In Vitro and in Situ Inhibition of Carotenoid Biosynthesis in *Capsicum annuum* by Bleaching Herbicides

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Pepper leaves treated with the herbicide J852 show an accumulation of phytoene and ζ-carotene, whereas treatment with norflurazon led to an accumulation of only phytoene. The effects of these herbicides were examined in vitro after the expression of carotenoid desaturases in *Escherichia coli*. Whereas norflurazon is a potent inhibitor of phytoene desaturase (PDS) ($I_{50} = 0.12\ \mu$M) but not of ζ-carotene desaturase (ZDS) ($I_{50} = 144\ \mu$M), J852 inhibits both PDS ($I_{50} = 23\ \mu$M) and ZDS ($I_{50} = 49\ \mu$M). The influence of PDS/ZDS inhibition on gene expression was examined by comparative RT-PCR. None of the examined genes, namely, encoding phytoene synthase, PDS, ZDS, or the terminal oxidase associated with phytoene desaturation, were induced upon herbicide treatment in pepper leaves or seedlings. This was unexpected because inhibition of carotene desaturation led to an up-regulation of the carotenoid biosynthetic capacity (higher amounts of accumulating precursors plus remaining colored carotenoids are present in treated tissues versus control).

INTRODUCTION

In plants, two closely related desaturases catalyze the conversion of phytoene to maximally desaturated lycopene with ζ-carotene as an intermediate (Sandmann 1994; Albrecht et al., 1995). Different herbicidal inhibitors are known to interfere with both enzymes. Phytoene desaturase (PDS) is the target for several commercially important herbicides such as norflurazon, fluridone, and diflufenican (Böger and Sandmann, 1990). They interact with PDS in a noncompetitive manner and cause the accumulation of phytoene in leaves at the expense of colored carotenoid of the photosynthetic apparatus. Deprived of protective carotenoids, chlorophyll and other components of the thylakoids are susceptible to photo-oxidation. The subsequent enzyme ζ-carotene desaturase (ZDS) is inhibited by direct interaction with pyrimidine derivatives (Chollet et al., 1990) such as J852 and the dihydropyrone LS80707 (Sandmann et al., 1985). Upon treatment of plants with ZDS inhibitors, not only does ζ-carotene but also substantial amounts of phytoene accumulate (Chollet et al., 1990). Either ZDS inhibitors also inhibit PDS to a certain extent or the accumulation of ζ-carotene exerts feedback inhibition on PDS. Because suitable in vitro systems were not available until recently (Breitenbach et al., 1999), enzymatic studies to support one of these alternatives have not been carried out.

It was reported (Giuliano et al., 1993) that inhibition of carotenoid synthesis in tomato seedlings by bleaching herbicides was accompanied by an increase of the mRNA levels of *pds* and *psy*, which encode phytoene synthase (Ray et al., 1992; Bartley et al., 1992). Similar observations were made upon inhibition of cyclic carotenoid formation in daffodil flowers (Al-Babili et al., 1999). This up-regulation was not found in inhibition experiments with *Arabidopsis* leaves (Wetzel et al., 1998). Most investigations on the effect of bleaching herbicides were performed with seedlings. In the present study, pepper (*Capsicum annuum*) plants with developing leaves as well as seedlings were used to investigate the effects of herbicidal PDS and ZDS inhibitors on carotenoid composition and to measure any influence on the expression of carotenoid biosynthesis related genes, namely, *pds*, *zds*, *psy*, and *ptox*, which encode a cofactor for desaturation (Carol et al., 1999; Wu et al., 1999; Josse et al., 2000). In addition, to evaluate a possible multifunctionality of both types of inhibitors, their inhibition properties on PDS and ZDS in pepper were determined.

MATERIALS AND METHODS

Plant Material and Treatment. Pepper (*Capsicum annuum* cv. Yolo Wonder) plants were grown under controlled culture room conditions in 4 cm diameter pots in vermiculite until ~10 cm in height with two leaves. Vermiculite was then allowed to dry for 4−5 h. Plants were treated with water or water containing norflurazon (10^{-4} M) or J852 (10^{-3} M) for 2−3 days in high light (12000 lx units) at culture room temperature (24−26 °C). The leaves were harvested after 2−3 days when symptoms of photobleaching became apparent. Seedlings were germinated in vermiculite until ~1 cm in height with cotyledons fully open. Seedlings were then left to germinate fully in water or in water containing norflurazon (10^{-4} M). The seedlings were harvested when the cotyledons had fully developed. A circulation of air was maintained around the plants/seedlings by electric fan to equalize temperature and evaporation. Ten seedlings or 1−2 leaves

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(∼3 cm in length) were ground in a pestle and mortar cooled with liquid nitrogen and kept frozen until used for RNA extraction and carotenoid analysis.

Carotenoid Analysis. About one-tenth of the plant material was freeze-dried and extracted for 20 min with methanol containing 6% KOH at 60 °C. After partitioning into 10% petroleum ether, carotenoids were separated and quantified by HPLC analysis. The system used consisted of a Nucleosil 120-3 C18 column and isocratic elution with acetonitrile/methanol/2-propanol 85:10:5 (Breitenbach et al., 1999). Authentic standards were used for identification and quantification of the reaction products. A Kontron (Straubenhard, Germany) diode array detector 440 was used to record the spectra from the elution peaks.

Assay of PDS and ZDS Activity. The pepper ZDS cDNA and the PDS from *Synechococcus* were expressed in *Escherichia coli* and the cells broken in a French pressure cell. The supernatants after centrifugation were used as the enzyme sources. In vitro phytoene desaturase (Schneider et al., 1997) and ζ-carotene desaturase assays (Breitenbach et al., 1999) were performed as previously described. They involved 10 µg of substrate carotenes, phytoene, and ζ-carotene, respectively, and ∼30 µg of enzyme. Incubation was for 4 h under anaerobic conditions, which were established by the addition of glucose (2 mM), glucose oxidase (20 units/mL) and catalase (20000 units/mL) in a tightly sealed vessel. Determination of the reaction products was by HPLC as described above.

Extraction of Total RNA. Frozen ground material was added to 1 mL of extraction buffer (0.1 M Tris, pH 8.0, 10 mM EDTA, 0.1 M LiCl, 1% SDS) mixed with 1 mL of water-saturated phenol preheated to 65 °C and vortexed. The samples were centrifuged, and the aqueous phase was recovered and re-extracted with 1 mL of chloroform. The aqueous phase was collected upon centrifugation and precipitated overnight with 0.5 volume of 6 M LiCl. Following centrifugation, the pellet was washed with 70% ethanol and 100% ethanol, dried, resuspended in RNA resuspension buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS), and precipitated in 2 volumes of absolute ethanol and 0.1 volume of sodium acetate. RNA samples were treated with 20 µg/mL proteinase K in buffer (10 mM Tris, pH 7.0, 0.4% SDS) at 50 °C and repurified by phenol/chloroform extraction. Samples were checked for DNA contamination by PCR. The concentration and purity of total plant RNA were determined by spectrophotometric analysis. All RNA samples in each experiment were analyzed by formaldehyde agarose gel electrophoresis and visual inspection of rRNA bands upon ethidium bromide staining. Samples were treated with DNase in 25 µL of buffer (20 mM Tris, pH 7.0, 6 mM MgCl, 40 units of RNase inhibitor (RNaseOUT, BRL), and 0.1 unit of DNase I to remove DNA contamination.

Measurement of mRNA by RT-PCR. Reverse transcription was carried out using 500 ng of total RNA and oligo-dT as a primer. The reaction mixture included 1 mM dNTPs, 0.5 µM oligo-dT, 20 units of RNase inhibitor, 10 pg of control RNA (rabbit globin mRNA from reticulocyte polyribosomes, BRL), 10 mM DTT, 1× RT buffer, and 150 units of M-MLV reverse transcriptase (BRL) in a total volume of 20 µL (Josse et al., 2000). Each reaction was carried out in duplicate. The reaction mixture was incubated for 10 min at 20 °C, for 35 min at 37 °C, and then for 15 min at 42 °C. Duplicate samples were pooled to give a final volume of 40 µL for PCR.

The PCR reaction contained 0.6−2.0 µg of each primer, 1.3× *Taq* polymerase buffer, 5 mM MgCl, 0.30 mM dNTPs, 1.5 units of *Taq* polymerase (BRL), and 10 µL of RT reaction mixture (25 ng of RNA/µL) in a total volume of 50 µL. Final concentration was 5 ng/µL of reaction mixture. The amplification reactions included 27 cycles of 30 s at 94 °C, 20 s at 50 °C (*ptox*) or 55 °C (*psy*, *pds*, *zds*, and *fib*), and 20 s at 72 °C (Josse et al., 2000). PCR products were fractionated on 1.5% agarose gel.

Table 1. Carotenoids (Milligrams per Gram of Dry Weight) in Leaf Samples Treated with Norflurazon (10⁻⁴ M) and J852 (10⁻³ M)^a

	phytoene	ζ-carotene	other carotenoids	total
		Set 1		
control	0	0	1.32	1.32
norflurazon	1.48	0	1.00	2.48
J852	0.55	0.19	0.86	1.60
		Set 2		
control	0	0	1.30	1.30
norflurazon	1.68	0	1.15	2.83
		Set 3		
control	0	0	1.40	1.40
norflurazon	1.94	0	0.48	2.42
		Seedlings		
control	0	0	1.21	1.21
norflurazon	1.34	0	0.91	2.24

^{a} Values are means of two to three determinations. Standard deviations were in the range of 6−9% of the mean. Phytoene values represent the sum of phytoene and its oxidation products (Sandmann and Albrecht, 1990).

RESULTS

Effects of Herbicide Treatment on Carotenoid Content in Pepper Leaves. Plants treated for 2 days with J852 showed an accumulation of the carotenoid precursors phytoene and ζ-carotene, indicating that J852 inhibited both phytoene desaturation and ζ-carotene desaturation in vivo. Plants treated with norflurazon showed an accumulation of phytoene as expected from the specific inhibition of phytoene desaturation by this herbicide (Böger and Sandmann, 1990). Plants treated with either J852 or norflurazon also showed a decrease in other (colored) carotenoids. However, it can be seen (Table 1, plant set 1) that the total amount of carotenoids (precursors plus other carotenoids) in the treated plants is higher than seen in the control plants, suggesting an up-regulation of this biosynthetic pathway. These data were confirmed using several sets of plants, a second one being shown in Table 1 (set 2), and using younger leaves treated for a 72 h period (Table 1, set 3). In all instances leaves treated with J852 or norflurazon showed significant effects of photo-oxidative bleaching visibly affecting chlorophyll levels (more severe bleaching and areas of cell death were observed in younger leaves). Therefore, it seems likely that the observed up-regulation in the carotenoid pathway upon herbicide treatment is understated due to the photodestruction of carotenoids and accumulating precursors.

In Vitro Inhibition of ZDS and PDS. The effect of J852 and norflurazon on pepper ZDS could be demonstrated in vitro after expression in *E. coli*. From ∼5 µM on, J852 shows a concentration-dependent inhibition of ζ-carotene conversion to neurosporene and lycopene (Figure 1). In addition, this reaction was also inhibited by norflurazon, which is a very sensitive inhibitor of PDS. The effect of J852 and norflurazon on pepper PDS could not be investigated because this protein could not be heterologously produced in an amount comparable to ZDS (not shown). Instead, the structurally related PDS from *Synechococcus* was used, and we found that J852 is also an inhibitor of PDS. For a quantitative comparison, I_{50} inhibition values (indicating a concentration that allows half-maximum activity of PDS and ZDS in an untreated control) were determined for norflurazon and J852 (Table 2). Inhibition of ZDS with J852 resulted in an I_{50} value of 49 µM. This compound

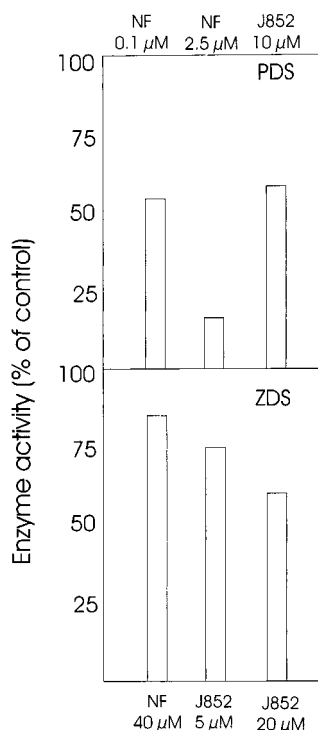**4678** *J. Agric. Food Chem.,* Vol. 48, No. 10, 2000

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Figure 1. In vitro inhibition of phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) activity by norflurazon and J852. Specific activities of the controls were 0.12 μg h^{-1} mg^{-1} of protein for PDS and 5.3 μg h^{-1} mg^{-1} of protein for ZDS.

Table 2. I_{50} **Values (Micromoles) for the Inhibition of PDS and ZDS by Norflurazon and J852**[a]

	PDS	ZDS
norflurazon	0.12	144
J852	23	49

[a] I_{50} values were calculated from a linear plot of herbicide concentrations versus the inverse enzyme activities. Regression coefficients were all >0.9.

is an even better PDS inhibitor as indicated by an I_{50} value of 23 μM. Norflurazon is a very potent inhibitor of PDS with an I_{50} value of 0.12 μM. The I_{50} value for the inhibition of ZDS by norflurazon was 144 μM, which is 1200-fold higher than for PDS inhibition.

Expression of Carotenoid Biosynthetic Genes. To study further the up-regulation of the carotenoid pathway, RNA was extracted (as described under Materials and Methods) from the same plant samples used for carotenoid analysis, and transcript levels were compared by RT-PCR. These experiments indicated no significant difference in *zds* or *pds* transcript levels between leaves of plantlets treated with either J852 or norflurazon for 48 h and control plantlets in any experiment. Parts a and b of Figure 2 show the data with plant sets 1 and 2, respectively (carotenoid content shown in Table 1). In addition, no significant difference in PTOX (Josse et al., 2000) or PSY (Römer et al., 1993) transcript levels was observed between norflurazon-treated and control leaves (Figure 2a). Because the leaves used for the above experiments did not show total bleaching during the duration of the experiments (even with higher herbicide concentrations), smaller leaves were used (carotenoid data shown in Table 1, plant set 3). Although these leaves showed more severe bleaching, a higher accumulation of phytoene, and a significant reduction in other carotenoids, in this case also, no induction of *zds* or *pds* was observed (not shown).
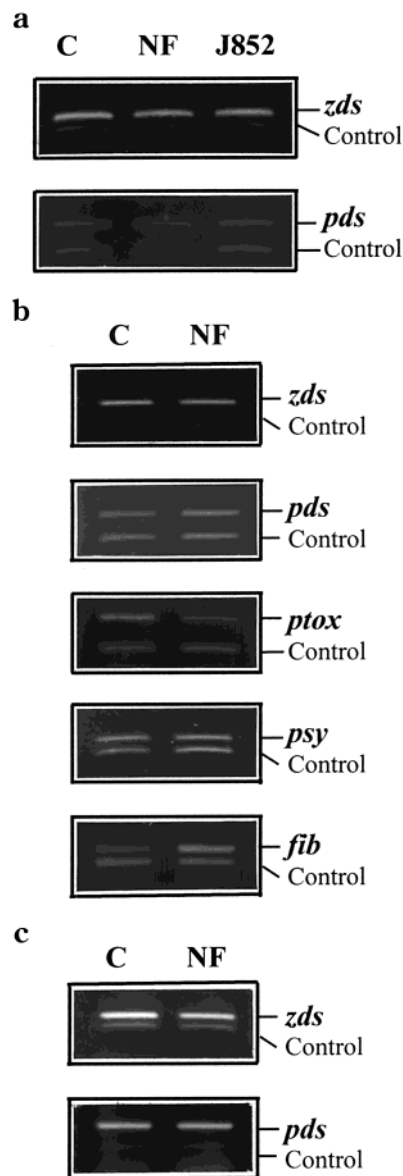

Figure 2. Gene expression studied by comparative RT-PCR in herbicide-treated plantlets. The expressions of *pds* and *zds* genes (a) and *pds, zds, ptox, psy,* and *fib* genes (b) in pepper leaves and of *pds* and *zds* genes in seedlings (c) were monitored. Carotenoid content for plants in panel a can be seen in Table 1 (plant set 1). Carotenoid content for plants in panel b can be seen in Table 1 (plant set 2). Carotenoid content for seedlings panel c can be seen in Table 1 (lower part). Equal amounts of total RNA were used in each reaction. PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Amplification of globin mRNA (added to RT reaction mix) was used as a control for the RT-PCR reaction (see Materials and Methods). C, control; NF, norflurazon.

Pepper seedlings were also treated with norflurazon after germination and, like leaves, these samples showed phytoene accumulation, a reduction in colored carotenoids, and an apparent increase in total carotenoids (Table 1, lower part). Here also no up-regulation of either *pds* or *zds* genes was observed (Figure 2c).

These data indicate that the regulation of *zds, pds, ptox,* and *psy* gene expression is not affected by a decrease in colored carotenoids or by an accumulation of the precursors phytoene or ζ-carotene in tissues due to a block in carotenoid biosynthesis. These data indicate that the increase in total carotenoids appears to

be independent of the regulation of these transcript levels.

Expression of the Fibrillin Structural Protein Gene Differs from That of Carotenoid Genes. Fibrillin is a structural protein involved in the assembly of carotenoid-storing lipoprotein structures in some chromoplast type, and its gene (*fib*) is induced during fruit ripening by redox regulatory mechanisms (Kuntz et al., 1998). This gene is also induced in leaves from pepper (Chen et al., 1998) and other species (Gillet et al., 1998) submitted to stress conditions. Reactive oxygen species produced by perturbations in photosynthetic electrons transport upon stress are involved in *fib* induction in leaves (Manac'h and Kuntz, 1999). Therefore, *fib* expression is a positive control for gene induction due to photo-oxidative stress. RT-PCR experiments with the previously used samples (which showed no increase in carotenoid gene expression) showed an increase in *fib* transcript in response to herbicide treatment (Figure 2b).

DISCUSSION

Upon plant treatment with the pyridine derivative J852, not only was ZDS inhibited as indicated by ζ-carotene accumulation but also substantial amounts of phytoene accumulated (Table 1, upper part). Direct inhibition of pepper ZDS was demonstrated in vitro by enzymatic investigations (Figure 1). The concentration of J852 used for inhibition of ZDS in the leaves was ~20-fold higher than the I_{50} value for half-maximum in vitro inhibition of the enzyme that should ensure a block of ζ-carotene conversion. Accumulation also of phytoene in plants after application of J852 can be explained in two ways: (i) Because PDS and ZDS are structurally closely related (Albrecht et al., 1995), a ZDS inhibitor may possess the potential also to inactivate PDS and vice versa. (ii) ζ-Carotene may exert a feedback inhibition on PDS, a mechanism that exists for the synthesis of other carotenes in the fungus *Phycomyces* (Bramley and Davies, 1976). In vitro inhibition studies with a PDS from S*ynechococcus*, which is closely related to the pepper enzyme, demonstrated that J852 inhibits PDS even better than ZDS (Figure 1). Therefore, we can assume that the accumulation of phytoene in J852-treated pepper leaves is caused by inhibition of PDS in addition to and independent of ZDS inhibition. In contrast to J852, norflurazon was rather specific for PDS inhibition and a very poor inhibitor of ZDS (Table 2).

Inhibition of carotenoid biosynthesis at the level of phytoene or ζ-carotene desaturation leads to a moderate decline in the levels of colored carotenoid after 2−3 days in developing pepper leaves or in seedlings while a concomitant accumulation of precursors was observed (Table 1). Because moderate decreases in carotenoid levels are likely to occur in plants under natural conditions, the existence of mechanisms able to up-regulate this pathway (to restore carotenoid levels) can be expected. The fact that the total amount of remaining carotenoids and precursors is higher than the carotenoid amount found in control leaves suggests that an up-regulation of this metabolic pathway did occur. However, the conclusion of our results is that carotenoid content is not a critical factor affecting the expression of *zds* or *pds* in pepper leaves or seedlings. Their expression is not noticeably influenced by a decrease of yellow carotenoid products or by an accumulation of either phytoene precursors or ζ-carotene. Numerous

reports have shown an important influence of transcriptional regulation for this pathway [see Ronen et al. (1999) and references therein], so it was unexpected to observe no up-regulation of *zds* and *pds* genes under our experimental conditions (Figure 2). An alternative and potentially regulatory level for carotenoid desaturation could be PTOX, a redox cofactor for PDS activity (Carol et al., 1999; Wu et al., 1999; Josse et al., 2000). However, this gene was not found to be up-regulated either. Neither was *psy*, which encodes the first dedicated enzyme of the pathway. It should be mentioned that the oligonucleotides used for *psy* mRNA detection were based on highly conserved DNA stretches [when the pepper sequence was compared to the two tomato *psy* genes (Ray et al., 1992; Bartley and Scolnik, 1993)] and would most likely amplify a second (yet unknown) *psy* gene from pepper. Thus, it is unlikely that the pathway up-regulation can be controlled at the level of a second *psy* gene.

The carotenoid levels reflect a steady state governed by biosynthesis and degradation, especially in photosynthetic organisms (Steiger et al., 1999). Therefore, an increase of total carotenoids including precursors upon treatment with inhibitors could be caused by higher (photo)stability of the precursors with a shorter polyene chain versus the carotenoid end products. This is definitely the case for phytoene (Steiger et al., 1999), and we cannot exclude that this phenomenon participates in the apparent increase in total carotenoids upon herbicide treatment. However, as mentioned above, one can speculate that other mechanisms, such as translational, post-translational, or enzymatic mechanisms may be involved in the up-regulation of carotenoid biosynthesis. The existence of regulatory mechanisms independent of transcription is also suggested by a recent study using fruits (Fraser et al., 1999).

As far as the expression of *pds* is concerned, our conclusions are in agreement with those of Wetzel et al. (1998) using norflurazon treatment or mutants. These authors concluded that there is no correlation between pigmentation and *pds* mRNA levels in *Arabidopsis* leaves or seedlings. In contrast, Giuliano et al. (1993) reported 2- and 10-fold increases in *psy* and *pds* mRNA levels, respectively, in tomato seedlings treated with norflurazon. They concluded that the control of *pds* and *psy* expression is mediated by either photo-oxidative stress and/or by the end products of carotenogenesis. Using transgenic tobacco plants, data from the same laboratory pointed to an end-product regulatory mechanism for control of the *pds* promoter (Corona et al., 1996). Our results do not support a similar conclusion using pepper (which is also a Solanaceae plant) leaves or seedlings. It should be mentioned, however, that the pepper seedlings were treated after germination (and not directly at the imbibition state, which in pepper and in our hands leads to a negative effect on seedling development). Therefore, a possible explanation for the differences in *pds* expression between pepper and tomato may lie in the fact that the pepper seedlings (and leaves) did not totally bleach upon herbicide treatment and may not have lost enough carotenoids to reach the threshold levels required for gene induction. We consider this potential explanation unlikely because these seedlings did show a significant loss of chlorophyll and areas of cell death. In addition, when using smaller leaves and longer treatment (leading to more severe bleaching symptoms), we were unable to demonstrate an induction of the studied genes. Furthermore, because

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total bleaching is not a common event in nature, gene induction under such severe conditions may not reflect a physiologically meaningful situation.

We did, however, observe an increase in the expression of the *fib* gene in plants treated with norflurazon and J852. This is consistent with the results reported by Manac'h and Kuntz (1999), who showed that *fib* is induced by various abiotic stresses that cause photo-oxidative stress and the production of reactive oxygen species. This indicates that in the present experiments, the plants were under sufficient stress (as also suggested by visual observation and a depletion in carotenoid content). These data suggest that *fib* expression is controlled in leaves by a regulatory mechanism different from that of the carotenoid biosynthetic genes studied here. This was unexpected because, during fruit ripening, similar mechanisms apparently control up-regulation of both *fib* and a carotenoid biosynthetic gene, namely, capsanthin/capsorubin synthase (Kuntz et al., 1998).

ABBREVIATIONS USED

PSY, phytoene synthase; PDS, phytoene desaturase; PTOX, plastid terminal oxidase; ZDS, ζ-carotene desaturase; fib, fibrillin; NF, norflurazon.

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