Functional analysis reveals effects of tobacco alternative oxidase gene (NtAOX1a) on regulation of defence responses against abiotic and biotic stresses

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Synopsis

Mitochondrial AOX (alternative oxidase) is the terminal oxidase of the CN (cyanide)-resistant alternative respiratory pathway in plants. To investigate the role of the tobacco AOX gene (NtAOX1a) under deleterious conditions which could induce ROS (reactive oxygen species) accumulation, we generated and characterized a number of independent transgenic tobacco (N. tabacum) lines with altered NtAOX1a gene expression and AP (alternative pathway) capacity. AOX efficiently inhibited the production of low-temperature-induced H_2O_2 and might be a major enzyme for scavenging H_2O_2 at low temperature. Furthermore, NtAOX1a may act as a regulator of KCN-induced resistance to TMV (tobacco mosaic virus) through the regulation of H_2O_2. Notably, a moderate accumulation of H_2O_2 under the control of NtAOX1a was crucial in viral resistance. Analysis of seed germination indicated an important role for NtAOX1a in germination under H_2O_2-induced oxidative stress when the CP (cytochrome pathway) was inhibited. These results demonstrate that NtAOX1a is necessary for plants to survive low temperature, pathogen attack and oxidative stress by scavenging ROS under these adverse conditions when the CP is restricted.

Key words: alternative oxidase, low temperature, Nicotiana tabacum AOX1a (NtAOX1a), reactive oxygen species (ROS), transgenic plant, viral resistance

INTRODUCTION

The mitochondrial respiratory chain in higher plants consists of the CN (cyanide)-sensitive CP (cytochrome pathway) and the CN-resistant AP (alternative pathway) [1]. AOX (alternative oxidase) is the terminal oxidase of the AP and is encoded by a small family of nuclear genes [2]. As a part of the electron transport chain, AOX can catalyse oxygen-dependent oxidation of ubiquinol [3]. The expression of AOX genes is induced in response to diverse biotic and abiotic stresses [4]. It has been suggested that AOX plays a crucial role in maintaining homoeostasis under varying growth conditions [3] and in protecting plants against the lethal effects of ROS (reactive oxygen species) [5,6].

All organisms produce a range of ROS and mitochondria are a major source of ROS in eukaryotic cells. It is known that ROS play a dual role depending on their accumulation levels. High intracellular concentration of ROS leads to extensive cell injury or death. A moderate accumulation of ROS functions as a key inducer for secondary programmed metabolism, defence signals and activation of MAPKs (mitogen-activated protein kinases), leading to environmental stress tolerance [7]. Several biotic and abiotic stresses increase ROS production in various tissues, and frequently result in a concomitant increase in AOX expression in higher plants [4]. The function of AOX in preventing ROS production has been shown in tobacco cells in culture [5] and isolated mitochondria [8]. Also, in transgenic Arabidopsis plants with antisense AOX cDNA, there is an increase in the level of ROS when the CP is inhibited by KCN treatment [9].

AOX is thought to contribute to the acclimation of respiration to low temperature [10]. The enhancement of the AP capacity in higher plants by exposure to low temperature is partly due to

Abbreviations used: AS1 etc., antisense line 1 etc.; AOX, alternative oxidase; AP, alternative pathway; CAT, catalase; CP, cytochrome pathway; CN, cyanide; DAB, diaminobenzidine; E1 etc., empty vector line 1 etc.; HR, hypersensitive response; HR9, horseradish peroxidase; HSD, manganese superoxide dismutase; MS medium, Murashige and Skoog medium; Nt, Nicotiana tabacum; ROS, reactive oxygen species; S2 etc., sense line 2 etc.; SA, salicylic acid; SHAM, salicylhydroxamic acid; TMV, tobacco mosaic virus.

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enhanced expression of the AOX genes at the transcriptional level [11]. Exposure of plants to low temperature impaired electron flow through the CP and caused an increase in H₂O₂ production [12]. A down-regulation of the CP is accompanied by an increase in AOX capacity [13].

AOX has been implicated in plant defence pathway against viruses. SA (salicylic acid) induces increased viral resistance and AOX expression [14,15]. It has been reported that SA-induced TMV (tobacco mosaic virus) resistance resulted from the activation of multiple mechanisms, a subset of which are inducible by AA (antimycin A) and influenced by AOX [16]. TMV vector-driven transient high-level expression of AOX enhanced virus spread and symptoms of infection in plants [17]. Although some results suggested that AOX was not a critical component of plant viral resistance, it might play a role in the HR (hypersensitive response) [18]. In spite of these previous studies showing that AOX is involved in viral resistance, to date, the underlying mechanism of the involvement of AOX in signalling during pathogen resistance has not been fully elucidated.

Germination of plant seeds after imbibition is a dynamic process requiring the activation of a number of metabolic enzymes, which is accompanied by a rapid increase in oxygen consumption through mitochondrial respiration. It has been reported recently that the expression profiles of respiratory components are associated with mitochondrial biogenesis during germination and early seedling growth in wheat [19]. Furthermore, AOX can support embryo germination and early seedling growth in conjunction with Complex I in wheat when the CP is restricted [19].

To elucidate the relationship of AOX, ROS and adverse stresses, we generated and characterized a series of transgenic tobacco lines with altered AOX expression profiles [14,15]. Mitochondrial biogenesis during germination and early seedling growth in wheat [19]. Furthermore, AOX can support embryo germination and early seedling growth in conjunction with Complex I in wheat when the CP is restricted [19].

To elucidate the relationship of AOX, ROS and adverse stresses, we generated and characterized a series of transgenic tobacco lines with altered NtAOX1α (where Nt is Nicotiana tabacum) expression. The results of the present study indicate that NtAOX1α plays a crucial role in protecting plants against a variety of abiotic and biotic stresses which lead to ROS accumulation.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Seeds from the tobacco cultivar Samsun (NN genotype, TMV resistant) and from Samsun (NN genotype) transgenic lines were germinated in soil and maintained under the following growth conditions: 16 h light/8 h dark photoperiod (light intensity of approx. 200 μmol·m⁻²·s⁻¹), constant air temperature of 26°C and relative humidity of 60–75%.

**Vector construction and plant transformation**

The NtAOX1α coding sequence was obtained from NN-type Samsun tobacco by RT-PCR (reverse transcription-PCR) using primers based on the published AOX1α sequence from Bright Yellow tobacco [20].

The cDNA was inserted either in sense or antisense orientation under the control of CaMV35S (35S cauliflower mosaic virus promoter) in the expression cassette of pBI121. An empty transformation vector control was also generated. The expression cassettes were introduced into Agrobacterium tumefaciens (strain LBA4404) and then used for tobacco leaf disc transformation [21]. Primary transformants were selected on kanamycin (100 mg/l) plates before they were transferred into soil.

**Northern blot analysis**

NtAOX1α transcript levels were assessed by Northern blot analysis using methods previously described [22]. These experiments were repeated twice using independent samples and representative results are shown.

**Mitochondrial isolation**

Mitochondria used for Western blot analysis were isolated from 50 g (fresh weight) of tobacco leaf tissue. After harvest, the leaves were homogenized using a mortar and pestle in 120 ml of grinding medium [20 mM Hapes/Tris (pH 7.6), 0.4 M sucrose, 5 mM EDTA, 0.6% (w/v) insoluble PVPP (polyvinylpyrrolidone), 0.3% (w/v) BSA and 25 mM potassium metabisulfite]. The subsequent material was then filtered through eight layers of gauze. The method was performed according to standard protocols described previously [23].

**Western blot analysis**

The proteins isolated from mitochondria of wild-type and transgenic plants were separated by SDS/PAGE according to the method of Laemmli [24], and were subsequently electrotransferred on to a PVDF membrane. Immunoblot analysis was performed as previously described [25]. A monoclonal antibody against AOX was developed previously in our laboratory and used at 1:100 (v/v) dilution. The anti-AOX antibody was detected using a goat anti-rat IgG (Dingguo, Beijing, China) conjugated to HRP (horseradish peroxidase) and was used at 1:5000 (v/v) dilution. The binding was visualized using a chemiluminescent HRP substrate.

**Measurement of respiratory capacity**

The respiration capacity was measured by obtaining cells (with cell walls still intact) from leaf strips using 0.5% (w/v) macerase (Macerzyme R-10; Yakult, Tokyo, Japan) in 0.7 M mannitol at 26°C, using the method developed by Gilliland et al. [16] and authenticated as valid by Pasqualini et al. [23]. This cell production procedure was based on the first step of a published protoplast purification method [26]. Cells were added to the reaction medium containing 50 mM KH₂PO₄ (pH 7.2), 0.4 M mannitol, 10 mM KCl, 10 mM MgCl₂ and 0.1% (w/v) BSA [FA (fatty acid)-free]. This mixture was then transferred to an oxygen electrode (Oxytherm; Hansatech, King’s Lynn, U.K.). Measurements of oxygen consumption were performed in the absence or presence of 2 mM KCN, 2 mM SHAM (salicylhydroxamic acid, an AOX inhibitor), 1 μM FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone, an uncoupler) or in the presence of both types of inhibitors. As residual respiration (oxygen uptake in the presence of KCN and SHAM) was often
not detectable, it was assumed to be equal to zero. Measurements were carried out in the dark to prevent photosynthetic oxygen production. For each cell line examined, at least five sets of measurements were carried out to obtain statistically valid comparisons of AP capacity between wild-type and transgenic lines.

**TMV inoculation of plants pre-treated with or without KCN**
Non-transgenic and transgenic plants (8-weeks-old) were used for experiments. The foliage was sprayed with 2 mM KCN or water daily for 2 days before inoculation with TMV (strain U1). TMV (2 μg/ml in water) was inoculated on to one lower leaf per plant using a cotton bud soaked in the virus suspension. The leaves destined for inoculation with TMV were sprinkled with carborundum before application of the virus suspension to enhance the efficiency of infection.

**Histochemical detection of H2O2**
H2O2 was visually detected in the leaves of plants by using DAB (diaminobenzidine) [27]. After treatment, leaves were sampled from plants and infiltrated with 1 mg/ml DAB (Sigma) solution (pH 3.8) for 6 h under light at 25°C and then treated with 95% ethanol to remove chlorophyll. This treatment decolorized the leaves except for the deep brown polymerization product produced by the reaction of DAB with H2O2.

**Measurement of H2O2 and antioxidative indices**
H2O2 was extracted from 1 g of leaf ground in 5 ml of acetone. Enzymes were extracted using 50 mM phosphate buffer (pH 7.8). All of these indices were measured with kits produced by the Nanjing Jiancheng Bioengineering Institute (Nanjing City, People’s Republic of China).

**RESULTS**

**Generation and identification of transgenic lines**
A number of independent transgenic tobacco lines harbouring the NtAOX1a cDNA either in a sense or antisense orientation as well as the cell line expressing the empty vector were produced.

Primary transformants (T0) were isolated on MS medium (Murashige and Skoog medium) containing kanamycin. Seeds of eight T0 plants, including three sense lines [S2 (sense line 2), S3 and S6], three antisense lines [AS1 (antisense line 1), AS4 and AS5] and two empty vector control lines [E1 (empty vector line 1) and E7], were chosen for germination. T-DNA (transfer DNA) inheritance was scored by kanamycin segregation analysis in the T1 generation. Resistance of T1 seedlings was segregated as a single dominant gene, and results are shown in Table 1. NtAOX1a transcript accumulation of T1 lines was analysed using Northern blotting (Figure 1). Compared with wild-type plants, the expression levels of NtAOX1a were significantly up-regulated in S2, S3 and S6, especially in S2 and S6. In contrast with a slight reduction in AS4, the transcript accumulation in AS1 and AS5 was reduced to an almost invisible level. Moreover, NtAOX1a expression in E7 was similar to that in wild-type and much higher than that in E1. Taken together, we selected S2, S6, AS1, AS5 and E7 for further analysis, because they displayed representative phenotypes of their transgenic lines. T2 plants were also selected by kanamycin segregation. Similarly, homozygous T3 individuals were used for further characterization.

**Characterization of T3 lines**
Analysis of NtAOX1a gene expression and protein accumulation was carried out by Northern and Western blotting respectively. The five T3 plants mentioned above were used, including E7, S2,
S6, AS1 and AS5. It was observed that high levels of *NtAOX1a* transcript and protein accumulated in the two sense lines and low levels of *NtAOX1a* were observed in the two antisense lines (Figures 2A and 2B), which is consistent with that in T1 plants. Accordingly, *NtAOX1a* expression was inherited stably from the T1 to the T3 generation.

Since AP capacity is an indicator of the potential activity of AOX [28], the respiratory characteristics of T1 plants were examined to determine whether transformation with *NtAOX1a* cDNA constructs affected their AP capacities. As expected, AP capacity was enhanced in the sense lines and reduced in the antisense lines (Figure 2C). In addition, we noticed that there were no significant differences in *NtAOX1a* expression and AP capacity between the two sense lines (S2 and S6), as well as the two antisense lines (AS1 and AS5), so we only selected S6, AS5 and E7 for further analysis.

**Effects of low temperature on H$_2$O$_2$ accumulation and antioxidant enzymes**

To analyse the effects of low temperature on transgenic and wild-type plants, tobacco seedlings were grown under normal conditions for 4 weeks and then transferred to low temperature conditions (12°C) for 24 h. Proteins were isolated from leaf mitochondria at the end of treatment (24 h). Western blot analysis indicated that NtAOX protein was induced by low temperature treatment in all lines except AS5 (Figure 3).

Exposure to low temperature always impairs electron flow through the CP and results in an increase in H$_2$O$_2$ production [12]. Thus we examined H$_2$O$_2$ accumulation in wild-type and transgenic plants under this condition. Leaves were collected from plants kept at 12°C for 0, 2, 6, 12 and 24 h. The DAB staining assay showed the appearance of H$_2$O$_2$ at different levels in the leaves of four genotypes at low temperature (Figure 4A). Specifically, the level of H$_2$O$_2$ in S6 was significantly lower than that in wild-type, E7 and AS5 from 2 to 24 h. AS5 contained the highest level of H$_2$O$_2$ at all time points except for 0 h, suggesting that the *NtAOX1a* overexpression efficiently inhibited low-temperature-induced ROS production (mainly H$_2$O$_2$). For an accurate quantification of the H$_2$O$_2$ level across the treatment times, H$_2$O$_2$ levels were measured. The result was in accordance with that from the DAB coloration assay (Figure 4B).

Since cellular ROS level is dependent upon both the rate at which ROS are generated by various cellular processes and the rate at which ROS are scavenged by the various antioxidant defences [29], we assumed that expression of the antioxidant enzymes might change in the leaves kept at 12°C treatment, and thus examined the activity of MSD (manganese superoxide dismutase) and CAT (catalase) during this period. MSD is known to be localized to mitochondria. CAT is targeted to the peroxisome or mitochondria. Strikingly, both the MSD and CAT activities were decreased in all genotypes under low temperature conditions, but they were at the highest level in AS5 throughout (Figure 5), accompanied by the highest H$_2$O$_2$ accumulation. Thus the higher activity of the antioxidant enzymes in AS5 did not effectively reduce the ROS formation caused by low temperature.

**Analysis of viral resistance responses in transgenic plants**

To analyse viral resistance responses in wild-type and transgenic tobaccos, 8-week-old plants were used for inoculation with
Figure 4 Analysis of H$_2$O$_2$ accumulation in wild-type and transgenic plants at low temperature

(A) DAB coloration assay of H$_2$O$_2$ accumulation. Plants (4-weeks-old) were treated at 12°C for 0, 2, 6, 12 and 24 h respectively. Leaves were sampled and infiltrated in 1 mg/ml DAB solution (pH 3.8) for 6 h, and then treated with 95% (v/v) ethanol for chlorophyll removal. (B) Measurement of H$_2$O$_2$ accumulation. H$_2$O$_2$ was extracted from 1 g of low-temperature-treated leaves in acetone and then measured using a measuring kit as stated in the Materials and methods section (Nanjing Jiancheng Bioengineering Institute). Results are means ± S.E.M. (n = 6) from four independent experiments, and data points with the same letter are not significantly different, as determined by the Tukey test (P < 0.05). fw, fresh weight; WT, wild-type.

Figure 5 Changes of antioxidant enzyme activities in the leaves of wild-type and transgenic plants at low temperature

Frozen leaf segments were homogenized in potassium phosphate buffer. The homogenate was centrifuged at 12 000 g for 20 min at 4°C and the supernatant was collected for the enzyme assays. The activities of MSD and CAT were measured using a measuring kit as stated in the Materials and methods section (Nanjing Jiancheng Bioengineering Institute). (A) Activity of MSD. (B) Activity of CAT. For each measurement, there are four replicate leaf samples. (A,B) Results are means ± S.E.M. (n = 6) from five independent experiments, and data points with the same letter are not significantly different, as determined by the Tukey test (P < 0.05). U, unit; WT, wild-type.

TMV. HR was expected to be induced in the NN-type Samsun tobacco.

Initial experiments were carried out to examine the effect of AOX expression on resistance responses under normal conditions. It was observed that all S6, wild-type, E7 and AS5 plants generated well-defined circular HR lesions approx. 3 days after TMV inoculation, but no difference was observed between plant lines in the number and size of lesions produced.

Resistance of plants to TMV can be activated using CN [15], and thus we investigated KCN-induced viral resistance in transgenic tobacco. Plants were sprayed with 2 mM KCN for 2 days and then inoculated with TMV. As a result, different HR lesions appeared in the four lines. Specifically, yellow haloes revealed the appearance of much more lesions in S6 than in wild-type or E7. Also, S6 lesions were significantly larger than the lesions observed on the other lines. Compared with the above three lines, AS5 displayed the least severe disease symptoms (Figure 6A). To quantify the lesions, we counted the number of lesions and measured their diameter at 7 days post-inoculation (Figures 6B and 6C). The results are consistent with that defined visually. In addition, all of the lines treated with KCN displayed smaller and fewer lesions in comparison with the corresponding untreated plants (Figures 6B and 6C), suggesting an enhanced resistance to TMV in response to KCN treatment.

Given the different HR lesions of the four lines and the function of moderate ROS accumulation as a defence signal, the H$_2$O$_2$ level in each KCN-treated line after TMV inoculation was measured (Figure 7). We observed that accumulation of H$_2$O$_2$ in AS5 was significantly higher than in wild-type and E7. S6 contained the lowest level of H$_2$O$_2$. This may account for the different HR...
lesions observed in the four lines. When plants were inoculated with TMV, rapid generation of H$_2$O$_2$ necessitated the activation of additional defences. Thus the lower concentration of H$_2$O$_2$ in S6 made it less capable of activating defence mechanisms than AS5. In addition, MSD and CAT activities were also examined in the four lines, but there was no notable difference (results not shown).

Germination of transgenic plant seeds under H$_2$O$_2$ stress

Germination is a critical period in the life cycle of a plant and can be affected by a number of stresses, including oxidative stress. To analyse \textit{NtAOX1a} function during seed germination under specific conditions, T$_3$ seeds were germinated and grown on MS medium supplemented with 20 mM H$_2$O$_2$. All of the genotypes showed delayed germination by 1 day. But the different seeds did not differ in their germination percentages, with all of them exhibiting $>90\%$ germination (Figure 8A). Considering the function of KCN in inhibiting the CP, we germinated the seeds on MS medium containing 20 mM H$_2$O$_2$ and 2 mM KCN. In response to the treatment, germination was further delayed. More importantly, the four genotypes exhibited distinct germination percentages. S6 germination did not plateau until 8 days and was reduced to 70\% germination relative to that on H$_2$O$_2$-containing MS medium. Subjecting AS5 seeds to the same treatment resulted in a $>70\%$ reduction in germination (Figure 8B). Meanwhile, as a negative control, the germination test was also performed under conditions using KCN only, with no notable difference observed among the four lines (results not shown). These results clearly demonstrate an important role for \textit{NtAOX1a} in germination under H$_2$O$_2$-induced oxidative stress when the CP is inhibited.
AOX expression is responsive to low temperature and has also been implicated in resistance to viruses. The issues of whether AOX affects ROS accumulation at low temperature and pathogen attack, and whether there exist additional functions for AOX, have yet to be determined. In the present study, we used transgenic plants with altered *NtAOX1a* expression to further verify the possible correlation of AOX and ROS under these conditions and the potential role for AOX in seed germination.

A lower growth temperature increases AP capacity and AOX protein in tobacco [30]. Also, low temperature treatment impairs AOX function in seed germination. Furthermore, ROS have been suggested to participate in the plant defence system in a variety of ways, including acting as signalling agents [32]. Increasing evidence suggests that the ROS network is essential to induce disease resistance [33]. Thus we hypothesize that ROS accumulation in mitochondria is most probably the resistance-inducing signal under the control of AOX. Measurement of H$_2$O$_2$ accumulation supports our hypothesis and provides us with new insight into the appearance of different HR lesions in the four KCN-treated lines. Although ROS are potentially harmful to cells, plants can use them as secondary messengers in signal transduction regulating defence mechanisms. When leaves are inoculated with TMV, rapid generation of H$_2$O$_2$ may be essential to activate pathogenesis-related proteins and to provide enough protection against the virus. In this process, AOX may function as a regulator of ROS-mediated signalling in the mitochondria and this potential AOX-regulated signalling mechanism appears to be involved in the activation of a subset of CN-inducible antiviral defence. Therefore, it may be premature to conclude, as Ordog et al. [18] have, that AOX does not play a role in the induction of viral resistance. However, the level of AOX did not influence TMV infection under non-KCN conditions. A possible explanation for this result is that, under this condition, the electron did not flow to AOX, and thus the accumulation of the signalling agent H$_2$O$_2$ may be mainly affected by some other factors rather than AOX expression, which results in almost identical HR lesions being produced in the four lines. Further investigation is of great importance to uncover a more comprehensive picture of the functional roles and action mechanism of AOX in viral resistance.

AOX function in seed germination was determined by subjecting the seeds of different genotypes to oxidative stress in the presence or absence of KCN. The obvious difference in germination profiles among the four lines was observed only with medium supplemented with both H$_2$O$_2$ and KCN (Figure 8). However, in this situation, it is also possible that application of KCN, not
AOX, leads to a different respiratory rate, which determines the final differences in germination profiles. So we performed a negative control, a germination test using KCN only. But we found no difference among the four lines and eliminated the possibility. Hence the up-regulation of \(NaAOX1a\) expression in S6 did contribute to the enhancement of oxidative stress tolerance in seed germination.

In conclusion, the present study supports the idea that AOX plays a role in regulating defence responses against abiotic and biotic stresses by preventing the production of ROS when the CP is restricted.

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