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Regulation of Proteinase Inhibitor Synthesis in Tomato Leaves[1, 2]

IN VITRO SYNTHESIS OF INHIBITORS I AND II WITH mRNA FROM EXCISED LEAVES INDUCED WITH PIIF (PROTEINASE INHIBITOR INDUCING FACTOR)

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ABSTRACT

Messenger RNA was isolated from young excised tomato leaves, induced to accumulate proteinase Inhibitors I and II with the proteinase inhibitor inducing factor (PIIF), and translated *in vitro* in a rabbit reticulocyte lysate system. Translatable messenger RNAs specific for Inhibitors I and II were present in PIIF-induced leaves but were not present without PIIF induction. The nascent *in vitro*-synthesized inhibitors migrated with an apparent molecular weight 2,000 to 3,000 daltons larger than that of the two inhibitors isolated from leaves. The molecular weights of the preinhibitors were identical whether translated from mRNA from PIIF-induced leaves or translated from mRNA isolated from wounded leaves. Incubation of excised PIIF-induced plants in CO_2-free air doubled the rate of *in vivo* synthesis of Inhibitor I over that in normal air (Ryan CA 1977 Biochem Biophys Res Commun 77: 1004-1008) but did not affect the rate of *in vivo* Inhibitor II accumulation. The rate of incorporation of $^{35}SO_4^{2-}$ into soluble proteins was 70% less when leaves were incubated in CO_2-free air rather than normal air. Messenger RNA isolated from PIIF-induced plants incubated in the presence or absence of CO_2 was translated *in vitro*. The amount of *in vitro*-translatable mRNA present for each inhibitor (per microgram total mRNA) was the same in leaves incubated in either atmosphere. Therefore, the increased rate of synthesis and accumulation of Inhibitor I in a CO_2-free atmosphere does not appear to result from an increased level of mRNA but appears to be controlled at a posttranscriptional level.

Mechanical wounding of leaves of tomato plants causes the accumulation of large amounts of two proteinaceous inhibitors of chymotrypsin and trypsin called Inhibitors I and II (1, 2). These inhibitors can account for up to 2% of the soluble proteins of tomato leaves within 3 days when leaves are severely and repeatedly injured and are thought to be part of an allelochemic response of plants to invading pests (12). The wound response is mediated by a putative wound hormone, PIIF[4] (10). Incubation of excised plants in solutions containing PIIF induces the synthesis and accumulation of both inhibitors (10). In PIIF-induced excised tomato leaves, the rate of Inhibitor I synthesis was found to double when the leaves were incubated in CO_2-free air (11).

The effects of the CO_2-free air on Inhibitor I accumulation is not understood but, because Inhibitor I is synthesized and not degraded (3), it was thought that the CO_2-free air was affecting the rate of synthesis of Inhibitor I, perhaps by increasing the levels of mRNA for the inhibitor.

Recently, messenger RNA was isolated from leaves of wounded tomato plants and translated *in vitro*. Analysis of the translation products showed that both Inhibitors I and II are initially synthesized as precursors 2,000 to 3,000 daltons larger than the inhibitors found *in vivo* (5). The inhibitors have been shown to accumulate in leaf cell vacuoles (13) and the additional amino acid sequence in the preinhibitors may be important for the transport of the inhibitors into the central vacuole (or lysosome compartment) of tomato leaf cells. Little, if any, translatable mRNA for the two inhibitors was found in leaves of unwounded tomato plants.

The study presented here was undertaken to determine if PIIF treatment of excised tomato plants would induce the appearance in the leaves of mRNA for precursors of Inhibitor I and II, as observed after wounding. With the ability to monitor the levels of translatable mRNA for both inhibitors, we herein assess the effects of CO_2-free air on the levels of Inhibitor I and II in relation to the levels of the translatable mRNA for two proteinase inhibitors in PIIF-induced tomato leaves.

MATERIALS AND METHODS

Plants. Young (13-day-old) tomato plants (*Lycopersicum esculentum*, var. Bonnie Best) were grown under 2,000 ft-c light with 17 h days (30 C) and 7 h nights (20 C). Leaves of tomato plants were wounded as described (13). Alternatively, tomato plants were excised and the main stem was supplied with crude PIIF (1 g autoclaved, lyophilized, and chloroform-methanol extracted tomato leaves/20 ml H_2O) (10) for 30 min. The same crude preparation was purposely employed in all experiments to assure reproducibility throughout the study. Although PIIF has not been fully purified, it is consistently associated with pectin-like polysaccharide fractions of tomato leaves (12) and is highly active in partially purified preparations.

Plants were rinsed and the stems were incubated in water under 1,500 ft-c continuous light in air-tight Plexiglas boxes (28 × 27 × 12 cm). Test atmospheres were continuously swept through an inlet and outlet on opposite sides of the incubation box at a rate of 2 l/min, resulting in a complete turnover of the atmosphere every 6 min. The CO_2-free air was prepared by forcing compressed air through 2 liters 10 N KOH, followed by a water vapor trap of anhydrous $CaCl_2$ and a CO_2 trap of ascarite (A. H. Thomas, Co., Philadelphia), and finally by rehumidifying the CO_2-free air by forcing it through 1 liter of H_2O. In addition, trays of 10 N KOH

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[2] Dedicated to the memory of Noe Higinbotham, good friend and colleague.

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[4] Abbreviation: PIIF, proteinase inhibitor inducing factor.

were placed in the Plexiglas boxes to minimize CO_2 accumulation due to plant respiration. Normal air was purged through 1 liter H_2O before introducing it to the Plexiglas boxes.

Isolation of mRNA. Messenger RNA was purified from the leaves of either wounded or PIIF-treated plants incubated in CO_2-free or normal air. Frozen leaf-tissue powder (5 g) was homogenized in a chaotropic buffer followed by extraction with phenol-chloroform and chromatography on oligo(dT)-cellulose (5).

***In Vitro* Translation.** Aliquots (7 μg) of poly(A)$^+$ RNA were translated in a rabbit reticulocyte lysate system using [^{35}S]methionine for 60 min at 30 C. Methods of *in vitro* translation product analysis have been previously described (5) except for the following modifications. Translations were terminated by the addition of 100 μl buffer A [10 mM Na-phosphate (pH 7.4) containing 1% Triton X-100 (v/v), 0.15 M NaCl, 50 μM methionine, and 1% w/v sodium deoxycholate]. Each translation sample was transferred into a tube containing a preformed antibody pellet washed with buffer A (purified immunoglobulin G fraction from anti Inhibitor I or II rabbit serum precipitated with swine antirabbit immunoglobulin G). The samples were shaken overnight, pelleted through a sucrose pad, and washed, and the immunological precipitates were analyzed on SDS-urea polyacrylamide slab gels [10–20% (w/v) acrylamide]. Purified Inhibitor I and II (50 μg) were added directly to the samples to identify their migration distances. After electrophoresis, the gels were sliced and digested in 30% H_2O_2, and the ^{35}S was quantified in the presence of Scintiverse (Fisher).

Measurement of *In Vivo* Inhibitor and Protein Synthesis. The $H_2{}^{35}SO_4$ (specific radioactivity equal to 43 Ci/ml) was purchased from New England Nuclear and diluted with 0.2 mM Na_2SO_4 to a final concentration of 5 μCi/ml. Twenty-four h after the excised plants were treated with PIIF and inhibitor accumulation was in a linear phase, the plants were transferred to vials containing the radiolabel. Incubation continued for 6, 8, 10, or 12 h, after which the plants were removed and rapidly frozen in powdered dry ice. Inhibitors I and II were purified according to Gustafson and Ryan (3), from heat-treated extracts of frozen leaves, by ammonium sulfate precipitation and chromatography on Sephadex G-75. Measurement of ^{35}S incorporation into Inhibitors I and II allowed the rates of inhibitor synthesis to be compared to rates of incorporation of ^{35}S into total protein. Rates of inhibitor synthesis and accumulation (nonradiolabel) were quantified through use of an immunological radial diffusion assay (9). To measure total soluble protein, 10 leaves were macerated in 10 ml 100 mM Tris-HCl (pH 8.5) containing 1 M KCl, 250 mM ascorbate, and 20 mM sodium *meta*-bisulfite. The homogenate was centrifuged at 14,000*g* for 11 min. The supernatant was decanted and saved, and the pellet was washed twice more with 10 ml extraction buffer. The combined supernatants were adjusted to 15% (w/v) trichloroacetic acid. The resulting precipitate was removed by centrifugation. The protein pellet was dissolved in 0.5 N NaOH and reprecipitated by the addition of trichloroacetic acid to a final concentration of 15% (w/v). This washing procedure was repeated three times. The final precipitate was redissolved in 0.5 N NaOH and ^{35}S incorporation was measured. Protein was quantitated through use of a modified Lowry assay (3).

RESULTS AND DISCUSSION

Synthesis of PIIF-induced mRNA Specific for Proteinase Inhibitors. *In vitro* translation of mRNA originating from PIIF-induced plants results in the production of precursor proteins which are approximately 2,000 to 3,000 daltons larger than inhibitors found *in vivo* (Fig. 1). These precursor inhibitor proteins are indistinguishable to those pre-inhibitor proteins resulting from translation of mRNA from wounded plants (5).

PIIF-induced excised tomato plants accumulate proteinase inhibitors for several days at rates of synthesis of about 3 to 7 μg/h. Since the inhibitors are not degraded, they can represent more

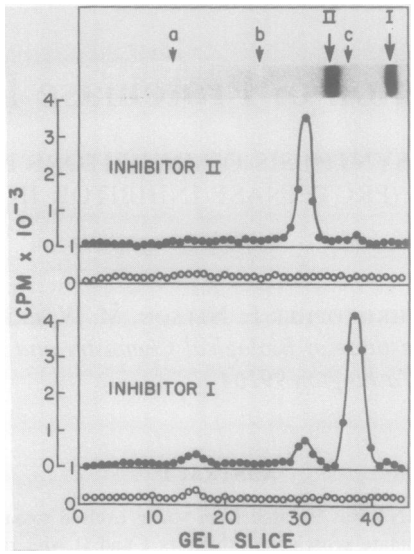

FIG. 1. Radioactivity from [^{35}S]methionine in 2-mm slices after electrophoresis of specific immunoprecipitates for Inhibitor I or Inhibitor II from translation mixtures directed by mRNA from leaves of PIIF-induced tomato plants (●——●) and from leaves of excised plants incubated in water (○——○). The mobilities of Inhibitors I and II and marker proteins are shown: a, heavy chain immunoglobulin G; b, light chain immunoglobulin G; C, Cyt *c*.

Table I. *Levels of In Vitro Translatable Inhibitors I and II mRNA Compared with In Vivo Inhibitors I and II Accumulation in Leaves of PIIF-induced Tomato Plants*

Young excised tomato plants were supplied with PIIF for 30 min and transferred to water under conditions described in the text. Each data point is an average of three separate translations.

Time after PIIF Induction	*In Vitro* Translatable mRNA: Incorporation of [^{35}S]Met		*In Vivo* Accumulation	
	Inhibitor I	Inhibitor II	Inhibitor I	Inhibitor II
h	*cpm*		*μg/g tissue*	
0	0	0	0	0
12	1800	3220	32	32
24	4220	4480	126	68
48	7380	2570	189	75
72	3440	1830	333	180

than 10% of the soluble protein by 70 h as the protein levels of the excised leaves decrease (3). In Table I, the levels of mRNA specific for proteinase Inhibitors I and II in PIIF-induced plants were estimated by measuring the amount of specific inhibitor products translated from mRNAs isolated from plants at varying times after PIIF treatment. Specific mRNA for Inhibitor I increases steadily during the first 48 h following PIIF induction and then significantly decreases. In contrast, the translatable mRNA for Inhibitor II initially rises and then drops off gradually after 24 h. The decline in translation ability in excised leaves may be due to leaf senescence. By 72 h, after leaf excision, when total *in vitro* incorporation declines both ribulose-1,5 bisP carboxylase and catalase activities decrease 90% (7) and the leaves become yellow. The time course of levels of the inhibitor mRNAs in excised leaves is in contrast to the pattern observed in intact leaves after wounding (6) where senescence is not occurring. In leaves of wounded plants, during the first 4 to 12 h after wounding, an increase in mRNA levels for both inhibitors occurs but, by 14 h, these mRNA levels decrease to <40%.

That the PIIF-induced accumulation of proteinase inhibitors apparently requires the formation of new mRNA was confirmed by the effects of cordecepin, an inhibitor of poly(A) synthesis (4), and emetine, an inhibitor of aminoacyl tRNA binding. Each inhibitor severely reduces the accumulation of the proteinase inhibitors. PIIF-induced accumulation of proteinase inhibitors had previously been shown to be inhibited by actinomycin D and cycloheximide, but not by chloramphenicol or rifampin (10).

Effect of CO_2-free Air on Proteinase-Inhibitor Accumulation and Protein Synthesis in PIIF-induced Leaves. The ability to measure levels of translatable mRNA specific for proteinase inhibitors has enabled us to probe the effect of CO_2-free air on specific mRNA levels in excised PIIF-induced leaves. Accumulation of Inhibitor I in PIIF-induced plants doubles when the plants are incubated in CO_2-free air compared to normal air (11). In comparing the effect of a CO_2-free atmosphere on the accumulation of Inhibitors I and II in PIIF-induced plants, we found no significant change in the rate of accumulation of Inhibitor II in the same PIIF-induced plants in which the rates for Inhibitor I doubles when CO_2 is absent from the atmosphere (Figs. 2 and 3). The data indicated that, although the rate of synthesis of one inhibitor protein was significantly increased in CO_2-free air, another inhibitor protein was not, even though both were induced by PIIF. The rate of synthesis of total soluble proteins (Fig. 4) and of Inhibitors I and II were compared under conditions of normal and CO_2-free air by following the incorporation of $^{35}SO_4^{2-}$ for 6, 8, and 12 h, starting 24 h after PIIF induction. In Table II, the rates of incorporation of ^{35}S into soluble proteins and into Inhibitors I and II are presented. Although the rate of incorporation of ^{35}S into soluble proteins was depressed by 70%, the rate of label incorporated into Inhibitor I nearly doubled in CO_2-free air over that in normal air and the rate for Inhibitor II remained virtually unchanged. The rates of synthesis of Inhibitors I and II in CO_2-free air represent nearly 10% of the total incorporation into proteins in CO_2-free air.

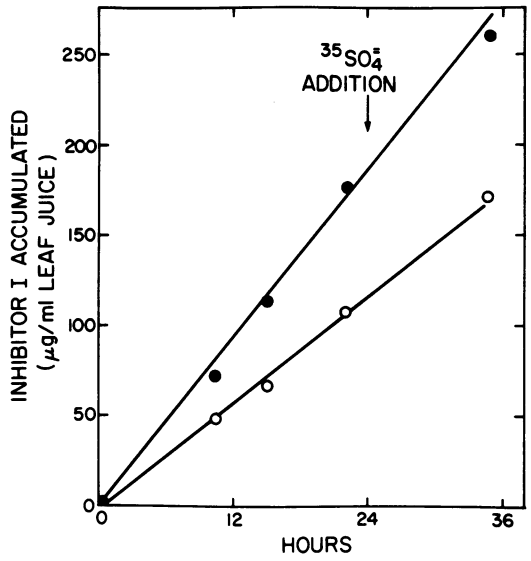

FIG. 2. The rate of *in vivo* proteinase Inhibitor I accumulation in normal (O——O) and CO_2-free (●——●) air. Incubations were carried out as outlined under "Materials and Methods." Amounts of inhibitor were quantitated through use of an immunoradial diffusion assay (9). The arrow designates when $^{35}SO_4^{2-}$ was added.

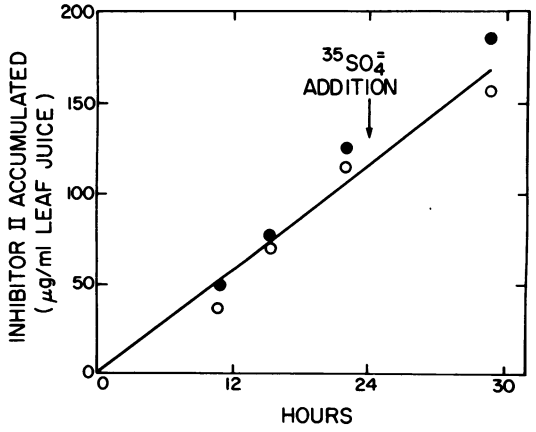

FIG. 3. The rate of *in vivo* proteinase Inhibitor II accumulation in normal (O——O) and CO_2-free (●——●) air. Legend is as described for Figure 2.

***In Vitro* Translation of mRNA from PIIF-induced Plants Incubated in Normal and CO_2-free Air.** To determine if the CO_2 effects on inhibitor accumulation were reflected in the levels of mRNA, mRNA was isolated from leaves of PIIF-treated plants incubated under the two atmospheres and translated. The total translatable mRNA for each inhibitor almost doubled between 12 and 24 h. This increase was not reflected in the rates of *in vivo* inhibitor synthesis, which remained constant (Table III). Computation of a ratio of the over-all rates of *in vivo* inhibitor synthesis to the incorporation of [^{35}S]methionine into each pre-inhibitor protein in the *in vitro* translation system reflects the apparent efficiency of translation for each inhibitor (6). In CO_2-free air, the apparent efficiency of Inhibitor I mRNA nearly doubled, whereas that of

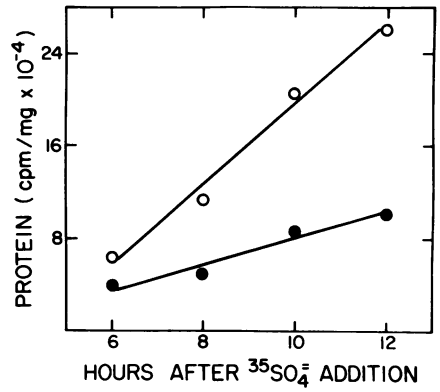

FIG. 4. The rate of $^{35}SO_4^{2-}$ accumulation into protein in leaves incubated in normal (O——O) and CO_2-free (●——●) air. Plants were treated with PIIF and incubated for 24 h in either normal or CO_2-free air. The plants then were placed in 1 ml 0.2 mM Na_2SO_4 containing 5 μCi $H_2{}^{35}SO_4$ and incubated 6, 8, 10, or 12 h, after which times the plants were removed and total protein was isolated and quantified.

Table II. *Effects of Normal or CO_2-free Air on PIIF-induced Rates of Protein Synthesis and Proteinase Inhibitor Synthesis in Leaves of Tomato Plants*

$^{35}SO_4^{2-}$ was supplied for 6, 8, or 12 h, at 24 h after PIIF induction. The values represent the average incorporation per h calculated from a plot of incorporation *versus* time.

Atmospheric Conditions	$^{35}SO_4^{2-}$ Incorporated into Protein			Soluble Protein Synthesis Represented by	
	Soluble protein	Inhibitor I	Inhibitor II	Inhibitor I	Inhibitor II
	cpm/mg·h			*% ^{35}S*	
Normal air	33,000	169	809	0.66	3.17
CO_2-free air	12,000	246	876	2.06	7.38

Table III. *Effect of Normal or CO_2-free Air on Apparent Translation Efficiency of mRNA Specific for Proteinase Inhibitors Isolated from Leaves of PIIF-induced Tomato Plants*

Inhibitor and Incubation Time	Atmosphere	*In Vivo* Rate of Inhibitor Synthesis (A)	*In Vitro*-translatable Inhibitors (B)	Apparent Translation Efficiency (A/B)
h		*μg/h*	*cpm/7 μg poly(A)$^+$ RNA*	*ratio × 10^3*
Inhibitor I				
12	CO_2-free air	7.7	1720	4.5
12	Normal air	5.0	1800	2.8
24	CO_2-free air	7.7	4050	1.9
24	Normal air	5.0	4420	1.1
Inhibitor II				
12	CO_2-free air	3.2	2740	1.2
12	Normal air	3.2	3220	1.0
24	CO_2-free air	3.2	3970	0.8
24	Normal air	3.2	4480	0.7

Inhibitor II was not affected. Between 12 and 24 h, there was a general decrease in the apparent translational efficiency for both inhibitors, regardless of the incubation atmosphere. This may have been due to the senescing condition of the excised plants.

Thus, rather than affecting levels of translatable Inhibitor I mRNA, the effect of CO_2 seems to be post-transcriptional. It is not known whether this control is translational or post-translational.

One possibility may be a post-translational control of pre-inhibitor protein degradation which becomes significantly slower in a CO_2-free atmosphere. Alternatively, control of translational efficiencies may involve mRNA-ribonucleoprotein complexes, such as those found in sea-urchin embryos (8). When *in vivo* translation occurs in normal air, significant amounts of Inhibitor I mRNA may be complexed in inefficiently translated RNP particles. CO_2-free atmospheres may somehow allow Inhibitor I mRNA to become associated preferentially with polyribosomes. The mechanism may become more apparent as more information is obtained concerning the structure of the two pre-proteins and of the events of compartmentation and processing of the vacuolar forms of the two inhibitors.

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