The Electron Partitioning between the Cytochrome and Alternative Respiratory Pathways during Chilling Recovery in Two Cultivars of Maize Differing in Chilling Sensitivity[1]

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Chilling effects on respiration during the recovery period were studied in two maize (*Zea mays* L.) cultivars differing in their tolerance to chilling: Penjalinan, a chilling-sensitive cultivar, and Z7, a chilling-tolerant cultivar. Both cultivars were exposed to 5°C for 5 d, after which measurements were taken at 25°C. Chlorophyll fluorescence analysis in dark-adapted leaves showed less damage in cv Z7 than in cv Penjalinan during recovery from the chilling treatment. Studies of the electron partitioning between the cytochrome and the alternative respiratory pathways during chilling recovery using the oxygen isotope fractionation technique showed that, although total leaf respiration was not affected by the chilling treatment in either of the two cultivars, electron partitioning to the alternative pathway was significantly increased in the more stressed chilling-sensitive cv Penjalinan, suggesting that increased activity of the alternative pathway is not related to the plant tolerance to chilling. These results suggest a possible role of the alternative pathway in plants under stress rather than specifically contributing to plant resistance to chilling.

The cyanide-resistant alternative pathway is one of the special features of plant respiration. This pathway, which shares electrons from the ubiquinone pool with the cyanide-sensitive cytochrome pathway, is not coupled to ATP synthesis and its function has been the center of discussion for many years (Ordentlich et al., 1991; Purvis and Shewfelt, 1993; Wagner and Krab, 1995; Vanlerberghe and McIntosh, 1996). The only known function for the alternative respiratory pathway is related to the thermogenesis during the anthesis of *Arum* spadices (Meeuse,

1975). This thermogenic function, combined with more recent observations that low temperature increases the amount of the alternative oxidase protein and cyanide-resistant respiration in plant mitochondria (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992; Gonzàlez-Meler et al., 1999), led to the hypothesis that the alternative pathway might play a role in plants grown at low temperatures or under chilling conditions (Stewart et al., 1990; Ordentlich et al., 1991; Moynihan et al., 1995). It is thought that the alternative pathway might play a role in preventing the formation of toxic oxygen species when the normal activity of the cytochrome pathway is restricted by low temperature (Purvis and Shewfelt, 1993; Wagner, 1995).

The effect of chilling on plant respiration has been widely studied, and different roles have been attributed to the alternative oxidase under this stress (Leopold and Musgrave, 1979; Smakman and Hofstra, 1982; Stewart et al., 1990; Purvis and Shewfelt, 1993; Moynihan et al., 1995; Collier, 1996). Some studies have indicated the potential for the alternative pathway to ameliorate chilling stress based on the loss of energy as heat when the alternative pathway is active (Ordentlich et al., 1991; Purvis and Shewfelt, 1993; Moynihan et al., 1995; but see Breidenbach et al., 1997). Both the levels of alternative oxidase protein and the rates of cyanide-resistant respiration, the so-called "capacity" of the alternative pathway, increase in plant maize seedlings and tobacco cell cultures exposed to low temperatures (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992). Recently, a similar response has been observed in mature leaves of mung bean and pea (Gonzàlez-Meler et al., 1998, 1999). However, an increase in levels of alternative oxidase protein does not necessarily indicate an increase in the actual electron flow through the alternative pathway in the absence of inhibitors as has been demonstrated in leaves in which the levels of alternative oxidase protein were modified with salicylic acid (Lennon et al., 1997) or growth temperature (Gonzàlez-Meler et al., 1999).

It has long been recognized that chilling causes photooxidative damage under illuminated conditions (Foyer et al., 1994; Wise, 1995) by increasing the formation of harmful active oxygen species in chilling-sensitive species (Wise and Naylor, 1987; Jahnke et al., 1991). It has also been postulated that the alternative pathway can stabilize the

reduction state of the ubiquinone pool when the cytochrome pathway is restricted (Millenaar et al., 1998), attenuating the formation of reactive oxygen species in the mitochondria (Purvis and Shewfelt, 1993). Moreover, there is a hypothesis that active oxygen species can enhance the expression of alternative oxidase genes (Wagner, 1995; Vanlerberghe and McIntosh, 1996).

Plant respiration studies have undergone significant changes in the last few years. It has been demonstrated that the alternative pathway can, under certain conditions, compete for electrons from the ubiquinone pool with an unsaturated cytochrome pathway (Hoefnagel et al., 1995; Ribas-Carbo et al., 1995). This challenges the classical interpretations of the results obtained when inhibitors were used to estimate the actual activities of both the cytochrome and the alternative pathways (Millar et al., 1995; Day et al., 1996). The only reliable methodology presently available with which to study electron partitioning between the cytochrome and alternative pathways in the absence of inhibitors is the use of oxygen isotope fractionation techniques that can be performed with either intact tissues (Robinson et al., 1992, 1995) or isolated mitochondria and enzymes (Ribas-Carbo et al., 1995, 1997). The purpose of the present study was to assess the control of electron partitioning between the cytochrome and alternative respiratory pathways during recovery from chilling stress using the oxygen isotope fractionation technique in two maize cultivars with different tolerances to chilling. Simultaneously, the degree of stress generated by the chilling treatment was assessed by chlorophyll fluorescence analysis.

MATERIALS AND METHODS

Plant Material

We used two maize lines with different chilling tolerance: cv Z7, a chilling-tolerant line from European cold regions and cv Penjalinan, a chilling-sensitive line from warm tropical regions (Stamp et al., 1983). Seeds were surface-sterilized with 0.02% (w/v) $HClO_4$ and then germinated at 25°C on wet perlite. Plant seedlings were then placed in 0.2-L volume pots (one plant per pot) and were irrigated with modified Hoagland solution (Downs and Hellmers, 1975). Plants were placed in growth chambers at 25°C on a 12-h/12-h (light/dark) regime at 300 μmol photons $m^{-2} s^{-1}$. For the chilling treatment, plants at the fourth leaf stage (fully expanded third leaf) were placed for 5 d at 5°C at the same light regime. After this period, plants were returned to 25°C at the same light regime for 24 h. Respiration and chlorophyll fluorescence were measured the day after the chilling treatment was terminated. All measurements were made on the third fully expanded leaf at 25°C.

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence was measured using a photosynthesis yield analyzer (MINI-PAM, Heinz Walz, Effel-trich, Germany). Measurements were carried out at 25°C on the fully expanded third leaf the day after the chilling treatment was terminated.

The minimal (F_o) and maximal (F_m) fluorescence in the dark-adapted leaves were measured in the dark before dawn. Maximal variable fluorescence (F_v) was calculated as $F_m - F_o$, and the optimal quantum yield of photosystem II (PSII) was calculated as ratio F_v/F_m, as previously described (Schreiber et al., 1994).

Respiration and Oxygen Isotope Fractionation

Oxygen isotope fractionation was measured in the third fully developed leaf. Three maize leaf section squares were taken from separate plants, weighed, and floated in a reaction mixture consisting of 10 mM N-Tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES) (0.2 mM $CaCl_2$) buffer, pH 7.2, for 15 min in the dark to stabilize respiration. Leaf slices were then surface-dried, placed into a dark, 4-mL closed cuvette at 25°C, and allowed to equilibrate with the inlet vent open. After equilibration the inlet vent was closed.

Oxygen isotope analysis were performed as described in Robinson et al. (1995) with modifications (Gonzàlez-Meler et al., 1999). At regular time intervals an air sample was taken into a 100-μL loop and directed into the helium flow of the gas chromatography-mass spectrometry unit. Carbon dioxide and water vapor were removed and the oxygen, argon, and nitrogen gases were separated by gas chromatography (NA 1500, Carlo Erba Instrumentazione, Milan) using a 915- $\times$ 6-mm-diameter molecular sieve (pore size 5A, 80–100 mesh, Varian Chrompack Benelux, Bergen ap Zoom, The Netherlands) column heated to 50°C at a flow rate of 30 mL min^{-1} He carrier gas. The components were detected using a thermal conductivity detector and integrated (model 3394 integrator, Hewlett-Packard, Palo Alto, CA). The isotope ratio $^{18}O/^{16}O$ was measured directly from the ratio of masses 32 and 34 using an isotope ratio mass spectrometer (SIRA series II, VG ISOGAS, Middlewich, UK) operated in continuous flow mode.

Oxygen isotope fractionation and electron partitioning were calculated as described by Guy et al. (1989) without forcing the relationship between $-\ln f$ and $\ln(R/Ro)$ to go through zero. We also discarded all experiments in which the r^2 of the slope was lower than 0.995 with a minimum of six data points and consuming at least 30% of the initial oxygen concentration.

For inhibitor treatments, either 2.0 mM KCN (in water) or 10 mM salicylhydroxamic acid (SHAM) (in water from a 1.0 M stock in DMSO) were added to the TES-$CaCl_2$ solution. These concentrations were used after previous inhibitor titration tests showed that they were the most efficient inhibitor concentrations. All stocks were freshly prepared before use. Total respiration, cyanide-resistant and SHAM-resistant respiration, and residual oxygen uptake were also measured in a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) as described in Lambers et al. (1993).

RESULTS AND DISCUSSION

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence analysis is a fast, non-destructive technique that allows the determination of different degrees of stress and has become widely used to study physiological stress (Schreiber et al., 1994). The most sensitive fluorescence parameters to screen chilling tolerance or damage are F_v and the optimal quantum yield of PSII (F_v/F_m) (Krause, 1994). A decrease in these parameters indicates the extent of photoinhibition caused by the chilling treatment (Krause, 1994). F_v and F_v/F_m decreased in both cultivars (Table I), but the decrease was more pronounced in cv Penjalinan, indicating a larger level of photoinhibition in the chilling-sensitive cv Penjalinan than in the chilling-tolerant cv Z7.

A decrease in F_v has previously been correlated with photooxidative damage caused by chilling (Van Hasselt and Van Berlo, 1980): the larger the decrease in F_v the greater the photoxidative damage. The larger decrease in F_v seen in cv Penjalinan compared with that of cv Z7 would therefore indicate a greater photooxidative damage in cv Penjalinan than in cv Z7. Moreover, cv Penjalinan leaves also had lower F_m values than cv Z7 after the chilling treatment (Table I). F_o was unchanged by the chilling treatment in cv Penjalinan, but increased by 130% in cv Z7 (Table I). Similar results were also found by Bergantino et al., (1995) during and after chilling treatment in two cultivars of maize differing in their chilling tolerance. This increase of F_o in cv Z7 can be explained by a decrease in the transfer of excitation energy from the light-harvesting complex of PSII to the PSII reaction center, preventing the overexcitation of the PSII reaction center (Ögren and Öquist, 1984; Krause, 1988; Bergantino et al., 1995).

In summary, our fluorescence results show that after recovery from chilling, the chilling-tolerant cv Z7 is in a much better physiological condition than the chilling-sensitive cv Penjalinan, as expected from previous studies on the same cultivars (Stamp et al., 1983; Pérez de Juan et al., 1997). Furthermore, during the recovery period, cv Z7 plants (chilling-tolerant) had positive growth while cv Penjalinan plants (chilling-sensitive) had no net growth (data not shown; Pérez de Juan et al., 1997).

Respiration and Electron Partitioning

It has been reported that exposing plants, including maize (Stewart et al., 1990), to low temperatures often results in an increase in alternative oxidase protein levels (Vanlerberghe and McIntosh, 1992; Gonzàlez-Meler et al., 1999). Increases in total alternative oxidase protein levels are generally correlated with increases in the cyanide-resistant respiration rates, the so-called "capacity" of the alternative pathway (Obenland et al., 1990; Stewart et al., 1990; Vanlerberghe and McIntosh, 1992, 1996; Rhoads and McIntosh, 1993; Lennon et al., 1997; Fiorani et al., 1998; Gonzàlez-Meler et al., 1999). The cyanide-resistant respiration rate (V_{KCN}) increased after the chilling treatment from 18.3 and 22.0 nmol O_2 g^{-1} dry weight s^{-1} to 27.3 and 30.8 nmol O_2 g^{-1} dry weight s^{-1} in cv Penjalinan and cv Z7, respectively, with no significant difference between the two cultivars (Table II). The increase in the cyanide-resistant respiration rate seen in both cultivars after the chilling treatment likely represents an increase in the amount of alternative oxidase protein present.

The oxygen isotope fractionation by the cytochrome pathway measured in the presence of 10 mM SHAM and the alternative pathway measured in the presence of 2 mM KCN were 19.3 ± 0.3 and 29.9 ± 0.8, respectively, for the two cultivars. These fractionation values were constant throughout the experiment and were not affected by the chilling treatment. They were also similar to other fractionation values of respiration obtained in leaves in the presence of SHAM or KCN (Robinson et al., 1992; Lennon et al., 1997; Ribas-Carbo et al., 1997; Gonzàlez-Meler et al., 1998, 1999). Consequently, these values were used as end points to calculate electron partitioning between the cytochrome and alternative pathway in the absence of inhibitors (Fig. 1).

There were no differences between cv Penjalinan and cv Z7 in the measured respiratory characteristics when plants were grown under control conditions (25°C; Table II). The oxygen isotope fractionations measured in the absence of inhibitors (Δn) were 21.9‰ and 22.1‰ for cv Penjalinan and cv Z7 respectively (Fig. 1). Therefore, respiration through the cytochrome pathway (v_{cyt}) were 19.3 and 21.8 nmol O_2 g^{-1} dry weight s^{-1}, and respiration through the alternative pathway (v_{alt}) were 6.3 and 7.8 nmol O_2 g^{-1} dry weight s^{-1}, for cv Penjalinan and cv Z7, respectively (Table II). Residual respiration was very similar between the two species and did not change significantly after recovery from the chilling treatment (Table II; see also Ribas-Carbo et al., 1997, for residual respiration).

Total leaf respiration, although slightly higher in cv Z7, was not significantly different between the two cultivars after recovering from the chilling treatment (Table II). Ox-

Table I. *Chlorophyll fluorescence analysis in dark-adapted leaves growing at 25°C (control) or after 5 d at 5°C (chilled)*

Measurements were made as described in "Materials and Methods" in the third fully expanded leaf. Values are means ± SE of four replicates. *, Statistically significant differences with a $P < 0.05$.

	cv Z7			cv Penjalinan		
	Control	Chilled	% Control	Control	Chilled	% Control
F_v/F_m	0.671 ± 0.003	0.446* ± 0.040	67	0.660 ± 0.022	0.312* ± 0.068	47
F_v	774 ± 38	417* ± 84	54	761 ± 35	212* ± 71	28
F_m	1153 ± 50	907 ± 101	79	1151 ± 31	633* ± 77	55
F_o	379 ± 13	490* ± 17	130	391 ± 11	421 ± 18	108

Table II. *Leaf respiration rates expressed as nanomoles of O_2 per gram dry weight per second of plants growing at 25°C (control) or after 5 d at 5°C (chilled)*

All measurements were made in the third fully expanded leaf. v_{cyt} and v_{alt} were calculated with the τa values shown in Figure 1. Values are means $\pm$ SE of three to eight replicates. Asterisks indicate statistically significant differences with a $P < 0.05$.

	n	cv Z7		cv Penjalinan	
		Control	Chilled	Control	Chilled
V_t [a]	8	29.6 ± 1.7	34.9 ± 1.8	25.6 ± 1.6	28.2 ± 0.7
v_{cyt}	8	21.8 ± 1.3	22.7 ± 1.2	19.3 ± 1.2	11.5* ± 0.3
v_{alt}	8	7.8 ± 0.5	12.2* ± 0.6	6.3 ± 0.4	16.8* ± 0.4
V_{SHAM} [b]	4	22.2 ± 1.4	29.7* ± 2.1	20.3 ± 1.0	26.0* ± 1.7
V_{KCN} [c]	4	22.0 ± 2.8	30.8* ± 4.2	18.3 ± 0.7	27.3* ± 1.1
V_{res} [d]	4	10.13 ± 1.6	15.7 ± 2.0	10.5 ± 1.8	12.6 ± 1.7

[a] V_t, Total respiration.   [b] V_{SHAM}, Respiration measured in the presence of 10 mM SHAM. [c] V_{KCN}, Respiration measured in the presence of 2 mM KCN.   [d] V_{res}, Residual respiration (only measured using the Clark-type electrode).

ygen isotope fractionation measured in the absence of inhibitors (Δn) increased significantly in the two cultivars after recovering from chilling treatment (Fig. 1), although the increase was more dramatic for cv Penjalinan (Fig. 1). This change in Δn indicates an increased relative contribution of the alternative pathway to total respiration (τa) in the two cultivars after recovering from chilling treatment (Fig. 1). In cv Penjalinan, v_{alt} increased more than 2-fold after recovering from chilling treatment (Table II), whereas v_{cyt} was reduced by about 40%, resulting in no net effect on Vt (Table II). Furthermore, cv Z7 plants had an increase in v_{alt} of 56% after recovering from chilling but no change on v_{cyt} (Table II), although such an increase in v_{alt} was not sufficient to significantly increase respiration rates (Table II).

The increase in alternative pathway respiratory flow after recovering from chilling treatment was much greater in cv Penjalinan than in cv Z7. The increase in v_{alt} in cv Penjalinan after recovering from chilling treatment was not coupled with any increase in total respiration (Table II), specially due to a decrease in v_{cyt} (Table II). Several authors

Figure 1. Oxygen isotope fractionation (Δn) and electron partitioning between the cytochrome and alternative pathway (τa) during respiration in the absence of inhibitors in two different maize lines, the chilling-sensitive cv Penjalinan (black bars) and the chilling-tolerant cv Z7 (gray bars). τa was calculated using the obtained end points Δa = 29.9‰ and Δc = 19.3‰. Bars represent SE (n = 3).

have indicated that the cytochrome pathway is more susceptible to damage at chilling temperatures than the alternative pathway (Leopold and Musgrave, 1979; Smakman and Hofstra, 1982; Prasad et al., 1994). cv Penjalinan plants were severely stressed after recovering from chilling treatment (Table I), restraining the normal activity of the cytochrome pathway during the recovery phase of the chilling treatment at 25°C (Table II; Fig. 1). Under these conditions, substrate supply to respiration (i.e. photosynthesis, see Pérez de Juan et al., 1997) may recover faster from chilling than the cytochrome pathway, requiring an increase in the activity of the alternative pathway to avoid the over-reduction of the ubiquinone pool, especially in the more stressed chilling-sensitive cultivars. Our results seem to confirm this idea, because the activity of the cytochrome pathway decreased in the chilling-sensitive cv Penjalinan but remained unchanged in the chilling-tolerant cv Z7.

Although previous measurements have suggested that the relative activity of the alternative pathway can decrease as temperature is lowered, this response is not only dependent on species but also on the growth history of the plant (Gonzàlez-Meler et al., 1998, 1999). It would have been important to measure the electron partitioning between the cytochrome and alternative at 5°C to assess the direct effect of chilling on respiration in these two cultivars. However, at 5°C, total respiration is lower than allowed for the current sensitivity of our experimental design for oxygen fractionation measurements and therefore we were unable to do those experiments within the acceptable margin of error. We are currently working on a new design that should provide reliable measurements of oxygen fractionation with much less oxygen being consumed.

CONCLUSIONS

The changes in the respiratory behavior of maize plants after recovering from chilling can be separated in two parts. On the one hand, the increase in V_{KCN}, which is likely to be related to the increase of total AOX protein. This increase could be caused by the decrease in growth temperature, as it has already been described (Stewart et
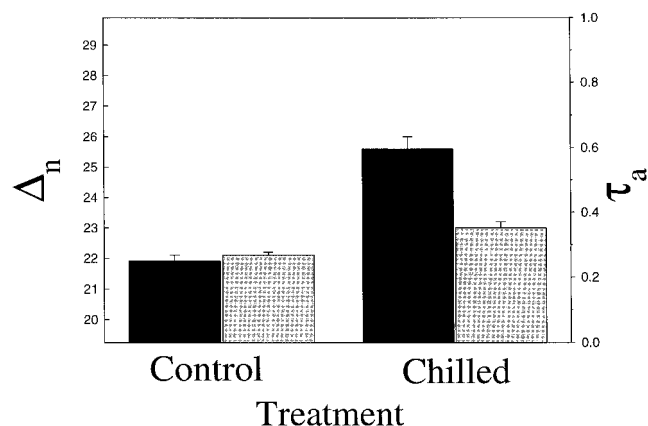
al., 1990; Vanlerberghe and McIntosh, 1992; Gonzàlez-Meler et al., 1999). On the other hand, the increase in v_{alt} strongly correlates with the level of stress sustained after recovering from chilling. This increase in the alternative pathway activity is more important in the most severely damaged cv Penjalinan. Based on a previous hypothesis (Purvis and Shewfelt, 1993; Wagner, 1995; Vanlerberghe and McIntosh, 1996), the increase in the actual activity of the alternative pathway in the stressed plants could be related to preventing an increase in mitochondrial formation of reactive oxygen species.

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