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FINAL REPORT

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DOE funding our research concentrated on the investigation of the role of respiration and oxidative stress in plant biology.

(I). Initially we concentrated on the possible role of cyanide-insensitive respiration in counteracting the deleterious effects of chilling stress. Although plants are considered to be poikilotherms, there are a few examples of thermogenesis, in which the tissue temperature increases well above ambient. We suggested that differences between thermogenic and non-thermogenic plants may be quantitative rather than qualitative, and that heat from increased respiration may have a local protective effect on the mitochondria, slowing or reducing the effects of chilling. We proposed that this is accomplished by a large increase in respiration, predominantly via the alternative pathway. We measured the increases in respiration, particularly via the alternative pathway, in response to chilling. We have also quantified the associated increases in heat evolution in response to chilling in a number of plant species using a microcalorimeter. For example, after 8 h exposure to 8°C, heat evolution in chilling-sensitive species increased 47-98%, compared to 7-22% for the chilling-resistant species. No increase in heat evolution was observed in the extremely chilling-sensitive ornamental *Episcia cupreata* (Hook). Increases in heat evolution were observed when plants were chilled in constant light or in the dark, but not when plants were chilled at high humidity. Heat evolution by mitochondria isolated from potato tuber slices were also measured. These values, together with measurements of the heat capacity of isolated mitochondria and counting of the mitochondria by flow cytometry, allow calculation of theoretical maximal rates of heating and the heat produced per mitochondrion. The obtained data was consistent with the protective role of respiratory heat production in cold-stressed plants.

The results of this research were published in: Moynihan, M. R., R. Linzer, A. Ordentlich, and I. Raskin. 1995. Chilling-induced heat evolution in plants. Plant Physiol. 108, 995-999.

(II). Pathogenesis is another stress commonly encountered by plants that is associated with increased respiration. The oxidative burst, produced as a result of this increase and the associated production of active oxygen species is one of the earliest events in pathogenesis. We observed that hydrogen peroxide, commonly produced during the oxidative burst induced the accumulation of free benzoic acid (BA) and salicylic acid (SA) in tobacco (*Nicotiana tabacum* L. cv Xanthi-nc) leaves. SA is an important signaling compound that activates defense responses in infected plants. Six

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hours after infiltration with 300 mM H_2O_2, the levels of BA and SA in leaves increased five-fold over the levels detected in control leaves. The accumulation of BA and SA was preceded by the rapid activation of benzoic acid 2-hydroxylase (BA2H) in the H_2O_2-infiltrated tissues. This enzyme catalyzes the formation of SA from BA. We observed that the enzyme activation was not a result of changes in the amount of BA2H protein and could be reproduced *in vitro* by addition of H_2O_2 or cumene hydroperoxide to the assay mixture. H_2O_2 was most effective *in vitro* when applied at 6 mM concentration. *In vitro* activation of BA2H by peroxides was inhibited by the catalase inhibitor 3-amino-1,2,4-triazole. We suggested that H_2O_2 activates SA biosynthesis via two mechanisms. Firstly, H_2O_2 stimulates BA2H activity directly or via the formation of its substrate, molecular oxygen, in a catalase-mediated reaction. Secondly, H_2O_2 releases BA from the conjugated pool. Higher BA levels induce the accumulation of BA2H protein in the cells and provide more substrate for this enzyme.

The results of this research were published in: León, J., M. A. Lawton, and I. Raskin. 1995. Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiol. 108, 1673-1678.

(III). During the last funding year we made substantial progress in understanding the role of oxidative stress in the resistance response of TMV-inoculated tobacco plants. The most important results are mentioned below:

We are developing a method for measurement of hydroxyl radicals in tobacco leaves during pathogenesis. This method utilizes the ability of hydroxyl radicals to non-enzymatically hydroxylate aromatic compounds, such as salicylic acid (SA). The dihydroxylated compounds formed are 2,3 and 2,5 dihydroxybenzoic acid (DHBA), which can be quantified by HPLC- EC. This method has been successfully used in studying oxidative injury in mammalian organs. We wanted to test whether we could monitor the production of ·OH in TMV-inoculated and healthy tobacco plants by using endogenous SA as a hydroxylation substrate. We succeeded in adopting the HPLC-EC system to measure DHBAs in extracts from healthy and TMV-inoculated tobacco leaves.

Three different experimental treatments were used to determine the kinetics of DHBA production; TMV-inoculation of tobacco, and two abiotic treatments that are known to generate active oxygen species: paraquat (a commonly used herbicide) and UV-irradiation of tobacco. Both paraquat and UV produced a large increase in DHBAs in tobacco leaves infiltrated with SA. A temperature shift system was also employed, whereby tobacco plants inoculated with TMV were kept at 32°C for 4 days and then shifted to 24°C. The temperature shift allows much stronger manifestation of hypersensitive response that follows TMV inoculation of Xanthi-nc tobacco. The levels of free DHBAs in TMV-infected tobacco leaves increased more than ten-fold (21.4 ng/gFW) at 10

hours after temperature shift, paralleling the increase in free SA. No increase was detected in mock-inoculated plants shifted to 24°C. Our data indicate that SA functions as a scavenger of hydroxyl radicals produced during the hypersensitive response. The formation of dihydroxybenzoic acids in the tissue may be a result of non-enzymatic hydroxylation of SA and can be used to quantify the severity of the oxidative stress.

The results were published in: León, J., M.A. Lawton, and I. Raskin. 1995. Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiol. 108, 1673-1678.
and in Sharma, Y.K., J. León, I. Raskin, K.R. Davis. 1996. Ozone-induced responses in Arabidopsis thaliana: The role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. Proc. Natl. Acad. Sci. USA.93, 5099-5104.