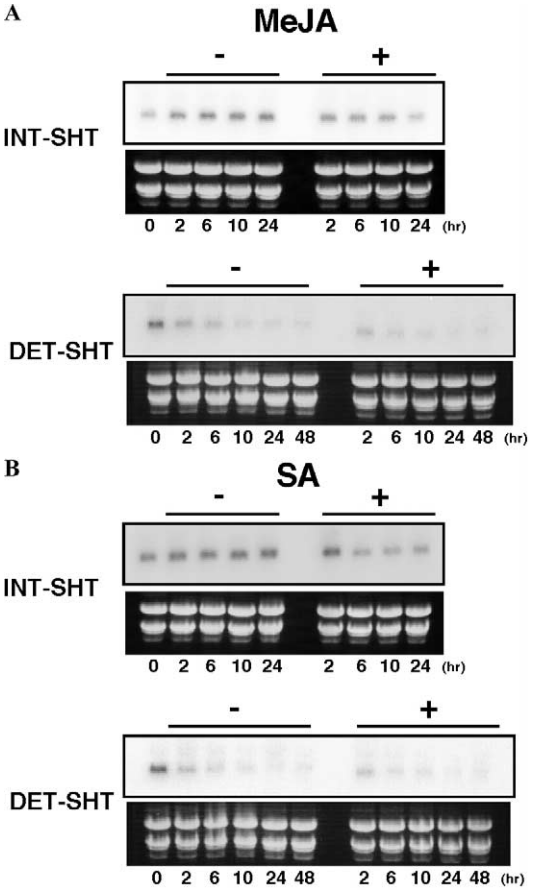

Fig. 4. Expression of *Tad1* in response to methyl jasmonate (MeJA) (A) and salicylic acid (SA) (B). In the upper panels (INT-SHT), hydroponically grown five-day-old seedlings were treated with 5 mM SA, 100 μM MeJA, or distilled water (−). In the lower panels (DET-SHT), detached whole shoots from five-day-old seedlings were floated on 5 mM SA, 100 μM MeJA or distilled water (−). Each shoot sample was collected at designated time for isolation of total RNA and analyzed by Northern blotting analysis.

was conserved with other known plant defensins (Fig. 2), supporting that *Tad1* is a novel plant defensin gene.

Overwintering plants encounter multiple biotic and abiotic stress factors during overwintering. Winter cereals are known to develop disease resistance in addition to freezing tolerance during cold acclimation [15,40]. Recent reports have shown that some of the apoplastic antifreeze proteins that accumulate during cold acclimation have sequence similarity to PR proteins, such as chitinases, β-1,3-glucanases, and thaumatin-like proteins [41]. Since enzyme activities for chitinase and β-1,3-glucanase were detected with the purified respective proteins, these proteins were considered to be bifunctional in regard to induced resistance against freezing and pathogens during CA [41]. In this study, we have shown for the first time that a novel class of plant defensin is also induced during cold acclimation. The recombinant rmTAD1 protein showed an IC_{50} of 25 μg/ml against *P. cichorii*, a plant pathogenic bacterium, suggesting that TAD1 protein functions as a defense protein. This is additional evidence that plants develop resistance against pathogens during cold acclimation through inducing defense proteins. When cold-induced chitinases, CHT9 and CHT46, from winter rye were produced in *E. coli*, the recombinant proteins exhibited antifreeze activity [11]. However, recombinant TAD1 protein did not show antifreeze activity, suggesting the TAD1 protein may solely function as an antipathogenic protein. Plant defensins are considered to be secreted to the apoplast or localized in cell wall [42]. Although it is to be determined if TAD1 localizes in the apoplast, discrimination in antifreeze activity of plant defense proteins that are specifically induced by low temperature is relevant for understanding functions of each proteins.

It is interesting to determine how cold-induced defense proteins are regulated. In plant defense mechanisms, divergent antimicrobial proteins (AMPs) are produced. The AMP genes encoding PR-1, PR-2, and PR-5 are induced by application of endogenous SA, whereas *pdf1.2* gene encoding a plant defensin as well as genes for PR-3 and PR-4 were induced by MeJA and not by SA in *Arabidopsis* [31–33,43]. Since MeJA and SA are the major factors that induce plant defense responses, we examined the response of *Tad1* to MeJA

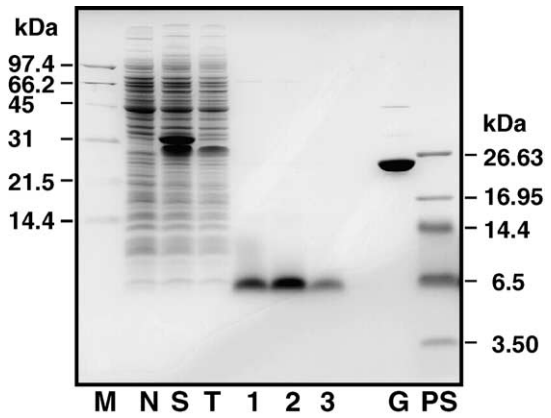

Fig. 5. Production and purification of recombinant mature TAD1 polypeptide (rmTAD1). Total soluble fraction was prepared from *E. coli* BL21 (DE3) strain containing pGEX-mTAD1 without (lane N) or with (lane S) IPTG induction, respectively. The IPTG-induced fraction (lane S) was subjected to glutathione–Sepharose 4B column and a flow-through fraction (lane T) and elution fractions after PreScission Protease digestion (lanes 1–3) were collected. Tricine–SDS–PAGE [24] was performed for separation of each fraction. Each molecular size of protein standard (M) and peptide standard (PS) is given on left and right, respectively.

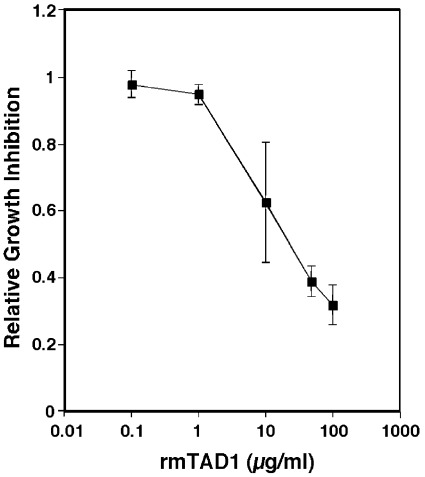

Fig. 6. Inhibitory effect of purified rmTAD1 polypeptide on the growth of *P. cichorii*. Growth of *P. cichorii* in the presence of indicated concentration of purified rmTAD1 polypeptide was monitored by measuring the OD_{595}. Average and standard errors of triplicate measurements are indicated.

and SA. Exogenously applied MeJA and SA did not increase the level of *Tad1* mRNA in the shoots of wheat seedlings (Fig. 4). This result suggested that *Tad1* induction possibly occurs independent from defense signaling pathways. Previously, it had been determined that cold and pathogen stimuli independently trigger accumulation of different classes of PR proteins in rye apoplast [44]. Our results are in good accordance with the previous observation, where *Tad1* is specifically induced by cold. Although ABA has been shown to be

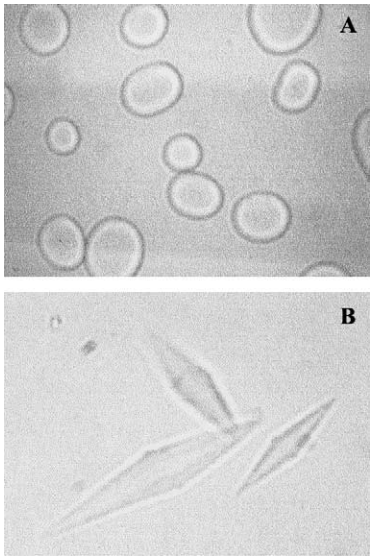

Fig. 7. Measurement of antifreeze activity. (A) Morphology of ice crystals formed in the presence of rmTAD1 (12.5 mg/ml) was determined using a Leica DMLB 100 photomicroscope equipped with a Linkam LK600 temperature controller. (B) Morphology of ice crystals formed in the presence of a type III antifreeze protein from antarctic eelpout (*R. dearborni*) (3 mg/ml).

involved in many cold-induced gene expression [4], cold-induced *Tad1* expression is independent of ABA signaling pathway.

For winter cereals, resistance to pathogens is an important trait for overwintering. Increased resistance to pathogen infection as well as freezing temperature is an important trait to be acquired through cold acclimation. This study demonstrates that cold-induced acquisition of pathogen resistance can be explained by the production of a subset of AMPs which is specifically induced by low temperature signals.

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