

Plant peptide deformylase: a novel selectable marker and herbicide target based on essential cotranslational chloroplast protein processing

Cai-Xia Hou¹, Lynnette M. A. Dirk¹, Sitakanta Pattanaik², Narayan C. Das², Indu B. Maiti², Robert L. Houtz¹ and Mark A. Williams^{1,*}

¹Department of Horticulture, Plant Physiology/Biochemistry/Molecular Biology Program, University of Kentucky, Lexington, KY 40546-0091, USA

²Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY 40546-0236, USA

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*Correspondence (fax 859-257-2859;
e-mail mawillia@uky.edu)

Summary

Transgenic tobacco plants expressing three different forms of *Arabidopsis* plant peptide deformylase (*AtDEF1.1*, *AtDEF1.2* and *AtDEF2*; EC 3.5.1.88) were evaluated for resistance to actinonin, a naturally occurring peptide deformylase inhibitor. Over-expression of either *AtDEF1.2* or *AtDEF2* resulted in resistance to actinonin, but over-expression of *AtDEF1.1* did not. Immunological analyses demonstrated that *AtDEF1.2* and *AtDEF2* enzymes were present in both stromal and thylakoid fractions in chloroplasts, but *AtDEF1.1* was localized to mitochondria. The highest enzyme activity was associated with stromal *AtDEF2*, which was approximately 180-fold greater than the level of endogenous activity in the host plant. Resistance to actinonin cosegregated with kanamycin resistance in *Atdef1.2-D* and *Atdef2-D* transgenic plants. Here, we demonstrate that the combination of plant peptide deformylase and peptide deformylase inhibitors may represent a native gene selectable marker system for chloroplast and nuclear transformation vectors, and also suggest plant peptide deformylase as a potential broad-spectrum herbicide target.

Keywords: deformylase, herbicide, protein processing, selectable marker.

Introduction

Peptide deformylase (DEF; EC 3.5.1.88) catalyses the hydrolysis of the *N*-formyl group from the initiating methionine in newly translated proteins, and is essential for all subsequent *N*-terminal protein processing as well as cell survivability. Originally thought to be restricted to prokaryotic organisms, this enzyme was recently discovered in several plant species as a consequence of genome sequencing efforts. In all plants thus far examined, there are two peptide deformylase genes (*DEF1* and *DEF2*), and both have been extensively characterized in *Arabidopsis thaliana* (Giglionne *et al.*, 2000; Dirk *et al.*, 2001; Serero *et al.*, 2001). Although both genes encode polypeptides with peptide deformylase activity, *AtDEF1* and *AtDEF2* have different biochemical characteristics, subcellular localizations and phenotypes resulting from T-DNA insertional mutations. *AtDEF2* exhibits a strong polypeptide sequence preference for the *N*-terminus of the D1 polypeptide subunit of the photosystem II complex, as evident by a 240-fold increase in catalytic efficiency (k_{cat}/K_m)

compared with other polypeptide sequences, whereas *AtDEF1* shows little change in catalytic efficiency with polypeptide substrate sequence (Dirk *et al.*, 2002). In addition, *AtDEF1* is less sensitive to the inhibitory effects of the known peptide deformylase inhibitor actinonin, with a twofold weaker *in vitro* binding for *AtDEF1* than *AtDEF2* (Dirk *et al.*, 2001). Subcellular localization studies suggest that both *AtDEF1* and *AtDEF2* are found in chloroplasts and mitochondria (Giglionne *et al.*, 2000; Dinkins *et al.*, 2003); however, the presence of two translational start codons in *AtDEF1* potentially gives rise to two translation products: *AtDEF1.1* and *AtDEF1.2*. Translation from the upstream start codon results in *AtDEF1.1*, which is restricted to mitochondria, and translation from the downstream start codon results in *AtDEF1.2*, which is localized to both mitochondria and chloroplasts (Dinkins *et al.*, 2003). Peptide deformylase inhibitors, such as actinonin, are lethal to all plants (Hou *et al.*, 2006), and recent evidence suggests that DEF inhibition results in incomplete and/or incorrect cotranslational processing of the D1 polypeptide (Hou *et al.*, 2004). Thus, the essentiality of

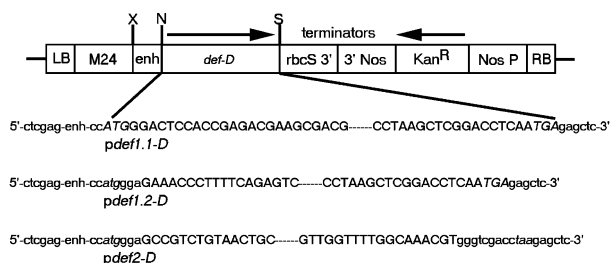


Figure 1 Schematic diagrams of the plant expression constructs containing chimeric *Atdef-D* genes. The modified full-length transcript promoter (M24) of the *Mirabilis mosaic virus* (Dey and Maiti, 1999a,b) directs the coding sequences of the respective peptide deformylase genes (*DEF*). A translational enhancer sequence (enh), a 35-nucleotide long 5' untranslated region of AIMV alfalfa mosaic virus RNA 4, was fused with the gene. LB, left T-DNA border; RB, right T-DNA border; Kan^R, neomycin phosphotransferase II marker gene directed by nopaline synthase promoter (Nos P). The 3' terminator sequences (terminators) of the ribulose bisphosphate carboxylase (*rbcS 3'*) and nopaline synthase (3'-Nos) genes are also shown. For each chimeric gene, the DNA sequences at the N and C termini are shown. The *DEF* sequence is shown in uppercase, and the coding sequence not derived from *DEF* is shown in lowercase. The positions of the *Xho*I (X), *Sst*I (S) and *Nco*I (N) restriction sites used to assemble these expression vectors are shown.

peptide deformylase in plants is probably a consequence of its role in the cotranslational processing of vital chloroplast proteins.

The essentiality of plant peptide deformylase, together with the large pool of chemical compounds representing peptide deformylase inhibitors, and the ever growing interest in alternatives to antibiotic-based selectable markers in transformation vectors, all suggest that an examination of plant peptide deformylase and its role in cotranslational protein processing could result in the development of a new selectable marker system as well as a new class of broad-spectrum herbicides. Here, we demonstrate that peptide deformylase in conjunction with a peptide deformylase inhibitor serves both of these roles.

Results

Nicotiana transformation with *Arabidopsis def* constructs

Chimeric *Atdef1.1-D*, *Atdef1.2-D* and *Atdef2-D* genes were introduced into tobacco (*Nicotiana tabacum* cv. Samsun NN) plants, and 12 independent transgenic tobacco lines were generated for each construct (Figure 1). A vector control, consisting of full-length β -glucuronidase (GUS) in pKM24 (Dey and Maiti, 1999a), was also introduced. Transgenic plants generated from each construct (T₁ and T₂ progeny, second and third generation) were screened for gene integration, transcription and translation by polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), real-time quantitative

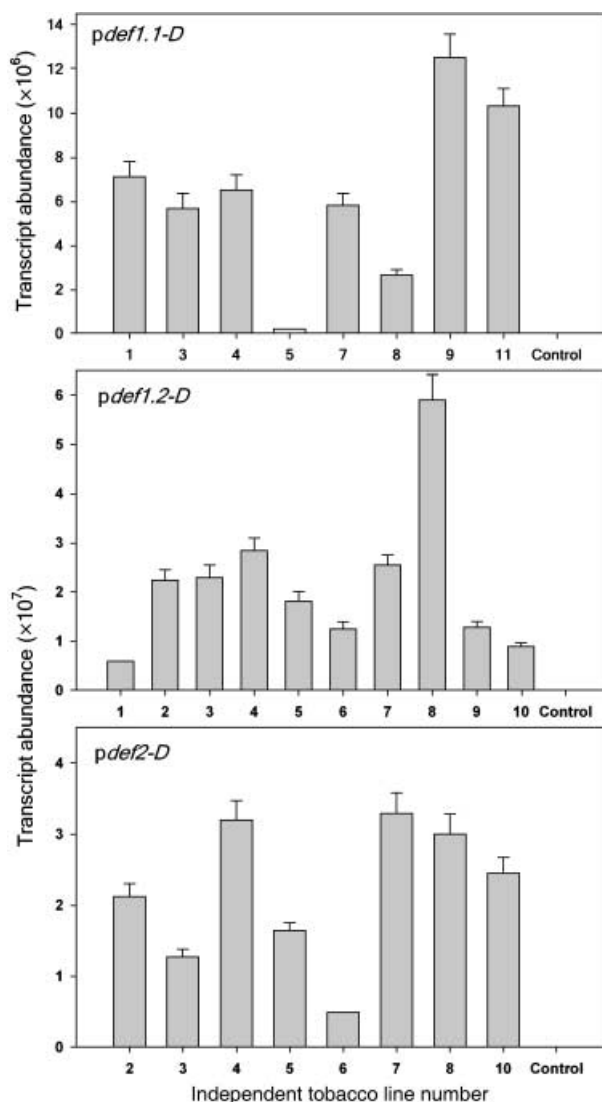


Figure 2 Expression analysis of plants containing *Atdef-D* genes by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in stably transformed transgenic tobacco plants. For each construct, independent transgenic lines (T₁ progeny) with KanR : KanS = 3 : 1 were selected. The average numbers of peptide deformylase (*DEF*) transcripts per 100 ng of total RNA in transgenic lines were derived for the constructs as indicated. Samsun NN was used as the untransformed control.

RT-PCR (qRT-PCR) and immunological analyses. Real-time qRT-PCR revealed different amounts of *DEF* transcripts with an abundance ranging from 1.7×10^5 to 5.9×10^7 transcripts per 100 ng of total RNA (Figure 2).

Resistance of *def-D*-expressing plants to the peptide deformylase inhibitor actinonin

Representative results from independent lines exhibiting a high level of expression of *DEF* are shown in Figure 3. The

Table 1 *Arabidopsis thaliana* peptide deformylase (AtDEF) accumulation and activity in transgenic tobacco lines. Accumulation of AtDEF in transgenic tobacco lines was quantified in stromal and thylakoid extracts by enzyme-linked immunosorbent assay (ELISA) using AtDEF1- and AtDEF2-specific antibodies. Stromal extracts were evaluated on the basis of total protein concentration and DEF activity was quantified using a continuous spectrophotometric assay. Thylakoid extracts were evaluated on the basis of chlorophyll content and were assayed for activity in a discontinuous assay. Data represent the mean \pm standard deviation ($n = 6$)

Genotype	DEF accumulation ($\mu\text{g}/\text{mg}$ protein or mg chlorophyll)	k_{cat} (s^{-1})	DEF activity ($\text{nmol}/\text{min}/\text{mg}$ protein or mg chlorophyll)
Stromal extracts			
WT*	ND	–	0.31 ± 0.08
<i>def1.1-7D</i> *	ND	–	0.22 ± 0.04
<i>def1.2-6D</i> *	0.46 ± 0.07	0.44 ± 0.05	0.52 ± 0.06
<i>def2-2D</i> †	0.67 ± 0.06	35 ± 5.1	57 ± 8.4
Thylakoid extracts			
WT†	ND	–	4.7 ± 1.1
<i>def1.1-7D</i> †	ND	–	7.7 ± 1.8
<i>def1.2-6D</i> †	2.1 ± 1.4	1.8 ± 0.17	9.7 ± 0.94
<i>def2-2D</i> §	7.9 ± 1.13	41 ± 7.0	800 ± 130

Assays were conducted with the following: *2 mg protein; †141 μg chlorophyll; ‡0.6 mg protein; §20 μg chlorophyll.

ND, not detectable; WT, wild-type untransformed tobacco.

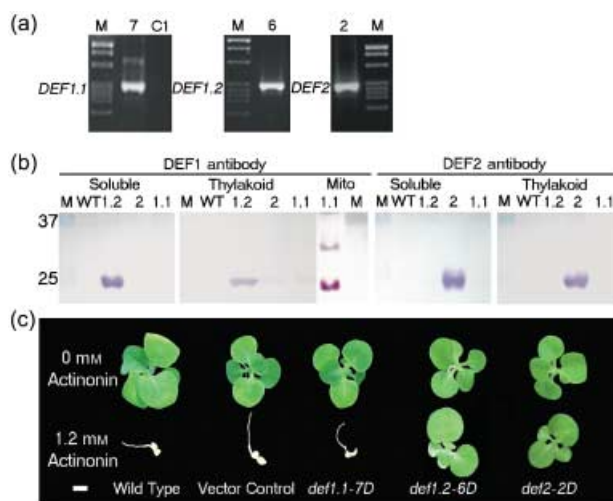


Figure 3 Expression of *Atdef1.1-D*, *Atdef1.2-D* and *Atdef2-D* in transgenic tobacco. (a) Analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) amplification products from independent T_2 lines (*def1.1-7D*, *def1.2-6D* and *def2-2D*) stained with ethidium bromide after electrophoresis on a 1% agarose gel. C1, non-transgenic tobacco control; M, marker DNA (100-bp ladders). (b) Western blot analysis of stromal (15 μg per lane), thylakoid (2 μg chlorophyll per lane) and mitochondrial (50 μg per lane) extracts from *def1.1-7D* (lane 1.1), *def1.2-6D* (lane 1.2) and *def2-2D* (lane 2) transgenic plants probed with AtDEF1- and AtDEF2-specific antibodies. WT, non-transgenic tobacco control; M, marker (kDa). (c) Tolerance to the peptide deformylase inhibitor actinonin by transgenic tobacco plants over-expressing *Atdef-D* genes. Seeds from the wild-type, vector control (no *Atdef-D* insert), *def1.1-7D*, *def1.2-6D* and *def2-2D* plants were germinated on T^+ medium [Murashige and Skoog basal salts (Sigma), vitamin B5 (Sigma), 3 mM CaCl_2 , 0.1 mM FeSO_4 , 2% sucrose, pH 5.8] containing 0 or 1.2 mM actinonin, and the seedlings were grown for 35 days. The bar represents 3 mm.

Atdef-D genes were stably integrated and transcribed into mRNA at the expected size (Figure 3a), and a single immunoreactive band was observed at the expected molecular mass when stromal and thylakoid extracts from *Atdef1.2-D* and *Atdef2-D* transgenic lines were screened by Western analysis with antibodies specific for either AtDEF1 or AtDEF2 (Figure 3b). Western analysis of *Atdef1.1-D* plants using the AtDEF1 antibody, which is reactive to both AtDEF1.1 and 1.2 proteins, revealed a strong band at the expected size in mitochondrial lysates and a weak signal in the thylakoid fraction, probably caused by a small amount of mitochondrial contamination (Figure 3b). Transgenic tobacco plants over-expressing either *Atdef1.2-D* or *Atdef2-D* were resistant to actinonin at all stages of plant growth (Figure 3c, and data not shown), providing strong evidence that peptide deformylase is the *in vivo* target of actinonin and, probably, all peptide deformylase inhibitors. Over-expression of *Atdef1.1-D*, however, did not result in resistance to actinonin, probably as a consequence of mitochondrial localization. Transgenic *Atdef1.2-D* and *Atdef2-D* plants grown to maturity were phenotypically normal and fertile (data not shown).

Quantification of peptide deformylase expression and kinetic analysis in transgenic plants

The detection of peptide deformylase activity has not been reported previously from plants. In this study, we detected endogenous DEF activity in chloroplasts from wild-type tobacco leaf tissues (Table 1), and this activity was stabilized in buffer with Ni^{2+} . Compared with endogenous tobacco DEF activity, the largest increase in transgenic peptide deformylase

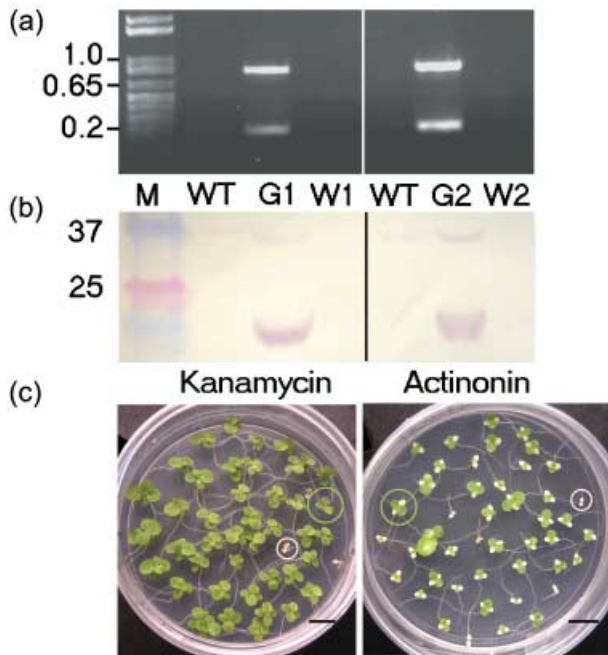


Figure 4 Comparison of *Arabidopsis thaliana* peptide deformylase (*Atdef2-D*) and neomycin phosphotransferase type II (*nptII*) used as selectable markers. (a) Analysis of polymerase chain reaction (PCR) amplification products from *Atdef2-D* and *nptII* wild-type (WT), transformed [G1, as circled in green in (c), left] and segregating, untransformed [W1, as circled in white in (c), right] plants after rescue from selection on 100 mg/L kanamycin (left) or 1.2 mM actinonin (right). Expected amplicon sizes of 819 and 197 bp for *Atdef2-D* and *nptII*, respectively, were visible only for the green plants. (b) Western blot analysis of AtDEF2-D from 40 µg per lane of leaf extracts from WT, transformed and segregating, untransformed plants from kanamycin-selected (left) and actinonin-selected (right) plants. The immunoreactive band of the expected size (23 kDa) was visible only in green plants. (c) Selection of T₁ progeny transformed with p*Atdef2-D*, which includes *nptII*, on 100 mg/L kanamycin (left) or 1.2 mM actinonin (right), 23 days after plating. The bar represents 1 cm.

activity occurred in the *def2-D* plants, with a 170- to 184-fold increase in activity in the thylakoid and stromal fractions, respectively (Table 1). Enzyme-linked immunosorbent assay (ELISA) quantification and subsequent calculation of k_{cat} values for *def2-D* thylakoid and stromal fractions revealed values that were 23- and 80-fold higher than those for *def1.2-D*, respectively, suggesting that DEF2 may be the dominate form in nascent protein N-terminal processing. This is consistent with previous results showing that insertional mutants in *AtDEF1* do not exhibit an identifiable phenotype, whereas *Atdef2* mutants grow slowly and are bleached in appearance (Gigliione *et al.*, 2003). Although *def1.2-D* plants showed a smaller amount of DEF accumulation and much lower k_{cat} values than *def2-D* plants (Table 1), these plants were equally resistant to the phytotoxic effects of actinonin, indicative of the higher actinonin tolerance of DEF1, as reported previ-

ously (Dirk *et al.*, 2001). *In vitro* measurements of DEF activity in the presence and absence of actinonin are also indicative of increased DEF protein levels, with significant increases in the level of actinonin required for 50% inhibition of enzyme activity (data not shown). Consistent with a mitochondrial localization, there was no detectable increase in activity in *def1.1-D* transgenics in the stromal fractions, and only a slight increase in the thylakoid fractions, which may have resulted from mitochondrial contamination.

Correlation of actinonin resistance with peptide deformylase subcellular localization

Plant DEF1 and DEF2 have been described as functionally redundant in studies demonstrating the rescue of a temperature conditional DEF mutant *Escherichia coli* cell line (Gigliione *et al.*, 2000). Although that report indirectly confirmed the DEF activity of AtDEF1 and AtDEF2, determining the functional redundancy in plants will be confounded by the different subcellular localization of plant DEF1.1, DEF1.2 and DEF2. Furthermore, mitochondrially localized forms of peptide deformylase are found in many eukaryotic species, including humans, have poor catalytic activity (Nguyen *et al.*, 2003) and may not be biologically active, as evident by the current testing of potent peptide deformylase inhibitors in phase II and III clinical trials as broad-spectrum antibiotics (Ramanathan-Girish *et al.*, 2004; Fritsche *et al.*, 2005). Therefore, in plants, it might be expected that a strictly mitochondrially localized form of peptide deformylase (DEF1.1) would not result in actinonin resistance, whereas forms of peptide deformylase localized to chloroplasts (DEF1.2, DEF2) would. The pattern of actinonin resistance observed (Figure 3c) is consistent with chloroplast localization for DEF1.2 and DEF2 and mitochondrial localization for DEF1.1, as reported previously from green fluorescent protein (GFP) fusion studies (Dinkins *et al.*, 2003).

def as a selectable marker

During these studies, it became apparent that the combination of *DEF* expression and DEF inhibitors could also serve as a selectable marker system for plant transformation. Therefore, we evaluated the feasibility of using *Atdef2-D* as a selectable marker. A comparison between transgenic plants grown on kanamycin or actinonin confirmed that *Atdef2-D* allows the selection of transgenic plants with an efficiency equal to kanamycin (Figure 4). Both green and bleached plants were screened for the cosegregation of the kanamycin gene and the *Atdef2-D* gene. Only green plants contained both genes (Figure 4a) and accumulated DEF (Figure 4b). As

with kanamycin selection, plants germinated in the presence of actinonin, which were not transgenic, were developmentally arrested at the cotyledonary stage (Figure 4c). For plants expressing the *AtDEF2* transgene, cotyledons remained white, but all subsequent growth was normal (see also Figure 2).

Conclusions

The essentiality and widespread conservation of DEF in plants makes it an attractive molecular target for the design of a new class of broad-spectrum herbicides. Indeed, actinonin has been shown to have broad-spectrum herbicidal activity against a wide range of plants, including many agriculturally important weed species (Hou *et al.*, 2006). The acute phytotoxic effects of actinonin, resulting from the inhibition of DEF, and the complete protection from this compound by the over-expression of either *DEF1.2* or *DEF2* are indicative of the potential of this technology as a novel broad-spectrum weed control system, as well as a selectable marker using native plant genes. Although the agricultural utility of peptide deformylase inhibitors has yet to be established, the potential effectiveness of this system warrants further investigation, especially in the light of recent structural studies which have identified major species-specific differences between peptide deformylases, thus providing the opportunity for the design of inhibitors specific to the plant forms of peptide deformylase. Specific and potent inhibitors of bacterial peptide deformylase have been designed and synthesized based on the numerous crystal structures available for this enzyme (Fieulaine *et al.*, 2005). Recent structural analyses suggest that *AtDEF1* is distinct from other peptide deformylases and most representative of eukaryotic mitochondrial peptide deformylases, whereas *AtDEF2* is restricted to plant plastids and *Apicomplexa* (Fieulaine *et al.*, 2005). Therefore, it seems plausible that a compound specific for plant DEF2 without antibiotic activity could be developed.

The resistance to actinonin by the over-expression of *AtDEF1.2* and *AtDEF2* also confirms the potential suitability of plant peptide deformylase as a selectable marker. This observation is highly significant in the light of the major biosafety concerns associated with the use of transgenic plants harbouring foreign DNA, with the potential for horizontal transfer of antibiotic resistance genes. Although not confirmed in field situations (Broothaerts *et al.*, 2005), these concerns have resulted in a call for the development of new selection technologies that do not depend on bacterially derived genes for antibiotic resistance. Alternative systems have been developed, such as marker-free selection (de Vetten *et al.*, 2003), marker removal using site-specific recombination (Baszczynski *et al.*, 2003) and plant genes capable of providing selection through either herbicide

resistance (Arias *et al.*, 2006) or antibiotic resistance (Mentewab and Stewart, 2005). Ideally, selection should not involve the expression of foreign DNA or the use of antibiotic compounds, and our studies suggest that plant peptide deformylase and associated specific inhibitors may be able to meet these criteria.

Experimental procedures

Construction of plant expression vectors *pdef1.1-D*, *pdef1.2-D* and *pdef2-D*

DNA fragments corresponding to the coding sequences of *DEF1.1*, *DEF1.2* and *DEF2* were PCR amplified from plasmids containing cDNAs of the *Arabidopsis* peptide deformylase genes *AtDEF1* (GENBANK accession no. AF250959) and *AtDEF2* (GENBANK accession no. AF269165). *DEF1.1* was amplified starting from the first ATG codon in *AtDEF1* using the forward primer 5'-GCGGGCTCGAGACCATGGGAC TCCACCGAGACGAAGCGACGGCTATGGAACCTTTTCAGAG-3' containing *XhoI* and *NcoI* restriction sites and the reverse primer 5'-ATGCAGGAGCTCTATTGAGGTCCGAGCTTAGG-3' containing an *SstI* restriction site and stop codon. *DEF1.2* was amplified starting from the second downstream ATG codon in *AtDEF1* using the forward primer 5'-GCGGGCTCGAGACCATGGGAGAAACCTTTTCAGAGTC-3' containing *XhoI* and *NcoI* sites and the same reverse primer as used for the *DEF1.1* amplification. The *DEF2* fragment was amplified using the forward primer 5'-GCGGGCTCGAGACCATGGGAGCCGTCTGTAAC TGC-3' containing *XhoI* and *NcoI* restriction sites and the reverse primer 5'-ATGCAGGAGCTCTTAGGTGACCCACGTTTGCCAAAAC CAAC-3' containing *SstI* and *SalI* restriction sites and a stop codon. PCR amplification was carried out for 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. PCR-amplified fragments were cloned into pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced by the dideoxy chain terminator method (Sanger *et al.*, 1977). Respective *DEF* fragments were digested with *NcoI*-*SstI*, gel purified and cloned into the corresponding sites of pBS-alfalfa mosaic virus AIMV5' (Maiti *et al.*, 1993) containing the 5' untranslated region of AIMV RNA 4. From these resulting plasmids, the *XhoI*-*SstI* fragments of respective *DEF* genes were cloned separately into the corresponding sites of the expression vector pKM24 (Dey and Maiti, 1999a,b). The resulting expression constructs were designated *pdef1.1-D*, *pdef1.2-D* and *pdef2-D*.

Plant transformation

The expression constructs *pdef1.1-D*, *pdef1.2-D* and *pdef2-D* were introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by triparental mating. Tobacco plants (*N. tabacum* cv. Samsun NN) were transformed with the engineered *Agrobacterium* as described previously (Maiti *et al.*, 1993). Regenerated plants were grown in a glasshouse in Metro Mix® 280 (The Scotts Company, Marysville, OH) at 30 ± 5 °C with both natural and supplementary lighting (minimum photon flux density, 300 µmol/m²/s) provided in a 17 h/7 h day/night cycle. T₁ lines with KanR : KanS = 3 : 1 segregation were selected for further analysis. Transgenic tobacco seeds (T₁) were germinated in the presence of kanamycin (200 mg/L) and positive transformants were selected.

Integration and transcription of the *Arabidopsis* peptide deformylase constructs *def1.1-D*, *def1.2-D* and *def2-D* into transgenic plants (T₁)

were confirmed by PCR, RT-PCR, and real-time qRT-PCR amplification using appropriately designed gene-specific primers. Genomic DNA from untransformed control and transformed plants was isolated using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Total cellular RNA from transgenic tobacco seedlings expressing constructs *pdef1.1-D*, *pdef1.2-D* and *pdef2-D* was isolated using an RNeasy Plant Mini Kit (Qiagen). Total RNA (2 µg) was treated with RNase-free DNase (Sigma, St Louis, MO) according to the manufacturer's instruction. For RT-PCR, a Superscript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used for the synthesis of first-strand cDNA in a total volume of 20 µL following the manufacturer's recommendations.

The expression level in transgenic plants *def1.1-D*, *def1.2-D* and *def2-D* was evaluated by real-time qRT-PCR following the procedures described previously (Bhattacharyya *et al.*, 2003; Pattanaik *et al.*, 2004). Briefly, a plasmid containing the full-length peptide deformylase cDNA was used as an external control. Serial dilution (10^8 – 10^3 copies/µL) of the control plasmid was used to generate a standard curve. An internal control (α -tubulin), which is present at a constant amount in all samples, was used to normalize for any minor variation in the samples. PCR amplification was performed in a DNA Engine Opticon™ 2 System for continuous fluorescence detection (MJ Research Inc., Reno, NV) in a total volume of 20 µL containing 1 µL of cDNA, using the DyNamo™ SYBER Green qPCR Kit (MJ Research Inc.).

Actinonin treatment of transgenic tobacco seedlings

Tobacco seeds from T₂ transgenic lines were germinated at 22 °C with continuous light (photon flux density, 50 µmol/m²/s) in the wells of 96-well microtitre plates containing 200 µL T⁻ medium [Murashige and Skoog basal salts (Sigma), vitamin B5 (Sigma), 3 mM CaCl₂, 0.1 mM FeSO₄, 2% sucrose, pH 5.8] with 0.8%–1% (w/v) phytoigel in the absence or presence of 1.2 mM actinonin (Sigma).

Selection of transgenic tobacco seedlings on actinonin and kanamycin

Tobacco seeds from T₁ transgenic lines were germinated on T⁻ medium with 0.8%–1% (w/v) phytoigel in the presence of 1.2 mM actinonin or 100 mg/L kanamycin monosulphate (Sigma). For PCR and Western analysis, representative white or green seedlings were transferred after 15 days to medium without actinonin or kanamycin to restore growth, and then subsequently transferred to Metro Mix® 280 for 2 weeks. For PCR amplification of the neomycin phosphotransferase type II gene (*nptII*), the forward primer 5'-ATGGCATACCTTATCCGCAATTC-3' and reverse primer 5'-TCAGAAGAAGCTCGTCAAGAAGGCG-3' were used. For amplification of *AtDEF2*, the forward primer 5'-TAAATTAGTACCGTTTGATGAAGGATG-3' and reverse primer 5'-TCATTCTGTCAAAGAAGAGAACTCCCT-3' were used. Both sets of primers were added together in the PCR to allow simultaneous amplification of both genes.

Chloroplast protein fractionation

Total soluble tobacco leaf protein was extracted by grinding in 150 mM boric acid, 10 mM MgCl₂, 1% (w/v) polyvinylpyrrolidone, pH 8.0. Intact chloroplasts were isolated according to Mills and Joy (1980) and subsequently lysed in 25 mM tris(hydroxymethyl)aminomethane (Tris), 100 µM NiCl₂, 5 mM sucrose, 5 mM NaCl, 1 mM ethylenediami-

netetraacetic acid (EDTA), 10 mM MgCl₂, pH 8.0. Stromal protein and thylakoid membrane fractions were separated by centrifugation for 15 min at 1000 *g* at 4 °C. The chlorophyll concentration of the thylakoid extracts was quantified according to Arnon (1949), and the protein concentration of the stromal extracts was determined using a modified Bradford reagent (Coomassie Plus, Pierce, Rockford, IL). For solubilization of the thylakoid membrane proteins, thylakoid membranes (based on 10 µg of chlorophyll) were resuspended in 200 µL of 3 M urea, 500 mM Tris, pH 6.8, and incubated overnight at room temperature. Following a 5-min centrifugation at 16 000 *g*, the supernatant containing the thylakoid membrane proteins was collected.

Immunodetection and quantification of peptide deformylase in transgenic plants

For Western blot analysis, total leaf (40 µg), stromal (15 µg), thylakoid membrane (2 µg chlorophyll) and mitochondrial (50 µg) proteins were separated by 15% (w/v) acrylamide sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 2 M urea, and electroblotted to polyvinylidene difluoride membranes (Immobilon™, Millipore, Billerica, MA). *AtDEF1* and *AtDEF2* specific rabbit polyclonal antibodies, elicited with purified inclusion bodies, were used to probe Western blots, as described previously (Dirk *et al.*, 2001).

For ELISA quantification, stromal proteins (0.4–1.2 µg in 0.2-µg increments in 50 mM Tris, 5 mM MgCl₂, 1 mM EDTA, 500 mM NaCl, pH 8.2) and solubilized thylakoid membrane proteins (0.4–1.2 µg in 0.2-µg increments in 0.6 M urea, 140 mM Tris, 4 mM MgCl₂, 0.8 mM EDTA, 400 mM NaCl, pH 8.2) were incubated in wells of high-binding enzyme immunoassay/radio-immunoassay (EIA/RIA) plates (Corning Costar, Corning, NY) for 1–2 h at 4 °C. After washing with sterile water, the wells were blocked with 3% (w/v) bovine serum albumin (BSA) in TTBS [0.1 M Tris, pH 7.5, 0.5 M NaCl, 0.05% (v/v) Tween-20] for 40 min at room temperature. *AtDEF1* and *AtDEF2* antibodies, diluted 1 : 1000 in blocking buffer, were added and the samples were incubated for 45 min at room temperature. After at least five washes with TTBS at room temperature, secondary antibody (1 : 5000 goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate in blocking buffer) was added and the samples were incubated for 45 min at room temperature. Both TTBS and sterile water washes were conducted prior to development with freshly prepared 6 mM *p*-nitrophenyl phosphate in 200 mM Tris, pH 9–10. Reactions were terminated with 50 µL of 3 M NaOH, and the absorbance (405 nm) was determined with a Uniskan® I plate reader (Labsystems, Helsinki, Finland). Standard ELISAs (using purified *AtDEF1* and *AtDEF2* proteins) were linear for increases in A₄₀₅ with increasing DEF proteins from 100 pg to 1 ng. Under these conditions, no immunological signal could be observed for endogenous tobacco DEF, as a consequence of its low abundance and the low level of target protein samples used in the ELISAs. In *Arabidopsis*, immunological detection of DEF proteins using Western blots requires a sample of 250 µg of total protein, and suggests a relative protein abundance of approximately 0.004% (Dirk *et al.*, 2001).

Peptide deformylase enzyme activity assays

In vitro spectrophotometric assays using *N*-formyl-Met-Leu-*p*-nitroanilide (f-ML-*p*NA; BACHEM Bioscience Inc., King of Prussia, PA) as a substrate were conducted at 23 °C.

Continuous assay

Stromal extracts were incubated in reaction buffer (50 mM Tris, 5 mM MgCl₂, 0.2 mM EDTA, 100 μM NiCl₂, pH 8.0) with 1.0 U *Aeromonas proteolytica* aminopeptidase (Sigma), and reactions were initiated by the addition of 200 μM f-ML-pNA. The release of *p*-nitroaniline was monitored using a Cary 50 UV-visible spectrophotometer (Varian Australia Pty Ltd., Palo Alto, CA), and the initial velocities were calculated from the linear portion of the reaction curve.

Discontinuous assay

Thylakoid extracts were incubated in reaction buffer, and reactions were initiated with 200 μM of substrate. After terminating the reaction by heating at 95 °C for 5 min, thylakoid membranes were removed by centrifugation at 10 000 *g* for 5 min, prior to incubating with 1.0 U aminopeptidase for 5 min to completely hydrolyse the deformylated substrate. The substrate to product conversion was kept to < 20% for all reactions.

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