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Expression of a Flax Allene Oxide Synthase cDNA Leads to Increased Endogenous Jasmonic Acid (JA) Levels in Transgenic Potato Plants but Not to a Corresponding Activation of JA-Responding Genes

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Both jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are thought to be significant components of the signaling pathway regulating the expression of plant defense genes in response to various stresses. JA and MeJA are plant lipid derivatives synthesized from α-linolenic acid by a lipoxygenase-mediated oxygenation leading to 13-hydroperoxylinolenic acid, which is subsequently transformed by the action of allene oxide synthase (AOS) and additional modification steps. AOS converts lipoxygenase-derived fatty acid hydroperoxide to allene epoxide, which is the precursor for JA formation. Overexpression of flax AOS cDNA under the regulation of the cauliflower mosaic virus 35S promoter in transgenic potato plants led to an increase in the endogenous level of JA. Transgenic plants had six- to 12-fold higher levels of JA than the nontransformed plants. Increased levels of JA have been observed when potato and tomato plants are mechanically wounded. Under these conditions, the proteinase inhibitor II (*pin2*) genes are expressed in the leaves. Despite the fact that the transgenic plants had levels of JA similar to those found in nontransgenic wounded plants, *pin2* genes were not constitutively expressed in the leaves of these plants. Transgenic plants with increased levels of JA did not show changes in water state or in the expression of water stress-responsive genes. Furthermore, the transgenic plants overexpressing the flax AOS gene, and containing elevated levels of JA, responded to wounding or water stress by a further increase in JA and by activating the expression of either wound- or water stress-inducible genes. Protein gel blot analysis demonstrated that the flax-derived AOS protein accumulated in the chloroplasts of the transgenic plants.

INTRODUCTION

Jasmonic acid (JA) is synthesized in plants from linolenic acid by an oxidative pathway similar to that leading to the synthesis of eicosanoids in animals. Indeed, the chemical structure of JA is very similar to that of prostaglandins (Vick and Zimmermann, 1987a). In mammals, eicosanoid synthesis is triggered by the release of arachidonic acid from membranes into the cytosol, where it is metabolized into stress-related second messengers (Needleman et al., 1986). Analogously, metabolites of linolenic acid might function as plant stress second messengers, released during defense responses to wounding or pathogen attack (Farmer and Ryan, 1992a). According to this model, release of the peptide systemin as a result of tissue injury may lead to activation of a lipase in the plasma membrane; the subsequent release of linolenic acid would then produce a rapid accumulation of JA. Fatty acid hydroperoxides (lipoxygenase products) have been shown to be metabolized to allene epoxides, precursors of more stable end products such as JA.

Recent evidence obtained following purification of the enzyme allene oxide synthase (AOS) from flax seed has allowed the identification of this enzyme as a member of the cytochrome P450 family of hemoproteins and thus implicates this type of oxygenase in the JA biosynthetic pathway (Song and Brash, 1991). Its relationship to the P450 gene family was established from the proposed primary structure as deduced from the cDNA. The full-length transcript encodes a protein of 536 amino acids containing a 58–amino acid leader sequence that has the features of a mitochondrial or chloroplast transit peptide

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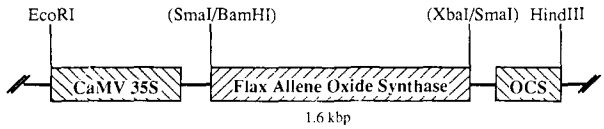

Figure 1. Structure of the Chimeric AOS Gene Construct Used for Transformation of Potato Plants.

A cDNA encoding AOS (the full coding sequence) from flax seed was inserted in the sense orientation between the cauliflower mosaic virus (CaMV) 35S promoter and the terminator of the octopine synthase (OCS) gene.

(Song et al., 1993). In our attempts to characterize the role of endogenous JA in the wound response, we manipulated the internal levels of this phytohormone. To this end and to verify the involvement of flax AOS in the biosynthesis of JA, we introduced the flax AOS full-length cDNA clone under the regulation of the cauliflower mosaic virus 35S promoter into potato plants. Because of the postulated role of JA as a stress-related compound (Farmer and Ryan, 1990; Parthier, 1991; Reinbothe et al., 1994a; Wasternack et al., 1994), the expression of wound- and water stress–responsive genes as well as the endogenous levels of JA were examined in transformed and nontransformed potato plants.

Our results showed that transgenic potato plants overexpressing flax AOS contain six- to 12-fold higher levels of JA than the nontransformed plants. Similar levels of this phytohormone (six- to eightfold higher than normal) were found in nontransgenic plants following wounding, a condition under which proteinase inhibitor II (*pin2*) genes are expressed in potato and tomato leaves (Peña-Cortés and Willmitzer, 1995). Although the increased levels of JA found in transgenic plants correspond to those observed in wounded nontransgenic plants, *pin2* genes were not expressed in potato leaves overexpressing flax AOS. Mechanical wounding or desiccation activated gene expression that corresponded to the stress and triggered an additional accumulation of JA in the transgenic potato lines. These results suggest that an increase in the endogenous levels of JA alone, at least under these conditions, is not able to trigger a constitutive expression of wound- or water stress–responsive genes.

RESULTS

Construction of a Chimeric AOS Gene

To increase AOS activity in transgenic potato plants, an overexpression approach was used. To this end, a 1.6-kb cDNA encoding AOS (the full coding sequence) from flax seed was inserted in the sense orientation between the cauliflower mosaic virus 35S promoter and the terminator of the octopine synthase gene of pTiACH5 (Figure 1). The resulting gene was ligated into the plant transformation vector pBin19 (Bevan, 1984) to create pBin–AOS. This construct was used to transform potato plants via Agrobacterium (Rocha-Sosa et al., 1989). Transgenic plants were screened for integration of the chimeric gene by DNA gel blot analysis, and only plants containing the intact gene were used for further analysis (data not shown).

Screening for Transgenic Potato Plants Containing the Flax AOS mRNA and Protein in Their Leaf Tissue

Transgenic potato plants maintained in tissue culture were visually indistinguishable from nontransformed control plants. Forty transgenic plants were randomly selected, transferred to soil, and grown under standardized greenhouse conditions (see Methods). To screen for plants having increased levels of AOS mRNA and protein, leaf samples were taken from 4-week-old transgenic and nontransformed control plants and subjected to RNA and protein gel blot analysis using a radioactively labeled AOS cDNA or an antibody raised against AOS polypeptide of flax, respectively (Song et al., 1993). Five independent transformants were selected for further analysis. Whereas the flax AOS cDNA did not recognize the corresponding potato AOS mRNA (Figure 2A), the flax AOS antibody cross-reacted unspecifically with a potato protein. This band was also observed in protein gel analysis of total proteins from flax leaves (Figure 2B, flax lane). The size of the cross-reacting protein (~55 kD) and the length of the AOS homologous mRNA (~1.6 kb) detected in the transgenic plants are in agreement with the length reported for the flax seed enzyme (Song and Brash, 1991; Song et al., 1993). Analysis of AOS activity in crude extracts from leaves revealed that potato plants overexpressing the flax AOS gene convert 13-hydroperoxylinolenic acid (13-HPLA) into 12-oxophytodienoic acid (12-oxo-PDA) more efficiently than do the nontransformed plants (Figure 2C), suggesting that the transformed plants possess increased AOS activity, which is probably due to the detected flax AOS protein. Despite the strong increase in both AOS mRNA and AOS enzyme activity in the leaves of transgenic plants, no dramatic change in the phenotype of the transgenic potato plants was observed during the first 4 to 6 weeks after transfer to the greenhouse.

Overexpression of AOS Leads to an Increase in the Endogenous JA Level

Lipoxygenase products (fatty acid hydroperoxides) are metabolized to allene oxides by a type of dehydrase that has been detected in several organisms, including plants (Vick and Zimmermann, 1983, 1984). The allene oxides are unstable epoxide precursors of more complex products, such as the phytohormone JA. In the principal sequence of reactions, linolenic acid is oxygenated by a lipoxygenase, the resulting 13-hydroperoxide is converted to an allene oxide by the AOS, and finally, the allene oxide is the substrate of an allene oxide cyclase, giving a direct analog and metabolic precursor of JA

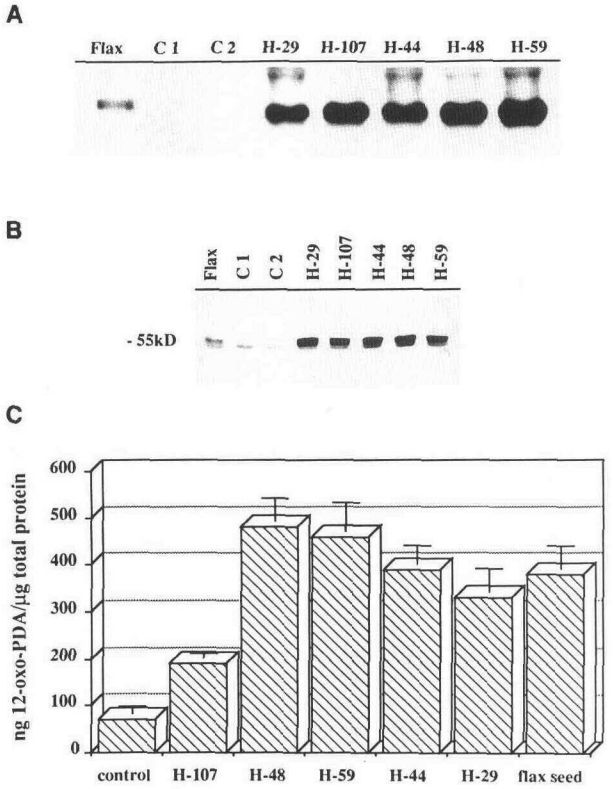

Figure 2. The AOS Gene Is Transcribed and Translated in Leaves of Transgenic Potato Plants.

(A) AOS transcript levels in leaves of five transgenic plants (H-29, H-107, H-44, H-48, and H-59) were detected by RNA gel blot analysis (10 µg of total RNA per lane). Leaf samples were taken from 4-week-old plants and immediately frozen in liquid nitrogen before the RNA was extracted. The RNA on the membrane was hybridized with a ^{32}P-labeled DNA probe from the flax AOS cDNA. Total RNA from flax leaves (flax) and wild-type potato plants (C1 and C2) served as controls.

(B) Total protein (30 µg per lane) from leaves of the same transgenic plants was separated by SDS-PAGE. AOS protein levels were detected by gel blot analysis using an anti-AOS antibody. Proteins from wild-type potato plants served as a negative control (C1 and C2). AOS from flax leaves served as a positive control (Flax). The molecular mass of the cross-reacting protein is 55 kD.

(C) In vitro AOS activity in transgenic potato leaves. Specific AOS activity in potato leaf extracts is given in nanograms of 12-oxo-PDA per microgram of total protein. Extracts from wild-type potato plants served as a negative control (control), whereas extracts from flax seed served as a positive control.

(Hamberg and Gardner, 1992). To investigate whether the overexpression of the flax AOS gene in potato plants influences endogenous JA levels, the concentration of this plant growth regulator in nontransformed and transformed potato plants was determined. To this end, leaves from 4-week-old healthy green plants were harvested and extracted, and the JA content was determined as described by Knöfel et al. (1990). The results obtained from these measurements are shown in Figure 3.

Transgenic potato plants overexpressing the flax AOS gene exhibited clearly increased levels of jasmonate. The levels in these plants were eight- to 12-fold higher (600 to 1400 pmol per gram fresh weight of tissue) than those amounts found in nontransformed plants (~60 to 90 pmol per gram fresh weight of tissue), indicating that the overexpression of AOS leads to an increase in the endogenous jasmonate levels.

Increased Endogenous Levels of Jasmonate Do Not Influence Wound-Induced *pin2* Gene Expression

JA and methyl jasmonate (MeJA) have been implicated in several physiological processes in plant metabolism (Parthier, 1990, 1991). Several studies have suggested a role for these substances as second messengers in a signal transduction pathway involved in the expression of plant defense genes in response to various stresses (Farmer and Ryan, 1992a; Staswick, 1992; Sembdner and Parthier, 1993). For instance, the exogenous application of either JA or MeJA initiates the accumulation of *pin2* mRNA in both potato and tomato plants (Farmer and Ryan, 1990, 1992b). These genes are constitutively expressed in potato tubers and in tomato and potato floral buds (Peña-Cortés et al., 1991). Mechanical wounding as well as exogenous application of JA activate the expression of *pin2* genes in potato and tomato leaves (Peña-Cortés and Willmitzer, 1995). Furthermore, mechanical damage leads to an increase in the JA level that has been correlated with wound-induced *pin2* gene expression (Peña-Cortés et al., 1993). Together, these results suggest the involvement of an increase in JA in the signal transduction chain regulating wound-induced *pin2* gene expression in potato and tomato leaves.

Transgenic potato plants overexpressing the flax AOS gene exhibited increased JA levels similar to those found in nontransformed plants after mechanical wounding (~600 to 800 pmol per gram fresh weight of tissue; Figure 3). Because under these conditions, the *pin2* genes are expressed in tomato and

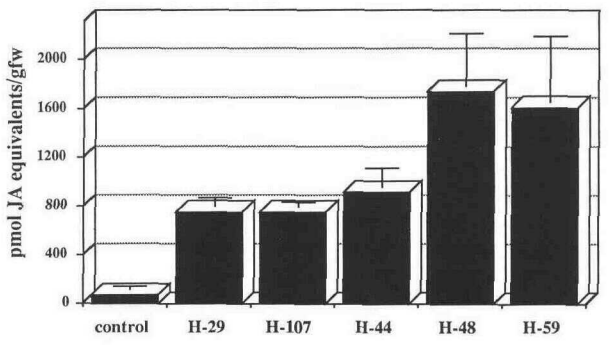

Figure 3. Endogenous Levels of JA in Potato Plants.

JA was isolated from leaves of nontransformed (control) and transformed (H-29, H-107, H-44, H-48, and H-59) potato plants that were 4 weeks old. It was measured by ELISA using a polyclonal antibody as described by Knöfel et al. (1990). gfw, grams fresh weight of tissue.

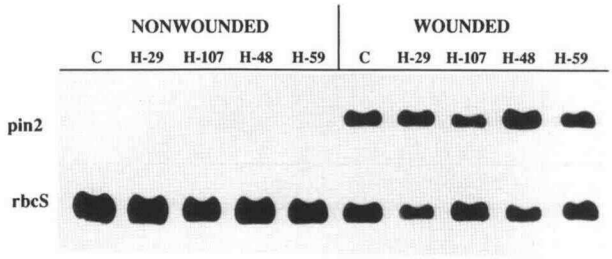

Figure 4. Elevated Levels of JA Do Not Increase *pin2* Transcript Levels.

pin2 transcript levels in leaves of wild-type (C) and transgenic (H-29, H-107, H-48, and H-59) plants before (nonwounded) or 6 hr after (wounded) mechanical damage were detected by RNA gel blot analysis (10 μg of total RNA per lane). The RNA was hybridized with a ^{32}P-labeled DNA probe from a potato *pin2* cDNA and a tobacco *rbcS* cDNA.

potato leaves, we decided to determine whether the increased JA levels in the transgenic plants could activate *pin2* gene expression in the absence of any mechanical damage. To this end, total RNA was isolated and examined by RNA gel blot analysis for the expression of *pin2* genes (Sanchez-Serrano et al., 1986). The same blot was reprobed with the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*) cDNA (Eckes et al., 1986). Figure 4 shows that *pin2* genes in transgenic plants, similar to nontransformed plants, are not expressed in nonwounded conditions (Figure 4, nonwounded). Mechanical wounding of the leaves, however, resulted in activation of *pin2* gene expression in both transformed and nontransformed plants (Figure 4, wounded). Conversely, *rbcS* gene expression was appreciably reduced after mechanical wounding in both transgenic and nontransformed plants. These results suggest that the increase in the endogenous JA levels as a result of the overexpression of the flax AOS gene in potato plants is not capable of triggering *pin2* gene expression.

Transgenic Potato Plants Containing Increased Levels of JA Do Not Display an Alteration in Water Status or Water-Induced Gene Expression

Osmotic (sorbitol or mannitol) and desiccation stresses cause the induction of several proteins, such as the JA-induced proteins, as well as an increase in the content of endogenous JA (Parthier, 1991; Reinbothe et al., 1994b). Furthermore, certain water stress–induced genes, such as some vegetative storage protein genes, can be induced by the exogenous application of JA (Mason and Mullet, 1990; Bell and Mullet, 1991). Because transgenic potato plants overexpressing the flax AOS gene have increased levels of JA, we decided to examine the expression pattern of certain genes that are usually induced under water stress conditions. To this end, total RNA was isolated from transgenic and nontransgenic plants before and after desiccation treatments. Expression of sucrose synthase (*Susy*; B. Müller-Röber, personal communication), *TAS-14* (Godoy et al., 1990), and *pin2* genes was examined by RNA gel blot analysis. *Susy* mRNA accumulates following mechanical damage, JA treatment, or water stress (B. Müller-Röber, personal communication), whereas *TAS-14* gene expression is strongly activated after water stress and weakly induced by wounding. This latter gene does not respond to JA treatment in potato leaves (Hildmann et al., 1992). The same or similar blots were also probed with the *rbcS* cDNA (Eckes et al., 1986). Both transgenic and nontransgenic plants showed no expression of these genes under normal conditions (Figure 5, nontreated). Conversely, desiccation stress activated the expression of *Susy* and *TAS-14* in both transformed and nontransformed potato plants. Following desiccation, there was no accumulation of *pin2* mRNA, as was previously demonstrated (Peña-Cortés et al., 1989), although expression of the *rbcS* gene clearly decreased (Figure 5, treated).

The influence of JA on stomatal movement has not been well characterized and remains unknown. Horton (1991) reported that MeJA is effective in causing stomatal closure only when a very high concentration is directly applied to the transpiration stream. Conversely, plants treated with JA show a decrease in the rate of photosynthetic CO_2 fixation and an increase in stomatal resistance of ~70% relative to control plants (Popova et al., 1988). Furthermore, the application of JA to the transpiration stream of potato and tomato leaves leads to a partial closure of stomates affecting assimilation (CO_2 fixation) and transpiration rates (H. Peña-Cortés, unpublished results). To determine whether increased levels of JA would affect the water status in plants, the water potential in leaf material was measured before and after desiccation treatment. Table 1 shows that transgenic plants with increased levels of JA have a water potential similar to that observed in control plants under

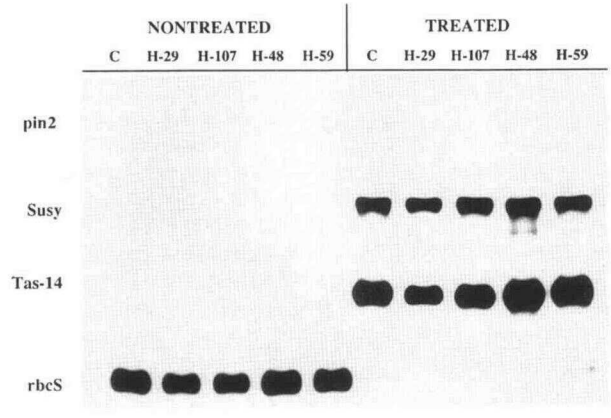

Figure 5. Elevated Levels of JA Do Not Alter the Expression of Water Stress–Responsive Genes.

Total RNA was isolated from leaves of wild-type (C) and transgenic (H-29, H-107, H-48, and H-59) potato plants before (nontreated) and after (treated) desiccation. The autoradiogram shows the result of a RNA gel blot hybridization of total RNA (10 μg per line) against radioactive *pin2*, *Susy*, *Tas-14*, and *rbcS* cDNA probes.

Table 1. Water Potential in Wild-Type and Transgenic Potato Plants

	Nontreated Water Potential (MPa)	Treated Water Potential (MPa)
Control 1	−0.6	−1.0
Control 2	−0.5	−1.5
H-48	−0.7	−1.4
H-44	−0.6	−1.1
H-59	−0.5	−1.1
H-29	−0.6	−1.5

Each given level is the mean value obtained from three independent determinations. Values for water potential are given in megapascal units.

normal conditions. After desiccation treatment, the water potential decreased to similar values in both transformed and nontransformed plants. These results suggest that the increased levels of endogenous JA in transgenic plants do not influence water status, gene expression, or the capacity of the plants to respond to water stress.

Mechanical Damage and Water Stress Lead to an Increase in JA Levels in Both Transformed and Control Plants

Increased levels of JA have been observed following mechanical wounding in soybean (Creelman et al., 1992) and tomato (Peña-Cortés et al., 1993) plants and after desiccation treatment of leaf segments (Reinbothe et al., 1994b). The effects of mechanical damage and desiccation on JA levels were analyzed. To this end, transformed and control plants were either mechanically wounded or subjected to desiccation. The highest wound-induced JA accumulation in nontransgenic plants occurred 4 to 8 hr following mechanical damage (Peña-Cortés et al., 1993). For this reason, endogenous levels of JA were examined 6 hr after mechanical wounding. Material for JA determination following desiccation was harvested from plants after the fresh weight had decreased by 25%. Nontransformed potato plants accumulated JA after mechanical wounding (Figure 6, control), reaching levels that were eight- to 10-fold higher (600 to 800 pmol per gram fresh weight of tissue) as compared with nonwounded conditions (60 to 90 pmol per gram fresh weight of tissue; Figure 3, control). Transgenic potato plants overexpressing the flax AOS gene also exhibited an increase in JA levels after wounding. This JA level was two- to fivefold higher (1200 to 5500 pmol per gram fresh weight of tissue) than that in the nonwounded state (600 to 1400 pmol per gram fresh weight of tissue). Similarly, desiccation led to an increase in the level of JA in both transgenic and nontransgenic plants, reaching values that were one- to twofold higher than those in nontreated conditions (Figure 7). These results suggest that both transformed and nontransformed plants react to wounding and to desiccation by accumulating JA.

Flax AOS Is Located in the Chloroplasts of Transgenic Potato Plants

The organelle in which JA biosynthesis takes place remains unknown, and no conclusive evidence has been provided to show where this phytohormone is produced or accumulated. Some data suggest that JA biosynthesis occurs in the chloroplast. For instance, Vick and Zimmermann (1987b) demonstrated that the spinach enzymes that metabolize fatty acid hydroperoxides are localized mainly in the chloroplast. Enzymes such as hydroperoxide lyase and AOS (also called hydroperoxide dehydrase) are associated with the membranes of the chloroplast. In addition, Song et al. (1993) reported that the flax cDNA encodes a 58–amino acid signal sequence characteristic of a mitochondrial or chloroplastidic transit peptide. Because the transformation of potato plants was performed by using the flax full-length cDNA clone encoding AOS with the putative signal peptide sequence, we used protein gel blot analysis to determine whether flax AOS is localized in the chloroplast. To this end, cytoplasmic and chloroplastic protein fractions were isolated from leaves of transgenic potato plants and subjected to protein gel blot analysis using a polyclonal antibody raised against the flax protein (Song et al., 1993). Figure 8 clearly shows that the flax protein overexpressed in potato plants is present mainly in the chloroplast fraction (lane CH). However, the flax protein could also be detected in the first supernatant, probably because this cytoplasmic fraction was contaminated by broken chloroplasts (lane 1). In the second supernatant from the last step of the chloroplast isolation, no protein able to cross-react with the flax antibody was detected (lane 2).

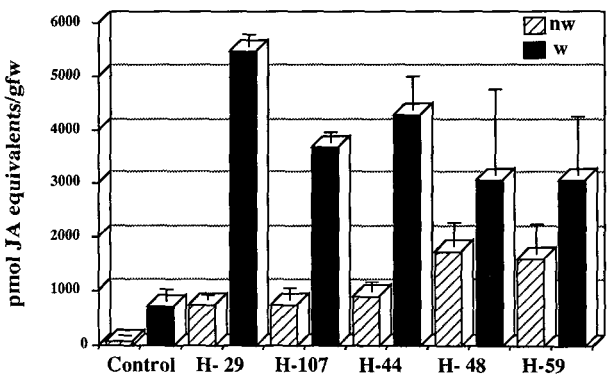

Figure 6. Effect of Mechanical Damage on Endogenous Levels of JA.

JA was isolated from leaves of wild-type (control) and transgenic (H-29, H-107, H-44, H-48, and H-59) potato plants. Internal levels of JA were analyzed before (nw) and 6 hr after (w) mechanical damage. gfw, grams fresh weight of tissue.

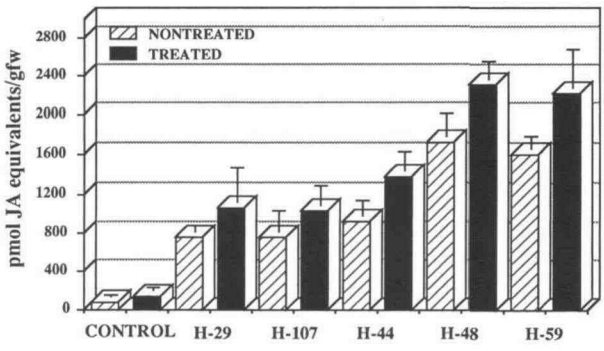

Figure 7. Effect of Desiccation on Endogenous Levels of JA.

JA was isolated from leaves of nontransformed (control) and transformed (H-29, H-107, H-44, H-48, and H-59) potato plants. Internal levels were analyzed before (nontreated) and after (treated) desiccation. gfw, grams fresh weight of tissue.

DISCUSSION

To determine whether AOS is involved in the de novo biosynthesis of the phytohormone JA and is capable of producing increased endogenous JA levels, a flax cDNA full-length clone encoding the complete AOS open reading frame driven by the 35S cauliflower mosaic virus promoter was introduced into potato plants. Forty independent transformants exhibited considerable amounts of flax AOS transcript and protein. Five of the highest expressing transgenic lines (4 weeks old) were chosen for detailed study and used for all of the experiments reported herein. Enzymatic assays for AOS activity revealed that the overexpressed flax protein in potato is active. Indeed, protein extracts from leaves of transgenic potato plants have the capability to convert 13-HPLA into 12-oxo-PDA more efficiently than nontransgenic potato plants (Figure 2C). Measurements of JA content showed that the transgenic lines had eight- to 12-fold higher levels of JA than the nontransformed plants. A similar increase in JA concentration has also been observed in potato and tomato plants following mechanical wounding (Peña-Cortés et al., 1993; Peña-Cortés and Willmitzer, 1995). The fact that the transgenic lines overexpressing flax AOS have JA levels similar to those observed upon wounding in nontransgenic plants suggests that the tissue may possess sufficient substrate (that is, linolenic acid) and enzymes to form 13-HPLA, which is subsequently converted into 12-oxo-PDA, a precursor of JA, by the action of AOS activity. This also suggests that the tissue does not require a previous stimulus to release the substrate and that the AOS activity may represent the major limiting step in the JA biosynthetic pathway. This agrees with earlier studies that have proposed that AOS is the key enzyme of the JA pathway (Vick and Zimmermann, 1987b).

Convincing evidence has been reported showing the involvement of JA in several stress responses of higher plants (see Sembdner and Parthier, 1993). A model has been proposed by Farmer and Ryan (1992a) in which the release of the peptide systemin as a result of tissue damage may lead to an activation of a lipase in the plasma membrane. The subsequent release of linolenic acid would then produce a rapid accumulation of JA, which in turn may trigger the activation of wound-responsive genes. Based on this proposed model, we started our work assuming that increases in the endogenous concentration of JA could result in alterations in the expression of such genes as wound- and desiccation-responsive ones. These alterations can be induced by the exogenous application of JA. Despite an elevation of eight- to 12-fold in internal JA levels in transgenic plants overexpressing the flax AOS gene, wound-inducible genes, such as *pin2*, or water stress–responsive genes were not constitutively expressed in the leaves of these plants. Interestingly, JA levels in transgenic plants were similar to those observed in wounded nontransformed plants. Under these conditions, *pin2* genes were activated and expressed in wild-type leaves but not in the transgenic lines. The fact that *pin2* genes were not expressed in these plants, even though some of them contained more JA than the wounded nontransgenic plants, suggests that the JA is either converted into an inactive form(s) or sequestered in some compartment, thus avoiding interaction with its putative receptor and the subsequent activation of *pin2* gene expression. We cannot rule out the possibility that the JA is sequestered in certain cells that do not produce proteinase inhibitor II (that is, epidermal cells). JA may be produced slowly and transported to the site of storage over a long period of time without exceeding the threshold level in the cytoplasm important for the interaction with the putative receptor and the subsequent activation of the expression of *pin2* genes.

The endogenous JA content was measured by using a polyclonal antibody raised against JA; this antibody also cross-reacts with the amino acid conjugates of JA. Therefore, the values of JA are given as JA equivalents. Because conjugates are formed via JA, the equivalents are indicative of the total amount of de novo synthesized JA. Separation of JA and JA conjugates from transgenic potato plants by HPLC and subsequent ELISA measurement showed that (−)-JA represents 86 to 95% of the total JA equivalents, whereas JA conjugates

Figure 8. Location of AOS in Leaves of Transgenic Potato Plants.

Chloroplast (CH) and cytoplasmic (supernatant from the first centrifugation step, lane 1; supernatant from the second centrifugation step, lane 2) fractions were isolated from leaves of transgenic potato plants. The presence of AOS was detected with an anti–flax AOS antibody by protein gel blot analysis. L, a total protein extract from leaves of transgenic potato plants.

represent only 5 to 15% of the measured amounts (data not shown). These data suggest that the transgenic plants overexpressing the flax AOS gene synthesize an active and functional form of JA that is sequestered by an unknown mechanism preventing the activation of genes previously shown to be induced by higher JA levels. It has been documented that JA treatment leads to a reduction in proteins involved in photosynthetic carbon assimilation (Müller-Uri et al., 1988; Wasternack, 1994). For instance, the steady state levels of *rbcS* are clearly diminished in barley leaves, following JA treatment (Weidhase et al., 1987; Roloff et al., 1994). Furthermore, the application of this phytohormone leads to a reduction in the accumulation of *rbcS* mRNA in potato and tomato leaves (H. Peña-Cortés, unpublished data) similar to that observed upon mechanical wounding (Figure 4). The fact that the transgenic lines display amounts of *rbcS* mRNA similar to those observed in nontransformed plants strongly supports the existence of a sequestered form of JA unable to affect gene expression.

The organelle in which JA biosynthesis takes place remains uncertain, and no conclusive evidence has yet been provided to show where this phytohormone is produced or accumulated. Some studies propose that JA biosynthesis occurs in the chloroplast. For instance, a lipoxygenase cDNA, recently isolated from rice leaves, encodes a protein of 923 amino acids that introduces molecular oxygen exclusively into the C-13 position of linoleic and linolenic acid. This protein has a putative transit peptide sequence at the N terminus, suggesting that the enzyme is located in the chloroplast (Peng et al., 1994). Furthermore, Vick and Zimmermann (1987b) demonstrated that the spinach enzymes metabolizing fatty acid hydroperoxides, such as hydroperoxide lyase and AOS, are associated with the membranes of the chloroplast.

In addition, Song et al. (1993) reported that the flax cDNA encodes a 58–amino acid signal sequence characteristic of a mitochondrial or chloroplastic transit peptide. The authors propose that this flax AOS enzyme is a type I cytochrome P450 and is most likely located in the chloroplast. Transformation of potato plants was performed by using the flax full-length cDNA clone encoding AOS with the signal peptide sequence. Protein gel blot analysis confirmed that flax AOS, when overexpressed in transgenic potato plants, accumulates in the chloroplast. The cross-reacting protein observed in the first supernatant by the chloroplast isolation may have resulted from the protein either associated with ribosomes and/or moving into the chloroplast or released from destroyed chloroplasts. The observation that flax AOS accumulates principally in the chloroplast supports the hypothesis that the biosynthesis of JA or at least a part of it occurs in this compartment.

However, the final steps in JA biosynthesis are β-oxidation reactions. In animal cells, peroxisomes as well as mitochondria are capable of degrading lipids via β-oxidation (Jedlitschky et al., 1993). Nevertheless, there are important differences between the two systems. For instance, peroxisomal β-oxidation does not degrade fatty acids completely but acts as a chain-shortening system, catalyzing only a limited number of β-oxidation cycles (Mannaerts and van Veldhoven, 1993). If the β-oxidation reactions required for JA production in plants occur in the peroxisomes, we must assume that 12-oxo-PDA can move from the chloroplast to the peroxisomes. Thus, the phytohormone may accumulate and remain sequestered either in the chloroplasts or in the peroxisomes, thus preventing the interaction of this phytohormone with the putative receptors, probably located in the cytoplasm, and thereby the activation of either wound- or desiccation-responsive genes is avoided.

The capacity of the transgenic plants to accumulate JA further following either mechanical wounding or desiccation conditions suggests that additional linolenic acid is released and converted into JA. Because an activation of wound- or desiccation-responsive genes was observed only under stress conditions, we propose that mechanical damage or water stress either activates an alternative biosynthetic pathway of JA located in the cytoplasm or facilitates the movement of the sequestered JA from the chloroplast or peroxisome to the cytoplasm by an unknown mechanism, thereby interacting with the corresponding receptors and leading to activation of gene expression.

Constitutive *pin2* gene expression in tomato leaves was recently described by McGurl et al. (1994). Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. Mechanical wounding is not required to expose prosystemin to the processing enzyme(s), and systemin would be continuously released. According to the proposed model (Farmer and Ryan, 1992a; Ryan, 1992), a continuous supply of systemin may activate the biosynthesis of JA. Thus, transgenic tomato plants overexpressing the prosystemin genes may have increased levels of this phytohormone. If this is the case, the overexpression of the prosystemin gene in tomato plants either would induce JA biosynthesis in the chloroplast and prevent the sequestration of JA in this organelle (or in the peroxisome) or would be able to induce directly an alternative wound-inducible JA biosynthetic pathway located in the relevant compartment (that is, the cytoplasm), thereby allowing *pin2* gene activation. According to the model (Farmer and Ryan, 1992a), two potential pathways for the synthesis of JA were postulated, one located in the cytoplasm and the other in plastids (Creelman and Mullet, 1995). The lack of constitutive *pin2* gene expression in transgenic potato plants containing increased levels of JA strongly suggests that these plants are different from tomato plants overexpressing the prosystemin gene (McGurl et al., 1994) with respect to the site of synthesis of JA, the site at which this substance accumulates, or indeed the mechanism that releases JA and subsequently activates *pin2* gene expression.

In summary, we report the increase in JA levels in transgenic potato plants that was accomplished by overexpressing a flax AOS cDNA. The transgenic lines exhibited higher levels of JA than did the nontransformed potato plants. Although the levels of JA in the transgenic potato plants were similar or even higher than those in nontransgenic wounded plants, the wound- or water stress–responsive genes examined were not expressed constitutively. Either mechanical wounding or

desiccation led to an activation of these genes and to a further increase in the endogenous levels of JA. Flax AOS is located in the chloroplast of the transgenic potato plants, suggesting that JA biosynthesis, or at least the formation of certain JA intermediates, occurs in this organelle. The results presented in this work suggest that an increase in JA as a consequence of the overexpression of AOS in the chloroplast is not sufficient to lead to changes in the expression of the JA-responsive genes.

METHODS

Plant Material and Growth Conditions

Solanum tuberosum cv Desiree was obtained from Vereinigte Saatzuchten eG (Ebstorf, Germany). Plants in tissue culture were kept under a 16-hr-light/8-hr-dark period on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2% (w/v) sucrose at 22°C. In the greenhouse, plants were grown at 22°C during the light period (16 hr) and 15°C during the dark period (8 hr). Plants were cultivated in separate pots and watered daily.

Potato Transformation

Cultivar Desiree was transformed via *Agrobacterium tumefaciens* according to Rocha-Sosa et al. (1989).

RNA and Protein Gel Blot Analysis

For RNA isolation and protein extraction, plant material was frozen in liquid nitrogen directly after harvest. Total RNA was extracted from the frozen leaf material according to Logemann et al. (1987). Blotting and hybridization conditions were as described by Müller-Röber et al. (1992). Proteins were extracted from frozen leaf material in 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM β-mercaptoethanol and 0.1% (w/v) Triton X-100. Protein concentrations were determined according to Bradford (1976). The proteins were separated by gel electrophoresis (Laemmli, 1970) and analyzed by protein gel blotting (Sambrook et al., 1989) using a polyclonal antibody raised against flax allene oxide synthase (AOS; Song and Brash, 1991).

Assay of AOS Activity

Formation of 12-oxophytodienoic acid (12-oxo-PDA) was assayed in 50 mM sodium phosphate buffer, pH 6.3, containing 0.1 mM 13-hydroperoxylinolenic acid (13-HPLA) as the substrate. The reaction was started by the addition of 0.1 mL of crude leaf extract (1 mg/mL total protein). After an incubation time of 90 sec at 25°C, the mixture was acidified with 0.1 M HCl to pH 2.8, and the lipids were extracted twice with two volumes of diethylether. The organic phase was dried over sodium sulfate, the solvent was removed under vacuum, and the remaining lipids were dissolved in 0.4 mL of methanol.

For 12-oxo-PDA analysis, the aliquots were injected into a reverse phase HPLC column on a Nucleosil C-18 column (KS-system, 250 × 4 mm, with a 5000-nm particle size; Macherey-Nagel, Düren, Germany) with a guard column (30 × 4 mm, with a 5000-nm particle size). A solvent system of methanol–water–acetic acid (80:20:0.1) and a flow rate of 1 mL/min were used. Chromatography was followed by absorbance at 235 nm with a diode array detector (1040 A; Hewlett Packard, Roseville, CA). 12-oxo-PDA was identified by retention time in a reverse phase HPLC column (coinjection with an authentic standard) and by UV spectroscopy during the chromatographic run. Chromatograms were quantified by integration of peak areas. 13-HPLA, which was used as substrate for 12-oxo-PDA formation, was prepared by oxidizing 9,12,15-(*Z,Z,Z*)-octadecatrienoic acid (linolenic acid) using soybean lipoxygenase and purified by straight phase HPLC (Vick and Zimmermann, 1987b).

Extraction and Analysis of Jasmonates

Jasmonates were determined by ELISA, using an antiserum with the properties described by Knöfel et al. (1990). The assay is highly specific for the methyl ester of (−)-jasmonic acid (JA), prepared by the methylation of plant extracts, and for (−)-JA S-amino acid conjugates.

Water Stress

Four-week-old, well-watered plants were subjected to water stress by carefully removing the soil from the roots, taking care to prevent any kind of wounding. After fresh weight determination, the plants were kept under light at 22°C. Leaf material was harvested when the plants had lost 25% of their original weight, that is, after 5 to 7 hr.

The water potential of leaf discs (diameter of 4 mm) was determined by using the dew point method and a dew point microvoltmeter (model HR-33T, measuring sensor C 52; Wescor, Logan, UT). The microvoltmeter was equilibrated with 0.5 M NaCl.

Wounding

Leaves of potato plants were wounded as described by Peña-Cortés et al. (1992). Leaf material was harvested 6 hr after wounding.

Isolation of Chloroplasts from Potato Leaves

All the steps of the isolation procedure were performed at 4°C. Chloroplasts for the protein gel blot analysis were isolated as described by Skrukrud at al. (1991), except that the resuspension buffer in the final working step was 0.33 M sorbitol, 25 mM Hepes, pH 6.8, 25 mM 2-(*N*-morpholino)ethane sulfonic acid, and 1 mM EDTA. The pelleted chloroplasts were stored at −20°C prior to use.

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