Photosynthesis Research 20: 235–247, 1989
© 1989 Kluwer Academic Publishers. Printed in the Netherlands.

The use of chlorophyll fluorescence as a screening method for cold tolerance in maize

A.H.C.M. SCHAPENDONK,[1] O. DOLSTRA[1] & O. VAN KOOTEN[2]
[1]Foundation for Agricultural Plant Breeding (SVP), P.O. Box 117, 6700 AC Wageningen, The Netherlands; [2] Department of Plant Physiological Research, Agricultural University, Generaal Foulkesweg 72, 6703 BW Wageningen, The Netherlands

Received 1 June 1988; accepted in revised form 30 August 1988

Key words: breeding, chlorophyll fluorescence, chlorosis, cold tolerance, maize, *Zea mays* L

Abstract. Chlorosis in maize (*Zea mays* L.) is a common phenomenon in the 12 to 17°C temperature range. A newly developed chlorophyll-fluorescence technique was used to elucidate the underlying subcellular processes of resistance to chlorosis. Four populations were used that were developed by divergent mass selection for contrasting resistance to chlorosis in a cold-tolerant *dent* and a cold-tolerant *flint* population. Young plants from the four populations were kept for six days at 17/10, 15/10 and 13/10°C (day/night). After 1, 3 and 6 days various chlorophyll-fluorescence parameters were determined. The measurements were done on leaf 4. Differences were not uniform for all fluorescence properties. The resistant and susceptible populations of the two sets differed for the Q-quenching which is related to the electron transport rate in the chloroplast. For the E-quenching which is related to the Calvin cycle activity, the resistant *dent* differed significantly from the other three populations. The ratio Fm/Fo (related to the transfer of absorbed light-energy from antennae pigments to reaction centers in the chloroplast) was higher for the resistant *dent* population than for the susceptible one. The *flint* types did not differ for this property.

Apparently, divergent mass selection for chlorosis resistance resulted in various changes at the subcellular level that are not necessarily comparable for *flint* and *dent* types.

When after 6 days the temperature was raised from 13°C to 17°C, the fluorescence signals led to the conclusion that there was a full recovery of various processes after two days, except for the metabolic activity of the susceptible *flint*.

Introduction

The efforts of maize breeders to select for genotypes that are resistant to chlorosis at low temperatures are directed to improve low temperature adaptation and growth at low temperatures. As such it is important to consider the backgrounds of chlorosis and its impact on yield. Maize seedlings have a lower chlorophyll content when they are grown at low temperatures (Alberda 1969). The low chlorophyll content (chlorosis) can be seen as a indication for a deficiency of the photosynthetic capacity of the

leaves. Unfortunately there is no consensus about the mechanism that leads to chlorosis (Wang 1982) nor to what extent it suppresses growth (Miedema et al. 1987). In the temperature range between 10°C and 15°C newly formed chlorophyll is presumably destroyed by rapid photo-oxidation before it is complexed in the chloroplast lamellae (McWilliam and Naylor 1967). The photo-oxidation is amplified by high light intensities (Taylor and Craig 1971). At temperatures lower than 10°C, the effects are more pronounced because also membrane-bound chlorophyll may be destroyed by free radicals of oxygen despite the protective action of carotenoids (van Hasselt and van Berlo 1980, Baker et al. 1983, Wise and Naylor 1987, Smillie et al. 1987) Alternatively, Hodgins and van Huystee (1986) showed that chill-induced chlorosis in maize seedlings was partly the result of two metabolic blocks in the porphyrin path leading to chlorophyll synthesis. The temperature range of the impaired chlorophyll synthesis coincides with the temperature range for chlorosis (i.e., 17–10°C).

In addition to the effect on the level of chlorophyll properties, low temperature has an important effect on metabolic and respiratory processes (Miedema et al. 1987). Thus low-temperature effects on growth are not necessarily exhibited by chlorosis and it is not evident to what extent cold tolerance is linked with resistance to chlorosis. The search for genetic differences for cold resistance should therefore take into account the different underlying processes. However, the procedures to distinguish genotypical differences at such level is complicated and time consuming. The latest progress in nondestructive analyses of different photosynthetic processes seems very promising (Krause et al. 1982, Schreiber et al. 1985, Bilger and Schreiber 1986, Björkman, 1987). The present study shows the implementation of this technique (pulsed amplitude modulated fluorescence measurements) in a breeding program for resistance to chlorosis in maize. With this nondestructive method it is possible to study the relation between various fluorescence parameters, chlorosis and the effects of low temperatures on fundamental processes and the extent of genetic variation for these processes.

Materials and method

Plant material

The plant material used in this study consisted of two sets of subpopulations produced by two cycles of mass selection for either chlorosis resistance or chlorosis susceptibility (Dolstra et al. 1988). The first set, being SVP-

PE3GP2 and SVP-PE3GM2, originated from the cold tolerant *dent* population SVP-PE3. In general, *dent* types grow more vigourously than *flint* types but they are more susceptible to low temperatures. The second set, being SVP-PD6AG1GP2 and SVP-PD6AG1GM2 originated from a cold-tolerant *flint* population SVP-PD6AG1. The respective subpopulations are denoted as resistant and susceptible *dent* and resistant and susceptible *flint*.

Experimental design

On 6 April seeds of the four populations were sown in pots in the greenhouse. Within each pot the plants were arranged in squares with the plants from the corresponding populations at the opposite corners. The day/night temperatures were 20/15°C. On 28 April the plants were transferred to growth cabinets with day/night temperature regimes of 17/10, 15/10 and 13/10°C respectively and a light period of 16 hours at a quantum flux density of 270 μmol m^{-2} s^{-1} photosynthetic active radiation (about 60 W m^{-2}) at the top of the plants. The relative humidity was 70%. Plants were watered daily. Each treatment consisted of 4 blocks with 9 pots. The duration of the temperature treatments was 6 days. Then the temperatures were reset to the control regime (17/10°C), to allow for recovery.

Leaf elongation rates and chlorophyll fluorescence were measured at day 1, 3, 6 and 8 after the start of the treatments. The responses on day 8 presented the effects of recovery from the imposed stress. The results were statistically analyzed by the ANOVA method.

Chlorophyll fluorescence

Chlorophyll fluorescence was measured at room temperature (22°C) in a completely darkened room. The plants were illuminated for a period of 12 h prior to the transfer from the growth cabinets to the measuring room. There, leaves were adapted to the dark for a period of 25 min prior to the fluorescence measurements. These measurements were done with intact leaves. Part of the leaf (1.5 cm^2), at 1/3 from the base, was clamped in an aluminium cuvette, flushed with humidified air with 2% oxygen. The variation of the fluorescence signals measured at twelve places from the base to the top was less than 4%. The illumination of the leaf and the guidance of the fluorescence signals to the detection apparatus was achieved with fiber optics. The measuring technique was based on a pulse amplitude modulation fluorometer (model PAM 101: H Walz, Effeltrich, West Germany). The measurements and calculations were described by Schreiber and Bilger (1987). The frequency of the saturating light pulses was either 0.05 Hz for the experiment

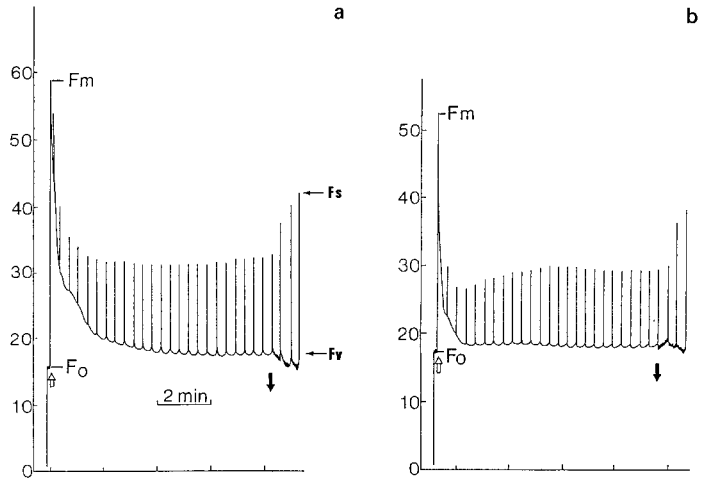


Fig. 1. Time course of the fluorescence induction curves of a maize leaf (susceptible *flint*) at 17°C (a) and after a temperature treatment of 3 days at 15°C (b). Fo designates the basic fluorescence. F_m is the maximum fluorescence induced by a saturating light flash after a dark period. F_v is the fluorescence, measured just before the light-saturating flashes. F_s is the fluorescence induced by light-saturating flashes. The open and closed arrows indicate the onset and the termination of the photosynthetically active radiation. The light saturating pulses are fired at a frequency of 0.05 Hz.

depicted in Fig. 1 or 0.25 Hz for all other experiments. The intensity of the photosynthetic active radiation was 105 μmol m^{-2} s^{-1}.

Examples of the fluorescence reponses in the control and 15°C treatments are given in Fig. 1:

(1) Chlorophyll was excited with a measuring beam of a very weak quantum flux density assuring that photosynthesis was not yet activated. The resulting basic fluorescence (F_o) could be attributed to the radiative loss of excitation energy due to improper energy transfer from the antennae pigments to the photosystems when all reaction centers are in an open state. F_o was higher in the cold treated plants (Fig. 1b) than in the control plants (Fig. 1a).

(2) F_m is the maximum fluorescence after a light flash with a saturating intensity. The ratio F_m/F_o gives insight in the potential efficiency of photosystem 2, determined by the rate of non-radiative energy dissipation and the donor side activity. Comparison of Figs. 1a and 1b reveals a decrease of the ratio F_m/F_o in the latter, which is in agreement with a cold-induced decrease of the photosystem 2 activity.

(3) Illumination with photosynthetically active light gave rise to variable fluorescence (F_v) induction curves (Kautsky effect), composed of an initial increase of the fluorescence to a peak level and a subsequent decay through

a number of phases that were attributed to changes in energy transfer between photosystem 2 and photosystem 1. The energy transfer between both photosystems is correlated with the amount of oxidized acceptor of photosystem 2 (quantified as Q-quenching; $(F_s - F_v)/(F_s - F_o)$). A cold treatment enhances the Q-quenching (compare Figs. 1a and 1b)

(4) Successive saturating light pulses were fired repetitively (0.05 Hz) to close all traps in order to study fluorescence changes that were not directly related to redox reactions. These non-photochemical reactions were shown to be caused by fluctuations of a pH gradient across the thylakoid membranes, driving ATP synthesis (Krause and Weis 1984). It is quantified by the relation: $(F_m - F_s)/(F_m - F_o)$. From Figs. 1a and 1b it seems evident that a cold treatment leads to an increase of the E-quenching, which might be the result of an inhibited Calvin cycle activity with a feedback to the pH-gradient (Weis and Berry 1987).

Results

Figure 2 shows the effect of temperature on the leaf elongation rates, averaged over all genotypes. Lowering the temperature from 17°C to 15°C evidently inhibited leaf elongation more than a decrease from 15°C to 13°C. The elongation rate of the leaves of the susceptible *dent* was significantly lower (with a probability higher than 99%: $P < 0.01$) at all temperatures

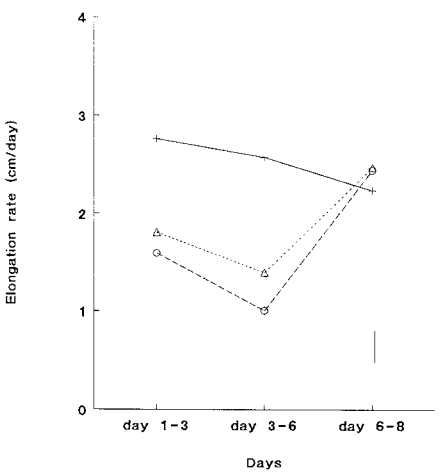


Fig. 2. Mean elongation rates of the fourth leaves of the different populations at 17°C (+) 15°C (Δ) and 13°C (0). The third interval covers the recovery period. The standard error of differences (SED) is indicated by the bar.

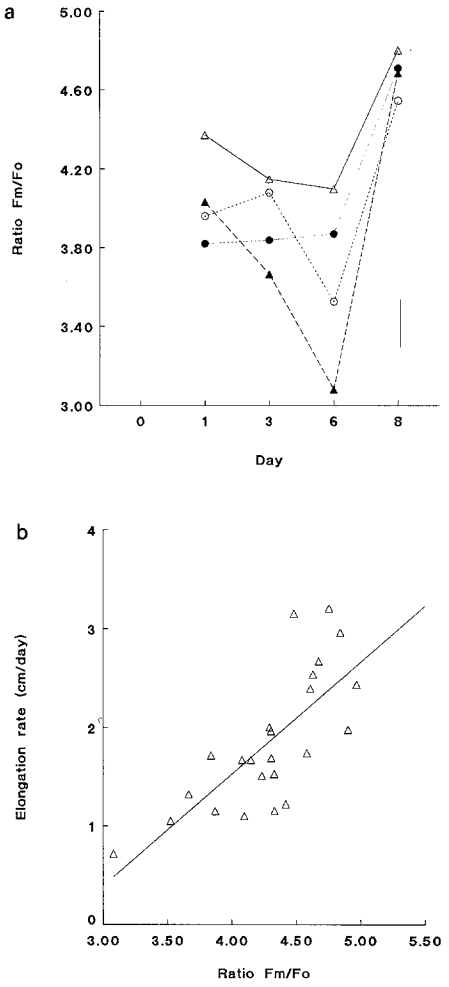


Fig. 3. Temperature effects on the ratio F_m/F_o and leaf elongation rate. (a) Ratios of F_m/F_o for the 13°C treatment as a function of time. The measurement at day 8 represents the effect of recovery at a temperature of 17°C. Susceptible *dent* (▲), tolerant *dent* (△), susceptible *flint* (●), tolerant *flint* (○). (b) The correlation between the ratio F_m/F_o, and the elongation rate for the low temperature treatments, i.e., 13°C and 15°C; $r = 0.74$. The SED is indicated by the bar.

(not shown). Among the other populations no significant differences were found. After alleviation of the temperature stress, recovery of the elongation rate was fast and complete (day 6–8).

The basic fluorescence *Fo* and the maximum fluorescence F_m were higher for the *flint* than for the *dent* populations in all treatments. This is probably due to morphological differences in the leaves. The ratios F_m/F_o for the 13°C

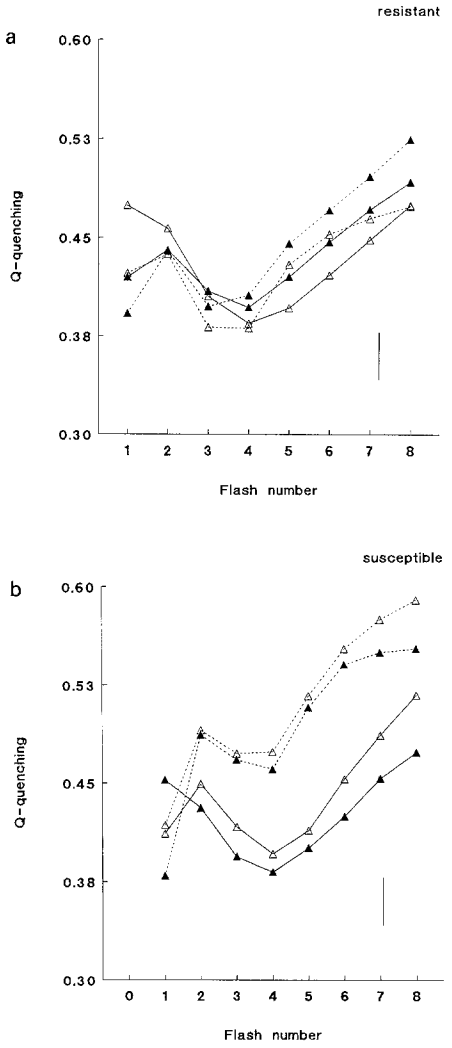


Fig. 4. Temperature effects on Q-quenching. The response on the first flash to determine F_m is not considered. (a) Q-quenching of populations resistant to chlorosis at 13°C and 17°C. (b) Q-quenching of populations susceptible to chlorosis at 13°C and 17°C. Evaluations were performed three days after the start of the treatments. The 13°C treatments are indicated by broken lines and the 17°C treatments by continuous lines. The *dent* and *flint* populations are indicated by open and closed triangles respectively. The bars indicate the SED.

treatment are depicted in Fig. 3a. In contrast to the differences observed between the *flint* populations, those between the *dent* populations were very significant ($P < 0.01$). After a recovery period of 2 days at 17°C, the ratios corresponded with those of the control treatment which indicated that, for the F_m/F_o attribute, all genotypes completely recovered very rapidly from

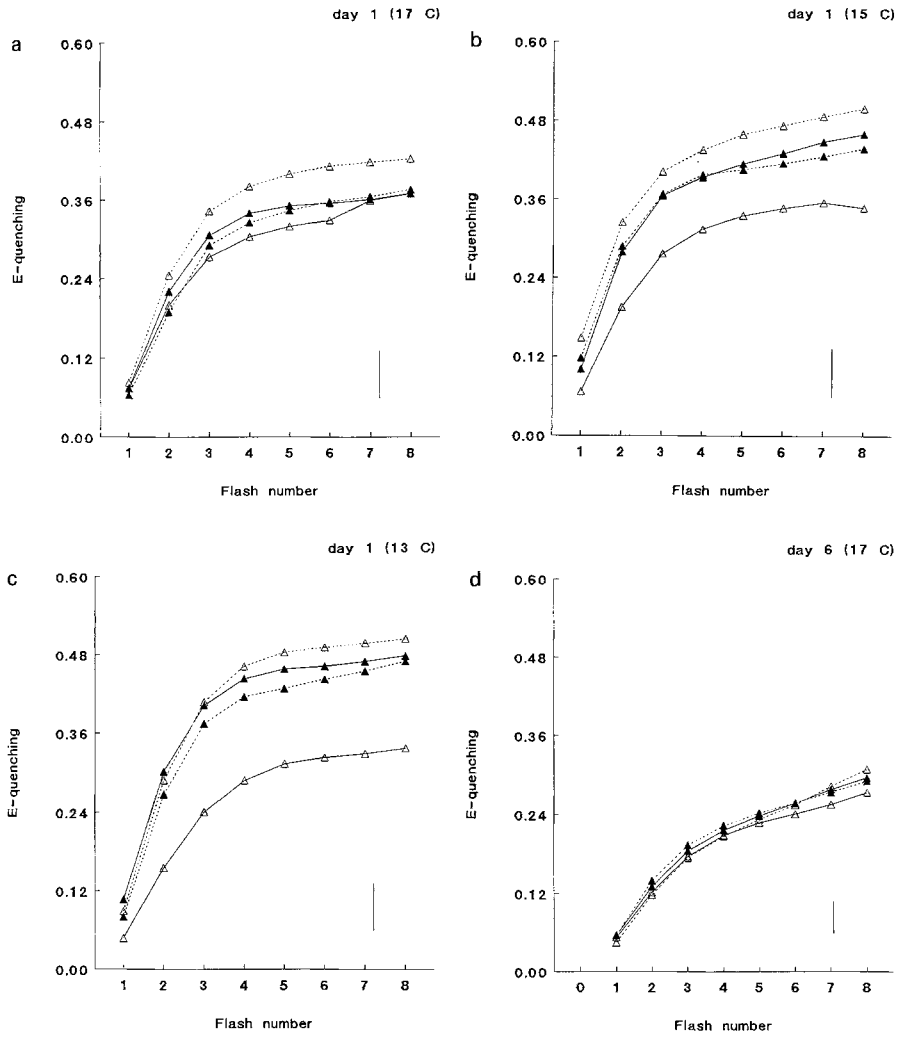
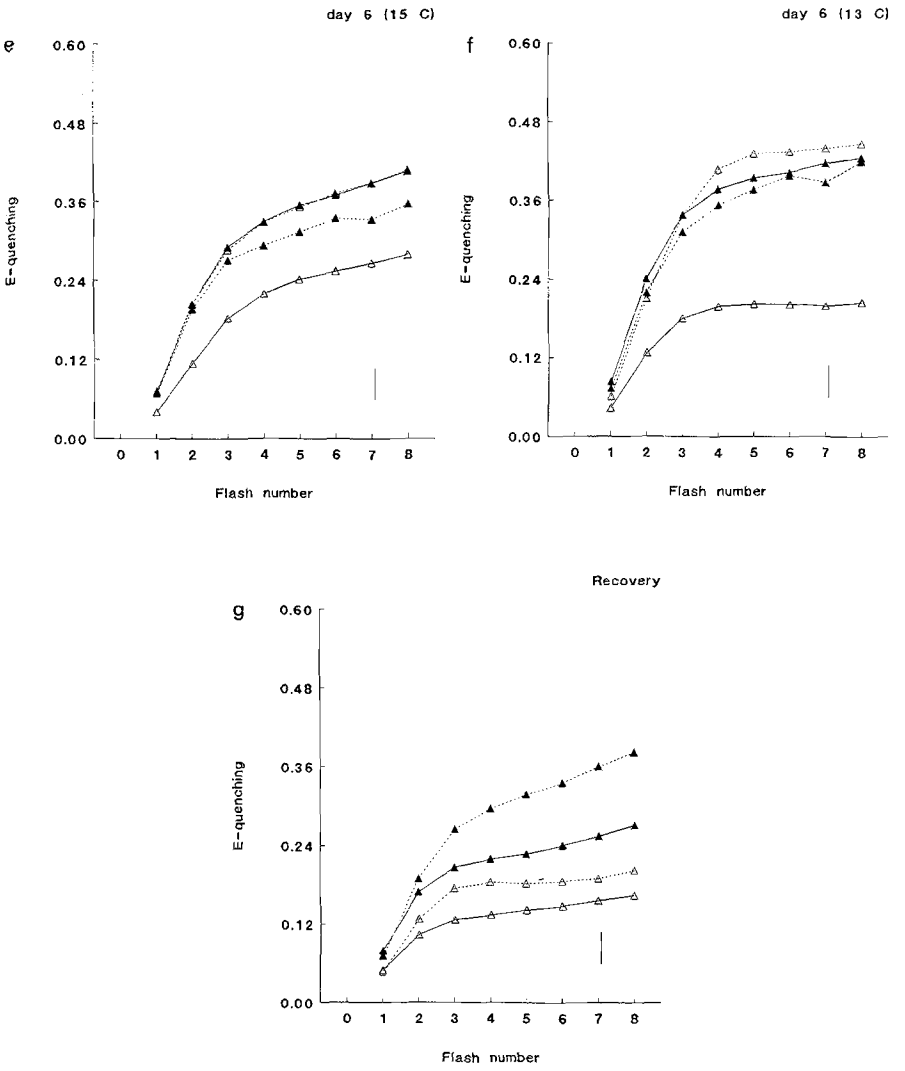


Fig. 5. E-quenching, 1 (a, b, c) and 6 days (d, e, f) after the start of the experiment respectively. The temperatures were 17°C (a, d), 15°C (b, e) and 13°C (c, f). The signals after recovery (day 8) are depicted in g. The tolerant populations are indicated by continuous lines and the susceptible popluations by the dotted lines. The open triangles are for the *dent* populations and the closed triangels are for the *flint* population. The bars indicate the SED.

the cold treatment. For the 15°C treatment the trends were similar but the differences were less pronounced and only significant for the *dent* populations at day 3 and day 6. Figure 3b shows the relations between the leaf elongation rate and the ratio F_m/F_o for all genotypes in the 13°C treatments, measured on day 3 and day 6. The correlation coefficient was 0.74.

The induction kinetics of the Q-quenching differentiated very clearly



between the resistant (Fig. 4a) and the susceptible populations (Fig. 4b). After a treatment of 3 days, the Q-quenching of the susceptible populations was much higher for the 13°C treatment than for the 17°C treatment. In contrast, no temperature effects were found for the resistant populations. The effects were accentuated by a prolonged treatment at 13°C (not shown). At 15°C, only the susceptible *dent* population showed a significantly higher Q-quenching than at 17°C but only after a treatment duration of 6 days. The Q-quenching of the other populations was not affected at 15°C.

The induction kinetics of the E-quenching are presented in Fig. 5. The

effects were evidently dependent on the temperature and the duration of the treatments. Low temperature caused an increase of the energy quenching of all but the resistant *dent* population. This was already ovserved after 1 day exposure to a temperature of 15°C (Fig. 5b). The differences were bigger at 13°C (Fig. 5c) and after a prolonged exposure to 15°C (Figs. 5e, f). The recovery of the E-quenching after the cold stress was only partial (Fig. 5 g), and from the heights of the E-quenching it appeared that the susceptible *dent* recovered better than both *flint* populations. The opposite effects were displayed by the tolerant *dent*, that was not affected at all and the susceptible *flint* that showed a strong temperature effect but hardly any recovery within the interval allowed for.

Discussion

The interpretation of the various fluorescence parameters is complicated because of various interactions. Electron flow is coupled with proton translocation and as such linked to both Q-quenching and E-quenching (Krause and Weis 1984). However, E-quenching is more affected by the low temperature treatment than Q-quenching. Already after one day at 15°C or 13°C, a significant increase ($P < 0.01$) of E-quenching occurs in all populations except in the resistant *dent*. Enhanced E-quenching is an indirect effect of restraints in the CO_2 assimilation causing a surplus of ATP and consequently a high proton gradient across the thylakoid membranes. This delay may be due to a change in stomatal regulation or a decrease of the enzyme activity in the Calvin cycle. The E-quenching of the resistant *dent* population, in contrast to the others, seems hardly affected, neither at 15°C nor at 13°C. The cold tolerance of this population may be caused by its capacity to sustain a vivid carbon metabolism at low temperatures. The recovery of the E-quenching at an elevated temperature was fast (within two days) for all genotypes except the susceptible *flint*. The recovery of the susceptible *dent* was faster than of both *flint* types. This and the indifferent reactions of the resistant *dent* suggest a fast adaptation to fluctuating temperatures which might be beneficial for growth. This seems in accordance with the vigorous growth of the *dent* populations in field experiments.

To evaluate the results in more detail, associated effects of temperature on both Q-quenching and E-quenching should be considered. Processes that lead to an elevated E-quenching enhance thermal deactivation in photosystem 2 and this leads to a lower linear charge separation and a consequent increase of the Q-quenching (Weis and Berry 1987). Another complication is the possibility of a stress-induced activation of cyclic electron flow around

photosystem 2 via a low redox potential form of cytochrome *b*559 (Schreiber personal communication). In the present case this would mean that the susceptible populations tend to decrease the quantum efficiency of photosystem 2 in the light more than the resistant populations due to this cyclic electron flow, that might provide a protection to photo-oxidation, when the Calvin cycle is inhibited and toxic O_2 radicals are formed. If operative at all, this mechanism was apparently not adequate to protect the susceptible *dent* from damage of the photosystem 2, as may be derived from its low ratio F_m/F_o (Fig. 3a). It was evident that photo-inhibition of the water splitting enzyme complex (a decrease of F_m, van Hasselt, 1988) occurred at 13°C. In addition Fo increased which may be caused by a deficient energy transfer from the light-harvesting complex to photosystem 2, associated with the incorporation of a pro-protein in the pigment complex (Hayden et al. 1986) or by destruction of the D-1 protein of photosystem 2 due to oxidation of proteins in its vicinity (Kyle 1987, Öquist et al. 1987).

The relation between the ratio F_m/F_o and the leaf elongation rate is suggestive for a common basis for both phenomena. For breeding purposes this ratio would be a suitable screening attribute because it can be measured within a few seconds.

An attempt to summarize the findings, leads first of all to the conclusion that selection for resistance to chlorosis has led to a *dent* population that upholds both an active metabolism and an intact photochemistry at low temperature. For the *flint* population, the selection responses are less clear. The differences between the corresponding selections are confined to the increased Q-quenching of the susceptible population at low temperatures and the unaltered Q-quenching of the tolerant population. Analogously to the *dent* populations this suggests that the selection resulted in a divergent sensitivity of the photochemistry. The effect of a cold treatment on the E-quenching was not significantly different between the *flint* populations. However, the susceptible *flint* kept a much higher E-quenching during the recovery stage, which is indicative for a long period of low metabolic activity after a period of low temperature.

The presented method may be valuable for selection studies because the measurements are non-destructive and significant effects of low temperature have been detected shortly after the start of the treatment when no visible damage of the leaves was seen. However, it is difficult to prove that the observed responses of the chlorophyll fluorescence are indeed directly related to chlorosis. This however, is of secondary importance because the main interest is breeding for cold tolerance and chlorosis is a derivative of this property just as chlorophyll fluorescence. However, the kinetics of chlorophyll fluorescence are directly related to changes in the yield potential

through CO_2-assimilation and membrane integrity. For chlorosis, this linkage is less clear. Thus chlorophyll fluorescence provides a tool to classify the impact of low temperature on basic physiological processes. As such the method may improve the basis for genetic analyses of yield reduction due to low-temperature treatments.

References

Alberda T. (1969) The effect of low temperature on dry matter production, chlorophyll concentration and photosynthesis of maize plants of different ages. Acta Bot Neerlandica 18: 39–49

Baker N.R., East T.M. and Long S.P. (1983) Chilling damage to photosynthesis in young Zea mays. II. Photochemial function of thylakoids in vivo. J Exp Bot 34: 189–197

Bilger W. and Schreiber U. (1986) Energy dependent quenching of dark level chlorophyll fluorescence in intact leaves. Photosynth. Res 10: 303–308

Björkman O. (1987) Low temperature chlorophyll fluorescence in leaves and its relation to photon yield of photosynthesis in photoinhibition. In: Kyle D.J., Osmond C.D. and Arntzen C.J. (eds) Photoinhibition, pp 123–144. Elsevier

Dietz K.J., Schreiber U. and Heber U. (1985) The relationship between the redox state of Q_A and photosynthesis in leaves at various carbon dioxide, oxygen and light regimes. Planta 166: 219–226

Dolstra O., Jongmans M.A. and Jong K. de (1988) Divergent mass selection of resistance to low-temperature damage in maize (*Zea mays*L.). I. Resistance to chlorosis. Euphytica (in press)

Hasselt Phr van and Berlo H.A. van (1980) Photooxidative damage to the photosynthetic apparatus during chilling. Physiol Plantarum 50: 52–56

Hasselt Phr van (1988) Light induced damage during chilling. In: Chilling injury of horticultural crops. CRC press (in press)

Hayden D.B., Baker N.R., Percival M.P.P. and Beckwith B.P. (1986) Modification of photosystem II light-harvesting chlorophyll *a/b* protein complex in maize during chill-induced photoinhibition. Biochim Biophys Acta 851: 86–92

Hodgins, R. and Huystee R. van (1986) Porphyrin metabolism in chill stressed maize (*Zea mays* L.). J Plant Physiol 25: 326–336

Krause G.H., Briantais J.M. and Vernotte C. (1982) Photoinduced quenching of chlorophyll fluorescence in intact chloroplast and algae. Resolution into two components. Biochim Biophys Acta 679: 116–124

Krause G.H. and Weis E. (1984) Chlorophyll fluorescence as a tool in plant physiology. II Interpretation of fluorescence signals. Photosynth Res 5: 139–157

Kyle D.J. (1987) The biochemical basis for photoinhibition of photosystem II. In: Kyle D.J., Osmond C.D. and Arntzen C.J. (eds) Photoinhibition, pp 197–226. Elsevier

Miedema P., Post J. and Groot P.J. (1987) The effects of low temperature on seedling growth of maize genotypes. Agricultural Research Reports 926 Pudoc Wageningen

McWilliam J.R. and Naylor A.W. (1967) Temperature and plant adaptation. I Interaction of temperature and light in the synthesis of chlorophyll in corn. Plant Physiol. 42: 1711–1715

Öquist G., Greer D.H. and Ögren E. (1987) Light stress at low temperature. In: Kyle D.J., Osmond C.D. and Arntzen C.J. (eds) Photoinhibition, pp 67–87. Elsevier

Schreiber U. and Bilger U. (1987) Rapid assessment of stress effects on plant leaves by

chlorophyll fluorescence measurements. In: Tenhunen et al. (eds) Plant Response to Stress, pp 27–53. NATO ASI series Vol G15.

Schreiber U., Schliwa U. and Bilger W. (1985) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulated fluorometer. Photosynth Research 10: 51–62

Smillie R.M., Nott R., Hetherington S.E. and Öquist G. (1987) Chilling injury and recovery in detached and attached leaves measured by cholorophyll fluorescence. Physiol. Plantarum 69: 419–428

Taylor A O and Craig A S (1971) Plants under climatic stress. II Low temperature high light effects on chloroplast ultrastructure. Plant Physiol 47: 719–725

Wang C.Y. (1982) Physiological and biochemical responses of plants to chilling stress. Hort. Science 17: 173–186

Weis E. and Berry J.A. (1987) Quantum efficiency of Photosystem II in relation to 'energy'-dependent quenching of chlorophyll fluorescence. Biochim Biophys Acta 894: 198–208

Wise R.R. and Naylor A.W. (1987) Chilling enhanced photooxidation. Plant Physiol 83: 278–282