This is to certify that the

dissertation entitled

DEVELOPMENTAL AND SALICYLIC ACID REGULATED EXPRESSION OF THE ALTERNATIVE OXIDASE OF HIGHER PLANTS

presented by

David Michael Rhoads

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date 9/6/92

DEVELOPMENTAL AND SALICYLIC ACID REGULATED EXPRESSION OF THE ALTERNATIVE OXIDASE OF HIGHER PLANTS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1992

ABSTRACT

DEVELOPMENTAL AND SALICYLIC ACID REGULATED EXPRESSION OF THE ALTERNATIVE OXIDASE OF HIGHER PLANTS

BY

David Michael Rhoads

Alternative respiration was described sixty years ago as respiration that is not sensitive to cyanide. But, to this day, the exact nature and function of alternative respiration remains a mystery. Research in just the last 10 years has provided a compelling argument that a terminal oxidase protein is involved. The flow of electrons through the alternative pathway is much less energy conserving than the electron flow through the cytochrome pathway. The purpose for such a potentially "wasteful" pathway is a source of great controversy, which will only be settled by future experimentation. Molecular-genetic analyses may provide new ways to study the function of the alternative pathway in plant cell metabolism. Chapter 2 of this thesis describes the first isolation and characterization of a cDNA clone encoding an alternative oxidase protein. The sequence of this cDNA has provided insight into the possible structure of the protein that it encodes. It has also provided a molecular probe with which to isolate genes from other species and investigate the regulation of expression of alternative oxidase genes. The results presented in Chapter 3 show that the developmental and salicylic-acid-

directed increases in alternative pathway capacity are accompanied by the accumulation of alternative oxidase proteins and transcripts in the appendix tissue of *Sauromatum guttatum* Schott (an aroid plant that uses the heat generated by the "lost" energy of alternative respiration to volatilize putrid compounds that attract insect pollinators). Chapter 4 describes the isolation and analysis of a genomic clone that corresponds to the cDNA and, therefore, encodes the putative alternative oxidase precursor protein of *S. guttatum*. A highly conserved region of the gene, which encodes a domain of the protein that is predicted to embed the mature protein in the inner mitochondrial membrane, is contained in one of the four exons. These data suggest that this region of the protein is functionally important and was established early in its evolution. The promoter region of this gene is of particular interest since the promoter region of other salicylic acid-"responsive" genes have been analyzed. Chapter 5 shows the changes in alternative oxidase expression and alternative pathway and cytochrome pathway capacities in suspension cultured tobacco cells at various stages after subculturing and following the addition of salicylic acid.

To Neera

and

My Parents,

for their love and support.

ACKNOWLEDGEMENTS

I thank my mentor, Dr. Lee McIntosh, for the support and guidance he has given me over the last five years. Although I did not always understand his ways, I believe he taught me more about being a top scientist and running a lab than most others could have. He will always have my respect and deep gratitude.

I greatly appreciate the help and guidance I received from each of the individuals who served on my advisory committee: Dr. Andrew Hanson; Dr. Zachery Burton; Dr. Mike Thomashow; Dr. John Wilson; and Dr. N. Edward Tolbert.

I thank those I consider to be my honorary guidance committee members because of all the support and guidance that each has given me: Dr. David McConnell; Dr. Hans Kende; and, especially, Dr. Thomas Elthon.

I thank all the past and present members of the Plant Research Laboratory and the Biochemistry Department who have helped me with their assistance in administrative and personal matters.

I thank my colleagues who provided technical advice and friendship: especially Dr. John Sherwood, Dr. Thea Wilkins, Dr. Mike Mansfield, Dr. David Hondred, Dr. John Scott-Craig, Dr. John Shanklin, Dr. Thomas Newman; Dr. Ellen Johnson, Dr. Timothy Casper, Dr. Thomas Johnson, Dr. Arun Goyal, John Jacobs, Crispin Taylor, Todd Black, Frank van de Loo, Sharon Thoma, Dr. Robert Meeley, Dr. Christoph Benning and Susanne Hoffmann, Thèrése Best, Kristen Rorer, Dr. Linda Briggs, Lydia Herdies, Dr. Greg Vanlerberghe, Dr. Idah Sithole, Dr. Neil Bowlby, Dr. Carrie Hiser, and Roxy Nickels.

I am especially thankful to Dr. John Fitchen, Vladimir Orbovic, Dr. Ellen Kearns, Dr. Ursula Hect, Dr. Beate Drechsler-Thielmann, Dr. Shawn Anderson and Denny Gerber, Larry Smart and Chris Durban, Dr. David Lerner, Harley Seeley, and Kurt Stepnitz for their close friendship.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	basepairs (of DNA)
BSA	bovine serum albumin
cDNA	complementary DNA (reverse-transcribed from RNA)
cv	cultivar
DEP	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate, disodium salt
EPR	electron paramagnetic resonance spectroscopy
FCCP	*p*-trifluoromethoxycarbonyl cyanide
GRP	glycine rich protein
HEPES	N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase pairs (of DNA)
kD/kDa	kilodaltons
MOPS	3-(n-morpholino)propanesulfonic acid
mRNA	messenger RNA
NADH	reduced nicotinamide adenine dinucleotide
nt	nucleotides
PAGE	polyacrylamide gel electrophoresis
PR	pathogenesis related
pv	pathovar
PVPP	polyvinylpolypyrrolidone
RBCS	ribulose bisphosphate carboxylase small subunit
RNA	ribonucleic acid
rRNA	ribosomal RNA
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate (sodium lauryl sulfate)
SHAM	salicylhydroxamic acid
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
Tween-20	polyoxyethylenesorbitan monolaurate

CHAPTER 1: INTRODUCTION

CHARACTERIZATION OF THE ALTERNATIVE RESPIRATORY PATHWAY

Plant thermogenesis was reported as early as 1778 when Lamarck demonstrated that heat was produced during the flowering of some aroid plants (referenced in 57). Garreau later linked this heat production to an increase in the rate of oxygen consumption (referenced in 57). In the 1930's van Herk determined that this elevated rate of respiration associated with heat production during flowering of the aroid species *Sauromatum guttatum* Schott (voodoo lily) was not inhibited by cyanide (referenced in 45,57). These early studies using aroid plants and similar studies by Genevois using sweet pea (referenced in 49) initiated scientific interest in cyanide-resistant respiration, which is now commonly referred to as alternative respiration.

Alternative respiration is insensitive to cyanide, azide, carbon monoxide, and antimycin A. Azide, carbon monoxide, and cyanide inhibit cytochrome pathway respiration by interfering with complex IV (see references 16,49 for reviews of cytochrome pathway components and inhibitors). Antimycin A inhibits cytochrome pathway respiration by binding to a protein in complex III (16).

These inhibitors have been valuable in establishing that the alternative pathway diverges from the cytochrome pathway after the ubiquinone pool (4,77,88). Some evidence suggests that the cytochrome and alternative pathways interact with different pools of ubiquinone in the mitochondrial membranes (2,32,62,75,80,83). The alternative pathway is inhibited by iron-chelating compounds (49), disulfuram (27) and propyl gallate (68,85). The most widely used inhibitors are the hydroxamic acid derivatives, especially SHAM (salicylhydroxamic acid)(82). SHAM inhibits the alternative pathway respiration and lipoxygenase activity completely at a concentration of 1 mM, but does not inhibit cytochrome pathway respiration below 2 mM (12).

The general nature of alternative respiration has, until recently, been a source of great controversy (49). Cyanide-resistant respiration has been proposed to be due to: 1) free radicals formed in mitochondrial membranes (49,78); 2) lipoxygenase (49,68); 3) a protonmotive ubiquinone cycle (49,77) cytochrome b_7 (49); and 5) a discrete terminal oxidase (49,77). Several lines of evidence now provide a compelling argument in favor of cyanide-resistant respiration occurring via a discrete terminal oxidase (7), which is called the alternative oxidase. The alternative oxidase has also been localized to the matrix surface of the inner mitochondrial membrane (74). There have been numerous proposals (49,83,89) concerning the composition of the alternative oxidase. Flavin has been proposed as a cofactor (83); however, this seems unlikely since the affinity of mitochondrial flavoproteins for O_2 is too low and the kinetics of reoxidation are too slow (4,83). Also, auto-oxidizable flavoproteins produce H_2O_2 (83,89), whereas the alternative

pathway produces H_2O (49,83,89). Nonheme iron (4,49,83) is an unlikely cofactor since no clear electron paramagnetic resonance (EPR) signal from a nonheme iron has been correlated with the presence of the alternative pathway (49). Copper (83,89), is likewise an unlikely candidate because of the lack of a clear EPR signal (63). Nevertheless, iron and copper cannot yet be ruled out completely since it is possible that an EPR signal from one or the other is masked (49,89) as in the case of coupled binuclear copper sites, designated as "type 3" copper (33). The determination of the exact composition of the alternative oxidase will, obviously, require a great deal more research.

PARTITIONING OF ELECTRONS BETWEEN RESPIRATORY PATHWAYS

The partitioning of electrons between the alternative pathway and cytochrome pathway must be regulated because this has significant consequences for the physiology of the organism. The mechanisms to control the partitioning of electrons between the two pathways can be divided into two general categories: those that control the capacity of each pathway and those that control the degree of engagement of each pathway.

The capacity of each pathway defined as the maximum number of electrons that can flow through the pathway. The capacity of the cytochrome pathway (V_{cyt}) is the rate of oxygen uptake sensitive to cyanide in the presence of SHAM using an exogenously added substrate. The capacity of the alternative pathway (V_{alt}) is measured as the rate of oxygen uptake sensitive to SHAM in the presence of

cyanide using an exogenously added substrate. The necessity for SHAM to be present in determining the capacity of the cytochrome pathway is debatable and based on the argument of whether or not inhibition of the cytochrome pathway results in increased electron flow through the alternative pathway (49,60,62). Although it is generally accepted that inhibition of the alternative pathway by SHAM does not cause an increase in electron flow to the cytochrome pathway (and will not cause erroneous determination of alternative pathway capacity), this assumption may not always be valid (60,62,99).

The factors that could influence the capacity of each pathway include: 1) the amount of each component that is present in the mitochondria, which is determined by transcription rates, RNA stability, and translation rates for protein components and synthesis of cofactors associated with the components; 2) targeting of components to their proper locations in the mitochondria; 3) assembly of protein complexes; 4) interaction of the pathway components with the proper electron donors and acceptors; and 5) the presence or absence of pathway regulators.

The activity of the alternative pathway (v_{alt}) is the rate of O_2 reduction that is occurring via the alternative oxidase in vivo. The activity of the alternative pathway is related to the capacity by the following relationship: $v_{alt} = \rho V_{alt}$. The engagement of the pathway, ρ, is the portion of the alternative pathway capacity that is being utilized and has values of 0 (no engagement) to 1 (full engagement). Methods for measuring alternative pathway activity include: 1) the simple addition of SHAM (to whole cells or tissues) and determination of the difference in the

rate of O_2 consumption before SHAM addition and the rate after SHAM addition (60), assuming that inhibition of the alternative pathway does not divert electrons to the cytochrome pathway; 2) the Bahr and Bonner method, which utilizes titration with SHAM (1,12) and assumes that inhibition of the alternative pathway does not result in an increase in electron flow to the cytochrome pathway (see above), and that the cytochrome pathway is always engaged to its maximum capacity (which has also been questioned [84,91]); and 3) the Lambowitz *et al.* method, in which the ATP/O ratios are determined for a given substrate in the presence and absence of inhibitors of the alternative and cytochrome pathways and used to calculate the fraction of electrons flowing through each pathway using equations that take into account the different amounts of ATP produced by electrons from different substrates flowing through each pathway (47,49). Recently, a non-invasive method for measuring the engagement of the alternative pathway was introduced (28). This method relies on the observation that the alternative oxidase discriminates against ^{18}O to a much greater extent than does cytochrome oxidase (28).

The factors that could influence the *in vivo* activity (or engagement) of the alternative pathway include the presence or absence of inhibitors and activators of the pathway, the identity of the substrates being oxidized (13,49,62), the physical arrangement of the components of each pathway, and the reduction level of the ubiquinone pool (2,11,19). Succinate (even in the presence of malonate, an inhibitor of succinate dehydrogenase) and malate (even in the presence of oxaloacetate, which inhibits malate oxidation) stimulate the rate of NADH

oxidation via the alternative pathway above the levels predicted by simple addition of the rates for the single substrates in *Petunia hybrida* callus mitochondria and potato tuber callus mitochondria (96). However, in mitochondria that poorly oxidize NADH via the alternative pathway, this stimulation was not observed (96) and succinate inhibits NADH oxidation via the alternative pathway in soybean cotyledon mitochondria (13). In addition, malonate, which inhibits succinate dehydrogenase because it is structurally similar to succinate, is not capable of stimulating NADH oxidation via the alternative pathway (96). These observations argue against the hypothesis that succinate and/or malate affect respiration via the alternative pathway by interacting with the alternative oxidase (96).

Electrons from succinate may flow through the alternative pathway more readily than electrons from NADH in the mitochondria from some sources, such as wounded potato tuber slices (49,96); while electrons from succinate seem to have little access to the alternative pathway in mitochondria from other sources, such as corn and fresh potato tuber slices (49). These studies illustrate the complex and poorly understood partitioning of electrons from different substrates to the alternative and cytochrome pathways in various tissues of assorted plant species. At least four hypotheses have been proposed to explain the observations: 1) there is a longer diffusion path between external NADH dehydrogenase and the alternative oxidase than between succinate dehydrogenase and the alternative oxidase (13,62); 2) there is a direct interaction between succinate dehydrogenase and the alternative oxidase (13,62); 3) there is compartmentation of distinct alternative oxidase pools (13,96); and 4) there is compartmentation of distinct

quinone pools associated with different dehydrogenases and/or oxidases (2,11,13,49,62,80,91). The actual situation in each plant mitochondrion may be a specific combination of these various possibilities suited to the metabolism of the cell in which the mitochondrion exists (13).

The partitioning of electrons to the alternative pathway may depend upon the reduction level of the ubiquinone pool (2,11,19,91). It has been postulated that the ubiquinone pool must be completely reduced before electrons will be diverted to the alternative pathway (2). A second model postulates that the partitioning of electrons is based upon the relative rate constants for the reactions between reduced ubiquinone and the cytochrome and alternative oxidase (11,91). In soybean cotyledon mitochondria, a quinone reduction level of 35-40% was sufficient to allow engagement of the alternative pathway (19), which argues against the first model. It must also be kept in mind that the reduction level of the quinone pool itself depends on the relative rates of electron flow into and out of the quinone pool, which depends on the substrate and the ADP level (18,19).

ALTERNATIVE PATHWAY IN AROIDS

Since electron flow through the alternative pathway does not result in proton translocation at complex III or complex IV (4,61,90), much of the potential energy of the system is not conserved as chemical energy and is lost as heat (90). Specific floral tissues of some aroid plants develop such a high level of the alternative pathway that they become thermogenic (12,57,70). The day the

inflorescence of the aroid species *S. guttatum* blooms (D-day), the capacity of the alternative pathway in the appendix tissue is 300-600 natoms O/min/mg protein and the temperature of the tissue increases about 9-12°C above ambient temperature (23,70,72). In *S. guttatum*, the heat produced during blooming is used to volatilize foul smelling compounds, such as indoles, which attract insect pollinators (56). Other aroids have also been good sources of mitochondria with which to study the alternative respiratory pathway. Mitochondria from *Arum maculatum* and *Symplocarpus foetidus* (skunk cabbage) have been used extensively for biophysical experiments (1,33,35,36,76,90) and in attempts to purify the alternative oxidase (6,34,38).

Three mitochondrial proteins with apparent molecular masses 35-, 36-, and 37 kilodaltons (kD) strongly correlate with the alternative pathway in *S. guttatum* (21). Several lines of evidence suggest that the alternative oxidase is comprised of at least one of these proteins. The initial identification was made by solubilizing the active mitochondrial proteins (20), followed by CM-Sepharose and phenyl-Sepharose column chromatography to attain a 166-fold purified preparation of the alternative oxidase activity (21). Mouse polyclonal antibodies raised to this preparation inhibited alternative oxidase activity and immunoprecipitated the 35-, 36- and 37 kD proteins which copurify with the activity. Three distinct monoclonal antibodies were raised to the 36 kD protein: the AOA monoclonal antibodies recognize all three proteins; the AOU antibodies recognize primarily the 37 kD protein, but do recognize the 35- and 36 kD proteins to a lesser extent; and the AOL antibodies recognize the 35- and 36 kD

proteins primarily and the 37 kD protein to a lesser extent (22). The observation that the three proteins are immunologically related is consistent with the hypothesis that these three proteins are posttranslationally modified versions of a single gene product. Secondly, the levels of the 35-, and 36 kD proteins correspond to changes in the level of alternative oxidase activity at various stages of development of the appendix tissue. The protein levels also increase after application of salicylic acid, which is known to cause an increase in alternative pathway activity and has been shown to be an endogenous "trigger" of thermogenesis in appendix tissue (70, see below). In addition, the AOA monoclonal antibodies recognize proteins in other tissues known to have high levels of alternative pathway activity (21). At least two mitochondrial proteins in specific tissues of all aroid plants investigated are also recognized by the AOA monoclonal antibodies (21,22). Finally, the AOA monoclonal antibodies recognize a protein that is correlated with alternative oxidase activity in *Neurospora crassa* mitochondria (46). These data provide strong evidence that the 35-, 36- and 37 kD proteins are components of the alternative oxidase of *S. guttatum.*

ALTERNATIVE PATHWAY IN OTHER ORGANISMS

The alternative pathway has been studied in a variety of plants other than aroids (3,4,84) as well as in algae (26,98), fungi (29,59,81,91,102), and "lower" animals including a *Paramecium* and some of the *brucei* group of African trypanosomes (8,83). The genetic studies using the fungus *Neurospora* have

contributed to the identification of the branch point of the alternative pathway (95), the identification of the alternative oxidase, and early hypotheses on the location of the genes. A great deal of biochemical, biophysical, and physiological work has been done with corn, mung bean, pea, potato, and soybean mitochondria (14,31,39,49,65,84,86,87,99). A single alternative oxidase protein that is recognized by the AOA monoclonal antibodies has been identified in mitochondrial proteins from specific tissues of potato (22), mung bean (22), tobacco callus (22), soybean (39), and siratro (39). These results suggest that only one protein is required for alternative oxidase activity. Also, cDNA clones encoding alternative oxidase proteins have been isolated from potato (30), rice (R. Nickels and L. McIntosh, personal communication), *Arabidopsis* (D. Söll, personal communication), and yeast (81). These data will provide information on the evolutionary conservation of the alternative oxidase. The protein produced from the *Arabidopsis* cDNA clone allows CN-resistant, SHAM sensitive respiration in *E. coli*, indicating that a single alternative oxidase protein is sufficient for alternative respiration (D. Söll, personal communication). *Hansenula anomala* may be a rich source of the alternative oxidase (81) to be used for biophysical analysis.

The role of the alternative pathway in non aroid plants is unknown (45,84). Over the years there have been many theories to explain the existence of the (presumably) wasteful alternative pathway in the mitochondria of non-aroid higher plants (45,reviewed in reference 84). It has been proposed that the pathway developed as a protection against periodic bursts of cyanide in cyanogenic plants (43,84), but this is generally disputed (48,84). Increased electron flow through the

alternative pathway is implicated in response to environmental stresses such as: wounding (15); pathogen attack, based on the observations of increased salicylic acid levels in plants during pathogen attack (see below); and low temperatures (10,45,54,55,86,87,94,101). While no direct evidence yet exists to support the idea that thermogenesis is an adaptive response to low temperatures, many of these studies support the hypothesis that respiration, at least in part through the alternative pathway, may be involved with plant responses to lowered temperature.

Since leaf mitochondria preferentially oxidize glycine during photorespiration, the alternative oxidase may remove excess NADH during this process (24). Reoxidation of excess NADH generated during glycine oxidation in isolated soybean mitochondria from greening cotyledons was mainly via the alternative pathway (24). However, separate studies using pea or spinach leaf mitochondria did not show a correlation between glycine oxidation and the alternative pathway (18,25).

A second association between alternative pathway respiration and substrate oxidation has been proposed for malate (49). In this scheme, excess reducing power can be removed without control by the phosphate potential or the energy charge by malate oxidation via malic enzyme and the alternative respiratory pathway (49). Such an association has been observed (79,80), although the interpretation of these data has been disputed (50).

The "energy overflow" hypothesis asserts that alternative pathway respiration provides increased respiratory capacity when the cytochrome pathway is saturated, allowing oxidation of cellular substrates in excess of those needed for

growth, osmoregulation, storage as carbohydrate, or for ATP production (44,45). The arguments in favor of and against this hypothesis will not be discussed since they have been extensively reviewed elsewhere (17,44,45,84).

Finally, another hypothesis for the function of the alternative oxidase asserts that the pathway allows a high rate of flux through the tricarboxylic acid cycle for the continued production of carbon skeletons required for growth and maintenance when the cytochrome pathway is inhibited by a high energy charge (45,92) or by a lack of cytochrome pathway components (42,45). This hypothesis implies that the cytochrome pathway must be saturated before the alternative pathway is engaged. However, one study indicated a non-linear relationship of quinone pool reduction with electron flow through the alternative pathway and demonstrated that a quinone pool reduction of 35-40% was sufficient for the engagement of the alternative pathway (19).

SALICYLIC ACID IN AROIDS

Salicylic acid has been shown to be an endogenous "trigger" of thermogenesis in *S. guttatum* appendix tissue (70). Salicylic acid is produced in the male floral region of the inflorescence and moves into the appendix beginning early on D-1 and "triggers" thermogenesis at about noon of D-day (70,72). The concentration of salicylic acid in the appendix increases from below 100 ng/g fresh weight as late as D-2 to over 1.0 μg/g fresh weight on D-day (72). Salicylic acid has also been shown to dramatically increase both the alternative pathway activity

and the levels of the 35-, 36-, and 37 kD alternative oxidase proteins when it is applied to *S. guttatum* appendix tissue sections (23). Prior to the application of salicylic acid, the 37 kD protein is expressed at a moderate level in immature appendix tissue (23). All three alternative oxidase proteins are present in great abundance in the tissue sections following incubation in a phosphate-buffered solution containing 1.0 mM salicylic acid (23). In addition, Raskin *et al.* (72) showed that light is required for salicylic acid to cause thermogenesis in appendix tissue sections.

SALICYLIC ACID IN OTHER ORGANISMS

Application of salicylic acid (and some of its derivatives) induces flowering in many species of the Lemnaceae family (9,37,40,41), in *Impatiens balsamina* (64), and *Pistia stratioles* L. (69). However, the concentration and function of endogenous salicylic acid in these organisms is unknown. The endogenous level of salicylic acid is high in the heat-producing tissues of many plants during thermogenesis and in the non-thermogenic flowers of *Passiflora caerulea* L. during anthesis (71). The highest recorded concentration of endogenous salicylic acid is found in the sporophylls of male cones of *Dioon edule*, where it approaches 0.1 mg/g fresh weight during heat production (71). The highest level of salicylic acid in a non-thermogenic plant is found in the leaves of *Oryza sativa* (rice), where it ranges between 24 and 68 μg/g fresh weight depending on the cultivar (71). Again, the function of the endogenous salicylic acid in these organisms has not

been established. Addition of salicylic acid to high CO_2-grown *Chlamydomonas* cultures in the log phase of growth also caused an increase in alternative pathway capacity, but it is not known if salicylic acid functions as a regulator *in vivo* (26).

Salicylic acid may also be a messenger in systemic acquired resistance (SAR) in tobacco plants infected with tobacco mosaic virus (TMV)(51,100) and in cucumber plants infected with: 1) tobacco necrosis virus (TNV)(58); 2) the fungal pathogen *Colletotrichum lagenarium* (58); or 3) *Pseudomonas syringae* pv *syringae*, a bacterial pathogen (73). TMV infection induces the accumulation of pathogenesis related proteins (PR proteins)(66,100) and the transcripts that encode them (5,51,52,53,97,100) in leaves of resistant tobacco plants. The level of salicylic acid increases in the infected leaves and the upper, non-infected leaves of a resistant tobacco plant that has been inoculated with TMV (51,100). Likewise, the level of salicylic acid increases in the phloem of a cucumber plant following an inoculation with *C. lagenarium*, to which the plant has become resistant by initial infection with TNV or *C. lagenarium* (58). Furthermore, application of salicylic acid to the leaves of TNV-resistant tobacco plants induces the accumulation of the some of the acidic PR1 proteins (5,52,53,66,97,100), including PR1a, and a glycine rich protein (GRP8) and their corresponding transcripts (5,51,97,100). Accumulation of the transcripts corresponding to other "SAR genes" has also been observed in primary and secondary leaves following inoculation with TMV or salicylic acid (97). Therefore, salicylic acid appears to act in disease resistance by regulating gene expression since application of salicylic acid has been shown to cause the accumulation of pathogenesis related (PR) proteins, and their

corresponding transcripts (51,66,97). The promoter regions of the genes encoding GRP8 and PR1a have been analyzed in order to identify salicylic acid responsive, *cis*-acting sequence elements (67,93). Although some of the data on the region of the PR1a promoter that conveys salicylic acid responsiveness is contradictory, a sequence motif found in both the PR1a promoter and the GRP8 promoter has been identified (67,93).

In summary, these data provide evidence that salicylic acid is involved in SAR, at least in tobacco resistance to TMV. Therefore, identification of the mechanism(s) by which salicylic acid regulates gene expression will be valuable in understanding the role(s) of salicylic acid in various plant tissues, the physiology of plant responses to pathogens, and the physiology of thermogenic tissues.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF A cDNA CLONE ENCODING AN ALTERNATIVE OXIDASE PROTEIN OF *Sauromatum guttatum* (Schott)

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Published in *Proc. Natl. Acad. Sci.* USA **88**: 2122-2126 (1991).

ABSTRACT

Polyclonal and monoclonal antibodies, which recognize the 35, 36, and 37 kDa alternative oxidase proteins of *Sauromatum guttatum* (Schott) were used to isolate a cDNA clone, pAOSG81, from an *S. guttatum* cDNA expression library. A fusion protein with an apparent molecular mass of 48 kDa was expressed from a pUC119 derivative of pAOSG81 in *E. coli* cells and was recognized by the monoclonal antibodies. When the *in vitro* translated and immunoprecipitated products made from mRNA hybrid-selected by pAOSG81 were analyzed, a single band corresponding to a protein with an apparent molecular mass of 42 kDa was observed. DNA sequence characterization showed that pAOSG81 contains the entire coding region of a protein with a calculated molecular mass of 38.9 kDa, a putative 63 amino acid transit peptide, and a nine amino acid match to authentic N-terminal sequence of the 36 kDa alternative oxidase protein. Analyses of the deduced amino acid sequence indicate: 1) that the transit peptide is predicted to form amphiphilic helices; and 2) that three regions of the processed protein are likely to form transmembrane alpha-helices. We conclude from these data that pAOSG81 represents a nuclear gene, *aox1*, encoding a precursor protein of one or more of the alternative oxidase proteins of *S. guttatum*.

INTRODUCTION

All higher plants that have been investigated, as well as many fungi, algae, and some protists (1), contain two respiratory pathways: the cytochrome pathway and the alternative pathway (2). The alternative pathway diverges from the cytochrome pathway after the ubiquinone pool (3,4,5). Since electron flow through the alternative pathway does not result in the formation of a proton gradient at the cytochrome b-c_1 complex or at the cytochrome a-a_3 complex (3,6,7), much of the potential energy of the system is not conserved as chemical energy and is lost as heat. Some plant tissues that express a high level of the alternative pathway are thermogenic (8). The role of thermogenesis in the appendix tissue of the Aroid species *Sauromatum guttatum* Schott (voodoo lily) is to volatilize foul-smelling compounds which attract insect pollinators (9). However, the role of the alternative respiratory pathway in non-thermogenic plants, and most other organisms, is problematic.

Alternative pathway respiration may function to increase respiration when the cytochrome pathway is "restricted", allowing a high rate of flux through the tricarboxylic acid cycle, thus producing carbon skeletons required for growth (10,11). This hypothesis implies that the cytochrome pathway must be saturated before the alternative pathway is engaged. However, a recent study indicated a non-linear relationship of quinone pool reduction with electron flow through the alternative pathway and demonstrated that a quinone pool reduction of 35-40% was sufficient for the engagement of the alternative pathway (12). Increased

electron flow through the alternative pathway has also been implicated in response to low temperatures (13). While no direct evidence yet exists to support the idea that alternative pathway thermogenesis is an adaptive response to low temperatures, it has been proposed that increased respiration in response to low temperatures may be required; possibly to prevent accumulation of toxic metabolites (14).

Three mitochondrial proteins with apparent molecular masses, as determined on denaturing polyacrylamide gels, of 35-, 36-, and 37 kilodaltons (kDa) strongly correlate with alternative oxidase activity in *S. guttatum* (15). Previous results also showed that a single monoclonal antibody recognized all three of these polypeptides and was capable of inhibiting alternative oxidase activity *in vitro* (16). Taken together, these data strongly indicate that the three alternative oxidase proteins are closely related, possibly as post-translationally modified versions of a single gene product. The same monoclonal antibody raised to the *S. guttatum* alternative oxidase also recognizes this oxidase from *Neurospora crassa* and has been employed to characterize mutants of the oxidase in this organism (17). The structural component of the *N. crassa* alternative oxidase is probably encoded by a single nuclear gene (18,19), thus lending support to the hypothesis that the three proteins correlated with alternative oxidase in *S. guttatum* are the products of a single gene.

Salicylic acid has been shown to increase alternative oxidase activity in isolated *S. guttatum* appendix sections dramatically (20). The 37 kDa protein is present in small amounts prior to the increase and all three polypeptides are

present in great abundance in the tissue sections following a 20 hour incubation in a solution of salicylic acid (21). These results suggest that *de novo* synthesis of alternative oxidase is required for the increased activity in *S. guttatum* appendix tissue, and that posttranslational modifications of the alternative oxidase may also be needed (*i.e.* for the appearance of the 35 kDa and 36 kDa proteins).

MATERIALS AND METHODS

Plant Material. *Sauromatum guttatum* Schott (voodoo lily) plants were maintained in a glasshouse at 27°C ± 4°C under long-day conditions, as previously described (22). The day the appendix region of the spadix heats is referred to as D-day (see ref. 9 for anatomy of *S. guttatum* inflorescence). Other developmental stages of the plant are indicated as the number of days before or after D-day (D-1 being the day before and D+1 the day after D-day).

Isolation of Mitochondria. Mitochondria were isolated by a modification of the procedure of Schwitzguebel and Siegenthaler (23). After the first centrifugation at 19600 x *g* for 10 minutes the mitochondrial pellet was resuspended directly in reaction medium (250 mM sucrose, 30 mM MOPS, pH 6.8).

Antisera. Antibodies used were as follows: M13 polyclonal antibodies, which recognize approximately 13 proteins on Western blots of total *S. guttatum* mitochondrial proteins (14); M9 polyclonal antibodies, which only recognize the

35-, 36-, and 37 kDa proteins (15); and the AOA, AOU, and AOL monoclonal antibodies (15). The AOA antibodies recognize all three polypeptides, AOU antibodies recognize primarily the 37 kDa polypeptide, and AOL antibodies recognize primarily the 35- and 36 kDa polypeptides (15).

Gel Electrophoresis, Immunoblotting, and Fluorography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was performed using the buffer system of Laemmli (24) as previously described (14). Immunoblotting was done according to the procedure of Blake *et al.* (25) except that antibody incubations were for 1 hour at room temperature. After SDS-PAGE, the gels were subjected to sodium salicylate fluorography using the procedure of Chamberlin (26).

Protein Sequencing. The 36 kDa alternative oxidase protein was electroeluted from polyacrylamide gel slices and SDS was extracted from the samples by the procedure of Konigsberg and Henderson (27). The N-terminus of the protein was sequenced following the procedure of Vandekerckhoue *et al.* (28). However, the first three amino acids were not identified.

Poly (A)+ RNA Isolation. Total RNA was isolated from frozen (in liquid N_2) *S. guttatum* appendices by the procedure of McIntosh and Cattolico (29), except that the tissue was homogenized with a mortar and pestle, 0.015 M EDTA was used in the extraction buffer, and the RNA was extracted three times with phenol,

three times with phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol), and three times with chloroform. The poly (A)+ fraction was isolated using a poly U Sepharose (Sigma, St. Louis, MO) column by the method of Cashmore (30).

***In Vitro* Translation of Poly (A)+ RNA and Immunoprecipitation.** Poly (A)+ RNA and hybrid-selected RNA transcripts were translated *in-vitro* using a rabbit reticulocyte lysate system (Promega, Madison, WI) with [^{35}S]-methionine (Amersham, Arlington Heights, IL) as the radioactive label. The protocol of the manufacturer (Promega, Madison, WI) was used, except that 165 mM potassium acetate and 1.05 mM magnesium acetate (final concentrations) were used in a 25 μl final reaction volume and incubations were done at 37°C. Specific *in vitro* translation products were immunoprecipitated by the addition of M9 polyclonal antibodies or preimmune antiserum and formalin-fixed *Staphylococcus aureus* cells using a modification of the procedure of Anderson and Blobel (31) as described in Hondred *et al.* (32).

Isolation of cDNA Clones. A *S. guttatum* cDNA expression library was constructed in the EcoRI sites of lambda ZAPII (33) by Stratagene (La Jolla, CA). Poly (A)+ RNA from the appendix of a D-day plant and a mixture of oligo dT and random primers were used to synthesize the cDNA. Both M13 polyclonal sera and a mixture of AOA, AOU, and AOL monoclonal antisera were employed to screen the cDNA expression library following Stratagene's *pico*Blue Immunodetection protocol. Inserts from phage isolates which produced proteins

that were recognized by both polyclonal and monoclonal antisera were subcloned by Stratagene's excision procedure as described by Short *et al.* (33). Small and large scale phagemid isolation and subcloning into pUC119 were done using standard procedures (34).

Hybrid Selection. Phagemids pAOSG81, pAOSG83, and pBLUESCRIPT (see Results for descriptions) were used to select homologous RNA transcripts from total poly (A)+ RNA by the procedure given in Maniatis *et al.* (34). The selected RNA was precipitated by addition of $MgCl_2$ to 10 mM, KAc to 100 mM, and cold ethanol to 67% (v/v) and incubation at -20°C.

Expression of Phagemid Inserts in *E. coli*. Liquid cultures of *E. coli* strain TG1 cells containing phagemids pAOSG81-119, pAOSG81i-119, or pUC119 (see Results for descriptions) were used for expression experiments. The insert of the phagemid pAOSG81-119 is in-frame with the *lacZ* gene in pUC119 so that a fusion protein was produced after addition of isopropyl-B-D-thiogalactopyranoside (IPTG) to the growth media. After one hour of growth at 37°C in LB medium (see 34 for description) containing 25-50 μg ampicillin/ml, IPTG was added to experimental cultures to a final concentration of 25 μg/ml; no IPTG was added to negative control cultures. Growth was continued for 8 hours, at which time the cells were pelleted and resuspended in 30 μl of SDS-PAGE sample buffer (24).

DNA Sequencing and Analysis. Single-stranded deletions of pAOSG81-119 and pAOSG81i-119 were made using the procedure of Dale (35). Sequencing of the phagemid inserts was done by the dideoxy method of Sanger *et al.* (36) using Sequenase® T7 DNA Polymerase (U.S. Biochemical Corporation, Cleveland, OH). Protein sequence was deduced from the nucleotide sequence using the Editbase DNA sequence analysis program. Helical wheel projections of the deduced amino acid sequence were made according to von Heijne (37). The structure prediction plot of the deduced amino acid sequences was made using the Seqanal Predictor program (38), a modified version of the Chou-Fasman protein secondary structure predictor program which is adapted for membrane proteins.

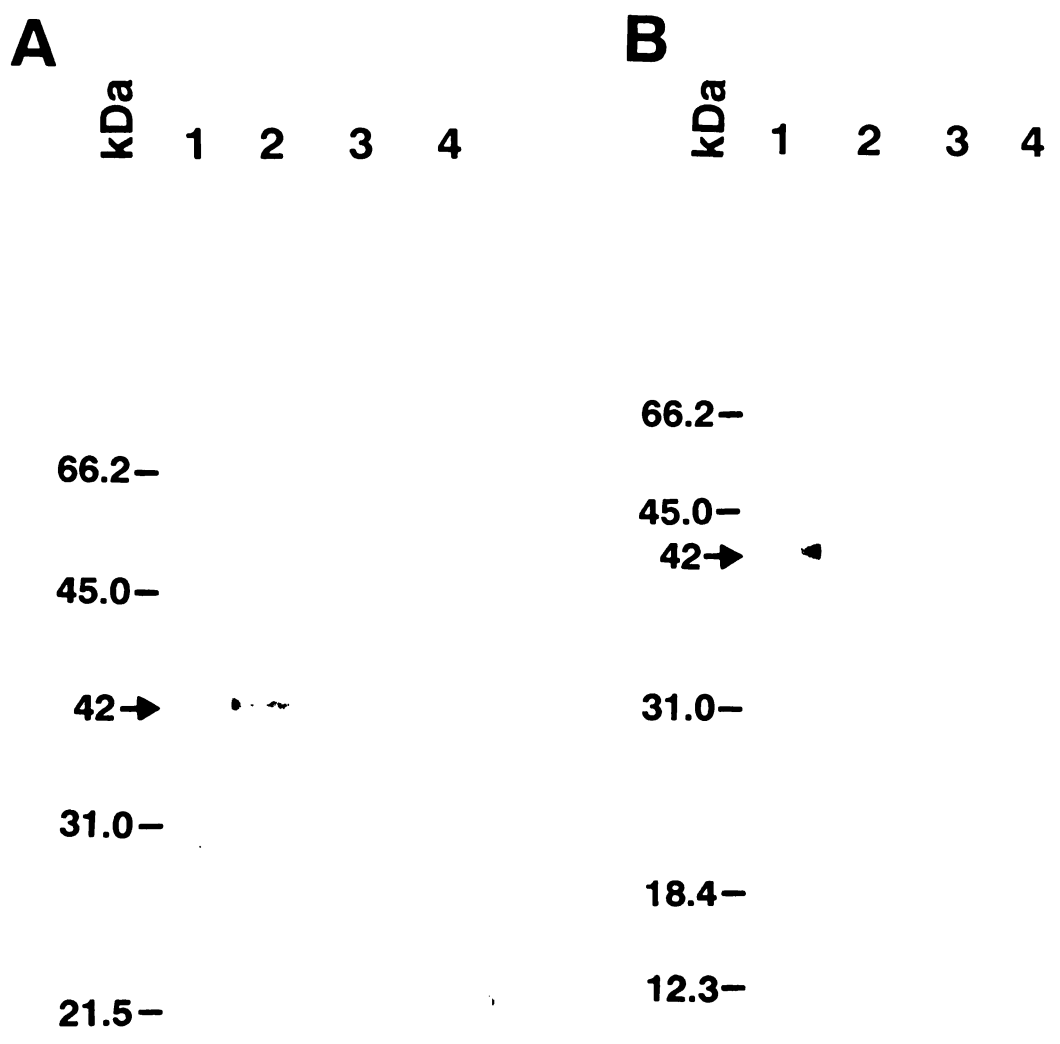
RESULTS

***In Vitro* Translation of Total Poly (A)+ RNA and Immunoprecipitation.** An *in vitro* translation product with an apparent molecular mass of 42 kDa was readily immunoprecipitated from total *in vitro* translation products made from poly (A)+ RNA with the M9 polyclonal antibodies (Fig. 2-1A & 2-1B, lane 1), but not with preimmune antiserum (data not shown). The alternative oxidase proteins previously identified (15) have apparent molecular masses of 35-, 36-, and 37 kDa, suggesting that the 42 kDa protein is a nuclear-encoded precursor of one or more of the alternative oxidase proteins.

Isolation of cDNA Clones. After two rounds of screening, five plaques which produced proteins that were recognized by both polyclonal and monoclonal antisera were selected for further analysis. The inserts from these clones were "rescued" as Bluescript phagemids. Phagemids from two of these putative alternative oxidase clones were designated pAOSG81 and pAOSG83 and contained inserts of 1400 base pairs and 1100 base pairs in length, respectively. The inserts of pAOSG81 and pAOSG83 were subcloned into pUC119 and the resulting vectors were designated pAOSG81-119, pAOSG83-119, and pAOSG81i-119 (pUC119 containing the insert of pAOSG81 in an inverted orientation).

***In Vitro* Translation and Immunoprecipitation of Hybrid Selected RNA.** Phagemids pAOSG81, pAOSG83, and pBLUESCRIPT were used to select homologous transcripts from total poly (A)+ RNA isolated from D-day appendix tissue. Upon *in vitro* translation of the selected RNA, SDS-PAGE, and fluorography, several diffuse bands representing proteins of widely distributed molecular masses were observed (Fig. 2-1A). These bands probably represent proteins translated from poly (A)+ RNA that bound nonspecifically to the nitrocellulose paper since they are present in all lanes. However, in the lanes corresponding to pAOSG81 and pAOSG83 a very abundant protein with an apparent molecular mass of 42 kDa was observed (Fig. 2-1A, lanes 2 & 3). This protein was not observed among the *in vitro* translation products from RNA selected by vector DNA alone (Fig. 2-1A, lane 4) and it comigrated with the protein made from total poly (A)+ RNA that was also immunoprecipitated with

Figure 2-1. Products from *in vitro* translation of hybrid-selected poly (A)+ RNA (**Panel A**) which could also be immunoprecipitated with M9 polyclonal antibodies (**Panel B**). **Panel A:** total poly (A)+ RNA and hybrid-selected poly (A)+ RNA transcripts were translated *in vitro* in the presence of [^{35}S]-methionine. The products were seperated by SDS-PAGE and visualized by fluorography. Lane 1; total poly (A)+ RNA translated *in vitro* and immunoprecipitated (see Materials and Methods) to indicate the position of the 42 kDa protein. The remaining lanes contained only products translated *in vitro* from RNA selected by the following phagemid samples: Lane 2, pAOSG81; Lane 3, pAOSG83; and Lane 4, pBLUESCRIPT. **Panel B:** total poly (A)+ RNA and hybrid selected poly (A)+ RNA transcripts were translated *in vitro*, immunoprecipitated, and visualized by fluorography (see Materials and Methods). The *in vitro* translation products were made from the following RNA samples proir to immunoprecipitation: Lane 1; total poly (A)+ RNA, Lane 2; pAOSG81 selected poly (A)+ RNA, Lane 3; pAOSG83 selected poly (A)+ RNA, and Lane 4; pBLUESCRIPT selected poly (A)+ RNA.


A
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1
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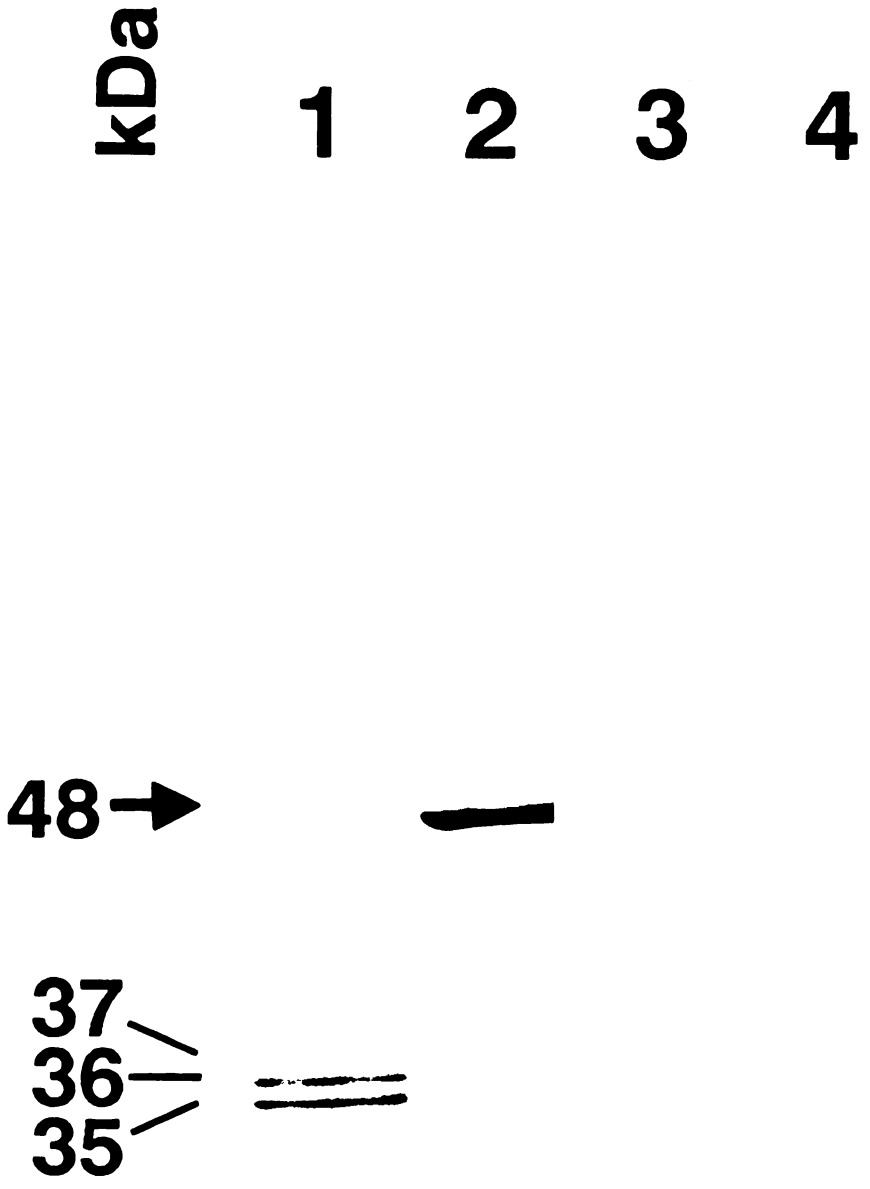
45.0
42
31.0
18.4
12.3

M9 polyclonal antisera (Fig. 2-1A, lane 1).

Fluorography of an SDS-polyacrylamide gel demonstrated that the 42 kDa protein translated from poly (A)+ RNA selected by phagemids pAOSG81 and pAOSG83 could also be immunoprecipitated by the M9 polyclonal antibodies (Fig. 2-1B, lanes 2 & 3). These data indicate that clones pAOSG81 and pAOSG83 encode a 42 kDa protein that is immunologically related to the 35-, 36-, and 37 kDa alternative oxidase proteins.

Expression of Plasmid Inserts in *E. coli*. Fig. 2-2, lane 2 shows that the AOA monoclonal antibodies recognize the protein encoded by pAOSG81-119 when it is expressed in *E. coli*. The protein has an apparent molecular mass of about 48 kDa, and was not expressed in uninduced cells (Fig. 2-2, lane 3), in cells that contained only pUC119 (Fig. 2-2, lane 4), or in cells that contained pAOSG81i-119, which has the insert from pAOSG81-119 inverted relative to the *lacZ* gene (data not shown). The increased size of the polypeptide arose as a result of cloning. A portion of the cDNA for *aox1*, a region which does not encode protein in the isolated gene, was inserted in such a manner so that it is now "in frame" and recognized as coding sequence behind the *lacZ* gene. DNA sequence analysis later showed that pAOSG81-119 is predicted to express a fusion protein of 44,794 Da and consist of 21 amino acids of B-galactosidase, 24 amino acids now encoded by the 5' non-coding region of the insert, and the 349 amino acids of the precursor alternative oxidase protein.

Figure 2-2. Western blot of the 48 kDa alternative oxidase/B-galactosidase fusion protein expressed in *E. coli.* Cell cultures containing phagemids were grown in the presence or absence of IPTG. An equal number of cells from each culture were pelleted, solubilized, and seperated by SDS-PAGE. The proteins were transferred onto nitrocellulose paper and detected using AOA monoclonal antibodies and alkaline-phosphatase conjugated goat anti-mouse IgG. Lane 1; total mitochondrial proteins from *S. guttatum* appendix tissue. The remaining lanes contained proteins from *E. coli* cultures grown in the presence (Lanes 2 & 4) or absence (Lane 3) of IPTG and which contained the following phagemids: Lane 2; pAOSG81-119, Lane 3; pAOSG81-119, Lane 4; pUC119.


kDa
1
2
3
4
48
37
36
35

DNA Sequence Analysis. The insert of pAOSG81-119 contains an open reading frame of 1047 base pairs which encodes a deduced polypeptide sequence of 349 amino acids (Fig. 2-3) with a calculated molecular mass of 38,931 Da. There is also a nine amino acid match between the deduced amino acid sequence of pAOSG81-119 and N-terminal amino acid sequence data that had been previously obtained using gel-purified 36 kDa alternative oxidase protein. Because of some ambiguity in protein sequencing, it was not possible to determine the specific N-terminal amino acid. However, the sequencing data do indicate that Ala-64 is the most likely N-terminal amino acid, and our analyses are based upon this assumption. The nine amino acid match begins at Leu-67 of the protein deduced from pAOSG81, as indicated in Fig. 2-3. If the first 63 amino acids of the deduced protein were removed during import into the mitochondria, then the mature protein would contain 286 amino acids and have a calculated molecular mass of 32,200 Da and a calculated isoelectric point of about 6.6, which is in relative agreement with the observed isoelectric point of 7.2-7.3 (39). Helical wheel projections of the putative transit peptide of the protein deduced from the sequence of pAOSG81 indicate that this region of the protein is capable of forming segments of amphiphilic helices (data not shown). Three adjacent regions of the deduced protein are predicted to be in alpha helical conformations and are likely to be membrane spanning as determined by the statistical Rao-Argos protein structure analysis program (Fig. 2-4). These three regions are as follows: a region from amino acids 171 to 202 (numbering based on the unprocessed protein), a region from amino acids 207 to 228, and a region from amino acids

233 to 262. Furthermore, between these regions of predicted helical conformation there are regions which are predicted to form turns in the protein backbone (40).

DISCUSSION

We have isolated a cDNA clone, pAOSG81, representing a *S. guttatum* nuclear gene, which we have called *aox1*, encoding a precursor alternative oxidase protein with a calculated molecular mass of 38.9 kDa and an apparent molecular mass of 42 kDa. The fusion protein expressed from pAOSG81 in *E. coli* has an apparent molecular mass of 48 kDa and was recognized by monoclonal antibodies to the alternative oxidase proteins. The observation that the 42 kDa precursor protein is nuclear-encoded is consistent with the results of Bertrand *et al.* (41), which indicate that a single nuclear gene encodes the alternative oxidase in *Neurospora crassa*. Our results do not rule out the possibility that there is more than one gene encoding the alternative oxidase in *S. guttatum*. However, this is unlikely since 1) it appears that a single 42 kDa protein was immunoprecipated from the products made *in vitro* from total poly (A)+ RNA (Fig. 2-1A & 2-1B, lane 1); 2) transcripts of a single size were homologous to the insert of pAOSG81 on Northern blots of total RNA from *S. guttatum* appendix tissue (data not shown); and 3) Southern blots of genomic DNA probed with pAOSG81 also indicate that the nuclear gene, *aox1*, is present in a single copy (data not shown). It is also possible that one of the alternative oxidase proteins is encoded by the mitochondrial genome. However, this is likewise unlikely since cycloheximide (an

Figure 2-3. Nucleotide and deduced amino acid sequence of the insert of pAOSG81-119. Both strands of the insert of pAOSG81-119 were sequenced by making unidirectional, single-stranded deletions of pAOSG81-119 and pAOSG81i-119 and sequencing by the dideoxy chain termination method. The triangle indicates the assumed start codon and first amino acid of the protein. The open box indicates the region of the deduced amino acid sequence which matches the amino acid sequence previously obtained by chemical sequencing. The first in-frame stop codon is indicated by the star.

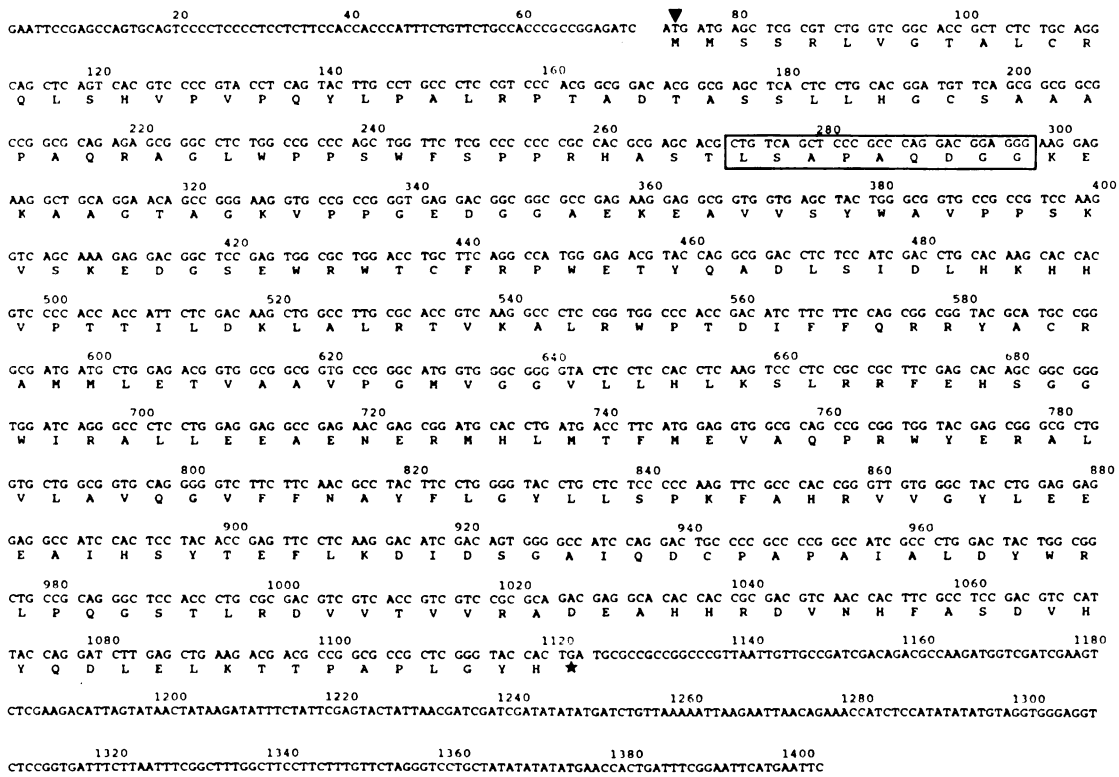
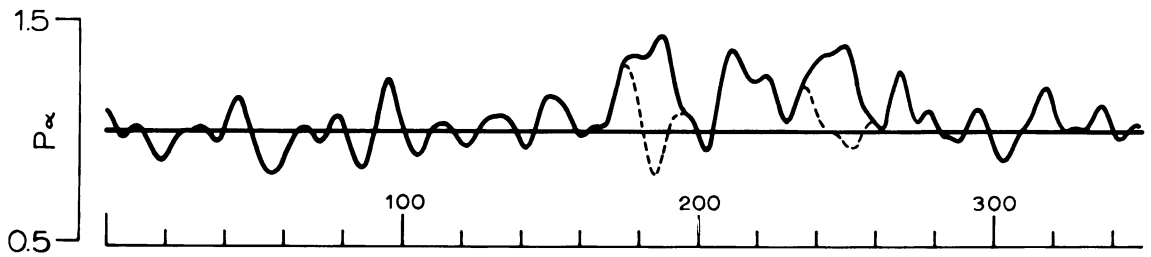

20 40 60 80 100
GAATTCCGAGCCAGTGCAGTCCCCTCCCCTCCTCTTCCACCACCCATTTCTGTTCTGCCACCCGCCGGAGATC ATG ATG AGC TCG CGT CTG GTC GGC ACC GCT CTC TGC AGG
M M S S R L V G T A L C R
120 140 160 180 200
CAG CTC AGT CAC GTC CCC GTA CCT CAG TAC TTG CCT GCC CTC CGT CCC ACG GCG GAC ACG GCG AGC TCA CTC CTG CAC GGA TGT TCA GCG GCG GCG
Q L S H V P V P Q Y L P A L R P T A D T A S S L L H G C S A A A
220 240 260 280 300
CCG GCG CAG AGA GCG GGC CTC TGG CCG CCC AGC TGG TTC TCG CCC CCC CGC CAC GCG AGC ACG CTG TCA GCT CCC GCC CAG GAC GGA GGG AAG GAG
P A Q R A G L W P P S W F S P P R H A S T L S A P A Q D G G K E
320 340 360 380 400
AAG GCT GCA GGA ACA GCC GGG AAG GTG CCG CCG GGT GAG GAC GGC GGC GCC GAG AAG GAG GCG GTG GTG AGC TAC TGG GCG GTG CCG CCG TCC AAG
K A A G T A G K V P P G E D G G A E K E A V V S Y W A V P P S K
420 440 460 480
GTC AGC AAA GAG GAC GGC TCC GAG TGG CGC TGG ACC TGC TTC AGG CCA TGG GAG ACG TAC CAG GCG GAC CTC TCC ATC GAC CTG CAC AAG CAC CAC
V S K E D G S E W R W T C F R P W E T Y Q A D L S I D L H K H H
500 520 540 560 580
GTC CCC ACC ACC ATT CTC GAC AAG CTG GCC TTG CGC ACC GTC AAG GCC CTC CGG TGG CCC ACC GAC ATC TTC TTC CAG CGG CGG TAC GCA TGC CGG
V P T T I L D K L A L R T V K A L R W P T D I F F Q R R Y A C R
600 620 640 660 680
GCG ATG ATG CTG GAG ACG GTG GCG GCG GTG CCG GGC ATG GTG GGC GGG GTA CTC CTC CAC CTC AAG TCC CTC CGC CGC TTC GAG CAC AGC GGC GGG
A M M L E T V A A V P G M V G G V L L H L K S L R R F E H S G G
700 720 740 760 780
TGG ATC AGG GCC CTC CTG GAG GAG GCC GAG AAC GAG CGG ATG CAC CTG ATG ACC TTC ATG GAG GTG GCG CAG CCG CGG TGG TAC GAG CGG GCG CTG
W I R A L L E E A E N E R M H L M T F M E V A Q P R W Y E R A L
800 820 840 860 880
GTG CTG GCG GTG CAG GGG GTC TTC TTC AAC GCC TAC TTC CTG GGG TAC CTG CTC TCC CCC AAG TTC GCC CAC CGG GTT GTG GGC TAC CTG GAG GAG
V L A V Q G V F F N A Y F L G Y L L S P K F A H R V V G Y L E E
900 920 940 960
GAG GCC ATC CAC TCC TAC ACC GAG TTC CTC AAG GAC ATC GAC AGT GGG GCC ATC CAG GAC TGC CCC GCC CCG GCC ATC GCC CTG GAC TAC TGG CGG
E A I H S Y T E F L K D I D S G A I Q D C P A P A I A L D Y W R
980 1000 1020 1040 1060
CTG CCG CAG GGC TCC ACC CTG CGC GAC GTC GTC ACC GTC GTC CGC GCA GAC GAG GCA CAC CAC CGC GAC GTC AAC CAC TTC GCC TCC GAC GTC CAT
L P Q G S T L R D V V T V V R A D E A H H R D V N H F A S D V H
1080 1100 1120 1140 1160 1180
TAC CAG GAT CTT GAG CTG AAG ACG ACG CCG GCG CCG CTC GGG TAC CAC TGA TGCGCCGCCGGCCCGTTAATTGTTGCCGATCGACAGACGCCAAGATGGTCGATCGAAGT
Y Q D L E L K T T P A P L G Y H ★
1200 1220 1240 1260 1280 1300
CTCGAAGACATTAGTATAACTATAAGATATTTCTATTCGAGTACTATTAACGATCGATCGATATATATGATCTGTTAAAAATTAAGAATTAACAGAAACCATCTCCATATATATGTAGGTGGGAGGT
1320 1340 1360 1380 1400
CTCCGGTGATTTCTTAATTTCGGCTTTGGCTTCCTTCTTTGTTCTAGGGTCCTGCTATATATATATGAACCACTGATTTCGGAATTCATGAATTC

Figure 2-4. Secondary structure prediction for the protein deduced from the nucleotide sequence of the insert of pAOSG81-119. The solid line represents the values obtained using the Seqanal Predictor program. The dashed line represents the values obtained using the Chou-Fasman program. Abscissa indicates amino acid position starting from the first amino acid of the putative precursor protein. Ordinate indicates P_α with a window of 15 amino acids (52).


1.5
P∝
0.5
100
200
300

inhibitor of cytoplasmic translation but not of mitochondrial translation) blocks expression of the alternative oxidase proteins in *S. guttatum* appendix tissue sections which were treated with salicylic acid (Rhoads and McIntosh, in preparation). This raises the intriguing possibility that the 35-, 36-, and 37 kDa proteins are all posttranslationally-modified products of the 42 kDa protein.

From the nucleotide sequence of pAOSG81 and the amino acid sequence of the 36 kDa alternative oxidase protein we determined that the N-terminus of the 36 kDa protein is internal to the amino acid sequence of the 38.9 kDa protein deduced from the nucleotide sequence, though the exact site of processing is not clear since the exact N-terminal amino acid of the 36 kDa protein was not determined from the sequencing. The putative transit peptide possesses many properties which are common to previously described mitochondrial transit peptides (42). It has a high content of amino acids considered to be either alpha helix formers (a total of 28) or neutral (a total of 22) relative to the number of helix breakers (a total of 13). The transit peptide also has regions that are likely to form amphiphilic helices and it has a net positive charge. Similar to many other transit peptides of mitochondrial proteins, there is a high abundance of the amino acids Ala, Leu, Arg, and Ser and with low abundance of Asp, Glu, Ile, and Lys (42). These data are consistent with the hypothesis that the 42 kDa protein is a precursor of one or more of the alternative oxidase proteins with a 63 amino acid transit peptide which is removed as the protein is transported into the mitochondria.

It has been shown that the alternative oxidase of another member of the Aroid family, *Arum maculatum*, is associated with the inner surface of the inner mitochondrial membrane (43). Analysis of our sequence data indicates that the region of the 42 kDa alternative oxidase precursor protein that is most likely associated with the mitochondrial membranes of *S. guttatum* is between amino acids 108 to 199. This region contains three stretches of amino acids which are predicted to form transmembrane alpha helices.

There have been numerous proposals (44,45,46) concerning the identity of the higher plant alternative oxidase. Flavin has been proposed as a cofactor (46); however, this seems unlikely since the affinity of mitochondrial flavoproteins for O_2 is too low and the kinetics of reoxidation are too slow (3,46). Also, auto-oxidizable flavoproteins produce H_2O_2 (45,46), whereas the alternative pathway produces H_2O (44,45,46). Nonheme iron (3,44,46) is an unlikely cofactor since no clear electron paramagnetic resonance (EPR) signal from a nonheme iron has been correlated with the presence of the alternative pathway (47). Copper (44,45), is likewise an unlikely candidate because of the lack of a clear EPR signal (48). Nevertheless, iron and copper cannot yet be ruled out completely since it is possible that an EPR signal from one or the other is masked (44,46) as in the case of coupled binuclear copper sites, designated as "type 3" copper (49). Since there is little precise structural data on the active sites of enzymes containing these cofactors, the deduced amino acid sequence of the alternative oxidase of *S. guttatum* does not allow us to draw many conclusions about possible cofactors of this enzyme. However, we can conclude from the sequence that this protein

alone does not contain consensus iron-sulfur protein sequence motifs which form Cys ligands in the 4Fe-4S ferredoxins and the 2Fe-2S type of ferredoxins (50,51).

It has also been proposed (45) that the alternative oxidase itself may transfer electrons directly from ubiquinone to oxygen and that it may, therefore, contain a ubiquinone binding site. Since the structures of quinone binding sites are still unknown, we cannot determine, on the basis of deduced protein sequence alone, if the alternative oxidase protein encoded by pAOSG81 contains such a site.

The isolation of an alternative oxidase cDNA clone is a new step toward answering questions concerning the role of the oxidase in higher plants and other organisms. While the mechanism and function of the alternative oxidase cannot be deduced in the absence of more biochemical and physiological data, it is now possible to use the gene to investigate the developmental regulation of alternative pathway appearance in *S. guttatum* and other higher plants. We intend to explore the role of the alternative oxidase in non-thermogenic plants through genetic manipulations that produce plants modified in their expression of the alternative oxidase.

ACKNOWLEDGEMENTS

We would like to thank the following: Dr. B. J. D. Meeuse for providing the *S. guttatum* plants; Dr. Thomas E. Elthon and Roxy Nickels for isolating the 36 kDa protein and for helpful advice; Dr. J. F. Vandekerckhoue for sequencing the 36 kDa protein; Dr. David Hondred for providing the *S. aureus* cells and for helpful advice; Dr. John Fitchen for helpful advice; Dr. Niels Nielson for the use of the Editbase program; and Dr. A. Crofts for the use of the Seqanal Package. This work was supported in part by U. S. Department of Energy contract DE-AC02-76ERO-1338 and National Science Foundation grant DCB-8904518.

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CHAPTER 3

SALICYLIC ACID REGULATION OF RESPIRATION IN HIGHER PLANTS: ALTERNATIVE OXIDASE EXPRESSION

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In Press in *The Plant Cell.*

ABSTRACT

Alternative respiratory pathway capacity increases during the development of the thermogenic appendix of a voodoo lily inflorescence. The levels of the alternative oxidase proteins increased dramatically between D-4 (four days prior to the day of anthesis) and D-3 and continued to increase until the day of anthesis (D-day). The level of salicylic acid in the appendix is very low early on D-1, but increases to a high level in the evening of D-1. Thermogenesis occurs after a few hours of light on D-day. Therefore, the initial accumulation of the alternative oxidase proteins precedes the increase in salicylic acid by 3 days, indicating that other regulators may be involved. A 1.6-kb transcript encoding the alternative oxidase precursor protein accumulated to a high level in the appendix tissue by D-1. Application of salicylic acid to immature appendix tissue caused an increase in alternative pathway capacity and a dramatic accumulation of the alternative oxidase proteins and the 1.6-kb transcript. Time course experiments showed that the increase in capacity, protein levels, and transcript level corresponded precisely. The response to salicylic acid was blocked by cycloheximide or actinomycin D, indicating that de novo transcription and translation are required. However, nuclear, in vitro transcription assays indicated that the accumulation of the 1.6-kb transcript did not result from a simple increase in the rate of transcription of *aox1*.

INTRODUCTION

All higher plants examined contain two pathways for mitochondrial electron flow: the cytochrome respiratory pathway and the alternative respiratory pathway (Bendall, 1958; Bendall and Bonner, 1971). The alternative pathway diverges from the cytochrome pathway after the ubiquinone pool (Bendall and Bonner, 1971; Rich and Moore, 1976; Storey, 1976). Therefore, electron flow through the alternative pathway is not coupled to ATP synthesis at the two sites of proton gradient formation (complex III and complex IV) that are downstream of the ubiquinone pool (Storey and Bahr, 1969; Moore and Bonner, 1982). The energy of electron flow through the alternative pathway is not conserved as chemical energy, but is lost as heat (Storey and Bahr, 1969). Specific floral tissues of some aroid plants develop such a high level of the alternative pathway that they become thermogenic (Meeuse, 1975; Day et al., 1980; Raskin et al., 1987). The day the inflorescence of the aroid species voodoo lily blooms (D-day), the capacity of the alternative pathway in the appendix tissue is 300-1000 natoms O/min/mg protein and the temperature of the tissue increases about 9-12°C above ambient temperature (Raskin et al., 1987). Immature appendix tissue (8 to 2 days prior to blooming, D-8 through D-2) has a relatively low alternative pathway capacity, 50-100 natoms O/min/mg protein. In voodoo lilies, the heat produced during blooming is used to volatilize foul smelling compounds, such as indoles, which attract insect pollinators (Meeuse, 1966). The role of the alternative pathway in non aroid plants is unknown (Lambers, 1985).

The appearance of three antigenically related mitochondrial proteins with apparent molecular masses of 35-, 36-, and 37 kD strongly correlates with the activity of the alternative oxidase, the terminal oxidase of the alternative respiratory pathway, in voodoo lily appendix tissue (Elthon and McIntosh, 1987). A 42 kD protein that is a putative precursor of all three of these alternative oxidase proteins has been identified (Rhoads and McIntosh, 1991). Since only the 42 kD protein was immunoprecipitated from products made by in vitro translation of total voodoo lily RNA, it is likely that the 35-, 36-, and 37 kD proteins are post-translationally modified products of the 42 kD protein. A cDNA clone, pAOSG81, corresponding to the nuclear gene, *aox1*, encoding the 42 kD protein has been isolated and characterized (Rhoads and McIntosh, 1991).

Salicylic acid has been shown to be an endogenous "trigger" of thermogenesis in voodoo lily appendix tissue (Raskin et al., 1987). Salicylic acid is produced in the male floral region of the inflorescence and moves into the appendix beginning early on D-1 and "triggers" thermogenesis at about noon of D-day (Raskin et al., 1987, 1989). The level of salicylic acid in the appendix increases from below 100 ng/g fresh weight as late as D-2 to more than 1.0 μg/g fresh weight in the evening of D-1 (Raskin et al., 1989). Salicylic acid has also been shown to dramatically increase both the alternative pathway activity and the levels of the 35-, 36-, and 37 kD alternative oxidase proteins when it is applied to voodoo lily appendix tissue sections (Elthon et al., 1989b). Prior to the application of salicylic acid, the 37 kD protein is constitutively expressed and is present at a moderate level in immature appendix tissue. All three alternative oxidase proteins

are present in great abundance in the tissue sections following incubation in a phosphate-buffered solution containing salicylic acid. In addition, Raskin et al. (1989) showed that light is required for salicylic acid to cause thermogenesis in appendix tissue sections.

Salicylic acid may be involved in flowering in many plant species (Raskin, 1992) and in systemic acquired resistance (SAR) in others (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991). Furthermore, and the endogenous level of salicylic acid is very high in a few species of higher plants, including rice (Raskin et al. 1990). However, the pathway by which salicylic acid is produced and the mechanisms by which salicylic acid acts remain unknown. Therefore, identification of the mechanism(s) by which salicylic acid regulates gene expression in voodoo lilies will be valuable in understanding the role(s) of salicylic acid in various plant tissues, the physiology of plant responses to pathogens, and the physiology of thermogenic tissues.

MATERIALS AND METHODS

Plant Material. Voodoo lily (*Sauromatum guttatum*) plants were maintained as previously described (Elthon and McIntosh, 1987). The day the inflorescence blooms and the appendix tissue of the spadix heats is referred to as D-day (for anatomy see Meeuse, 1966). Other developmental stages of the plant are indicated as the number of days before or after D-day (D-1 being the day before and D+1 the day after D-day). Between the time the inflorescence begins to

develop and the end of D-2, the inflorescence is referred to as "immature."

Salicylic Acid and Inhibitor Treatment of Tissue Sections. At 8 AM (time 0) of D-6, D-5, or D-4 the appendix tissue of a voodoo lily plant was cut into 1.0-1.5 cm sections. Sections were placed into 10 mL beakers containing 1.0 mL of phosphate buffer (10 mM KH_2PO_4 and 50 μg/mL streptomycin) and a drop of buffer was placed on top of each section.

For the time course experiments, one set of sections at each time point was incubated in phosphate buffer alone and one set in phosphate buffer containing 1.0 mM salicylic acid. The portion of the appendix remaining on the plant until the inflorescence bloomed is referred to as the D-day tissue sample. Application of calorigen served as a positive control for the induction of the accumulation of the alternative oxidase proteins (Raskin et al., 1987). Calorigen, a crude extract of the male floral region of the inflorescence, was prepared as described by Raskin et al. (1987).

For the inhibitor experiments, actinomycin D (60 μg/mL final concentration; Sigma), an inhibitor of transcription, was added to one of four sets of sections and cycloheximide (15 μg/mL final concentration; Sigma), an inhibitor of translation by 80S ribosomes, was added to a second set. After 2 hr of incubation at room temperature, salicylic acid was added (1.0 mM final concentration) to the two experimental sets of sections and the positive control set. The negative control set was incubated in phosphate buffer for the duration of the experiment. The beakers were covered and incubated at room temperature

for an additional 22 hr.

For the nuclear, in vitro transcription experiments, six to eight sections from all along the length of an appendix were incubated in phosphate buffer for 24 hr while other sections from the same appendix were incubated in phosphate buffer containing 1.0 mM salicylic acid.

Isolation of Mitochondria and Respiration Assays. Mitochondria were isolated by a modification of the procedure of Schwitzguebel and Siegenthaler (1984) as described previously (Rhoads and McIntosh, 1991). The oxygen content of air-saturated water was estimated according to Estabrook (1987). Respiration rates were measured as oxygen uptake using a Rank Brothers (Cambridge, UK) oxygen electrode. The capacity of the alternative pathway was measured in 1.0 mL of reaction medium (250 mM sucrose, 30 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 6.8) at 25°C with 1 mM NADH as the substrate. Carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP) was added to 0.5 μM after NADH in order to diminish the electrochemical gradient prior to measurement of the pathway capacity (Elthon et al., 1986). The capacity of the alternative pathway was taken as the rate of oxygen uptake sensitive to 1 mM salicylhydroxamic acid in the presence of 1 mM KCN (Elthon et al., 1987). The capacities were standardized by dividing each value by the total amount of protein in the reaction (which is directly proportional to the number of mitochondria in the assay). The amount of total protein in each sample was determined by the procedure of Larson et al. (1986).

Gel Electrophoresis, Immunoblotting, and Antisera. SDS-PAGE and immunoblotting were performed as described previously (Elthon et al., 1987). Antibodies used were the AOA monoclonal antibodies raised to the 36 kD alternative oxidase protein of voodoo lily, and which recognize all three alternative oxidase proteins of voodoo lily (Elthon et al., 1989a). Since the same amount of total mitochondrial protein was loaded onto each lane of each polyacrylamide gel, the protein detected on protein blots represents the proportion of total mitochondrial proteins that constitutes the alternative oxidase proteins.

Plasmid Insert Isolation and Radiolabeling. The 1400-bp EcoRI insert of pAOSG81 (Rhoads and McIntosh, 1991) was purified by as described in Maniatis et al. (1989) except that the elution buffer was 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.05% SDS, and 0.3 M LiCl and the insert was electrophoresed through two successive polyacrylamide gels. The purified fragment was resuspended to a concentration of 60 ng/μl in Tris-EDTA. The purified inserts were used to make DNA radiolabeled with α-^{32}P-dATP (Amersham Corporation) by the random primer method (Feinberg and Vogelstein, 1983). Specific activity of the DNA probes was routinely about 10^7 cpm/μg template, as determined by the sodium phosphate wash method (Maniatis et al., 1989).

RNA Isolation and Blots. Total RNA was isolated from frozen (in liquid N_2) voodoo lily appendix tissue by the procedure of McIntosh and Cattolico (1978) as described by Rhoads and McIntosh (1991. RNA was separated on agarose gels

containing formaldehyde as described by Ausubel et al. (1987), and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) as described by Maniatis et al. (1989). All RNA blots were probed with radiolabeled DNA made using the purified insert of pAOSG81 as described above. Hybridization was done under high-stringency hybridization conditions as described by Maniatis et al. (1989). Autoradiography using Kodak diagnostic film XAR-5 was used to view the hybridization results as described by Ausubel at al. (1987). The amount of radiolabeled probe that hybridized to each sample was determined using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Since each lane of each RNA blot contained the same amount of total RNA, the amount of hybridized probe detected represents the proportion of total RNA that constitutes the alternative oxidase transcript.

Isolation of Nuclei and In Vitro Transcription. Nuclei were isolated following method II of Luthe and Quatrano (1980a) except that the discontinuous gradients contained 25, 50, and 80% (v/v) Percoll layers, all on top of a 2.0 M sucrose cushion and the centrifugation for the washes was done at 1100*g*. Most of the nuclei were found at the interface between the 80% Percoll and 2.0 M sucrose layers as determined by fluorescence microscopy of a 50/50 (v/v) mixture of nuclei and stain [20 mg/mL 4',6-diamidino-2-phenylindole (DAPI), 0.1X phosphate-buffered saline, 0.05% (w/v) sodium azide, 90% (v/v) glycerol]. Nuclei were isolated from the appendix tissue of voodoo lily plants on D-6, D-5, D-4, or on D-day. In a separate experiment, tissue sections from a D-6 plant were incubated

for 24 hr in phosphate buffer or in phosphate buffer containing 1.0 mM salicylic acid. Nuclei were then isolated from the appendix tissue sections. In vitro RNA synthesis was performed essentially as described by Luthe and Quatrano (1980b). The final concentrations of ATP and GTP were both 0.5 mM, 3-5 μL of α-^{32}P-UTP (20 mCi/mL, 800 Ci/mmol) was used in each synthesis, and the final volume was 200 μL.

Isolation of In Vitro Synthesized RNA. After the in vitro synthesis, RNase-free DNase I was added to a final concentration of 0.5 μg/μL and the mixture was incubated at 26°C for 5 min. An equal volume of proteinase K mix (200 μg/mL proteinase K, 2% SDS, 10 mM EDTA, 20 mM Tris-HCl pH 7.4, and 200 μg/mL yeast tRNA) was added to each sample and the samples were incubated at 37°C for 30 min. Each sample was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1[v/v]). The RNA was precipitated twice using ammonium acetate and ethanol as described by Maniatis et al. (1989) and resuspended in 50 μL of STE (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). NaOH was added to a final concentration of 0.2 N, and the RNA was incubated on ice for 10 min, followed immediately by the addition of 1 M Hepes to a final concentration of 0.25 M. The RNA was ethanol precipitated and resuspended in 50 μL of Tris-EDTA. The total number of counts in each RNA sample was determined by the sodium phosphate wash method (Maniatis et al., 1989). To compare gene expression at different developmental stages, we used the same number of total counts per minute of the RNA made from immature

appendix tissue nuclei as the number of total counts per minute of the RNA made from the D-day appendix tissue nuclei. To compare gene expression in salicylic acid treated and untreated tissue, we used the same number of total counts per minute of the RNA made from untreated appendix tissue nuclei as the number of total counts per minute of the RNA made from the nuclei from the salicylic acid-treated appendix tissue.

Hybridization of In Vitro Synthesized RNA to DNA Probes. The unlabeled DNA samples used to determine the levels of the specific transcripts produced by in vitro transcription in isolated nuclei are as follows: 1) the single-stranded form of the phagemid pAOSG81, corresponding to the antisense of the 1.6-kb alternative oxidase transcript; 2) a single-stranded form of phagemid p25SSG10; and 3) denatured pSAc3 DNA (a generous gift of Dr. R. B. Meagher). Phagemid p25SSG10 is a cDNA clone that has been partially sequenced and corresponds to a voodoo lily 25S ribosomal RNA gene based on the observation that it has >90% sequence similarity to bases 846 to 1982 of the rice 25S rRNA gene (Takaiwa at al., 1985). Plasmid pSAc3 is a genomic clone that represents a soybean actin gene (Shah at al., 1982). The DNA samples were denatured and applied to a nitrocellulose membrane as described by Ausubel et al. (1987). Prehybridization, hybridization, and washing conditions were essentially as described by Ausubel et al. (1987), except that 50 mM sodium phosphate was used instead of potassium phosphate, 25 μg/mL of salmon sperm DNA, and 1X Denhardt's solution (1X Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA)

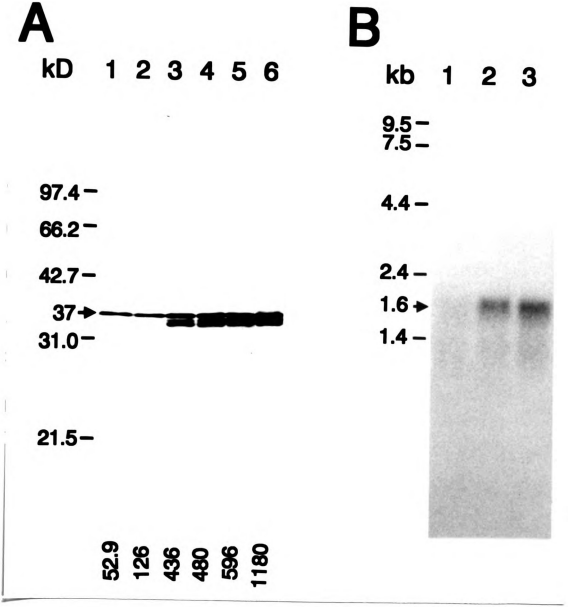
were used in the prehybridization and hybridization solutions, and 12.5% dextran sulfate was used in the hybridization solution. Hybridization was performed at 42°C for 72 hr and the filters were washed 4 times in 2X SSC (1X SSC is 0.15 M NaCl, 0.015 sodium citrate) containing 0.1% (v/v) SDS for 5 min at room temperature and once in 0.1X SSC containing 0.1% (v/v) SDS for 5 min at 50°C. The amount of radiolabeled RNA that hybridized to each probe was visualized by autoradiography (Maniatis at al., 1989).

RESULTS

Developmental Expression of the Alternative Oxidase Gene of Voodoo Lily. Figure 3-1A shows that the 37 kD alternative oxidase protein was constitutively expressed in the appendix tissue and was present at about the same level in the appendix on D-5 and D-4 (lanes 1 and 2). The level increased significantly by 10 AM of D-3 (lane 3) and reached a peak on D-day (lane 6). The levels of the 35- and 36 kD proteins were very low in the appendix tissue at D-5 and D-4 (lanes 1 and 2) and increased dramatically by 10 AM of D-3 (lane 3). The levels continued to increase steadily on D-2 (lane 4) and D-1 (lane 5), and were the highest on D-day (lane 6). It is interesting to note that the levels of the alternative oxidase proteins were already fairly high by D-3, even though thermogenesis does not occur until about noon on D-day.

Figure 3-1B shows that the 1.6-kb alternative oxidase transcript accumulated to a very high level in the appendix tissue of the voodoo lily

Figure 3-1. Developmental Expression of the Alternative Oxidase Proteins and Transcript in Voodoo Lily Appendix Tissue. **Panel A:** Immunoblot of total mitochondrial proteins from a single voodoo lily appendix at different days during inflorescence development: D-5, lane 1; D-4, lane 2; D-3, lane 3; D-2, lane 4; D-1, lane 5; D-day, lane 6. Mitochondria were isolated at 10 AM each day and assayed for alternative pathway capacity. The mitochondrial proteins (30 μg per lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the AOA monoclonal antibodies. Alternative pathway capacity in natoms O/min/mg protein of each mitochondrial sample is indicated at the bottom. Apparent molecular masses in kilodaltons are indicated to the left. **Panel B:** RNA blot of total RNA from a second appendix. All RNA was isolated from the appendix tissue at 10 AM of each day during inflorescence development: D-2, lane 1; D-1, lane 2; D-day, lane 3. The RNA (10 μg per lane) was separated by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose. The radiolabeled probe was made from the purified insert of pAOSG81. Transcript lengths in kilobases are indicated to the left.


A
kD 1 2 3 4 5 6
97.4
66.2
42.7
37
31.0
21.5
52.9
126
436
480
596
1180
B
kb 1 2 3
9.5
7.5
4.4
2.4
1.6
1.4

inflorescence by 10 AM of D-1 (lane 2). Comparatively little of the 1.6-kb transcript was present at 10 AM of D-2 (lane 1). The level of the 1.6-kb alternative oxidase transcript, relative to the total RNA of the appendix tissue, was significantly higher at 10 AM of D-day (lane 3) than it was at 10 AM of D-1 (lanes 2).

Time Course of Salicylic Acid Induced Alternative Oxidase Expression. Figure 3-2 shows that the capacity of the alternative pathway in immature appendix tissue sections treated with salicylic acid reached a peak by about 16 hr after salicylic acid application. The capacity was slightly lower by 24 hr after application. Quantitatively the capacity at the 16 hr time point was almost three higher than the capacity at time 0, and the capacity decreased about 20% by the 24 hr time point.

Figure 3-3A shows that the levels of the alternative oxidase proteins, particularly the 35- and 36 kD proteins, at each time point corresponded to the alternative pathway capacity (compare to Figure 3-2). A slight increase in the levels of the 35- and 36 kD proteins, compared to a time 0 and negative control, was observed as early as 5 hr after salicylic acid application (D. M. Rhoads and L. McIntosh, unpublished results). The peak in the accumulation of the proteins always occurred by 16 hr after salicylic acid application (lanes 16- and 16+), as did the peak in the capacity. Qualitatively, the levels of the proteins remained constant between the 16 and the 24 hr time points (lanes 24- and 24+), although the capacity usually decreased slightly (Figure 3-2).

Figure 3-2. Time Course Graph of Alternative Pathway Capacity in Response to Applied salicylic acid. Voodoo lily appendix tissue sections were incubated in phosphate buffer (triangles and broken line) or buffer plus 1.0 mM salicylic acid (circles and solid line). Mitochondria were isolated at each time point, and the capacity was determined using a Rank Brothers oxygen electrode. Results are averages of three experiments.

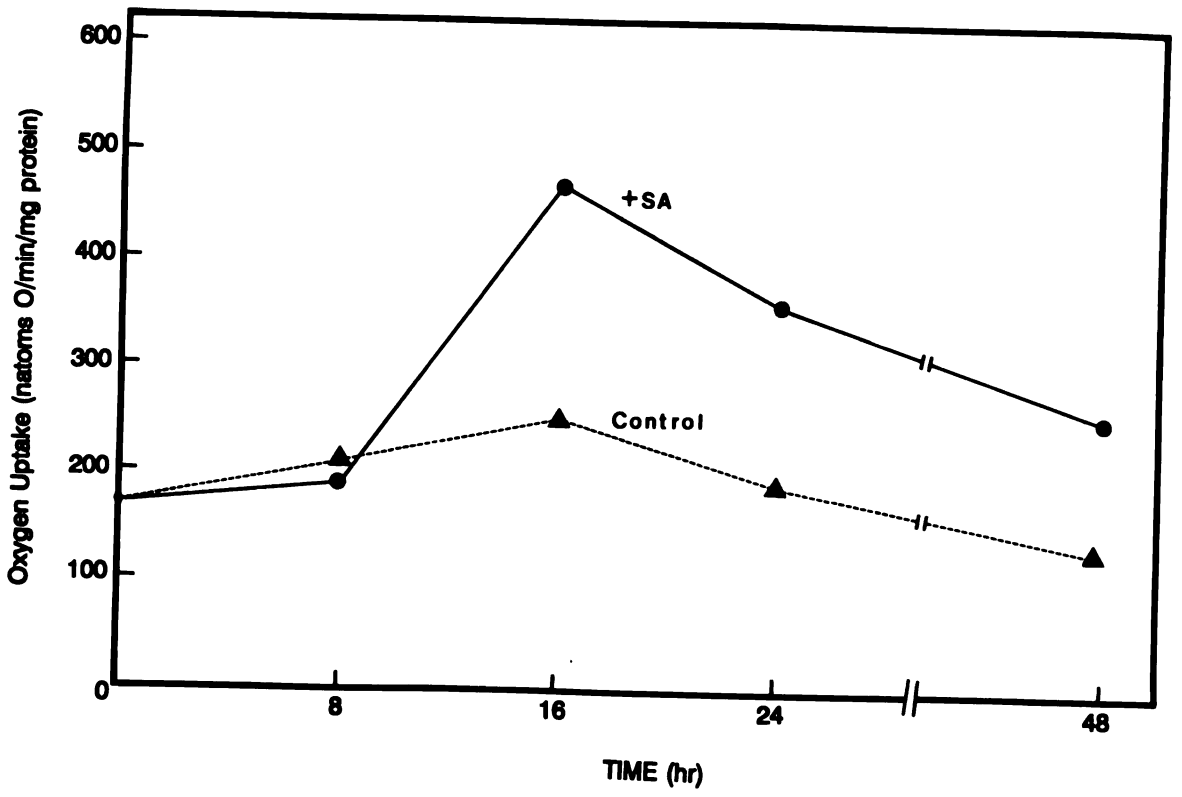

600
500
400
300
200
100
0
Oxygen Uptake (natoms O/min/mg protein)
+SA
Control
8
16
24
48
TIME (hr)

Figure 3-2. Time Course Graph of Alternative Pathway Capacity in Response to Applied salicylic acid. Voodoo lily appendix tissue sections were incubated in phosphate buffer (triangles and broken line) or buffer plus 1.0 mM salicylic acid (circles and solid line). Mitochondria were isolated at each time point, and the capacity was determined using a Rank Brothers oxygen electrode. Results are averages of three experiments.

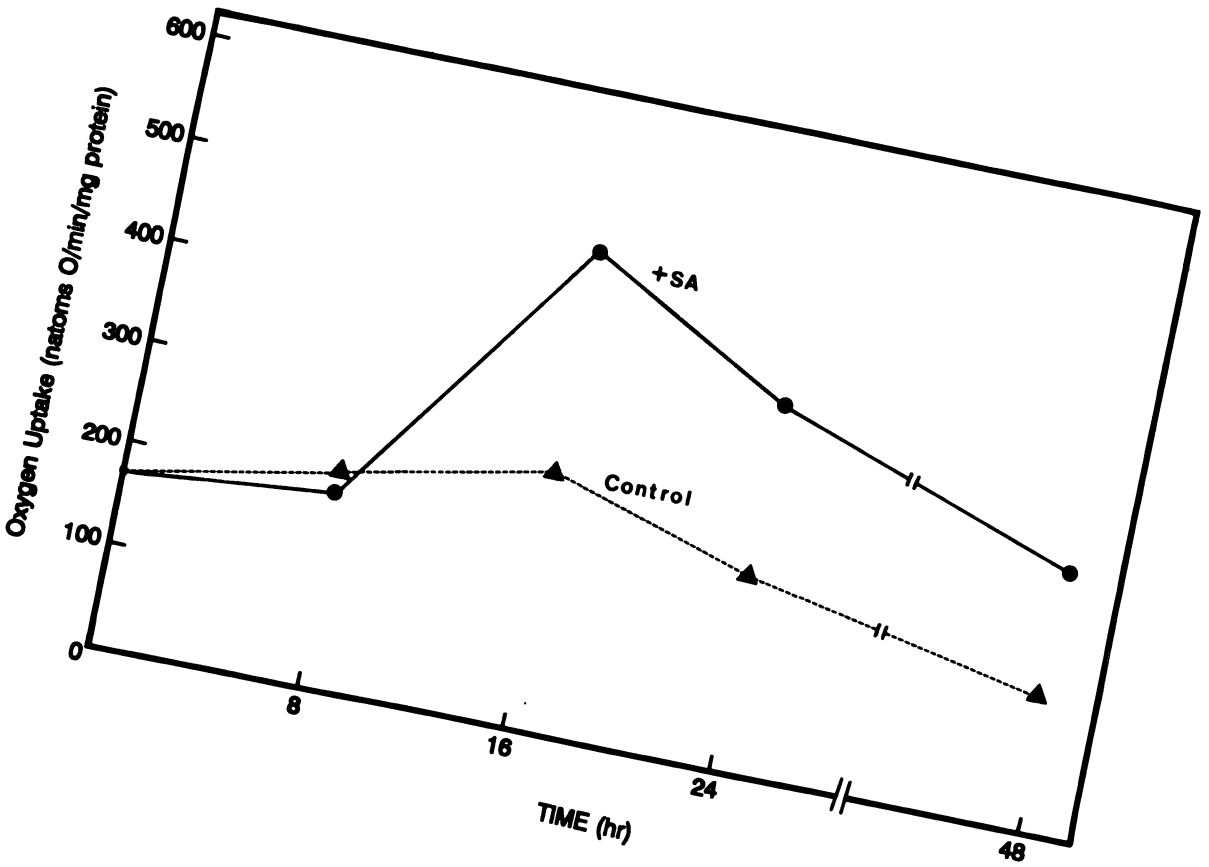
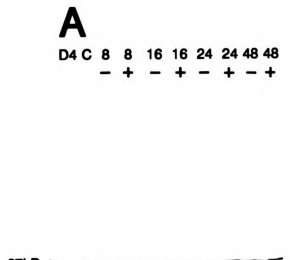

Oxygen Uptake (natoms O/min/mg protein)
600
500
400
300
200
100
0
+SA
Control
8
16
24
48
TIME (hr)

Figure 3-3B shows that the accumulation of the 1.6-kb alternative oxidase transcript corresponded precisely to the accumulation of the alternative oxidase proteins. Transcript accumulation above the control level was apparent by 4 hr after the application of salicylic acid (lanes 4- and 4+). The accumulation was increased by the 8 hr time point (lanes 8- and 8+). The highest level of the transcript was reached by 16 hr after salicylic acid application (lanes 16- and 16+), and the peak level was maintained until at least 24 hr after the application (lanes 24- and 24+). Lanes D5 and D0 of Figure 3-3 show the level of the alternative oxidase transcript that was present at D-5 and D-day, respectively. It is interesting to note that the amount of the 1.6-kb transcript was slightly higher at time 0 (lane D5) than in the negative control at the 4 hr time point (lane 4-). Furthermore, the level of the alternative oxidase transcript increased steadily over the course of the experiment rather than rapidly at a specific time point.

Inhibition of the Effects of Applied Salicylic Acid. Figure 3-4A shows that application of salicylic acid to immature (D-4) appendix tissue sections for 24 hr induced the accumulation of the alternative oxidase proteins, particularly the 35- and 36 kD proteins (lane 2), relative to the amount present in untreated tissue sections (lane 1). The accumulation of the proteins was significantly blocked by incubating the tissue sections for 2 hr in a cycloheximide solution (lane 4) or for 2 hr in an actinomycin D solution (lane 3) prior to the addition of salicylic acid for 22 hr. Slightly more of the 35- and 36 kD proteins was present in the sample treated with actinomycin D than in the sample treated with cycloheximide. This

Figure 3-3. Time Course of Accumulation of Alternative Oxidase Proteins and Transcript after Salicylic Acid Application. **Panel A:** Immunoblot showing the levels of the alternative oxidase proteins at specific time points after salicylic acid application. Voodoo lily appendix tissue sections were incubated in phosphate buffer (-) or buffer plus 1.0 mM salicylic acid (+) or buffer plus calorigen (lane C). Mitochondria were isolated from the tissue sections from a D-4 plant at time 0 (lane D4) or at 8 hr (lanes 8- and 8+), 16 hr (lanes 16- and 16+), 24 hr (lanes C, 24-, and 24+), or 48 hr (lanes 48- and 48+) after the application of salicylic acid. The capacity of the alternative pathway was determined. The mitochondrial proteins from each sample (30 μg per lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the AOA monoclonal antibodies. **Panel B:** RNA blot showing the level of the 1.6-kb alternative oxidase transcript at specific time points after salicylic acid application. Tissue sections from a D-5 plant were frozen in liquid N_2 at time 0 (lane D5); 4 hr (lanes 4- and 4+), 8 hr (lanes 8- and 8+), 16 hr (lanes 16- and 16+), or 24 hr (lanes 24- and 24+) after salicylic acid application; and at 10 AM of D-day (lane D0). Total RNA was then isolated, separated by formaldehyde-agarose gel electrophoresis (10 μg per lane) and transferred to nitrocellulose. The radiolabeled probe was made from the purified insert of pAOSG81.


A
D4 C 8 8 16 16 24 24 48 48
– + – + – + – +
37kD
B
D5 D0 4 4 8 8 16 16 24 24
– + – + – + – +
1.6kb

would be expected if actinomycin D did not completely inhibit transcription. Figure 3-4B shows that application of salicylic acid to immature (D-4) appendix tissue sections for 24 hr induced the accumulation of the 1.6-kb alternative oxidase transcript (lane 2) relative to the level of the transcript found in untreated tissue sections (lane 1). Again, the accumulation of the 1.6-kb transcript was blocked by preincubation in either cycloheximide (lane 4) or actinomycin D (lane 3).

Nuclear, In Vitro Transcription. Figure 3-5A shows that nuclear, in vitro transcription of the voodoo lily rRNA gene(s) occurred in nuclei isolated from D-6 appendix tissue (slot I1), but in vitro synthesis of the alternative oxidase transcript was very low in the nuclei isolated from appendix tissue at this stage of development (slot I2). Figure 3-5B shows that in vitro transcription of rRNA gene(s) also occurred in nuclei isolated from D-day (mature) appendix tissue (slot M1), but in vitro synthesis of the alternative oxidase transcript was very low in these nuclei as well (slot M2). Figure 3-5C shows that in vitro transcription of rRNA gene(s) (slot C1) and the actin gene(s) (slot C2) occurred in nuclei isolated from control D-4 tissue, but in vitro synthesis of the alternative oxidase transcript was very low in these nuclei (slot C3). Finally, Figure 3-5D shows that in vitro transcription of rRNA gene(s) (slot D1) and the actin gene(s) (slot D2) also occurred in nuclei isolated from D-4 tissue treated with salicylic acid, but that in vitro synthesis of the alternative oxidase transcript was very low in these nuclei as well (slot D3).

Figure 3-4. Inhibition of Salicylic Acid Induced Expression of Alternative Oxidase Proteins and Transcript. **Panel A:** Immunoblot of the salicylic acid induced expression of the alternative oxidase and inhibition of expression. Tissue sections from a single voodoo lily appendix from a D-4 plant were incubated for 24 hr as follows: in phosphate buffer, lane 1; in buffer containing 1.0 mM salicylic acid, lane 2; in buffer plus actinomycin D for 2 hr followed by the addition of salicylic acid for 22 hr, lane 3; in buffer plus cycloheximide for 2 hr followed by the addition of salicylic acid for 22 hr, lane 4. Mitochondria were isolated after the 24 hr incubations and the capacity of the alternative pathway was determined for each sample. For the protein analysis, the mitochondrial proteins from each sample (30 μg per lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the AOA monoclonal antibodies. Alternative pathway capacity in natoms O/min/mg protein of each mitochondrial sample is indicated at the bottom. The results are representative of four experiments. **Panel B:** RNA blot of the salicylic acid induced expression of the alternative oxidase and inhibition of expression. The tissue sections from a separate appendix from a D-5 plant were treated as follows for 24 hr: in phosphate buffer, lane 1; in buffer containing 1.0 mM salicylic acid, lane 2; in buffer plus actinomycin D for 2 hr followed by the addition of salicylic acid for 22 hr, lane 3; in buffer plus cycloheximide for 2 hr followed by the addition of salicylic acid for 22 hr, lane 4. Total RNA from each sample (10 μg per lane) was separated by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose. The radiolabeled probe was made from the purified insert of pAOSG81. Results are representative of two experiments.

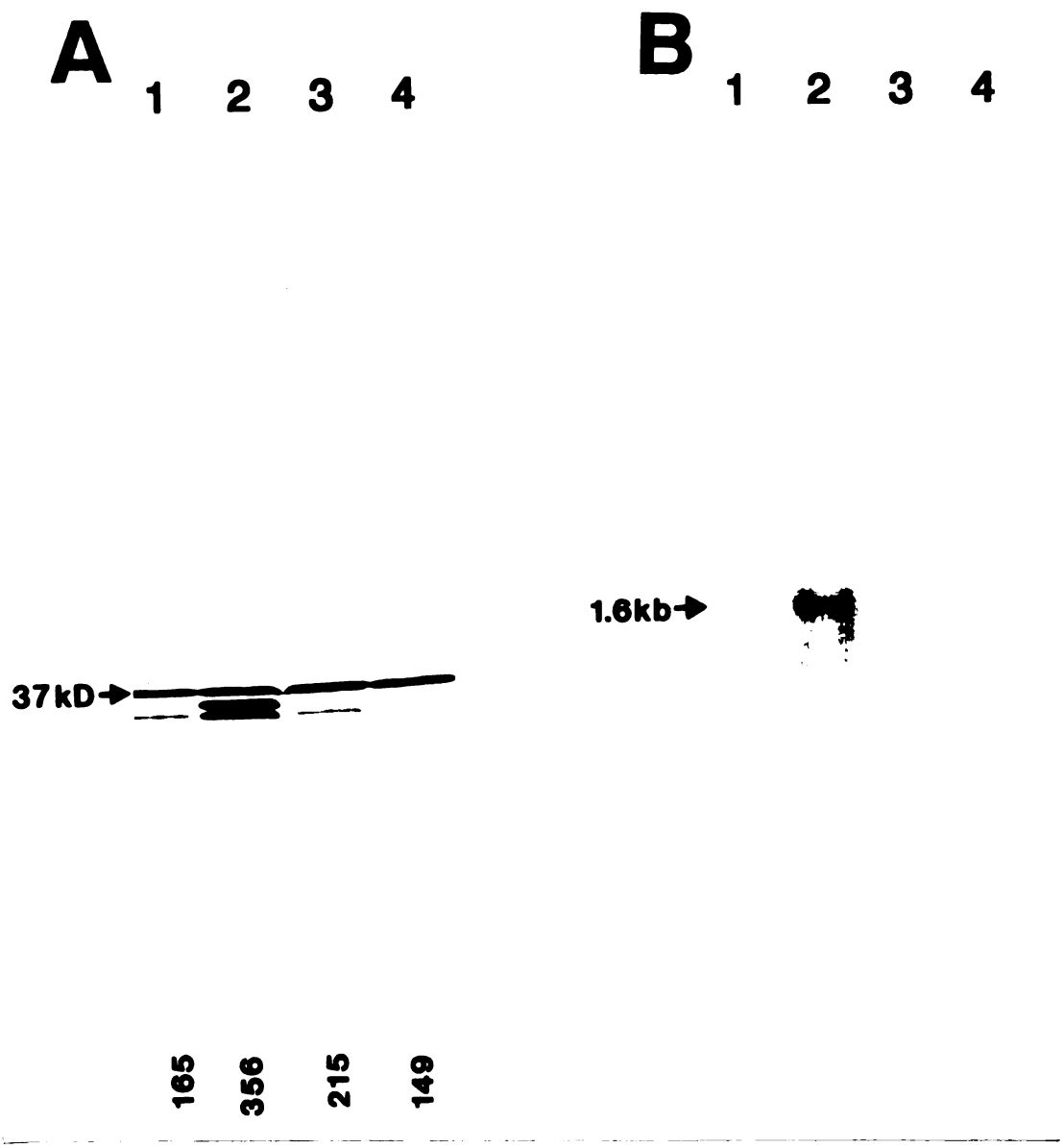
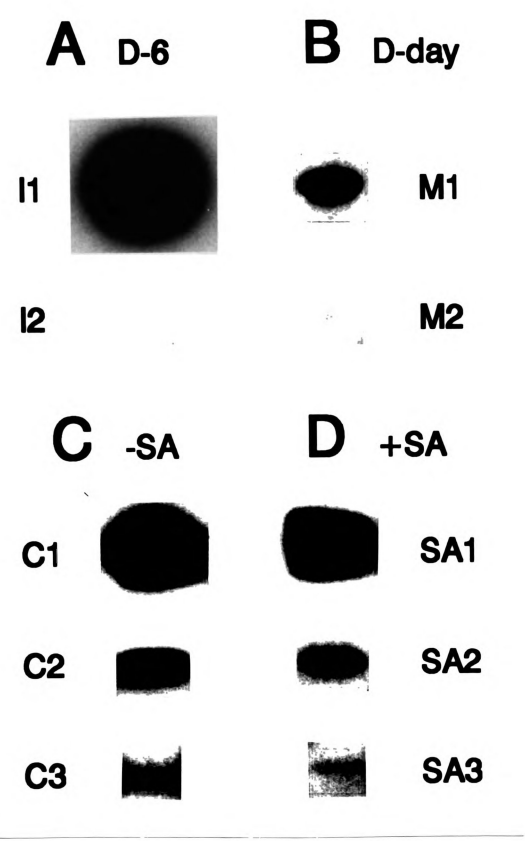

A
1 2 3 4
B
1 2 3 4
1.6kb
37kD
165
356
215
149

Figure 3-5. Nuclear, in vitro Transcription Assays. **Panel A:** Radiolabeled RNA made from D-6 nuclei was hybridized to nitrocellulose-bound, single-stranded DNA probes corresponding to the antisense strand of p25SSG10, slot I1; or pAOSG81, slot I2. **Panel B:** Radiolabeled RNA made from D-Day nuclei was hybridized to nitrocellulose-bound, single-stranded DNA probes corresponding to the antisense strand of p25SSG10, slot M1; or pAOSG81, slot M2. **Panel C:** Radiolabeled RNA made from nuclei from untreated D-4 appendix tissue was hybridized to nitrocellulose-bound, single-stranded DNA probes corresponding to the antisense strand of p25SSG10, slot C1; pAOSG81, slot C2; or a double-stranded, denatured HindIII-MspI fragment of pSAc3, slot C3. **Panel D:** Radiolabeled RNA made from nuclei from salicylic acid treated D-4 appendix tissue was hybridized to nitrocellulose-bound, single-stranded DNA probes corresponding to the antisense strand of p25SSG10, slot SA1; pAOSG81, slot SA2; or a double-stranded, denatured HindIII-MspI fragment of pSAc3, slot SA3.

Nuclei isolated from the appendix tissue of a single voodoo lily plant on D-6 and on D-day were used for the experiment yielding the results shown in **Panel A** and **Panel B**. Nuclei isolated from appendix tissue sections of a single D-4 voodoo lily plant were used for the experiment yielding the results shown in **Panel C** and **Panel D**. The sections for **Panel C** were incubated in phosphate buffer for 24 hr, while the sections for **Panel D** were incubated in buffer plus 1 mM salicylic acid for 24 hr.


A D-6
B D-day
I1
M1
I2
M2
C -SA
D +SA
C1
SA1
C2
SA2
C3
SA3

DISCUSSION

In the appendix tissue of the voodoo lily inflorescence, salicylic acid is an endogenous signal that "triggers" an increase in the capacity of alternative respiratory pathway, which results in thermogenesis (Meeuse, 1966; Raskin et al., 1987). The levels of the alternative oxidase proteins, particularly the 35- and 36 kD proteins, present at each day (from D-5 until D-day) in the development of the inflorescence corresponded to the level of the alternative pathway capacity. The level of the 1.6-kb transcript, which encodes the 42 kD alternative oxidase precursor protein, increased from a relatively low level at 10 AM on D-2 to a high level on D-1. The level of the 1.6-kb transcript remained high until at least 10 AM of D-day, when the level of each of the alternative oxidase proteins was highest and when thermogenesis of the appendix tissue is approaching a peak (Raskin et al., 1987, 1989). However, Raskin et al. (1989) have demonstrated that salicylic acid begins to appear in the appendix tissue early on D-1, but does not reach a peak until late in D-1 or during the dark period between D-1 and D-day.

Taken together these data indicate that the capacity of the alternative pathway and the levels of the alternative oxidase proteins in the appendix tissue begin to rise relatively early in the development of the inflorescence (D-3), when the level of salicylic acid is, presumably, quite low. In contrast, the amount of the 1.6-kb transcript is relatively low until at least 10 AM of D-1, at which time there is a dramatic increase in the level. This increase may correspond to the appearance of salicylic acid in the appendix tissue. We have not determined the

level of salicylic acid in the appendix tissue used for this these experiments, but the peak in the salicylic acid level usually occurs late on D-1 (Raskin et al., 1989). The basal level (Fig. 3-3, lane D5) of the transcript probably accounts for the constitutive expression of the 37 kD protein and allows for the steady rise in the accumulation of the 35- and 36 kD proteins. We have not determined if the level of the 1.6-kb transcript changes between D-5 and D-2.

Because the accumulation of the alternative oxidase proteins in developing appendix tissue seems to precede the dramatic rise in the salicylic acid levels observed by Raskin et al. (1989), it is possible that the developmental regulation of expression of the alternative oxidase gene, *aox1*, of voodoo lily appendix tissue is controlled by one or more additional regulators, not by salicylic acid alone. salicylic acid may then act as a light-induced (Raskin et al., 1987, 1989) "booster" of the transcript and protein levels at the critical time of D-1 through D-day so that thermogenesis occurs at precisely the proper time. The developmental experiments and the time course studies indicate that the accumulation of the alternative oxidase transcript is the first step in the dramatic burst of alternative pathway capacity between D-1 and D-day, which results in thermogenesis. Therefore, it is likely that salicylic acid functions at the level of transcript accumulation.

Applied salicylic acid causes immature appendix tissue sections to become thermogenic between 16 and 24 hr after application. We have demonstrated that thermogenesis is accompanied by an increase in the capacity of the alternative pathway, which peaks at 16 hr after application. The accumulation of the

alternative oxidase proteins, which began as early as 5 hr after the application of salicylic acid, also peaked at 16 hr and remained essentially constant until 24 hr after application. The level of the 1.6-kb alternative oxidase transcript began to rise by about 4 hr after the application of salicylic acid, continued to rise to a peak at 16 hr after the application, and remained essentially constant until 24 hr. Thus, the time course for increase in alternative pathway capacity, the time course for the accumulation of the alternative oxidase proteins, and the time course for the accumulation of the transcript coincided precisely. Furthermore, these results agree with a developmental time course in which salicylic acid, released from the male floral region on D-1, "boosts" the levels of the alternative oxidase transcript and proteins, resulting in thermogenesis at about noon on D-day. In addition, these results demonstrate that salicylic acid alone (in lighted conditions) was sufficient to cause these changes.

It is difficult to derive a clear picture of how salicylic acid regulates the accumulation of the alternative oxidase proteins in the mitochondria of the appendix tissue from the inhibitor data presented here. It is possible that the effects of cycloheximide are due to the inhibitor simply blocking the synthesis of a protein(s) involved in general translation or a protein(s) involved in general transcription. Likewise, it is possible that actinomycin D blocks the transcription of a gene(s) whose product(s) is involved in general translation or transcription. Although we do not know the components of the salicylic acid signal transduction pathway that are eliminated by cycloheximide and/or actinomycin D, it is clear that both de novo transcription and de novo translation are required for the

accumulation of the alternative oxidase transcript and proteins. If the inhibitors are not having general effects, then it appears that salicylic acid is affecting the accumulation of the 1.6-kb alternative oxidase transcript because actinomycin D does block the accumulation of the transcript when salicylic acid is present.

The nuclear, in vitro transcription data presented indicate that the rate of transcription of *aox1* was the same at D-6 and D-day. Likewise, nuclear, in vitro transcription assays using nuclei isolated from untreated, immature (D-5) appendix tissue and sections from the same appendix treated with salicylic acid for 24 hr indicate that the rate of transcription of *aox1* was the same in the treated tissue and the untreated tissue. It is possible that the level of detection of the nuclear, in vitro transcription assays was not sensitive enough to detect the levels of the alternative oxidase transcript produced in these tissues. However, this possibility seems unlikely because the amount of the transcript is extremely high in the appendix tissue at D-day and after treatment with salicylic acid. Furthermore, the rate of transcription of *aox1* may increase at a specific time in the development of the appendix and at a specific time after the application of salicylic acid, and we may not have isolated nuclei from the appendix tissue at one of these specific times.

Based on the data presented, there are several possible mechanisms by which salicylic acid is regulating the expression of the alternative oxidase proteins and the capacity of the pathway. Because salicylic acid does induce an accumulation of the 1.6-kb alternative oxidase transcript, we assume that this is the ultimate purpose of salicylic acid in regulating the alternative pathway in the

appendix tissue. However, this does not eliminate the possibility that salicylic acid also regulates the accumulation of the alternative oxidase proteins in the mitochondria by increasing the production of an unidentified protein(s) involved in transport of the precursor protein to the inner mitochondrial membrane. Alternatively, salicylic acid may cause a stabilization of the 1.6-kb transcript by binding to a protein that stabilizes the 1.6-kb *aox1* transcript or initiating a complex series of events that eventually results in a stabilization of the *aox1* transcript. Any of these mechanisms would be inhibited by both actinomycin D and cycloheximide if the protein(s) involved must be made by de novo transcription and translation. A putative, soluble salicylic acid-binding protein in tobacco has recently been identified (Chen at al., 1991). It is possible that this protein is involved in some pathway leading to a response to salicylic acid in tobacco.

Finally, it is likely that a complex mechanism accounts for the salicylic acid regulation of the alternative respiratory pathway. However, once the mechanism is elucidated, it will be very interesting to determine if salicylic acid regulates the expression of other proteins in other higher plants, such as the PR and SAR proteins in tobacco and cucumber, via the same mechanism.

ACKNOWLEDGEMENTS

This study was supported in part by the U.S. Department of Energy contract DE-AC02-76ERO-1338 (L.M.) and the National Science Foundation DCB-8904518 (L.M.). We thank the following: Dr. Bastiaan J. D. Meeuse for providing the *S. guttatum* plants; Dr. Richard B. Meagher for providing the pSAc3 DNA; Dr. Ilya Raskin, Dr. Thomas E. Elthon, and Dr. Michael A. Mansfield for helpful advice and discussions.

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CHAPTER 4

ISOLATION AND CHARACTERIZATION OF A GENOMIC CLONE CONTAINING THE *SAUROMATUM GUTTATUM* ALTERNATIVE OXIDASE GENE *AOX1*

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FOOTNOTES

[1]Supported in part by U.S. Department of Energy contract DE-AC02-76ERO-1338 (L.M.) and the National Science Foundation DCB-8904518 (L.M.).

[2]Abbreviations: PR, pathogenesis related; GRP, glycine rich protein; SAR, systemic acquired resistance; RBCS, ribulose bisphosphate carboxylase small subunit; Mops, 3-(*N*-morpholino)propanesulfonic acid; FCCP, carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone; SHAM, salicylhydroxamic acid

ABSTRACT

We have isolated and characterized a genomic clone, λAOSG11, corresponding to *aox1*, which encodes the 42 kD alternative oxidase precursor protein of *Sauromatum guttatum* Schott. The sequence of λAOSG11 revealed that *aox1* consists of four exons separated by three short introns. Exon three contains the region of *aox1* that: 1) is highly conserved in the corresponding genes of potato, rice, and yeast; and 2) encodes a region of the deduced protein that is predicted to form two transmembrane α-helices, which are predicted to embed the mature protein in the inner mitochondrial membrane. Southern blot analysis of restriction endonuclease digested genomic DNA, indicated that *aox1* is a single, nuclear-encoded gene in *S. guttatum*. We have determined the transcriptional start site of *aox1* using nuclease protection and primer extension experiments. Comparison of the putative promoter region of *aox1* to promoters of PR1a and GRP8 revealed some sequence similarity.

INTRODUCTION

The alternative and cytochrome respiratory pathways exist together in the mitochondria of higher plants, fungi, algae, and protista (10,40). If electrons flow through the cytochrome pathway, a proton gradient is established at three sites along the pathway (3,41,24). The potential energy of the proton gradient is used to produce chemical energy in the form of ATP (25). If electrons flow through the alternative pathway, a proton gradient is formed from, at most, one site (3,35,41). Therefore, the partitioning of electrons between the two pathways must be regulated because it may have significant consequences on cellular energy metabolism (18,40).

The energy of electron flow through the alternative pathway that is not coupled to proton gradient formation is released as heat (22). Some plant tissues that express a high level of the alternative pathway are thermogenic (29). The role of thermogenesis in the appendix tissue of the Aroid species *Sauromatum guttatum* Schott (voodoo lily) is to volatilize foul-smelling compounds which attract insect pollinators (21). The appearance of three mitochondrial proteins with apparent molecular masses of 35-, 36-, and 37 kD correlates with the activity of the alternative oxidase, the terminal oxidase of the alternative respiratory pathway, in *S. guttatum* appendix tissue (4). A 42 kD protein that is a putative precursor of all three of these alternative oxidase proteins has been identified and a cDNA clone, pAOSG81, corresponding to the nuclear gene, *aox1*, encoding the 42 kD protein has been isolated and characterized (33). One of the factors that may

determine the alternative pathway activity (the actual rate of O_2 reduction that is occurring via the alternative pathway *in vivo*) is the amount of the alternative oxidase present in the mitochondria. The amount of alternative oxidase in the mitochondria may be determined, in part, by the level of expression of the corresponding gene(s). We demonstrated that the level of the alternative pathway capacity (the maximum rate of O_2 reduction of the pathway in isolated mitochondria) in developing *S. guttatum* appendix tissue correlates well with the levels of the 35- and 36 kD alternative oxidase proteins (6,34) and the level of the 1.6 kb alternative oxidase transcript (34). The accumulation of the transcript may be due to an increase in the rate of transcription of *aox1* or an increase in the stability of the transcript (34). Previous work failed to show an increased rate of transcription of *aox1* (34). However, since these results are not direct proof that RNA stability is the mechanism by which the transcript accumulates, it is possible that an increase in the rate of transcription of *aox1* does play a role in the transcript accumulation.

Salicylic acid is an endogenous "trigger" of thermogenesis in voodoo lily appendix tissue (29). Salicylic acid is produced in the male floral region of the inflorescence and moves into the appendix beginning early on D-1 and "triggers" thermogenesis at about noon of D-day (29,30). Raskin *et al.* (30) showed that light is required for salicylic acid to induce thermogenesis in appendix tissue sections. Salicylic acid induces an increase in alternative pathway capacity in *S. guttatum* appendix tissue by causing an accumulation of the alternative oxidase transcript, resulting in an accumulation of the alternative oxidase proteins (34).

Salicylic acid may also be a messenger in systemic acquired resistance (SAR) in tobacco and cucumber (19,20,31,46). Salicylic acid may act in disease resistance by regulating gene expression since application of salicylic acid caused the accumulation of pathogenesis related (PR) proteins, including PR1a and a glycine rich protein (GRP) called GRP8, and their corresponding transcripts in tobacco leaves, which have increased levels of salicylic acid following innoculation of certain pathogens (19,26,45). The promoter regions of the genes encoding GRP8 and PR1a have been analyzed in order to identify salicylic acid responsive, *cis*-acting sequence elements (27,43). One study indicated that only the first 300 basepairs (bp) of the 5'-upstream region of PR1a gene were sufficient to allow the increased expression of a reporter gene (β-glucuronidase gene) in transgenic tobacco plants following infection with tobacco mosaic virus (TMV) or application of salicylic acid (27). A separate study indicated that the first 643 bp were required for an increase in expression of the same reporter gene in transgenic tobacco (43). However, a sequence motif found in both the PR1a promoter and the GRP8 promoter has been identified (42).

Salicylic acid may participate in thermogenesis in aroids and the response to pathogen attack in tobacco and cucumber by regulating the expression of (apparently) unrelated genes, *aox1* and the PR genes. Therefore, identification of the mechanism(s) by which salicylic acid regulates gene expression will be valuable in understanding the role of salicylic acid in various plant processes. In this paper we report on the identification and characterization of *aox1*, the salicylic acid "responsive" gene encoding the alternative oxidase of *S. guttatum*.

MATERIALS AND METHODS

Plant Material and Salicylic Acid Treatment. *Sauromatum guttatum* Schott (voodoo lily) plants were maintained in a glasshouse at 27°C ± 4°C under long-day conditions, as previously described (4). D-day is the day the inflorescence blooms and the appendix tissue of the spadix heats (see reference 21 for description). Other developmental stages of the plant are indicated as the number of days before or after D-day (D-1 being the day before and D+1 the day after D-day). Between the time the inflorescence begins to develop and the end of day D-2, the inflorescence is referred to as "immature". For the salicylic acid treatment experiments, appendix tissue of a *S. guttatum* plant was cut into 1.0-1.5 cm sections. Sections were placed into each of two 10 ml beakers containing 1.0 ml of phosphate buffer (10 mM KH_2PO_4 and 50 μg/ml streptomycin). Salicylic acid was added to a final concentration of 1.0 mM to one beaker and a drop of buffer or buffer plus salicylic acid was placed on top of each section.

Isolation of Mitochondria and Respiration Assays. Mitochondria were isolated as described previously (4). Respiration rates were measured as oxygen uptake using a Rank Brothers (Cambridge, UK) oxygen electrode. The oxygen content of air-saturated water was estimated according to Estabrook (7). The capacity of the alternative pathway was measured in 1.0 mL of reaction medium (250 mM sucrose, 30 mM 3-(*N*-morpholino)propanesulfonic acid [Mops], pH 6.8) at 25°C with 1 mM NADH as the substrate. Carbonylcyanide *p*-trifluoro-

methoxyphenylhydrazone (FCCP) was added to 0.5 μM after NADH in order to diminish the electrochemical gradient prior to measurement of the pathway capacity (4). The capacity of the alternative pathway was taken as the rate of oxygen uptake sensitive to 1 mM salicylhydroxamic acid (SHAM) in the presence of 1 mM KCN (4). The amount of total protein in each sample was determined by as described previously (4).

Gel Electrophoresis, Immunoblotting, and Antisera. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously (4). The antibody used was the AOA monoclonal antibody raised to the 36 kD alternative oxidase protein of voodoo lily, and which recognize all three alternative oxidase proteins of voodoo lily (5). Since the same amount of total mitochondrial protein was loaded onto each lane of each polyacrylamide gel, the protein detected on protein blots represents the proportion of total mitochondrial proteins that constitutes the alternative oxidase proteins.

Plasmid Insert Isolation and Radiolabeling. The 1400-bp EcoRI insert of pAOSG81 (33) was purified by as described in Sambrook *et al.* (37) except that the elution buffer was 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.05% SDS, and 0.3 M LiCl and the insert was electrophoresed through two successive polyacrylamide gels. The purified fragment was resuspended to a concentration of 60 ng/μl in Tris-EDTA and was used as a template to make DNA radiolabeled

with α-^{32}P-dATP (Amersham Corporation) by the random primer method (8). Specific activity of the DNA probes was routinely about 10^7 cpm/μg template, as determined by the sodium phosphate wash method (37).

RNA Isolation, Blots, and Autoradiography. Total RNA was isolated from frozen (in liquid N_2) *S. guttatum* appendix tissue as described previously (33). RNA was separated on agarose gels containing formaldehyde as described (1) and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) as described in Sambrook *et al.* (37). RNA blots were probed with radiolabeled DNA made using the purified insert of pAOSG81. Hybridization was done under high stringency hybridization conditions (37). Autoradiography using Kodak diagnostic film XAR-5 (Eastman Kodak Company, Rochester, NY) was used to view the hybridization results (1).

Genomic DNA Isolation and Southern Blot Analysis. *S. guttatum* plants were grown in the dark for 24 h prior to genomic DNA isolation. Genomic DNA was isolated from *S. guttatum* leaves by the procedure of Ausubel *et al.* (1). Southern blotting was performed according to the procedure of Sambrook *et al.* (37) using 10 μg per lane of restriction endonuclease digested genomic DNA and radiolabeled DNA probes made using the insert of pAOSG81, as described above.

Isolation of Genomic Clones and Subcloning. A library of the genomic DNA partially digested with Sau3A was constructed in the Bam HI site of lambda

EMBL3 vector by Stratagene (LaJolla, CA). Library screening and all subcloning were performed according to the procedures of Sambrook *et al.* (37). The 2.2 kbp Nco I fragment (see Figure 4-3 for location of sites) and the 2.9 kbp Bam HI/Sal I fragment were subcloned from λGAOSG11 into pUC119 to form pGAOSG-N22 and pGAOSG-BS29, respectively. The 2.3 kbp Sma I/Sac I fragment from λGAOSG11 was subcloned into pBluescript (Stratagene, LaJolla, CA) to form pGAOSG-SS23.

DNA Sequencing and Analysis. Sequencing of the inserts of phagemids pGAOSG-N22 and pGAOSG-BS29 was done by the dideoxy method of Sanger *et al.* (38) using Sequenase® T7 DNA Polymerase version 2.0 (U.S. Biochemical Corporation, Cleveland, OH). Primers for sequencing and primer extension experiments were made in the laboratory of Dr. C. S. Sommerville or the Michigan State University Macromolecular Facility. All sequence analysis was performed using the EDITBASE DNA sequence analysis program.

Synthesis of Radiolabeled Transcripts and Mapping of *aox1* mRNA with RNase. Radiolabeled transcripts used to map the 5' end of the mRNA encoding the 42 kD alternative oxidase protein were synthesized *in vitro* by the procedure of Sambrook *et al.* (37). The template used for synthesis was Xmn I digested pGAOSG-SS23 (see Fig. 4-3 for location of sites). T7 RNA polymerase was used to synthesize a 544 nucleotide transcript that contained 10 bases of pBluescript sequence, 173 bases of sequence of pGAOSG-SS23 that is the same as the 5' end

of the insert of pAOSG81, and 361 bases of pGAOSG-SS23 preceding the first base that corresponds to the 5' end of the insert of pAOSG81. The radiolabeled transcripts were hybridized to 10 μg of total *S. guttatum* RNA isolated from D-day appendix tissue and digested with RNase T1 (BRL, Gaithersburg, MD) and RNase A (Sigma Chemical Co., St. Louis, MO) following the procedure of Sambrook *et al.* (37). Radiolabeled RNA protected from digestion was analyzed by electrophoresis through a 7% (w/v) polyacrylamide sequencing gel and autoradiography. The products of the sequencing reactions using primer FDR52-4, which corresponds to bases 1349 to 1366 of the insert of λGAOSG11, were loaded into adjacent lanes as markers. Each of these products (DNA) migrates through the sequencing gel at the same rate as an RNA molecule that is 5%-10% shorter (37).

Primer Extension Mapping of *aox1* mRNA. Primer extension mapping of the *aox1* transcript was done by the procedure of Sambrook *et al.* (37) using a 25 base oligonucleotide, called PE-25, of the sequence 5'-GGTACGGGGACGTGACTGA GCTGCC-3', corresponding to the complementary strand of bases 118 to 142 of λGAOSG11 and, therefore, complementary to the *aox1* transcript. Products of the primer extension were analyzed by electrophoresis through a 7% (w/v) polyacrylamide sequencing gel and autoradiography.

RESULTS

Salicylic Acid-Regulated Expression of the Alternative Oxidase. The alternative pathway capacity and the amounts of the 35- and 36 kD alternative oxidase proteins are increased by 5 h following the application of salicylic acid to day D-5 *S. guttatum* appendix tissue sections (Fig. 4-1A, lane 2) as compared to the levels before salicylic acid application (lane 1). The capacity and amount of each of the three alternative oxidase proteins are greatly increased by 24 h following salicylic acid addition (lane 3). The amount of the 1.6 kb alternative oxidase transcript increased by 4 h following salicylic acid addition to day D-5 appendix tissue (Fig. 4-1B, lane 2) as compared to the amount present before salicylic acid application (lane 1). The amount of the transcript is greatly increased by 24 h following salicylic acid (lane 3).

Southern Blot Analysis to Determine Gene Copy Number. The copy number of alternative oxidase genes was determined by Southern blot analysis of *S. guttatum* genomic DNA digested with various restriction endonucleases. Figure 4-2 shows that single DNA fragments were detected when genomic DNA digested with either Bam HI (lane 1), Hind III (lane 3), Nco I (lane 4), Sal I (lane 7) or Xba I (lane 8) was probed with radiolabeled DNA made using the insert of pAOSG81. Two bands were detected when the genomic DNA was digested with either Eco RI (lane 2), Pst I (lane 5), or Sac I (lane 6). Based on the sequence of λGAOSG11, there should not be two bands recognized by the probe when

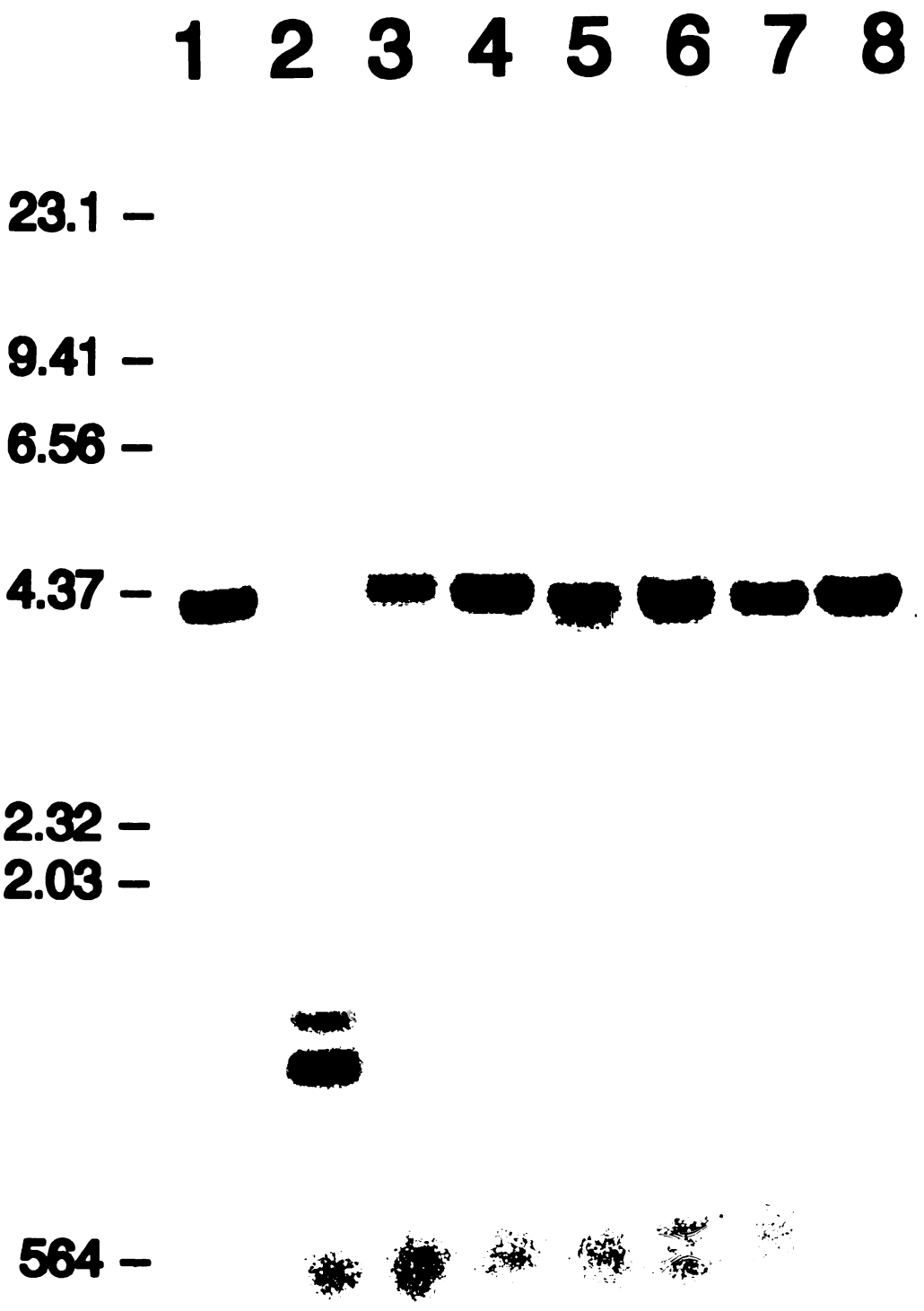
genomic DNA is digested with EcoRI. We can only explain this as an error in fidelity in the production of clone λGAOSG11. All other bands agree with the sequence data. Southern blot data obtained using genomic DNA digested with Cla I, Hinc II, Kpn I, Nde I, and Sal I also agreed with the sequence data (data not shown).

Sequence of *aox1*. Figure 4-3 shows a schematic diagram of the exon/intron structure of *aox1* deduced from the sequence of λGAOSG11 and the locations of key restriction endonuclease recognition sites. The sequence of the phagemid subclones, pGAOSG-N22 and pGAOSG-BS29, derived from genomic clone λGAOSG11, revealed that *aox1* is organized as four exons separated by three short introns (Fig. 4-4). Exon 1, exon 2, exon 3, and exon 4 are 452 bp, 133 bp, 493 bp and approximately 570 bp in length, respectively. They are separated by intron 1, intron 2, and intron 3, which are 117 bp, 79 bp, and 119 bp in length, respectively. Exon 3 contains the entire region of *aox1* that is highly conserved among the alternative oxidase cDNA clones from potato (11), *Hansenula anomala* (36), and rice (Dr. L. McIntosh and R. Nickels, personal communication). This region of *aox1* encodes a region of the deduced protein that is predicted to form two transmembrane α-helices (33). The putative TATA box with the sequence TATAAA is located at bases -32 to -27. Two putative CAAT boxes are at bases -83 to -79 (CCCAT) and at bases -67 to -62 (CAAAAT). Many *cis*-acting transcriptional elements (see 12,14, and 15 for descriptions) were identified in the promoter of *aox1*, but only the potential zinc finger (GATA) and GT-1/GT-2

Figure 4-1. Immunoblot and RNA blot analysis of salicylic acid induction of alternative oxidase expression. **Panel A:** Total mitochondrial proteins were separated by SDS-PAGE and transferred to nitrocellulose. The immunoblot was probed with the AOA monoclonal antibody. The mitochondrial proteins were from D-5 *S. guttatum* appendix tissue sections before application of salicylic acid (lane 1), following incubation in phosphate buffer containing 1.0 mM salicylic acid for 5 h (lane 2), and 24 h following salicylic acid application (lane 3). Alternative pathway capacity in natoms O/min/mg protein is indicated at the bottom of each lane. Protein relative molecular masses in kD are indicated at the side. **Panel B:** Total RNA (10 μg per lane) was electrophoresed through 1.2% (w/v) agarose-formaldehyde gels and transferred to nitrocellulose. The blot was probed with radiolabeled DNA made using the insert of pAOSG81 by the random primer method and hybridization that occurred at high stringency was detected by autoradiography. The RNA samples were from appendix tissue from a D-5 *S. guttatum* appendix tissue sections before application of salicylic acid (lane 1), following incubation in phosphate buffer containing 1.0 mM salicylic acid for 4 h (lane 2), and 24 h following salicylic acid application (lane 3). Transcript length in kilobases is indicated at the side.


A
B
1 2 3
1 2 3
37
36
35
1.6
110
142
392

Figure 4-2. Southern blot analysis of genomic DNA. *S. guttatum* genomic DNA (10 μg) was digested with individual restriction endonucleases, electrophoresed through a 0.7% (w/v) agarose gel, and transferred to nitrocellulose. The blot was probed with radiolabeled DNA made using the insert of pAOSG81 by the random primer method. Hybridization at high stringency was detected by autoradiography. The DNA samples were digested with the following restriction endonucleases: Bam HI (lane 1), Eco RI (lane 2), Hind III (lane 3), Nco I (lane 4), Pst I (lane 5), Sac I (lane 6), Sal I (lane 7), and Xba I (lane 8). DNA marker sizes in base pairs are indicated to the side.


1 2 3 4 5 6 7 8
23.1 –
9.41 –
6.56 –
4.37 –
2.32 –
2.03 –
564 –

(GTGG) target sequences were identical to the consensus sequence of each (Fig. 4-3). Regions with sequence similarity to the following classes of promoter elements were identified: bZIP (TGACG, GACGTG, CACGTG, GACGTG), at bases -695 to -689 (matched 5/6 bases), bases -195 to -190 (5/6), bases -152 to -147 (5/6), and bases -57 to -51 (5/6); HLH (CAGGTGC), -1281 to -1275 (6/7); heat shock (GAAnnTTC), at bases -1446 to -1439 (7/8), bases -1183 to -1174 (7/8), bases -1064 to -1057 (7/8), bases -1043 to -1036 (7/8), bases -860 to -853 (7/8), bases -597 to -590 (7/8), bases -265 to -258 (7/8), and bases -161 to 154 (7/8); and SV40 (GTGGA/TA/TA/TG), at bases -606 to -599 (7/8) and bases -139 to -132 (7/8). Sequences similar to each of the "boxes" identified in the promoter of the gene encoding the small subunit of ribulose bisphosphate carboxylase (RBCS) were also detected: Box I at bases -1458 to -1446 (10/13), bases -903 to -897 (6/7), bases -759 to -753 (6/7), bases -366 to -360 (6/7), bases -278 to -271 (6/7), and bases -117 to -111 (6/7); Box II at -138 to -128 (10/14); Box II* at bases -234 to -223 (9/12); Box III at bases -378 to -369 (8/10); Box IV at bases -806 to -797 (8/10), bases -784 to -775 (8/10), bases -634 to -624 (8/10), and bases -99 to -90 (8/10); and Box V at bases -611 to -601 (8/11) and bases -109 to -99 (8/11). A region of the *aox1* promoter, from base -524 to base -468 can be aligned with the -50 to +1 regions the PR1a and GRP8 promoters (42) as follows:

```
aox1 -524 GTaTATtTg-TCtA-AT--gTAgtTAT-AAGtcAaAACaaCTT-tTAGTAAATC-AaTaTC-TC-A -468
          :| :|: :  || | ||   |:  ||| ||:  : :||  |::  :||||:||: | | |: || |
GRP8  -54 TT-TATGCATTCAA-ATCA-TA-CTATAAAG--AGAAC-CC--AAGAGTACATA-AGTTTCTTC-A +1
           |  |  : ||||| ||   |  ||||||      || ||     |||| ||  ||||| ||| |
PR1a  -56 GT-GAAATCTTCAAGATTTCTC-CTATAAA-----TAC-CCTTGGTAGTAAATCTAGTTT-TTCCA +1
```

in which capital letters in the *aox1* sequence dots between the sequences indicate bases that are present in either the GRP8 sequence or the PR1a sequence and

Figure 4-3. Schematic diagram of genomic clone λGAOSG11. Exons are shown as open boxes, introns as filled boxes, the untranscribed regions as lines, and the arms of the lambda phage as hatched boxes. The positions of recognition sites of the following restriction endonucleases are shown: Bam HI, indicated as B; Nco I, N; Sac I, S; Sal I, Sa; Sma I, Sm; and Xmn I, X.

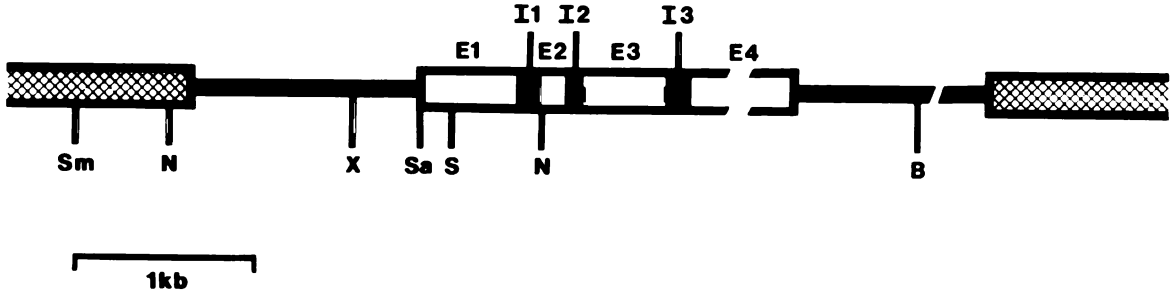
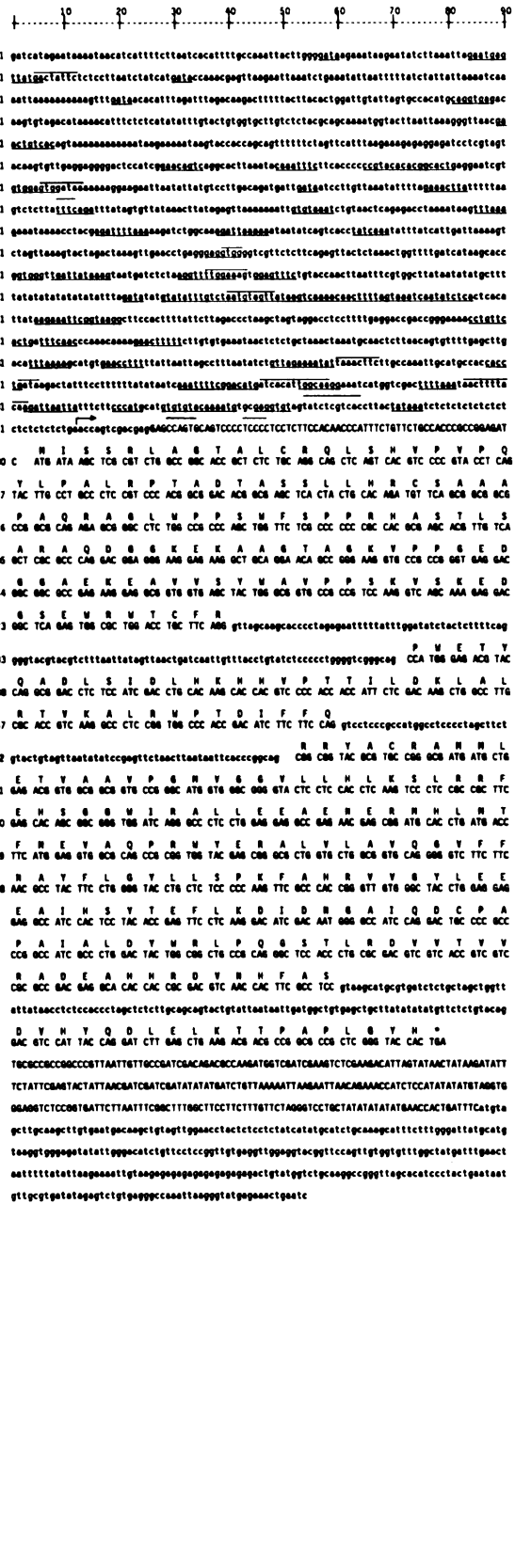

I1
I2
I3
E1
E2
E3
E4
Sm
N
X
Sa
S
N
B
1kb

Figure 4-4. Nucleotide and deduced amino acid sequence of the *aox1* gene of *S. guttatum*. The sequence of cDNA clone pAOSG81 is shown in capital letters. The transcription start site (+1) is marked by a bent arrow. The putative TATA box, potential CAAT boxes, and other potential *cis*-acting transcriptional elements are underlined or overlined and discussed in the text.



lines between sequences indicate nucleotides that are present in all three sequences. In addition, the *aox1* promoter has three regions that have sequence similarity to the -689 to -643 region of the PR1a promoter and the promoter of GRP8. These regions are bases -146 to -134 (10/13), bases -139 to -128 (10/11), and bases -442 to -457 (11/16).

Mapping of *aox1* mRNA Using RNase. A large amount of the 544 nucleotide, radiolabeled transcript was made by the *in vitro* system (Figure 4-5, lane 6). The largest fragment of the 544 nucleotide transcript that is protected from RNase digestion by hybridization to the *aox1* transcript was about 200 nucleotides in length (lane 5). The transcriptional start site that corresponds to a fragment of this size is base +1 of λGAOSG11, which is marked with a bent arrow in Figure 4-4. The other bands present in lane 5 correspond to RNA molecules of about 199, 198, 185, 173, 172, 171, 169 and 129 nucleotides.

Primer Extension Mapping of *aox1* mRNA. A large amount of primer PE-25 was end labeled with α-^{32}P and that there may have been some contamination with oligonucleotides of about 50 nucleotides in length (Figure 4-6, lane 6). The most abundant product made by primer extension was 142 nucleotides (lane 5) and corresponds to a transcription start site at base +1 of λGAOSG11, which is marked with a bent arrow in Figure 4-4. Bands corresponding to products of about 141, 138, 91, 90, and 89 nucleotides were also detected.

Figure 4-5. RNase protection mapping of the 5' end of the *aox1* transcript. An *in vitro* synthesized, 544 nucleotide transcript was hybridized to 10 μg of total RNA from D-day appendix tissue and digested with RNase T1 and RNase A. Radiolabeled RNA samples were analyzed by electrophoresis through a 7% (w/v) polyacrylamide sequencing gel and autoradiography. A protected fragment of about 200 nucleotides (lane 5) is indicated by an arrow. The undigested, radiolabeled probe transcript (lane 6) is also indicated by an arrow. Sequencing products made using the single stranded form of phagemid pGAOSG-BS29 and primer FDR52-4 (lanes 1-4) were used as size markers. The positions of some of the sequencing products are indicated in DNA base pairs at the side. Each of these products (DNA) migrates through the sequencing gel at the same rate as an RNA molecule that is 5%-10% shorter (37).

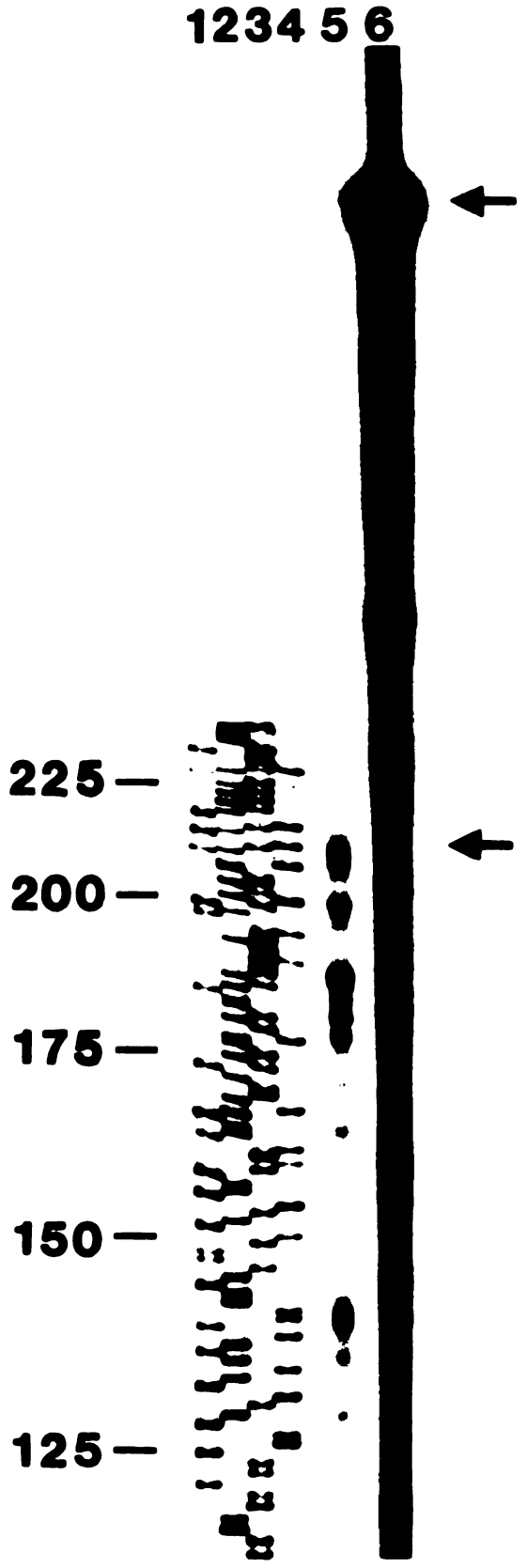
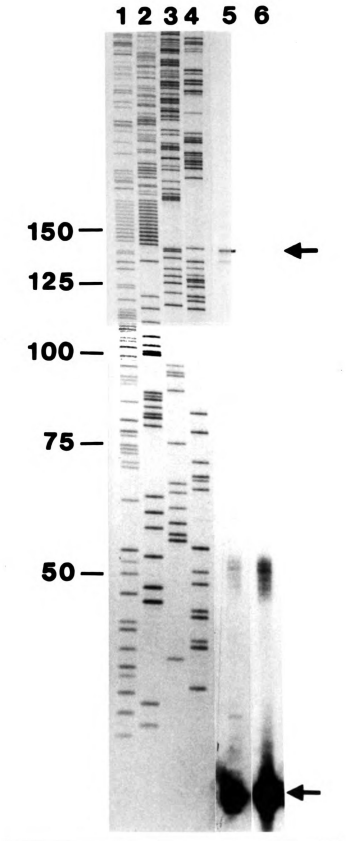

1234 5 6
225
200
175
150
125

Figure 4-6. Primer extension mapping of the 5' end of the *aox1* transcript. Primer extension was performed using oligonucleotide RDR22-7, which is complementary to the *aox1* transcript and corresponds to bases 1661 to 1685 of λGAOSG11. The products of primer extension (lane 5), the unextended oligonucleotide primer (lane 6), and products of sequencing reactions using the same primer and the single stranded form of phagemid pGAOSG-SS23 were analyzed by electrophoresis on a 7% (w/v) polyacrylamide gel and autoradiography. DNA molecule sizes in base pairs are indicated at the side. The most abundant primer extension product (142 nucleotides) is indicated by an arrow.


1 2 3 4 5 6
150
125
100
75
50

DISCUSSION

The amount of alternative oxidase present in plant mitochondria is one of the factors that will determine the partitioning of electrons between the alternative and cytochrome pathways. The data presented here indicates that alternative pathway capacity and the amounts of the 35- and 36 kD alternative oxidase proteins were increased by 5 h following salicylic acid application to immature *S. guttatum* appendix tissue. The amount of the 1.6 kb alternative oxidase transcript increased by 4 h following salicylic acid application. It is likely that the expression of the alternative oxidase in *S. guttatum* appendix tissue is affected by salicylic acid at the level of transcript accumulation (34). In order to better understand this regulation, we have isolated a genomic clone corresponding to *aox1*, which encodes the 42 kD alternative oxidase precursor protein. Three lines of evidence suggest that this gene is the only expressed alternative oxidase gene in *S. guttatum* appendix tissue and is present in a single copy in this organism: 1) single DNA fragments were detected on Southern blots when genomic DNA was digested with several different restriction endonucleases; 2) a single, 1.6 kb transcript is detected on RNA blots; and 3) a single, 42 kD protein is immunoprecipitated from products made by *in vitro* translation of poly $(A)^+$ RNA from appendix tissue (33).

The *aox1* gene is organized as four exons that are separated by three short introns. One interesting feature of this gene structure is that exon 3 contains the entire region of *aox1* that: 1) is very highly conserved among several higher plant

species, including potato (11) and rice (R. Nickels and L. McIntosh, unpublished results), and yeast (36); and 2) encodes the region of alternative oxidase protein that is predicted to embed the protein in the inner surface of the inner mitochondrial membrane (33). Each intron begins with GU and ends with AG, a pattern typical of eukaryotic introns (2). Each intron is AU-rich, a characteristic of dicots (9), and contains the YURAY sequence common to introns of higher plants (13).

We have mapped the 5' end of the *aox1* transcript by RNase protection and primer extension experiments. The results from each of these suggest that the first base of the *aox1* transcript corresponds to the adenine marked as base +1 on Fig. 4-4, which is 14 bp upstream of the 5' end of the start of the match with pAOSG81. The smaller RNA fragments that appear in the RNase protection experiment may be due to multiple transcription start sites that are not detected by RNA blot analysis due to the transcripts differing by a small number of nucleotides. Alternatively, the bands could have resulted from imperfect conditions during the RNase digestion, which could result in slight separation of the strands at the ends. The primer extension experiment also showed multiple products that are shorter than the main product. These products may have resulted from premature termination of reverse transcription. The first base of the proposed TATA box is 32 bases from the start site, which is the distance often observed for the TATA box of a higher plant gene transcribed by RNA polymerase II (44). The most likely CAAT site occurs at bases -67 to -62, which agrees with the position of the CAAT site of other plant genes (44).

Several sequence regions in the promoter of *aox1* are similar to the plant transcription elements that have been identified (see references 12,14,15,17,28 for element descriptions), but only the potential zinc finger and GT-1/GT-2 target sequences were identical to the consensus sequence of each of these *cis*-acting regulatory elements. Regions with sequence similarity to the following classes of promoter elements are located in the promoter of *aox1*: bZIP, HLH, heat shock, and SV40. Since light is required for applied salicylic acid to cause thermogenesis in immature *S. guttatum* appendix tissue sections (30), it is interesting to note that several regions were identified that are similar to the "boxes" of the light-regulated RBCS promoter (16). A region of the *aox1* promoter, from base -524 to base -468 can be aligned with the -50 to +1 regions the PR1a and GRP8 promoters (42). In addition, the *aox1* promoter has three regions that have sequence similarity to the -689 to -643 region of the PR1a promoter and the promoter of GRP8. It is possible that one or more of these regions plays a role in the potential salicylic acid controlled regulation of these genes. The polyadenylation site of the *aox1* transcript is not known and cannot be accurately deduced from the sequence of λGAOSG11.

In summary, our results are consistent with the hypothesis that salicylic acid "triggers" thermogenesis in *S. guttatum* appendix tissue by causing, at least in part, an increase in the accumulation of the 1.6 kb *aox1* transcript, which results in increased accumulation of the 35- and 36 kD alternative oxidase proteins and increased capacity of the alternative pathway. Accumulation of the 1.6 kb transcript may be through an increase in transcription rate or an increase in RNA

stability.

Salicylic acid may play a role in SAR by causing an accumulation in the transcripts encoding certain PR and SAR genes, although relative transcription rates have not been determined for these events. The promoters of PR1a and GRP8 have been analyzed to determine regions that are important for salicylic acid directed gene regulation. It may now be possible to use λGAOSG11 to: 1) identify any protein(s) that may interact with the *aox1* promoter in nuclei of *S. guttatum* appendix tissue; and 2) determine the regions of the *aox1* promoter that may be responding to salicylic acid using a transgenic system. Results from such experiments may be useful in determining the general mechanism by which salicylic acid regulates gene expression

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CHAPTER 5

CYTOCHROME AND ALTERNATIVE PATHWAY RESPIRATION IN TOBACCO: EFFECTS OF SALICYLIC ACID

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FOOTNOTES

[1]Supported in part by U.S. Department of Energy contract DE-AC02-76ERO-1338 (L.M.) and the National Science Foundation DCB-8904518 (L.M.).

[2]Abbreviations: FCCP, *p*-trifluoromethoxycarbonylcyanide; SHAM, salicylhydroxamic acid

ABSTRACT

In suspension cultures of NT1 tobacco (*Nicotiana tabacum* L. cv. bright yellow) cells the cytochrome pathway capacity increased between day 3 and day 4 following subculturing, reached the highest level observed on day 7, decreased significantly by day 10, and was at the same level on day 14. Both alternative pathway capacity and the amount of the 35 kD alternative oxidase protein increased significantly between day 5 and day 6, reached the highest point observed on day 7, remained constant until day 10, and decreased by day 14. The highest points of the alternative and cytochrome pathway capacities and the largest amount of the 35 kD protein were attained on the day cell cultures reached a stationary phase of growth. Addition of salicylic acid to cell cultures on day 4, caused a significant increase in alternative pathway capacity and a dramatic accumulation of the 35 kD protein by 12 hours. The capacity and the protein level each reached the highest point observed by 16 h after salicylic acid addition and the cytochrome pathway capacity was at about the same level at each time point. The accumulation of the 35 kD protein was significantly decreased by addition of actinomycin D one hour before salicylic acid and was blocked by addition of cycloheximide. These results indicate that *de novo* transcription and translation were necessary for salicylic acid to cause the maximum accumulation of the 35 kD protein.

INTRODUCTION

The regulation of electron flow between the alternative and cytochrome respiratory pathways in higher plants may be important in determining the overall carbon balance of plant cells (8,23). If electrons flow through the cytochrome pathway, a proton gradient is established from three coupling sites (complex I, complex III, and complex IV) along the pathway (1,14,24). The potential energy of the proton gradient is used to produce chemical energy in the form of ATP (reviewed in reference 15). If electrons flow through the alternative pathway, only complex I can contribute to establishing a proton gradient, but this depends on the substrate being oxidized (1,20,25). The energy of electron flow that is not used to produce ATP in the alternative pathway is released as heat, which is used in the reproductive physiology of the aroid plants such as the species *Sauromatum guttatum* Schott (12,13). The role of the alternative pathway in non-aroid plants is not yet defined (8,23).

The appearance of three mitochondrial proteins with apparent molecular masses of 35-, 36-, and 37 kD strongly correlates with the activity of the alternative oxidase, the terminal oxidase of the alternative pathway, in *S. guttatum* appendix tissue (2). These proteins are believed to be posttranslationally modified products from a single, nuclear-encoded precursor protein (18). A monoclonal antibody that recognize all three alternative oxidase proteins of *S. guttatum* was prepared and named AOA monoclonal antibody (3). This antibody also recognizes a single, putative alternative oxidase protein of 35 kD in tobacco and a single

mitochondrial protein from other non-aroid higher plants (2,3). A 42 kD protein that is a putative precursor of all three proteins has been identified (18). A cDNA clone, pAOSG81 corresponding to the gene, *aox1*, encoding the precursor protein has been isolated and characterized (18).

Salicylic acid is an endogenous "trigger" of thermogenesis in *S. guttatum* appendix tissue (16). Raskin *et al.* (17) also showed that light is required for salicylic acid to "trigger" thermogenesis in appendix tissue sections. We previously used pAOSG81 and the AOA monoclonal antibody to study the developmental regulation of alternative oxidase expression and the expression of the alternative oxidase after application of salicylic acid in *S. guttatum* appendix tissue sections (19). Salicylic acid caused an increase in alternative pathway capacity in *S. guttatum* appendix tissue by causing an accumulation of the alternative oxidase transcript, which leads to an accumulation of the alternative oxidase proteins in the mitochondria (19).

We are now establishing a system to study the regulation of the two respiratory pathways using suspension cultures of NT1 tobacco cells. Alternative pathway capacity increases and a 35 kD alternative oxidase protein accumulates in these tobacco cells when they are transferred from the normal growth temperature of 30°C to 18°C (26) or when antimycin A, an inhibitor of the cytochrome pathway, is added to the culture medium (27). We report here on the developmental regulation of alternative oxidase expression and cytochrome and alternative pathway capacities in NT1 cells and on the effects of exogenous salicylic acid on the capacities and expression of the alternative oxidase.

MATERIALS AND METHODS

Tobacco Cultures. Suspension cultures of tobacco (*Nicotiana tabacum* L. cv.bright yellow) cells were grown in batch culture in LS medium (10) containing 0.2 μg/ml 2,4-D on a rotary shaker at 150 rpm and 30°C under heterotrophic conditions. In order to determine the cell density of a culture, a 5 mL aliquot was removed and the cells were pelleted at 1876*g* in a Beckman RT6000B centrifuge. The supernatant was carefully removed, and the packed cells were weighed. The cell density was calculated as the grams of cells per 100 mL of growth medium and given as a percentage. The cell cultures were subcultured to a final density of 4% (w/v). The day following subculturing is referred to as day 1, the second day as day 2, and so on.

Salicylic Acid and Inhibitor Treatment of Suspension Cultured Cells. For the time course experiments, salicylic acid was added to a final concentration of 1.0 mM to day 3 or day 4, and the cultures were then grown under normal conditions for 2 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, and 48 h.

For each inhibitor experiment, actinomycin D (50 μg/mL final concentration; Sigma, St. Louis, MO), an inhibitor of transcription, was added to one of four cultures and cycloheximide (100 μg/mL final concentration; Sigma, St. Louis, MO), an inhibitor of translation by 80S ribosomes, was added to a second culture. After one hour of incubation at 30°C, salicylic acid was added (1.0 mM final concentration) to the two cultures containing the inhibitors and the positive

control culture. The cultures were incubated at 30°C for an additional 15 h. The negative control culture was grown under normal conditions for 16 h and each experiment was started on day 3.

For each concentration experiment, salicylic acid was added to final concentrations of 1.0 mM, 100 μM, 10 μM, or 1.0 μM to independent cultures. The cultures were incubated at 30°C for 16 h after addition of salicylic acid and experiments were started on day 3 or day 4.

Isolation of Mitochondria and Respiration Assays. Suspension cultured cells were washed twice in fresh growth medium at room temperature. Mitochondria were isolated by a modification of the procedure of Schwitzguebel and Siegenthaler (22) as previously described (18). The cells were disrupted in a commercial blender by three bursts of 3 s each. Respiration rates were measured as oxygen uptake using a Rank Brothers oxygen electrode and 1.0 mL of reaction medium at 25°C. The oxygen content of air-saturated water was estimated according to Estabrook (5). Capacities of the cytochrome and alternative pathways were determined as described by Elthon *et al.* (2). The capacity of the alternative pathway is the rate of oxygen uptake that is sensitive to SHAM in the presence of KCN. The capacity of the cytochrome pathway is the rate of oxygen uptake that is sensitive to KCN in the presence of SHAM. Total respiratory capacity is the sum of the alternative and cytochrome pathway capacities plus residual respiration (respiration in the presence of SHAM and cyanide), which was very low in the suspension cultured tobacco cells. The amount of total protein in each sample

was determined by the procedure of Larson *et al.* (9).

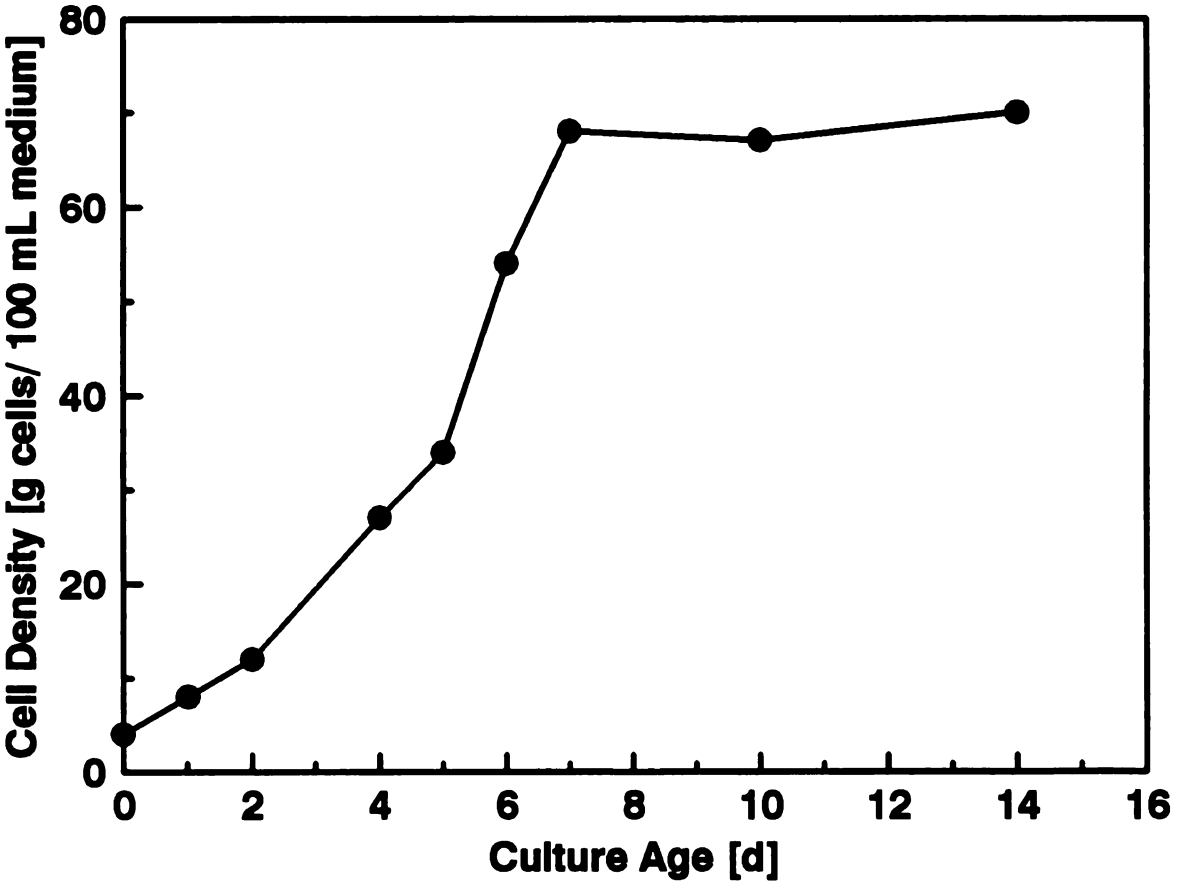
Gel Electrophoresis, Immunoblotting, and Antisera. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (2). The antibody used was the AOA monoclonal antibody, which was raised to the 36 kD alternative oxidase protein of *S. guttatum.* This antibody recognizes all three alternative oxidase proteins of *S. guttatum* as well as putative alternative oxidase proteins from other higher plants, including tobacco (2,3).

RESULTS

Growth of Suspension Cultured Tobacco Cells. Figure 5-1 shows a growth curve for the suspension cultured NT1 tobacco cells following subculture. The cell density increased by a factor of 1.5 each day from day 1, when the density was about 8%, until reaching a density of about 68% and a stationary phase at day 7. The cells remained in this stationary phase (at a density of about 70%) until at least day 14.

Respiratory Pathway Capacities During Growth of Suspension Culture Tobacco Cells. Changes that occurred in the capacities of the cytochrome and alternative pathways, measured in isolated mitochondria, during the growth of the suspension cultured tobacco cells are shown in panel A of Figure 5-2. The cytochrome

Figure 5-1. Growth curve for suspension cultured NT1 tobacco cells. Cell density of each culture was determined by removing a 5 mL aliquot, pelleting the cells, removing the supernatant, and weighing the packed cells. The cell density, calculated as the grams of cells per 100 mL of growth medium, was plotted as a function of the number of days after subculturing. Results are averages from four experiments.


Cell Density [g cells/ 100 mL medium]
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Culture Age [d]

pathway capacity increased rapidly between day 3 and day 4, and continued to increase until it reached the highest level observed on day 7. The capacity decreased between day 7 and day 10 and was at about the same level on day 14 as it was on day 10. The capacity of the alternative pathway was relatively high the day after the cells were subcultured, but decreased rapidly between day 1 and day 3. The capacity then increased rapidly between day 5 and day 6, reached the highest observed level on day 7, was at the same level on day 10 as it was on day 7, and decreased by day 14. Panel B of Figure 5-2 shows that the net result was that the percentage of the total respiratory capacity (see Materials and Methods for definition) of the cells that was attributable to the alternative pathway increased from 12% on day 5 to 36% on day 7.

Developmental Regulation of Alternative Oxidase Expression in Suspension Cultured Tobacco Cells. Figure 5-3 shows that the amount of the 35 kD alternative oxidase protein in the mitochondria used for the capacity measurements (Fig. 5-2) increased dramatically between day 6 (lane 5) and day 7 (lane 6), increased slightly between day 7 and day 10 (lane 7), and decreased substantially by day 14 (lane 8). This pattern parallels the changes that occurred in the capacity of the alternative pathway during the growth of the cells (Fig. 5-2) and the dramatic increase occurred at the same time that the cells were approaching the stationary phase of growth.

Figure 5-2. Respiratory pathway capacities of suspension cultured NT1 tobacco cells. Mitochondria were isolated at each time point, and the capacities were determined using a Rank Brothers oxygen electrode. **Panel A:** The capacity of the alternative pathway (solid circles) and the cytochrome pathway (solid triangles) were plotted as functions of the number of days after subculturing. **Panel B:** The percentage of the total respiration that was attributable to the alternative pathway was plotted as a function of the number of days after subculturing. Results are averages of three experiments.

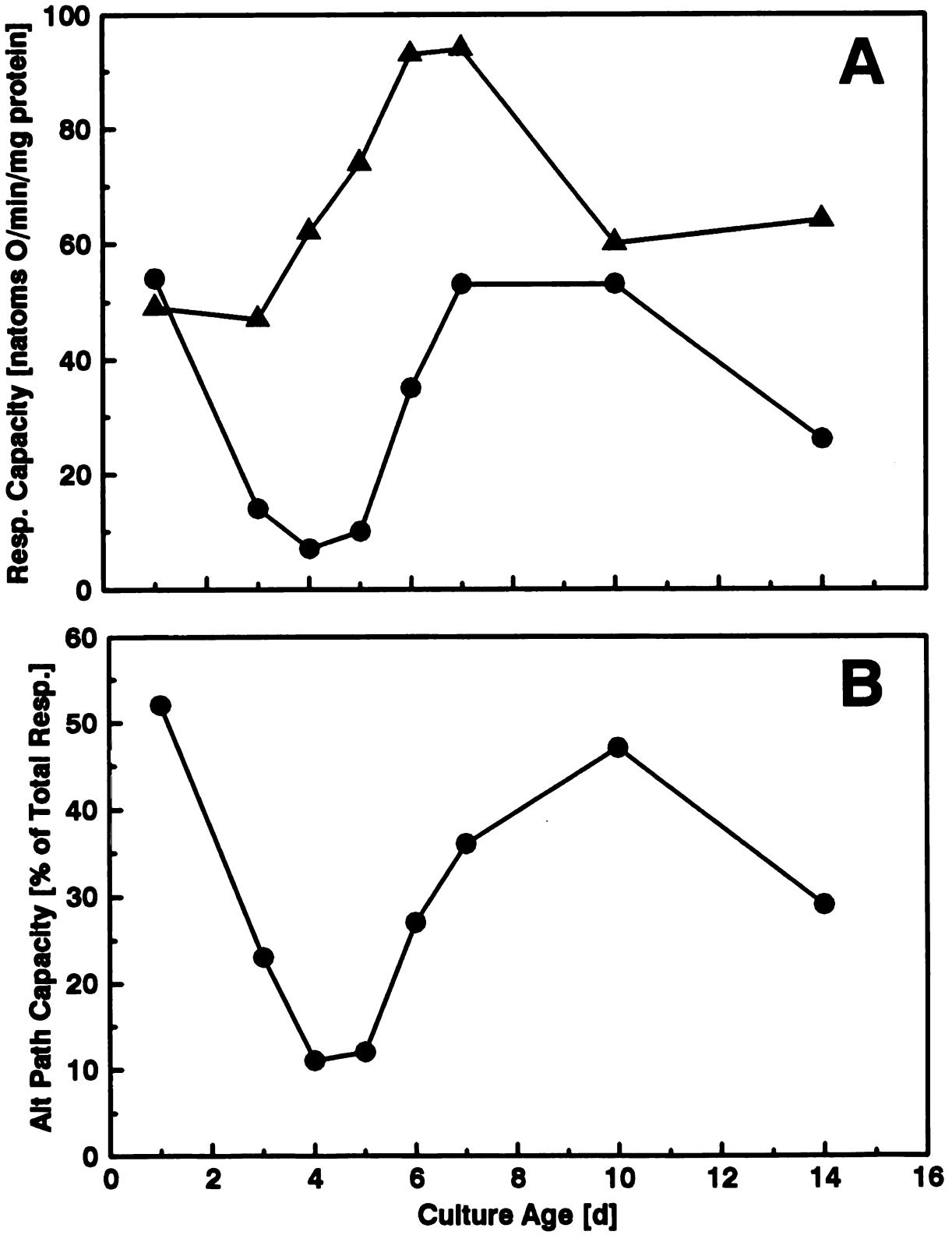
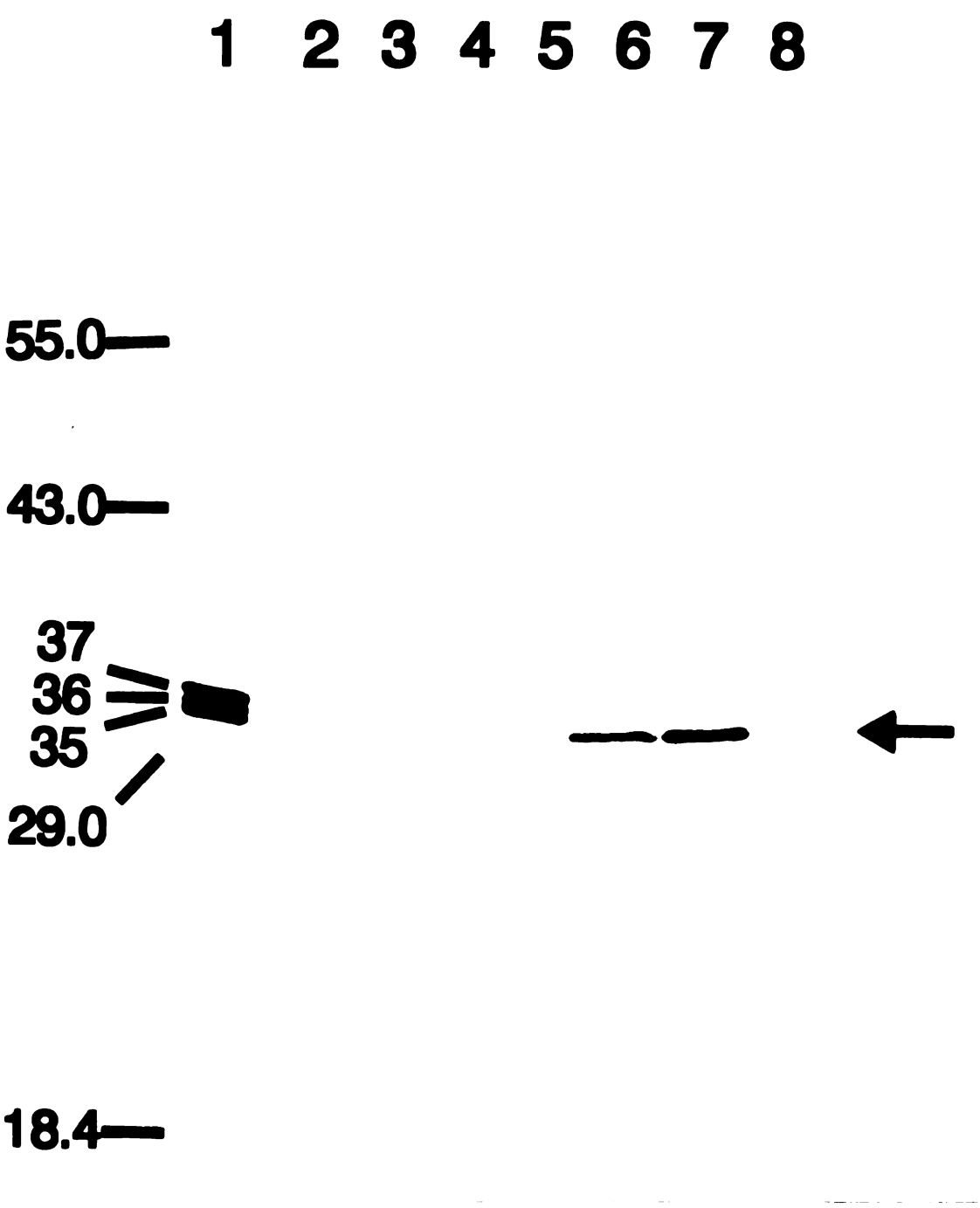

A
Resp. Capacity [natoms O/min/mg protein]
100
80
60
40
20
0
B
Alt Path Capacity [% of Total Resp.]
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
Culture Age [d]

Figure 5-3. Immunoblot showing accumulation of tobacco alternative oxidase at various times following subculturing. Mitochondria were isolated from suspension cultured NT1 tobacco cells at various times after subculturing and the respiratory pathway capacities were determined (see Fig. 5-2). The mitochondrial proteins (100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and the alternative oxidase proteins were identified using the AOA monoclonal antibody (3) and alkaline phosphatase linked to a secondary antibody. Lane 1: *S. guttatum* mitochondrial proteins; lane 2: day 3 NT1 tobacco mitochondrial proteins; lane 3: day 4; lane 4: day 5; lane 5: day 6; lane 6: day 7; lane 7: day 10; lane 8: day 14. Mitochondria were isolated from *S. guttatum* appendix tissue as previously described (18) and used as a positive control (lane 1). Protein molecular masses in kilodaltons are indicated at the side. Results are representative of four experiments.


1 2 3 4 5 6 7 8
55.0
43.0
37
36
35
29.0
18.4

Time Course of Salicylic Acid Induced Changes in Respiratory Pathway Capacities. Panel A of Figure 5-4 shows that the capacity of the alternative pathway, measured in isolated mitochondria, remained relatively constant through 8 h after the addition of salicylic acid to a day 3 culture. The capacity then increased dramatically between 8 h and 12 h after salicylic acid addition, continued to increase until it reached the highest observed level at 16 h, decreased between 16 h and 20 h, and was at the same level at the 48 h time point as it was at the 20 h time point. During this experiment, the capacity of the cytochrome pathway did not increase as it did in the cultures that did not contain salicylic acid (Fig. 5-2, Panel A). Panel B of Figure 5-4 shows that the net result of the salicylic acid-induced changes in the capacities was that the percentage of total respiratory capacity (see Materials and Methods for definition) of the cells that was attributable to the alternative pathway increased from 22% before salicylic acid was added to a maximum of 53% at the 16 h time point.

Salicylic Acid Induced Accumulation of Alternative Oxidase. The increase in the accumulation of the 35 kD alternative oxidase protein in the mitochondria used to determine the respiratory pathway capacities (Fig. 5-4) is shown in Figure 5-5. The amount of the protein remained relatively low until about 8 h (lane 6) after addition of salicylic acid to 1.0 mM. The amount of the protein was dramatically higher at 12 h than at 8 h (lane 7), was at a slightly higher level by 16 h (lane 8), was at a decreased level by 20 h (lane 9), was at about the same level at 24 h (lane 10), and was at a lower level at 48 h (lane 11).

Figure 5-4. Time course of salicylic acid effects on alternative and cytochrome pathway capacities. Salicylic acid was added to 1.0 mM to cell cultures on day 3. **Panel A:** The capacities of the alternative (solid circles) and the cytochrome pathways (solid triangles) were plotted as functions of the number of hours after salicylic acid addition. The solid circle surrounded by a circle and the solid circle surrounded by a square represent alternative pathway capacity measured in uninduced cells at the 24 h time point and the 48 h time point, respectively. The solid triangle surrounded by a circle and the solid triangle surrounded by square represent cytochrome pathway capacity in untreated cells at the 24 h time point and the 48 h time point, respectively. **Panel B:** The percentage of the total respiration that was attributable to the alternative pathway was plotted as a function of the number of days after subculturing. The solid circle surrounded by a circle and the solid circle surrounded by a square represent the percentage of total respiration attributed to the alternative pathway at 24 h and 48 h time points, respectively. Results are averages of three experiments.

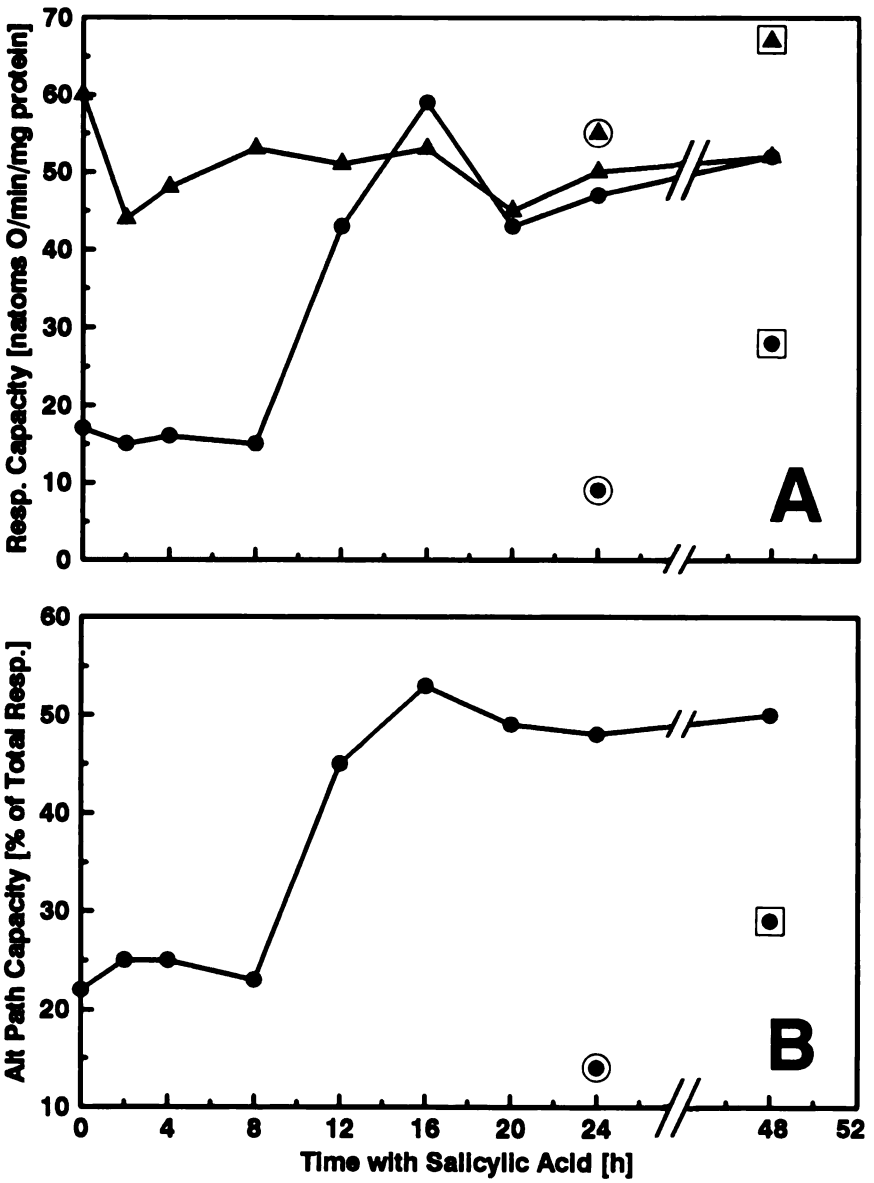

Resp. Capacity [natoms O/min/mg protein]
70
60
50
40
30
20
10
0
A
Alt Path Capacity [% of Total Resp.]
60
50
40
30
20
10
B
0
4
8
12
16
20
24
48
52
Time with Salicylic Acid [h]

Figure 5-5. Immunoblot showing the time course of salicylic acid induction of increased alternative oxidase accumulation. Salicylic acid was added to 1.0 mM to cell cultures on day 3. Mitochondria were isolated at each time point, and the respiratory pathway capacities were determined (see Fig. 5-4). The mitochondrial proteins (100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and the alternative oxidase proteins were identified using the AOA monoclonal antibody (3) and alkaline phosphatase linked to a secondary antibody. Mitochondrial proteins were from the following sources; lane 1: day 3 suspension cultured tobacco cells as time zero control; lane 2: day 4 as control; lane 3: day 5 as end point control; lane 4: cells treated for 2 h; lane 5: cells treated for 4 h; lane 6: cells treated for 8 h; lane 7: cells treated for 12 h; lane 8: cells treated for 16 h; lane 9: cells treated for 20 h; lane 10: cells treated for 24 h; lane 11: cells treated for 48 h. Protein molecular mass in kilodaltons is indicated at the side. Results are representative of three experiments.

1 2 3 4 5 6 7 8 9 10 11

35→

Inhibition of Salicylic Acid Induced Accumulation of Alternative Oxidase. Figure 5-6 shows that the accumulation of the alternative oxidase protein between the time when salicylic acid was added (lane 2) and 16 h after addition (lane 4) was decreased by the addition of actinomycin D (lane 5) 1 h prior to salicylic acid and blocked by addition of cycloheximide (lane 6) 1 h prior to salicylic acid.

Salicylic acid Concentration Required for Increase in Alternative Oxidase and Capacity. Addition of salicylic acid to 1.0 mM (lane 3) for 16 h caused an increase in the capacity and accumulation of the 35 kD alternative oxidase protein (Figure 5-7). Addition of salicylic acid to 100 μM (lane 4) also caused a slight increase in alternative pathway capacity (data not shown) and a slight increase in the accumulation of the 35 kD alternative oxidase protein above the control level (lane 4). However, addition of salicylic acid to 10 μM (lane 5) or 1 μM (lane 6) for 16 h did not result in a significant increase in alternative pathway capacity (data not shown) or in an accumulation of the protein that was greater than that of the negative control (lane 2).

Figure 5-6. Immunoblot showing inhibition of salicylic acid induced increase in alternative oxidase expression. Actinomycin D (lane 5) or cycloheximide (lane 6) was added to day 3 suspension cultured cells. One hour later salicylic acid was added to 1.0 mM to the cultures, which were then incubated under normal growth conditions for 16 h. For positive control samples salicylic acid alone was added. Mitochondria were isolated after each treatment and the respiratory pathway capacities were determined. The mitochondrial proteins (100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and the alternative oxidase proteins were identified using the AOA monoclonal antibody (3) and alkaline phosphatase linked to a secondary antibody. Mitochondrial proteins were from the following sources; lane 1: *S. guttatum* appendix tissue; lane 2: untreated day 3 cells as a time zero control; lane 3: untreated day 4 cells; lane 4: salicylic acid treated cells; lane 5: actinomycin D and salicylic acid treated cell culture; lane 6: cycloheximide and salicylic acid treated cell culture. Mitochondria were isolated from *S. guttatum* appendix tissue as previously described (18) and used as a positive control (lane 1). Protein molecular masses in kilodaltons are indicated at the side. Results are representative of two experiments.

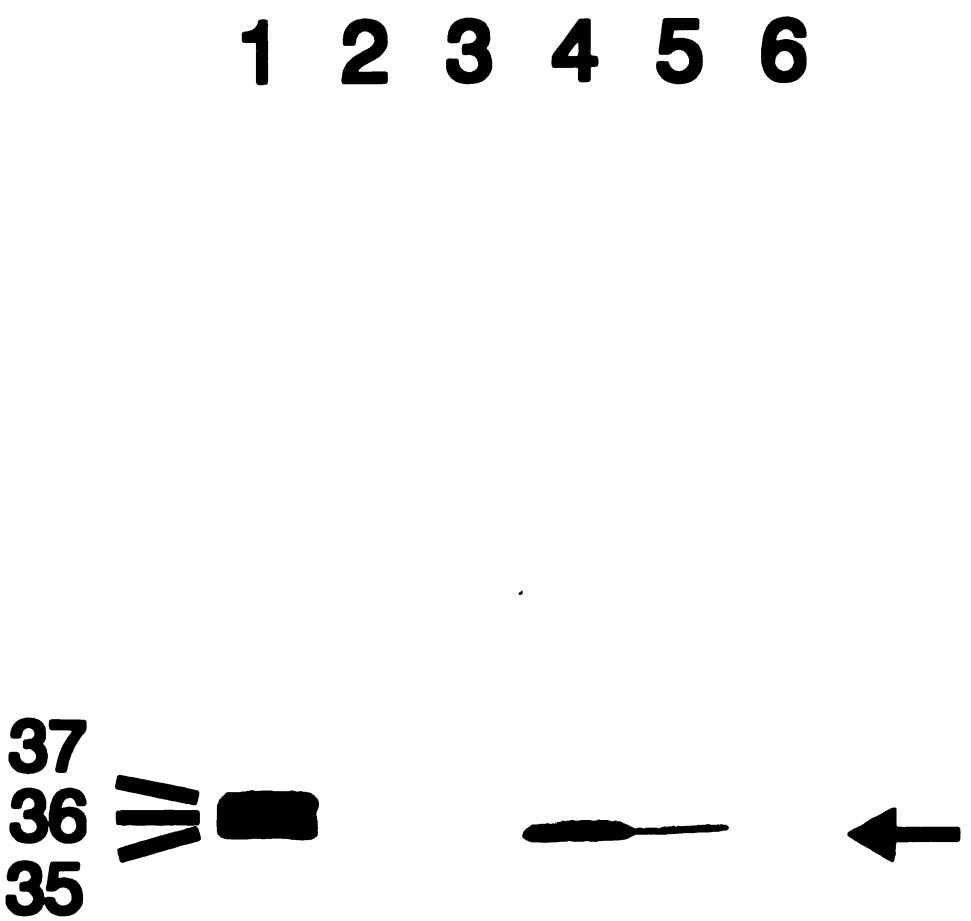
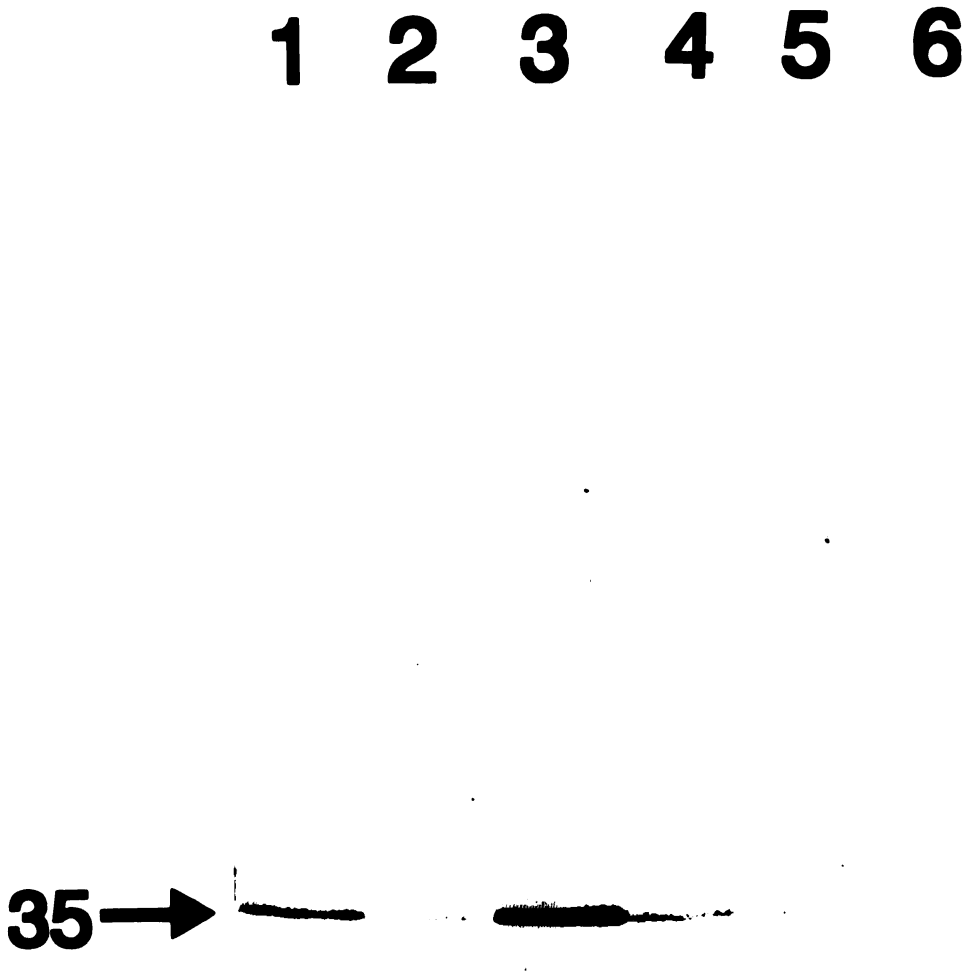

1 2 3 4 5 6
37
36
35

Figure 5-7. Immunoblot showing alternative oxidase accumulation caused by various concentrations of salicylic acid. Salicylic acid was added to the cells to a final concentration of 1.0 mM (lane 3), 100 μM (lane 4), 10 μM (lane 5), and 1.0 μM (lane 6) and the cells were grown under normal conditions for 16 h. Mitochondria were isolated after each treatment and the respiratory pathway capacities were determined. The mitochondrial proteins (100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and the alternative oxidase proteins were identified using the AOA monoclonal antibody (3) and alkaline phosphatase linked to a secondary antibody. Mitochondrial proteins were from the following sources; lane 1: untreated day 3 cell culture as a time zero control; lane 2: untreated day 4 cells; lane 3: 1.0 mM salicylic acid treated cells; lane 4: 100 μM salicylic acid treated cells; lane 5: 10 μM salicylic acid treated cells; lane 6: 1.0 μM treated cells. Protein molecular mass in kilodaltons is indicated at the side. Results are representative of two experiments.


1 2 3 4 5 6
35

DISCUSSION

The data presented here demonstrate that the capacities of both the alternative and the cytochrome pathway, measured in isolated mitochondria, varied greatly over the growth cycle of suspension cultured cells of *Nicotiana tobaccum* L. cv bright yellow. Specifically, the cytochrome pathway capacity increased dramatically between day 3 and day 4 after subculturing, while alternative pathway capacity increased dramatically between day 5 and day 6. The capacities of both pathways their highest observed levels on day 7, which is the day the cell cultures reached a stationary phase of growth. However, the net result of the changes in the capacities of the pathways was that the percentage of the total respiratory pathway that was attributed to the alternative pathway increased significantly as the cells approached and reached the stationary phase of growth. In contrast, another study, using the *N. glutinosa* L. cells, showed that the level of the alternative pathway was greatest during the lag and log phases of growth (6). The capacity measurements presented by Horn and Mertz used whole cells and measured alternative pathway capacity as O_2 uptake that was sensitive to SHAM only; not in the presence of cyanide (6). The difference between the results presented here and the results of Horn and Mertz (6) may be due to the use of different lines of suspension cultured tobacco cells. Our protein immunoblot data indicated that the level of the 35 kD alternative oxidase protein of the NT1 tobacco cells coincided with the pathway capacity throughout the growth cycle. Each increased dramatically between day 5 and day 6 and reached the highest level observed on day 7.

We have not measured the amount of sucrose at the various stages of the growth of the culture cells, but we assume that the amount was much lower at the stationary phase than it was in the early stages. Based on this assumption, the data presented here are contradictory to the overflow hypothesis (8), which postulates that the alternative pathway is most active when there is a supply of carbohydrate that exceeds the demand. Indeed, it seems illogical that the culture cells would partition electrons to the alternative pathway, with the inherent decrease in energy conservation, when the carbon supply is becoming limited. In this case, it may be a way for the cells to slow their growth during periods of decreased carbohydrate supply. When the alternative pathway was increased by the addition of salicylic acid, the density of the cells after 24 h of growth was 23 ± 4%, compared to 33 ± 2% for the control cultures. We do not know if the slight decrease in growth was caused by an increase in alternative pathway respiration or the presence of salicylic acid in the medium.

Salicylic acid may play a role in systemic acquired resistance (SAR) in tobacco (11,30). Chemicals that mimic the effects of salicylic acid are being actively pursued as possible agents for crop protection (28,29). Studies to determine the effect of salicylic acid upon the regulation of expression of the alternative oxidase will add to our understanding of the consequences of increased levels of salicylic acid and salicylic acid-mimicking chemicals in plant cells. In the experiments presented here, 1.0 mM salicylic acid caused a dramatic accumulation of the 35 kD alternative oxidase protein in suspension cultures of tobacco cells. The amount of the protein increased dramatically between 8 h and 12 h following

the addition of salicylic acid to the growth medium. The greatest accumulation occurred 16 h after the addition of salicylic acid. It is interesting to note that a very similar time course was observed for the response of *S. guttatum* appendix tissue to salicylic acid application (19). As in the experiments using *S. guttatum* appendix tissue, the increased protein accumulation in the tobacco cells coincided with a striking increase in the capacity of the alternative pathway. The capacity of the cytochrome pathway in these tobacco cells remained unchanged. Therefore, the percent of the total respiratory pathway that was attributed to the alternative pathway increased greatly. Since cytochrome pathway capacity increased in control culture cells during the same time, it seems that salicylic acid caused an inhibition of the normal, developmental increase in cytochrome pathway capacity. Again, a similar phenomenon occurs during the development of the appendix tissue of *S. guttatum* (4). While the capacity of the alternative pathway increases dramatically as the appendix tissue becomes thermogenic, the capacity of the cytochrome pathway is greatly decreased (4). The connection between the presence of salicylic acid in the observed effect on cytochrome pathway capacity in these systems remains unknown.

In an attempt to dissect the mechanism of regulation of the expression of alternative oxidase in suspension cultured tobacco cells, we have demonstrated that actinomycin D significantly decreased the salicylic acid-directed accumulation of the 35 kD protein in the mitochondria of these cells. In addition, cycloheximide completely inhibited the accumulation of the protein. These data indicate that *de novo* transcription and translation were necessary for salicylic acid to cause an

increase in the accumulation of the 35 kD alternative protein in suspension cultured tobacco cells. The observation that actinomycin D greatly inhibited the accumulation of the protein is consistent with the theory that salicylic acid regulates alternative oxidase expression in tobacco cells at the level of transcription.

We have also demonstrated that a concentration of 100 μM salicylic acid caused a slight increase in alternative pathway capacity (data not shown) and a slight increase in the accumulation of the 35 kD alternative oxidase protein, but that concentrations of 10 μM or less did not cause any significant increases. By comparison, Kapulnik *et al.* (7) reported that a salicylic acid concentration of only 20 μM was sufficient to increase alternative pathway capacity in whole cells of *Nicotiana tobaccum* cv. Xanthi-nc. Since we do not know the efficiency with which salicylic acid is taken up by the suspension cultured tobacco cells and directed to its place of action, it is difficult for us to draw any conclusions from our results. Furthermore, all our cultures were grown in the dark, leaving the possiblity that a light component may be required for salicylic acid to have a full effect, as is the case in *S. guttatum* appendix tissue (17).

This is the first thorough investigation of the developmental and salicylic acid induced changes in the respiratory pathway capacities and alternative oxidase expression in isolated mitochondria of suspension cultured tobacco cells. Future experiments will focus on identifying the changes in metabolism and cell growth resulting from increased and decreased alternative oxidase expression in order to learn more about the function of the alternative oxidase in higher plant cells.

ACKNOWLEDGEMENTS

The authors thank Dr. Gynheung An for the suspension cultured tobacco cells and Dr. Thomas E. Elthon, Dr. Beate Drechsler-Thielmann and Dr. Greg Vanlerberghe for helpful advice and discussions.

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SUMMARY OF THESIS

The major goals of my research project were to isolate cDNA and genomic clones encoding the alternative oxidase of *Sauromatum guttatum* and to study the developmental and salicylic acid induced expression of the alternative oxidase in *S. guttatum*. The research expanded to encompass the developmental and salicylic acid induced expression of alternative oxidase in suspension cultured tobacco cells.

CHAPTER 2: ISOLATION OF A cDNA CLONE ENCODING THE ALTERNATIVE OXIDASE OF *SAUROMATUM GUTTATUM*

The Results

The first major achievement from my thesis research was the isolation of a cDNA clone encoding an alternative oxidase protein of *S. guttatum*. This work benefitted greatly from the prior isolation of polyclonal and monoclonal antibodies that recognize only the 35-, 36-, and 37 kD alternative oxidase proteins on immunoblots of total mitochondrial proteins from *S. guttatum*. This cDNA clone has already been used to: 1) study the developmental and salicylic acid induced expression of the alternative oxidase in *S. guttatum* (CHAPTER 3); 2) isolate a

genomic clone of the alternative oxidase gene, *aox1*, of *S. guttatum* (CHAPTER 4); and 3) isolate alternative oxidase cDNA clones from several other higher plants. The identification of a single, 42 kD product of *in vitro* translation that is immunoprecipitated by polyclonal antibodies, supports our hypothesis that the 35-, 36-, and 37 kD alternative oxidase proteins are post-translationally modified products of a single, nuclear-encoded protein.

The Future

The isolation of the cDNA clone encoding an alternative oxidase protein of *S. guttatum* has provided a tool for the isolation of alternative oxidase cDNA clones from other organisms. The comparison of the sequence of these various cDNA clones will provide information about the evolution of the alternative oxidase gene.

CHAPTER 3: DEVELOPMENTAL AND SALICYLIC ACID REGULATION OF ALTERNATIVE OXIDASE EXPRESSION IN *SAUROMATUM GUTTATUM*

The Results

Exploring the salicylic acid regulation of the alternative respiratory pathway and, to some extent, the cytochrome pathway was the most interesting aspect of

my thesis research. This research provided evidence that salicylic acid regulates the expression of the alternative oxidase in *S. guttatum* by causing an increase in the accumulation of the 1.6 kb transcript. However, the mechanism resulting in this accumulation remains a mystery and an exciting area of research. Research on pathogenesis related proteins indicates that the transcripts for many of these proteins accumulate following salicylic acid application. Analysis of transgenic plants containing constructs with the promoters of each of these genes fused to reporter genes is consistent with the hypothesis that salicylic acid acts at the level of transcription. Thus, it is attractive to hypothesize that salicylic acid functions at the level of transcription to regulate alternative oxidase expression. However, the research in this thesis provides no direct evidence to support this hypothesis.

The Future

One way of attempting to prove this hypothesis would be to create transgenic tobacco plants containing a construct with the promoter region of the *S. guttatum* alternative oxidase gene, *aox1*, fused to a reporter gene. These transgenic plants could be sprayed with salicylic acid and analyzed to determine if there is increased accumulation of the transcripts of the reporter gene. Alternatively, the existence of a protein that binds to the promoter region may be confirmed by using nuclear extracts mixed with a DNA corresponding to the promoter region, followed by gel retardation assays. The binding of a protein in the presence of salicylic acid, but not the absence, would support this hypothesis.

Another hypothesis to explain the accumulation of the alternative oxidase transcript is that salicylic acid causes an increase in the stability of the alternative oxidase transcript. In order to investigate this hypothesis it would be helpful to: 1) establish an *in vitro* assay system to analyze the rate of transcript degradation; and 2) construct individual vectors to produce transcripts consisting of individual regions of the 1.6 kb alternative oxidase transcript fused to transcripts that are stable in the *in vitro* system.

CHAPTER 4: ISOLATION AND CHARACTERIZATION OF A GENOMIC CLONE CONTAINING THE *SAUROMATUM GUTTATUM* ALTERNATIVE OXIDASE GENE *AOX1*

The Results

The *S. guttatum* alternative oxidase gene, *aox1*, is a single copy gene that consists of four exons separated by three short introns. Exon 3 contains the region of the deduced precursor protein that is predicted to form two transmembrane helices. It is likely that this arrangement has some evolutionary significance in terms of protein structural and/or functional domains. The promoter of *aox1* has sequences that resemble previously identified promoter elements that are associated with transcriptional regulation resulting from various stimuli. More importantly, the promoter contains sites with homology to sites of other promoters that convey salicylic acid responsiveness to their respective genes.

The Future

The significance of the putative promoter elements present in the *aox1* promoter may be determined directly by "dissection" of the promoter and analyses using transgenic tobacco plants. Isolation of genomic clones encoding the alternative oxidase of other organisms may assist in the determination of the evolutionary significance of the structure of *aox1.*

CHAPTER 5: CYTOCHROME AND ALTERNATIVE PATHWAY RESPIRATION IN TOBACCO: EFFECTS OF SALICYLIC ACID

The Results

The capacity of the alternative pathway at various time points during the growth of suspension cultures tobacco cells was determined. The results indicate that both the cytochrome pathway capacity and the alternative pathway capacity reach the highest level observed as the cells enter the stationary phase of growth. These results are inconsistent with the "overflow" hypothesis, which postulates that the alternative pathway is most active when there is a supply of carbohydrate that exceeds the demand. For these cells it may be a way for the cells to slow their growth during periods of decreased carbohydrate supply. Salicylic acid caused an increase in the alternative pathway capacity and increased expression of the 35 kD alternative oxidase protein in these cells. Even if salicylic acid is not a normal, *in*

vivo regulator of the pathway in these cells, this result is important since the level of salicylic acid rises in tobacco leaf cells following inoculation of some pathogens. This will be useful information for researchers who are considering the use of salicylic acid mimicking chemicals as agents to increase the resistance of plants to pathogens.

The Future

The suspension cultured tobacco cells may prove to be a useful model system for studying the function of the alternative respiratory pathway in higher plants. Comparison of cell cultures with high alternative oxidase expression (by fusion of an alternative oxidase cDNA clone to an "inducible" promoter) to cell cultures with lowered expression (by production of anti-sense alternative oxidase transcripts) and cell cultures with normal expression may provide information regarding the contribution by alternative pathway respiration to whole cell. Furthermore, the suspension cultured tobacco cells may be a used as a model system to study the mechanism of salicylic acid regulation of alternative oxidase expression, alternative pathway capacity, and cytochrome pathway capacity in higher plants.