Phenylalanine Ammonia-lyase Inhibitors Do Not Prevent Russet Spotting Lesion Development in Lettuce Midribs

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ABSTRACT. Russet spotting is a physiological disorder of lettuce (*Lactuca sativa* L.) caused by exposure to hormonal levels ($<1 \mu\text{L}\cdot\text{L}^{-1}$) of ethylene in air at $\approx 5^\circ\text{C}$. Enhanced phenolic metabolism and the accumulation of phenolic compounds accompany the appearance of brown, oval lesions on the leaf midrib. Phenylalanine ammonia-lyase (PAL) is the first committed enzyme in the phenylpropanoid pathway. Three inhibitors of PAL activity [2-aminoindan-2-phosphonic acid (AIP), α -aminooxyacetic acid (AOA), and α -aminooxi- β -phenylpropionic acid (AOPP)] greatly reduced the accumulation of phenolic compounds and browning of lesions. At a concentration of $50 \mu\text{M}$, AIP inhibited the formation of chlorogenic and dicaffeoyl tartaric acids in cut midribs of iceberg lettuce by 92% and 98%, respectively. AIP competitively inhibited PAL activity from a lettuce midrib homogenate with an apparent K_i of 22 nM. While the formation of phenolic compounds was strongly inhibited by AIP, the number of lesions associated with russet spotting was not affected. Only the color of the lesions was affected by AIP. In control midribs the russet spotting lesions were brown while those in the AIP-treated midribs were initially olive green and after 3 to 7 days these lesions turned the characteristic brown color. No tyrosine ammonia-lyase activity was detected in a homogenate of lettuce midrib tissue. These results indicate that the early development of russet spotting lesions is independent of the increase in PAL activity and phenolic compounds rather than an effect of these increases as previously suggested. However, accumulation of phenolic compounds does contribute to the subsequent browning symptoms indicative of russet spotting.

Russet spotting (RS) is a physiological disorder of iceberg lettuce that manifests itself as oval, brownish spots or lesions, mainly on the achlorophyllous midribs (Link and Gardner, 1919; Lipton, 1961; Lipton et al., 1972). RS occurs in lettuce that has been exposed to hormonal levels ($<1 \mu\text{L}\cdot\text{L}^{-1}$) of ethylene in air at $\approx 5^\circ\text{C}$ (Rood, 1956). Yet biotic and abiotic stresses during growth or after harvest can predispose lettuce to develop RS lesions even when the lettuce has not been exposed to exogenous ethylene. It has been suggested that this results from the endogenous production of stress ethylene (Ke and Saltveit, 1988; Lipton et al., 1972; Moline and Lipton, 1987; Ritenour et al., 1996).

Several studies have described a relationship between the activity of phenylalanine ammonia-lyase (PAL, the first committed enzyme in the phenylpropanoid pathway) in iceberg lettuce leaf tissue and the development of RS symptoms (Hyodo et al., 1978; Ke and Saltveit, 1986, 1988, 1989a, 1989b; Ritenour et al., 1996). Hyodo et al. (1978) observed that an ethylene-induced increase in PAL activity paralleled the appearance of RS symptoms. They also measured an increase in total phenolic compounds. It has been proposed that ethylene induces PAL activity and the resulting accumulation of phenolic compounds in cells leads to their discoloration and eventual death (Hyodo et al., 1978; Ke and Saltveit, 1988, 1989b). However, although increased

phenolic metabolism and the accumulation of phenolic compounds could be induced by physical stress, induction of characteristic RS lesions did not occur unless the tissue had been exposed to ethylene at the inducing temperature. Obviously, ethylene had other effects in addition to stimulating phenolic metabolism, and these effects might be paramount in the formation of RS lesions.

Compounds such as 2-aminoindan-2-phosphonic acid (AIP), α -aminooxyacetic acid (AOA), and α -aminooxi- β -phenylpropionic acid (AOPP) have been well characterized as PAL inhibitors in several tissues (Amrhein, 1979; Amrhein and Gödeke, 1977; Hanson and Havir, 1981; Zon and Amrhein, 1992) and no inhibitory activity of phenylalanine-tRNA synthetase was found with AOPP or AIP (Leubner-Metzger and Amrhein, 1994; Zon and Amrhein, 1992). However, these PAL inhibitors have not been used to demonstrate a direct link between PAL activity and the development of RS in lettuce.

Therefore, the purpose of this study was to examine the relationship between PAL activity and the development of RS using PAL inhibitors. We present evidence indicating that an ethylene-induced increase in PAL activity is not a prerequisite for the development of the russet spotting lesions, though the brown discoloration of the cells does require PAL activity.

Materials and Methods

LETTUCE SAMPLES AND TISSUE PREPARATION. Iceberg lettuce (*Lactuca sativa*) was obtained from a local supermarket (unknown cultivar), a wholesale distributor ('Salinas', 'Honcho-2') or a shipper/processor in Arizona ('Desert Storm'). Whole lettuce heads were used immediately after arrival at the lab or stored at 2.5°C until used.

Lettuce wrapper leaves were discarded and the next five to eight leaves were gently removed from the core and the white midribs were excised. Midrib segments were trimmed to produce 5×7 -cm sections. Sections were rinsed with 50 ppm chlorinated water (5%

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Fig. 1. Effect of 0 and 50 μM 2-aminoindan-2-phosphonic acid (AIP) on chlorogenic (upper panel) and dicaffeoyl tartaric (lower panel) acid concentrations on a fresh mass basis in lettuce midribs held at 5 °C in air. The phenolic compounds were measured using reverse-phase high-performance liquid chromatography as described in Materials and Methods. The vertical bars represent the standard deviations.

commercial bleach) and centrifuged with a manual salad spinner to remove excess solution. Pieces were randomly mixed and treated with appropriate inhibitor solutions (see below). After treatment, 10 midrib pieces were placed in 2-L plastic containers and connected to air flow-through systems to maintain CO_2 levels <0.2% and to provide atmospheres of humidified air $\pm 5 \mu\text{L}\cdot\text{L}^{-1}$ ethylene at 5 °C.

CHEMICALS. The PAL inhibitor 2-aminoindan-2-phosphonic acid (AIP) was kindly provided by Nikolaus Amrhein. α -Aminooxyacetic acid (AOA) and α -aminooxy- β -phenylpropionic acid (AOPP) were purchased from Sigma Chemical Co. (St. Louis) and Cambridge Research Biochemicals Ltd. (Northwich, Cheshire, U.K.), respectively.

TREATMENTS. Midrib sections were soaked for 1 h at 20 °C in aqueous solutions of the PAL inhibitors. After treatment, pieces were centrifuged with a manual spinner to remove excess solution.

LESIONS ASSOCIATED WITH RUSSET SPOTTING. Lesions or spots, whether olive green or brown, were subjectively rated using a 1 to 9 hedonic scale as described previously for russet spotting where 1 = no lesions and 9 = severe lesion development (Ke and Saltveit, 1986).

PHENOLIC COMPOUNDS. High-performance liquid chromatography (HPLC) analysis was done with a Hewlett Packard Series 1050 gradient system (Linc Quantum Analytics, Foster City, Calif.)

with a photodiode array detector. Separations were conducted with a Nucleosil C-18 reverse-phase column (Phenomenex, Torrance, Calif.) using 0.05 M ammonium phosphate buffer (pH 2.8) (solvent A) and 4 methanol : 1 ammonium phosphate buffer (v/v) (Solvent B). An authentic standard (Sigma Chemical) of chlorogenic acid was used for peak identification by co-chromatography and for quantification by its absorbance at 320 nm. Since no standard for dicaffeoyl tartaric acid was available it was identified based on its UV spectra and co-chromatography with the major phenolic compound from green Belgium endive (*Cichorium endivia* L.) tissue, which has been characterized as dicaffeoyl tartaric acid (Wöldecke and Herrmann, 1974). Dicaffeoyl tartaric acid was quantified based on the chlorogenic acid standard and corrected for the difference in absorbance using the molar absorptivity at 330 nm of $1.95 \times 10^4 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for chlorogenic acid and $3.16 \times 10^4 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for 3,5-dicaffeoyl quinic acid (Trugo and Macrae, 1984).

ENZYME ASSAYS. PAL activity was measured in lettuce tissue extracts as previously described (Ke and Saltveit, 1986). Tyrosine ammonia-lyase (TAL) activity was measured by a similar procedure with L-tyrosine replacing L-phenylalanine in the assay.

UV-VISIBLE SPECTRA MEASUREMENT. Sections of midrib tissue with a large number of lesions were extracted in 50% ethanol, filtered and the UV-visible spectra measured on a Shimadzu (Kyoto, Japan) UV-160A spectrophotometer.

Results

EFFECTS OF INHIBITORS ON PHENOLIC LEVELS. There were large increases in chlorogenic acid and dicaffeoyl tartaric acids in the ethylene-free, air control tissue by day 6. AIP at 50 μM inhibited the increase of these two compounds by 92% and 98%, respectively

Fig. 2. A Lineweaver-Burk plot of phenylalanine ammonia-lyase (PAL) activity (cinnamic acid formed, $\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) at various substrate (phenylalanine) concentrations and with 20 and 80 nM AIP. Enzyme extracts were prepared and assayed as described in Materials and Methods. AIP competitively inhibited PAL with an apparent K_i of 22 nM.

Fig. 3. Scores for lesion development, whether olive green or brown, in midribs treated with 0, 50, and 100 μM 2-aminoindan-2-phosphonic acid (AIP). After treatment the midribs were held at 5 °C in air (upper panel) or 5 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene in air (lower panel). Description of scoring system is given in Materials and Methods; score = 1, no lesions and score = 9, severe lesion development. The vertical bars represent the standard deviations.

(Fig. 1). We observed over 90% reductions in these two phenolic compounds with AOPP. Other unidentified peaks were also present in the control samples and AIP also strongly inhibited their formation (data not shown). AIP inhibits phenylpropanoid metabolism (e.g., PAL activity) in other tissues at similar concentrations (Keller et al., 1990; Sato et al., 1993). These data suggest that these two compounds inhibit *in vivo* PAL activity in lettuce and prevent the production of phenolic compounds that could lead to brown polymerized compounds on oxidation.

Additionally, we measured the effect of AIP on PAL activity in the supernatant fraction from a lettuce midrib tissue homogenate and determined the apparent K_i to be ≈ 22 nM (Fig. 2). As has been previously reported, AIP acted as a competitive inhibitor of PAL (Zon and Amrhein, 1992).

EFFECT OF PAL INHIBITORS ON RUSSET SPOTTING DEVELOPMENT. We did not observe a marked difference in RS development between the air and ethylene treatments; the air samples usually developed RS to a similar extent as the ethylene treated samples. This suggests that in most cases the lettuce had been exposed to

stress conditions in the field or after harvest to induce elevated phenolic metabolism and RS.

In general, the number of spots or lesions in the control and inhibitor-treated midribs in both the air- and ethylene-treated samples were similar (Fig. 3). In the air samples there appeared to be a slight reduction in the number of lesions with 100 μM AIP, yet in the ethylene treatment, the number of lesions with both 50 and 100 μM AIP was the same as in the control. There was variability in the response of the tissue to AIP treatments. In one experiment, the number of lesions on 100 μM AIP treated midribs was the same in both the air and ethylene treatments (data not shown). Yet in another experiment, we observed a large number of lesions (score = 5) in the AIP treated tissue at day 3 when there were no lesions in the control tissue. At day 6 the control tissue had developed lesions and by day 9 the number of lesions in the AIP treated and control tissues was similar.

Although we observed little difference in the number, size or shape of spots associated with RS that developed in the control and inhibitor-treated midribs, we did observe a marked difference in the color development of the spots. In the control tissue the lesions were brown, while those in the inhibitor-treated tissue initially were olive green. In the inhibitor-treated tissue, the olive green lesions eventually turned brown 3 to 7 d after their appearance, at which point they were indistinguishable from the lesions in the control tissue.

Areas of midribs exhibiting olive green lesions from AIP-treated and brown lesions from control midribs were extracted in 50% ethanol and their spectra were measured (Fig. 4). The absorption peak near 520 nm in the extract from the control tissue was absent in the extract from the AIP-treated tissue.

We examined the development of the spots in the air controls (without inhibitors) using a video microscope with 100 $\times$ lens. In the early stage of RS development, we observed individual olive-green epidermal cells. The development of RS from individual cells has been previously reported, although a description of their color development was not given (Ilker et al., 1977). By noting the position of these individual olive-green cells on the midrib we were able to observe the same cells for several days (≈ 15 cells were followed). From an individual olive-green cell the color began to spread to adjacent cells usually within 1 to 3 d, after which the central part of the spot turned brown. In no case did we observe the development of RS from a single cell that was brown rather than

Fig. 4. UV-visible absorption spectra of 50% ethanol extracts of midrib tissue 6 d after treatment with 0 or 50 μM 2-aminoindan-2-phosphonic acid (AIP). Midribs from the 0- μM treatment had brown lesions while those from the 50- μM treatment had olive-green lesions.

olive green. In control tissue, when the spots were observable without magnification, the spots appeared brown. In the midribs treated with PAL inhibitors, browning of the spots was delayed such that olive-green spots of the same size as the brown spots in controls were readily visible without magnification.

Discussion

Two major phenolic compounds in stressed and ethylene exposed lettuce are chlorogenic acid and dicaffeoyl tartaric acid (Tomas-Barberan et al., 1997; Winter and Herrmann, 1986). The three PAL inhibitors significantly inhibited an increase in these two phenolic compounds (Fig. 1). That these compounds inhibited PAL activity and reduced the accumulation of phenolic compounds is supported from another study where we observed that these same chemicals inhibited the brown discoloration of cut lettuce midribs. In the present study, although the production of phenolic compounds was almost completely inhibited, there was no inhibition of lesion formation associated with RS (Fig. 3). Yet there was an effect on the color formation in the lesions. In tissue treated with the inhibitors the lesions remained olive green several days longer than lesions in the controls. These results with PAL inhibitors indicate that the formation of the lesions and browning of the lesions (presumably from the oxidation and polymerization of phenolic compounds) can be temporally separated. This suggests that an increase in PAL activity and the production of phenolic compounds are probably a secondary effect of the formation of RS lesions and not the primary cause as has been previously proposed (Ke and Saltveit, 1988, 1989b).

An alternative interpretation of our results could be that even though PAL activity was significantly inhibited, another enzyme such as TAL might be unaffected by the PAL inhibitors and lead to the formation of enough phenolic compounds to directly cause the RS lesions. However, this is unlikely since we were unable to measure an increase in phenolic compounds in the presence of AIP (Fig. 1) and AIP also inhibits TAL activity (Zon and Amrhein, 1992). Also, we were unable to detect any TAL activity in a lettuce midrib tissue homogenate.

When the lesions first appeared on the inhibitor-treated midribs, they were olive green. Yet after 3 to 7 d, these lesions began to slowly turn the characteristic brown color of RS lesions. In the presence of AOPP, the amount of PAL enzyme (Amrhein and Gerhardt, 1979; Noé and Seitz, 1982) and PAL mRNA (Bolwell et al., 1988) levels are greatly increased, due primarily to the lack of feedback regulation by cinnamic acid (Bolwell et al., 1988; Mavandad et al., 1990). Presumably AIP would have a similar effect on PAL enzyme and mRNA levels. Therefore, 3 to 7 d after the olive-green lesions had appeared, enough PAL enzyme may have been produced to overcome the inhibition and form enough phenolic compounds to produce the brown lesions typical of RS. After 9 d, however, phenolic compounds were still at very low levels in the AIP-treated tissue.

The available evidence indicates that ethylene is important for the development of RS lesions, though it is unclear what process is initiated by ethylene that causes the lesions. Auxin application can inhibit RS lesion development (Ke and Saltveit, 1988), yet Ritenour et al. (1996) were unable to establish a relationship between endogenous IAA levels and RS development. RS development in midrib sections treated with calcium was also inhibited (Ke and Saltveit, 1986). We know little about the olive-green color observed in the lesions from the AIP-treated midribs and in the controls in the very early stages of lesion development. Greenish compounds could be formed from the reaction of amino acids with

phenolic compounds such as chlorogenic acid (Adams, 1994). However, the olive-green lesions from midribs treated with AIP were probably not from chlorogenic acid since the absorption maximum for a chlorogenic acid–glutamine complex was at 680 nm, while there was essentially no absorption beyond 540 nm in the lettuce extract (Fig. 4). Also, AIP inhibited the formation of chlorogenic acid (Fig. 1). Yet other greenish-colored complexes between amino acids and gallic acid, whose synthesis would not be inhibited by AIP, can form in plant tissues (Robinson, 1967). Further work is required to identify the compound(s) responsible for the olive-green color.

In conclusion, we present evidence that inhibitors of PAL activity block the formation of phenolic compounds, yet this did not block the formation of RS lesions. This indicates that ethylene induces some other process(es) in addition to the induction of PAL, with the subsequent formation of phenolic compounds. It is this other process that causes the initiation of the RS lesions, and not the phenolic compounds themselves, as has been previously suggested. The role of PAL induction and phenolic metabolism in RS development is to contribute to the subsequent brown discoloration of the RS lesions.

Literature Cited

- Adams, J. B. 1994. Green colour development in potato cooking water. *Food Chem.* 49:295–298.
- Amrhein, N. 1979. Novel inhibition of phenylpropanoid metabolism in plants, p. 173–182. In: M. Luckner and L. Schreiber (eds.). *Regulation of secondary products and plant hormone metabolism*. Pergamon Press, Oxford.
- Amrhein, N. and J. Gerhardt. 1979. Superinduction of phenylalanine ammonia-lyase in gherkin hypocotyls caused by the inhibitor, L- α -aminooxy- β -phenylpropionic acid. *Biochim. Biophys. Acta* 583:434–442.
- Amrhein, N. and K.H. Gödeke. 1977. α -Aminooxy- β -phenylpropionic acid—A potent inhibitor of L-phenylalanine ammonia-lyase in vitro and in vivo. *Plant Sci. Lett.* 8:313–317.
- Bolwell, G.P., M. Mavandad, D. Millar, K. Edwards, W. Schuch, and R. Dixon. 1988. Inhibition of mRNA levels and activities by *trans*-cinnamic acid in elicitor-induced bean cells. *Phytochemistry* 27:2109–2117.
- Hanson, K. and E. Havir. 1981. Phenylalanine ammonia-lyase, p. 577–625. In: P.K. Stumpf and E.E. Conn (eds.). *The biochemistry of plants. A comprehensive treatise*, vol. 7. Academic Press, New York.
- Hyodo, H., H. Kuroda, and S.F. Yang. 1978. Induction of phenylalanine ammonia-lyase and increase in phenolics in lettuce leaves in relation to the development of russet spotting caused by ethylene. *Plant Physiol.* 2:31–35.
- Ilker, Y., A.A. Kader, R. Ilker, and L.L. Morris. 1977. Anatomy of lettuce tissue affected by three physiological disorders. *J. Amer. Soc. Hort. Sci.* 102:426–428.
- Ke, D. and M.E. Saltveit. 1986. Effects of calcium and auxin on russet spotting and phenylalanine ammonia-lyase activity in iceberg lettuce. *HortScience* 5:1169–1171.
- Ke, D. and M.E. Saltveit. 1988. Plant hormone interaction and phenolic metabolism in the regulation of russet spotting in iceberg lettuce. *Plant Physiol.* 88:1136–1140.
- Ke, D. and M.E. Saltveit. 1989a. Developmental control of russet spotting, phenolic enzymes, and IAA oxidase in cultivars of iceberg lettuce. *J. Amer. Soc. Hort. Sci.* 114:472–477.
- Ke, D. and M.E. Saltveit. 1989b. Regulation of russet spotting, phenolic metabolism, and IAA oxidase by low oxygen in iceberg lettuce. *J. Amer. Soc. Hort. Sci.* 114:638–642.
- Keller, B., D. Jierhaus-Wunderwald, and N. Amrhein. 1990. Deposition of glycine-rich structural protein in xylem cell walls of french bean seedlings is independent of lignification. *J. Structural Biol.* 104:144–149.
- Link, G.E. and M.W. Gardner. 1919. Market diseases of vegetables. *J. Amer. Soc. Hort. Sci.* 123(4):687–691. 1998.

- Phytopathology 9:497–520.
- Lipton, W.J. 1961. Anatomical observations on russet spotting and pink rib of lettuce. *Proc. Amer. Soc. Hort. Sci.* 78:367–374.
- Lipton, W.J., J.K. Stewart, and T.W. Whitaker. 1972. An illustrated guide to the identification of some market disorders of head lettuce. *USDA Mktg. Res. Rpt.* 950.
- Leubner-Metzger, G. and N. Amrhein. 1994. Phenylalanine analogues: potent inhibitors of phenylalanine ammonia-lyase are weak inhibitors of phenylalanine-tRNA synthetases. *Z. Naturforsch.* 49C:781–790.
- Mavandad, M., R. Edwards, X. Liang, C. Lamb, and R. Dixon. 1990. Effects of trans-cinnamic acid on expression of the bean phenylalanine ammonia-lyase gene family. *Plant Physiol.* 94:671–680.
- Moline, H.E. and W.J. Lipton. 1987. Market diseases of beets, chicory, endive, escarole, globe artichokes, lettuce, rhubarb, spinach, and sweet potatoes. *USDA Hdbk.* 155.
- Noé, W. and H.U. Seitz. 1982. Induction of de novo synthesis of phenylalanine ammonia-lyase by L- α -aminooxy- β -phenylpropionic acid in suspension cultures of *Daucus carota* L. *Planta* 154:454–458.
- Ritenour, M., E.G. Sutter, D.M. Williams, and M.E. Saltveit. 1996. Indole-3-acetic acid (IAA) content and auxillary bud development in relation to russet spotting in harvested iceberg lettuce. *J. Amer. Soc. Hort. Sci.* 121:543–547.
- Robinson, T. 1967. *The organic constituents of plants.* 2nd ed. Burgess Publishing Co., Minneapolis.
- Rood, P. 1956. Relation of ethylene and postharvest temperature to brown spot of lettuce. *Proc. Amer. Soc. Hort. Sci.* 68:296–303.
- Sato, Y., M. Sugiyama, R. J. Górecki, H. Fukuda, and A. Komamine. 1993. Interrelationship between lignin deposition and the activities of peroxidase isoenzymes in differentiating tracheary elements of *Zinnia*. *Planta* 189:584–589.
- Tomas-Barberan, F., J. Loaiza-Velarde, A. Bonfanti, and M.E. Saltveit. 1997. Early wound- and ethylene-induced changes in phenylpropanoid metabolism in harvested lettuce. *J. Amer. Soc. Hort. Sci.* 122:399–404.
- Trugo, L.C. and R. Macrae. 1984. Chlorogenic acid composition of instant coffees. *Analyst* 109:263–266.
- Winter, M. and K. Herrmann. 1986. Esters and glucosides of hydroxycinnamic acids in vegetables. *J. Agr. Food Chem.* 34:617–620.
- Wöldecke, M. and K. Herrmann. 1974. D-(+)-dicaffeoyl tartaric acid from endives (*Cichorium endivia* L.) *Z. Naturforsch.* 29C:360–361.
- Zon, J. and N. Amrhein. 1992. Inhibitors of phenylalanine ammonia-lyase: 2-Aminoindan-2-phosphonic acid and related compounds. *Liebigs Ann. Chem.* p. 625–628.