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Involvement of components of the phospholipid-signaling pathway in wound-induced phenylpropanoid metabolism in lettuce (*Lactuca sativa*) leaf tissue

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In plant tissue, a wound signal is produced at the site of injury and propagates or migrates into adjacent tissue where it induces increased phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity and phenylpropanoid metabolism. We used excised mid-rib leaf tissue from Romaine lettuce (*Lactuca sativa* L., Longifolia) as a model system to examine the involvement of components of the phospholipid-signaling pathway in wound-induced phenolic metabolism. Exposure to 1-butanol vapors or solutions inhibited wound-induced increase in PAL activity and phenolic metabolism. Phospholipases D (EC 3.1.4.4), an enzyme involved in the phospholipid-signaling pathway is specifically inhibited by 1-butanol. Re-wounding tissue, in which an effective 1-butanol concentration had declined below active levels by evaporation, did not elicit the normal wound response. It appears the 1-butanol-treated tissue developed resistance to wound-induced increases in phenylpropanoid metabolism that persisted even when active levels of 1-butanol were no longer present. However, a metabolic product of 1-butanol, rather than 1-butanol itself, may be the active compound eliciting persistence resistance. Inhibiting a subsequent enzyme in the phospholipid-signaling pathway, lipoxygenase (LOX; EC 1.13.11.12) with 1-phenyl-3-pyrazolidinone (1P3P) or reducing the product of LOX activity with diethyldithio-carbamic acid (DIECA) also inhibited wound-induced PAL activity and phenolic accumulation. The effectiveness of 1-butanol, DIECA, and 1P3P declined as the beginning of the 1-h immersion period was delayed from 0 to 4 h after excision. This decline in effectiveness is consistent with involvement of the inhibitors in the production or propagation of a wound signal. The wound signal in lettuce moves into adjacent tissue at 0.5 cm h^{-1}, so delaying application would allow the signal to move into and induce the wound response in adjacent tissue before the delayed application inhibited synthesis of the signal. Salicylic acid (SA) inhibits allene oxide synthase (AOS, EC 4.2.1.92), another enzyme in the phospholipid-signaling pathway. Exposure to 1 or 10 m*M* SA for 60 min reduced wound-induced phenolic accumulation by 26 or 56%, respectively. However, 1 m*M* SA lost its effectiveness if applied 3 h after excision, while 10 m*M* SA remained effective even when applied 4 h after excision. At 1 m*M*, SA may be perturbing the wound signal

Abbreviations – 1P3P, 1-phenyl-3-pyrazolidinone; 12-OPDA, 12-oxy-phytodienic acid; 13-HPOT, 13 (S)-hydroperoxylinolenic acid; AOS, allene oxide synthase; DAG, diacylglycerol; DEICA, diethyldithio-carbamic acid; LA, linolenic acid; LOX, lipoxygenase; PA, phosphatidic acid; PLD, phospholipase D; SA, salicylic acid; WIPA, wound-induced phenolic accumulation.

through inhibition of AOS, while at 10 m*M* it appears to have some generally inhibitory effect on subsequent phenolic metabolism. These data further implicate the phospholipid-signaling pathway in the generation of a wound signal that induces phenolic metabolism in wounded leaf tissue.

Introduction

Abiotic stresses (e.g. physical and mechanical wounds) encountered during growth, harvest, and subsequent processing elicit physiological responses that include elevated rates of respiration, ethylene production, and phenolic metabolism (Brecht et al. 2004). Wounds accompanying excision of lettuce leaf tissue stimulate phenylpropanoid metabolism with the production and accumulation of soluble phenolic compounds (Tomás-Barberán et al. 1997). The de novo synthesis and increased activity of phenylalanine ammonia lyase (PAL, EC 4.3.1.5) (Campos-Vargas et al. 2004), the first committed enzyme in the phenylpropanoid pathway (Dixon and Paiva 1995), is an initial response to wounding in lettuce as it is in many other plant tissues.

A signal that transduces the physical wound into a physiological response is produced at the site of injury and migrates or propagates into adjacent, non-injured tissue where it induces increased phenolic production (Choi et al. 2005, Ke and Saltveit 1989). In lettuce leaf tissue, the signal arises within 30 min of wounding and moves into adjacent non-injured tissue at 0.5 cm h^{-1} (Ke and Saltveit 1989). A kinetic analysis of wound-induced increases in ethylene production and PAL activity showed that increased PAL activity did not proceed through the induced synthesis and action of ethylene. While high concentrations of a number of putative wound signal chemicals [i.e. abscisic acid, jasmonic acid (JA), methyl jasmonate, and salicylic acid (SA)] slightly increased PAL activity and phenolic accumulation in non-wounded leaves, none of them produced the many-fold increase seen in excised lettuce leaf tissue (Campos-Vargas and Saltveit 2002).

SA increases in stressed plants and can have dual functions in the plant's response to stress. In *Arabidopsis thaliana*, it induces the development of systemic acquired resistance and other pathogen defense genes, while it inhibits wound-inducible genes (Wendehenne et al. 2004). In the C4 monocot sorghum (*Sorghum bicolor* L.), SA induced genes of the octadecanoic acid pathway for JA synthesis which resulted in higher JA content (Salzman et al. 2005). When compared, SA and JA exhibited a range of activities in the induction of other stress-related genes from synergistic to mutually antagonistic.

Proposed phospholipid-signaling pathways are complex, interrelated, and are composed of numerous enzymes and substrates (Wang 2004). Products of the phospholipid-signaling pathway [e.g. phosphatidic acid (PA) and JA] are part of the wound signal complex in a number of plants (Creelman and Mullet 1997, Peña-Cortés and Willmitzer 1995, Turner et al. 2002). Mechanical injuries promote increased levels of JA in soybean (Creelman et al. 1992), tomato plants (Peña-Cortés et al. 1993), and potato plants (Peña-Cortés et al. 1995). The release of PA from membrane phospholipids by phospholipase D (PLD, EC 3.1.4.4) is thought to be one of the first reactions in this lipid-signaling pathway (Creelman and Mullet 1997, Leon et al. 2001, Wang 2004). Subsequent steps produce a myriad of phytoactive compounds that participate in tissue responses to biotic and abiotic stresses (Fig. 1) (Farmer and Ryan 1992, Farmer et al. 1998, Munnik 2001, Schaller 2001).

We previously showed that exposing excised mid-rib leaf tissue to vapors of n-alcohols from ethanol to heptanol [e.g. 10 μmol/g fresh weight (FW) of 1-butanol] inhibited wound-induced increases in PAL activity and phenolic accumulation (Choi et al. 2005). 1-Butanol is a selective inhibitor of PLD activity, whereas 2- and 3-butanol have no such inhibitory effect (Gardiner et al. 2003, Lee et al. 2001, Munnik et al. 1995). 1-Butanol interacts with PLD producing phosphatidic butanol instead of PA. PLD-dependent signaling in plants has been studied using 1-butanol as a specific PLD inhibitor; e.g. pollen germination and tube growth in tobacco was stopped by 1-butanol, whereas 2- and 3-butanol were ineffective (Potocky et al. 2003). While 1-butanol was effective in reducing wound-induced increases in phenylpropanoid metabolism in our lettuce leaf system, the 2- and 3-isomers were ineffective (Choi et al. 2005). Similar results with the butanol isomers were used to indicate that phospholipid signaling, mediated through PLD action, was involved in pollen tube growth, microtubule organization, and gene activation (Gardiner et al. 2003, Lee et al. 2001, Potocky et al. 2003).

Lipoxygenase (LOX) converts linolenic acid into the hydroperoxide 13-HPOT [13 (S)-hydroperoxylinolenic acid] (Porta and Rocha-Sosa 2002). LOX activity increases because of wounding or physical stress (Bell et al. 1995, Leon et al. 2001, Porta and Rocha-Sosa

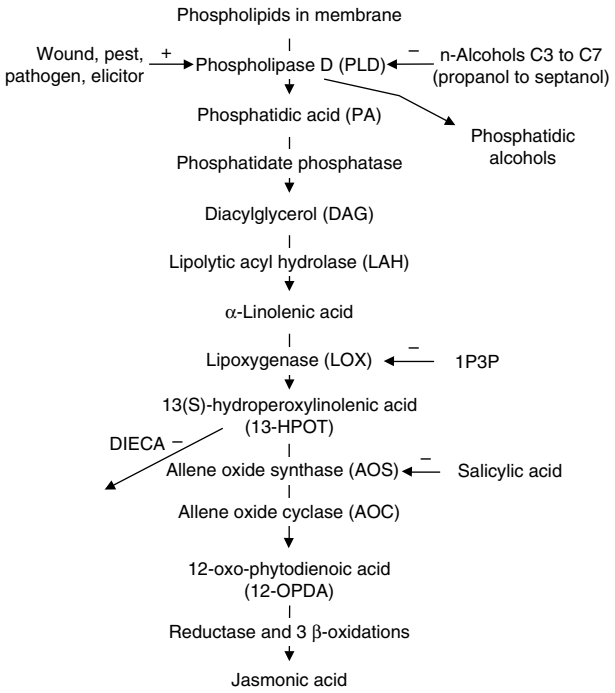

Fig. 1. Hypothetical model for the phospholipid-signaling pathway with enzymes, products, and inhibitors (adapted from Devoto and Turner 2003, Feussner and Wasternack 2002, Turner et al. 2002, Wasternack and Hause 2002).

2002). Inhibitors of LOX include 1-phenyl-3-pyrazolidinone (phenidone, 1P3P) (Farmer et al. 1994). 1P3P is oxidized during LOX activity, and one or more of its metabolites covalently interact with and inactivates LOX (Cucurou et al. 1991, Farmer et al. 1994). In contrast, diethyldithio-carbamic acid (DIECA) effects the pathway by oxidizing the products of LOX activity, thereby reducing the pool of 13-HPOT available for subsequent reactions.

SA increases in plants in response to a number of stresses, and its application can elicit responses similar to those induced by certain stresses (Klessig and Malamy 1994, Senaratna et al. 2000). SA inhibits certain stress responses by blocking JA biosynthesis (Doares et al. 1995, Harms et al. 1998, Peña-Cortés et al. 1993). SA inhibits the conversion of 13-HPOT to 12-oxy-phytodienic acid (12-OPDA) by interfering with the activity of allene oxide synthase (AOS; EC 4.2.1.92) (Creelman and Mullet 1997, Harms et al. 1998). Both 12-OPDA and JA overcame the inhibitory effect of SA (Peña-Cortés et al. 1993).

The diverse action of SA may contribute to its activity over a wide range of concentrations. Besides inhibiting AOS, SA can modulate catalase activity (Chen et al. 1993, Durner and Klessig 1996) and act as a non-specific inhibitor of cellular kinases (Frantz and O'Neill 1995). Physiological responses were elicited by <0.1 m*M* SA in cell suspension cultures (Chen et al. 2002, Quiroz-Figueroa et al. 2001, Sappl et al. 2004), by 0.5–3 m*M* applied to plant roots (Janda et al. 1999, Kang and Saltveit 2002, Singh and Usha 2003), and by 1–50 m*M* SA applied to whole plants or excised leaf tissue (Harms et al. 1998, Kang and Saltveit 2002, Mori et al. 2001, Ward et al. 1991).

We previously suggested (Choi et al. 2005) that enzyme involved in the phospholipid-signaling pathway may be involved in producing the wound signal responsible for increased wound-induced PAL activity, phenolic accumulation, and browning in excised lettuce leaf tissue. Research reported in this paper was undertaken to further characterize the involvement of enzymes in this pathway in wound-induced phenylpropanoid metabolism in excised lettuce leaf tissue.

Materials and methods

Plant material

Romaine lettuce (*Lactuca sativa* L. Longifolia) was purchased from local commercial vendors. Outer damaged leaves were discarded, and undamaged leaves were carefully detached from the stem. The leaf blade was removed and 5- or 10-mm length segments of the mid-rib tissue were excised starting 20 mm from the base and extending 80 mm up the mid-rib. The freshly excised segments were randomly distributed among treatments, and 10 g were placed in 20 × 100-mm diameter plastic Petri dishes.

Measure of phenolic content

Three grams of tissue from each Petri dish was put into a 50-ml plastic centrifuge tube along with 20 ml of high-performance liquid chromatography grade methanol. The tissue was ground, and the absorbance at 320 nm of a clarified aliquot was measured (Campos-Vargas and Saltveit 2002, Ke and Saltveit 1989, Loiza-Velarde et al. 1997) and expressed as absorbance per gram FW.

Measure of PAL activity

Phenylalanine ammonia-lyase (PAL) activity was measured as previously described by Ke and Saltveit (1989), with slight modifications (Campos-Vargas and Saltveit 2002).

Chemicals used

All chemicals used were reagent grade or better and were purchased from Sigma-Aldrich (St. Louis, MO).

Exposure to butanol

Freshly excised mid-rib segments were either exposed to 1-butanol vapors or immersed in shaken aqueous solutions of 1-butanol. After immersion with gentle shaking in 20 or 100 ml of the appropriate solution for 1 or 2 h, the segments were drained, blotted with paper tissues to remove excess solution, and placed in 20 × 100-mm diameter Petri dishes. The dishes were placed in 20 × 15 × 10-cm plastic tubs lined with wet paper towels, the top of each tub was loosely covered with aluminium foil, and the tubs placed in a 10° C incubator.

Tissue was exposed to 1-butanol vapor by placing the bottom portion of a 7 × 20-mm diameter plastic Petri dish in the center of the 20 × 100-mm diameter Petri dish and applying the appropriate amount of 1-butanol (0–4.0 μl g^{-1} FW) to a filter paper disk inserted in the smaller dish. The top of the larger dish was put in place, the dish enclosed in a plastic bag, and the plastic bag shut and sealed. The assembled dish and bag were put one layer deep on a tray and the tray placed in a 10° C walk-in cold-room. The dish was removed from the plastic bag after 1 or 2 h, the smaller dish with filter paper removed, and the dish replaced on the tray which was then covered with wet paper towels and returned to the 10° C incubator. Air movement past the exposed dishes facilitated diffusion of the previously applied 1-butanol away from the treated tissue.

Measure of 1-butanol tissue concentration

Three grams of tissue previously exposed to 1-butanol vapors for 2 h at 10° C was enclosed in a 16-ml, 16 × 100-mm long glass test tube, the tube capped with a rubber serum stopper, and frozen at −20° C. After warming to 45° C for 2 h, the head space was sampled and 1 ml of gas was injected in a gas chromatograph (Shimadzu model GC-8A, Pleasanton, CA). The GC used a capillary column (Alltech, Deerfield, IL; Porapak Q 80/100 6 feet × 1/8 inches outside diameter × 0.085 inches internal diameter) with injector at 120° C, oven at 80° C, and FID detector at 120° C. Nitrogen was the carrier gas. A standard curve was constructed using aqueous butanol solutions and tissue spiked with known amounts of 1-butanol.

Statistical analysis

All treatments were replicated two times within each experiment, and each experiment was done three times. The treatments were applied as a completely randomized design. Means and standard deviation were calculated for each treatment within an experiment and compared across experiments. An analysis of variance was done on the combined replicates for an experimental procedure, and when appropriate, LSD 5% values were calculated from the mean square error term.

Results

Applying inhibitors of LOX or AOS activity

Treatment with inhibitors that either affected LOX activity (1P3P) or reduced the pool of product (DIECA) reduced WIPA (wound-induced phenolic accumulation). Immersion in 1 m*M* 1P3P or DIECA for 60 min reduced WIPA by 69 and 32%, respectively, while antipyrine and DIEP were ineffective at this concentration (Fig. 2). Increasing the concentration to 10 m*M* produced 17 and 33% reduction in WIPA by antipyrine and DIEP, respectively.

Immersion in 0–10 m*M* SA solutions (pH 7.0) for 1 h first caused a quadratic decline in WIPA (Fig. 3). The maxim level of inhibition (52%) occurred at 10 m*M*. SA treatments suppress both PAL activity measured 24 h after excision and phenolic content measured 48 h after excision (Fig. 4). There was a strong correlation ($R^2 = 0.97$) between PAL activity 24 h after excision and phenolic content 48 h after (Fig. 4 inset).

Timing of the application of inhibitors of LOX or AOS activity

Immersion of 5-mm mid-rib segments in 2.0 or 10 m*M* SA for 60 min at 20° C reduced WIPA by 26 ± 6 or 56 ± 8%, respectively (Fig. 5A). At 2 m*M*, SA lost its effectiveness when applied 3 or more hours after excision, while 10 m*M* SA remained effective even when applied 4 h after excision. In contrast, the ability of 0.5 m*M* 1P3P to reduce WIPA was lost as the time between excision and application increased from 0 to 4 h (Fig. 5B). WIPA was reduced 48% by a 1-h immersion in 0.5 m*M* 1P3P that started immediately after excisions. Applying the same 1-h treatment 1 or 2 h after excision produced only a 32 and 10% reduction, respectively. Further delaying the start of the treatment steadily decreased the effectiveness of 1P3P until there was no significant inhibition of WIPA with a 4-h delay.

Measurement of 1-butanol in tissue

The gas chromatograph response to increasing 1-butanol concentration in 3 g of an aqueous 1-butanol solution was proportional and linear, with 1-butanol concentration

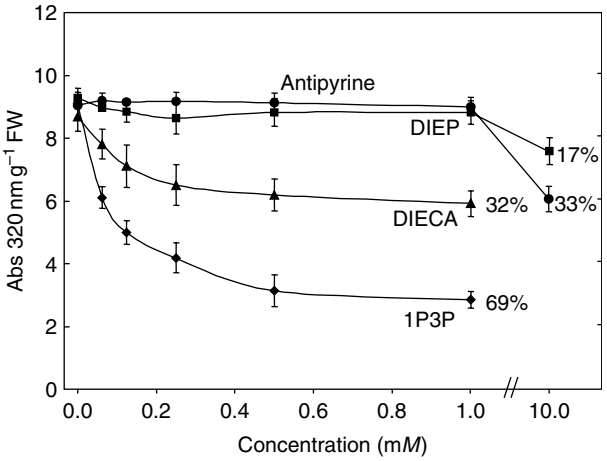

Fig. 2. Phenolic content of mid-rib segments treated with various inhibitors of lipoxygenase. Segments (5-mm long) were immersed in various concentrations of antipyrine, diethyldithio-carbamic acid (DIECA), potassium diethyl dithiophosphate (DIEP), and 1-phenyl-3-pyrazolidinone (1P3P) for 60 min. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue at 10° C for a total of 48 h after the initial excision. Vertical lines associated with each mean represent the standard deviation about that mean.

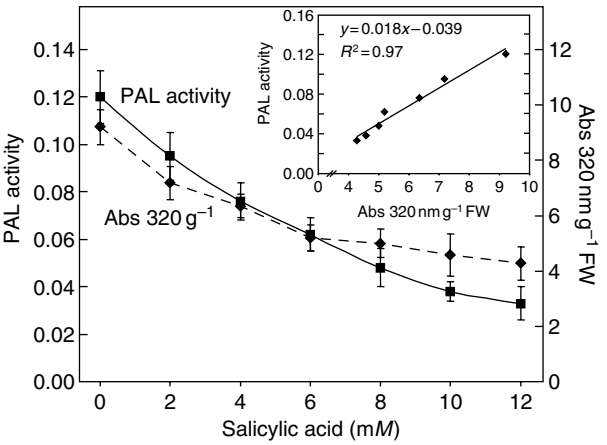

Fig. 4. Phenylalanine ammonia lyase (PAL) activity and phenolic content of mid-rib segments treated with salicylic acid (SA). Segments (5-mm long) were immersed in a 0–12 m*M* SA (pH 7.0) for 2 h at 10° C. PAL activity was measured as described in *Materials and methods*. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for a total of 48 h after the initial excision. The inset shows the relation between PAL activity 24 h after excision and abs 320 g^{-1} FW 48 h after excision. Vertical lines associated with each mean represent the standard deviation about that mean.

(m*M*) equal to 0.15 times the detector response with an $R^2 > 0.99$ (Fig. 6A). The response of the gas chromatograph was also proportional and linear to the butanol concentration in excised 5-mm sections of mid-rib tissue previously exposed to various concentrations of butanol vapors for 2 h at 10° C (Fig. 6B). If all the 1-butanol applied as vapor was uniformly distributed in the tissue, then tissue exposed to 1.0 µl/g FW of 1-butanol vapor would have an internal concentration of 10.9 m*M*. The calculated concentration of 1-butanol in tissue exposed to 1.0 µl/g FW 1-butanol vapor was 10.8 m*M*, so the GC protocol detected 98.8% of the applied 1-butanol. Taken together, these results validate the calculation of the butanol concentration in tissue from the detector response.

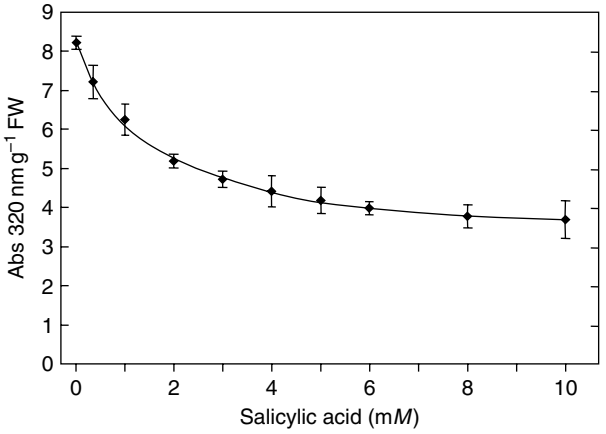

Fig. 3. Phenolic content of mid-rib segments treated with salicylic acid (SA). Segments (5-mm long) were immersed in a 0–10 m*M* SA (pH 7.0) for 1 h at 10° C. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for a total of 48 h after the initial excision. Vertical lines associated with each mean represent the standard deviation about that mean.

Butanol was lost from tissue previously exposed for 2 h at 10° C to 2.5 µl/g FW of 1-butanol in an exponential fashion (Fig. 7A). The concentration of butanol in the mid-rib tissue declined 91% from 25 ± 3 m*M* immediately after treatment to 2.3 ± 0.6 m*M* after a total of 24 h from the start of the 2-h exposure. The decline followed an exponential decline with an R^2 of 0.96.

WIPA was reduced by 1-butanol

Exposing 5-mm mid-rib segments to vapors of 1-butanol for 2 h at 10° C reduced the subsequent wound-induced rise in phenolic accumulation (Fig. 7B). The level of inhibition increased as the initial concentration of 1-butanol in the tissue increased, reaching 50% inhibition at 32 m*M* which was produced by a 2-h exposure to 3.0 µl/g FW 1-butanol vapors at 10° C. The level of inhibition is given by the equation [(2.97 × 1-butanol tissue concentration) − 0.0454 × (1-butanol tissue

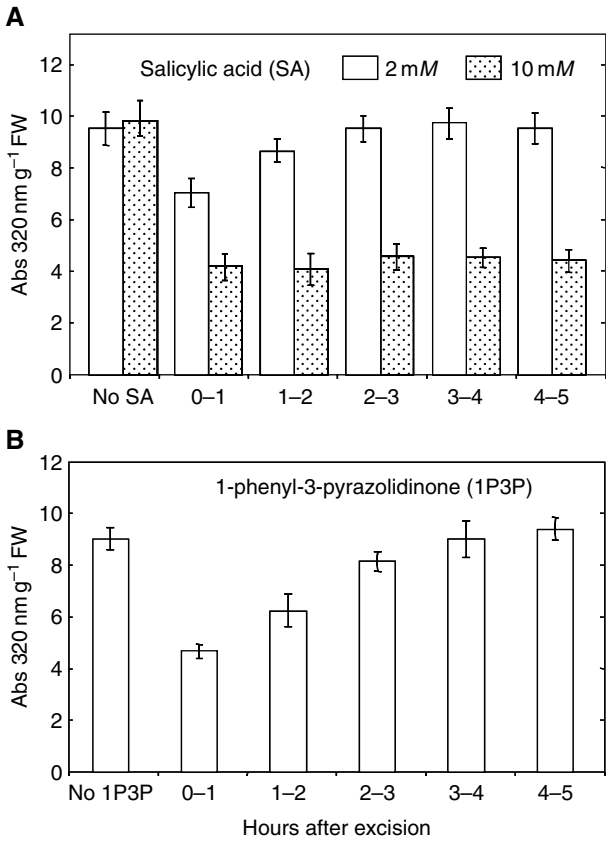

Fig. 5. Phenolic content of mid-rib segments treated with salicylic acid (SA) or 1-phenyl-3-pyrazolidinone (1P3P) at different times after excision. Segments (5-mm long) were immersed in (A) 2 or 10 m*M* SA or (B) 0.5 m*M* 1P3P solution (pH 7.0) for 60 min starting immediately after excision or at 0, 1, 2, 3, or 4 h after excision. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for a total of 48 h after the initial excision. Vertical lines associated with each bar represent the standard deviation about that mean.

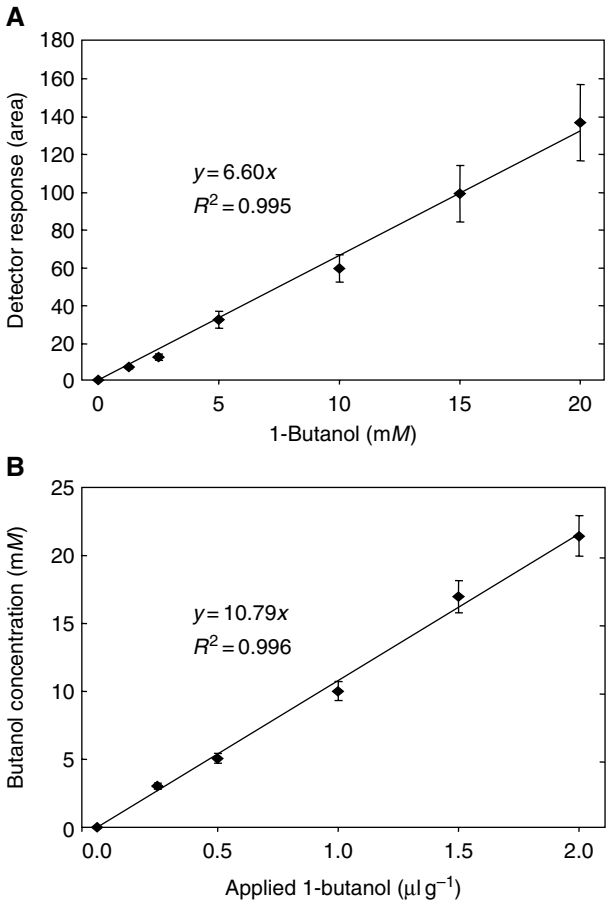

Fig. 6. Gas chromatograph responses to 1-butanol vapors. (A) Three grams of 0–20 m*M* 1-butanol aqueous solutions was put into a 16 × 100-mm glass test tube, capped with a rubber serum stopper, heated to 45° C for 2 h, and then 1-ml head-space injected into the gas chromatograph. (B) Three grams of tissue exposed to various concentrations of 1-butanol vapors for 1 h at 10° C was put into a 16 × 100-mm glass test tube, capped with a rubber serum stopper, heated to 45° C for 2 h, and then 1-ml head-space injected into the gas chromatograph. Vertical lines associated with each mean represent the standard deviation about that mean.

concentration)^2] with an R^2 of 0.95. From this equation we can calculate that an initial tissue concentration of 2.3 m*M* would produce a 6.6% reduction in WIPA.

Re-wounding tissue previously treated with 1-butanol

Cutting 10-mm segments into 5-mm segments 24 h after the initial excision further increased phenolic concentration (measured after an additional 48 h) over that of tissue initially cut into 5-mm segments (Fig. 8). The 45% increase following re-wounding declined linearly to 8.8% as the initial concentration of 1-butanol in the tissue following the 2-h exposure increased from 0 to 32 m*M*.

Discussion

Inhibition of WIPA

We previously showed that application of n-alcohols to segments of excised mid-rib lettuce tissue inhibited the increase in wound-induced PAL activity and the synthesis and accumulation of phenolic compounds that was consistent with their known effect on PLD activity (Choi et al. 2005). The inhibitory effect increased with increasing chain length from methanol (C2) to heptanol (C7), after which 1-octanol (C8) and 1-nonanol (C9) were ineffective. While the 1-isomers of the C2 to C7 alcohols were effective inhibitors, the 2- and 3-isomers

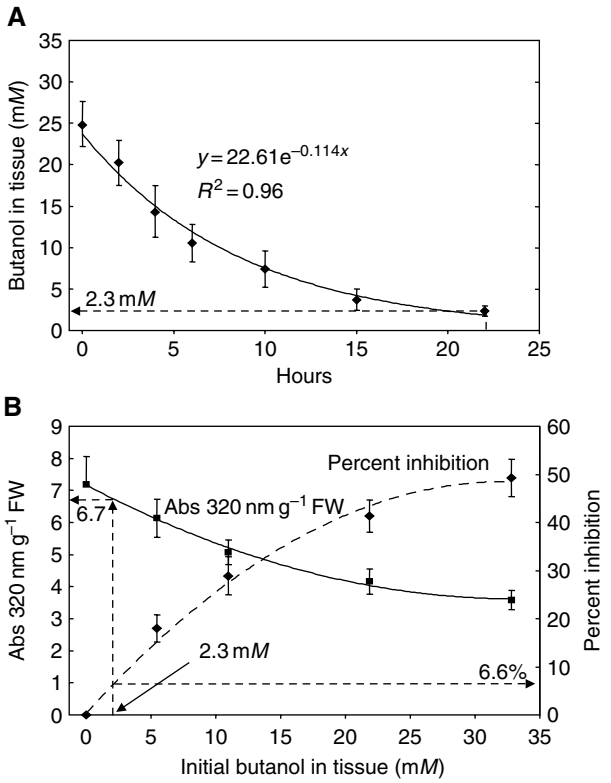

Fig. 7. Decline in the tissue concentration of 1-butanol in mid-lettuce rib segments previously exposed to 1-butanol vapors, and inhibition of WIPA in lettuce mid-rib tissue exposed to increasing concentration of 1-butanol vapor. (A) Decrease in butanol in 5-mm segments exposed to 2.5 µl g^{-1} FW for 2 h at 10° C before being transferred to 10° C air. Three grams of tissue was put into a 16 × 100-mm glass test tube, capped with a rubber serum stopper, heated to 45° C for 2 h, and then 1-ml head-space injected into the gas chromatograph. (B) Freshly excised 5-mm segments were exposed to butanol vapors for 2 h at 10° C before being transferred to 10° C air. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for a total of 48 h after excision. Vertical lines associated with each mean represent the standard deviation about that mean.

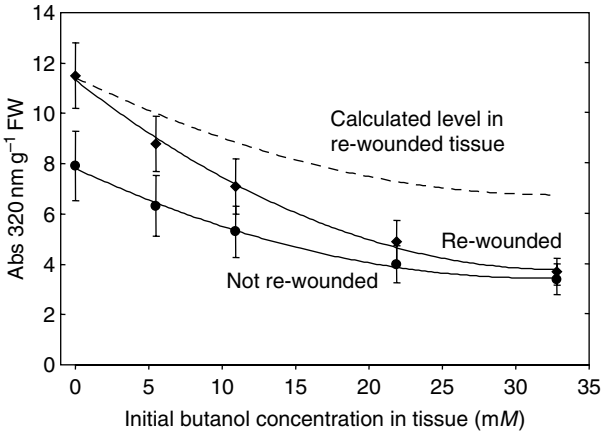

Fig. 8. Phenolic content of mid-rib segments exposed to various levels of butanol vapor. Segments (10-mm long) were exposed to 1-butanol vapors for 2 h at 10° C, held in air for 22 h at 10° C, and then cut into 5-mm segments. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for a total of 48 h after the final cut. Vertical lines associated with each mean represent the standard deviation about that mean.

of the same alcohols were ineffective. The effectiveness of n-alcohols to inhibit PLD activity with increased chain length up to 1-octanol and the ineffectiveness of 2- and 3-alcohols was also observed in other plant and mammalian systems (Ella et al. 1997).

Applying the active alcohols (e.g. 1-butanol) for a period of 2 h immediately after excision produced a 55% reduction in WIPA, but delaying the 2-h application for 4 h rendered the effective n-alcohols ineffective (Choi et al. 2005). A wound signal appears to be produced at the site of injury within 30 min of injury and migrates or propagates into adjacent non-injured tissue at about 0.5 cm h^{-1} where it induces increased phenolic metabolism (Campos-Vargas and Saltveit 2002, Ke and Saltveit 1989). Applying the inhibitor after the wound signal had been synthesized and dispersed into adjacent tissue would account for the reduced effectiveness of 1-butanol applied 2 h after excision.

The n-alcohols appear to interfere with the production of a wound signal that is responsible for wound-induced increases in PAL activity and thereby increases in phenolic synthesis and accumulation. Phenolic content measured 48 h after excision was closely related to PAL activity measured 24 h after excision (Campos-Vargas and Saltveit 2002, Choi et al. 2005, Loaiza-Velarde et al. 1997).

Effect of LOX and AOS inhibitors

Three known inhibitors of the phospholipid-signaling pathway were effective at reducing WIPA (0.5 m*M* 1P3P produced a 69% inhibition, 0.5 m*M* DIECA produced a 32% inhibition, and 2 m*M* SA produced a 26% inhibition) (Figs 2 and 3). LOX catalyzes the conversion of linolenic acid to 13-HPOT (Porta and Rocha-Sosa 2002), and products of 1P3P oxidization interact with and inactivate LOX (Cucurou et al. 1991, Farmer et al. 1994). DIECA reduces the product of LOX activity, thereby decreasing the precursor pool for AOS (Creelman and Mullet 1997). SA inhibits the conversion of the product of LOX activity (13-HPOT) to 12-OPDA by inhibiting the activity of AOS (Harms et al. 1998).

If these inhibitors are exerting their effect by interfering with the wound signal, then delaying their application until the wound signal had time to be synthesized

and moved into adjacent tissue should diminish their effectiveness. 1-Butanol is known to interfere with the activity of PLD, and a delay of 4 h in the application of 1-butanol eliminated its effectiveness as an inhibitor of WIPA (Choi et al. 2005). Delaying the 1-h application of 1P3P for 1–4 h steadily decreased its effectiveness until there was no significant inhibition of WIPA with a 3-h delay (Fig. 5B). A similar response was observed for DIECA (data not shown). Reduction of WIPA using 1P3P and DIECA suggests that the product of LOX activity is necessary for generation of the wound signal.

In contrast, the inhibitory effect of SA was dependent on its concentration, and at 10 m*M*, its activity was not diminished when applied even 4 h after excision (Fig. 5A). SA is an aromatic carboxylic acid (a carboxyl group at the 1 position and a hydroxyl at the 2 position on the aromatic ring), and we have shown that carboxylic acids and their salts inhibit WIPA in lettuce leaf tissue (Saltveit et al. 2005). The C2–C9 mono-carboxylates were equally effective at reducing WIPA whether applied immediately after excision or 5 h later. SA is effective when applied to plant roots and excised tissue at 0.5–3 m*M* (Janda et al. 1999, Kang and Saltveit 2002, Mori et al. 2001, Singh and Usha 2003) and at higher concentrations (1–50 m*M*) when applied as sprays to whole plants (Harms et al. 1998, Ward et al. 1991). SA may inhibit AOS activity in cut lettuce leaves as it does in other plant tissue (Creelman and Mullet 1997) at the lower concentrations (<3 m*M*), while at higher concentrations it may have an additional mode of action similar to the mono-carboxylates that reduce WIPA (Saltveit et al. 2005). For example, SA could modulate catalase activity (Chen et al. 1993, Durner and Klessig 1996) or act as a non-specific inhibitor of cellular kinases (Frantz and O'Neill 1995). These additional functions could account for the persistent effectiveness of SA when applied 4 h after excision, when the wound signal would have been synthesized and migrated into adjacent tissue.

Effect of re-wounding on WIPA

If 1-butanol inhibited the accumulation of wound-induced phenolic compounds by interfering with the formation or dispersion of the wound signal, then re-wounding tissue once the initially applied 1-butanol had diffused away to a low level should elicit a response of the same magnitude as it would in tissue initially treated with that low level of 1-butanol. Initial 1-butanol concentrations of 0.0, 5.5, 10.9, 21.9, and 32.8 m*M* produced reductions of 0, 20, 33, 49, and 57%, respectively, in WIPA (Figs 6B and 7). A quadratic curve fitted the data with an $R^2 > 0.99$ (Fig. 7).

The level of phenolic compounds (abs 320 nm g^{-1} FW) produced by cutting 10-mm segments into 5-mm segments (re-wounding) decreased with increasing prior exposure to 1-butanol (Fig. 7). The reduction in phenolic accumulation in re-wounded tissue that had initial 1-butanol concentrations of 0.0, 5.5, 10.9, 21.9, and 32.8 m*M* was 0.0, 23.5, 38.3, 57.5, and 67.8%, respectively. A quadratic curve fitted the data with an $R^2 > 0.99$ (Fig. 7).

The loss of 1-butanol from the tissue was calculated from the equation derived from data presented in Fig. 6A and confirmed with gas chromatographic measurements. Tissue with initial concentrations of 0.0, 5.5, 10.9, 21.9, and 32.8 m*M* 1-butanol at the end of the 2-h treatment had 1-butanol levels of 0.0, 1.3, 2.6, 5.1, and 7.6 m*M* after an additional 22 h in air. These lower concentrations would produce an inhibition of 0.0, 1.3, 2.6, 5.1, and 7.6%, respectively, in WIPA (Fig. 6B).

The phenolic content of tissue is relatively stable from 48 to 72 h after excision (Campos-Vargas and Saltveit 2002, Choi et al. 2005). The additional phenolic compounds synthesized upon re-wounding 24 h after excision should therefore be additive, with the proviso that their synthesis would be inhibited by the level of 1-butanol remaining in the tissue at the time of re-wounding. When this reduction was factored in, the calculated level of phenolic compounds in the re-wounded tissue was substantially higher than the level actually measured in the tissue (Fig. 7). The level actually measured was 0.0, 11, 19, 34, and 45% lower than those calculated. The greater inhibition in phenolic content than would be expected from the remaining levels of 1-butanol in the tissue when it was re-wounded suggests that prior treatment with 1-butanol induced an inhibition of WIPA that persisted even when concentrations of 1-butanol had decreased to minimally active levels.

1-Butanol is thought to exert its inhibitory effect by interacting with PLD to form phosphatidic butanol, instead of PA. For this reaction to occur there must be sufficient 1-butanol present in the tissue to serve as substrate for this alternate reaction. Because WIPA was still inhibited in 1-butanol-treated tissue when the level of 1-butanol in the tissue had declined to levels that would produce only a fraction of the inhibition observed, it appears that the 1-butanol treatment is inducting persistent resistance to WIPA.

While stresses usually stimulate phenolic metabolism, not decrease it, some abiotic stresses reduce WIPA. A persistent decrease in WIPA was induced in lettuce by chemical, heat, or osmotic shocks. The phenolic content of wounded lettuce tissue was reduced by

immersion in aqueous hypertonic solutions immediately after cutting (Kang and Saltveit 2003a). Re-wounding tissue 1 day after excision did not produce a second rise in PAL activity or phenolic accumulation in tissue that had previously received an osmotic stress. A heat-shock treatment also induced persistent resistance to WIPA (Loaiza-Velarde and Saltveit 2001), and this resistance required the induced synthesis of heat-shock proteins (Kang and Saltveit 2003b). While the heat-shock treatment did not interfere with the wound signal or the induction of wound-induced PAL mRNA, it did prevent translation of the message into PAL protein and an active enzyme (Campos-Vargas et al. 2004). Besides its inhibitory effect on the formation of the wound signal, 1-butanol exposure may produce a stress response similar to that induced by other abiotic stresses that increased tissue resistance to WIPA by inducing the synthesis of stress proteins (e.g. hsps) and thereby interfering with the translation of the wound-induced PAL mRNA. However, 1-butanol itself may not be the active chemical.

There are two lines of evidence that suggest that it is not the 1-butanol per se, but a metabolic product (e.g. phosphatidic butanol) of the n-alcohols that is increasing resistance to WIPA. The 2- and 3-isomers of butanol (and of other n-alcohols) are chemically similar, yet they differ significantly in their physiological activity (e.g. their ability to reduce WIPA). There is an increasing effectiveness with chain length for the n-alcohols and the disappearance of effectiveness when the chain length exceeded seven carbons, yet the chemical differences between heptanol (C7) and octanol (C8) are slight. Because the osmotic or chemical stress produced by the different isomers and the C7 and C8 n-alcohols would be minimal, it does not appear that the persistent resistance could have been caused by 1-butanol itself exerting a simple osmotic or chemical stress.

Production of phosphatidic butanol, or the phosphatidic alcohols of the other active C2 to C7 n-alcohols, is the proposed mode of action by which PLD is inhibited by 1-butanol. The Application of PA can elicit some wound-and-stress response (Munnik 2001), but there is no information on tissue responses to application of phosphatidic alcohols which are thought to be metabolically inert. The production or persistence of phosphatidic alcohols in the wounded tissue may be responsible for their persistent induced resistance to WIPA. Because the concentration of 1-butanol had decreased to minimally active levels, normal levels of PA would be produced, but the persistence of PA-butanol may have interfered with subsequent reactions with the newly synthesized PA.

Conclusion

Compounds produced by the phospholipid-signaling pathway are involved in a number of wound-and-stress responses in plants that include mechano-transduction, growth inhibition (Dathe et al. 1981), and accelerated senescence (Ueda and Kato 1980). An initial step in this pathway may be the release of LA from cellular membranes by the action of PLD and the production of PA. Published literature supports the following sequence of reactions in the phospholipid-signaling pathway: PLD produces PA through the hydrolysis of structural phospholipids (Arisz et al. 2003, Jong et al. 2004, Potocky et al. 2003), PA is converted to DAG by phosphatidate phosphatase (Katagiri et al. 2005), and DAG is converted to LA by a lipolytic acyl hydrolase (Ryu and Wang 1998). While further processing of LA is thought to contribute to the production of phyto-active compounds, PA has also been shown to be physiologically active. We have constructed a hypothetical working model from these reports and the results of our own studies (Fig. 1).

Treatment of whole non-injured lettuce leaves with JA or MeJA did induce slight increases in PAL activity and WIPA in mid-rib tissue, but the increase was small in comparison with that induced by wounding (Campos-Vargas and Saltveit 2002). This does not eliminate the JA pathway as the source of the wound signal, because other phytoactive compounds are also produced during the synthesis of JA. We have shown that the suppression of WIPA by 1-butanol, an inhibitor of PLD activity, persists even when the concentration of 1-butanol has declined to levels that could not account for the inhibition. Experiments with inhibitors of specific steps in the phospholipid-signaling pathway further implicated this pathway in the generation of the wound signal and indicate that 1-butanol and SA may have alternate modes of action to that ascribed to them as inhibitors of PLD and AOS activity, respectively.

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