Reports

Salicylic Acid: A Natural Inducer of Heat Production in *Arum* Lilies

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For more than 50 years the identity of "calorigen," the agent that triggers pronounced heat production in the flowers and inflorescences of some thermogenic plants, remained obscure. Mass spectroscopic analysis of highly purified calorigen extracted from the male flowers of *Sauromatum guttatum* Schott (voodoo lily) revealed the presence of 2-hydroxybenzoic (salicylic) acid. Application of salicylic acid at 0.13 microgram per gram (fresh weight) to sections of the upper part of the plant's immature spadix, known as the appendix, led to temperature increases of as much as 12 Celsius degrees. These increases duplicated, in both magnitude and timing, the temperature increases produced by the crude calorigen extract. The sensitivity of appendix tissue to salicylic acid increases daily with the approach of anthesis and is controlled by the photoperiod. Thus, at least in some *Arum* lilies, salicylic acid functions as an endogenous regulator of heat production.

THERMOGENICITY (HEAT PRODUCTION) in plants, first described by Lamarck (*1*) in 1778 for the genus *Arum*, is now known to occur in the male reproductive structures of cycads and in the flowers of inflorescences of some species of angiosperms belonging to the families Annonaceae, Araceae, Aristolochiaceae, Cyclanthaceae, Nymphaeaceae, and Palmae. The heating is believed to be associated with a large increase in the cyanide-insensitive, nonphosphorylating electron transport pathway that is unique to plant mitochondria (*2–5*). The increase in this alternative respiratory pathway is so dramatic that oxygen consumption in the inflorescences of *Arum* lilies at the peak of heat production is as high as that of a hummingbird in flight (*6*).

In one of the *Arum* lilies, *Sauromatum guttatum* Schott (voodoo lily), the inflorescence develops from a large corm and can reach 80 cm in height. Very early on the day of anthesis a large bract (spathe) that surrounds the central column of the inflorescence (spadix) unfolds to expose the appendix, which is the upper part of the spadix (see cover). Soon thereafter, the appendix starts to generate heat, which facilitates the volatilization of amines and indoles (*7*) whose putrescent odor is attractive to insect pollinators. By early afternoon the temperature of the appendix may have increased by 14°C above ambient, but it returns to ambient in the evening. Later in the night a second episode of heating occurs in the lower portion of the spadix hidden inside the pollination chamber (*8*).

In 1937 van Herk (*9, 10*) suggested that the burst of metabolic activity in the appendix of the voodoo lily is triggered by "calorigen," a water-soluble substance produced in the male (staminate) flower primordia just below the appendix. Van Herk believed that calorigen begins to enter the appendix on the day preceding the day of anthesis. Van Herk's ideas have encountered some skepticism, partially because attempts to isolate and characterize calorigen have not been successful (*11–13*). The calorigen purification procedure described here was based on procedures reported earlier and expanded with high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) (*14*). As a result, a highly purified calorigen preparation, which contained at least 50% of the thermogenic activity of the crude extract, was obtained. A mass spectral analysis showed that the sample of purified calorigen contained a compound with an apparent molecular ion at a mass-to-charge ratio (*m/z*) of 138 (M^+), with the base peak (100%) at *m/z* 120 ($M^+ - 18$) and unique ions at *m/z* 92 (*m/z* 120 − 28) and *m/z* 64. This spectrum indicated the presence of 2-hydroxybenzoic acid, commonly known as salicylic acid, and closely matched the spectrum obtained from the authentic salicylic acid as well as the reference spectrum for this compound (*15*). Both pure salicylic acid and the thermogenically active band had the same R_F values in the TLC system used in the final purification step.

The identification of salicylic acid as calorigen was further confirmed when different concentrations of authentic salicylic acid duplicated the effects of the crude calorigen extract in sections of immature appendix (Fig. 1). Both the extract and salicylic acid caused warming of the appendix tissue when applied on the day preceding the day of temperature measurements. The temperature increase of 8.2°C caused by 200 μl of 15 μ*M* salicylic acid (0.13 μg/g fresh weight of appendix tissue) was similar to that produced by the crude calorigen extract. The heat production in sections treated with crude calorigen and salicylic acid always coincided with a release of strong putrescent odor. The peak of heat and odor production in all sections treated with the crude calori-

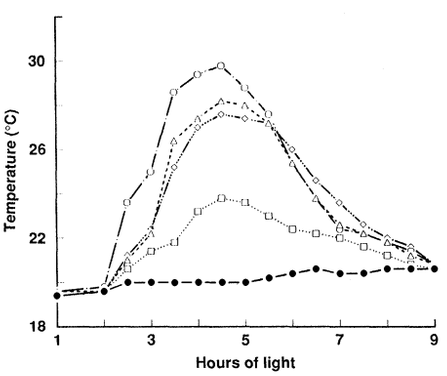

Fig. 1. Dose response of appendix tissue to salicylic acid and calorigen extract. The corms were placed in environmentally controlled growth chambers at least 4 days before anthesis and grown at 19°C and 60% relative humidity under a 15-hour photoperiod with a light intensity of 150 μE m^{-2} sec^{-1}. Two days before anthesis and 4 hours after the beginning of the light period, the central portion of the appendix was sliced transversely into 3-cm-long cylindrical sections, each weighing approximately 3 g. The inflorescence with the remainder of the appendix was kept in the growth chamber so that the estimated day of anthesis could be verified retroactively. Crude calorigen extract or salicylic acid solution (200 μl) was transferred by pipette into a circular cavity carved into the top of each section. Water was used for the control sections. The sections were placed upright on moist filter paper and incubated overnight in the growth chamber. The next morning the highest surface temperature of each of the sections was measured every 30 minutes with a Thermal Video System (model TVS-4300, Hughes Aircraft), which is capable of visualizing and quantifying surface temperatures with 0.1°C resolution. The standard deviation from four experiments varied from 0 to 5.3% of the mean. Symbols: (●) control; (□) 1.5 μ*M* salicylic acid; (△) 15 μ*M* salicylic acid; (○) 150 μ*M* salicylic acid; (◇) crude extract. Only one symbol is shown when data points overlap.

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gen extract or salicylic acid occurred after 4.5 hours of light exposure; this timing was independent of the concentration of salicylic acid applied. In sections treated with the same amount of salicylic acid, the magnitude of the thermogenic response was strongly affected by the timing of compound application on the previous day (Fig. 2). Application of 200 μl of 15 μ*M* salicylic acid 1 hour after the lights were turned on in the growth chamber caused the largest increase in temperature the following day, whereas later applications induced progressively smaller thermogenic activity. Applications of salicylic acid after 13 or 16 hours of continuous light caused essentially no heat production on the next day. Remarkably, maximum heat was always generated after 4.5 hours of light on the day of anthesis, independent of the timing of salicylic acid application on the previous day. These data suggest that the calorigen-triggered thermogenesis in the voodoo lily spadix is under strict photoperiodic control.

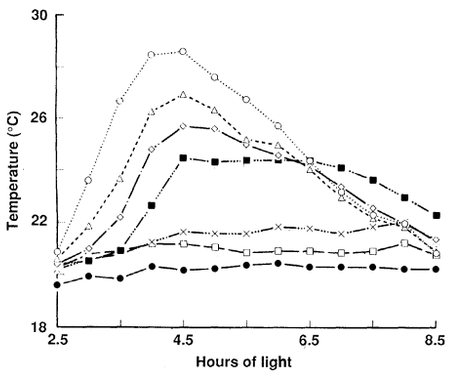

Fig. 2. Diurnal changes in the sensitivity of appendix tissue to salicylic acid. The assay was performed as described in the legend to Fig. 1, except that 200 μl of a 15 μ*M* aqueous solution of salicylic acid (approximately 0.13 μg/g fresh weight) was applied to the sections at six different times during the light period preceding the day of temperature measurements. The time of the treatment was measured from the beginning of the light period. Water was added to the control sections 1 hour after the beginning of the light period. Each data point is the average of the highest surface temperatures of three sections excised from different plants. The standard deviation from three experiments varied from 0 to 6.4% of the mean. Symbols: (●) control; (○) 1 hour; (△) 4 hours; (◇) 7 hours; (■) 10 hours; (×) 13 hours; (□) 16 hours. Only one symbol is shown when data points overlap.

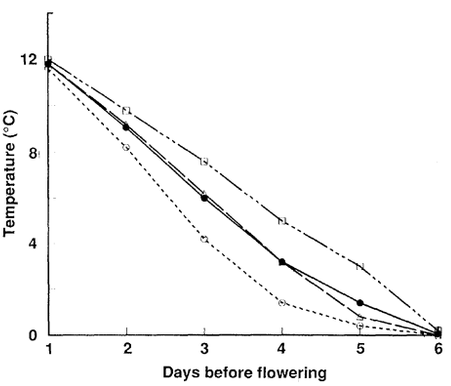

Fig. 3. Sensitivity of appendix tissue to salicylic acid as a function of days before anthesis. On each of 6 days preceding anthesis, two 3-cm-long sections were cut off the top of the appendices of three different inflorescences. One section from each pair was treated with 200 μl of a 15 μ*M* aqueous solution of salicylic acid 3 hours after the beginning of the light period while the other was used as the water control. The temperature of the sections was measured after a 4.5-hour light exposure as described in the legend to Fig. 1. The numbers on the vertical axis are the differences between the highest surface temperature of salicylic acid–treated and water control sections. This experiment was repeated three times with similar results. Symbols: (●) average; (□) plant 1; (○) plant 2; (△) plant 3.

The sensitivity of maturing appendix tissue to salicylic acid increases daily with the approach of anthesis (Fig. 3). The appendices of three different inflorescences started to respond to salicylic acid 5 days before anthesis, with an average temperature increase of 1.4°C. Administration of salicylic acid on the day preceding the day of anthesis resulted in an increase of as much as 12°C in tissue temperature. On that day the appendix had to be severed from the spadix after no more than 3 hours of light because sections excised later that day produced heat and putrescent odor regardless of the treatment. This suggests that by midmorning endogenous salicylic acid had already moved from the male flowers into the appendix.

These data support van Herk's original idea about the existence of an endogenous developmental regulator called calorigen and identifies it as salicylic acid. This compound induced concentration-dependent and photoperiodically controlled heat and odor production in the appendix tissue of *Sauromatum guttatum*. Salicylic acid probably serves as a natural thermogenic trigger in other plants, since a compound partially purified from the staminate flowers of four species of Araceae had physical properties and thermogenic capacities indistinguishable from those of *Sauromatum guttatum* calorigen (*11, 12*). Salicylic acid is also known to stimulate flowering in Lemnaceae (*16–20*) and in *Impatiens balsamina* (*21*). The stimulatory effect of salicylic acid on heat-generating, cyanide-insensitive respiration is particularly interesting because its analog salicylhydroxamic acid and some other aromatic hydroxamates are potent inhibitors of the alternative respiration (*22*). Salicylic acid also inhibits the biosynthesis of the plant hormone ethylene (*23*), stomatal closure (*24*), and ion uptake in barley roots (*25*). All this raises the possibility that salicylic acid plays a much broader regulatory role in plants than previously appreciated.

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14. The initial steps of the calorigen purification protocol were adapted from existing procedures (*11–13*). During purification the thermogenic activity was followed by means of the bioassay described in the legend to Fig. 1. Cylindrical sections of the spadix-containing male flowers were collected on the morning of flowering and stored at −80°C. For the calorigen extraction, 12 sections (40 g fresh weight) were ground in water (1 ml/g fresh weight), and the extract was centrifuged at 10,000*g* for 10 minutes. After lipid-like material was removed from the surface, the supernatant was diluted 1:4 (v/v) with acetone, stored overnight at −20°C, centrifuged at 10,000*g* for 10 minutes, and reduced by rotary evaporation at 45°C to 40 ml. The resulting extract was partitioned in a 2:1:1 (v/v) mixture of chloroform:methanol:extract. The upper phase was reduced in volume by rotary evaporation at 45°C to 40 ml and ultrafiltered through a 1000 molecular weight cutoff membrane at 4°C. This collected filtrate is called crude calorigen extract in the text. The extract was diluted 1:9 (v/v) with acetonitrile and centrifuged at 10,000*g* for 10 minutes. The supernatant was lyophilized to dryness and then suspended in 4 ml of acetonitrile:water (4:1 v/v). The upper, colorless phase (500 μl) of the resulting two phases was subjected to HPLC on an amino-bonded column (25 cm by 4.6 mm internal diameter) with acetonitrile:water (4:1 v/v) as mobile phase at a flow rate of 1 ml/min. All calorigen activity was collected in a 4- to 6-ml fraction. This fraction was lyophilized and subjected to TLC on silica gel 60 with fluorescent indicator. The mobile phase was a 3:5:1:1 (v/v) mixture of methylethylketone:ethyl acetate:ethanol:water. Essentially all thermogenic activity was detected in a faint fluorescence-quenching band with an R_F value of 0.52. The band was eluted from the gel with 50% acetone, and the eluant was lyophilized. A subsample of the purified calorigen was analyzed by solid-probe electron-impact mass spectroscopy.
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