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OVERPRODUCTION OF SWEET POTATO PEROXIDASES IN TRANSGENIC TOBACCO PLANTS

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Key Word Index—*Ipomoea batatas*; Convolvulaceae; *Nicotiana tabacum*; Solanaceae; transgenic plants; peroxidase; superoxide dismutase.

Abstract—Two peroxidase (POD) cDNAs, anionic (swpa1) and neutral (swpn1) POD derived from suspension cultures of sweet potato (*Ipomoea batatas*), were used to express high levels in transgenic plants. Two sweet potato PODs were overproduced in two cultivars of transformed tobacco (*Nicotiana tabacum*) plants, cv. Bel W3 and cv. Samsun by introducing a chimeric gene composed of the cauliflower mosaic virus 35S promoter and POD cDNA. Young fully expanded leaves of transgenic plants of both cultivars showed higher POD activity than those of nontransgenic plants. Bel W3 transgenic plants carrying anionic POD had total POD activity *ca* five times higher than control plants. However, no significant differences were observed in superoxide dismutase activity between transgenic and nontransgenic plants. Transgenic Bel W3 and Samsun tobacco plants with either anionic or neutral POD isoenzyme did not show distinctive phenotypes compared with untransformed plants. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Peroxidase (POD, EC 1.11.1.7) has been implicated in numerous developmental and growth processes in plants in response to stresses. In addition, plant PODs are also widely used as an important reagent for industrial applications because of its high sensitivity [1, 2]. Most higher plants possess a number of POD isoenzymes. These enzymes are useful markers for environmental stresses since POD activity in plants is affected by factors that include air pollution, low temperature, ozone, heavy metals, wounding, pathogen, salts, drought, and UV radiation [3]. In all of these physiological processes the POD isoenzyme patterns are often complex. This complexity has caused difficulty in understanding the specific function of these enzymes *in vivo* and their specific roles in plant growth and in adaptation to environmental stress [4]. Therefore, it is important to isolate the genes that encode each isoenzyme, and characterize their expression and properties of the gene products *in vivo*.

The tobacco anionic POD (M_r 36000, pI 3.5) is the best characterized of all PODs. Transgenic tobacco and tomato plants that overproduce tobacco anionic POD showed a distinctive phenotype of chronic wilting [5, 6]. These plants wilt due to severely reduced root mass which was a consequence of limited root branching. Different phenotypes seen in plants that overexpress or underexpress anionic POD may relate to overall plant growth and development [7].

As a first step to understanding the physiological functions of POD isoenzymes that are highly produced in suspension cultures of sweet potato (*Ipomoea babatas*), we have purified and characterized three anionic PODs [8] and isolated cDNAs that encode both anionic (swpa1) and neutral (swpn1) POD from sweet potato suspension-cultured cells [9]. Expressions of these genes are very high in cultured cells, although their expression patterns are different during cell growth [9]. In addition, the swpa1 and swpn1 transcripts were induced by wounding in intact plants, and the genes responded differently to chilling-induced oxidative stress. The two POD isoenzymes may contribute to protection from wounding- or chilling-induced oxidative stresses. Thus, we are interested in the possible physiological roles of these two isoenzymes *in vivo*. In this paper, transformed plants that overproduce either the anionic or neutral POD, encoded by swpa1 or swpn1, respectively, were generated, and POD and superoxide dismutase (SOD) activities in these plants were examined.

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RESULTS AND DISCUSSION

Construction of chimeric genes encoding the anionic or neutral POD

The POD cDNAs (swpa1 and swpn1) were previously isolated from a cDNA library of sweet potato cultured cells [9]. The recombinant plasmids carrying the POD cDNAs were constructed as follows. The 1334 bp fragment of swpa1 including 43 bp 5′-untranslated region (5′UTR), an open reading frame (ORF) encoding anionic POD and 199 bp 3′UTR was amplified by PCR. The sense primer was 5′-(TGCTCTAGAGCA)GATCCATGGGTTCAAACATCA-3′ and antisense primer was 5′-(CGGGGTACCCCG)TATAGGAGTAAGTACACTATA-3′. The 1252 bp fragment of swpn1 including 12 bp 5′UTR, an ORF encoding neutral POD and 168 bp 3′UTR was amplified by PCR. Sense and antisense primers were 5′-(TGCTCTAGAGCA)AGTCAACCCCTTCTTCTTTAT-3′ and 5′-(CGGGGTACCCCG)AACAAAGGCAGATTAAGATTA-3′, respectively. The sense and antisense primers contained additional sequences that included Xba I (TGC/TCTAGA/GCA) and Kpn I (CGG/GGTACC/CCG) restriction sites, respectively. The PCR products were ligated in Xba I and Kpn I sites of binary vector pMBP1 for overexpression of POD cDNAs and the resulting plasmids were called pMBP1-swpa1 and pMBP1-swpn1. pMBP1 induces the neomycin phosphotransferase (NPT II) expression cassette as a selectable maker, 35S promoter of cauliflower mosaic virus (P_{35S}) and nopaline synthase terminator (Tnos). The complete gene construct is shown in Fig. 1.

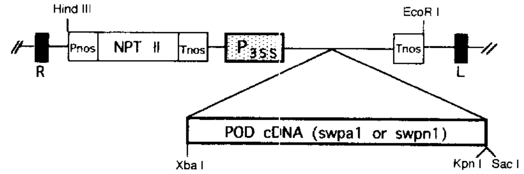

Fig. 1. A partial map of the plant expression vectors, pMBP1-swpa1 and pMBP1-swpn1. Both cDNAs were inserted in sense orientation P_{35S}: cauliflower mosaic virus 35S promoter; Pnos and Tnos: nopaline synthase promoter and terminator sequences, respectively; NPT II: neomycin phosphotransferase II; L and R: T-DNA left and right borders, respectively.

Transformation and regeneration of tobacco plants

The recombinant binary vectors, pMBP1-swpa1 and pMBP1-swpn1, were separately introduced into *Agrobacterium tumefaciens* strain LBA4404. Tobacco leaf discs of Bel W3 and Samsun were cocultivated with *Agrobacterium* carrying either the pMBP1-swpa1

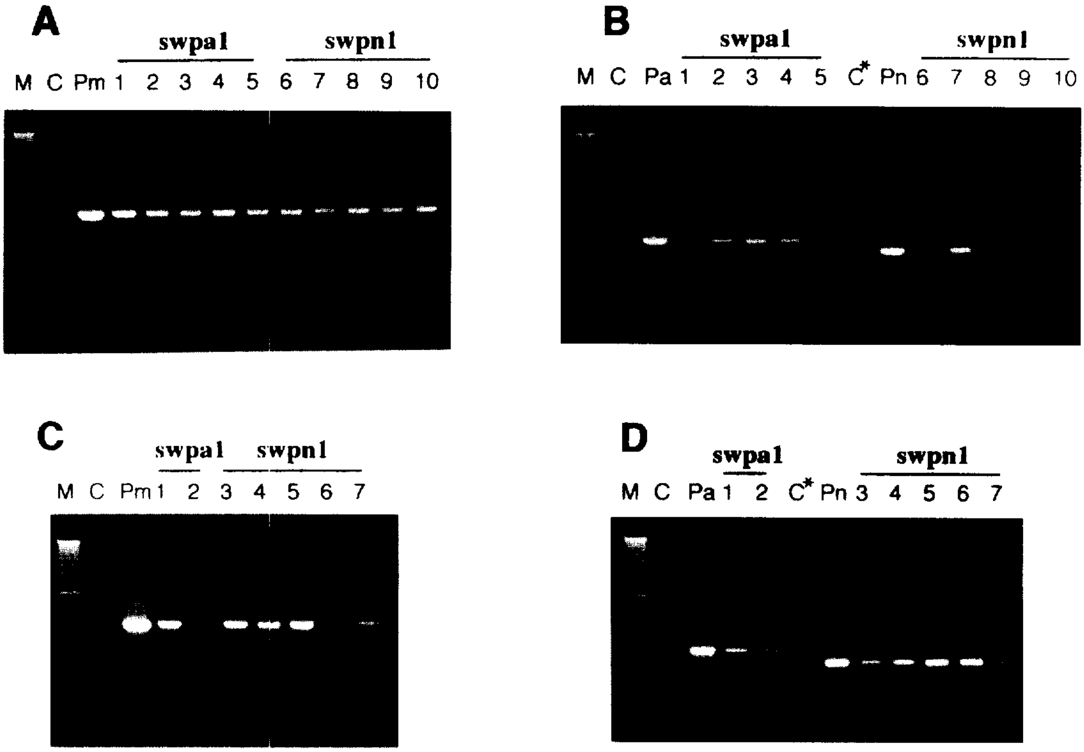

Fig. 2. PCR analysis for the NPT II gene and POD cDNAs in transformed Bel W3 (A and B) and Samsun (C and D) tobacco plants. M: 1 kb DNA ladder; C: control tobacco plants; Pm: pMBP1 plasmid DNA; Pa: swpa1 plasmid DNA; Pn: swpn1 plasmid; 1–10: transformed tobacco plants with pMBP1-swpa1 and pMBP1-swpn1. (A) PCR with NPTII primers; (B) PCR with swpa1-specific primers (C, Pa, and lanes 1–5) or swpn1-specific primers (C*, Pn and lanes 6–10); (C) PCR with NPTII primers; (D) PCR with swpa1-specific primers (C, Pa, and lanes 1 and 2) or swpn1-specific primers (C*, Pn and lanes 3–7).

or pMBP1-swpn1. Transformed shoots were regenerated directly on the cut edges of tobacco leaf discs on MS medium supplemented with 0.1 mg l^{-1} NAA, 1 mg l^{-1} BAP, 100 mg l^{-1} kanamycin, and 250 mg l^{-1} carbenicillin and rooted *in vitro*. Regenerated tobacco plants harboring the NPT II gene were normal in morphology and growth rate (Data not shown here).

Integration of POD gene in the tobacco genomic DNA

To examine the integration of POD gene in the transformed plant, PCR was performed. Genomic DNA was prepared from grown leaves of non-transformed and transformed plants (cv Bel W3 and cv Samsun) carrying swpa1 or swpn1. Both NPT II and POD genes were detected in transformed Bel W3 and Samsun plants (Fig. 2), respectively. These results confirmed the presence of the foreign POD genes in the transformed tobacco plants.

Overproduction of sweet potato POD isoenzymes in transgenic plants

First generation (T_0) Bel W3- and Samsun-based transgenic plants were used for the analysis of enzyme overproduction. Transgenic plants from all lines were selected on kanamycin-containing media before transplanting to soil. POD overproduction in each transgenic line was analysed. Fully expanded leaves (the third leaf from the top) were sampled and total POD activity was assayed. Bel W3 transformant lines that express the anionic POD (swpa1) (BTA1, BTA2, BTA3, BTA4 and BTA5) showed from 4.1 to 6.8 times higher POD specific activity (units mg^{-1} protein) than control plants (Table 1). Five Bel W3 lines containing the neutral POD (swpn1) (BTN1–BTN5) showed POD specific activities *ca* 2 times higher than control plants. In the Samsun-based transformants, STA1 and STA2 that carry anionic POD gene showed POD activities similar to that of control plants, whereas five lines carrying neutral POD gene (swpn1) (STN1–STN5) showed 2.0 to 3.8 times higher activity than control plants. Therefore, the average POD overproduction in transgenic plants is as follows: Bel W3 transformants carrying swpa1 showed the highest POD level (*ca* 4.8 times), followed by Samsun harboring swpn1 (*ca* 2.9 times), Bel W3 transformants with swpn1 (*ca* 2.3 times), whereas Samsun transformants carrying swpa1 showed a slight increase (*ca* 1.1 times) compared to control plants (Fig. 3). These results indicated that the cultivars and gene sources may be a significant factor in POD overproduction. Next, we examined whether the expression of POD in these transgenic plants affects SOD activity. The SOD activity was slightly decreased in swpa1 transformants and slightly increased in swpn1 transformants regardless of the cultivars, even though there was no statistically significant difference in SOD activity between transformed and nontransformed tobacco plants (Fig. 3).

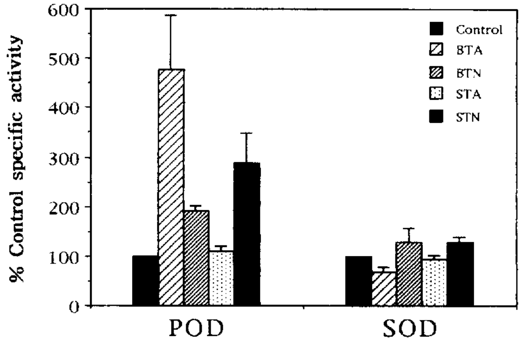

Fig. 3. Overall comparison of POD and SOD activities in POD transgenic plant lines. Anionic POD transgenic lines (BTA, STA) and neutral POD transgenic lines (BTN, STN) were assayed for enzyme activity in 2-month-old plants. Data are means ± S.E. of 5 independent plant lines except for STA (2 lines) and shown as percentage enzyme specific activity (units mg^{-1} protein) of control plants.

Table 1. POD and SOD activities of anionic (swpa1) and neutral (swpn1) POD transgenic lines in two cultivars (Bel W3, Samsun) were assayed using the fully expanded young leaves when plants were 2-month-old. Data are means of three replicates

Bel W3 Tobacco						Samsun Tobacco					
	swpa1			swpn1			swpa1			swpn1	
	POD	SOD		POD	SOD		POD	SOD		POD	SOD
	units mg^{-1} protein (fold to control)										
*CON	2.1	33.2		3.2	39.8		1.7	35.0		3.4	35.6
BTA1	14.4 (6.8)	26.2 (0.7)	BTN1	7.2 (1.8)	34.5 (0.8)	STA1	2.1 (1.2)	34.8 (1.0)	STN1	9.8 (2.8)	45.0 (1.2)
BTA2	8.8 (4.1)	19.0 (0.5)	BTN2	7.5 (1.9)	50.6 (1.2)	STA2	1.8 (1.0)	32.2 (0.9)	STN2	9.9 (2.9)	47.0 (1.3)
BTA3	9.7 (4.6)	31.7 (0.9)	BTN3	8.1 (2.1)	50.0 (2.1)		—	—	STN3	13.1 (3.8)	38.2 (1.0)
BTA4	8.7 (4.1)	22.5 (0.6)	BTN4	7.5 (1.9)	61.6 (1.5)		—	—	STN4	6.9 (2.0)	50.0 (1.4)
BTA5	8.9 (4.2)	25.0 (0.7)	BTN5	7.4 (1.9)	69.1 (1.7)		—	—	STN5	10.0 (2.9)	53.8 (1.5)

*CON: nontransformed control plant; BTA1–BTA5: Bel W3 plants transformed by anionic POD; BTN1–BTN5: Bel W3 plants transformed by neutral POD; STA1–STA2; Samsun plants transformed by anionic POD; STN1–STN5: Samsun plants transformed by neutral POD; —: not determined.

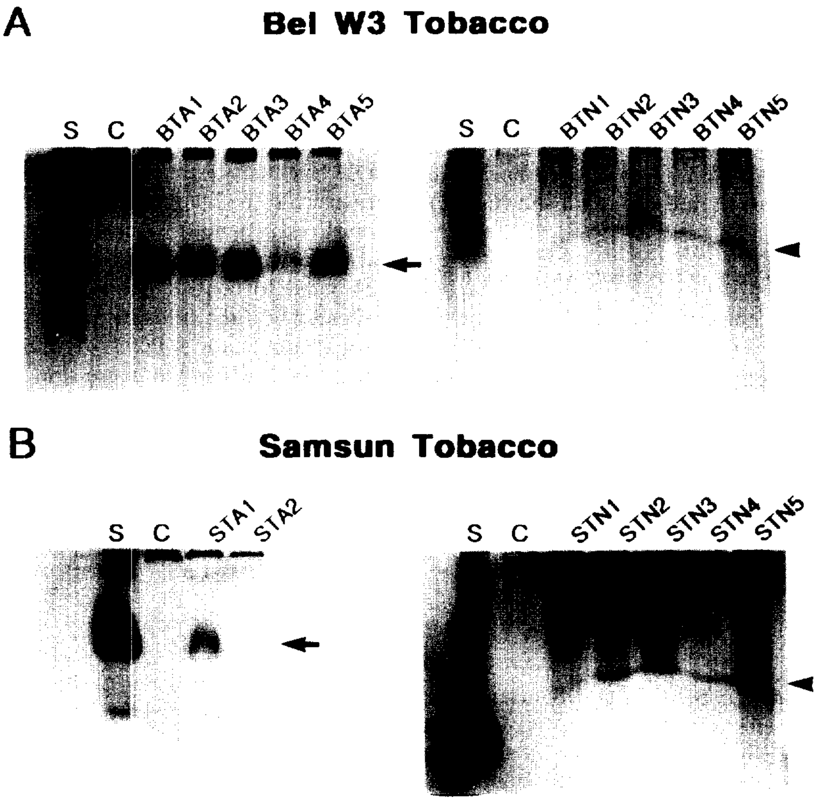

Fig. 4. Native gel stained for the POD activity from two transgenic tobacco clutivars, Bel W3 and Samsun. Equal amount of protein (60 μg) was loaded on the gel. (A) S: sweet potato suspension culture cells (SP-47 cell line) [9]; C: nontransformed Bel W3 plant; BTA1–BTA5: Bel W3 plants transformed by anionic POD; BTN1–BTN5; Bel W3 plants transformed by neutral POD; (B) C: nontransformed Samsun plant; STA1 and STA2: Samsun plants transformed by anionic POD; STN1–STN5: Samsun plants transformed by neutral POD. Arrows and arrowheads indicate acidic and neutral POD isoenzymes, respectively.

To characterize the foreign sweet potato protein in more detail, soluble extracts from tobacco leaves was subjected to native gel electrophoresis and subsequently stained for POD activity. Extracts from transformed Bel W3 and Samsun tobacco plants with anionic or neutral POD isoenzyme had a novel band, which was not found in control plants (Fig. 4). This result indicated that the elevated POD activity was a consequence of the introduced POD chimeric gene.

From the native gel assay of POD tranformants, it appears that the isoenzyme encoded by the swpa1 cDNA does not have the same electrophoretic mobility as the major POD isoenzyme (A-2) in suspension cultures of sweet potato [8]. This is surprising since the deduced amino acid sequence of swpa1 closely matched the *n*-terminal sequence of A-2 isoenzyme, and the corresponding mRNA is highly expressed in the cultured cells [9]. All of the major anionic isoenzymes (A-1, A-2 and A-3) in suspension cells of sweet potato have much greater electrophoretic mobility than isoenzymes encoded by swpa1 or swpn1 on the native PAGE gel (Data not shown). These results suggest that two cDNAs encode minor isoenzymes of sweet potato. However, it is also possible that the enzymes are not processed in the same way in tobacco as they are in sweet potato. For example, since most PODs are secreted, there could be differences in glycosylation or other processing steps that could affect their mobility.

Transcripts levels in transgenic tobacco

The steady-state accumulation of anionic or neutral POD mRNA in Bel W3 and Samsun transgenic tobacco plants, and control plants was analysed by Northern blot hybridizations. Total RNA was prepared from tobacco leaves. The tobacco anionic POD mRNA was found at extremely low levels in leaf tissues, as previously reported by Lagrimini *et al.* [10]. Although transcript level of spwa1 or swpn1 from individual tobacco plants was manifold, the expression levels from transgenic plant lines were higher than those from nontransgenic plant lines, which indicates active transcription of the introduced gene in transgenic tobacco plants (Fig. 5). Those transformant plants with anionic or neutral POD isoenzyme in Bel W3 and Samsun tobacco did not show distinctive phenotypes such as wilting or overall plant growth represented in the transgenic plants overproducing tobacco anionic POD, compared with untransformed plants [5, 6].

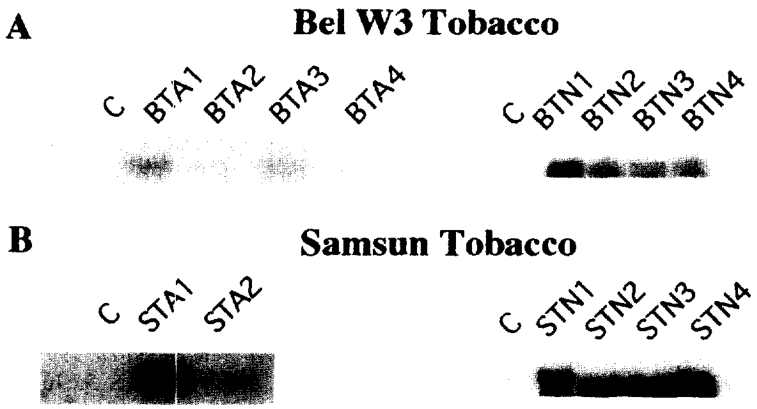

Fig. 5. Northern blot analysis of RNA isolated from nontransformed and transformed plants. RNA was extracted from fully expanded leaves of transformed or control plant lines. Equal amount of each sample was loaded in each lane. Swpa1 or swpn1 cDNA was used as a probe. The legends of (A) and (B) are the same as Fig. 4.

EXPERIMENTAL

Plant and bacteria. Two tobacco cultivars (*Nicotiana tabacum* cv Samsun and cv Bel W3) were grown in a greenhouse and used as plant materials for transformation. *Agrobacterium tumefaciens* strain LBA4404 were used for tobacco transformation.

Plant transformation and regeneration. The tobacco cultivars were transformed with *A. tumefaciens* strain LBA4404 harboring pMBP1-swpa1 or pMBP1-swpn1 vector (Fig. 1) by the leaf disc method [11]. Transformants were selected on MS (Murashige and Skoog) [12] medium supplemented with 100 mg l^{-1} kanamycin and 250 mg l^{-1} carbenicillin. Kanamycin resistant shoots were directly formed on cut edges of leaf discs. Rooting of kanamycin resistant plantlets was carried out in the selectable MS medium without growth regulators. After acclimation, the plants were transplanted to potting soil and maintained in a greenhouse. The fully grown transgenic plants were self-pollinated using envelopes made of oil paper.

PCR analysis. Chromosomal DNA was isolated from the leaves of kanamycin resistant tobaccos as described in ref. [13]. Transgenic plantlets were checked by PCR with NPTII primers (sense, 5′-GAGGCTATTCGGCTATGACTG-3′ and anti-sense, 5′-ATCGGGAGCGGCGATACCGTA-3′) and POD-specific primers 5′-GGCTACCTTCACCGGAGAT-3′ and 5′-TCGATTCTTCACATGGAT-3′ for swpa1, and 5′-CATGACTGCTTCGTCGACCGC-3′ and 5′-ATGGTGGTCCTGGCGGGAGCA-3′ for swpn1, respectively). Temp. was cycled at 95° for 1 min (denaturation), then at 65° for NPT II or 52° for POD genes for 1 min (annealing), and at 72° for 1 min (extension) for 30 cycles.

Enzyme activity and gel assay. The leaves (1 g fr. wt) of transgenic tobacco plants were homogenized on ice with a mortar in 100 mM KPi buffer (pH 6, 1:2.5). The POD activity was assayed according to the method of ref. [8] using pyrogallol as a substrate. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0. POD gel assay was conducted according to the method of ref. [14] with modifications. The native-polyacrylamide gel electrophoresis (PAGE) was conducted for 3 hr at 30 mA using 9.8% gel for anionic POD isoenzyme, whereas using 3.5% gel for neutral POD isoenzyme. After native-PAGE, POD was stained with 1% benzidine and 1.5% H_2O_2. The SOD activity was measured according to the method of ref. [15] using xanthine, xanthine oxidase, and cytochrome c. Protein was determined according to the method of ref. [16] using Bio-Rad protein assay reagents.

Northern blot analysis. Total RNA was prepared from the leaves of tobacco plants using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instruction. Total RNA was fractionated on 1% agarose gel containing 0.67 M HCHO and blotted onto a Hybond-N nylon membrane (Amersham). The blots were hybridized to ^{32}P-labelled cDNA (swpa1 or swpn1) [1]. Hybridization was carried out in a sol of 6 × SSC, 0.5% SDS, 10 × Denhardt'sol and sonicated salmon sperm DBA (100 μg ml^{-1}) at 60°. The membranes were rinsed once with 2 × SSC containing 0.5% SDS, and then washed with twice 0.1 × SSC containing 0.1% SDS at 60°.

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