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Fast Upward Propagation of the Wound Signal That Systemically Elevates Phosphatidic Acid

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Phosphatidic acid (PA) increases in response to wounding at the neighboring unwounded leaf as well as at the wounded leaf of many plants (Lee et al., 1997). This indicates that a signal propagates from the wounded leaf to its neighboring leaves. In this paper, we report the speed and direction of propagation for a systemic wound signal that elevates PA. When a leaf of a soybean (*Glycine max*) seedling at the 2-leaf-stage was wounded, the PA level of the neighboring leaf did not change within the first min, but did increase significantly in 2 min, returning to the control level after 15 min. This implies that the systemic wound signal was generated at least within 2 min of wounding, and was propagated at a speed of at least 10 - 16 mm/min. When we wounded individual leaves of soybean and tobacco (*Nicotiana tabaccum*) seedlings that had 3 or 4 leaves, PA levels were elevated only in the younger leaves located above the wounded leaf, but not in the older, lower leaves. Thus, the PA-elevating wound signal preferentially moves upward in these plants.

Keywords: *Glycine max*, *Nicotiana tabaccum*, phosphatidic acid, systemic wound signal propagation

When plants are wounded, they develop a series of defense responses including transcriptional activation of genes that encode proteins with defensive functions (Lawton and Lamb, 1987; Brederode et al., 1991; Hemerly et al., 1993; Memelink et al., 1993). Interestingly, these defense responses occur not only at the wound site, but also systemically, i.e., at distant locations (Bowles, 1993). The induction of a systemic defense response implies that a signal is generated at the wound site, and propagates to other parts of the plant (Bowles, 1997, 1998).

A few signals are responsible for the systemic induction of proteinase inhibitor (PI) gene expression, the best characterized systemic wound response (Pearce et al., 1991; Ryan, 1992; Wildon et al., 1992; Malone and Alarcon, 1995). Wound signals differ chemically and in their speed of propagation. For example, McGurl et al. (1992) found that systemin, a peptide hormone comprising 18 amino acids, was generated enzymatically from its inactive precursor, prosystemin, upon wounding. Their investigations using ^{14}C-systemin revealed that it took 30 min for systemin to spread throughout the wounded leaf and 60 - 90 min to arrive at the petiole of the wounded leaf. Systemin has been found in the phloem, suggesting its movement is through that tissue (Narvaez-Vasquez et al., 1995). In tomato seedlings, an electrical signal that was recorded when the cotyledons were wounded spreads to the petiole of the lowest leaf (the first true leaf) within 5 min of wounding (Wildon et al., 1992). Hydraulic dispersal of chemicals, resulting from a hydraulic pressure change, occurs instantaneously after wounding as a mass flow. This pressure change can distribute chemicals applied to the cut-end to other leaves as rapidly as in 30 s (Malone et al., 1994; Malone and Alarcon, 1995).

Studies of wound signals have been mainly limited to identifying those factors that induce systemic PI gene expression. However, many other systemic wound responses exist, and some of them, such as activation of protein kinases (Bögre et al., 1997; Zhang and Klessig, 1998; Seo et al., 1999) and elevation of phosphatidic acid (PA) (Ryu and Wang, 1996; Lee et al., 1997), occur much faster than does PI gene expression (Graham et al., 1986; Lee et al., 1997). To understand the early steps of wound signaling, it is necessary to characterize the signals that elicit these fast systemic wound responses.

PA is elevated rapidly and systemically after wounding (Ryu and Wang, 1996; Lee et al., 1997), and has been shown in many organisms to play important roles in signaling. It is a signal mediator for ABA in the barley aleurone layer and in *Vicia faba* guard cells (Ritchie and Gilroy, 1998; Jacob et al., 1999). Moreover, PA is involved in host defense of phagocyte (Exton,

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1994) and proliferation (Fukami and Takenawa, 1992), an essential step in the wound- healing process of animal cells.

Because PA does not move to the neighboring leaf when applied to the wounded leaf (Lee et al., 1997), a wound signal evidently is propagated to the unwounded neighboring leaf, where the PA level then becomes elevated. In the current study, we addressed how fast and in what direction the PA-inducing factor was propagated in soybean and tobacco plants, as a step toward identifying the nature of the signal that elevates PA levels systemically in wounded plants.

MATERIALS AND METHODS

Plant Materials

Soybean (*Glycine max* L., cv Kent) and tobacco (*Nicotiana tabaccum* L., cv Xanthi) plants were grown in a greenhouse or a growth chamber at 23°C, with light/dark cycles of 16/8 h. Wounding was applied with a pair of pliers by pinching the distal area of a leaf across the vein. Wounded plants were incubated under constant light (200 - 300 μmol m^{-2} s^{-1}) for various lengths of time. The wounded leaves and their neighboring leaves were then cut from the plants and frozen immediately in liquid nitrogen.

Lipid Extraction

Lipids were extracted according to the Folch method (Folch et al., 1957) with some modifications. Briefly, the frozen leaf samples were ground in a mortar with 6 mL of Folch solvent (chloroform:methanol = 2:1, v/v). Afterward, 1.5 mL of 0.58% NaCl was added and the mixture was vortexed. The organic layer was taken, and washed once with 2 mL of pure upper-phase solvent containing chloroform:methanol:0.6% NaCl (3:48:47, v/v/v). The organic layer was taken, dried under nitrogen gas, and redissolved in 1:1 (v/v) mixture of chloroform and methanol.

Separation on TLC and Quantification of Lipids

The extracted lipids were separated with a two developing-solvent system on a TLC plate (Merck F254 60, Germany). First, the TLC plate was developed with the upper phase of ethylacetate:isooctane :acetic acid:water (110:50:20:100, v/v/v/v) to one-third of the plate. It was then dried, and developed again with the same solvent up to half way. After drying again, the plate was developed with hexane:diethyl ether:methanol:acetic acid (90:20:3:2, v/v/v/v) until the solvent passed slightly over the top of the TLC plate. The separated PA was identified by comparing it with the lipid standard, using iodine staining. The silica on TLC plate for PA was then scraped, and sonicated with 0.3 mL of 0.7 N HCl. PA was desorbed from the silica with 1.9 mL chloroform and 1.9 mL methanol. It was washed again with 1.9 mL of chloroform and 1.13 mL of 0.7 N HCl, evaporated at 70°C, and quantified by phosphate assay (Bartlett, 1959). The data were normalized with fresh weight of the sample leaves and presented as a percentage of the PA detected in control leaves of non-wounded plants.

RESULTS AND DISCUSSION

We tested how fast the systemic signal moved to elevate PA levels in the unwounded leaf in soybean seedlings at 2-leaf-stage. In many plants including soybean, systemic PA increases within 5 min after wounding (Lee et al., 1997). In the current experiment, we measured the PA content earlier than 5 min. In wounded leaves, the PA level did not increase much in the first min (111%, $P < 0.2$), but it did increase to 197% ($P < 0.05$) in 2 min, to 789% ($P < 0.005$) in 5 min, and 243% ($P < 0.1$) in 15 min, compared with levels measured in the control leaves of non-wounded plants. In the neighboring leaf (systemic site) on a wounded plant, PA content also did not change in the first min, but did increase to 162.4% ($P < 0.05$) after 2 min, and to 250% after 5 min ($P < 0.05$), compared with the control. By 15 min after wounding, the PA content had returned to the control level (Fig. 1). Thus the PA level increased significantly as early as 2 min after wounding, both in the wounded and in the neighboring leaves. This elevation was transient, as is expected for a signal mediator, returning to the basal level in 15 min. Moreover, the time course for elevation of the systemic PA content was similar to what we reported for wounded tomato seedlings at a similar developmental stage (Lee et al., 1997). Therefore, these results clearly indicate that the systemic wound signal that elevates the PA level is generated within 2 min after wounding in these plants.

Such rapid propagation of the wound signal for PA elevation in soybean seedling of 2-leaf-stage eliminates the possibility that systemin is the signal. The speed of propagation cannot be calculated accurately because of unknown factors. However, assuming that

Figure 1. Time course of phosphatidic acid increase in response to wounding in soybean seedlings. One leaf of the 2-leaf-stage soybean seedlings was wounded and incubated for various time periods, then both the wounded and the unwounded neighboring leaves were harvested. Lipids were extracted and PA band was scraped from the TLC plate and quantified by phosphate assay. Values represent the mean ± SE (n = 3 - 6) of PA content as % of PA in control leaves of non-wounded soybean seedlings.

1) no time elapses for the propagated signal to elevate PA level in the neighboring leaf and 2) the systemic signal is generated from the wound site immediately after wounding, then the speed of signal propagation is 10 - 16 mm/min. These estimates are based on the distance between the wound site and the neighboring leaf (from the wound site, the bottom of the blade of the neighbor leaf was about 20 mm, and the middle of the neighbor leaf was about 32 mm). However, it is also possible that the PA levelelevating wound signal arrived at the systemic site at the very instant of wounding, thus causing a simultaneous change in PA level both at the wounded and the neighboring leaves. In the latter scenario, the speed of propagation is too fast to measure. The possible involvement of either a hydraulic signal or an electrical signal in wound-induced systemic elevation of PA merits further study.

Next, to determine the direction of propagation for the systemic wound signal involved in PA elevation, we measured the PA levels in leaves above and below the wounded leaf. Soybean seedlings with 4 fully developed leaves and tobacco seedlings with 3 fully developed leaves were used. The soybean plants were wounded at the second leaf from the base, and 20 min later, the PA level was measured. PA contents were 6.6, 19.6, 8.8, and 9.9 nmole/g fresh weight, for the first, second, third, and fourth leaves, respectively. In wounded leaves, the PA level increased to 346% ($P < 0.05$) of that in the control at the same developmental stage. However, leaves below the wounded leaf did not show any change in PA level, compared with their own controls. Only the leaves above the wounded leaf tended to show an increase in the PA level, 197% ($P < 0.2$) of the control for the leaf directly above the wounded leaf and 429% ($P < 0.05$) for the youngest leaf at the top of the stem (Fig. 2A). Tobacco leaves showed similar responses. When the middle leaf of 3-leaf-stage tobacco was wounded, the PA level was elevated after 2 min in the leaf located above the wounded leaf, to 301% ($P < 0.05$) of the control. However, PA content did not change in the leaf below the wounded one (Fig. 2B). These results indicate that the systemic wound signal that elevated

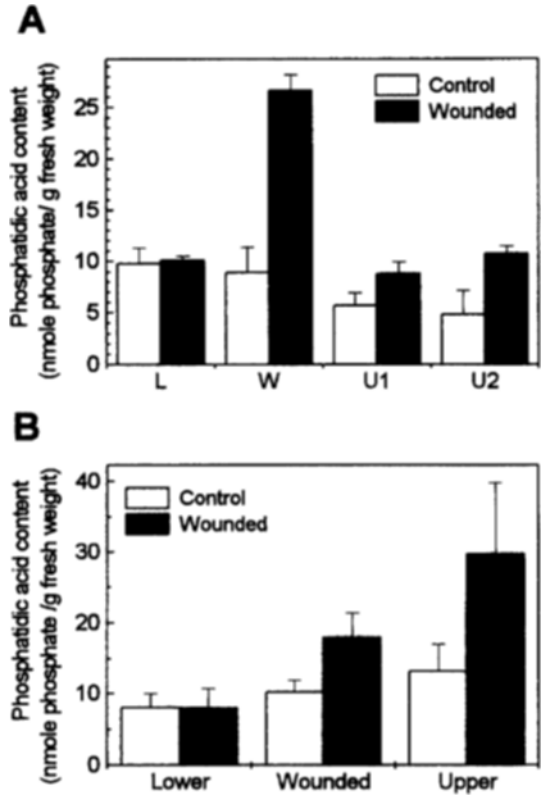

Figure 2. Upward propagation of wound signal in soybean and tobacco seedlings. The second leaf from the base of 4-leaf-stage soybean seedlings (A) and the middle leaf of 3-leaf-stage tobacco seedlings (B) were wounded. After 20 min (A) and 2 min (B) respectively, the wounded and neighboring leaves were harvested. Other experimental procedures were the same as described in Figure 1. Control values are from non-wounded plants of the same developmental stages. W, wounded leaf; L, the leaf located below the wounded leaf on the stem; U, the leaf located above the wounded leaf. U1 is the leaf directly above the wounded leaf and U2 is the youngest leaf at the top of the stem. Values represent the mean ± SE (n = 3 - 6).

PA levels was preferentially propagated upward both in tobacco and soybean seedlings at 3- and 4-leaf-stages, respectively. Previous studies on the direction of propagation of wound signals showed differences depending on the response. For example, PI I mRNA expression was induced only in leaves above the wounded one in tomato seedlings (Nelson et al., 1983). However expression of PI II mRNA was induced in both the upper and the lower leaves of wounded potato plants (Peña-Cortes et al., 1988).

How the systemic wound signal elevating PA level passes only to the leaves located above the wounded leaf is yet to be solved. Although phloem may be used for upward chemical movement, the speed of phloem flow (Narvaez-Vasquez et al., 1995) is too slow to elevate the PA within 2 min in unwounded leaves. Neither can rapid hydraulic chemical dispersal explain the exclusively acropetal PA-level elevation because this dispersal occurs basipetally as well (Malone et al., 1994).

In this study, we showed that the wound signal involved in systemic PA elevation moved acropetally at a speed of at least 10 - 16 mm/min. We are currently testing if PA has a role in wound signaling and/or wound healing, as is found in animal cells. Further studies on the relationship between PA-induced and wound-induced responses, and the identity of the systemic wound signal that elevates PA, will contribute to the understanding of wound signal transduction and defense response in plants.

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LITERATURE CITED

Bartlett GR (1959) Phosphorous assay in column chromatography. J Biol Chem 234: 466-468

Bögre L, Ligterink W, Meskiene I, Barker PJ, Herbert-Bors E, Huskisson NS, Hirt H (1997) Wounding induces the rapid and transient activation of a specific MAP kinase pathway. Plant Cell 9: 75-83

Bowles DJ (1993) Local and systemic signals in the wound response. Semin Cell Biol 4: 103-111

Bowles DJ (1997) The wound response of tomato plants: analysis of local and long-range signalling events. Essays Biochem 32: 161-169

Bowles DJ (1998) Signal transduction in the wound response of tomato plants. Philos Trans R Soc Lond B Biol Sci 353: 1495-1510

Brederode FT, Linthorst HJ, Bol JF (1991) Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol Biol 17: 1117-1125

Exton JH (1994) Phosphatidylcholine breakdown and signal transduction. Biochim Biophys Acta 1212: 26-42

Folch JM, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497-509

Fukami K, Takenawa T (1992) Phosphatidic acid that accumulates in platelet-derived growth factor-stimulated Balb/c 3T3 cells is a potential mitogen signal. J Biol Chem 267: 10988-10993

Graham JS, Hall G, Pearce G, Ryan CA (1986) Regulation of synthesis of proteinase inhibitors I and II mRNAs in the leaves of wounded tomato plants. Planta 169: 399-405

Hemerly AS, Ferreira P, de Almeida Engler J, van Montagu M, Engler G, Inzé D (1993) cdc2a expression in Arabidopsis is linked with competence for cell division. Plant Cell 5: 1711-1723

Jacob T, Ritchie S, Assmann SM, Gilroy S (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. Proc Natl Acad Sci USA 96: 12192-12197

Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol Cell Biol 7: 335-341

Lee S, Suh S, Kim S, Crain RC, Kwak JM, Nam H-G, Lee Y (1997) Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. Plant J 12: 547-556

Malone M, Alarcon JJ (1995) Only xylem-born factors can account for systemic wound signalling in the tomato plant. Planta 196: 740-746

Malone M, Alarcon J-J, Palumbo L (1994) A hydraulic interpretation of rapid long-distance wound signalling in the tomato. Planta 193: 181-185

McGurl B, Pearce G, Orozco-Cardenas M, Ryan CA (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. Science 255: 1570-1573

Memelink J, Swords KM, de Kam RJ, Schilperoort RA, Hoge JH, Staehelin LA (1993) Structure and regulation of tobacco extensin. Plant J 4: 1011-1022

Narvaez-Vasquez J, Pearce JG, Orozco-Cardenas ML, Franceschi VR, Ryan CA (1995) Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. Planta 195: 593-600

Nelson CE, Walker-Simmons M, Makus D, Zuroske G, Graham J, Ryan CA (1983) Regulation of synthesis and accumulation of proteinase inhibitors in leaves of wounded tomato plants, *In* PA Hedin, ed, Plant Resistance to Insects, Washington DC, American Chemical Society, pp 103-122

Pearce G, Strydom D, Johonson S, Ryan CA (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 153: 895-898

Peña-Cortes H, Sanchez-Serrano J, Rocha-Sosa M, Willmitzer L (1988) Systemic induction of proteinase-inhibitor-II gene expression in potato plants by wounding. Planta 174: 84-89

Ritchie S, Gilroy S (1998) Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. Proc Natl Acad Sci USA 95: 1697-2702

Ryan CA (1992) The search for the proteinase inhibitor-inducing factor, PIIF. Plant Mol Biol 19: 123-133

Ryu SB, Wang X (1996) Activation of phospholipase D and the possible mechanism of activation in wound-induced lipid hydrolysis in castor bean leaves. Biochim Biophys Acta 1303: 243-250

Seo S, Sano H, Ohashi Y (1999) Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. Plant Cell 11: 289-298

Wildon DC, Thain JF, Minchin PEH, Grubb IR, Reilly AJ, Skipper YD, Doherthy HM, ODonnell PJ, Bowles DJ (1992) Electrical signaling and systemic proteinase inhibitor induction in the wounded plants. Nature 360: 62-65

Zhang S, Klessig D (1998) The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. Proc Natl Acad Sci USA 95: 7225-7230