# Plant Peptide Deformylase: A Novel Selectable Marker and Herbicide Target Based on Essential Co-Translational Chloroplast Protein Processing

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Transgenic tobacco plants expressing three different forms of *Arabidopsis* plant peptide deformylase (*At*DEF1.1, *At*DEF1.2, and *At*DEF2; EC 3.5.1.88) were evaluated for resistance to actinonin, a naturally occurring peptide deformylase inhibitor. Overexpression of either *AtDEF1.2* or *AtDEF2* resulted in complete resistance to actinonin accompanied by a 3-5 fold increase in IC50, but overexpression of *AtDEF1.1* did not result in actinonin resistance. Immunological analyses demonstrated that *At*DEF1.2 and *At*DEF2 enzymes were equally distributed between stromal and thylakoid fractions in chloroplasts but *AtDEF1.1* was localized to mitochondria. Highest enzyme activity was associated with stromal *At*DEF2 which was 184-fold higher than wild type enzyme activity. Resistance to actinonin co-segregated 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 represents a native gene selectable marker system for chloroplast and nuclear transformation vectors, and also identify plant peptide deformylase as a viable broad-spectrum herbicide target.

Peptide deformylase (DEF; EC 3.5.1.88) catalyzes 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* [1-3]. While both genes encode polypeptides with peptide deformylase activity, *At*DEF1 and *At*DEF2 have different biochemical characteristics, subcellular localizations, and phenotypes resulting from T-DNA insertional mutations. Whereas *At*DEF2 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 to other polypeptide sequences, *At*DEF1 shows little change in catalytic efficiency with polypeptide substrate sequence [4]. Additionally, *At*DEF1 is less sensitive to the

inhibitory effects of the known peptide deformylase inhibitor actinonin, with a 2-fold weaker *in vitro* binding for *At*DEF1 compared to *At*DEF2 [1]. Subcellular localization studies suggest that both *At*DEF1 and *At*DEF2 are found in chloroplasts and mitochondria [2,5]; however, the presence of two translational start codons in *AtDEF1* potentially gives rise to two translation products: *At*DEF1.1 and *At*DEF1.2. Translation from the upstream start codon results in *At*DEF1.1 which is restricted to mitochondria, and translation from the downstream start codon results in *At*DEF1.2 which is localized to both mitochondria and chloroplasts [5]. Peptide deformylase inhibitors, such as actinonin, are lethal to all plants [6], and recent evidence suggests that DEF inhibition results in incomplete and/or incorrect co-translational processing of the D1 polypeptide [7]. Thus, the essentiality of peptide deformylase in plants is likely a consequence of its role in the co-translational processing of vital chloroplast proteins.

The essentiality of plant peptide deformylase along 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 an examination of plant peptide deformylase and its role in co-translational 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.

Chimeric *Atdef1.1-D, Atdef1.2-D* and *Atdef2-D* genes were introduced into tobacco (*Nicotiana tabaccum* cv. Samsun NN) plants and twelve independent transgenic tobacco lines generated for each construct (**Fig. 1**). A vector control, consisting of full length GUS in pKM24 [8], was also introduced. Transgenic plants generated from each construct ($T_1$ and $T_2$ progeny, $2^{nd}$ and $3^{rd}$ generation) were screened for gene integration, transcription, and translation by PCR, RT-PCR, real-time quantitative RT-PCR (qRT-PCR) and immunological analyses. qRT-PCR revealed different amounts of *DEF* transcripts with abundance ranging from $1.7 \times 10^5$ to $5.9 \times 10^7$ transcripts per 100 ng of total RNA (**Supplementary Fig. 1** online). Representative results from independent lines exhibiting a high level of expression of *DEF* are shown in Fig. 2. The *Atdef-D* genes were stably integrated and transcribed into mRNA at the expected size (**Fig. 2a**), and a single immuno-reactive 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 *At*DEF1 or *At*DEF2 (**Fig. 2b**). Western analysis of *Atdef1.1-D* plants using the *At*DEF1 antibody, which is reactive to both *At*DEF1.1 and 1.2 proteins, revealed a strong band at the expected size in mitochondrial lysates and a weak signal in the thylakoid fraction, likely caused by a small amount of mitochondrial contamination (**Fig. 2b**). Transgenic tobacco plants overexpressing either *Atdef1.2-D* or *Atdef2-D* were completely resistant to actinonin at all stages of plant growth (**Fig. 2c**) providing strong evidence that peptide deformylase is the *in vivo* target of actinonin and likely all peptide deformylase inhibitors. Overexpression of *Atdef1.1-D*, however, did not result in resistance to actinonin, likely as a consequence of mitochondrial localization.

Detection of peptide deformylase activity has not been previously reported from plants. In this study we detected endogenous deformylase activity in chloroplasts from wild type tobacco leaf tissues (Table 1), and this activity was stabilized in buffer with $Ni^{2+}$. Compared to endogenous tobacco DEF activity, the largest increase in transgenic peptide deformylase activity occurred in the *def2-D* plants, with a 170- to 184-fold activity increase in the thylakoid and stromal fractions, respectively (**Table 1**). ELISA quantification and subsequent calculation of $k_{cat}$ values for *def2-D* thylakoid and stromal fractions were 23- and 80-fold higher than those for *def1.2-D*, respectively, suggesting that DEF2 might 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 [9]. Although *def1.2-D* plants had a lower amount of DEF accumulation and much lower $k_{cat}$ values compared to *def2-D* (**Table 1**), these plants were equally resistant to the phytotoxic effects of actinonin, indicative of the higher actinonin tolerance of DEF1, as previously reported [1]. 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.

Plant DEF1 and DEF2 have been described as functionally redundant from studies which demonstrated rescue of a temperature conditional DEF mutant *E. coli* cell line [2]. Although that report indirectly confirmed deformylase activity of *At*DEF1 and 2, determining functional redundancy in plants would be confounded by the differential subcellular localization of plant DEF1.1, DEF1.2 and DEF2. Furthermore, mitochondrial localized forms of peptide deformylase are found in many eukaryotic species including humans, have poor catalytic activity [10], and are probably not biologically essential as evident by the current testing of potent peptide deformylase inhibitors in phase two and three clinical trials as broad-spectrum antibiotics [11,12]. In plants then, it might be expected that a strictly mitochondrial-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 (**Fig. 2c**) is consistent with chloroplast-localized forms of DEF1.2 and DEF2 and a mitochondrial localization for DEF1.1 as we have previously reported from GFP protein fusion studies [5].

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 and a comparison between transgenic plants grown on kanamycin or actinonin confirmed that *Atdef2-D* allows selection of transgenic plants with efficiency equal to kanamycin (**Fig. 3**). Both green and bleached plants were screened for co-segregation of the kanamycin gene and the *Atdef2-D* gene, and only green plants contained both genes (**Fig. 3A**) and accumulated DEF (**Fig. 3B**). As with kanamycin selection, plants germinated in the presence of actinonin which were not transgenic were developmentally arrested at the cotyledonary stage (**Fig. 3C**). For plants expressing the *AtDEF2* transgene, cotyledons remained white but all subsequent growth was normal (see also **Fig. 2**).

Additionally, western analysis revealed that there was a strong correlation between plant size and DEF2 expression level (data not shown).

The essentiality and widespread conservation of DEF in plants makes it an attractive molecular target for designing 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 [6]. The acute phytotoxic effects of actinonin resulting from the inhibition of DEF coupled with the complete protection from this compound by overexpression of either *DEF1.2* or *DEF2* is indicative of the potential of this technology as a novel broad-spectrum weed control system as well as 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 light of recent structural studies identifying 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 [13]. Recent structural analyses suggests that *At*DEF1 is distinct from other peptide deformylases and most representative of eukaryotic mitochondrial peptide deformylases, while *At*DEF2 is restricted to plant plastids and Apicomplexa [13]. Therefore it seems plausible that a compound specific for plant DEF2 without antibiotic activity could be developed.

The resistance to actinonin by overexpression of *AtDEF1.2* and *AtDEF2* also confirms the suitability of plant peptide deformylase as a selectable marker. This observation is highly significant in light of the major biosafety concerns associated with the use of transgenic plants harboring foreign DNA with the potential for horizontal transfer of antibiotic resistance genes. Although never confirmed in field situations [14], 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 [15], marker removal using site specific recombination [16], and using plant genes capable of providing selection either through herbicide resistance or most recently through antibiotic resistance [17]. Ideally selection would not involve 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.

## METHODS

**Construction of plant expression vectors p*def1.1-D*, p*def1.2-D* and p*def2-D*.** DNA fragments corresponding to the coding sequence 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'-GCGGGCTCGAGACCATGGGACTCCACCGAGACGAA GCGACGGCTATGGAAACCCTTTTCAGA-3'which contained *Xho*I and *Nco*I restriction sites, and the reverse primer 5'-ATGCAGGAGCTCTCATTGAGGTCCGAGCTTAGG-3' which contained a *Sst*I restriction site and stop codon. *DEF1.2* was amplified starting from the second downstream ATG codon in *AtDEF1* using forward primer 5'-GCGGGCTCGAGACCATGGGAGAAACCCTTTTCAGAGTC-3' containing *Xho*I and *Nco*I sites, and the same reverse primer used for the *DEF1.1* amplification. The *DEF2* fragment was amplified using the forward primer 5'-GCGGGCTCGAGACCATGGGAG CCGTCTGTAACTGC-3' containing *Xho*I and *Nco*I restriction sites, and reverse primer 5'-ATGCAGGAGCTCTTAGGTCGACCCACGTTTGCCAAAACCAAC-3'containing *Sst*I and *Sal*I restriction sites and a stop codon. PCR amplification was carried out for 30 cycles of 94°C for 30 sec, 55° C for 30 sec, and 72°C for 1 min. PCR amplified fragments were cloned into pGEM®-T Easy (Promega, USA) and sequenced by the dideoxy chain terminator method [18]. Respective *DEF* fragments were digested with *Nco*I-*Sst*I, gel purified and cloned into the corresponding sites of pBS-A1MV5' [19] containing the 5' untranslated region of AlMV RNA 4. From these resulting plasmids the *Xho*I-*Sst*I fragments of respective *DEF* genes were cloned separately into the corresponding sites of the expression vector, pKM24 [8,20]. The resulting expression constructs were designated p*def1.1-D*, p*def1.2-D* and p*def2-D*.

**Plant transformation.** Expression constructs p*def1.1-D*, p*def1.2-D* and p*def2-D* were introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by tri-parental mating. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were transformed with the engineered *Agrobacterium* as described previously [19]. Regenerated plants were grown in a greenhouse in Metro Mix ® 280 (The Scotts Company, USA) at 30±5°C with both natural and supplemental lighting (minimum 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photo flux density [PFD]) provided in a 17 / 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 selected.

Integration and transcription of the *Arabidopsis* deformylase constructs *def1.1-D*, *def1.2-D* and *def2-D* into transgenic plants (T1) was confirmed by PCR, RT/PCR, and real time quantitative RT-PCR amplification using appropriately designed gene-specific primers. Genomic DNA from untransformed control and transformed plants was isolated using DNeasy plant mini kit (Qiagen, USA). Total cellular RNA from transgenic tobacco seedlings expressing constructs p*def1.1-D*, p*def1.2-D* and p*def2-D* was isolated using the RNeasy plant mini kit (Qiagen, USA). The total RNA (2 µg) was treated with RNase free DNase (Sigma, USA) as per manufacturer's instruction. For RT-PCR, Superscript™ First

Strand Synthesis System (Invitrogen, USA) was used for the synthesis of first-strand cDNA in a total volume of 20 µl following the manufacturer's recommendation.

The expression level in transgenic plants, *def1.1-D*, *def1.2-D* and d*ef2-D*, was evaluated by real-time quantitative RT-PCR following the procedures described previously [21,22]. Briefly, a plasmid containing the full-length peptide deformylase cDNA was used as an external control. Serial dilution ($10^8$ to $10^3$ copies $\mu l^{-1}$) 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 samples. PCR amplification was performed in a DNA Engine Opticon™2 System for continuous fluorescence detection (MJ Research Inc., USA) in a total volume of 20 µl containing 1 µl of cDNA, using the DyNamo™ SYBER Green qPCR kit (MJ Research Inc., USA).

**Actinonin treatment of transgenic tobacco seedlings.** Tobacco seeds from $T_2$ transgenic lines were germinated at 22°C with continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PFD) in the wells of 96-well microtiter plates containing 200 µl $T^-$ media (Murashige and Skoog basal salts (Sigma, USA), vitamin B5 (Sigma, USA), 3 mM $CaCl_2$ , 0.1 mM $FeSO_4$, 2% sucrose, pH 5.8) with 0.8-1% (w/v) phytogel in the absence or presence of 1.2 mM actinonin (Sigma, USA).

**Selection of transgenic tobacco seedlings on actinonin and kanamycin.** Tobacco seeds from T1 transgenic lines were germinated on $T^-$ media with 0.8-1% (w/v) phytogel in the presence of 1.2 mM actinonin or 100 mg/l kanamycin monosulfate (Sigma, USA). For PCR and Western analysis, representative white or green seedlings were transferred after 15 days to media without actinonin or kanamycin to restore growth, and then subsequently transferred to Metro Mix ® 280 for 2 weeks. For PCR amplification of *NPTII* the forward primer 5'-ATGGCATACCTTATCCGCAACTTC-3' and reverse primer 5'-TCAGAAGAACTCGTCAAGAAGGCG-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 reaction 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 $MgCl_2$, 1% (w/v) polyvinylpyrolidine, pH 8.0. Intact chloroplasts were isolated according to Mills and Joy [23] and subsequently lysed in 25 mM Tris, 100 µM $NiCl_2$, 5 mM sucrose, 5 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 8.0. Stromal proteins and thylakoid membranes fractions were separated by centrifuging for 15 min at 1,000*g* at 4°C. Chlorophyll concentration of the thylakoid extracts was quantified according to Arnon [24], and protein concentration of the stromal extracts was determined using a modified Bradford reagent (Coomassie Plus, Pierce, USA). For solubilization of thylakoid membrane proteins, thylakoid membranes (based on 10 µg chlorophyll) were resuspensed in 200 µl 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 thylakoid membrane proteins was collected.

**Immunodetection and quantification of DEF 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 SDS-PAGE in the presence of 2 M urea, and electroblotted to polyvinylidene difluoride membranes (Immobilon™ , Millipore, USA). DEF proteins were then detected as previously described [1].

For ELISA quantification, stromal proteins (0.4 to 1.2 µg in 0.2 µg increments in 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 500 mM NaCl, pH 8.2) and solubilized thylakoid membrane proteins (0.4 to 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 EIA/RIA plates (Corning Costar, USA) for 1-2 hours at 4°C. After washing with sterile water, the wells were blocked with 3% (w/v) 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. *At*DEF1 or 2 antibodies, diluted 1:1000 in blocking buffer, were added and incubated for 45 min at room temperature. After at least 5 washes of TTBS at room temperature, secondary antibody (1:5000 goat anti-rabbit IgG alkaline phosphatase conjugate in blocking buffer) was added and the samples 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 3 N NaOH and the absorbance (405 nm) determined with a Uniskan® I plate reader (Labsystems, Finland).

**DEF enzyme activity assays.** *In vitro* spectrophotometric assays using N-formyl-Met-Leu-ρ-nitroanilide (f-ML-ρNA; BACHEM Bioscience Inc., USA) as a substrate were conducted at 23°C. Stromal and thylakoid extracts were preincubated with actinonin under reaction conditions for 5 min prior to initiation by addition of substrate [1].

***Continuous assay*** – Stromal extracts were incubated in reaction buffer (50 mM Tris, 5 mM $MgCl_2$, 0.2 mM EDTA, 100 µM $NiCl_2$, pH 8.0) with 1.0 U *Aeromonas proteolytica* aminopeptidase (Sigma, USA) and reactions initiated by the addition of 200 µM f-ML-ρNA. The release of ρ-nitroaniline was monitored using a Cary 50 UV-Vis spectrophotometer (Varian Australia Pty Ltd., USA) and initial velocities calculated from the linear portion of the reaction curve.

***Discontinuous assay***- Thylakoid extracts were incubated in reaction buffer and reactions initiated with 200 µM 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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## COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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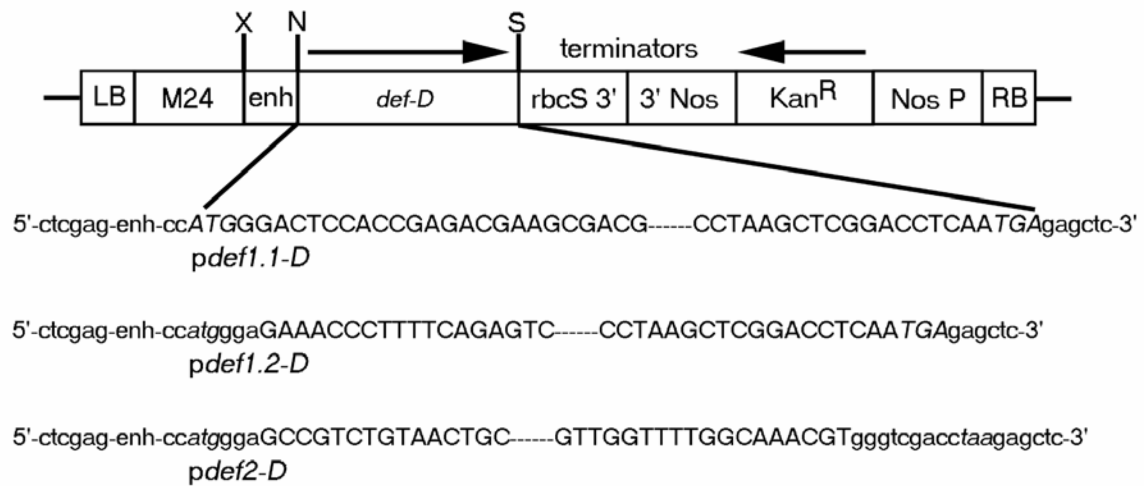



**Figure 1** Schematic of the plant expression constructs containing chimeric *Atdef-D* genes. The modified full-length transcript promoter (M24) of the *Mirabilis mosaic virus* [8,20] directs the coding sequences of respective peptide deformylase genes. A translational enhancer sequence (enh), 35-nt long 5'-untranslated region of AlMV 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 E9 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 shown in uppercase, coding sequence not derived from *DEF* are shown in lowercase. The position of the *Xho*I (X), *Sst*I (S) and *Nco*I (N) restriction sites used to assemble these expression vectors are shown.

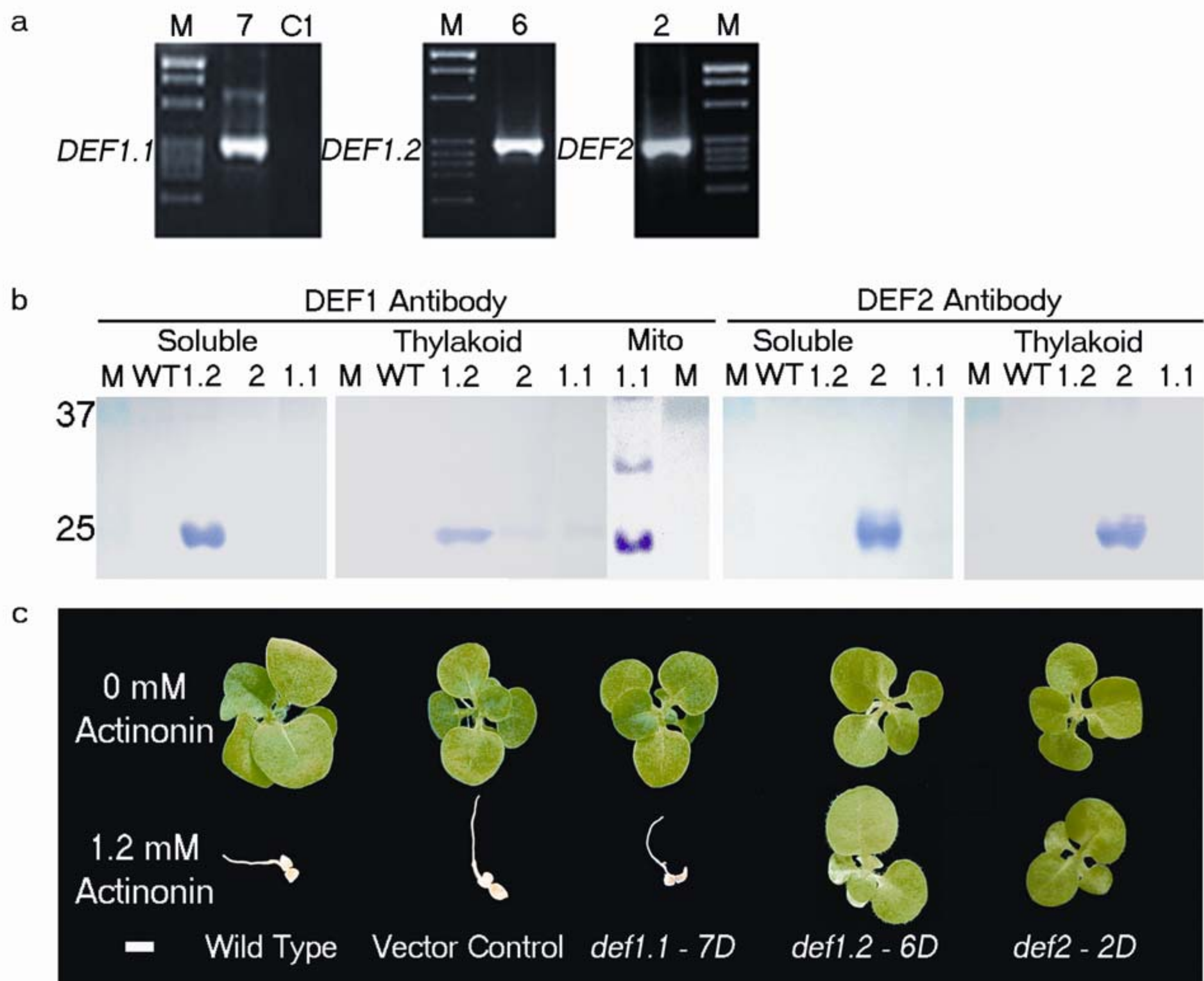



**Figure 2** Expression of *Atdef1.1-D*, *Atdef1.2-D* and *Atdef2-D* in transgenic tobacco. (**a**) Analysis of RT-PCR amplification products from independent $T_2$ lines (*def1.1-7D*, *def1.2-6D*, and *def2-2D*) stained with ethidium bromide after electrophoresis on a 1% agarose gel. C1, nontransgenic 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 *At*DEF1- and *At*DEF2-specific antibodies. WT, nontransgenic tobacco control; M, marker (kD). (**c**) Tolerance to the peptide deformylase inhibitor actinonin by transgenic tobacco plants overexpressing *Atdef-D* genes. Seeds from wild type, vector control (no *Atdef-D* insert), *def1.1 line 7D*, *def1.2 line 6D*, and *def2 line 2D* plants were germinated on $T^-$ medium containing 0 or 1.2 mM actinonin and seedlings grown for 35 days. Bar represents 3 mm.

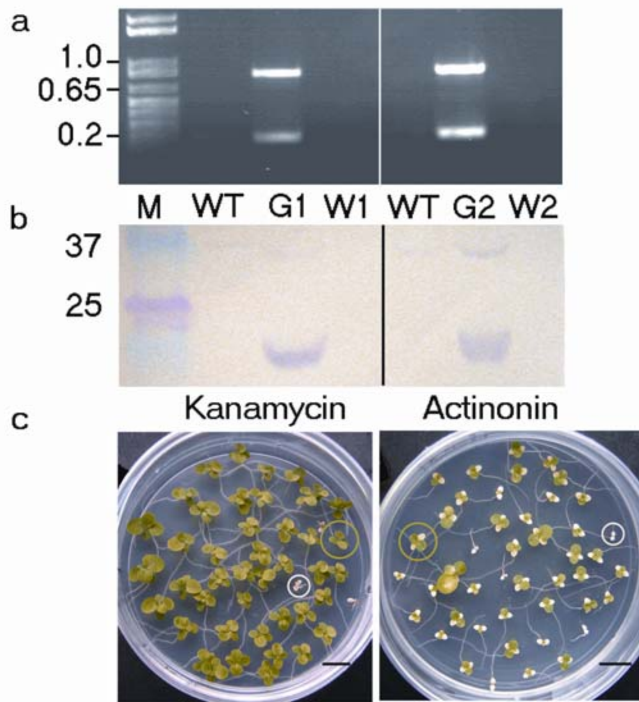
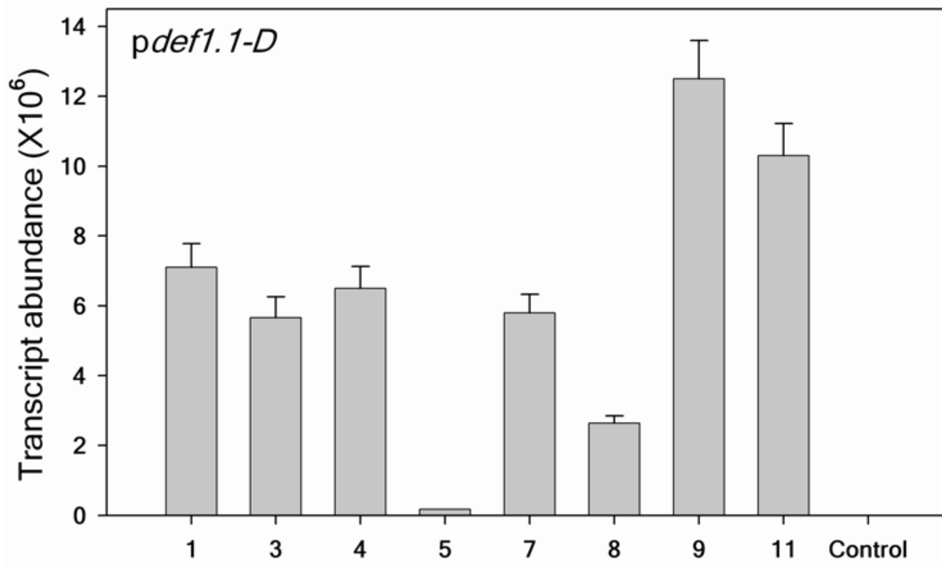
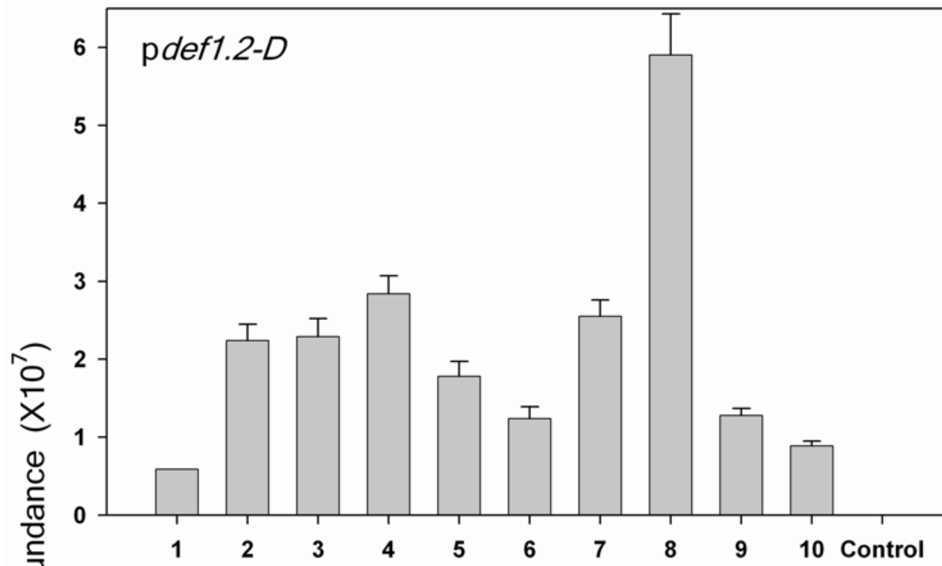


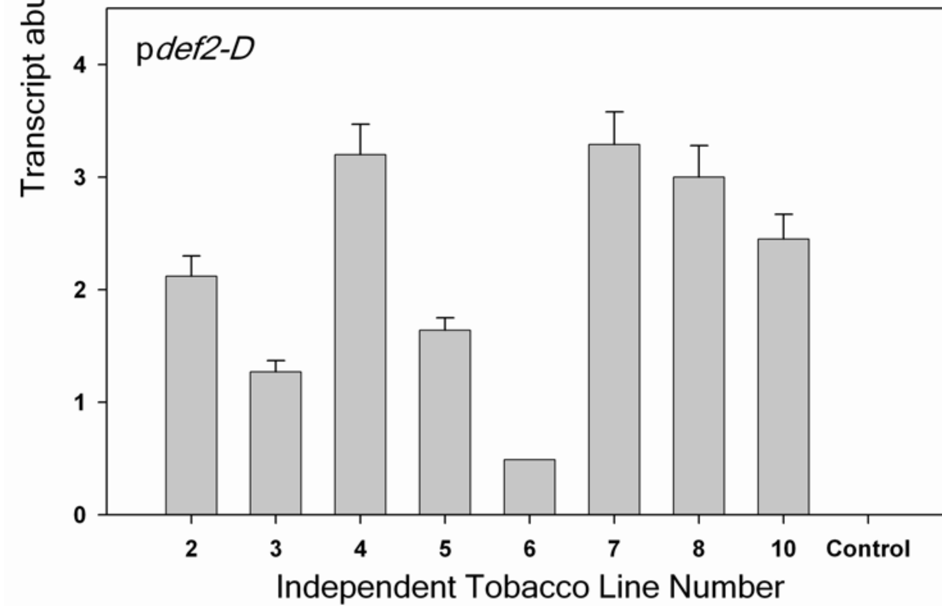

**Figure 3** Comparison of *Arabidopsis thaliana* peptide deformylase (*Atdef2-D*) and neomycin phosphotransferase type II (*nptII*) used as selectable markers. (**a**) Analysis of PCR amplification products from *Atdef2-D* and *NPTII* wild type (WT), transformed (G1, as circled in green in panel c, left), and segregating, untransformed plants (W1, as circled in white in panel c, right) after rescue from selection on 100 mg $l^{-1}$ 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 *At*DEF2-D from 40 µg per lane of leaf extracts from WT, transformed and segregating, untransformed plants from kanamycin-(left) and actinonin-selected (right) plants. The immunoreactive band of the expected size (23 kD) 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. Bar represents 1 cm.

| Genotype | DEF accumulation (µg $mg^{-1}$ protein, or $mg^{-1}$ chlorophyll) | $k_{cat}$ ($s^{-1}$) | DEF activity (nmoles $min^{-1}$ mg $protein^{-1}$ or mg $chlorophyll^{-1}$) | *IC* $_{50}$ (µM) |
|---|---|---|---|---|
| *Stromal Protein* | | | | |
| WT [a] | ND | - | 0.31 ± 0.08 | 0.04 ± 0.01 |
| *def1.1-7D* [a] | ND | - | 0.22 ± 0.04 | 0.04 ± 0.01 |
| *def1.2-6D* [a] | 0.46 ± 0.07 | 0.44 ± 0.05 | 0.52 ± 0.06 | 0.14 ± 0.02 |
| *def2-2D* [b] | 0.67 ± 0.06 | 35 ± 5.1 | 57 ± 8.4 | 0.14 ± 0.02 |
| *Thylakoid Protein* | | | | |
| WT [c] | ND | - | 4.7 ± 1.1 | 0.05 ± 0.02 |
| *def1.1-7D* [c] | ND | - | 7.7 ± 1.8 | 0.05 ± 0.02 |
| *def1.2-6D* [c] | 2.1 ± 1.4 | 1.8 ± 0.17 | 9.7 ± 0.94 | 0.2 ± 0.05 |
| *def2-2D* [d] | 7.9 ± 1.13 | 41 ± 7.0 | 800 ± 130 | 0.14 ± 0.02 |

**Table 1** *At*DEF accumulation and activity in transgenic tobacco lines. Accumulation of *At*DEF in transgenic tobacco lines was quantified in stromal or thylakoid extracts by ELISA using *At*DEF1- and *At*DEF2-specific antibodies. Stromal extracts were evaluated based on total protein concentration and DEF activity was quantified using a continuous spectrophotometric assay. Thylakoid extracts were evaluated based on chlorophyll content and assayed for activity in a discontinuous assay. Data represent mean ± standard deviation (n=6). Assays were conducted with the following: [a] 2 mg protein, [b] 141 µg chlorophyll, [c] 0.6 mg protein, [d] 20 µg chlorophyll. WT, wild type untransformed tobacco. ND, not detectable.


pdef1.1-D
Transcript abundance (X10^6)
14
12
10
8
6
4
2
0
1
3
4
5
7
8
9
11
Control


pdef1.2-D
Transcript abundance (X10^7)
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
Control


pdef2-D
4
3
2
1
0
2
3
4
5
6
7
8
10
Control
Independent Tobacco Line Number

**Supplementary Figure 1** Expression analysis of plants containing *Atdef-D* genes by real-time qRT-PCR in stably transformed transgenic tobacco plants. For each construct, independent transgenic lines (T1 progeny) with KanR : KanS = 3 : 1 were selected. Average number of 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.