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Nicole Christine Ott, having been admitted to the Carl and Winifred Lee Honors College in 1994, successfully presented the Lee Honors College Thesis on April 22, 1998.

The title of the paper is:

"The Effects of Rose Bengal Application on the Production of Salicylic Acid in nahG Tobacco Plants"

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THE EFFECTS OF ROSE BENGAL APPLICATION ON THE PRODUCTION OF SALICYLIC ACID IN *nahG* TOBACCO PLANTS

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Plants have the ability to develop resistance to a wide range of pathogens following exposure to a single bacterium, fungus, or virus. This resistance occurs in all tissues, not only those that were previously infected. This mechanism is termed systemic acquired resistance (SAR). The phenolic compound salicylic acid (SA) has been linked to SAR in tobacco, *arabidopsis*, and cucumber plants. Oxidative stress also causes SA production, which leads to SAR. An engineered tobacco plant (*nahG*) that does not have the ability to accumulate SA has been used as a research tool in investigations concerning SA activity. Rose Bengal (RB), a dye which causes singlet oxygen atoms to form in the leaf mesophyll, was applied to *nahG* and wild type tobacco plants. Ethylene diurea (EDU), an antioxidant, was sprayed on prior to RB applications in some treatments. SA production was measured at 24 hour time points for 3 days. SA was shown to reach a peak at 24 hours in wild type plants treated with RB. This peak was deferred and decreased in wild type plants treated with EDU and RB both, suggesting that oxidative stress produces an SA response.
INTRODUCTION

The term salicylic acid (SA) is derived from the Latin word salix, which is the genus name for the willow tree. The healing properties of the willow tree were independently discovered by the American Indians and the ancient Greeks. However, it was not until 1828 that a German chemist named Johann Buchner isolated salicin, a glucosidic derivative of SA. By 1878 Bayer had developed Aspirin. Aspirin is the trade name for acetylsalicylic acid1.

Acetylsalicylic acid consists of an SA molecule with an extra functional group attached. Its structure is compared with that of the SA molecule in Figure 1. SA belongs to the class of plant secondary compounds called phenolics. Other classes of secondary compounds include terpenes and alkaloids. Phenolic compounds, which consist of a benzene ring with a hydroxyl group attached, are derived from the shikimic acid pathway (figure 2). This pathway is also responsible for the production of phenylalanine, tyrosine, and tryptophan, the aromatic amino acids2.

SA has been linked to the phenomenon of thermogenesis. At peak reproductive times the inflourescence of the Arum lily can heat up to 14°C above the ambient air temperature. This increase in heat results in the volatilization of amines and indoles in the plant's spadix, the central column of the inflourescence. The odors created by these compounds attract insects for pollination1. This burst of heat, which can last up to seven hours, is created by the alternative oxidase pathway of the electron transport chain. The inner membrane of the mitochondria consists of four protein complexes which transport
electrons to one another, ultimately passing them to oxygen, and shuttle hydrogen ions across the membrane. A fifth complex allows the hydrogen ions to pass back across the membrane in accordance with the established concentration gradient. This hydrogen ion flow is coupled to the production of adenosine triphosphate (ATP). Cyanide, which is toxic to animals, causes plants to utilize an alternative electron carrier. With this pathway complexes III and IV are bypassed, resulting in the energy normally produced by the action of these complexes being given off as heat.

In the absence of cyanide, some "calorigen" must be present to activate the alternative oxidase pathway. In 1987 Van Herk purified this "calorigen" and ran it through a mass spectrometer. Analysis of this molecule suggested that it is SA. To support the hypothesis that SA is the "calorigen" that causes this burst of metabolic activity, which results in degrees of oxygen consumption comparable to those occurring in the wing muscles of a humming bird in flight, exogenous SA was applied to an immature spadix of the *Arum* lily. A 12°C rise in temperature resulted. Treatment with aspirin, which undergoes spontaneous hydrolysis to SA in aqueous solutions, produced similar results. Addition of 1.0 mM SA to isolated mitochondria further supported Van Herk's hypothesis. Concentrations of the alternative oxidase protein rose 2.5 fold from control levels within 5 hours of this treatment. The levels of this protein remained two fold of controls for three days. As further support, levels of endogenous SA on the day prior to blooming of the lily's inflorescence have been found to be 100 times those of vegetative plants.

Of greater interest to scientists is SA's role in plant disease resistance. The general defense action consists of two phases: the determinate phase in which the pathogen is recognized and the expressive phase in which the plant undergoes biochemical and structural changes to initiate or increase resistance. The initial biochemical response common to all plants is the flooding of calcium out of the organelles and into the cytoplasm. A universal structural response to a disruption of the epidermal cells is the modification of the cell wall, e.g. additional crosslinking of proteins or the production of extra lignin.

Tobacco plants exhibit a hypersensitive response (HR) following pathogen attack. The HR is a protective cell suicide in which the cells surrounding a cell infected with a virus, such as the tobacco mosaic virus (TMV), senesce to prevent the virus from utilizing them for its propagation. The plant can also initiate a systemic acquired resistance pathway.
(SAR) response following pathogen attack. SAR results in immunity to attack, not only by the original pathogen, but by many others, such as other viruses, bacteria, or fungi. The entire plant, not just the previously injured portions, exhibits this protection. The level of SA in plant tissues has been correlated with the level of SAR. This phenomenon can also be initiated (in the absence of pathogens) by applications of SA or aspirin to leaf tissues. In this way, a susceptible plant response can be converted to a resistant response by the application of SA.

SAR has also been linked to the expression and activity of a set of pathogenesis related (PR) proteins. Different subsets of these proteins are induced by various viral, fungal, and bacterial pathogens and by environmental stresses. In "NN" genotype tobacco plants (Xanthi-nc) exogenous applications of SA induce PR proteins. Levels of PR-1 following pathogen attack have been found to rise as endogenous levels of SA do so, suggesting that an accumulation of SA induces PR-1 gene expression, which leads to the synthesis of PR proteins. HR and SAR are consequently elicited in conjunction with SA and PR protein levels. Mechanical wounding, however, does not produce the same effect. Therefore, de novo synthesis of SA and expression of PR-1 genes is not a general response to cell death.

To further study the role of SA in SAR, transgenic tobacco plants have been engineered to express the bacterial nahG gene from Pseudomonas putida. This gene encodes salicylate hydrolase, an enzyme that catalyzes the decarboxylative hydroxylation of SA into an inactive form, catechol (figure 3). Thus, nahG tobacco plants accumulate little or no SA and are unable to induce SAR following TMV attack. To determine whether the nahG gene product successfully blocks SA accumulation, Gafney et al. inoculated three leaves of transgenic plants and wild type plants with TMV in 1993. The leaf tissues were assayed for SA and for the salicylate hydrolase enzyme seven days following infection. Wild type plants exhibited a 185 fold increase in SA over controls. Transgenic plants having high levels of the nahG enzyme exhibited low levels of SA. Those with intermediate levels of the enzyme had intermediate levels of SA. Therefore, expression of the nahG gene is correlated inversely with SA levels in transformed plants.

Figure 3: Degradation of SA to catechol by salicylate hydroxylase
Following the above treatment, the upper leaves of the same test plants were subjected to a subsequent TMV infection. Lesion size of these leaves five days later was inversely proportional to the amount of SA observed following the primary infection. The necrotic regions following a primary or secondary TMV infection are significantly larger in nahG plants than in "NN" plants. In the transformed plants this cell death proceeds to spread to the stem and enters all tissues, even the vascular regions, due to the fact that the HR is not elicited to prevent the virus from moving throughout the plant in a cell to cell manner. These results, and the fact that the exogenous applications of SA and aspirin can decrease lesion size and induce accumulation of PR proteins, suggest that SA is necessary for primary resistance responses and for SAR to be elicited. In addition, SAR and SA levels are strongly correlated with the expression of genes encoding PR proteins.

These tests have supported the claim that SA is responsible for SAR. But it remains unknown which molecule transmits the signal from the site of infection to uninfected tissues. SA does not remain at the site of infection. Free SA is most concentrated in this area and decreases further away from the HR lesion. However, exudates from cut petioles contain SA following TMV inoculation. The concentration of SA in the phloem is proportional to that at the infection site which is proportional to the concentration of TMV applied. This accumulation in the phloem is observed after HR but before SAR.

Because SA was found in the phloem exudates, it was thought to be the translocated signal. However, research conducted in 1994 by Vernooij et al. disproved this assumption. Test plants were prepared in which wild type scions were grafted onto either wild type or nahG rootstocks. The same procedure was performed with nahG scions (figure 4).

Figure 4: grafting experiment
X = wild type
N = nahG
Leaves on the rootstocks were infected with TMV. Seven days later the upper scion leaves were assayed for SAR by measuring SA and by performing a secondary TMV inoculation and measuring the resulting lesion sizes. Plants with wild type rootstocks and wild type scions exhibited SAR, demonstrating that the signal can travel through the graft. In the heterogeneous plants, those with \textit{nahG} rootstocks exhibited SAR in the wild type scions. Conversely, infection of wild type rootstocks did not induce SAR in the \textit{nahG} scions. These results suggest that SA accumulation at the site of infection is not necessary for SAR but is required in the uninfected tissues for protection to be elicited. Even though the \textit{nahG} rootstocks could not accumulate SA, they could transmit a signal. The exact nature of that signal remains to be elucidated\textsuperscript{11,12}.

Another loosely understood signalling sequence is that which occurs at the site of infection. A soluble 240 KD binding protein has been isolated from infected leaves. SA's dissociation constant for this protein is 14 \(\mu\text{M}\). SA reaches levels up to 15 \(\mu\text{M}\) following infection, suggesting that this protein binds to SA under conditions of pathogen attack. This binding protein is catalase, an enzyme that converts hydrogen peroxide to water and oxygen. When SA binds to catalase, it inhibits its activity by 80\%, allowing hydrogen peroxide levels to become and remain high during HR\textsuperscript{13}.

This leads to the prediction that hydrogen peroxide may be a secondary messenger in SAR. However, no accumulation of hydrogen peroxide has been found in uninfected tissues exhibiting SAR\textsuperscript{12}. Its role in protective mechanisms may be limited to the site of infection. Leaves injected with hydrogen peroxide have been found to induce PR-1 gene expression and to increase cell wall lignification. Therefore, it is probably active in the signal transduction pathway which leads to the HR. The rapid burst of hydrogen peroxide following pathogen attack may cause an oxidative stress that facilitates the destruction of pathogens, a localized cell death, and an activation of other biochemical responses\textsuperscript{13}. One of these responses could be the induction of SA. This hypothesis suggests a pathway in which hydrogen peroxide is upstream of SA. It may activate SA, which inhibits catalase, allowing more hydrogen peroxide to accumulate in lesion areas\textsuperscript{12}.

As SA inhibits or activates catalase (depending upon hydrogen peroxide levels in the cell) by donating or accepting a single electron, it may itself become a free radical, an unstable molecule with an unpaired electron. This SA free radical then causes the accumulation of lipid peroxidation products. Treatment with 500 \(\mu\text{M}\) SA has caused a 60-70\% rise in malondialdehyde\textsuperscript{14}. Free radicals are short lived. Therefore, one must
measure the products of their transformations, which are aldehydes in the case of membrane lipids. Exogenous applications of these lipid peroxidation products have resulted in an elevation of PR-1 gene expression, suggesting that they are active in the defensive signalling pathway. The observation that treatments with various antioxidants suppress PR-1 gene expression strengthens the conclusion that free radicals are vital defensive elements.

An example of an antioxidant that inhibits PR-1 gene expression is N-acetyl-L-cysteine (NAC). When applied with Rose Bengal (4,5,6,7 tetrachloro-2',4',5',7' tetraiodofluoroscien), NAC prevents PR proteins from accumulating. Rose Bengal is a water soluble xanthene dye which produces singlet oxygen atoms [or reactive oxygen species (ROS)]. In the absence of NAC, RB causes an increase in the concentration of PR-1 proteins. In plants, these singlet oxygen atoms are spontaneously converted to other ROS. Therefore the PR gene signalling pathway is not necessarily dependent upon singlet oxygen production, but upon the presence of other ROS. This ROS pathway also occurs when the photosynthetic apparatus is damaged by UV-B or UV-C radiation. Therefore UV light can create ROS, which induces PR gene expression.

SA's role in this mechanism is not fully understood. Measurements of endogenous SA following applications of Rose Bengal to wild type and nahG plants will be performed. Some of these treatments will be coupled with exposure to ethylene-diurea (EDU), a fungicide which acts as an antioxidant. These experiments should aid in the understanding of SA's contribution to tobacco plant defensive mechanisms.
MATERIALS AND METHODS

Plant Growth

*nahG-10* and NN seeds were sown into trays containing moistened Metro Mix. The trays were then maintained under fluorescent lights and at room temperature. The seeds germinated and continued to grow in the trays for two weeks. After this time seedlings were transplanted to individual pots to promote growth and root expansion.

Application of Rose Bengal

Upon reaching a size in which at least four large leaves were present (at about one month of age), treatment was initiated. Twenty four *nahG* plants were randomly assigned to the treatment or control groups. 40 mM Rose Bengal containing a few drops of the surfactant Tween-20 (to promote adhesion to the leaf surface) was applied with a paint brush to one lower leaf on each of the 12 treatment plants. The 12 control plants were treated in the same way, except deionized water was painted on instead of Rose Bengal. Leaves painted with water were fitted with twist ties around their petioles for identification. The NN plants received identical treatments.

Harvest of Samples

Samples were collected at 24 hour time points, beginning with a time 0 and ending with a time 72 hours. At each time point the treated leaf of three plants from each treatment group was removed. The leaf directly above the treated leaf was also removed. A tissue sample of 500 mg was obtained from each leaf and stored in a test tube at -80°C.

Application of EDU

EDU solution was prepared at a concentration of 1 gram/L and placed in a spray bottle. It was sprayed liberally on the leaves of 12 *nahG* plants. Rose Bengal was then applied the following day as above. Samples were harvested and stored as above. This procedure was then performed on the NN plants.

SA Extraction Technique

Stored samples were removed from the -80°C freezer and placed in liquid nitrogen. They were ground with a cold stirring rod. 2.5 ml of 90% methanol was added to each. The tubes were then centrifuged for 15 minutes at 3000 g.
The supernatant was poured into new tubes. 2.5 ml of 95% methanol was added to the precipitates. They were then centrifuged for 15 minutes at 3000 g. The resulting supernatants were combined with their corresponding supernatants from the first centrifugation. They were then spun in a speed vac overnight.

2.5 ml of 5% trichloroacetic acid was added to the resulting dry solids. They were scraped off of the sides of the test tubes with a glass rod. They were centrifuged for 15 minutes. The supernatant was poured into new tubes. 2.5 ml of extraction media (ethyl acetate and cyclopentane at a 1:1 volume ratio) was added to each supernatant. They were vortexed in order for the SA to dissolve in the resulting organic layers. These layers were drawn off with a pipette and added to new tubes. Another addition of 2.5 ml of extraction media was performed, and the resulting organic layers were withdrawn and added to their corresponding previous layers. These were then dried in a Speed Vac overnight.

The resulting dried solids were dissolved in 55% methanol [high performance liquid chromatography (HPLC) grade]. One µl of methanol was added for each mg of leaf tissue the samples were derived from. The samples were transferred to vials containing microsample filters having a 0.2 µm pore size. They were spun on a microcentrifuge to filter out any impurities. They were then transferred to HPLC sampling vials.

Quantification of SA

Standards were prepared at concentrations of 0, 25, 50, 100, 250, 500, and 1000 ng/ml. These standards and the samples were chromatographed using an HPLC at a flow rate of 0.5 ml/minute. The column was maintained at a temperature of 40°C. HPLC grade methanol and sodium acetate served as the mobile phase. A fluorescence detector measured absorbance of the 315 nm excitation and the 405 nm emission wavelengths.

Determination of Percent Recovery

Five spiked samples were prepared in which 500 mg of untreated leaf tissue was ground up in frozen tubes. Five hundred ng of SA was added to each along with the 2.5 ml of 90% methanol. The SA extraction technique and HPLC run were then performed on the spiked samples just as they were with the above samples. The amount of SA eluted from the HPLC was compared to the 500 ng added to determine the percent recovery achieved by the extraction technique.
RESULTS

The SA standard set was analyzed at the beginning of the HPLC run and also throughout the run at intervals of about every 15 samples to assure uniformity in measurements. A standard curve was constructed with the mean peak areas occurring around 2.8 minutes for each standard concentration. (SA elutes from the column between 2.7 and 2.9 minutes.) The following equation with which to calculate the samples' concentrations was created: \[ Y = 91.171 \times X - 564.01 \] (See figure 5). The variable "Y" represents the peak area. The variable "X" represents the concentration of SA in the sampling vials in ng/ml.

The spiked samples had a mean peak area of 33,693.2. The above equation was rearranged in order to solve for \( X \) \[ \frac{(Y + 564.01)}{91.171} = X \]. With the peak area substituted into the equation, the SA concentration in the vials was 375.7 ng/ml. The average volume of methanol in the vials was 542 \( \mu l \). \[ 375.7 \text{ ng/ml} \times .542 \text{ ml} = 203.6 \text{ ng} \]. 500 ng of SA was initially added to each leaf tissue sample. Therefore, the percent recovery for the extraction technique is \( \frac{203.6}{500} \times 100\% = 40.7\% \).

The mean peak areas for each time point of each treatment were calculated and placed in the above equation to determine SA concentration in the sampling vials in ng/ml. However, the concentration of SA in the leaf tissue is of interest. Because one \( \mu l \) of methanol was added for each mg of leaf tissue in the sample, the vial concentration of ng/ml is equivalent to a leaf tissue concentration of ng/g. The value then obtained for SA concentration in the leaf tissues was divided by .407 in order to account for that SA lost during the extraction process.

The SA concentrations over time for the treated and untreated leaves of the nah G plants subjected to treatments with RB, EDU + RB, or distilled water are plotted in figures 6-8, respectively. At most of the time points of each treatment, the untreated leaf contained a greater SA concentration than the treated leaf did. The SA concentrations in the leaves of the control plants climbed over time, becoming greater than those for the RB and EDU + RB treated leaves in some cases. The EDU + RB untreated leaf experienced a drop in SA concentration at 48 hours.
The results for the NN plants are plotted in figures 9-11 in a similar fashion to those for the *nahG* plants. The SA concentrations in the RB treated and control plants start around 200 ng/g at time 0, hit a peak at 24 hours, and then return to the initial levels at 72 hours. The EDU + RB plants follow a similar pattern but start at 600 ng/g, peak at 48 hours, and then drop down to the 200 - 400 ng/g range at 72 hours. In all three trials the changes over time of the SA concentrations in the untreated leaves mirror those of the treated leaves. However, in the control plants the values for the untreated leaves are greater than those for the treated leaves. Yet they are smaller than those for the treated leaves of the RB and the EDU + RB treatments. A comparison of the peak SA concentrations for both leaf types of all three treatments is displayed in figure 12. Note that measurements for the RB treated and the control plants were taken at 24 hours, while those for the EDU + RB treated plants were taken at 48 hours. These are the times that peak SA production was reached.
DISCUSSION

Due to the fact that the nahG plants contain the gene encoding salicylate hydrolase, they were not expected to accumulate SA. Therefore, it was surprising that concentrations reached as high as 700 ng/g. These high values were observed in the EDU + RB untreated leaves and in the treated control leaves. These unexpected results could be contributed to the transgenic nature of the plant. All of the properties of the gene encoding salicylate hydrolase may not yet be fully understood in the tobacco plant. Possibly, some other portion of the bacterial vector that was inserted into test plants during the engineering process is responsible for the observed results. A more likely explanation, however, is that the unexpected and inconsistent data can be attributed to experimenter error due to lack of experience.

The NN plants, whose extractions were performed at a later date than those for the nahG plants, displayed more consistent results. The SA concentrations start low, reach a peak, and then return to a low value. The RB treated plants obtained the highest SA concentrations. The untreated leaves' SA concentrations rose in conjunction with those of the treated leaves, suggesting a systemic response to the oxidative stress. It is surprising, however, that the control plants displayed a peak in SA concentrations at 24 hours for both treated and untreated leaves. Also the SA concentrations in the untreated leaves mirrored those of the treated leaves, just as in the RB and the EDU + RB treated plants. However, these untreated control leaves had greater SA concentrations than their treated counterparts, suggesting that something other than the distilled water application created the peak in SA production at 24 hours.

It is interesting that the EDU + RB application produced a smaller peak SA concentration than the RB treatment alone produced. The EDU also delayed the burst in SA production. That in the RB treated plant occurred at 24 hours, while that in the EDU + RB treated plant occurred at 72 hours. These results suggest that the presence of an antioxidant decreases and delays the SA response to oxidative stress. These results, coupled with those displaying SA production in response to RB (figure 9), lead to the conclusion that the formation of singlet oxygen atoms or other ROS activates SA production as a defensive response. The free radical event is occurring upstream of the SA production. However, it remains to be elucidated whether hydrogen peroxide and catalase play a part in this pathway and whether additional free radical products, such as lipid peroxides, are formed down stream of SA.
Figure 6: Comparison of RA treated leaf (b) with the leaf directly above (a) for the mung plants.
Figure 7: Plant was treated w/EDU prior to RB application. RB treated leaves (♦) are compared with the leaf directly above (•) for naph plants.
Figure 8: Comparison of leaf treated with distilled water (•) with the leaf directly above (*) for the

Leaf Plants.

Time (hours)
Figure 9: Comparison of RB treated leaf (•) with leaf directly above (♦) for "NN" type plants.
Figure 10: Plant was treated w/EDU prior to RB application. RB treated leaves (x) are compared with the least directly above (x) for the "NN" type plants.
Figure 11: Comparison of leaf treated with distilled water (a) with the leaf directly above (b) for the "NN" type plants.
Figure 12: Maximum SA levels are compared for the various treatments applied to the "NN" type plants. The value for the EDU+RB treatment was taken at 48 hours, while the values for the other 2 treatments were taken at 24 hours.
REFERENCES


