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TsPAP1 encodes a novel plant prolyl aminopeptidase whose expression is induced in response to suboptimal growth conditions

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ABSTRACT

A triticale cDNA encoding a prolyl aminopeptidase (PAP) was obtained by RT-PCR and has been designated as *TsPAP1*. The cloned cDNA is 1387 bp long and encodes a protein of 390 amino acids with a calculated molecular mass of 43.9 kDa. The deduced TsPAP1 protein exhibits a considerable sequence identity with the biochemically characterized bacterial and fungal PAP proteins of small molecular masses (~35 kDa). Moreover, the presence of conserved regions that are characteristic for bacterial monomeric PAP enzymes (the GGSWG motif, the localization of the catalytic triad residues and the segment involved in substrate binding) has also been noted. Primary structure analysis and phylogenetic analysis revealed that *TsPAP1* encodes a novel plant PAP protein that is distinct from the multimeric proteins that have thus far been characterized in plants and whose counterparts have been recognized only in bacteria and fungi. A significant increase in the *TsPAP1* transcript level in the shoots of triticale plants was observed under drought and saline conditions as well as in the presence of cadmium and aluminium ions in the nutrient medium. This paper is the first report describing changes in the transcript levels of any plant PAP in response to suboptimal growth conditions.

1. Introduction

Prolyl aminopeptidase (PAP, EC 3.4.11.5) is an enzyme that specifically removes N-terminal proline from peptides [1]. Although the proline aminopeptidase is the name recommended by the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/), other names such as Pro-X aminopeptidase, cytosol aminopeptidase V, proline aminopeptidase, proline iminopeptidase are also accepted and used. Some PAPs also have the ability to remove N-terminal hydroxyproline [2–5]. Studies using site-directed mutagenesis showed that despite a significant inhibition of prolyl aminopeptidase activity by cysteine peptidase inhibitors, serine plays a key role in the active centers of bacterial [6] and fungal PAPs [7]. Therefore, in the MEROPS database, bacterial and fungal PAPs have been classified in the family of serine peptidases (the S33 family). This family is divided into two subfamilies: S33.001, which more closely resembles the prolyl aminopeptidase from *Neisseria gonorrhoeae* [8], and S33.008, which more closely resembles the prolyl aminopeptidase from *Aeromonas sobria* [3]. The first subfamily contains small PAP proteins (30–35 kDa), which have been identified and characterized so far only in bacteria [8–12] and fungi [7]. With the exception of the PAP found in *Phanerochaete chrysosporium* [7], these proteins function as monomers. The second subfamily consists of multimeric proteins with masses ranging from 100 to 370 kDa, composed of 50 ± 5-kDa subunits, which are also found in bacteria [3,14–16] and fungi [2,13]. The plant PAPs that have been characterized are predominantly homotetramers of approximately 200 kDa [4,17–21], corresponding to the protein structure of the S33.008 subfamily. Among the PAP proteins characterized biochemically thus far, the amino acid sequences have been determined only for bacterial and fungal enzymes. The amino acid sequences of biochemically characterized plant PAPs have not yet been identified. In the last three years, the amino acid sequences of plant proteins that show by alignments significant similarity to the monomeric or multimeric sequences of bacterial or fungal PAPs appeared in the GenBank databases. However, there is no information regarding whether these genes undergo expression and under what conditions. In the current study, we cloned the gene encoding the triticale PAP protein. The deduced protein sequence contains conserved regions that are characteristic of the PAPs belonging to the S33.001 subfamily. For the first time, we also report an induction of the expression of a plant gene encoding a PAP under stress conditions, and we discuss its possible physiological role.

Abbreviations: PAP, prolyl aminopeptidase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; TsPAP1, *Triticosecale* prolyl aminopeptidase 1; UTR, untranslated region.

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2. Materials and methods

2.1. Plant material and experimental conditions

Seeds of triticale (x *Triticosecale* Wittm.) cv. Fidelio were obtained from the Breeding Station in Laski, Poland. The grains were surface-sterilized with 0.5% NaOCl for 30 min, washed several times with water and allowed to germinate. Stress treatments were imposed on 3-week old plants grown in containers filled with aerated Hoagland nutrient solution [22]. The salinity was supplied at a concentration of 50 mM NaCl, and the heavy metal ions were provided as 50 μM $AlCl_3$ or 50 μM $CdCl_3$. For the drought-stress treatment, the plants were removed from the nutrient solution and dehydrated on plastic trays. The experiments were conducted in a growth chamber under conditions described previously [4].

2.2. Cloning of the full-length TsPAP1 from triticale seedlings

The total RNA was isolated from the shoots of three-day-old triticale seedlings according to the method of Chomczynski and Sacchi [23]. First-strand cDNA synthesis, primed with an oligo $(dT)_{12–18}$ primer, was performed with avian myeloblastosis virus reverse transcriptase (AMV RT) following the manufacturer's protocol (Promega). Two oligonucleotide primers, F1 (5′-CACACCATCTACTACGAGC-3′) and R1 (5′-AGACAGCCAACTTTGATTG-3′), were used to amplify the internal region of *TsPAP-1* cDNA with High Fidelity PCR Enzyme Mix (Fermentas). The forward primer, F1, corresponded to a fragment of *Oryza sativa* and *Zea mays* cDNAs (GenBank ID CT831126 and AY105785, respectively) that exhibited a high sequence similarity to *Arabidopsis thaliana* prolyl iminopeptidase (GenBank ID NM_201725). The reverse primer, R1, was designed using a fragment of a *Secale cereale* EST (expressed sequence tag) clone (GenBank ID BE588175). The PCR amplification was performed under the following conditions: 2 min at 95 °C; 36 cycles of 30 s at 95 °C, 20 s at 52 °C, 1 min at 72 °C; and a final extension step for 5 min at 72 °C. The subsequent PCR product was ligated into the pGEM-T Easy vector (Promega), amplified in *Escherichia coli* JM 109, sequenced and analyzed.

The full-length cDNA of *TsPAP1* was obtained using the GeneRacer Kit (Invitrogen). The 3′ end was amplified using the gene-specific primer F2 (5′-AGTTTAAGGTGGTTCCAGATGCAGG-3′) and a GeneRacer 3′ Primer, provided with the kit. The PCR for 3′ end amplification was performed with Platinum® Taq DNA Polymerase (Invitrogen) under the following conditions: 2 min at 94 °C; 5 cycles of 30 s at 94 °C, 1 min at 72 °C; 5 cycles of 30 s at 94 °C, 1 min at 70 °C; 25 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 68 °C; and a final elongation time of 10 min at 68 °C. The 5′ end of the *TsPAP1* cDNA was amplified in two PCR stages using the Long PCR Enzyme Mix (Fermentas). The first round of the PCR was performed using the gene-specific primer R2 (5′-TGAACCGCCGAACACCTGCCACTCTG-3′) and a GeneRacer 5′ Primer (provided with the kit), under the following conditions: 2 min at 95 °C; 37 cycles of 30 s at 95 °C, 20 s at 58 °C and 45 s at 68 °C; and a final elongation time of 5 min at 68 °C. The PCR product from the first round of PCR was diluted 10-fold and was used as the template in the second PCR stage (nested PCR). The nested PCR for amplification of the longest 5′ end was performed with the gene-specific primer R3 (5′-GGTTCCCGGACTGCTCGTAGTAGAT-3′) and the GeneRacer 5′ Nested Primer (provided with the kit) under the following conditions: 2 min at 95 °C; 27 cycles of 30 s at 95 °C, 20 s at 58 °C and 30 s at 68 °C; and a final elongation time of 5 min at 68 °C.

The DNA sequencing was performed at the DNA Sequencing and Oligonucleotide Synthesis Laboratory, The Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

2.3. Real time RT-PCR analysis of TsPAP1

Aliquots of total RNA (1 ng) extracted individually from triticale shoots were used as the templates in Real-Time RT-PCR amplification carried out with gene-specific primers and the LightCycler – RNA Amplification Kit SYBR Green I (Roche) following the manufacturer's instructions ((55 °C for 10 min; 95 °C for 30 s; 45 cycles of 95 °C for 0 s (slope 20 °C/s), 60 °C for 10 s and 72 °C for 30 s; product length 248 bp; primers: 5′-GAGATTTTATTCCTGAG GACGAAAGG-3′ and 5′-GTGTGAGTCCGAGTCTAAG AATCC-3′)). The transcript level of *TsPAP1* under stress is expressed as the value relative to its transcript level determined in 3-week-old shoots harvested at the starting point of the stress treatment ($K = 1$), after being normalized to the triticale 18S rRNA level (amplification conditions as above; product length 427 bp; primers 5′-CCAGGTCCAG ACATAGTAAG-3′ and 5′-GTACAAAGGGCAGGGACGTA-3′). Three independent experiments were performed.

2.4. Computer analysis of DNA and protein sequences

The sequence similarity was verified by searching the database at the National Center for Biotechnology Information server using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). The global alignments of two nucleotide or amino acid sequences and the percentages of identity were calculated using the EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/emboss/). The alignment of the amino acid sequences was carried out using the Clustal W1.8 program (http://www.ebi.ac.uk/clustalw/).

3. Results and discussion

3.1. Isolation and sequence analysis of the TsPAP1 cDNA

Using the RT-PCR technique, the full-length cDNA of *PAP* was isolated and designated as *Triticosecale* prolyl aminopeptidase 1 (*TsPAP1*; GenBank ID JN808306). The first PCR, for the amplification of the internal region of *TsPAP1* cDNA, was performed with the F1 and F2 primers and produced a fragment 1056 bp in length. A nested PCR for the amplification of the longest 5′ end produced a cDNA fragment 310 bp in length. The PCR for the 3′ end amplification produced a cDNA fragment 306 bp in length.

The full-length *TsPAP1*cDNA is 1387 bp long and contains a 1173 bp open reading frame (ORF), a 5′ untranslated region (5′ UTR) of 12 bp and a 3′ UTR of 202 bp. The *TsPAP1* cDNA encodes a protein consisting of 390 amino acids (Fig. 1) with a calculated molecular mass of 43.9 kDa and an isoelectric point of 7.73.

3.2. Homology and phylogenetic analysis of functional and putative prolyl aminopeptidases

A GenBank database search revealed that the deduced TsPAP1 protein exhibits a sequence similarity to the biochemically characterized bacterial and fungal prolyl aminopeptidases with small molecular masses (~35 kDa). The TsPAP1 protein shares an overall sequence identity of ~45% with the PAP proteins of *Serratia marcescens* (GenBank ID BAA23336), *Neisseria gonorrhoeae* (GenBank ID AAW89640), *Xanthomonas axonopodis* (GenBank ID NP_641261), *Eikenella corrodens* (GenBank ID AAO42738) and *Phanerochaete chrysosporium* (GenBank ID ACS88344). Only a very limited sequence identity (15–19%) was detected between the TsPAP1 protein and the multimeric PAPs, which consist of subunits with higher molecular masses (~50–55 kDa).

To investigate the evolutionary relationship among different bacterial, fungal and plant prolyl aminopeptidases, a maximum-likelihood phylogenetic tree was constructed based on the amino

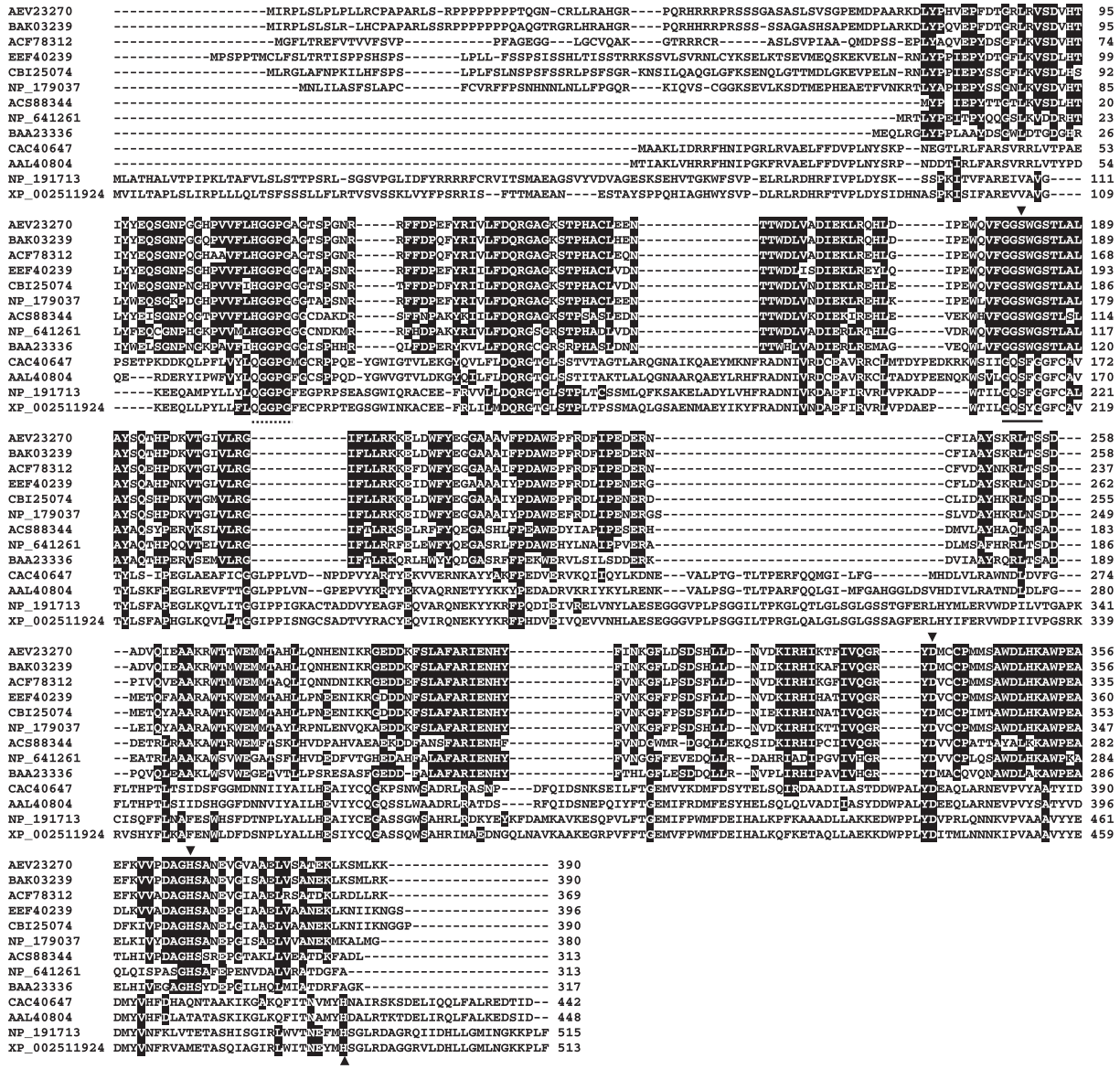

Fig. 1. The alignment of the deduced amino acid sequences of *TsPAP1* with functional (bacterial and fungal) or putative (plant) PAP proteins. Identical amino acids among the aligned sequences are shown on a black background. The conserved GXSXG ($G^{180}G^{181}S^{182}W^{183}G^{184}$ in TsPAP1) and HGGPG ($H^{113}G^{114}G^{115}P^{116}G^{117}$ in TsPAP1) motifs are underlined with continuous and dotted lines, respectively. The positions of the residues of the putative catalytic triad are marked by arrows (▼). The sequences of the following species were aligned: *Hordeum vulgare* (BAK03239), *Zea mays* (ACF78312), *Ricinus communis* (EEF40239), *Vitis vinifera* (CBI25074), *Arabidopsis thaliana* (NP_179037), *Phanerochaete chrysosporium* (ACS88344), *Xanthomonas axonopodis* (NP_641261), *Serratia marcescens* (BAA23336), *Aspergillus niger* (CAC40647), *Talaromyces emersonii* (AAL40804), *Arabidopsis thaliana* (NP_191713) and *Ricinus communis* (XP_002511924). The AEV23270 amino acid sequence represents TsPAP1 from x *Triticosecale*, obtained in this work.

acid sequences of biochemically characterized (bacterial and fungal) or putative (plant) PAP proteins. The aligned PAP sequences have been grouped into two large clusters (Fig. 2) supported with high bootstrap values.

One cluster (A) consists of proteins that are generally greater in length and occurs in bacteria and fungi (PAPs > 440 amino acids) as well as in plants (PAPs > 510 amino acids). Only bacterial and fungal members of this group have been characterized, and all of these proteins appeared to be multimeric enzymes [2,3,13–16]. The second cluster (B) also contains members from bacteria, fungi and plants, but these proteins are generally shorter in length (bacterial and fungal PAPs < 330 amino acids and plant PAPs < 400 amino acids). In this group, the vast majority of the enzymes function as monomers [8–12], although some exceptions exist [7]. In each cluster, the plant proteins are grouped into a single clade, suggesting that they share a common evolutionary ancestor. The division of the PAP proteins into two major clusters, revealed by the phylogenetic analysis, mirrors the classification proposed by the MEROPS database. The MEROPS database has grouped PAPs into two subfamilies on the basis of similarity to *Neisseria gonorrhoeae* PAP (S33.001) or *Aeromonas sobria* PAP (S33.0008). The PAP protein of *Neisseria gonorrhoeae* exists as a monomer of 35 kDa [8], whereas that of *Aeromonas sobria* is a homotetrameric enzyme of 205 kDa [3]. According to Mahon et al. [13], this classification correlates exactly with the presence of four additional helices (Mα1-4) in the secondary structures of all of the proteins from the S33.008 subfamily. According to the authors, an additional four helices are predicted to be unique to the multimeric PAPs and are not predicted to occur in monomeric enzymes. This feature might indeed be characteristic of all multimeric enzymes; however, it should be noted that the

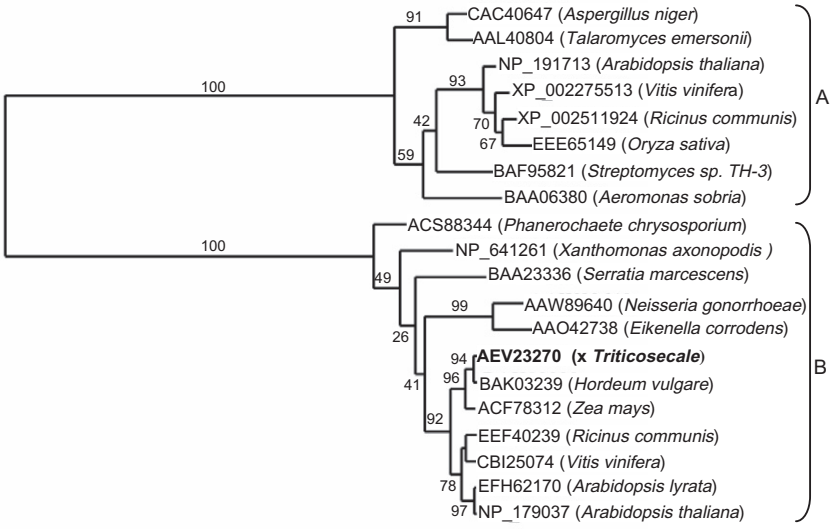

Fig. 2. The phylogenetic relationship among bacterial, fungal and plant prolyl aminopeptidases. The analysis was based on the ClustalW multiple amino acid sequence alignment of the functional (bacterial and fungal) or putative (plant) PAP proteins. The GenBank accession numbers of the amino acid sequences are indicated on the tree. The maximum-likelihood phylogenetic tree was created with the PhyML program [34] using the Jones–Taylor–Thornton (JTT) amino acid substitution model. The tree was displayed with TreeDyn. The bootstrap values are shown for each branch as a percentage of 100 replicates.

Phanerochaete chrysosporium PAP protein grouped in cluster B (Fig. 2), which is considered to be the counterpart of the S33.001 subfamily, occurs as a dimer [7].

3.3. Sequence analysis of the deduced TsPAP1 protein

When aligning the TsPAP1 amino acid sequence with representatives of the biochemically characterized monomeric and multimeric prolyl aminopeptidases, conserved regions characteristic for the first group were identified in the triticale sequence (Fig. 1). Bacterial and fungal PAPs are considered to be serine peptidases that possess a conserved catalytic triad consisting of Ser, Asp and His residues (Ser^{182}–Asp^{338}–His^{356} in TsPAP1). Whereas the positions of Ser and Asp are conserved between the mono- and multimeric enzymes, the His residue of the monomeric PAPs was aligned in localization other than in the latter group. The specific GXSXG motif, which contains the nucleophilic serine and is conserved among α/β-hydrolase fold serine peptidases [24], consists of the GGSWG amino acid sequence ($G^{180}G^{181}S^{182}W^{183}G^{184}$ in TsPAP1) in the monomeric enzymes, whereas in the multimeric ones, it comprises the GQSFG residues. In the PAP from *Xanthomonas campresis*, the residues from His^{41} to Gly^{46} play a putative role in substrate binding at the primed subsites [11]. Based on the sequence alignment, this segment was also found in TsPAP1 at the residues 113–118, with Ala instead of Gly at the last position. In the multimeric enzymes, the segment involved in substrate binding starts with Gln instead of His and ends with Phe or Met instead of Gly.

In the GenBank database, sequences encoding putative plant PAP proteins with high amino acid identity to bacterial and fungal PAPs, either of small or high molecular masses, are also present; however, their biochemical characteristics are not yet confirmed. Among these proteins, the highest identity with TsPAP1 was displayed by a sequence from *Hordeum vulgare* (92%) that encodes a protein with a calculated molecular mass of 44.1 kDa (GenBank ID BAK03239) and the sequence characteristics of the bacterial monomeric PAPs. It is interesting to note that plants, such as *Arabidopsis*, *Vitis* and *Ricinus* sp., possess in their genomes genes that encode two types of PAPs, whereas bacteria and fungi possess only one type of PAP, either monomeric or multimeric. To date, the biochemically characterized plant PAPs have turned out to be multimeric enzymes, primarily homotetramers, with a molecular weight of ~55 kDa per subunit. There is no information regarding the functioning of monomeric prolyl aminopeptidases in plant tissues, although the presence of other plant aminopeptidases (for example aromatic aminopeptidases) that exist as a monomers has been reported [25 and references therein].

3.4. Expression of TsPAP1 under stress conditions

The expression of *TsPAP1* under stress conditions was determined by real-time semi-quantitative RT-PCR, and the results were standardized relative to the triticale 18S rRNA gene.

Under drought conditions, a significant 2.5-fold increase in the *TsPAP1* transcript level was observed as early as the first day after the stress application (Fig. 3A). The upward trend continued until the fourth day (96 h), at which point the transcript accumulation was four times higher than at the beginning of the experiment. Thereafter, the *TsPAP1* transcript level declined slightly.

In plants grown on nutrient media supplemented with NaCl (Fig. 3B) or metal ions (Fig. 3C and D), a considerable increase in the expression of *TsPAP1* (by approximately 50%) was observed no earlier than 48 h after treatment application. The *TsPAP1* transcript level continued to increase until the fourth day (96 h), at which point the mRNA accumulation was approximately two times higher than at the beginning of the experiment. The expression of *TsPAP1* then decreased.

Changes in the expression of genes encoding PAPs in plants, under either physiological or stress conditions, have not yet been described in the literature. Similarly, the information concerning the changes in the activity of prolyl aminopeptidase in response to stress conditions is very scarce and is limited to observations made in shoots of triticale [4]. We have reported previously a significant increase in aminopeptidase activity against proline-β-naphthylamide (β-NA) in the presence of metal ions in the nutrient medium as well as under salinity and drought stresses. After the application of cadmium and aluminum to the nutrient medium, the most pronounced increase in activity was observed on the fifth day (7- and 11-fold, respectively); under drought conditions, the largest increase, five-fold, was observed on the fourth day (96 h); and in

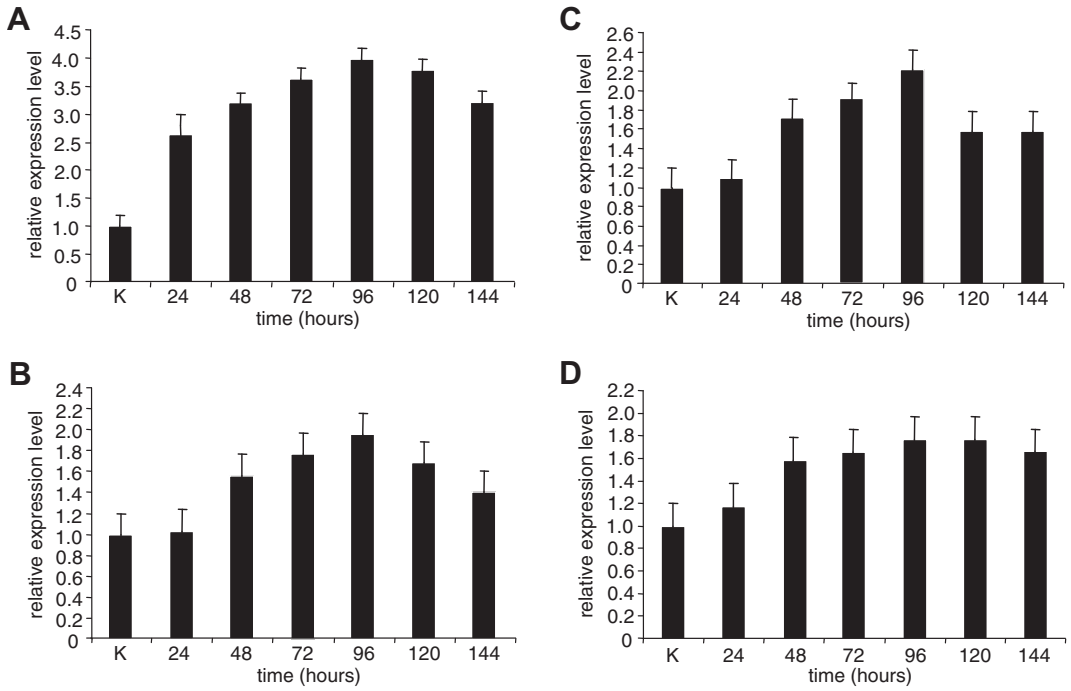

Fig. 3. The changes in the *TsPAP1* transcript levels in the shoots of triticale plants grown under drought (A), salinity (B), aluminum (C) or cadmium (D) treatments. The salinity was supplied at a concentration of 50 mM NaCl, and the heavy metal ions were provided as 50 μM $AlCl_3$ or 50 μM $CdCl_3$. The transcript level of *TsPAP1* under stress is expressed relative to its transcript abundance at the starting point of the stress treatment ($K = 1$), after being standardized to the 18S rRNA level. The results are the means ± SD from three independent reactions.

plants grown under salinity, the largest increase (15-fold) was observed on the sixth day [4]. The increases in the *TsPAP1* transcript levels observed in the present study were less spectacular than the activity changes observed previously [4], but positively correlated with previous findings with respect to time. Under all of the examined stress conditions, the increase in the *TsPAP1* transcript level always preceded in time the increase in activity toward Pro-β-NA, which was confirmed when both changes were tracked in parallel in one experiment. Because the unique structure of proline limits the hydrolysis of N-terminal peptide bonds by aminopeptidases other than PAP, the changes in activity toward Pro-β-NA, which were observed previously in crude extracts of triticale shoots, were mainly attributable to the multimeric form of PAP, which is commonly found in plants. However, the results presented here may suggest the possible contribution of the protein encoded by *TsPAP1* to the activity changes observed previously under stress. It is possible that another prolyl aminopeptidase was present among the aminopeptidases with low molecular weight, which were separated during the purification process from the multimeric PAP with high molecular weight (~200 kDa) [4].

To date, the studies conducted on changes in the activity level and mRNA level in response to adverse environmental conditions have focused primarily on leucine aminopeptidase and aminopeptidases with a broad substrate specificity. An increase in the level of protein and mRNA coding for leucine aminopeptidase type A was observed in tomato leaves subjected to drought and salinity stress [26]. In rice, salinity increased the activity against Leu-*p*-nitroanilide in the roots and lowered the activity in the shoots [27]. In wheat leaves, however, the conditions of acute water deficit caused a significant increase in the aminopeptidase activity (against Leu-*p*-nitroanilide), both in varieties resistant and susceptible to drought stress [28]. Very few studies have been published so far on the changes in aminopeptidase activity under the effect of heavy metal ions. In pea leaves under the influence of cadmium ions, an increase in the activity of two peroxisomal isoforms of leucine aminopeptidase was observed [29]. However, the same treatment caused a decline in the LAP activity in germinating rice seeds [30].

The role of aminopeptidases in response to adverse growth conditions has not been fully recognized yet. It is assumed that aminopeptidases can modify the level or the activity of proteins and regulatory peptides, regulate protein half-life, facilitate the turnover of oxidatively damaged proteins and take part in the osmotic regulation of plants by participating in the regulation of the free amino acid pool size [26,31,32]. Given the known role of proline as an osmoprotectant, one can assume that not only the rate of proline synthesis [33] but also the activity of prolyl aminopeptidase may determine the high levels of this amino acid under stress conditions.

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