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Effects of Jasmonic Acid and Wounding on Polyphenol Oxidase Activity in Senescing Tomato Leaves

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The effects of jasmonic acid(JA) and wounding on polyphenol oxidase(PPO) during leaf senescence was investigated by measuring the PPO activity in detached tomato(*Lycopersicon esculentum* Mill.) leaves of two-week-old seedlings. The PPO activity in the detached senescing leaves increased significantly in the dark. The leaf segments responded to the application of JA with accelerated senescence, as indicated by the loss of chlorophyll and rapid increase in the PPO activity. The senescence-promoting action of JA differed in the light and dark. Wounding the detached senescing leaves by scraping surface segments or making punctures with needles considerably delayed the loss of chlorophyll and had a significant effect on the PPO activity, the amounts of which were roughly proportional to the intensity of the wounding. In the dark, the combination of wounding plus JA resulted in stable levels of chlorophyll and PPO. JA and ABA acted similarly in both unwounded and wounded leaves, however, the amount of chlorophyll and PPO in the wounded segments was always higher than in the respective controls. JA was found to eliminate the senescence-retarding action of benzyladenine. In a histochemical localization test, the PPO activity was found to be localized in the cell walls of the parenchyma tissue, thereby indicating moderate cytoplasmic reactions. In the JA-treated plants, the PPO activity was intense in the cells of the cortex and phloem parenchyma. Accordingly, based on these observations it would appear that PPO is a component of a defense response maker, whereas JA plays an integral role in the intracellular signal transduction involved in inducible defense mechanisms.

Key words : detached leaves, jasmonic acid, polyphenol oxidase, senescence, tomato, wounding

1. Introduction

Plant defense strategies for adapting to an unpredictable environment include the rapid accumulation of anti-herbivore phytochemicals and proteins in response to insect attacks[1,2]. The wound response of the tomato is a well-established model system that has been used to study defense mechanisms and intracellular signalling in plants, thereby establishing that signals other than chemicals transported through the phloem are involved in wound-induced gene expression[3].

The major anti-nutritive protein induced by the tomato when wounding is systemin, and the octadecanoid pathway involves polyphenol oxidase. This enzyme oxidizes phenolic compounds into quinones, which are reactive molecules that can interact with a variety of biological molecules. Polyphenol oxidases(PPOs) have been studied in many different plant species and a recent survey identified that leaf PPO is essentially ubiquitous in higher plants[4]. Furthermore, due to its conspicuous reaction products, PPO is considered to have a possible defensive role against pathogens and herbivores. Infestation with pathogens, insects, and mechanical wounding results in the proliferation of many different chemical compounds and affect the concentration of individual PPOs[5]. PPO has also been implicated as a plant defensive protein functioning to cause an anti-nutritive modification of plant proteins upon cell disruption by insect herbivores[6]. Other proposed roles for PPO include the buffering of plastid oxygen levels, biosynthesis of phenolics, and wound healing[7]. However, no

conclusive evidence has yet been provided for such roles.

Circumstantial evidence could be obtained through demonstrating that plants respond to herbivore or pathogen damage by altering their expression of PPOs, as has been previously established for those pathogenesis-related proteins and protease inhibitors suggested to play a role in plant defense. In fact, a large number of studies have already demonstrated that PPO activity increases in response to biotic and abiotic injury.

Recently it was suggested that jasmonates play an integral role in the intracellular signal transduction cascade, that acts in an inducible defense mechanism that plants have developed against pathogens and insect attack through which plant cells counteract stresses in general[8~10]. This suggestion was based on evidence of molecular level similarities in the defense systems activated by jasmonates and wounding[11,12]. In addition, in several tissues, jasmonates have been found to induce the synthesis of proteins identical to those induced by stresses such as wounding, water deficit, and pot removal[13] or similar those induced by salt stress[14] and osmotic stressors[15].

Among jasmonates, a class of cyclopentanone compounds, mainly jasmonic acid(JA) and its methyl ester(MeJA) are widely distributed in plants and have recently been regarded to be putative regulators of plant growth and development[16,17]. Jasmonates are involved in the regulation of many processes[18,19], including promotion[20] and inhibition[21] of growth, wound response, elicitation of secondary product formation and further promotion of senescence, chilling tolerance[22], tuberization, and bulb formation[23]. The functional physiology underlying these phenomena is not yet clear, however, there is increasing evidence that jasmonates may interconnect with the classic plant growth regulators.

Jasmonates have chemical and physiological similarities to abscisic acid[13]. Exogenous jasmonates promote senescence based on an action similar to that observed with ABA. The observation that ABA and JA apparently induce the same abundant proteins in barley leaf segments, which may be important in acquired stress tolerance, is of special interest. Among the physiological processes influenced by jasmonates, the senescence syndrome in detached leaves appears to be a promising model system for studying the modes of action of the jasmonate group. Leaf senescence can be regarded as a genetically integral pattern of leaf development, usually characterized by the loss of chlorophyll, proteins, and nucleic acids, the disassembly of the membrane, and alterations in the gene expression program and enzyme activities[24]. There is good evidence of an interaction between plant growth regulators and light in leaf senescence, either regulating(cytokinins) or promoting(abscisic acid) the process. The choice of plant has varied, including monocots and dicots, and although some studies have used leaves attached to the plants, more have used detached leaves. When the effects of wounding have been studied, these were found to include the stimulation of cell division and changes in the metabolic pathways in the syndrome of leaf senescence. As such, since it would be interesting to determine the possible involvement of PPO in the regulation of JA-promoted senescence, this study describes the effect of jasmonic acid and wounding on PPO activity during the senescence of detached tomato leaves.

2. Materials and Methods

2.1. Plant material and treatments

Tomato(*Lycopersicon esculentum* Mill.) seedlings were grown in moist vermiculite in a growth chamber under 14 h of light($250\,\mu E \cdot m^{-2} \cdot s^{-1}$) at 26℃ and 10 h of dark at 18 ℃. Apical 3 cm segments from the first true leaves of two-week-old plants were excised and floated in a Petri dish containing 10 ml of a JA solution(10^{-4} M) or related compound. The incubation was carried out at 26℃ in darkness or in the light up to 96 h. The leaves were analyzed for PPO after various time intervals or after various experimental treatments. The primary leaves of two-week-old plants were cut and wounded using various methods. Under standard wounding conditions, the leaves received 24 needle punctures and were analyzed one day after wounding. Thereafter, the segments were thoroughly washed, dried with tissue cloth, weighed, and stored in a deep freezer until homogenization.

2.2. Methods of wounding

The mechanical wounding of the leaves was performed using six different methods : (a) the 3cm segments were subdivided into 5mm subsegments ; (b) the adaxial surface of the segments was gently scraped with a sharp blade ; (c) 50 roughly equidistant punctures were made with a sharp needle ; (d) whole segments were scraped with a sharp blade ; (e) the apical surface was half scraped ; (f) the basal surface was half scraped.

2.3. PPO extraction and assay

The leaf segments were homogenized in a prechilled mortar and pestle with a cold 0.1 M Na phosphate buffer, pH 7.2, containing 0.1%(w/v) SDS. The homogenate was then clarified by centrifugation at 12,000 g for 2 min. The supernatant was assayed for PPO activity spectrophotometrically at 490 nm and 30℃, in order to follow the conversion of DL 3,4-dihydroxyphenylalanine (DOPA) into quinone polymers. The assay solution consisted of 1 ml of 5mg ml^{-1} DOPA in 0.1 M Na phosphate, pH 7.2, which had been aerated for 5 min prior to the assay. To prevent any oxidation of the substrate by peroxidase, 280 units of catalase(from bovine liver, Sigma) in 0.1ml H_2O_2 was added. The assay was initiated by the addition of 0.05 ml of the enzyme extract[4].

2.4. Estimation of chlorophyll

Each preweighed leaf was homogenized with a mortar and pestle in 80%(v/v) acetone and centrifuged at 10,000g for 10 min at 0~4℃. The supernatant was brought up to a 1ml volume, then the absorbance of the acetone extract was measured at 663 and 645nm using a UV-visible spectrophotometer(UV-260, Shimadzu). The chlorophyll content was calculated according to the method of Arnon[25] and expressed in mg chlorophyll per g fresh weight.

All data were represented by the average of three replicate experiments.

2.5. Test for histochemical localization of PPO

Fresh shoot samples from two-week-old plants were collected as control and treated plants. These samples were immediately individually immersed in a cold neutral phosphate buffer solution. Ten μm-thick transections of the shoots were obtained in a sledge microtome. The sections were then floated on deionized water, rinsed, and incubated in an appropriate reagent medium to localize the polyphenol oxidase *in situ*[26]. Suitable control tests were performed for the cytochemical tests. The polyphenol oxidase was localized using a 0.04 M aqueous solution of catechol as the substrate by lightly misting the shoot slices with a sprayer[27].

3. Results

3.1. Effect of JA and wounding on PPO activity

The senescence of detached tomato leaves is characterized by a decrease in the chlorophyll and protein levels[28]. This decrease in the chlorophyll and protein levels has been the principal criterion of leaf senescence for most previous studies.

Fig. 1 shows the time course of the chlorophyll levels in the detached leaves floating on water or 10^{-4} M JA in the dark. The chlorophyll content increased up to the 3rd day, after which it started to decrease, however, the loss of chlorophyll was only slight or zero in the 4-day-old leaves. The JA treatment showed a small variation in the chlorophyll level, thereby indicating a promoted effect on senescence. Although, wounding had a significant effect on the chlorophyll level up to the 3rd day, a smaller decrease in the chlorophyll level was observed in the wounded leaves. The PPO activity as a function of the leaf age was studied in primary senescing tomato leaves(Fig. 2). The control showed a daily increase, while the wounded leaves showed an inhibited decrease in the enzyme activity, although only a small effect was detected. In contrast, a significant increase in the PPO activity was exhibited in the JA-treated leaves. Accordingly, it was clear that JA significantly promoted the senescence and increased the PPO levels in the detached leaves.

3.2. Wounding in presence of agents that modify senescence

The way in which foliar senescence is modified by plant growth substances has already been extensively studied. Three such substances, cytokinin, JA, and ABA, have been implicated in

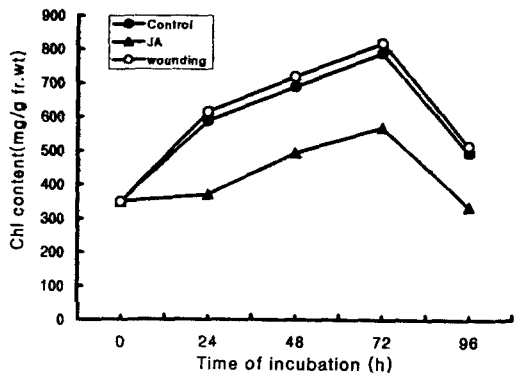

Fig. 1. Time course of changes in chlorophyll content in detached tomato leaves in the dark.

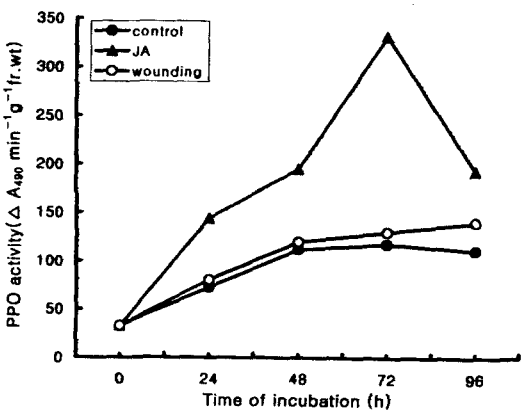

Fig. 2. Time course of changes in PPO activity in detached tomato leaves in the dark.

either delaying or promoting senescence by affecting the chlorophyll level. The effects of these substances on the senescence of wounded leaves was monitored in the current study(Figs. 3 and 4). Two compounds that were previously reported to promote chlorophyll loss, namely ABA and JA, had the same effect when the segments were wounded, although in each case the chlorophyll content was still increased by wounding(Fig. 3). In contrast, benzyladenine(BA) strongly inhibited the loss of chlorophyll in the control segments, yet failed to in the presence of wounding. The modifications in the PPO activity caused by wounding in the presence of a similar group of substances are shown in Fig. 4. JA and ABA, which have chemical and physiological similarities, had a significant effect on the PPO activity in the detached leaves. In general, the differences in the PPO activity due to wounding were comparable to those related to chlorophyll loss. With the exception of BA, the PPO levels of the wounded segments were higher than in their respective unwounded controls, whether the substances were promoters or inhibitors.

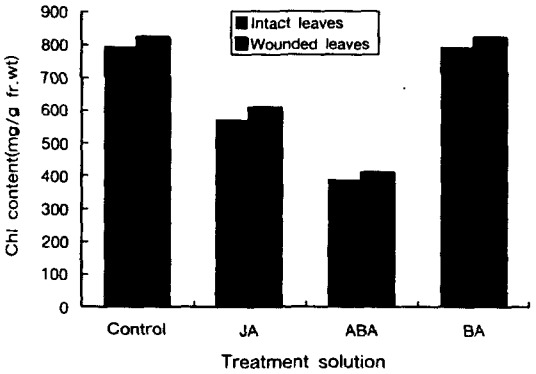

Fig. 3. Effect of JA, ABA and BA on chlorophyll content in intact and wounded tomato leaves in the dark.

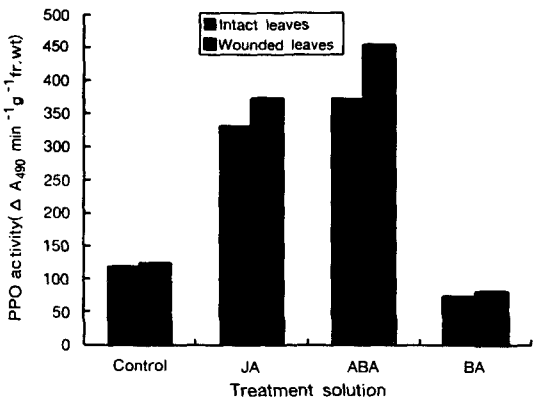

Fig. 4. Effect of JA, ABA and BA on PPO activity in intact and wounded tomato leaves in the dark.

It has been previously observed that, after 4 days in darkness when most of the visible chlorophyll has disappeared, a thin greenish line 1 to 2 mm wide remains along both the cut edges. To further investigate this effect, six types of wounding, as described above, were inflicted. When the 3 cm segments were subdivided into 5 mm segments, the cut edges of each subsegment showed a distinct retention of chlorophyll after 4 days in the dark. When the segments were scraped, so that the

wounding was uniform throughout, the whole segment remained green. When the wounding was done using methods (c) and (d), the PPO activity close to the cuts or punctures was clearly increased. Data on the PPO activity in this experiment is presented in Table 1. While the initial PPO activity increased 33% in the controls, all the wounded segments showed much higher values. The extent of the increased PPO activity was found to be roughly proportional to the area wounded, and scraping the apical half of the segments was the most effective.

Table 1. Effect of wounding types on PPO activity in detached leaves. The data was taken after 4 d in darkness

Type of wounding	PPO activity ($\triangle A_{490}$ min^{-1} g^{-1} fr.wt)
Initial values	32.8
Control(unwounded)	111.0
Wounded	
Method a	169.4
b	159.8
c	173.1
d	165.9
e	180.7
f	140.5

a. 3 cm segments were subdivided into 5 mm subsegments
b. The adaxial surface of the segment was gently scraped with a sharp blade
c. 50 roughly equi-distant punctures were made with a sharp needle
d. Whole segments scraped
e. Apical half scraped
f. Basal half scraped

3.3. Effect of light on PPO activity

Fig. 5 shows the time course for the chlorophyll behavior when the detached leaves were maintained in the light and dark. In the light, the chlorophyll loss occurred continuously after detachment. These results are in striking contrast to what happens in leaves when the chlorophyll decay is slow in the dark and more rapid in the light, thereby suggesting the photo-oxidative effect of chlorophyll degradation.

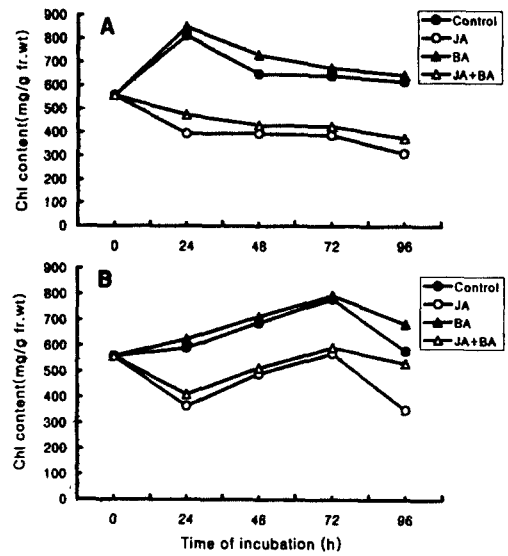

Fig. 5. Time course of chlorophyll content in detached tomato leaves floated on 10^{-4} M solutions of JA and benzyladenine, alone or in combination, in the light(A) and dark(B).

When the detached tomato leaves were floated on 10^{-4} M JA and BA solutions, alone or in combination in the light or dark, the chlorophyll content did not change markedly in the senescing leaves and BA only exhibited a slight senescence-retarding effect. In contrast, a significant decrease in the chlorophyll level was observed in the JA-treated leaves. This decrease was partially reversed by BA. The leaves yellowed quickly and 15 h after JA application the difference with the water control was already obvious.

Whatever the conditions, the excision and incubation of the leaves led to an increase in the PPO activity(Fig. 6). A sharp rise was observed in the illuminated leaves subjected to JA treatment. In the dark, there was a gradual increase in the PPO activity which lasted up to 72 h, thereafter the PPO activity slowly decreased. A comparison of the kinetics of both curves in the light and dark revealed that the enzyme activity was higher in the light than in the dark, thereby indicating the positive involvement of senescence under the influence of JA.

3.4. Interaction of JA and BA

As already shown in Fig. 5 for chlorophyll and

in Fig. 6 for the PPO activity, the senescence-promoting action of JA was overcome to a certain extent by BA, when both substances were added simultaneously in equimolar amounts. In other words, BA was able to at least partially eliminate the effect of JA in the illuminated leaf segments, however, it failed to prevent the chlorophyll loss caused by JA treatment in the light. This observation may reflect different velocities in the uptake of BA and JA, respectively. In order to test the rates of uptake indirectly, equimolar amounts of the two substances were added sequentially i.e. either BA was added 24 h prior to JA, or JA was added 24 h prior to BA. The results shown in Fig. 7 indicate that the drop in the chlorophyll content and rise in the PPO activity in the JA-treated leaves were unaltered when the tissues were pretreated with BA. In contrast, BA halted or even aided in the recovery of the senescence promoted by JA, when BA was added 24 h after the addition of JA.

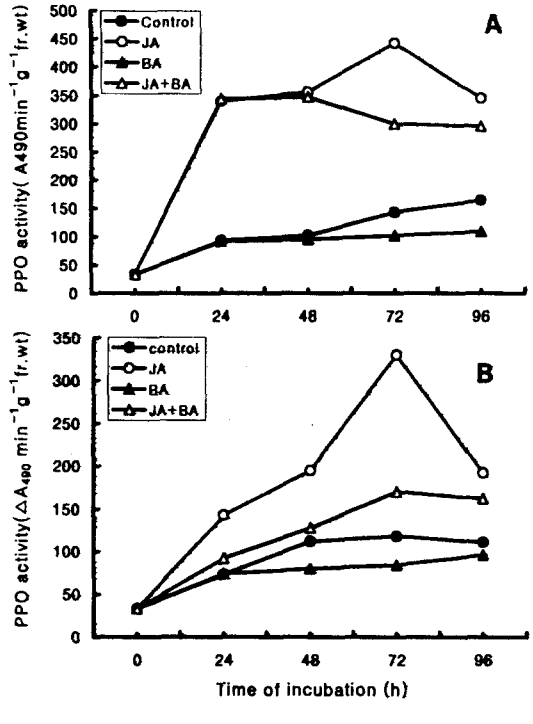

Fig. 6. Time course of PPO activity in detached tomato leaves floated on 10^{-4} M solutions of JA and benzyladenine, alone or in combination, in the light(A) and dark(B).

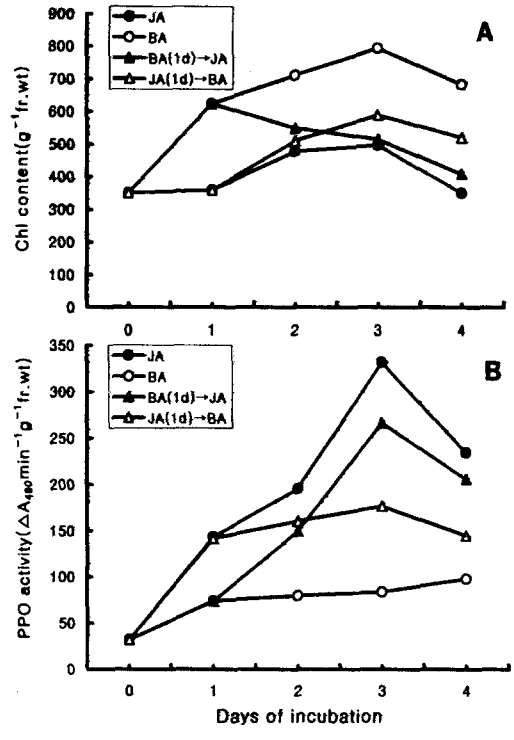

Fig. 7. Effects of sequential treatments of JA and benzyladenine on chlorophyll content(A) and PPO activity(B) in senescing leaves incubated in the dark.

3.5. Histochemical localization of PPO

An *in situ* study is highly reliable for interpreting and understanding cellular morphology and its functions since the histolocalization of enzymes provides valuable information regarding cellular reactions and metabolic processes. The details and degree of localized intensity of PPO in the control and treated plants are shown in Fig. 8.

The PPO activity was widely localized in the parenchyma tissues as granular and intense cytoplasmic reactions in the cell walls of the parenchymatous tissues, including the cortex, phloem, and pith parenchyma. The intensity of tissue localization was generally moderate to fairly rich in the control plants(Figs. 8A and B), whereas the PPO activity was fairly rich to intense in the parenchyma cells in the wounded plants. A strong PPO activity was observed in the cells of the outer cortex and phloem parenchyma in the JA-treated plants(Fig. 8D).

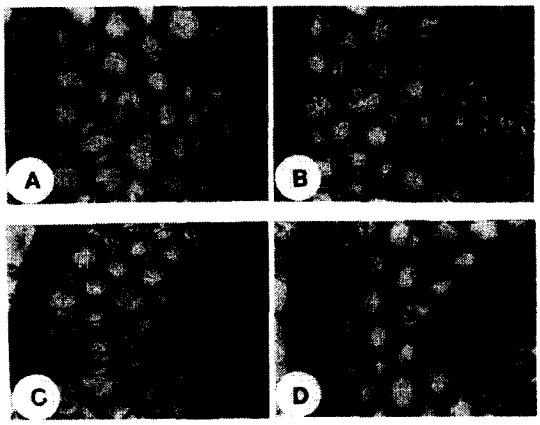

Fig. 8. Transverse section of shoot of two-week-old tomato seedling showing localization of polyphenol oxidase activity. x 100. A, control (untreated catechol) ; B, control(treated atechol) ; C, wounded plant ; D, JA-treated plant. E, epidermis ; CP, cortical parenchyma ; Ph, phloem ; X, xylem ; P, pith.

4. Discussion

Leaf senescence is a sequence of degradative processes leading to the remobilization of nutrients and eventual leaf death. The senescence process is highly regulated, involving photosynthetic decline, protein degradation, lipid peroxidation, and chlorophyll degradation[24]. The results from the current study clearly established that JA significantly promotes senescence and increases the PPO level in detached tomato leaves, which is in agreement with earlier reports[28,29]. It was also observed that, although the PPO activity increased in the control leaves during senescence, the JA-treated leaves had a higher PPO activity than the control leaves(Fig. 2).

The observations in the current study on the senescence-promoting action of JA in detached tomato leaves confirm those with detached barley and oat leaves[30,31]. In addition, the PPO activity was utilized as a sensitive measure of the senescence syndrome. Therefore, JA is clearly a potent tool for studying leaf senescence, since it can accelerate the processes going on in non-treated leaf segments at a much lower rate. This activity does not appear to be species-specific, as other plant species respond in a similar manner although less dramatically. JA appeared to be more active in chlorophyll and carotenoid degradation than MeJA when administered to primary leaves from intact barley seedlings[31]. MeJA has been found to accelerate senescence in detached barley leaf segments, as indicated by the loss of chlorophyll and rapid decrease in the activity and immunoreactive protein of ribulose-1,5-bisphosphate carboxylase[31]. It is suggested that MeJA acts on chloroplast senescence by promoting cytoplasmic events which eventually bring about the degradation of chloroplast constituents and a *de novo* synthesis of new abundant cytoplasmic polypeptides; these may be a cause rather than a consequence of the senescence syndrome.

The action mechanisms of JA and PPO in senescence promotion in isolated leaf tissues are unknown. In the former case, it is suggested that JA may act indirectly via a general speeding up of chloroplast senescence as a result of a catabolic process in the cytoplasm which is amplified or accelerated by jasmonates. Such a process could include a JA-triggered activation of the *de novo* synthesis of endopeptidases. Alternatively, JA may destroy the integrity of the chloroplasts by attacking their membranes, thereby degrading the chlorophyll level.

The pronounced effect of JA on leaf senescence in the light is interesting and points to an involvement of jasmonates in a light-dependent process. These substances may facilitate photooxidative effects on pigments. A light-promoted activation or transformation of JA into a biologically more active form is also possible. Conversely, the uptake of JA at the action sites could be accelerated in the light. This seems to merit further investigation.

Assuming that the two compounds JA and BA reach their site(s) of action using about the same uptake kinetics, the data in Fig. 7 can be explained based on a rapid activation and then inactivation of JA in the leaf segments. This assumption seems justified for two reasons : (i) BA pretreatment is unable to protect the leaf tissues against the senescence-promoting activity of JA. (ii) BA can stop or even partially restore the chlorophyll loss caused by JA when BA is added after JA has seemingly disappeared as an active compound. Both observations point to different modes of action for the two substances rather than competition for

the same target.

Jasmonates are biologically similar to ABA. Many of the physiological responses to jasmonates are similar to the effects caused by ABA[9]. To a certain extent, the action of ABA is similar to the inhibiting effect of ABA in a number of photosynthetic parameters and senescence.

The increased PPO activity during detached leaf senescence determined whether or not the tissue was capable of *de novo* synthesis in the plant species studied. This rise in enzyme activity was probably due to additional enzyme activity following detachment. Some controversial reports have suggested that the behavior of the enzyme increases during senescence in some species, whereas shows a decreasing pattern in others[32]. Although the tendency of PPO activity to increase has been noted during detached leaf senescence, its distribution is different in attached leaves ; this clearly shows that PPO activity cannot be taken an indicator of leaf senescence.

Senescence does not involve growth, although it is indeed an active process, since it depends on protein synthesis and stomatal movement, consumes energy, and is strongly modified by visible light. The fact that wounding delays or inhibits the senescence process is somewhat surprising, since a number of general indications would seem to indicate the reverse[33]. It is well known that wounding induces the formation of ethylene in most tissues and this is equally true of leaves. So far, the only clear relationship between wounding and the senescence of leaves is related to wound ethylene. It has been previously shown that a protein inhibitor is produced as a result of wounding and that it is formed in a leaf adjacent to the leaf that was actually wounded. The direct evidence that leaf proteinases are inhibited strengthens the possibility that the wound response does result from the formation of a protein inhibitor. Wounding does not simply result in the retention of existing proteins but also involves the appearance of some new ones.

When tomato plants were previously tested for the induction of foliar activity using wounding and JA, PPO activity was also detected in the unwounded controls. The almost ubiquitous occurrence of constitutive PPO in the plant kingdom has been previously reported by Sherman et al.[4]. The levels of measured PPO activity vary widely. This may partly be due to differing substrate preferences of PPOs from different plants. The species surveyed varied widely in their capacity to respond to wounding and JA with increased PPO activity[6]. The systemic induction of PPO by wounding and JA in tomato plants has been previously reported, along with the systemically wound-induced accumulation of PPO proteins in potato plants[34]. Interestingly, the wound-induction of PPO generally correlates with JA-induction. JA or closely related jasmonates are components of the tomato octadecanoid pathway which has been proposed to transduce wound signals inside the cell. Accordingly, PPO will be a very useful defense response maker in further analyses of the signalling pathways in these plants.

The histochemical localization of PPO in cell walls and in the cytoplasm of the parenchyma cells in tomato plants was identical with observations of other plants[26,27]. The increased PPO activity in the cytoplasm, cortex, and pith parenchyma indicates their association with respiratory activity, cellular differentiation, and ion transport. Plants oxidized by oxidases are highly reactive and are known to combine with the acids and proteins of pathogens or herbivores, rendering them biologically inactive[26]. As such, an increase in the phenolic compounds and PPO activity along with other metabolites can be considered to play a role in the restriction of a pathogen or herbivore attack.

It is already known that a small amount of foliar PPO can be induced as a result of mechanical damage, and likewise oxidative defenses including PPO are differentially induced depending on the type of damage. Alternatively, the lack of PPO activity in some plants may reflect specialized plant defense mechanisms, since these plants are still capable of activating other defenses. For example, *Brassica napus* accumulates significant levels of glucosinolates in wounded or MeJA-treated leaves[35], whereas the PPO activity remains unaffected by these treatments. Conserving the signalling pathways, while adapting responses to specific ecological conditions, may be one strategy of plants in the evolutionary race with insects.

In highly-inducible species such as tomato and tobacco, PPO is most likely to function as a defensive anti-nutritive protein. However, in species

with apparently little or no wound-inducible PPO, such a function is less certain ; perhaps PPO plays a different role in these plants. The almost ubiquitous distribution of PPO may indicate that this enzyme has evolved for another, as yet undiscovered role, while some plants have employed it as a defense against insect herbivores.

In addition, an increase in PPO activity following pathogen infection has been reported in old tomato leaves[36]. Mechanical wounding also results in both localized and systemic wound induction. Tomato plants overexpressing systemin exhibit elevated PPO levels, in addition to protein inhibitors. The finding that PPO accumulation is induced by wounding, systemin, and methy jasmonate suggests that tomato seedling PPO is regulated via the octadecanoid signalling pathway.

The disruption of plant cells by injury not only causes the release of PPOs from thylakoid association, thereby facilitating interaction with phenolic substances, but also systemically induces the *de novo* synthesis of PPOs in young leaf tissue, presumably to protect the growing part of the plant. Hence, PPO can be considered as both an inducible and constitutive component of plant defences. Further studies are necessary to determine whether such increases in PPOs afford a significantly protective effect against herbivores or pathogens.

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