Expression of Phospholipase D during Castor Bean Leaf Senescence

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Membrane deterioration in plant senescence is commonly associated with progressive decreases in membrane phospholipid content. This study investigated the expression and regulation of phospholipase D (PLD; EC 3.1.4.4) during senescence in castor bean (Ricinus communis L. cv Hale) leaf discs. The rate of leaf senescence was accelerated by 50 μM abscisic acid and was attenuated by 50 μM cytokinin during incubation at 23°C for up to 5 d. Leaf senescence was indicated by decreases in the content of total proteins, chlorophyll, and phospholipids. PLD activity in both membrane-associated and cytosolic fractions showed a gradual increase in the absence of phytohormones. Abscisic acid stimulated an increase in membrane-associated PLD and had little effect on the soluble form. On the other hand, cytokinin retarded the increase in membrane-associated PLD. Immunoblotting analysis using PLD-specific antibodies revealed that the changes in PLD activity were correlated with those of PLD protein. Analysis of PLD by nondenaturing PAGE showed the appearance of a PLD structural variant, PLD 3, in abscisic acid-treated leaf discs. Northern blotting analysis using a PLD cDNA probe revealed an increase in PLD mRNA in senescing leaf discs. These data indicate complex mechanisms for the regulation of PLD during senescence, which include increases in membrane-associated PLD, differential expression of PLD isoforms, and changes in amounts of PLD protein and mRNA. Such controlled expression points to a role for PLD in membrane deterioration and plant senescence.

PLD (EC 3.1.4.4) hydrolyzes glycerophospholipids at the terminal phosphodiesteric bond, leading to the formation of PA and a free amino alcohol group. PLD is active in many plant tissues, but its physiological significance in plant growth and development is unclear (Heller, 1978; Wang, 1993). Changes in PLD activity were observed in a number of physiological processes, including various stress injuries (Yoshida, 1979; Chetal et al., 1982; Willemot, 1983), senescence (Thompson et al., 1987), and seed aging and germination (Herman and Chrispeels, 1980; Di Nola and Mayer, 1986; Lee, 1989; Samama and Pearce, 1993; Wang et al., 1993). It has been proposed that PLD-mediated hydrolysis is a first step in membrane deterioration in senescing carnation flowers, tomato fruits, cabbage leaves, γ-irradiated cauliflower florets, and aging cucumber and onion seeds (Paliyath et al., 1987; Thompson et al., 1987; Cheour et al., 1992; McCormac et al., 1993; Samama and Pearce, 1993; Voisine et al., 1993). The resulting PLD product, PA, is further hydrolyzed by PA phosphatase and acyl hydrolases, followed by lipoxygenase. These reactions lead to the formation of oxy-free radicals and lipid peroxides that may cause membrane deterioration. Increased PLD activity may also result directly in membrane destabilization, since PA favors nonlamellar phase formation (Israeilachvilli et al., 1980; Samama and Pearce, 1993).

Crucial to the understanding of the cellular role for PLD is its regulatory mechanism and expression during plant growth and development. Decreases in lipid bilayer fluidity of plasma and microsomal membranes have recently been proposed to activate PLD in ripening fruits, thus facilitating phospholipid catabolism (McCormac et al., 1993). It has also been postulated that the increased PLD hydrolysis of membrane phospholipids under certain stresses results from decompartmentalization of PLD from its original intracellular stores (Yoshida, 1979; Willemot, 1983). These mechanisms assume that the increases in cellular PLD activity result from activation of preexisting PLD in plant cells. Recent studies using anti-PLD antibodies have found that after seed germination and during leaf development of castor bean (Ricinus communis L.), changes in PLD activity are correlated with the changes in PLD protein concentration, suggesting that PLD activity is regulated by synthesis and/or degradation of PLD protein (Wang et al., 1993; Dyer et al., 1994). Furthermore, multiple molecular forms of PLD have been identified in castor bean tissues (Dyer et al., 1994). The expression of specific PLD variants is associated with developmental and growth conditions, raising the possibility that the differential expression of PLD variants constitutes another mechanism for controlling PLD activity. The recent cloning of a castor bean PLD cDNA makes it possible to examine the regulation of PLD at the molecular level (Wang et al., 1994).

The purpose of the present study was to examine the expression of PLD in senescing leaf discs of castor bean; these were chosen because of the ease of uniform application of ABA and kinetin. The two phytohormones are known to have opposite effects on senescence in the excised tissues; ABA promotes senescence of detached tissues, whereas cytokinin attenuates it. Such hormone manipulation helps further define the expression and regulation of PLD in senescent leaves.

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Abbreviations: g, average gravity; PA, phosphatidic acid; PC, phosphatidylcholine; PLD, phospholipase D.
MATERIALS AND METHODS

Plant Materials and Sample Treatment

Coatless castor bean (Ricinus communis L. cv Hale) seeds were germinated in the dark in moist vermiculite for 3 d. The seedlings were individually transplanted into plastic pots containing a mixture of vermiculite and perlite (1:1, v/v) that were subirrigated with Hoagland nutrient solution. Plants were grown under cool-white fluorescent lights at 23 ± 2°C with a 14-h photoperiod. Fully expanded leaves from approximately 8-week-old plants were briefly rinsed with sterile, deionized water before leaf discs were excised with a cork borer (13 mm diameter). Leaf discs were placed adaxial side up in Petri dishes (85 mm) on two layers of 3-mm filter paper (Whatman No. 5) that were wetted with 7 mL of H2O, ABA (50 μM), or kinetin (50 μM). ABA was first dissolved in ethanol and then diluted in deionized water to the final concentration. Kinetin, which was in liquid form, was diluted to its final concentration. The ethanol concentration of all solutions was adjusted to 0.01%. Discs were incubated at 23 ± 2°C under a 14-h photoperiod.

Tissue Fractionation

Leaf discs were harvested after various intervals of incubation and homogenized with a mortar and pestle chilled on ice. Proteins were extracted with buffer A containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 0.5 mM PMSF, and 2 mM DTT. All the following steps were carried out at 4°C unless stated otherwise. The homogenate was centrifuged at 6,000g for 10 min, and the supernatant was saved as the soluble fraction. The pellet (microsomal fraction) was resuspended in homogenization buffer and centrifuged at 110,000g for 60 min. The resultant supernatant was saved as the soluble fraction. The pellet (microsomal fraction) was resuspended in homogenization buffer and centrifuged at 110,000g for 60 min to remove cytosolic contaminants. The resultant pellet was suspended by grinding with a glass homogenizer in buffer A. The soluble and microsomal proteins were either used immediately or stored at −80°C until use.

PLD Activity Assay

PLD was assayed for its hydrolysis and transphosphatidylcystosylation activity using 1-palmitoyl-2-[9,10-3H]palmitoyl-glycero-3-P-choline as substrate. The conditions for substrate preparation, activity assay, and the reaction-product determination have been detailed elsewhere (Wang et al., 1993). Briefly, radioactive PC (2.5 μCi) and 20 μmol of egg yolk PC were emulsified in 1 mL of H2O by sonication. A standard enzyme assay mixture contained 100 mM Mes/NaOH (pH 6.5), 25 mM CaCl2, 0.3 mM SDS, 2 mM PC, 1% ethanol, and enzyme preparations in a total volume of 0.2 mL. PC, PA, and phosphatidylethanol were separated by TLC and quantitated by scintillation counting. The amounts of protein used in each assay for soluble and microsomal fractions were typically 30 and 3 μg, respectively.

Electrophoresis and Immunoblotting

Both SDS-PAGE and nondenaturing PAGE used gels containing 8% (w/v) acrylamide (pH 8.8) in the resolving phase and 3.5% (pH 6.8) in the stacking phase (Wang et al., 1993). Fifty micrograms/lane and 20 μg/lane proteins were loaded for soluble and microsomal fractions, respectively. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes. The membranes were incubated with PLD antibodies (1:1000 dilution) in PBS containing 5% (v/v) nonfat dry milk. Polyclonal antibodies of PLD were raised in rabbits against a 92-kD protein purified from d-2 germinated endosperm (Wang et al., 1993). PLD was made visible with horseradish peroxidase conjugated with goat antibodies against rabbit immunoglobulin. PLD protein amounts on immunoblots were estimated by densitometric scanning of the band intensity with a video densitometer (Bio-Rad).

For nondenaturing PAGE, soluble proteins were precipitated with cold acetone to enhance PLD visibility (Dyer et al., 1994). Membrane samples were solubilized with octyl glucoside (30 mM). Protein samples were adjusted to 5 mM DTT and 5% (v/v) glycerol prior to loading. Eighty micrograms/lane and 30 μg/lane proteins were loaded for soluble and microsomal fractions, respectively. The gel was run at constant voltage of 80 V for 30 min and then increased to 120 V at 4°C for 60 min. For immunoblot analysis of PLD resolved on nondenaturing PAGE, after electrophoresis the gel was immersed in 0.05% SDS in a protein transfer buffer (12.5 mM Tris and 100 mM Gly) for 10 min. The SDS-soaked gel was briefly rinsed with the transfer buffer. Subsequent procedures for transferring the proteins onto polyvinylidene difluoride membranes were the same as previously reported for immunoblots of SDS-PAGE gels (Wang et al., 1993). The immunoblots were made visible using alkaline phosphatase conjugated to goat antibodies against rabbit immunoglobulin.

Northern Blot Analysis

Total RNA was isolated from the leaf discs of castor bean stored in liquid nitrogen using a cetyltrimethylammonium bromide extraction method described previously (Wang and Hildebrand, 1988). RNA concentration was determined spectrophotometrically, and that the same amounts of RNA were loaded was verified by ethidium bromide staining of the gel. RNA (20 μg/lane) was subjected to denaturing 1% formaldehyde/agarose gel electrophoresis and transferred onto a nylon membrane. After transfer, the RNA was fixed on the filters by cross-linking with UV illumination. The filters were prehybridized in a solution of 6× SSC, 0.5% SDS, 5× Denhardt's reagent, and 100 μg/mL salmon sperm DNA at 68°C. The probe was the 2840-bp EcoRI and KpnI fragment of PLD cDNA labeled with [α-32P]dATP by random priming (Wang et al., 1994). Hybridization was performed in the same solution at 68°C overnight. The blots were washed with 1× SSC and 0.1% SDS at 68°C and exposed to x-ray film.
Phospholipase D in Leaf Senescence

Figure 1. Changes in content of soluble (A) and membrane-associated (B) proteins, phospholipids (D), and Chl (C) of leaf discs incubated in water (O) and in the presence of 50 μM ABA (●) or 50 μM kinetin (△). Values are means ± SE of four experiments.

Contents of Chl, Phospholipids, and Protein

Chl was extracted from leaf discs with 100% methanol at ambient temperature, and its concentration was calculated according to Holden (1976). Total lipids were extracted from the leaf discs according to Bligh and Dyer (1959). Phospholipid content was calculated from the phosphorus content of the total lipids, which was determined based on its interaction with molybdate (Rouser et al., 1966). Protein content was determined by a dye-binding method (Bio-Rad).

RESULTS

Castor bean leaf discs senesced during the 5-d incubation, as indicated by the gradual losses of soluble protein, phospholipids, and Chl (Fig. 1). When compared with freshly excised leaf discs, the contents of soluble protein decreased by 22%, phospholipids by 21%, and Chl by 7% in the leaf discs that were incubated in water (referred to as control discs). Incubation of leaf discs with 50 μM ABA accelerated these losses by about 20% over the 5 d of incubation. Kinetin treatment slowed down the senescence. In the kinetin-treated tissue, the soluble protein content was 20% higher than that in water-treated control leaf discs after the 5 d of incubation, and phospholipid content was 40% higher than that of 5-d controls and was about 20% higher than that of freshly excised leaf discs. Such an increase in membrane lipid content is consistent with the reported stimulatory effect of kinetin on cell proliferation. After 5 d of incubation, yellowing appeared in the ABA-treated leaves, and the kinetin-treated leaves looked vigorous, but not as dark green as the control leaves. The difference in green color between the control and kinetin-treated leaves mimicked that between mature leaves and rapidly expanding young leaves on castor bean plants. This difference was reflected in Chl content, which in the kinetin-treated discs was about 6% lower than that of control discs (Fig. 1C). The amounts of membrane-associated proteins in the control and ABA- and kinetin-treated discs were similar, and they increased in the first 3 d, followed by a decline after 5 d of incubation.

PLD activity increased during senescence in the leaf tissue (Fig. 2). The disc homogenates were fractionated into soluble and microsomal fractions. The specific activity of membrane-associated PLD was about 9-fold higher than that of soluble PLD in fully expanded castor bean leaves, and approximately 70% of total PLD was associated with the membranes. After 5 d of incubation, the specific PLD activity of control leaf tissues increased about 1.5-fold in membrane-associated fractions and 2-fold in soluble fractions; the total PLD activity in each leaf disc increased about 1.5-fold in both fractions.

The differences in the specific activity of PLD in the control and ABA- and kinetin-treated leaf tissues occurred primarily in the membrane fractions (Fig. 2A). ABA treatment of discs resulted in further increases of membrane-associated PLD; the specific activity of PLD in the leaf tissue after 1, 3, and 5 d of treatment with ABA was about 20% higher than that of control discs. The membrane-associated PLD activity in kinetin-treated discs was significantly lower than that in the control leaf discs. After 5 d of incubation there was a small but not significant increase in the microsomal PLD activity in the kinetin-treated discs, whereas that in control and ABA-treated leaves increased approximately 40 and 60%, respectively. The differences in total PLD activity per leaf disc between the control and

Figure 2. Changes in microsomal (A) and soluble (B) PLD activities of leaf discs incubated in water (O) and in the presence of 50 μM ABA (●) or 50 μM kinetin (△). Microsomal PLD was from the pellet of 110,000g, centrifugation of the 6,000g, supernatant, and soluble PLD was from the supernatant after 110,000g, centrifugation for 60 min. Values are means ± SE of four experiments.

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ABA- and kinetin-treated tissues followed the same pattern as those in specific PLD activity because the contents of membrane-associated proteins per disc were similar in these tissues (Fig. 1B).

The extent of increases in the specific activity of soluble PLD was similar in control and ABA- and kinetin-treated leaves after 1 and 5 d of incubation, and there was a 2-fold increase in the three treatments after 5 d of incubation (Fig. 2B). A difference in the PLD activity was detected between control and kinetin-treated leaves incubated for 3 d; PLD activity in kinetin-treated tissue was about 20% higher than that of control leaves at this time. Because of the different rates of loss of soluble proteins in the three treatments (Fig. 1A), the total amount of PLD activity per disc in kinetin-treated discs was about 30% higher than that of control and ABA-treated discs after 5 d of incubation. After 5 d of incubation, the total PLD activity in ABA-treated discs was about 40% lower than that of control and kinetin-treated discs.

PLD protein content of the leaf discs was analyzed using anti-PLD specific antibodies. Consistent with the data of PLD activity measurement, the PLD protein content in the microsomal fraction was much higher than that in the soluble fraction (Fig. 3). Membrane-associated PLD gradually increased in the control discs by 5 d of incubation. The increases were 1.5- and 2-fold at 5 and 3 d compared to freshly excised discs. In the ABA-treated discs, about 2- and 2.5-fold increases of PLD occurred after 3 and 5 d of incubation. Compared to freshly excised leaves, there was no decrease in total membrane-associated protein (mg/disc) in the three treatments after 5 d of incubation (Fig. 1B). Therefore, the increase in PLD resulted from a higher content of PLD protein in membranes, rather than a decrease in other membrane proteins. In kinetin-treated discs, there was no increase in membrane-associated PLD. An increase in soluble PLD was apparent in all of the control and ABA- and kinetin-treated discs (Fig. 3), which was caused by both increased total PLD accumulation and a relative enrichment of PLD.

Figure 3. Immunoblot of microsomal and soluble PLD in leaf discs using PLD antibodies. Leaf discs were incubated in water, 50 μM ABA, and 50 μM kinetin. Soluble and microsomal proteins were prepared as described in Figure 2. Proteins were separated on 8% SDS-PAGE. Fifty micrograms/lane and 20 μg/lane proteins were loaded for soluble and microsomal fractions, respectively. Day 0 is freshly excised leaf tissues.

Specifically, the soluble PLD increase in the kinetin-treated leaves came primarily from elevated accumulation of total PLD protein, whereas the increased PLD accumulation and the decrease in total soluble proteins contributed almost equally to the higher content of PLD protein in the discs treated with ABA for 5 d. The trend of increases in PLD protein correlated with those of PLD activities, suggesting that an increase in PLD protein accumulation is responsible for the increased PLD activity.

Previous studies on castor bean showed the presence of three major structural variants of PLD, designated as PLDs 1, 2, and 3 according to their mobility resolved by nondenaturing PAGE (Dyer et al., 1994). To examine whether different PLD isoforms were present during leaf senescence, proteins from the control and ABA- and kinetin-treated discs were separated by nondenaturing PAGE, and PLD variants were made visible by immunoblotting with PLD antibodies. Only the PLD 2 band was detected in the proteins extracted from fully expanded castor bean leaves, and this result is consistent with a previous observation (Dyer et al., 1994). After 5 d of incubation, the control and kinetin-treated leaves still contained one PLD 2 band (Fig. 4, lanes W and K). In contrast, two PLD bands, PLDs 2 and 3, were visible in leaves treated with ABA (Fig. 4, lanes A). PLD 3 migrated slightly faster than PLD 2 and was present in both the soluble and microsomal fractions. The PLD 3 band was not the result of major proteolytic cleavage of PLD because only one PLD band was observed when the same protein extract was subjected to SDS-PAGE. This result showed that the occurrence of PLD 3 was associated with the more senescent, ABA-treated leaves.

PLD mRNA in the senescing tissues was monitored by northern blot analysis. Changes in the PLD transcript accumulation were evident in the control and ABA- and kinetin-treated leaves (Fig. 5). The highest amount of PLD mRNA (more than 5-fold compared to freshly excised leaves) was observed after 1 d of incubation. ABA resulted in the largest increase of PLD mRNA (about 8-fold). PLD mRNA decreased afterward in all the treatments, and the most marked decline occurred in the leaves that were incubated with kinetin for 5 d. After 5 d of incubation, ABA-treated leaf discs contained 3-fold more PLD mRNA
One may be that the newly synthesized PLD mRNA is not efficiently translated due to posttranscription modifications or translation control. Another possibility is that the rate of turnover of PLD protein increases during senescence, thus resulting in its low accumulation despite increased PLD synthesis. Protease activity is known to increase during senescence, and membrane perturbation makes membrane lipids and proteins more prone to hydrolysis (Duxbury et al., 1991).

Comparison of PLD expression between leaf discs in the presence or absence of ABA and kinetin has shown that the increase in membrane-associated PLD, but not in soluble PLD, is positively correlated to the rate of senescence in castor bean leaf discs as measured by loss of proteins, phospholipids, and Chl. ABA and kinetin are known to have opposite effects on senescence in excised tissues. The former promotes senescence, and the latter delays it. In the present study, ABA and kinetin effects on leaf senescence were shown clearly by the accelerated or attenuated losses, respectively, in total proteins and phospholipids in the ABA- and kinetin-treated discs. In terms of PLD specific activity and protein, the primary difference between the control and ABA- and kinetin-treated discs was in the microsomal-associated PLD. ABA treatment promoted the increase of membrane-associated PLD, whereas the membrane PLD level in the kinetin treatment remained the same as that in the freshly excised discs. The elevation of microsomal-associated PLD activity was also associated with phospholipid hydrolysis in γ-irradiated cauliflower (Voisine et al., 1993). It is conceivable that the increased membrane association of PLD increases the enzyme contact with its substrate, thereby promoting PLD hydrolysis and membrane deterioration.

The association of PLD with microsomes was also reported in other plants such as carnation flowers, tomato fruits, and broccoli (Thompson et al., 1987; McCormac et al., 1993). It remains to be elucidated how PLD associates with membranes and to what membrane(s) it is attached. Because the castor bean microsomal fraction was washed with extraction buffer, simple contamination of the microsomes with soluble proteins cannot explain the pattern of differences in membrane-associated PLD between the control and ABA- and kinetin-treated discs. Washing the microsomal fractions with 0.2 M KCl released considerable PLD from the membranes, suggesting that a portion of PLD is peripheral or loosely associated with the membranes (data not shown). The pattern of differences between those treatments remained essentially the same, indicating that the membrane-associated PLD that increases is both peripherally and tightly attached to microsomal membranes.

Recent immunocytochemical studies comparing the in situ intracellular distribution of PLD in the castor bean leaves of early and mature stages showed that PLD is found primarily in vacuoles in young leaves, and that in mature leaves considerable PLD is present in the cytoplasmic phase, associated with the ER (Xu et al., 1994). It is likely that the increased PLD in membrane fractions is associated with the ER.

Interestingly, the ABA and kinetin treatments of leaf discs produced an effect that mimics the changes in the soluble versus membrane-associated PLD during leaf development. For example, the induction of PLD in castor bean re-
revealed that the ratio of soluble versus membrane-associated PLD undergoes a gradual decrease during castor bean leaf development (Dyer et al., 1994). In early stages of leaf development, PLD is mostly soluble, and it becomes largely membrane associated as leaves mature and age. In the present study, kinetin treatment promotes growth as indicated by the increased protein and phospholipid contents, and it also increases the ratio of soluble versus membrane-associated PLD. On the other hand, ABA treatment that accelerates leaf senescence resulted in the lowest ratio of soluble versus membrane-associated PLD among the three treatments. After 3 d of incubation, the ratios of soluble versus microsomal PLD were 0.16, 0.14, and 0.24 for the control and ABA- and kinetin-treated tissues, respectively. The relationship of the ratio with growth stages indicates a role for the relative distribution of PLD between cytosolic and membranous fractions in cellular metabolism. The higher ratio of soluble versus membrane-associated PLD is related to rapidly growing leaves, whereas a lower ratio is involved in increased PLD hydrolysis of membrane lipids.

In addition, the present data provide support for the notion that differential expression of PLD isoforms plays a role in controlling PLD function during plant growth and development (Dyer et al., 1994). Three PLD isoforms are present in castor bean, and their expression is specific to growth and developmental stages (Dyer et al., 1994). Their roles in plant metabolism have been suggested based on the association of specific PLD variants with certain physiological stages. PLD 1 may play a role in membrane lipid turnover during the rapid growth of plants, since it is expressed in the early stages of seedling establishment and in young leaves. PLD 2 is a housekeeping enzyme, since it is found in all of the tissues and stages examined. PLD 3 is involved in membrane deterioration and senescence, since its expression is associated with senescent endosperms. In this study, PLD 3 was present in the control discs after a prolonged period of incubation (data not shown), and ABA treatment accelerated senescence and also the appearance of PLD 3. The occurrence of PLD 3 in the advanced senescence of tissues provides more evidence for its role in phospholipid degradation in senescing tissues. The molecular origin and chemical basis for the structural heterogeneity of PLD are under investigation.

The change in PLD mRNA in the detached leaves is complex and is likely to depend on a combination of plant responses to stress and senescence. Nonetheless, the present study has clearly shown that the expression of the PLD gene responds to the changes of growth stages and environments. When changes were compared within each treatment for the 5-d treatment, PLD mRNA was highest at 1 d of incubation and then declined at 3 and 5 d in all treatments. It is possible that the initial stress, which may be caused by wounding and changing growth conditions, was the overwhelming factor triggering the largest increase in PLD mRNA. Among the control, ABA, and kinetin treatments, the ABA-treated discs showed the highest amount of PLD mRNA at 1, 3, and 5 d of incubation. Thus, the wounding and ABA effects appear to be additive, implying that different signals are involved in the induced PLD expression. Kinetin treatment gave the lowest PLD mRNA, and the overall order of PLD mRNA (ABA > control > kinetin) is in agreement with that of the rate of senescence.

In summary, the present study demonstrates complex mechanisms for the regulation of PLD during plant development. These include changes in the intracellular distribution of PLD, differential expression of PLD isoforms, and changes in PLD protein and mRNA content. Judging from the observations that a relatively high amount of soluble PLD was found in young leaves (Dyer et al., 1994) and kinetin-treated leaf discs, a simple correlation between total cellular PLD protein and rate of PLD hydrolysis can be misleading. Rather, increased membrane association of PLD plays a role in membrane phospholipid breakdown, which leads to membrane deterioration and senescence. Such highly controlled expression of PLD in senescing leaves strongly suggests a role for this enzyme in membrane deterioration and plant senescence.

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