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研 究 论 文

不同低温胁迫对烟草愈伤组织抗氰交替途径诱导和交替氧化酶表达影响的比较①

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摘要　比较了不同低温（14℃和4℃）胁迫对烟草（*Nicotiana rustica* L.）愈伤组织抗氰交替途径诱导和交替氧化酶表达的影响。结果显示，不同低温胁迫处理能显著诱导烟草愈伤组织交替途径容量和实际运行的增加，且都呈现出基本相同的变化模式：在胁迫的初期（1～3 d）持续增加，在3 d时达到最高，而后下降到一个相对恒定的水平。但交替途径容量增加的幅度与温度下降的程度密切相关，而交替途径实际运行量的诱导程度在不同低温胁迫下的差异却很小。表明交替途径容量和实际运行对低温胁迫的响应是不同的。免疫印迹分析结果表明：低温胁迫明显诱导了交替氧化酶总蛋白的增加，且其随低温胁迫进程的变化与交替途径容量的变化基本一致；而对交替氧化酶单体与二聚体在低温胁迫下的含量变化检测结果则显示，烟草愈伤组织中交替氧化酶主要以二聚体形式存在，且这一存在形式并不随低温胁迫程度的加深而发生改变。两种形式的交替氧化酶蛋白含量都能被低温胁迫诱导增加，但其单体水平在两种不同的低温胁迫下并无明显差别，而4℃低温胁迫诱导的二聚体交替氧化酶蛋白含量明显高于14℃。表明不同程度低温对抗氰交替途径发生的不同影响主要是由于对交替氧化酶蛋白二聚体形式的不同诱导程度所致；而高活性的交替氧化酶单体形式则不因低温胁迫程度的加重而被明显诱导升高，使得抗氰交替途径的运行程度在两种不同的低温胁迫处理条件下无显著差异。

关键词　烟草愈伤组织，低温胁迫，抗氰交替途径，交替氧化酶表达

Comparison of the Effects of Different Low Temperature Stresses on the Induction of the Cyanide-Resistant Alternative Pathway and the Expression of Alternative Oxidase in Tobacco Callus

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Abstract　Effects of different low temperature stresses (14℃ and 4℃) on cyanide-resistant alternative pathway development and operation, as well as on alternative oxidase expression in plant calli of tobacco (*Nicotiana rustica* L.) were compared. The results showed that both alternative pathway

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capacity (V_{alt}) and its actual operation activity (rV_{alt}) were dramatically induced under the two different low temperature stresses, and the change patterns of V_{alt} and rV_{alt} were similar: both of them were induced at the early stage of stress treatment, the highest was found on 3 d and then declined to a relatively constant level. The increase of V_{alt} was in relation to the temperature decreased, while no significant difference of rV_{alt} induced between 14℃ and 4℃. This suggests that responses of V_{alt} and rV_{alt} to different temperature stress are different. The immunoblot results indicated that low temperature stress remarkably induced the content of total alternative oxidase (AOX) protein, and the changing mode throughout the stress was similar to V_{alt}. The results of variation in AOX monomer and homodimer contents under stress treatment showed that AOX is largely located in mitochondria membrane of tobacco callus as the oxidized form (homodimer), and the existing state could not be changed by low temperature stress. The amount of monomer and homodimer both induced by low temperature stress, but no appreciable differences in the content of monomer was observed between two different low temperatures, and the level of homodimer induced under 4℃ was higher than that under 14℃. Our results suggest that, effects of low temperature on the development of cyanide-resistant alternative pathway are mainly due to the differently induced content of AOX homodimer under different low temperatures. The content of highly active AOX monomer form is not obviously induced by aggravating low temperature stress, thus the operation degree of cyanide-resistant alternative pathway shows no notable difference between two kinds of low temperatures tested.

Key words Tobacco callus, Low temperature stresses, Cyanide-resistant alternative pathway, Expression of AOX

The higher plant mitochondria electron transport chain contains, in addition to the cytochrome chain which terminates with cytochrome oxidase, an alternative pathway that terminates with an alternative oxidase(AOX)(Siedow and Umbach, 1995; Vanlerberghe and McIntosh, 1997). Because of the special heat-instability, AOX is very difficult to be eluted from the inner membrane of mitochondria and maintained the activity(Liang and Liang,1998). Until 1987, AOX was successfully purified from mitochondria of *Sauromatum guttatum* Schott (voodoo lily) and the polyclonal antibodies raised to AOX were prepared(Elthon and McIntosh,1987). Two years later, monoclonal antibodies against AOX were produced and designated as AOA, AOU and AOL(Elthon *et al.*,1989). With the polyclonal and monoclonal antibodies against AOX, the studies on cyanide-resistant respiration were carried out in-depth at molecular level to elucidate the mechanisms of its development and operation. In addition, the anti-synthetic polypeptide antibodies against AOX using synthetic polypeptide were prepared in our lab and used to study the relationship between cyanide-resistant respiration and the expression of AOX in mung bean(Li *et al.*, 2000) and to confirm its detecting effects on AOX expression in tobacco callus as compared with AOX monoclonal antibody(Yan *et al.*,2002).

The reported studies showed cyanide-resistant respiration exists in a great variety of plant species (Moore and Siedow, 1991), and its development and operation are, in addition related with the internal development and physiological status of the plant(Liang and Liang,1997), affected by many external

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factors such as environmental stresses those can influence the ability of cyanide-resistant respiration or change the degree of its engagement (Jolivet *et al.*, 1990; Rycher *et al.*, 1992; Zhang *et al.*, 2001). Low temperature is a general environmental stress, and its effects on cyanide-resistant respiration have been investigated with different plant tissues or organs(Stewart *et al.*, 1990a; 1990b; Vanlerberghe and McIntosh,1997; Meler *et al.*, 1999). The results indicated that V_{alt} is higher in plants grown at low temperature (10-15℃) compared to those controls grown at near optimal temperature (25-30℃), so is the AOX protein level. However, in most of these studies, only one kind low temperature was applied, and merely examined the response of alternative pathway respiration to short-term low temperature stress. Moreover, some studies showed AOX located in inner membrane of mitochondria with two forms: oxidized homodimer and reduced monomer(Umbach and Siedow, 1993), and the activity of monomer is 4-5 folds higher than that of homodimer(Moore *et al.*, 1995). However, only the total AOX protein content induced by low temperature was investigated in most of the previous studies, the relative changes of AOX homodimer and monomer were not concerned. We have found the cyanide-resistant respiration existed obviously in tobacco callus(Liang *et al.*, 1984), and was significantly enhanced when treated with 4℃ low temperature(Zhou *et al.*, 2000). In the present study, tobacco callus were used to measure the dynamic changes of the cyanide-resistant respiration throughout the low temperature stresses of 14℃ and 4℃. The changes of the total AOX content and the relative amount of AOX homodimer and monomer were detected in the stress course. Our objective is to better compare the effects of different low temperature stresses on cyanide-resistant respiration and the relationship between the ability of cyanide-resistant respiration and the expression of AOX under low temperature stresses.

1 Materials and Methods

1.1 Plant material and low temperature treatment

Tobacco (*Nicotiana rustica* L.) callus was dedifferentiated from seedling leaves on a basal Murashige and Skoog medium supplemented with 2, 4-D(2 mg·L^{-1}), 6-BA(0.5 mg·L^{-1}), 3%(W/V) sucrose and 0.8%(W/V) agar(pH 5.8). The calli were maintained in a growth chamber at 25℃ and illuminated with a 12 h photoperiod at light intensity of 100 μmol·m^{-2}·s^{-1}, and subcultured once a month. When grown to the 15-day stage, tobacco calli were treated with low temperatures of 14℃ or 4℃ under the same photoperiod and light intensity. Control samples were maintained at 25℃, and all samples were grown for 1, 3, 5 and 7 d under the above light and temperature conditions. At different stages of low temperature stress, the respiration parameters were determined and the mitochondria were isolated in stressed or non-stressed tobacco callus.

1.2 Determination of tobacco callus respiration

The respiratory rates of tobacco callus were measured and calculated according to the equations described by Liang and Lü (1984) with Clark-type oxygen electrode at 25℃ in a dark room. The respiratory parameters were determined by Bahr and Bonners' hydroxamic acid titration method(Bahr and Bonner,1973), the data were calculated with the equation modified by Theologis and Laties(1978):

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$$V_t = r V_{alt} + r' V_{cyt} + V_{res}$$

Where, V_t is the total respiratory rate, $r' V_{cyt}$ is the cytochrome-mediated respiration, V_{res} is the residual O_2 uptake which is not inhibited by KCN plus SHAM, and r presents the fraction of the alternative pathway which is operating, and V_{alt} is the maximal capacity of the alternative pathway. Accordingly, rV_{alt} represents the extent of operation activity of the alternative pathway in the absence of inhibitor. Optimal concentrations of inhibitors used with tobacco calluses were: KCN 1 mmol·L^{-1}; SHAM 1 mmol·L^{-1}. The respiration rate was calculated as μLO_2·g^{-1} FW·h^{-1}. The data presented were the means of at least four independent measurements.

1.3 Isolation and purification of tobacco callus mitochondria

Mitochondria were isolated and purified from tobacco callus suffered from different degree of low temperatures based on the procedure of previous study(Wen and Liang,1993). Purified mitochondria were resuspended in washing medium devoid of bovine serum albumin and stored at -20℃. Protein concentration was measured according to the method of Bradford (1976).

1.4 SDS-PAGE and immunoblotting analysis of mitochondrial protein

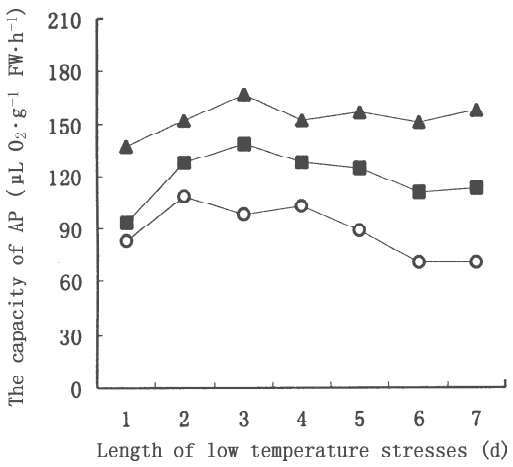
Mitochondria proteins were separated by reducing and nonreducing SDS-PAGE according to the methods described by Umbach and Siedow(1993) and Vanlerberghe *et al.* (1998). For reducing SDS-PAGE, mitochondria (50 μg of protein) were dissolved in equal volume sample buffer (10% [V/V] β mercaptoethanol, 20%[V/V] glycerol, 4%[W/V] SDS, 0.005%[W/V] bromophenol blue and 50 mmol·L^{-1} Tris, pH 6.8) and incubated in boiling water bath for 3 min. Nonreducing SDS-PAGE analysis was performed without β-mercaptoethanol in sample buffer. SDS-PAGE analysis was performed according to the standard method of Laemmli (1970). A 5% (W/V) polyacrylamide stacking gel and 13% polyacrylamide gradient resolving gel were used. The resolved proteins were then electrotransferred to a nitrocellulose membrane and probed with a 1∶100 dilution of a monoclonal antibody (AOA) raised against the *Sauromatum guttatum* AOX by the standard method of Sambrook *et al.* (1989). The secondary antibody was goat-anti-mouse IgG conjugated with alkaline phosphatase (diluted 1∶500). The color was developed by reaction with color buffer supplemented with ρ-nitro blue tetrazolium chloride and 5-bromo-4-chloro-indolyl phosphate.

2 Results

2.1 Effects of different low temperature stresses on capacity of alternative pathway in tobacco callus

The capacity of alternative pathway(V_{alt}) refers to its maximal engagement when cytochrome pathway (CP) is completely inhibited. It represents the maximal extent to which alternative pathway (AP) is engaged *in vivo*(Day *et al.*, 1995). AP operation is dependent upon the degree of its development (McIntosh, 1994). For this reason, we first examined the effects of low temperature (14℃ and 4℃) stresses on AP capacity. The results in Fig.1 indicate that, low temperature stress could dramatically induce the increase in V_{alt} during tissue recovering. However, the changing models of V_{alt} under different low temperatures were not so consistent. When the calli were treated with 4℃ for 1 d, V_{alt} gave rise to 137.1 μLO_2·g^{-1} FW·h^{-1} from 83.6 μLO_2·g^{-1} FW·h^{-1} in control. By contrast, V_{alt} at 14℃

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○. Control; ■. Treated with 14℃; ▲. Treated with 4℃

Fig.1 Effects of different low temperature stresses on capacity of AP in tobacco callus

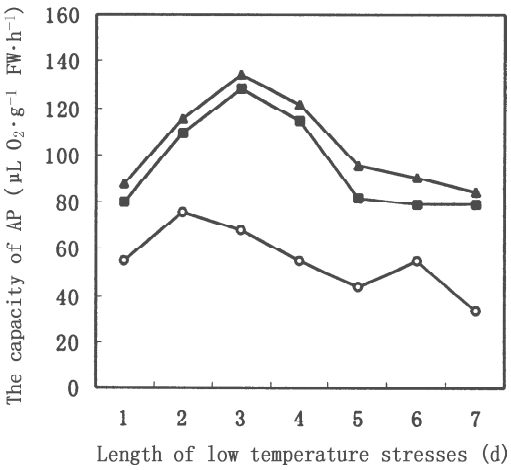
went up to 93.1 $\mu LO_2 \cdot g^{-1}$ FW·h^{-1}. It increased by 64.0% and 11.4% of control respectively. Following the time of stress, V_{alt} remained raising until it reached the highest level observed on 3 d of 166.4 $\mu LO_2 \cdot g^{-1}$ FW·h^{-1} under 4℃ and 137.8 $\mu LO_2 \cdot g^{-1}$ FW·h^{-1} under 14℃, increasing by 69.6% and 40.5%, respectively, in comparison to control. Thereafter, both of the V_{alt} under 14℃ and 4℃ declined slightly, and then kept on relatively stable level which is still significantly higher than the value of control.

2.2 Effects of different low temperature stresses on activity of AP in tobacco callus

The activity of AP (rV_{alt}) is the actual engagement when there is no inhibitor on cytochrome pathway, it represents the actual demanded extent of AP for plant tissue in specific physiological conditions(Day *et al.*, 1995). Though the operation of AP is dependent on its development, the developed alternative pathway respiration is not necessarily engaged totally. So, we also determined changes in rV_{alt} at different low temperatures. As shown in Fig. 2, there was a rapid increase in rV_{alt} in the first three days of low temperature treatment. On 3 d of the stress, rV_{alt} increased to 128.3 $\mu LO_2 \cdot g^{-1}$ FW·h^{-1} from 80.1 $\mu LO_2 \cdot g^{-1}$ FW·h^{-1} at 14℃ treatment and from 88.1 $\mu LO_2 \cdot g^{-1}$ FW·h^{-1} to 134.1 $\mu LO_2 \cdot g^{-1}$ FW·h^{-1} under 4℃, increasing by 60.2% and 52.2%, respectively. As compared with the control, rV_{alt} increased by 87.8% under 14℃ and 96.3% under 4℃ on 3 d of the low temperature stress. Unlike V_{alt}, rV_{alt} decreased soon after it reached its peak, and then maintained at about the same level of each. So, the induced pattern of rV_{alt} under different low temperatures was nearly the same, only for the extent induced under 4℃ is higher than that induced under 14℃.

2.3 Effects of different low temperature stresses on total AOX protein in tobacco callus

The changes in alternative pathway capacity in plant tissues were dependent upon the expression of AOX protein, and their changes were usually in parallel (McIntosh,



○. Control; ■. Treated with 14℃; ▲. Treated with 4℃

Fig.2 Effects of different low temperature stresses on AP activity in tobacco callus

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1994; Day *et al.*,1995). In order to analyze effects of different low temperature stresses on AOX protein expression, mitochondria were isolated from tobacco calli treated with low temperatures for 1, 3, 5 and 7 d and the controls grown for the same time. After separated by reducing SDS-PAGE, mitochondria proteins were probed with the monoclonal antibody of AOA. As shown in Fig.3A and Fig.3B, the change pattern of total AOX protein content under the two low temperatures were the same: there was an increase at the early stage of stress, maximal AOX amount was induced on 3 d, after then (on 5 d and 7 d), it showed no appreciable variation that was still higher than that in control. The changes of the total AOX protein content under different low temperature stress treatment showed excellent correlation with measurement of V_{alt} (Fig.1). The results of Fig.3 also indicate the level of the total AOX protein at each stress treatment time under 4℃ was higher than that under 14℃. This is also in good coincidence with our results of higher V_{alt} induced under 4℃.

2.4 Effects of different low temperature stresses on AOX homodimer and monomer in tobacco callus

To further examine changes in AOX homodimer and monomer under low temperature stresses, mitochondrial protein was separated by nonreducing SDS-PAGE and analyzed with immunoblotting. As shown in Fig. 4A and Fig. 4B, in tobacco callus grown at either low or normal temperature, the level of 70 kD AOX protein, i.e., the oxidized form, was higher than that of 35 kD protein of AOX monomer form. As far as change mode of each AOX form under low temperature stresses was concerted, 70 kD protein was markedly induced during the early three days. Afterwards, no obvious changes could be seen. To the contrary, though maximal levels of 35 kD protein were induced on 3 d, its expression declined slightly following the stress. However, if the comparison was made between total AOX protein content at the same time of different temperature stress treatment the same amount of 35 kD protein was found under 14℃ and 4℃. So, the changes of 35 kD AOX protein level under different temperatures were generally in accordance to rV_{alt} at 14℃ and 4℃.

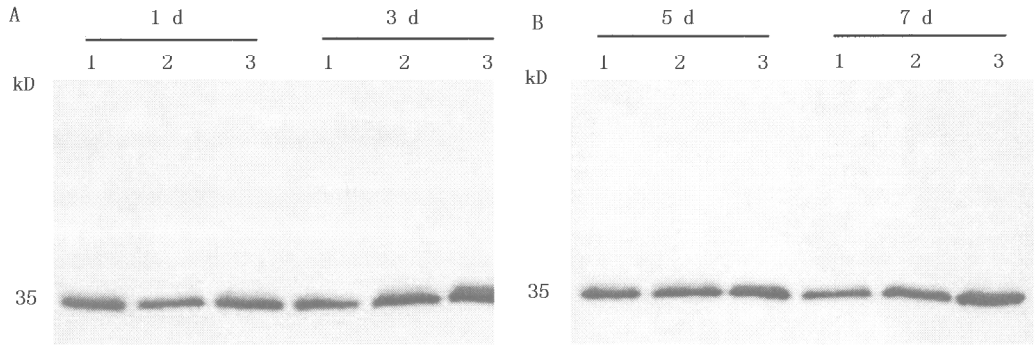


Fig. 3 Immunoblots of mitochondrial protein separated by reducing SDS-PAGE
Mitochondria were isolated from tobacco callus treated with different low temperature stresses for 1, 3, 5 and 7 d. 1. Control; 2. Treated with 14℃; 3. Treated with 4℃. Equal amounts (50 μg) of mitochondrial protein were loaded in each lane and blotted with the AOA monoclonal antibody

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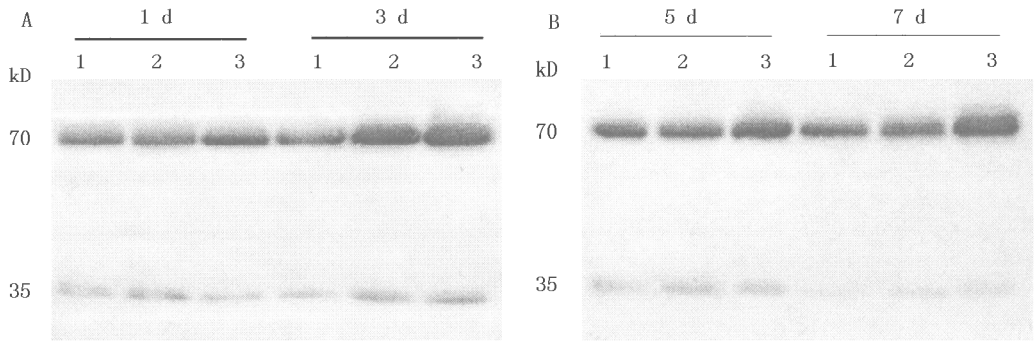


Fig. 4 Immunoblots of mitochondrial protein separated by nonreducing SDS-PAGE
Mitochondria were isolated from tobacco callus treated with different low temperature stresses for 1, 3, 5 and 7 d. 1. Control; 2. Treated with 14℃; 3. Treated with 4℃. Equal amounts (50 μg) of mitochondrial protein were loaded in each lane and blotted with the AOA monoclonal antibody

3 Discussion

Low temperature stress can significantly enhance the ability of the cyanide-resistant respiration in plants. Our results also showed that low temperature stresses markedly induced an increase in the capacity and activity of alternative pathway in tobacco callus. We further compared the dynamic changes of V_{alt} and rV_{alt} under different temperature stresses. The results showed that their change patterns were nearly the same: they increased at the early stage of low temperature treatment, reached its maximal level on 3 d and began to decline, then maintained at a stable level. This indicates that the induction of alternative pathway has an adaptation process. Though V_{alt} was induced at 14℃ and 4℃, and the induced extent at 4℃ was higher than that at 14℃, the actual operation(rV_{alt}) under the two temperatures had no significant difference, indicating that V_{alt} is inclined to be influenced by low temperatures, and the responses of alternative pathway development and operation to different low temperature are different.

AOX activity can be rapidly regulated by means of interconversion of its homodimer and monomer, and plant is capable of fast adapting to the changes of environmental conditions by this way(Rhoads *et al.*, 1998). Furthermore, the results of some previous works also indicated AP capacity(V_{alt}) and the level of AOX protein are in parallel (McIntosh, 1994; Day *et al.*, 1995; Vanlerberghe *et al.*, 1995). Here, we show that changes in total AOX protein content (Fig. 3) are in relation to V_{alt} throughout the stress. Moreover, the analysis of the changes in relative extent of AOX homodimer and monomer demonstrated that, though the difference of AOX homodimer content under different temperatures was remarkable, there was no significant difference in AOX monomer content. Because the activity of monomer was 4-5 folds higher than that of homodimer(Moore *et al.*, 1995), and rV_{alt} was mainly decided by the content of the monomer, the measurement of little difference in rV_{alt} at the two temperatures may be a result of nearly the same extent of AOX monomer induced. However, the increase in V_{alt} induced by low temperatures mainly depends upon the increase in total AOX protein, especially upon

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the increase in AOX homodimer. So, the results of our study not only find distinct effects of different low temperatures on plant alternative pathway development and operation, in addition, we further elucidate the mechanisms of the differences at AOX protein expression level.

The operation of alternative pathway could result in heat evolution(Kapulnik *et al.*, 1992; Wen and Liang, 1994; Vanlerberghe and McIntosh, 1997). Accordingly, plants can be classified into typical thermogenic plants and atypical thermogenic plants. For the AOX existing states in the mitochondria of the two kinds plants, Kinden and Akerlund implied that AOX exists with generally high-activity monomer in typical thermogenic plant mitochondria and with low-activity homodimer in atypical thermogenic plant(Lidén and Akerlund,1993). In our study, we found that, AOX in tobacco callus mitochondria was largely existed in its oxidized state (homodimer), and low temperature stress could not modify the status of AOX protein in mitochondria. It provided good direct evidence for the implication of Kinden and Akerlund that there was different AOX protein existing states in the two kinds of plants.

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