Communication

Pathogenesis-Related Proteins and Polyamines in a Developmental Mutant of Tomato, Epinastic

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ABSTRACT

The polyamine level and the accumulation of pathogenesis-related (PR) proteins were studied in the ethylene overproducing Epinastic (Epi) tomato (Lycopersicon esculentum Mill.) mutant, as compared with its parent, cv VFN8. Neither a decreased putrescine level nor an enhanced production of PR proteins were detected in Epi, contrary to what could be expected from our previous studies (JM Bellés, J Carbonell, V Conejero [1991] Plant Physiol 96: 1053–1059). However, treatment with the ethylene-releasing compound 2-chloroethylphosphonic acid (ethephon) or silver nitrate at high doses induced a decrease in putrescine content and an enhancement of the synthesis of PR proteins in Epi as ascertained by immunoblot analysis using antisera raised against Rutgers tomato PR proteins.

The exocitosis disease of citrus has been demonstrated to be produced by CEVd2 (14). CEVd can be transmitted from citrus to Gynura aurantiaca DC and tomato (Lycopersicon esculentum Mill. cv Rutgers) plants, which after inoculation develop in approximately 15 d the characteristic symptoms of the disease: stunting of the plant, severe epinasty and rugosity of the leaves, and a shortened root system. CEVd infection also induces an overproduction of ethylene and the de novo synthesis of PR proteins in both herbaceous hosts (6).

Notably, treatment of these plants with high doses (10 mM) of silver nitrate or ethephon (2-chloroethylphosphonic acid, an ethylene-releasing compound) produces an analogous developmental syndrome and the same PR proteins as those produced by CEVd infection (6). The viroid-like effects of silver ions have been attributed to their capacity to strongly elicit ethylene biosynthesis in these plants (6). The viroid-like syndrome was prevented by specific inhibitors of ethylene biosynthesis (aminooxyacetic acid and Co2+) and ethylene action (NBD) (2). Based on these findings, a view of viroid pathogenesis, in which viroid molecules are considered as pure replicative signals, has been proposed (6). In this hypoth-

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2 Abbreviations: CEVd, citrus exocitosis viroid; PR, pathogenesis-related; Epi, Epinastic; STS, silver nitrate:sodium thiosulfate; NBD, norbornadiene.

MATERIALS AND METHODS

Plant Material

Tomato (Lycopersicon esculentum Mill.) cv Rutgers seeds of Epi and its parent, cv VFN8, were obtained from Dr. Kent J. Bradford, Department of Vegetable Crops, University of California, Davis. Plants were grown as previously described (16).

Plant Treatments

Five-week-old greenhouse-grown plants of VFN8 and Epi were each sprayed until run-off with 10 mL of 10 mM aqueous solution of 2-chloroethylphosphonic acid (ethephon) adjusted to pH 5.5 containing 0.1% (v/v) Tween 80 as a wetting agent. Control plants were sprayed with water containing 0.1% (v/v) Tween 80. The inhibitors of ethylene action, STS (1 mM) and NBD (1 mL/L), were applied 5 h before ethephon treatment. STS solutions were prepared by mixing AgNO3 and sodium thiosulfate solution at a concentration ratio of 1:4 (v/v). The concentration reported refers to that of the AgNO3.

Protein Extraction and Electrophoretic Analysis

Apical leaves (apex + four youngest leaves) from 5-week-old plants were homogenized in cold (4°C) 50 mM of Mc-
Ilvaine’s citric acid-phosphate buffer (pH 2.8, at which PR proteins are soluble) containing 30 mM 2-mercaptoethanol (1 mL buffer/g fresh weight). The homogenate was filtered through cheesecloth and centrifuged at 20,000 rpm for 30 min at 4°C. Fractions of the supernatant were used for SDS-PAGE according to the procedure previously described (7). Protein content was measured by the method of Bradford (5) using BSA as a standard.

**Extraction and Analysis of Free Polyamines**

Extraction of free polyamines was based on the method described by Flores and Galston (8). Apical leaves from plants at different stages (19, 25, and 31 d after sowing) were homogenized with a pestle in a chilled mortar in 0.2 M perchloric acid (4 mL/g tissue) containing 1,6-diaminohexane as internal standard. After extraction for 1 h in an ice bath, homogenates were centrifuged in a JA21 rotor at 20,000 rpm for 30 min. Then, 0.5 mL aliquots from the supernatant were benzoylated and extracted for HPLC analysis as previously described (1).

**Table I. Free Polyamine Content in Apical Leaves (Apex + Four Youngest Leaves) of VFN8 and Epi Plants 19, 25, and 31 d after Sowing**

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Putrescine</th>
<th>Spermidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>VFN8</td>
<td>Epi</td>
<td>VFN8</td>
</tr>
<tr>
<td>put (nmol/mg protein)</td>
<td>put (nmol/mg protein)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>2.15 ± 0.2</td>
<td>2.11 ± 0.3</td>
</tr>
<tr>
<td>25</td>
<td>2.36 ± 0.3</td>
<td>2.10 ± 0.2</td>
</tr>
<tr>
<td>31</td>
<td>2.27 ± 0.3</td>
<td>2.40 ± 0.2</td>
</tr>
</tbody>
</table>

**Immunoblotting (Western Blots)**

We have used antisera raised against purified PR proteins from Rutgers tomato. These proteins were PR-P69 (18), PR-P32 (12), PR-P23 (Rodrigo et al., in preparation), and PR-P1 (p14) (19). The anti-PR-P32 also reacted with PR-P34.

Immunoblots were performed according to the method of Towbin et al. (15). Proteins were electroblotted onto a nitrocellulose membrane. PR proteins were detected with the appropriate antiserum, followed by goat anti-rabbit antibody coupled to horseradish peroxidase and then by the color-development reagent (4-chloro-1-naphthol) as previously described (19). All materials employed were obtained from Bio-Rad.

**RESULTS AND DISCUSSION**

We have investigated the polyamine content and the possible native accumulation of PR proteins in leaves of the ethylene-overproducing, developmentally altered *Epi* mutant 19, 25, and 31 d after sowing as compared with that of VFN8. On day 19, only small differences in phenotype between *Epi* and VFN8 were observed. Between days 19 and 25, the *Epi* phenotype became more pronounced and exhibited its epinastic characteristics. As in Rutgers tomato leaves (1), putrescine and spermidine were the major polyamines in leaves of both VFN8 and *Epi*, and the level of spermine was always very low. Table I shows that no significant differences in putrescine or spermidine content between *Epi* and VFN8 were observed at any stage of development (19, 25, and 31 d after sowing). Both putrescine and spermidine levels remained constant from day 19 until day 31 (Table I), with the levels of putrescine being slightly lower than those of spermidine in both genotypes.

We also have investigated the accumulation of PR proteins in *Epi* plants by SDS-PAGE and western blot analysis of crude extracts from *Epi* leaves. Soluble proteins from 5-week-old *Epi* and VFN8 plants were extracted at pH 2.8 and separated by SDS-PAGE. The electrophoretic pattern of *Epi* plants showed no significant differences with respect to that of VFN8 (Fig. 1). In addition, Figure 2 shows that none of the antiserum against PR proteins P69, P32, P23, and P1 (p14) from Rutgers tomato reacted with proteins present in *Epi* extracts on a western blot. This clearly indicates that no PR proteins are synthesized in *Epi* in response to the constitutively elevated ethylene synthesis.

Work of our laboratory (1, 6) indicated that the develop-
Table II. Effect of Ethephon on Free Polyamine Levels in Apical Leaves (Apex + Four Youngest Leaves) of Epi Plants, and Effect of Inhibitors of Ethylene Action on Polyamine Content in Ethephon-Treated Epi Plants

Five-week-old greenhouse-grown plants were sprayed with 10 mm ethphon or water with 0.1% Tween 80. Measurements were made prior to treatment and 48 h later. STS (1 mm) and NBD (1 mL/L) were applied 5 h before ethphon treatment. Values are means of five replicates ± sd.

<table>
<thead>
<tr>
<th>Time after Treatment</th>
<th>Control C2H4</th>
<th>C2H4 + STS</th>
<th>C2H4 + NBD</th>
<th>STS</th>
<th>NBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>nmol/mg protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Putrescine 0</td>
<td>2.19 ± 0.4</td>
<td>2.04 ± 0.2</td>
<td>1.95 ± 0.3</td>
<td>2.10 ± 0.1</td>
<td>2.09 ± 0.2</td>
</tr>
<tr>
<td>Spermidine 0</td>
<td>2.27 ± 0.3</td>
<td>0.78 ± 0.2</td>
<td>2.02 ± 0.3</td>
<td>1.88 ± 0.2</td>
<td>2.00 ± 0.3</td>
</tr>
<tr>
<td>48</td>
<td>2.50 ± 0.3</td>
<td>2.35 ± 0.3</td>
<td>2.75 ± 0.4</td>
<td>2.87 ± 0.4</td>
<td>2.85 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2.64 ± 0.4</td>
<td>2.20 ± 0.2</td>
<td>2.68 ± 0.3</td>
<td>2.79 ± 0.2</td>
<td>2.96 ± 0.3</td>
</tr>
</tbody>
</table>

The effect of ethphon on PR protein synthesis in Epi was investigated 48 h after the treatment. The pattern of ethphon-treated Epi plants showed additional bands with respect to the untreated controls. These proteins migrated with the same electrophoretic mobility as Rutgers tomato PR proteins found in extracts from leaves infected with CEVd (Fig. 2). Western blot analysis of SDS gels from ethphon-treated Epi leaf extracts revealed the presence of PR proteins corresponding to homologous Rutgers tomato PR proteins (Fig. 2). In addition, both ethphon and silver nitrate treatments induced in Epi a dramatic increase in ethylene production, as happens in Gynura and Rutgers tomato plants (2, 6). All these results suggested that the pathway for the decrease in putrescine content and the coordinated PR protein induction was responsive to the ethylene elicited in the Epi mutant after ethphon treatment. To directly assess this idea, we took advantage of two widely used inhibitors of ethylene action: STS and NBD (20). Neither STS nor NBD alone had any effect on either putrescine or spermidine content in Epi plants (Table II). However, the depletion in putrescine content caused by ethphon was totally prevented in both STS- or NBD-pretreated plants (Table II), and pretreatment with STS completely blocked the accumulation of PR proteins in ethphon-treated plants (Fig. 2). It is pertinent to note that 1 mM STS by itself did not produce any accumulation of PR proteins in Epi. NBD was also equally effective in preventing the accumulation of PR proteins (data not shown). Equivalent results on polyamine content and PR protein synthesis were obtained when plants were treated with silver ions at high doses (10 mm) (data not shown).

All this can be explained by admitting two different types of ethylene effects: as a growth factor and as a component of a pathway for transduction of pathogenic and stressing signals.
The relatively high level of ethylene in Epi would be involved in the production of the developmentally altered phenotype as a hormonal effect. However, this relatively high but “normal” level of ethylene in Epi would not constitute a stress signal. Only an induced increase over the normal level would be perceived as a signal leading to the reaction of the plant and hence to PR protein synthesis. It is also possible that ethylene is involved in neither the genesis of the distorted phenotype (9) nor in the synthesis of PR proteins (4). Nevertheless, these possibilities would be in contrast with our previous results in Rutgers tomato and in Gymura plants infected with CEVd or treated with silver ions or ethephon at high doses. In these systems, both developmental symptoms and PR synthesis proved to be associated and mediated by ethylene and putrescine (1). Plants manifest their homeostasis against the disorganizing effects of the environment through adaptive distortions in their developmental pattern, changes in their physiological state, and also by means of a series of more specific coordinated reactions (3, 6). One of these reactions against pathogens and other elicitors is the production of PR proteins, whose pathogenic or defensive role is not completely unraveled. Regarding this question, the defensive and adaptive character of several PR proteins has been attributed (13, 17). This, along with the fact that PR proteins are induced coordinately as an integrated response, strongly suggests a defensive role for all these proteins. Our finding that in Epi, PR proteins are not associated with the production of the developmental distortions, and that they only appear after being elicited by an external aggression such as ethephon or silver ions, further supports this idea. Thus, Epi constitutes an excellent model for studying separately two major components of the response of plants to pathogens: developmental alterations and the synthesis of PR proteins.

ACKNOWLEDGMENT

We thank Dr. Kent J. Bradford for kindly providing seeds of Epi and VFN8 tomato plants.

LITERATURE CITED