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Phospholipase D Involvement in the Plant Oxidative Burst

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Pathogen-triggered generation of reactive oxidants, termed the oxidative burst, contributes to disease resistance in both plant and animal kingdoms. Since phospholipase D plays a key role in the neutrophil oxidative burst signaling cascade and is highly abundant in plants, we investigated its participation in the plant oxidative burst. Thin layer chromatography of extracted phospholipids revealed no changes in phosphatidic acid levels in soybean cells undergoing oxidant production, and no changes in phosphatidyl-ethanol biosynthesis could be detected when ethanol was present during elicitation. An inhibitor of phosphatidic acid hydrolase, propranolol, did not modify burst parameters or phosphatidic acid levels during the burst, suggesting our inability to detect phosphatidic acid accumulation was not due to rapid elimination. Furthermore, exogenous phosphatidic acid did not elicit a burst or enhance elicitor-stimulated bursts. Finally, ethanol, a substitute nucleophile, did not abrogate the burst. With data showing the presence of phospholipase D in soybean cells, these data argue that soybean phospholipase D does not participate in signaling the oxidative burst. This constitutes the first major difference between the plant and animal oxidative burst signal transduction pathways.

The oxidative burst, the rapid production of hydrogen peroxide and other active oxygen species following elicitation, may mediate protection against pathogens and physical stress by a variety of mechanisms (for reviews, see 1-3). The elicited oxidants may catalyze reinforcement of cell walls (4, 5), serve directly as anti-microbial agents (6-9), mediate certain effects of salicylic acid (10, 11), induce biosynthesis of defense metabolites (12,13), and help initiate the hypersensitive response (14-16), although these latter two sequelae may depend on the co-existence of other factors (17). Because of the importance of the oxidative burst to plant defense, a more complete understanding of the signaling pathway(s) leading to its generation is clearly needed.

One useful model of the signal transduction pathways that connect elicitor binding at the cell surface to assembly of an oxidase complex on the plasma membrane has been provided by the human neutrophil (18). To date, all signaling components of the neutrophil oxidative burst that have been investigated in plants have been found to participate in the plant oxidative burst, including G proteins (19), protein kinases and phosphatases (20-21), phospholipase A (22), phospholipase C (23), and homologues of the neutrophil oxidase complex itself (21,24). In an effort to extend this analogy between neutrophils and plants, we have explored the possible activation of phospholipase D in elicitor-stimulated soybean cells.

Phospholipase D is a key signaling component of the neutrophil oxidative burst (25,26). The current model of phospholipase D activation begins with ligand binding to a G-protein coupled receptor, followed by activation of the associated G protein. This G protein is then thought to either directly stimulate phospholipase D or to activate the enzyme indirectly via a phospholipase C pathway. Although phospholipase D may be required for signaling the burst initiated by many neutrophil ligands, it is significant that neutrophil activation can still proceed in the absence of phospholipase D if formyl-Met-Leu-Phe is employed as the stimulating ligand (27). Thus, phospholipase D-independent signaling pathways clearly also exist in neutrophils.

Although phospholipase D was first discovered in plants (for review see 28) and has recently been cloned from castor bean (29) and rice (30), its physiological role in the plant kingdom is still not fully understood. Changes in extracted phospholipase D activity have been observed during germination of rice seedlings (31), storage of γ-irradiated cauliflower (32), and senescence of flower petals (33,34). However, the physiological roles of these changes have not been clearly demonstrated, and whether phospholipase D predominantly serves a metabolic or signal transduction role in the

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Abbreviation: OGA, oligogalacturonic acid.

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plant kingdom remains unresolved. A possible signaling role for phospholipase D activation in plants has recently been suggested, as mastoparan, a G protein stimulator, can activate phospholipase D activity in *Chlamydamonas* and carnation petals (35). Given the similarities of the neutrophil and plant oxidative burst signaling pathways, we decided to evaluate the activity of phospholipase D during induction of the plant oxidative burst. Because previous studies have demonstrated that harpin, oligogalacturonic acid, and an extract from the pathogenic fungus *Verticillium dahliae* induce the oxidative burst by independent signal transduction pathways (22), we have employed each of these elicitors to explore the possible participation of phospholipase D in their disparate pathways. The results described below argue strongly against the enzyme's involvement in the soybean oxidative burst.

MATERIALS AND METHODS

Plant cell culture. Cell suspension cultures of soybean (*Glycine max* Merr. cv Kent) were maintained in W-38 medium as described earlier (19). Briefly, 6 cm^3 of filtered cells were transferred into 100 mL fresh W-38 media every 10 days. All assays were conducted 16 h after transfer, when H_2O_2 generation was completely dependent upon elicitor addition.

Elicitor preparation. An oligogalacturonide (OGA) fraction that elicits the oxidative burst in soybean cells was prepared by an adaptation of the procedure of Nothanagel et al. (36). A crude digest was prepared from 16 g sodium pectinate (Sigma) by heating for 2h at 85°C in 800 mL 2 N trifluroacetic acid. The solution was filtered, evaporated to dryness and washed four times with 200 mL methanol. The crude digest was then separated by stepwise anion exchange on a 13.1 cm by 3.1 cm QAE-Sephadex A-25 column. The active fraction, which eluted between 0.5 and 0.75M imidazole, was then further purified by FPLC on a 60 cm by 1 cm QAE-Sephadex A-25 column with a gradient from 0.5 M to 1.0 M imidazole (ICN). Active fractions were pooled and desalted on a BioGel P10 column. The concentration of uronic acid equivalents was determined by the method of Blumenkrantz and Asboe-Hansen (37.) For elicitation, 2.5 μg OGA mL^{-1} cell suspension culture was used. A *Verticillium dahliae* 277 elicitor, applied at 6.75 μg protein mL^{-1} suspension, was prepared as described previously (38). Harpin elicitor was generously provided by Dr. Stephen Beer and employed for stimulation of an oxidative burst at 100 μg mL^{-1} cell suspension.

Oxidative burst assays. Hydrogen peroxide production was evaluated by monitoring the oxidative quenching of the fluorescent peroxidase substrate, pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; λ_{ex}=405 nm, λ_{em}=512 nm; Molecular Probes) as previously described (23). Since pyranine oxidation is dependent upon endogenous plant peroxidase activity, all modulators employed in this study were also tested for their direct effect upon peroxidase activity. No alteration was observed with any modulator used (data not shown).

Phospholipase D activity assays. Phospholipase D activity assays were based upon methods from the neutrophil field. To label endogenous soybean lipids, cells were incubated with 5 μCi [9, 10-^{3}H] myristic acid per mL cell culture for 3 h under normal cell maintenance conditions. Identical cells were also treated with an equivalent amount of non-radioactive myristic acid in ethanol and tested for oxidative burst activity to confirm that labeling does not affect activity (data not shown). Maximal labeling was achieved after 2 h and resulted in uniform labeling of cellular lipids. Because activation of phospholipase D can be assayed either by evaluating the production of its hydrolysis product, phosphatidic acid, or by determining the amount of phosphatidylethanol generated via transesterification in the presence of ethanol, 5 μL of ethanol was added, as indicated, to 1 mL cells directly prior to elicitation. At the desired times, enzymatic activity was quenched by homogenization of one mL of cell suspension in two mL of chloroform:methanol (2:1, v/v). Phases were separated by centrifugation at 3000g for 5 minutes. The upper phase was re-extracted with chloroform/methanol and lower phases were pooled and dried under a stream of argon. The dried extracts were dissolved in 80 μL of chloroform: methanol (2:1, v/v), and 35 μL of each sample was analyzed in duplicate by thin layer chromatography on Silica H plates from Analtech. The plates were developed using the organic phase of ethylacetate:isooctane:acetic acid:water (110:50:20:10, v/v; (39)), except in figure 2, where the solvent system of Munnik et al. (35), which is composed of the same components but in the ratios of 13:2:3:10 (v/v). After drying, the lipid bands were visualized by staining with iodine vapor or by En^{3}Hance (DuPont) flourography according to the manufacturer's directions. Bands were identified by comparison with standards (Avanti Polar Lipids) and quantitated by scraping and scintillation counting.

To demonstrate that phospholipase D is indeed present and active in the soybean cell cultures, both Western blots and assays of phospholipase D activity using 25 μM mastoparan were conducted. Western blots were performed as described by Wang et al. (29) using anti-phospholipase D antibodies generously provided by Dr. X. Wang. Phospholipase D activity assays with mastoparan and its inactive analogue, Mas-17, were conducted as described above.

Phosphatidic acid generation and degradation

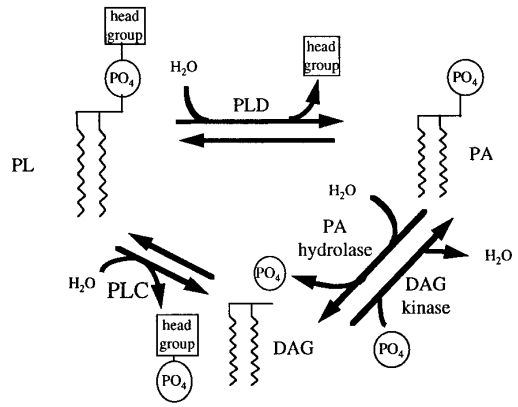

FIG. 1. Diagram of phosphatidic acid generation and degradation. Phosphatidic acid (PA) can be generated either directly by phospholipase D (PLD) or by the consecutive activation of phospholipase C (PLC) and diacylglycerol (DAG) kinase. Phosphatidic acid can be degraded by phosphatidic acid hydrolase to form diacylglycerol.

RESULTS

Phospholipase D Is Not Activated in the Elicitor-Induced Oxidative Burst

The action of phospholipase D on substrate phospholipids leads to formation of phosphatidic acid plus an alcoholic head group such as choline. Unfortunately, phosphatidic acid can also be produced by the combined action of phospholipase C (which is already known to be involved in the oxidative burst; 23) and diacylglycerol kinase (figure 1). Therefore, to ensure that any phospha-

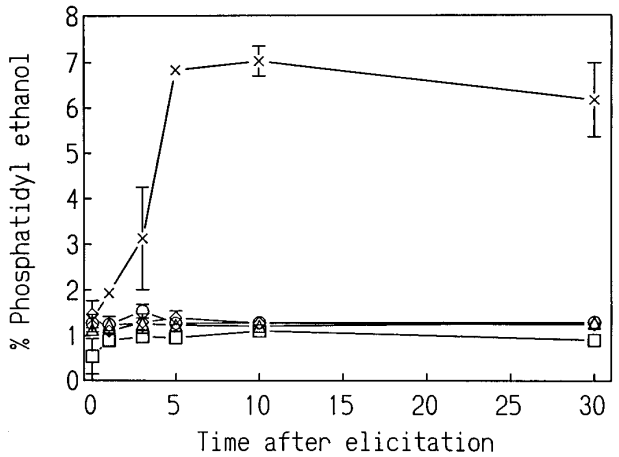

FIG. 2. Kinetics of production of phosphatidylethanol by soybean cells following elicitation in the presence of ethanol. ^{3}H-myristic acid-labeled soybean cells suspended in their growth medium were treated for 1 min with 0.5% ethanol and elicited to generate an oxidative burst by harpin (△), OGA (○), or *Verticillium dahliae* elicitor (□). The behavior of unelicited control cells (◇) and mastoparan stimulated cells (X) are also included for comparison. Production of phosphatidyl ethanol, a reaction product diagnostic of phospholipase D activation, was then followed with time as described under Materials and Methods. Data are expressed as percent of total lipid. Results represent the average of duplicate samples from two (harpin, *Verticillium dahliae*), or three (OGA, mastoparan) separate experiments +/− SEM.

tidic acid generated during the soybean oxidative burst was produced by phospholipase D, 0.5% ethanol was added to the cell suspension prior to elicitation. Even when present in such low quantities, ethanol readily replaces water as a nucleophile in the phospholipase D reaction and promotes formation of phosphatidylethanol. Since the consecutive activation of phospholipase C and diacylglycerol kinase cannot yield phosphatidylethanol, the production of this unnatural phospholipid constitutes a conclusive demonstration of phospholipase D activity. Furthermore, phosphatidylethanol is not readily degraded, and thus can indicate the cumulative magnitude of phospholipase D activity. This assay technique has been routinely used to study phospholipase D involvement in the neutrophil oxidative burst (27, 39) and in plant G protein stimulation (35).

To quantitate the production of phosphatidic acid and phosphatidylethanol, soybean cells were first labeled with [9,10-^{3}H]myristic acid and then stimulated with the desired elicitor in the presence of 0.5% ethanol. At the indicated times, phospholipids were separated and quantified by thin layer chromotography (figure 2). Importantly, no changes in the levels of phosphatidic acid or phosphatidylethanol were observed at any point during the oxidative burst, regardless of whether oxidant production was elicited by OGA (a plant cell wall elicitor), *Verticillium dahliae* (a fungal elicitor) extract, or harpin (a bacterial elicitor). Since the OGA fragment used in these initial studies was highly purified, a crude digest was also tested to verify that OGA oligomers of other sizes were also incapable of inducing phospholipase D activity. Results identical to those seen with the purified OGA fragment were again obtained. Furthermore, no changes in phospholipase D activity were detected for 9 h (data not shown), demonstrating that the absence of measurable activity was not a consequence of sampling the cells over too limited a time span.

To confirm that the lack of stimulatable phospholipase D activity was not due to the absence of the enzyme from soybean suspension cells or the insensitivity of the monitoring technique, several control experiments were conducted. First, soybean cell extracts were immunoblotted with an antibody to castor bean phospholipase D from Dr. Xuemin Wang, and the expected cross-reactive species were observed at 120, 90, and 70 kD (data not shown). Second, mastoparan, a wasp venom peptide previously shown to activate phospholipase D in carnation petals (35) was examined for its impact on the soybean suspension culture. As shown in figure 2, the peptide induced a six-fold elevation in phosphatidylethanol production, suggesting that the enzyme is both present and inducible in the cultured soybean cells. Finally, the above studies were repeated using an alternative solvent system to ensure that the phospholipase D reaction products were resolved. The analogous results, shown in figure 3, demonstrate that the lack of phosphatidylethanol accumulation is not due to technique ininaccuracy or insensitivity. Interestingly, an inactive mastoparan analogue, Mas-17,

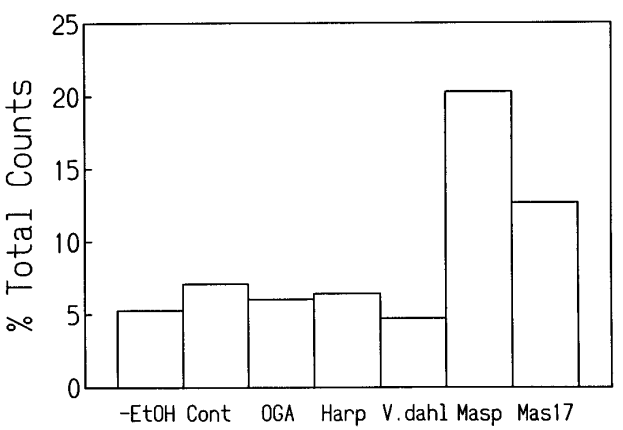

FIG. 3. A second chromatographic technique confirms the lack of phosphatidyl ethanol accumulation during the oxidative burst. ^{3}H-myristic acid-labeled soybean cells suspended in their growth medium were treated for 1 min with 0.5% ethanol prior to the addition of OGA, harpin (Harp), *Verticillium dahliae* extract (V.dahl), mastoparan (Masp), or Mas-17. Production of phosphatidyl ethanol, a reaction product diagnostic of phospholipase D activation, was monitored 10 minutes after elicitor addition by the method of Munnik et al. (1995) as described under Materials and Methods. Control (Cont) and cells not pre-treated with ethanol (-EtOH) are included for comparison.

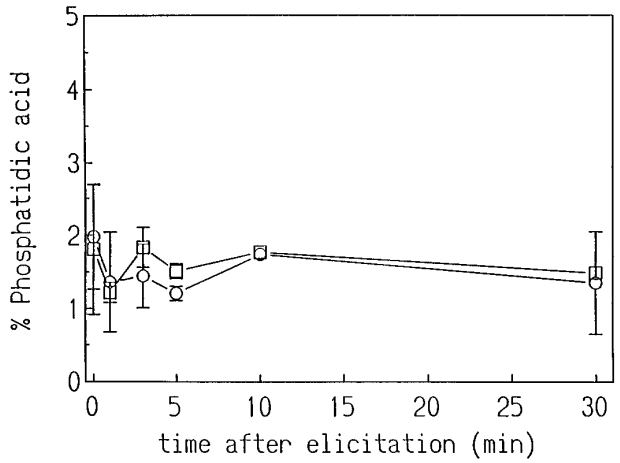

FIG. 4. Kinetics of production of phosphatidic acid by soybean cells following elicitation in the presence of propranolol. ^{3}H-myristic acid-labeled soybean suspension cells were treated for 1 min with 300 μM propranolol to inhibit any dephosphorylation of the initial phospholipase D reaction product, phosphatidic acid, as described under Materials and Methods. The cells were then elicited with OGA (○) to initiate the oxidative burst, and the appearance of phosphatidic acid was monitored as a function of time. The behavior of unelicited cells is included for comparison (□). Results represent the average of two independent experiments +/− SD.

was also able to stimulate phosphatidylethanol production three fold.

Propranolol, an Inhibitor of Phosphatidic Acid Hydrolase, Does Not Modify the Burst or the Accumulation of Phosphatidic Acid

One possible explanation for the lack of phosphatidic acid accumulation during the oxidative burst might be its rapid degradation to diacylglycerol by phosphatidic acid hydrolase (figure 1). To explore this possibility, propranolol, an inhibitor of phosphatidic acid hydrolase, was added prior to elicitation by OGA or harpin. As can be seen in figure 4, propranolol promoted no accumulation of phosphatidic acid, suggesting rapid removal of the reaction product is not responsible for its absence. Furthermore, if phosphatidic acid were important for signaling the burst, addition of propranolol prior to elicitation would have been expected to have changed the rate, quantity, or duration of H_2O_2 production. However, no alteration of any of these parameters was seen over a range of 0 to 300 μM (Table I). Since 200 μM propranolol maximally inhibits phosphatidic acid hydrolase in neutrophils (39), the total lack of inhibition at 300 μM provides further evidence that phospholipase D is not involved in the oxidative burst.

Ethanol, a Substitutive Nucleophile in the Phospholipase D Reaction, Does not Abrogate the Burst

Another method for determining the significance of phospholipase D activation in the oxidative burst involves looking for changes in the kinetics of H_2O_2 formation when primary alcohols are present during elicitation (40, 27). The primary alcohol, ethanol in this case, competes with water as a nucleophile and prevents the formation of phosphatidic acid. As shown in Table II, addition of ethanol had no impact on the rate of H_2O_2 biosynthesis, even though similar concentrations measurably reduce the quantity of phosphatidic acid generated in cells with activatable phospholipase D (27). These data provide further evidence that phosphatidic acid is not a second messenger in the oxidative burst signaling pathway.

TABLE I

Propranolol Does Not Affect the Rate, Magnitude, or Duration of the Oxidative Burst Elicited by OGA

Propranolol (μM)	Maximum rate[a] ± SD (F.U. min^{-1})	Magnitude[b] (F.U.)	Duration[c] (min)
0	22.0 ± 1.9	90.0	9.03
100	23.9 ± 0.1	90.0	9.06
200	22.5 ± 1.7	87.4	9.06
300	21.5 ± 0.9	89.8	8.95

Note. Assays were conducted as described under Materials and Methods. The data are averages of three independent experiments performed on separate days.

[a] Rate of H_2O_2 mediated quenching of pyranine by cell wall peroxidases as measured by fluorescence units per min (F.U. min^{-1}).

[b] Total quantity of dye quenched over the duration of the burst.

[c] Total duration of the oxidative burst.

Exogenous Phosphatidic Acid Does Not Elicit a Burst on Its Own or Enhance the Burst Induced by OGA

If phospholipase D were activated in the oxidative burst, it would be expected that its lipid hydrolysis product, phosphatidic acid, would either independently inaugurate a burst or increase the activity of an other-

TABLE II

Addition of Ethanol Does Not Alter Oxidative Burst Activity

Percent ethanol (v/v)	Average slope* ±SD	Percent of control
0	33.8 ± 0.6	100
0.5	35.3 ± 1.5	104
1.0	33.8 ± 1.3	100.1

Note. Assays were performed as described under Materials and Methods, with OGA as the elicitor. Similar results were obtained when harpin or *Verticillium dahliae* extract were used as the elicitor (data not shown). The data are averages of three independent experiments.

[a] Average slope refers to the rate of quenching of pyranine fluorescence upon stimulation of the oxidative burst and corresponds linearly to the initial rate of H_2O_2 biosynthesis.

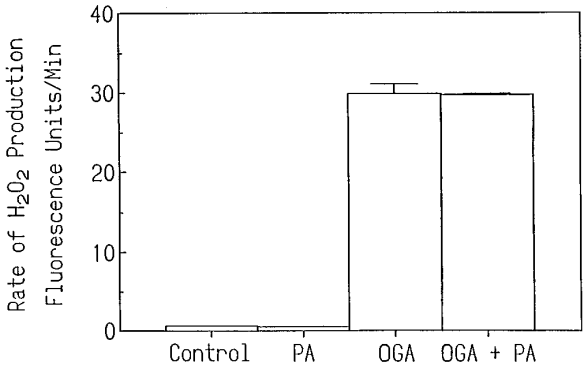

FIG. 5. Addition of exogenous phosphatidic acid does not activate the oxidative burst or increase the production of H_2O_2 upon elicitation with OGA. A stirred suspension of 1.5 mL of soybean cell culture were monitored for the production of hydrogen peroxide via the decrease in pyranine fluorescence upon treatment with 2.5 μg OGA (OGA), 15 μg phosphatidic acid alone (PA), 2.5 μg OGA + 15 μg phosphatidic acid (OGA + PA), or no additive (control). The results shown are the mean of three trials ± SD.

wise elicited burst. Importantly, no such activation or synergism was observed (Figure 5). This observation suggests that phosphatidic acid is neither sufficient for activation of H_2O_2 biosynthesis nor capable of augmenting other second messenger pathways in production of the burst.

DISCUSSION

Although phospholipase D is present and catalytically active in soybean cell suspension cultures, our data demonstrate that it is not involved in transducing the oxidative burst signal by any of three independent burst pathway elicitors. Evidence for this contention derives from i) the lack of change in phosphatidic acid levels during elicitation, ii) the absence of accumulation of phosphatidylethanol following induction of the burst in the presence of ethanol, iii) the lack of inhibition of the burst by primary alcohols (which reduce formation of phosphatidic acid), and iv) the absence of any burst enhancement by addition of either propranolol or exogenous phosphatidic acid. Since *Verticillium dahliae* and harpin also induce other defense responses such as phytoalexin biosynthesis and the hypersensitive response, the pathways leading to these defense responses must also operate in phospholipase D independent manners.

The apparent lack of phospholipase D participation in signaling the plant oxidative burst constitutes the first major distinction between the burst pathways of the plant and animal kingdoms. As noted in the Introduction, not only are the oxidase components of the two kingdoms similar, but the signaling components and second messengers leading to their assembly also appear closely related. The well documented abundance of phospholipase D in plants, taken together with our ability to stimulate at least a fraction of this activity with mastoparan, however, argue that the lack of involvement of phospholipase D does not stem from an inability to exploit the enzyme for signaling purposes. Instead, the most reasonable explanation of the soybean's lack of use of phospholipase D is that the soybean cells found no need for the enzyme in triggering oxidase assembly. While many explanations of this distinction between plants and animals are possible, it is conceivable that phospholipase D activation in neutrophils is required for processes not necessary in plants. Upon stimulation, neutrophils not only activate the oxidative burst, but they also initiate chemotaxis, adherence, aggregation, and phagocytosis. It also appears that phospholipase D plays a critical role in promoting secretion of cytotoxic agents from neutrophils (25, 41). In fact, it has even been hypothesized that phospholipase D activation is not required for the neutrophil oxidative burst, but rather is essential for mobilization of cytochrome b_{558}, a component of the oxidase complex, from subcellular stores (27). The localization of phospholipase D to the surfaces adjacent to *Xanthomonas oryzae* penetration in rice leaves (42) during resistant interactions suggests localization of the enzyme may play a role in other aspects of the plant defense response.

CONCLUSION

The functions of plant phospholipase D obviously remain uncertain. Since up-regulation of phospholipase D is seen *in vitro* during germination and senescence, it may be that phospholipase D is primarily involved in membrane remodeling and degradation. However, it would be very surprising if such an important animal second messenger were completely without signaling function in plants. Indeed, activation of phospholipase D by mastoparan, a wasp venom peptide that stimulates heterotrimeric G proteins, suggests its involvement in some type of signal transduction. Our data simply exclude a role for the enzyme in mediating three unrelated oxidative burst pathways. Further scrutiny will obviously be required before the function of this ubiquitous plant enzyme is fully understood.

ACKNOWLEDGMENTS

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