Induction of Mitochondrial Alternative Oxidase in Response to a Cell Signal Pathway Down-Regulating the Cytochrome Pathway Prevents Programmed Cell Death[1]

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Treatment of tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) cells with cysteine (Cys) triggers a signal pathway culminating in a large loss of mitochondrial cytochrome (cyt) pathway capacity. This down-regulation of the cyt path likely requires events outside the mitochondrion and is effectively blocked by cantharidin or endothall, indicating that protein dephosphorylation is one critical process involved. Generation of reactive oxygen species, cytosolic protein synthesis, and Ca^{2+} flux from organelles also appear to be involved. Accompanying the loss of cyt path is a large induction of alternative oxidase (AOX) protein and capacity. Induction of AOX allows the cells to maintain high rates of respiration, indicating that the lesion triggered by Cys is in the cyt path downstream of ubiquinone. Consistent with this, transgenic (AS8) cells unable to induce AOX (due to the presence of an antisense transgene) lose all respiratory capacity upon Cys treatment. This initiates in AS8 a programmed cell death pathway, as evidenced by the accumulation of oligonucleosomal fragments of DNA as the culture dies. Alternatively, wild-type cells remain viable and eventually recover their cyt path. Induction of AOX in response to a chemical inhibition of the cyt path (by antimycin A) is also dependent upon protein dephosphorylation and the generation of reactive oxygen species. Common events required for both down-regulation of the cyt path and induction of AOX may represent a mechanism to coordinate the biogenesis of these two electron transport paths. Such coordinate regulation may be necessary, not only to satisfy metabolic demands, but also to modulate the initiation of a programmed cell death pathway responsive to mitochondrial respiratory status.

Mitochondria play a central role in energy and carbon metabolism of eukaryotic cells, being the site of both the tricarboxylic acid cycle and oxidative phosphorylation pathways (Siedow and Day, 2000). Mitochondria have other important functions, such as taking an active role in programmed cell death (PCD) pathways of animals (Green and Reed, 1998) and possibly plants (Jones, 2000; Lam et al., 2001).

In plant mitochondria, the electron transport chain (ETC) supporting oxidative phosphorylation branches at ubiquinone (Siedow and Day, 2000; Vanlerberghe and Ordog, 2002). Electrons flow from ubiquinone through the cytochrome (cyt) pathway (including ubiquinol:cyt c oxidoreductase [Complex III], cyt c, and cyt oxidase) or to alternative oxidase (AOX). Electron flow from ubiquinone to AOX is not coupled to the generation of proton motive force. Thus, this pathway bypasses two of the three sites of energy conservation that otherwise support oxidative phosphorylation. Study of transgenic plant cells with altered levels of AOX supports the hypothesis that this protein dampens the mitochondrial generation of reactive oxygen species (ROS), presumably by preventing over-reduction of ETC components such as ubiquinone (Maxwell et al., 1999; Parsons et al., 1999; Yip and Vanlerberghe, 2001).

Mitochondrial biogenesis requires the expression of genes from both the mitochondrial and nuclear genomes. As such, mechanisms must exist in eukaryotes for two-way communication between the mitochondrion and nucleus (Poyton and McEwen, 1996). Such communication would maintain the functional state of the mitochondrion and ensure that as metabolic and other demands placed upon the mitochondrion changed (such as during development or in response to biotic and abiotic stress), gene expression could be adjusted to meet the new demands.

An effective means to induce expression of a nuclear gene encoding AOX is by artificial chemical inhibition of the cyt pathway by compounds such as CN and antimycin A (AA; Vanlerberghe and McIntosh, 1997). This suggests that when the capacity for cyt pathway respiration is altered, it signals coordinate changes in the capacity for AOX respiration. A natural example of such coordinate regulation occurs in the thermogenic inflorescense of Arum lilies such as *Sauromatum guttatum* (Meeuse, 1975). In these specialized floral organs, an extremely high rate of respiration generates heat to volatilize insect-attracting chemicals for pollination. This respiration occurs pre-

dominantly via AOX as a result of both a sharp decline in cyt path capacity and large increase in AOX capacity (Elthon et al., 1989a). Although there has been some progress toward identifying physiological signals important in regulating the capacity for AOX respiration (Vanlerberghe and McIntosh, 1996), to our knowledge, there is nothing known in plants about mechanisms by which cyt pathway capacity is regulated. It is probable that coordinate regulation of ETC components will involve pathways of communication between the mitochondrion and the rest of the cell.

Here, we define a system to trigger a down-regulation of the cyt pathway in tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) cells, showing that this down-regulation is dependent upon changes in protein phosphorylation and other cell processes. Down-regulation of the cyt pathway is accompanied by an increase in AOX capacity and we identify cell processes associated specifically with this induction. We also show that antisense cells unable to increase AOX capacity undergo PCD. Our results have implications for the coordinate biogenesis of ETC components and for the potential regulation of a PCD pathway responsive to mitochondrial respiratory status.

RESULTS

The Growth and Viability of Transgenic Cells Lacking AOX Are Highly Susceptible to Cys

Wild-type (wt) tobacco suspension cells (a 3-d-old culture) were supplied with different concentrations of Cys in their culture medium and growth (culture density) was periodically determined over a 72-h period. At a Cys concentration of 0.5 mM, growth was significantly delayed over a 24-h period, but then growth resumed (Fig. 1A). At higher concentrations (up to 5 mM), a similar pattern of delayed growth and recovery occurred, except that the length of the delay increased with increasing concentrations of Cys. Nonetheless, even at a concentration of 5 mM, some recovery of growth (increase in culture density) was observed by 72 h.

The growth of AS8 cells (transgenic cells lacking AOX due to the expression of an antisense AOX transgene) was much more sensitive to inhibition by Cys. At a Cys concentration of 0.5 mM, growth was delayed and showed some recovery only by 72 h (Fig. 1B). At 1 mM Cys (and all higher concentrations), culture density had decreased after 24 h and did not recover.
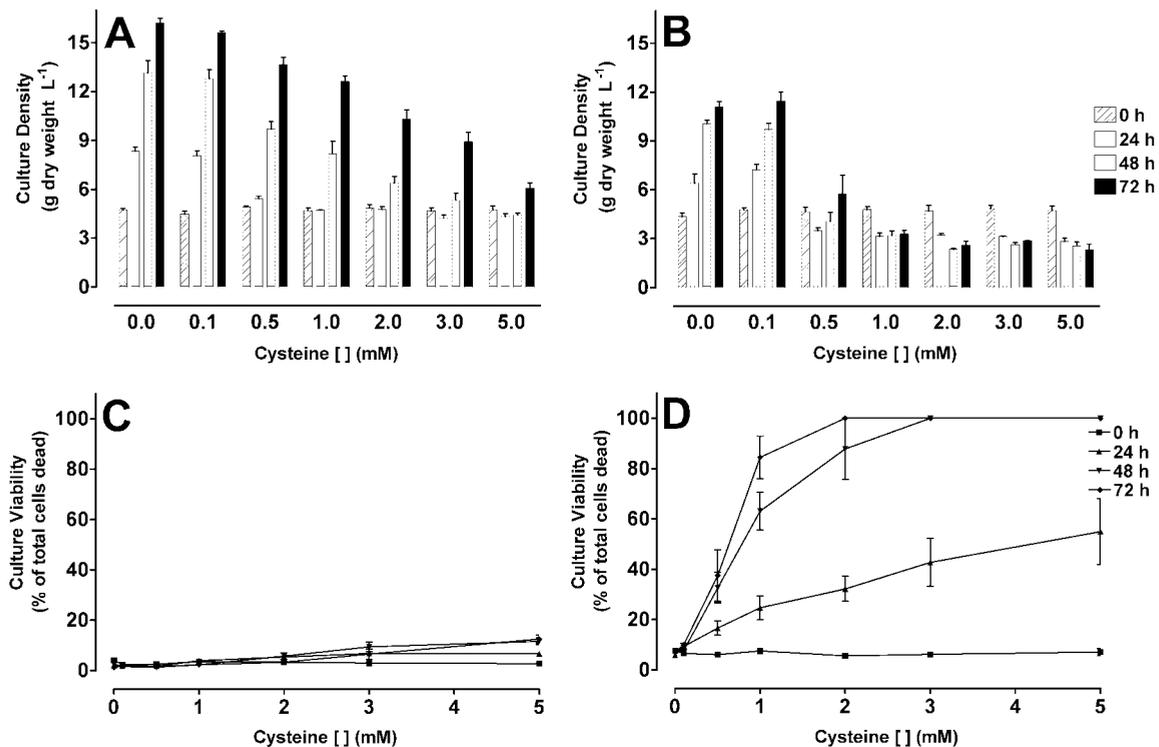

Figure 1. Growth and viability of wild-type (wt) and transgenic (AS8) tobacco cell cultures treated with different concentrations of Cys in their culture medium. Cys was added to wt (A and C) and AS8 (B and D) cultures at 3 d after subculture (time 0) and the culture density (A and B) and culture viability (C and D) were periodically determined over the following 72-h period (see legend on right). Data are the mean ± SE from three independent experiments. In some cases, error bars are smaller than the data symbols.

In parallel with the above experiments, we determined viability of the wt and AS8 cultures in response to Cys. In the wt culture, cell viability remained high over the entire 72-h period and at all Cys concentrations (up to 5 mM; Fig. 1C). In the AS8 culture, there were large losses in viability over time, even at 0.5 mM Cys (Fig. 1D). At 1 mM Cys, approximately 60% of cells were dead by 48 h and greater than 80% by 72 h. At 2 mM Cys (and at higher concentrations), all cells were dead by 72 h.

Cys Treatment Causes a Progressive Loss of Cyt Pathway

The respiratory characteristics of wt and AS8 cells were determined after treatment of cells with 1 mM Cys for up to 24 h. Surprisingly, we found that within 1 h of Cys treatment, the capacity of the cyt pathway in wt cells had decreased by approximately 70% (Fig. 2A) By 4 h, cyt pathway capacity was almost completely lost. AS8 cells suffered a similar loss of cyt pathway in response to Cys (Fig. 2A). However, although cyt pathway capacity in wt cells completely recovered by 24 h, no such recovery occurred in AS8.

Accompanying the loss of cyt pathway capacity, wt cells showed a large induction of AOX capacity over the first 4 h (Fig. 2B). No induction of AOX occurred in AS8 cells in response to the loss of cyt pathway due to the presence of the antisense AOX transgene in these cells.

Given the loss of cyt pathway and lack of AOX induction, AS8 cells had a dramatically reduced respiration rate after Cys treatment (Fig. 2C). Alternatively, the respiration rate of wt cells remained high at all time points and could be accounted for by the combined capacity of the cyt pathway and AOX (Fig. 2, A–C).

The changes in electron transport capacities being measured in whole cells (Fig. 2) could also be readily demonstrated in organello (Table I). Mitochondria were isolated from Cys-treated cells and electron transport capacities of these mitochondria were measured in the presence of several substrates (see "Materials and Methods"). Dithiothreitol and pyruvate were present as well to ensure maximal activation of AOX. Under these assay conditions, mitochondria isolated from Cys-treated wt cells showed a large loss of cyt pathway capacity and large induction of AOX capacity, compared with mitochondria isolated from untreated cells (Table I). Alternatively, mitochondria isolated from Cys-treated AS8 cells, although showing the large loss of cyt pathway, lacked any induction of AOX.

Protein analysis of isolated mitochondria showed that the large induction of AOX capacity in wt cells by a 4-h Cys treatment was the result of a large increase in AOX protein (Fig. 3). Alternatively, AOX protein was not detected in mitochondria from AS8 cells. Protein analysis also indicated that the level of
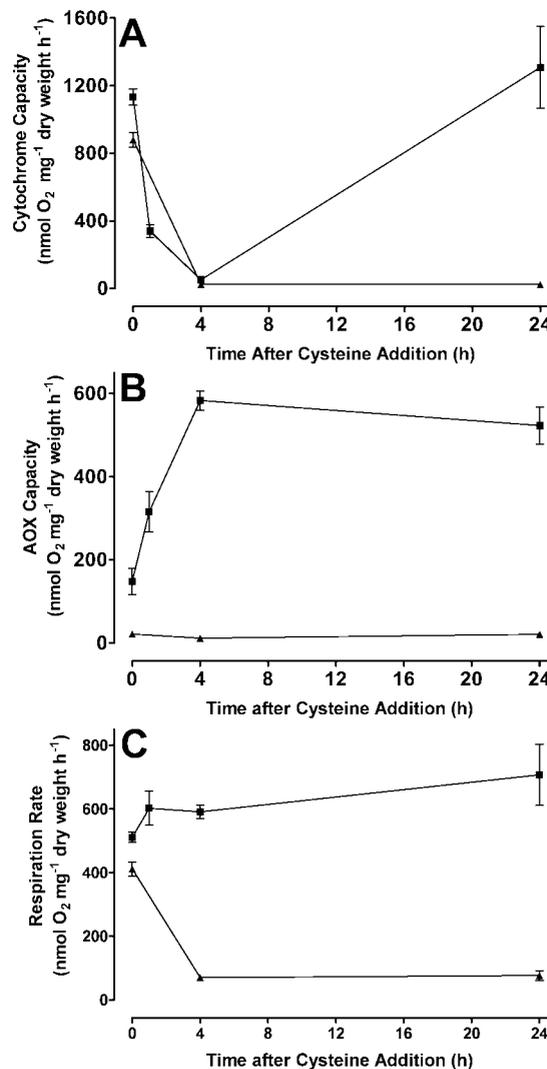

Figure 2. Respiratory characteristics of wt and transgenic (AS8) tobacco cell cultures treated with Cys in their culture medium. Cys (1 mM) was added to wt (squares) and AS8 (triangles) cultures at 3 d after subculture (time 0) and cyt pathway capacity (A), AOX capacity (B), and respiration rate (C) were periodically determined over the following 24-h period. Data are the mean ± SE from five to 13 independent experiments. In some cases, error bars are smaller than the data symbols.

two cyt pathway proteins (cyt c and cyt oxidase subunit II [cox II] were not dramatically effected in wt or AS8 cells by the 4 h Cys treatment; Fig. 3).

We compared the effects of Cys with that of other thiol- or S-containing compounds (Table II). None of the other compounds tested (1 mM Met, 1 mM dithiothreitol, and 1 mM reduced glutathione) had any dramatic effect on cyt pathway capacity in wt or AS8 cells in comparison with untreated cells. In accordance, these treatments did not readily induce wt AOX capacity or suppress AS8 respiration, two characteristic features of Cys treatment (Table II). We also found that treatment of wt cells with 0.4 mM cystine (the approximate maximal concentration of this com-

Table I. *Cyt and AOX capacities in mitochondria isolated from wt and transgenic (AS8) tobacco cells*

In some cases, cells were treated with 1 mM Cys for 4 h prior to mitochondrial isolation. See "Materials and Methods" for assay conditions. In some cases, results from two independent experiments are shown, and values with the same asterisks (* or **) represent data from the same experiment.

Cells	Mitochondria	Cyt Capacity	AOX Capacity
		nmol O_2 mg^{-1} protein min^{-1}	
Wt	From untreated cells	51.9*, 83.0**	12.7*, 41.1**
	From cells treated for 4 h with Cys	5.6*, 6.3**	63.3*, 136.6**
AS8	From untreated cells	44.6	1.8
	From cells treated for 4 h with Cys	6.4	2.8

pound in aqueous solution) had no effect on cyt or AOX capacity (data not shown).

Cys-Induced Down-Regulation of the Cyt Pathway Is Completely Blocked by Protein Phosphatase Inhibitors and Partially Blocked by Inhibitors of Other Cellular Processes

We took a pharmacological approach to identify processes involved in the Cys-induced down-regulation of the cyt pathway. Different pharmacological compounds were added to cells 15 min before the addition of 1 mM Cys and the capacity of the cyt pathway was determined after 4 h. Using wt cells, we found that the protein phosphatase inhibitors cantharidin and endothall were each able to provide almost complete protection against the Cys-induced loss of cyt pathway (Fig. 4A). Cantharidin and endothall also effectively blocked the accompanying induction of AOX capacity (Fig. 4B) and AOX protein (Fig. 5), but interpretation of this result is more complex (see below).

Cycloheximide (inhibitor of cytosolic protein synthesis) and ruthenium red (inhibitor of calcium flux from mitochondria and other intracellular stores) were other pharmacological compounds able to provide considerable protection against the Cys-induced loss of cyt pathway (Fig. 6A). Alternatively, chloramphenicol (inhibitor of mitochondrial protein synthesis) and LaCl (inhibitor of plasma membrane calcium flux) provided little or no protection (Fig. 6A). The protein kinase inhibitors genistein and staurosporine also provided no protection (data not shown). We also examined how the above treatments effected the accompanying induction of AOX (Fig. 6B). These results are discussed further later.

We investigated the possibility that generation of a ROS as a result of Cys auto-oxidation was an important initial event capable of activating the signal pathway that then down-regulates the cyt pathway. Two antioxidants were tested for their ability to protect against the Cys-induced loss of cyt pathway. Flavone provided considerable protection, whereas N-acetyl-Cys provided no protection (Fig. 6A). Also, the antioxidant enzymes catalase and superoxide dismutase (when added to the culture medium) pro-

vided little or no protection against cyt pathway loss. Again, we also examined how the above treatments effected the accompanying induction of AOX (Fig. 6B), results that are discussed further later. Auto-oxidation of thiols such as Cys may be particularly evident when in the presence of redox-active metal ions such as Fe. However, we found that Cys was equally effective at initiating the loss of cyt pathway capacity, regardless of whether Fe was present or not in the growth medium during the treatment (Table III).

Some of the above pharmacological compounds were also tested with AS8 cells and the results were qualitatively similar to that seen with wt cells. That is, protection against Cys-induced loss of cyt pathway was seen with endothall, cycloheximide, ruthenium red, and flavone, whereas no protection was seen with LaCl (Table IV). Interestingly, however, the level of protection of the AS8 cyt pathway afforded by compounds was never as great as seen with wt cells (compare Table IV with Figs. 4A and 6A).

The Effects of Cys on Viability and Growth Are Comparable with Those of AA

To confirm that the differential effects of Cys on the respiratory characteristics, viability, and growth of
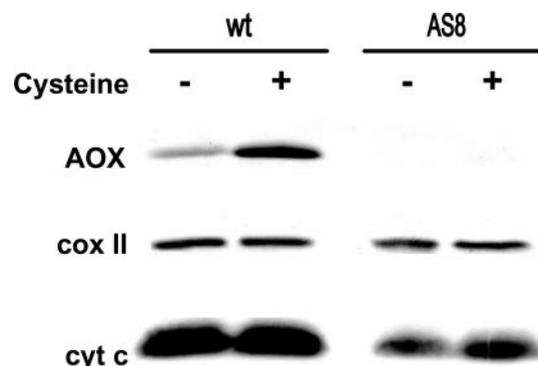

Figure 3. The level of ETC proteins in mitochondria isolated from wt and transgenic (AS8) tobacco cells. The cells had been either left untreated or treated for 4 h with 1 mM Cys before mitochondrial isolation. Mitochondrial protein (100 μg) was separated by reducing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies raised to AOX, cox II, or cyt c.

Table II. *Effect of different thiol-containing compounds (Cys, dithiothreitol, and reduced glutathione) and the S-containing amino acid Met on respiratory characteristics of wt and transgenic (AS8) tobacco cells*

Treatments were for 4 h and at a concentration of 1 mM. Data are the mean ± SE from three independent experiments.

Treatment	Respiration		Cyt Capacity		AOX Capacity	
	wt	AS8	wt	AS8	wt	AS8
			nmol O_2 mg^{-1} dry wt h^{-1}			
Control	468 ± 45	439 ± 30	1,085 ± 160	904 ± 82	90 ± 28	29 ± 4
Cys	550 ± 66	84 ± 8	39 ± 8	32 ± 6	522 ± 63	14 ± 3
Dithiothreitol	482 ± 59	386 ± 46	1,084 ± 123	725 ± 60	207 ± 30	12 ± 4
Glutathione	606 ± 64	430 ± 34	1,276 ± 105	823 ± 31	24 ± 6	11 ± 6
Met	462 ± 29	425 ± 41	1,100 ± 58	817 ± 38	69 ± 12	46 ± 20

wt versus AS8 cells were due to a large loss of cyt pathway (rather than some other effect of Cys), we compared the effects of Cys with that of AA (Figs. 7 and 8). AA is a well-known chemical inhibitor of the cyt pathway, inhibiting electron transfer downstream of ubiquinone at Complex III. Cells were treated with either 2 mM Cys or 10 μM AA and, as expected, both treatments resulted in a large loss of cyt pathway in wt and AS8 cells, when measured after 24 h (Fig. 7A). Also, both compounds caused a large suppression of AS8 respiration in comparison with similarly treated wt cells (Fig. 7C) and both compounds resulted in a large induction of AOX capacity in wt (but not AS8) cells (Fig. 7B). Note that by 48 h, the cyt pathway of wt cells treated with Cys had recovered and AOX capacity had again declined. This did not occur in the case of AS8 cells treated with Cys or with either wt or AS8 cells treated with AA.

Importantly, viability of the AS8 culture dropped in response to either the Cys or AA treatment and the kinetics of the decline over a 72-h period were very similar for both compounds (Fig. 8B). In wt cells, viability remained high in response to either treatment. We also showed that wt culture density increased slowly over time (during treatment with either Cys or AA) whereas AS8 culture density declined abruptly and did not recover in response to either treatment (Fig. 8A).

In summary, treatment of cells with AA generated similar patterns of growth, viability, and respiratory characteristics to that which were being generated by Cys, indicating that all of the differential effects of Cys on wt versus AS8 cells were due to the ability of Cys to induce a loss of cyt pathway capacity downstream of ubiquinone.

Identification of Cellular Processes Required for Induction of AOX after a Loss of Cyt Pathway

A strong induction of AOX occurred in wt cells in response to a loss of cyt pathway, regardless of whether cyt pathway loss resulted from activation of a signal pathway (such as is the case with Cys treatment) or was the result of an artificial chemical inhibition (such as is the case with AA treatment; Fig. 7). Just as we had taken a pharmacological approach to identify processes important in the Cys-induced down-regulation of the cyt pathway, we also wanted to identify processes involved specifically in the induction of AOX in response to a loss of cyt pathway. However, the pharmacological data on AOX induction in which Cys has been used to inhibit the cyt pathway (i.e. the data in Figs. 4B and 6B) is difficult to interpret because effects may be occurring for either of two reasons. The pharmacological compound may in fact be directly affecting a cellular process required for up-regulation of AOX in response to an inhibition of the cyt pathway. Alternatively, the pharmacological compound may be affecting the up-regulation of AOX only indirectly by having effected the ability of Cys to inhibit the cyt pathway. This is assuming that inhibition of the cyt pathway is a critical event in the AOX induction. To obtain less ambiguous data on the effects of pharmacological compounds on specifically the induction of AOX in response to a loss of cyt pathway, another approach was necessary. This approach was to examine the effect of pharmacological compounds on AOX induction in response to AA. Because chemical inhibition of the cyt pathway by AA is not dependent upon any cellular process, effects of pharmacological compounds on AOX induction in response to AA can be more readily interpreted.

As expected, a 3-h treatment of wt cells with AA caused a dramatic increase in the capacity of the AOX pathway (Fig. 9). On average in these experiments, AOX capacity would increase from approximately 29 nmol O_2 mg^{-1} dry weight h^{-1} to approximately 520 nmol O_2 mg^{-1} dry weight h^{-1} over the 3-h treatment period and this increased capacity correlated with a large induction of *Aox1* mRNA and AOX protein (Fig. 10). Of the compounds tested to block this induction, flavone was the most effective followed closely by endothall and cantharidin (Fig. 9). Figure 10 shows that the reduced AOX induction with endothall and cantharidin correlates with a lack of accumulation of *Aox1* mRNA and AOX protein. Alternatively, induction of AOX capacity by AA was only marginally blocked by ruthenium red or LaCl, whereas *N*-acetyl-Cys had no effect on the induction (Fig. 9).
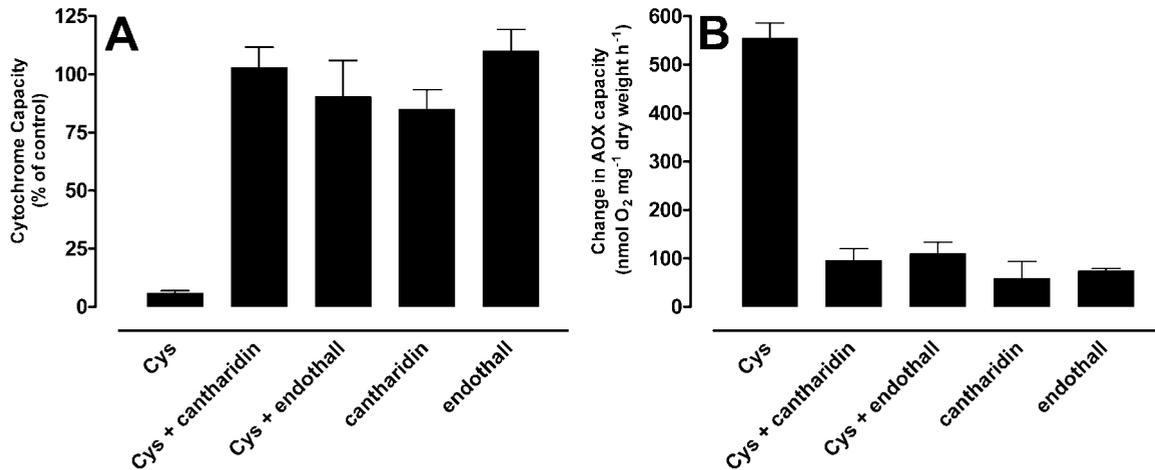
Figure 4. The effect of the protein phosphatase inhibitors cantharidin and endothall on the Cys-induced loss of cyt pathway (A) and the accompanying induction of AOX capacity (B). Cantharidin (20 μM) and endothall (100 μM) were added 15 min before Cys (1 mM) and respiratory capacities were determined after 4 h. Data are the mean $\pm$ SE from three to five independent experiments. The untreated (control) cells had a cyt capacity of 1,270 $\pm$ 62 nmol O_2 mg^{-1} dry weight h^{-1} and an AOX capacity of 67 $\pm$ 15 nmol O_2 mg^{-1} dry weight h^{-1} (mean $\pm$ SE).

The Loss of All Respiratory Capacity in Transgenic Cells Results in a Programmed Form of Cell Death

To determine whether AS8 cells experienced a necrotic or programmed form of cell death in response to the loss of cyt pathway, we examined genomic DNA extracted from cells at different times after treatment with Cys or AA. In response to either treatment, we saw an accumulation of oligonucleosomal fragments of DNA (and multiples thereof) as the culture died (Fig. 11). As would be expected, a 100-bp ladder estimated the size of these fragments at approximately 185, 390, 600, and 765 bp. This result is indicative of a programmed form of cell death. Alternatively, if cells were rapidly killed by treatment with a high concentration of H_2O_2, only a smear of DNA (indicative of a necrotic form of cell death) was seen (Fig. 11).

Protection against Cys-Induced Inhibition of the Cyt Pathway Prevents the Programmed Death of Transgenic Cells

Given the ability of ruthenium red to protect to some extent against the loss of cyt pathway in AS8 cells (Table IV), we examined whether ruthenium red would protect against the Cys-induced reductions in growth and viability of such cells. AS8 cells treated with Cys showed the characteristic loss of viability and growth after 48 h of treatment (Fig. 12). Alternatively, in AS8 cultures treated with Cys plus ruthenium red, viability remained high (similar to untreated cells) over the 48-h period (Fig. 12B). Ruthenium red alone had little effect on AS8 viability in comparison with untreated cells, although it did reduce growth significantly. Similarly, ruthenium red could only afford moderate protection against the Cys-induced growth decline (Fig. 12A).

DISCUSSION

A Cell Signaling Pathway Modulating Mitochondrial Cyt Pathway Capacity

Treatment of tobacco cells with Cys triggers an almost complete loss of mitochondrial cyt pathway capacity within a few hours (Fig. 2A). In wt cells, this loss is associated with a large induction of AOX capacity (Fig. 2B), due to a large increase in AOX protein (Fig. 3). The large induction of AOX allows these cells to maintain a high rate of respiration (Fig. 2C). Such cells remain viable (Fig. 1) and eventually recover their cyt pathway (Fig. 2A). The changes in electron transport capacities measured in intact cells (Fig. 2) were also confirmed in mitochondria isolated from Cys-treated cells, indicating that no artifactual
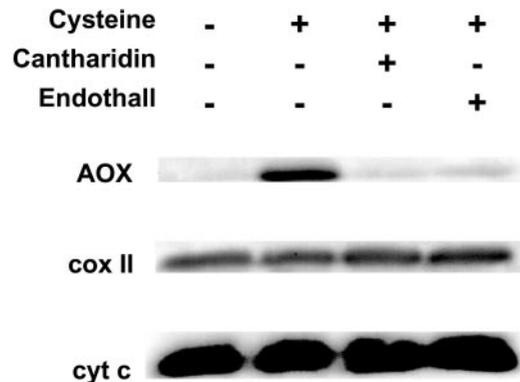

Figure 5. The level of ETC proteins in mitochondria isolated from wt cells. The cells had been either left untreated or treated for 4 h with Cys (1 mM) or with a combination of Cys and the protein phosphatase inhibitor cantharidin (20 μM) or endothall (100 μM) before the mitochondrial isolation. Mitochondrial protein (100 μg) was separated by reducing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies raised to AOX, cox II, or cyt c.
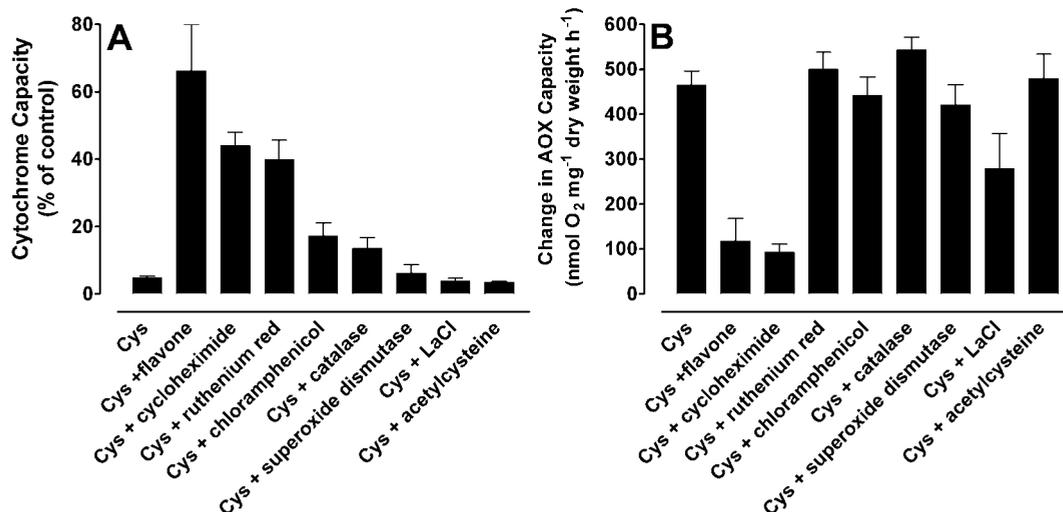
Figure 6. The effect of pharmacological compounds on the Cys-induced loss of cyt pathway capacity (A) and the accompanying induction of AOX capacity (B). Compounds were added 15 min before the addition of 1 mM Cys and respiratory capacities were determined after 4 h. Compounds were used at the following concentrations: flavone (1 mM), cycloheximide (360 μM), ruthenium red (100 μM), chloramphenicol (4 mM), catalase (3,000 units mL^{-1}), superoxide dismutase (150 units mL^{-1}), LaCl (1 mM), and N-acetyl-Cys (1 mM). At these concentrations, each of the compounds (when added alone to cells for 4 h) had only a small if any effect on cyt or AOX capacity (data not shown). Data are the mean ± SE from three to seven independent experiments. Untreated (control) cells had a cyt capacity of 1,196 ± 43 nmol O_2 mg^{-1} dry weight h^{-1} and an AOX capacity of 127 ± 25 nmol O_2 mg^{-1} dry weight h^{-1} (mean ± SE).

effects were hindering the in vivo measurements (Table I).

The above results differ from those in the yeast *Hansenula anomala* in which Cys treatment did not impact cyt pathway capacity, although it was an effective inducer of AOX (Minagawa et al., 1991). However, in that system, dithiothreitol, glutathione, and methionine were also effective at inducing AOX, again unlike here (Table II). These results suggest that the action of Cys in the fungal system is different from that in tobacco cells.

We took a pharmacological approach to identify cellular processes necessary for Cys-induced down-regulation of the cyt pathway. Cantharidin and en-

dothall are each potent inhibitors of the Ser/Thr protein phosphatases types 1 and 2A (Honkanen, 1993). Either of these inhibitors was able to almost completely block the Cys-induced loss of cyt pathway (Fig. 4A). In addition, either inhibition of cytosolic protein synthesis (using cycloheximide) or inhibition of Ca^{2+} flux from intracellular stores (using ruthenium red) was able to partially block against the loss of cyt pathway (Fig. 6A). These results indicate that Cys triggers a complex signal pathway, involving a range of different cellular activities, and that a protein dephosphorylation event(s) is a critical component of the pathway (Fig. 13). The dependence of Cys action on different cel-

Table III. *Effect of Fe in the medium on respiratory characteristics of wt tobacco cells*

Three-day-old cultures were washed twice and suspended with either their original (old) growth medium or with fresh one-half-strength growth medium (which contains 50 μM Fe-EDTA) or with fresh one-half-strength growth medium without Fe-EDTA. Cells were then left untreated for 4 h or were treated with 1 mM Cys for 4 h prior to measurement of respiratory characteristics. Data are the mean from two independent experiments, both of which showed similar results.

Treatment	Medium	Respiration	cyt Capacity	AOX Capacity
		nmol O_2 mg^{-1} dry wt h^{-1}		
Control	Old	577	1,227	56
	Fresh, +Fe	588	1,262	45
	Fresh, −Fe	599	1,279	33
Cys	Old	714	75	639
	Fresh, +Fe	566	64	537
	Fresh, −Fe	615	106	572

Table IV. *The effect of pharmacological compounds on the Cys-induced loss of cyt pathway capacity in transgenic (AS8) cells*

Compounds were added 15 min prior to Cys addition, and cyt capacity was determined after 4 h. Compounds were used at the following concentrations: Cys (1mM), endothall (100 μM), ruthenium red (100 μM), flavone (1 mM), cycloheximide (360 μM), and LaCl (1 mM). Data are the mean ± SE from three to five independent experiments. The untreated (control) cells had a cyt capacity of 1,132 ± 62 nmol O_2 mg^{-1} dry wt h^{-1} (mean ± SE).

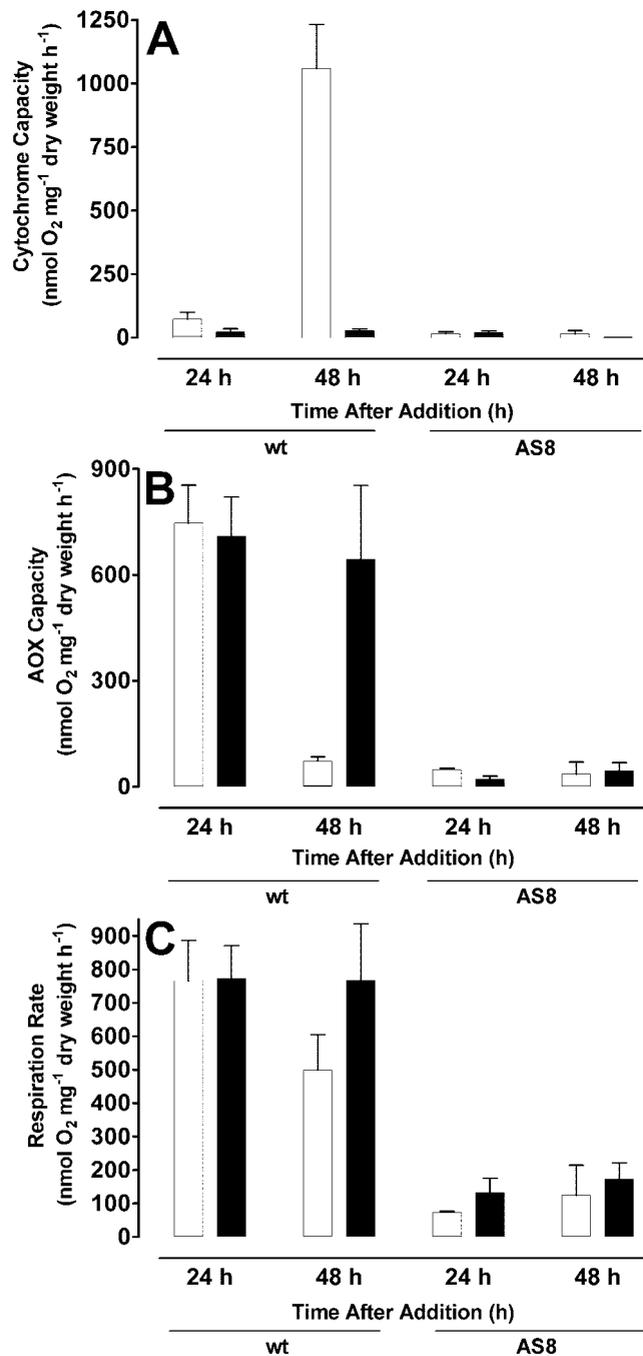| Treatment	cyt Capacity
	% of Control
Cys	3 ± 1
Cys + endothall	28 ± 8
Cys + ruthenium red	22 ± 4
Cys + flavone	19 ± 4
Cys + cycloheximide	12 ± 2
Cys + LaCl	2 ± 1

Figure 7. Respiratory characteristics of wt and transgenic (AS8) tobacco cells treated with Cys or AA. At time 0, 2 mM Cys (hatched bars) or 10 μM AA (solid bars) were added to the culture medium of wt and AS8 cells. After 24 and 48 h, cyt capacity (A), AOX capacity (B), and respiration rate (C) were measured. Data are the mean from two independent experiments and error bars represent the minimum and maximum values.

lular events is consistent with the observation that the loss of cyt pathway requires several hours to take full effect (Fig. 2A).

At present, we do not know the site of the lesion in the cyt pathway being induced by Cys treatment.

However, the lesion must reside downstream of ubiquinone because a lesion upstream of ubiquinone would also preclude any AOX respiration, which it clearly has not (Fig. 2B). Potential target sites then include Complex III, cyt c, and cyt oxidase. We also do not know the nature of the lesion in the cyt pathway. Possibilities would include degradation or modification of the above ETC component(s). We did not see any dramatic change in the level of cyt c or coxII protein after Cys treatment (Figs. 3 and 5), precluding that any comprehensive loss of ETC proteins had occurred.

The protein phosphatase activity necessary for down-regulation of the cyt pathway may reside within or outside the mitochondrion. Interestingly, recent work indicates the presence of a canthardin-sensitive phosphatase in plant mitochondria (Bunney et al., 2001). The ability of cycloheximide and ruthenium red to at least partially inhibit the loss of cyt pathway (Fig. 6A) indicates that events outside the mitochondrion are likely also necessary.
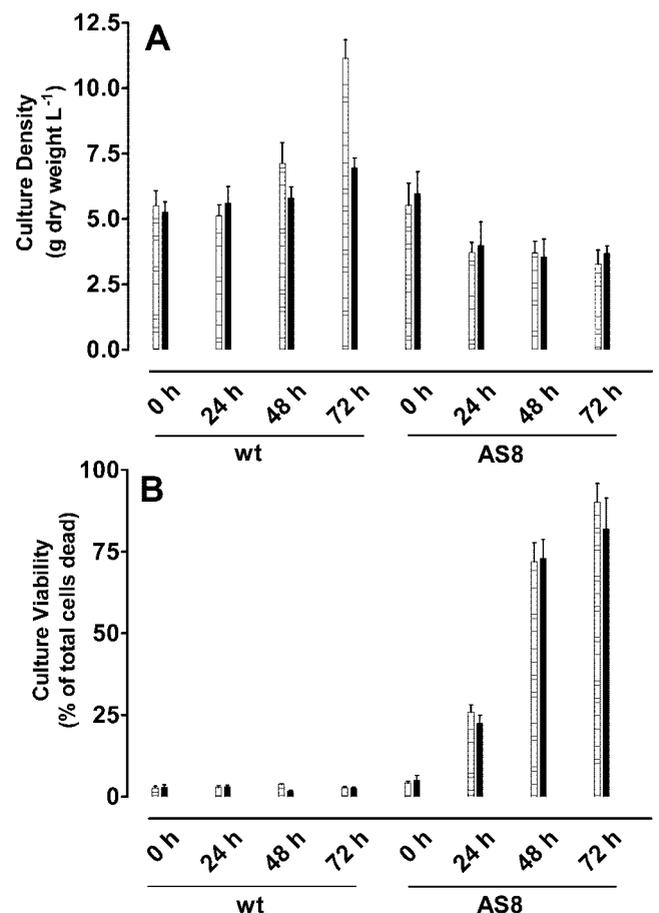

Figure 8. Growth and viability of wt and transgenic (AS8) tobacco cells treated with Cys or AA. At time 0, 2 mM Cys (hatched bars) or 10 μM AA (solid bars) were added to the culture medium and culture density (A) and culture viability (B) were periodically measured over the following 72 h. Data are the mean ± SE from three independent experiments.
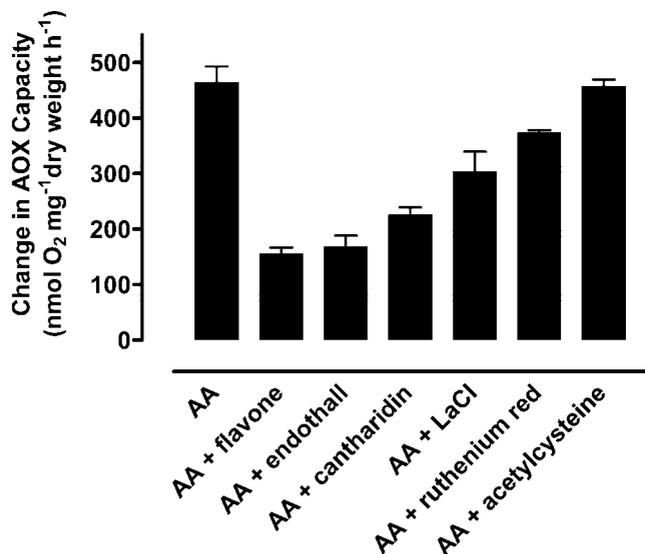
Figure 9. The effect of pharmacological compounds on the AA-induced induction of AOX capacity in wt tobacco cells. Compounds were added 15 min before AA addition and AOX capacity was determined after 3 h. Compounds were used at the following concentrations: AA, 2 μM; flavone, 1 mM; endothall, 100 μM; cantharidin, 20 μM; LaCl, 1 mM; ruthenium red, 100 μM; and N-acetyl-Cys, 1 mM. Data are the mean $\pm$ SE from four to six independent experiments. The untreated (control) cells had an AOX capacity of 29 $\pm$ 5 nmol O_2 mg^{-1} dry weight h^{-1} (mean $\pm$ SE).

Approaches were taken to investigate what might be the early initial mode of action of Cys, which allowed it to so effectively activate a complex signal pathway down-regulating the cyt pathway. One possibility was that Cys might act via a ROS. Thiols such as Cys readily undergo auto-oxidation, generating superoxide anion, and this pro-oxidant effect is greatly potentiated in the presence of the redox-active metal ion Fe (Yang et al., 2000). Such pro-oxidant activity may explain why thiol compounds such as Cys, although normally viewed as reducing agents, have often been paradoxically shown to induce the expression of antioxidant enzymes (Herouart et al., 1993). We found that externally supplied and membrane-impermeable catalase and superoxide dismutase did not affect the ability of Cys to down-regulate the cyt pathway (Fig. 6A). Removal of Fe from the medium before Cys treatment also did not hinder Cys action (Table III). These results suggest that any ROS generated outside the cell as a result of Cys auto-oxidation are not required for the action of Cys.

The above experiments do not preclude the possibility that Cys auto-oxidation within the cell generates ROS that are a component of the Cys action. This possibility was investigated by utilizing membrane-permeable ROS-scavenging chemicals. Phenolic compounds are well recognized for their antioxidant properties and because flavone was previously shown to effectively reduce the in vivo level of ROS in tobacco cells (Maxwell et al., 1999), it seemed a

good candidate for our study. N-acetyl-Cys, on the other hand, has been extensively utilized as an antioxidant compound in both plant and animal studies (Moldeus and Cotgreave, 1994). We found that although N-acetyl-Cys was completely ineffective at blocking the Cys-induced down-regulation of the cyt pathway, flavone was very effective in this regard (Fig. 6A). In fact, flavone was second only to the protein phosphatase inhibitors in its ability to block Cys action. We should note that N-acetyl-Cys treatment, in itself, had no effect on the cyt path (see legend to Fig. 6). The results support the idea that intracellular generation of superoxide anion, in particular, may be an important component of the signaling pathway. Flavone can likely scavenge superoxide anion (Rice-Evans et al., 1997), whereas N-acetyl-Cys is thought to primarily scavenge H_2O_2 and hydroxyl radical, while not readily interacting with superoxide anion (Moldeus and Cotgreave, 1994).

Cys action could alternatively involve it acting as a reducing agent, possibly capable of reducing a regulatory disulfide bond in a critical target protein. It is interesting, however, that dithiothreitol and reduced glutathione (both of which can act as reducing agents as well as being subject to auto-oxidation) could not mimic the effects of Cys (Table II). This suggests that Cys action might involve something other than its pro-oxidant or antioxidant effects. An alternative is that Cys action could involve it acting as a substrate. Cys is the primary precursor of all organic molecules containing reduced sulfur and its increased availabil-
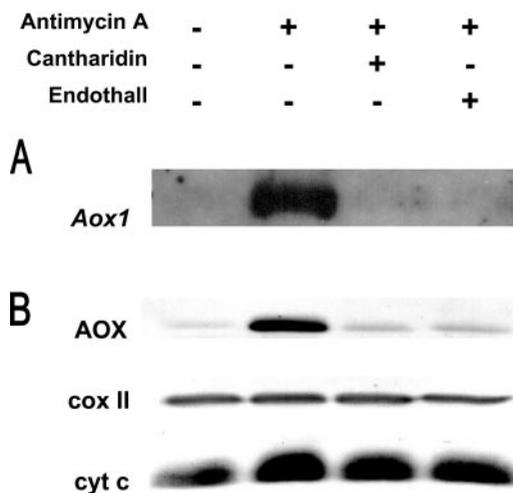

Figure 10. The effect of the protein phosphatase inhibitors cantharidin and endothall on AA-induced changes in the level of *Aox1* mRNA (A) and mitochondrial ETC proteins (B). The cells had been either left untreated or treated with AA (2 μM) or with a combination of AA and cantharidin (20 μM) or endothall (100 μM) before isolation of total RNA (after 2 h) or isolation of mitochondria (after 4 h). Total RNA (10 μg) was used for northern analysis using a cDNA clone of *Aox1* as hybridization probe. Mitochondrial protein (100 μg) was separated by reducing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies raised to AOX, cox II, or cyt c.

Figure 11. Agarose gel analysis of DNA isolated from transgenic (AS8) cells treated with 2 mM Cys, 10 μM AA, or 500 mM hydrogen peroxide (H_2O_2). In each case, 4 μg of DNA was separated in a 2% (w/v) agarose gel and stained with ethidium bromide. Numbers represent the hours of treatment before DNA isolation, and L marks lanes containing a 100-bp DNA ladder. In the Cys-treated culture, all cells were dead at the 48-, 72-, and 96-h time points. In the AA-treated culture, 72% of cells were dead at 48 h, 83% of cells were dead at 72 h, and 80% of cells were dead at 96 h. In the culture treated with 500 mM H_2O_2, all cells were dead at 4 h. Representative results are shown.
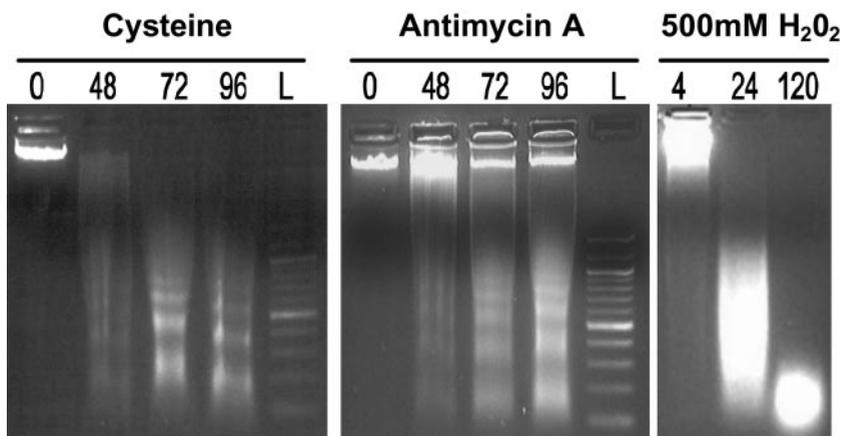
ity during our treatments might alter the level of a key cellular metabolite. For example, recent experiments indicate that enhanced biosynthesis of Cys in transgenic plants significantly increases the cellular level of glutathione, a key modulator of cellular redox balance (Harms et al., 2000). This might represent an indirect means by which Cys could act in a redox-dependent fashion.

Regardless of the initial mode of action of Cys, we favor the hypothesis that Cys is not the "natural" physiological trigger of the signal pathway able to modulate the Cyt pathway. Rather, we favor the view that Cys in our experimental system is able to inadvertently trigger this signal pathway, either by one of the mechanisms discussed above or some alternate means.

To our knowledge, this is the first example in plants of an experimental approach to study a signal pathway modulating cyt pathway capacity. In this regard, we have recently found that an 8-h treatment of wt cells with cantharidin alone increases the capacity of cyt pathway in isolated mitochondria by approximately 1.5-fold in comparison with mitochondria from untreated cells (G.C. Vanlerberghe,

unpublished data). One interpretation of the results is that activity of the cantharidin-sensitive protein phosphatase serves to counterbalance the basal activity of a protein kinase acting to enhance cyt pathway capacity.

There is growing evidence that the activity of Ser/Thr protein phosphatases is affected by the oxidation state of redox-sensitive functional groups on the protein (Rusnak and Reiter, 2000). This provides a mechanism by which the activity of these proteins could be modulated by ROS or a change in cellular or mitochondrial redox state, as described for Tyr phosphatases. Based on our results, it is intriguing to speculate that cyt pathway capacity in plant mitochondria is controlled by a phosphorylation cascade modulated by redox signals (Fig. 13).

A Mechanism to Coordinate the Biogenesis of Cyt and AOX Pathways in Plant Mitochondria

Using AA, we took a pharmacological approach to examine what signal events are important for the induction of AOX in response to a loss of cyt pathway. Of the compounds tested, we found that fla-
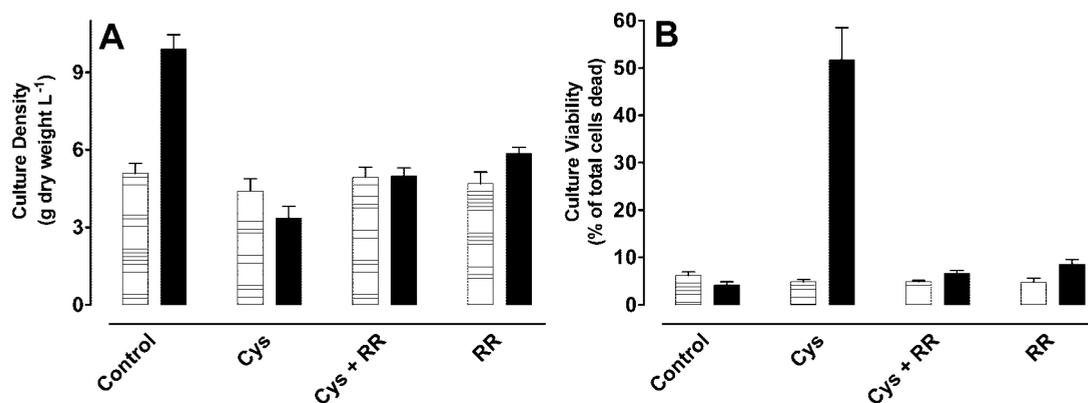
Figure 12. The effect of ruthenium red on the Cys-induced reductions in growth and viability of transgenic (AS8) cells. Cells were either left untreated (control) or treated with combinations of 1 mM Cys and 100 μM ruthenium red. Culture density (A) and culture viability (B) were measured just before the treatments (hatched bars) and after 48 h of treatment (solid bars). Data are the mean $\pm$ SE from three independent experiments.
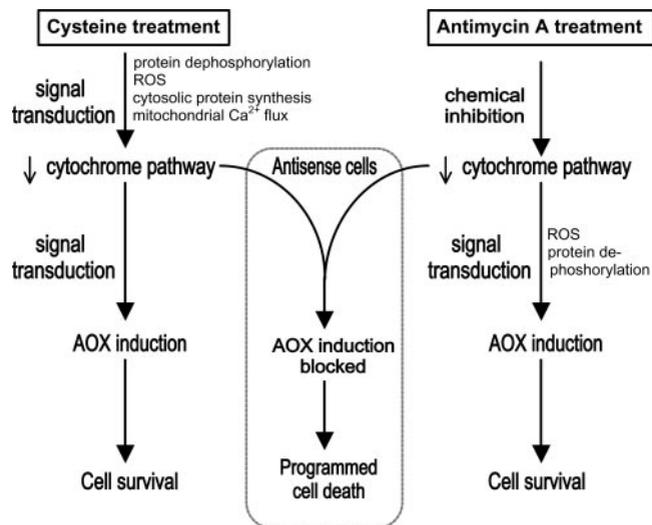
Figure 13. Reversible protein phosphorylation, the generation of ROS, and other cellular events are components of plant signal transduction pathways used to modulate the mitochondrial capacity for cyt and AOX respiration. For example, a dephosphorylation-dependent signal pathway can trigger a loss of cyt pathway, which then initiates a signal pathway to induce AOX. Under such conditions, high levels of AOX maintain high rates of respiration until the cyt pathway recovers. Conversely, antisense cells lacking AOX exhibit PCD in response to the loss of cyt pathway. Hence, coordinate regulation of the ETC pathways may be important, not only to satisfy metabolic demands, but also to modulate the initiation of a PCD pathway responsive to mitochondrial respiratory status.

vone and the two protein phosphatase inhibitors (cantharidin and endothall) were the most effective at blocking the induction of AOX capacity (Fig. 9). These compounds did so by effectively blocking AA-induced increases in *Aox1* mRNA and AOX protein (Figs. 9 and 10). Significantly, these same compounds were also the most effective at blocking the Cys-induced signal pathway responsible for downregulation of the cyt pathway (Figs. 4–6). Physiological data suggest that the cyt and AOX pathways are regulated in a coordinate manner (Elthon et al., 1989a; Vanlerberghe and McIntosh, 1992, 1994). Our data here suggest that such coordinate regulation is achieved by having common elements involved in the signal pathways required for both downregulation of the cyt pathway and induction of AOX (Fig. 13).

The ability of cyt pathway inhibitors (particularly AA) to induce AOX expression has been demonstrated in a number of plant species, as well as other organisms (Vanlerberghe and McIntosh, 1997). In the yeast *H. anomala*, AOX induction by AA was suppressed by low-oxygen conditions (Yoshimoto et al., 1989) or by the addition of scavengers of ROS, including flavone (Minagawa et al., 1992). We also found that flavone blocked AOX induction (Fig. 9). These results are consistent with the hypothesis that a ROS (possibly generated by the ETC itself as a direct consequence of cyt pathway inhibition) acts as

a signal to increase AOX expression (Fig. 13). That a ROS might represent an important signal for AOX expression is also supported by the observation that an exogenous treatment of plant or fungal cells with H_2O_2 can increase AOX expression (Vanlerberghe and McIntosh, 1996; Yukioka et al., 1998). Such a mechanism to regulate AOX expression is also consistent with the proposed function of AOX to dampen the mitochondrial generation of ROS (Maxwell et al., 1999; Parsons et al., 1999; Yip and Vanlerberghe, 2001). Under respiratory conditions in which ROS generation became excessive due to an overreduction of ETC components, the induced expression of AOX in response to the elevated level of ROS would act to relieve such over-reduction and lower the rate of ROS generation.

The best understood example of communication from mitochondrion to nucleus is the so-called retrograde regulation in the yeast *Saccharomyces cerevisiae* (Poyton and McEwen, 1996). In this case, mitochondrial dysfunction, due to either genetic or chemical inhibition, signals the expression of specific nuclear genes, the products of which modify metabolism to accommodate for the dysfunction. There is evidence that retrograde communication in response to mitochondrial dysfunction is also operative in other organisms (Amuthan et al., 2001; Borghouts et al., 2001). The ability of ETC inhibitors such as AA to induce AOX gene expression thus may represent a bona fide example of retrograde communication. However, it should be kept in mind that large changes in AOX expression, at least in plants, also occurs in the absence of any obvious mitochondrial dysfunction, indicating that the regulation of expression is more complex (Vanlerberghe and Ordog, 2002). It will be of interest to determine whether the signal events identified here as important to induce AOX in response to mitochondrial dysfunction (loss of cyt pathway) are also important for the induction of AOX under other conditions that do not inhibit the cyt pathway (such as citrate induction; Vanlerberghe and McIntosh, 1996).

Mitochondrial Electron Transport and a Plant PCD Pathway Responsive to Mitochondrial Respiratory Status

The mitochondrion plays an active role in many PCD pathways in animals (Green and Reed, 1998) and may also play a role in some plant PCD pathways although the evidence for this is only beginning to emerge (Jones, 2000; Lam et al., 2001). Here, we find that a loss of cyt pathway capacity in wt cells (induced by either Cys or AA) is accompanied by a strong induction of AOX capacity, which maintains respiration and cell viability (Figs. 1–3). Alternatively, a loss of cyt pathway in AS8 cells (induced by either Cys or AA) results in a complete loss of respiratory capacity (because these cells cannot induce AOX) and

results in massive cell death (Figs. 1–3). Results indicate that death of the AS8 cells is a programmed rather than necrotic event. Necrotic cell death is usually rapid and characterized by nonspecific breakdown of DNA, resulting in a smear of DNA fragments on an agarose gel. We could readily generate such death by exposure of cells to a very high concentration of H_2O_2 (Fig. 11). PCD is often delayed and characterized by an orderly cleavage of DNA resulting in the accumulation of oligonucleosomal fragments of predictable size that can be readily visualized as a "ladder" on agarose gels (Ryerson and Heath, 1996). This ladder was readily visible in AS8 cells after a loss of all respiratory capacity (Fig. 11).

All of the differential effects of Cys on the growth, viability, and respiratory characteristics of wt versus AS8 cells could also be generated by AA, the well-known chemical inhibitor of the cyt pathway (Figs. 7 and 8). Also, although the cyt pathway could be effectively inhibited by 1 mM Cys, resulting in the death of AS8 cells, wt cell viability was unaffected by Cys concentrations up to 5 mM. These data provide strong evidence that the cyt pathway was the critical (if not exclusive) target of Cys action in our experiments and that Cys had no other particularly adverse effect on the cells.

Based on our results with wt and AS8 cells, we suggest that a loss of cyt pathway downstream of ubiquinone can activate a PCD pathway, but that this activation can be strongly attenuated by the presence and/or induction of AOX. Such a model (Fig. 13) places AOX as an important modulator of PCD pathways, at least pathways in which a loss of cyt pathway capacity is a critical event. To our knowledge, no other study in plants has yet shown that a loss of cyt pathway capacity is necessarily a critical event inducing a PCD pathway. Nonetheless, PCD in plants can be associated with release of cyt c from the mitochondrion, a process that would reduce cyt pathway capacity (Balk and Leaver, 2001). Further, it has been shown that AOX is transiently induced during the hypersensitive response (Lacomme and Roby, 1999) and that higher levels of AOX protein after an anoxic treatment of soybean (*Glycine max*) cells correlated with increased resistance to H_2O_2-induced PCD (Amor et al., 2000). Although none of these studies provide definitive evidence for or against a model in which AOX acts to attenuate cell death, these studies are certainly suggestive that mitochondrial ETC components may play some role in PCD.

Many biotic and abiotic stress conditions have been shown to negatively impact the cyt pathway and induce AOX (Vanlerberghe and McIntosh, 1997; Vanlerberghe and Ordog, 2002). We suggest that, in some instances, such induction of AOX may represent an important mechanism to prevent activation of a PCD pathway responsive to mitochondrial respiratory status. Further, our results with Cys indicate that the plant cell maintains a signal pathway(s) able to actively regulate cyt pathway capacity, and that the fate of such a cell (survival or PCD) is dependent upon the level of AOX. Hence, in some cases, regulation of AOX gene expression (or activity?) may represent an important mechanism by which the cell could actively modulate entry into a PCD pathway, such as during development (Fig. 13).

One can hypothesize that the ability of AOX to attenuate cell death might relate to its ability to generate ATP (albeit inefficiently in terms of carbon consumption) or might relate to its ability to dampen the excessive mitochondrial generation of ROS by preventing over-reduction of ETC components (Maxwell et al., 1999; Parsons et al., 1999; Yip and Vanlerberghe, 2001). This latter function might be critical given that ROS are widely implicated in most PCD pathways in plants and animals (Jabs, 1999).

MATERIALS AND METHODS

Plant Material and Growth Conditions

The suspension cells used were derived from leaves of wt or transgenic tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) and were in culture for approximately 7 years before this study (Vanlerberghe et al., 1994). The transgenic cells (AS8) constitutively express an antisense construct of the nuclear gene *Aox1*, encoding a tobacco AOX. Hence, the normal expression of AOX in these cells is severely impaired.

Cell cultures (200-mL culture in 500-mL Erlenmeyer flask) were grown in the dark on a rotary shaker (140 rpm) at 28°C and were subcultured every 7 d by dilution in fresh growth medium. The growth medium (Linsmaier and Skoog, 1965) contains 3% (w/v) Suc as carbon source. Unless stated otherwise, experiments were always initiated using cells at 3 d after subculture and each individual experimental treatment was carried out with a separately grown 200-mL culture of cells. Each of these parameters was found to be critical because both culture age and any manipulation of culture volume during an experiment were found to impact the respiratory characteristics of the cells.

Chemicals

All experimental compounds to be added to cell cultures (*N*-acetyl-Cys, AA, cantharidin, catalase, chloramphenicol, cycloheximide, Cys, cystine, dithiothreitol, endothall, flavone, genistein, reduced glutathione, LaCl, Met, ruthenium red, staurosporine, and superoxide dismutase) were from Sigma-Aldrich Canada (Oakville, ON). Stock solutions were made fresh the day of use and filter sterilized when required. In cases in which the stock solution was made up in a solvent other than water, control experiments showed that addition of the solvent alone to cells (to a concentration which never exceeded 0.1% [v/v]) had no effect on any of the experimental parameters being measured (data not shown).

Culture Growth and Viability

To evaluate growth, an aliquot of the cell culture was washed twice with water, frozen, and lyophilized to determine cell dry weight. Cell viability was determined by microscopic observation of cells treated with Evans blue, which accumulates in dead cells as a blue protoplasmic stain (Baker and Mock, 1994). Typically, 500 to 750 cells were scored to establish viability of a culture.

Cell Respiratory Characteristics

Suspension cells (adjusted to 1–2.5 mg dry weight mL^{-1} in their culture medium) were placed in a Clark-type oxygen electrode cuvette (Hansatech,

King's Lynn, UK) at 28°C. Respiration rate refers to the control rate of O_2 uptake in the absence of any additions. Once a steady respiration rate was established (after 1–3 min), additions were made to the cuvette to measure the capacities of the cyt pathway and AOX. An uncoupler of oxidative phosphorylation (1 μM p-trifluoromethoxycarbonyl-cyanide) and inhibitors of cyt oxidase (1 mM KCN) and AOX (20 μM n-propyl gallate) were used. The cyt pathway capacity is defined as O_2 uptake in the presence of p-trifluoromethoxycarbonyl-cyanide and n-propyl gallate that was sensitive to KCN. The AOX capacity is defined as the O_2 uptake in the presence of KCN that was sensitive to n-propyl gallate. Residual respiration (O_2 uptake by cells in the presence of KCN and n-propyl gallate) was always low, ranging in most cases from 3% to 10% of the respiration rate. This residual rate has not been subtracted from respiration rates being reported here. The O_2 concentration in air-saturated water at 28°C was assumed to be 253 μM and dry weight was determined as described above.

Isolation and Assay of Mitochondria

Washed mitochondria were isolated from suspension cells (4 × 200 mL of culture) as previously described (Vanlerberghe and McIntosh, 1992). Immediately after isolation, O_2 uptake by mitochondria (0.2–0.5 mg protein mL^{-1}) was measured in an oxygen electrode cuvette (as described above) at 28°C in a reaction medium containing 10 mM N-Tris (pH 7.2), 0.25 M Suc, 5 mM KH$_2$PO$_4$, 2 mM MgSO$_4$, 0.1% (w/v) bovine serum albumin, and 0.1 mM each of NAD, NADP, ATP, and thiamine pyrophosphate.

Electron transport capacities were measured in the presence of a combination of substrates consisting of 2 mM ADP, 2 mM NADH, 10 mM succinate, 10 mM malate, and 10 mM Glu. In all cases, 1 mM pyruvate and 10 mM dithiothreitol were also present to ensure activation of AOX (Vanlerberghe et al., 1998). Under these assay conditions, cyt capacity is defined as the O_2 uptake that was sensitive to 16 μM myxothiazol in the presence of 100 μM n-propyl gallate. AOX capacity is defined as the O_2 uptake that was sensitive to 100 μM n-propyl gallate in the presence of 16 μM myxothiazol. Stock solutions of pyruvate, NADH, and dithiothreitol were made fresh the day of use.

Protein Analysis of Mitochondria

Reducing SDS-PAGE and immunoblot analysis of protein from isolated mitochondria was performed as previously described (Vanlerberghe et al., 1998). Monoclonal antibodies recognizing AOX (Elthon et al., 1989b), cox II (a gift from Dr. Alex Tzagaloff, Columbia University, New York), and cyt c (Pharmingen Canada, Mississauga, ON) were used. Antibodies were used at the following dilutions: AOX, 1:200 (v/v), cox II, 1:500 (v/v); and cyt c, 1:500 (v/v). Each antibody recognized a single prominent band of the expected size. A prestained broad range protein marker (New England Biolabs, Mississauga, ON) was used to estimate apparent molecular weights.

DNA and RNA Isolation and Analysis

Genomic DNA was isolated according to the method described by Mettler (1987) and was treated with RNase A. DNA was quantified using the agarose plate gel method (Sambrook and Russell, 2001). Then, 4 μg of DNA was separated on a 2% (w/v) agarose gel containing ethidium bromide, visualized on a UV transilluminator, and photographed. A 100-bp DNA ladder (New England Biolabs) was also run on the gel.

RNA was isolated by a miniprep procedure (Verwoerd et al., 1989), separated on agarose gels containing formaldehyde, transferred to membrane (Hybond-XL, Amersham-Pharmacia Biotech, Piscataway, NJ) using a vacuum blotting pump (Amersham-Pharmacia Biotech), and UV cross-linked. Northern analysis was then done as previously described, using a radiolabeled Aox1 cDNA as hybridization probe (Vanlerberghe and McIntosh, 1994).

Other Methods

Protein concentration was determined by a modified Lowry method (Larson et al., 1986). Statistical analyses were performed using Prism 3 (GraphPad Software, San Diego).

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