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Early Alterations of Chlorophyll Fluorescence by Light-Chilling in Cucumber (*Cucumis sativus*) Leaves and Their Usage as Stress Indicators

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오이 잎에서 저온 광저해에 의한 형광유도과정의 초기 변이와 스트레스 지표

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ABSTRACT

To investigate the early symptoms of light-chilling, alterations of chlorophyll fluorescence transients were monitored in cucumber (*Cucumis sativus* L. cv. Ilmichungjang) leaves. During 24 h chilling, decreases in (Fv)m/Fm, qE and qQ, and an increase in Fo were observed. The chilling effects were not recovered at room temperature, and a significant increase in Fo was observed during the recovery period. After 6 h chilling, 'dip' (D) level of the transients became obscure, and the negative slope after 'peak' (P) disappeared. The first derivative (dFv/dt) of the fast fluorescence rise curve was used to obtain more accurate information about the changes in the transients. The maximal rate of the fluorescence increase in the D-P rise curve (Fr) has been the most frequently used chilling stress indicator. However, a correct value of Fr could not be measured when the D level became obscure. This problem was overcome by introducing a new indicator, HFr (dFv/dt at Fv = 1/2 (Fv)m), and HFr gave very similar values to Fr. To monitor the changes in curvature around D level, another new parameter, ΔS(D-Fr), was also introduced. These three parameters decreased very sensitively during light-chilling. In addition, increases in these parameters were observed during the first 2 h chilling, but this increase in Fr was also observed in pea leaf discs dark-chilled for 15 min, suggesting that this very early change is a common response to chilling in both pea and cucumber leaves. Quenching coefficients were also very sensitive to chilling, especially qE. Discussion on the usage of these parameters as chilling stress indicators is given in the text.

Key words: Chlorophyll fluorescence, Cucumber, Fr, Light chilling, Photosynthesis, Stress indicator

INTRODUCTION

It is well-known that chilling-sensitive plants are more severely damaged while they are exposed to low temperatures under irradiation than in darkness (Neuner and Larcher 1991, Chun *et al.* 1993, Ha *et al.* 1996). Many attempts have been made to quantify chilling injury, which include changes in the rate of electrolyte leakage, lipid fluidity, respiration, or photosynthesis. However, most of the biochemical techniques are not suitable for use in chilling-tolerance screening, because they have been shown to be either very time consuming or unreliable and destructive to the plants under investigation (Wilson and Greaves 1990).

The chlorophyll (Chl) fluorescence measurement technique is a diagnostic tool that allows a simple, rapid, nondestructive and reliable quantitative assessment of the injuries of plants to chilling stresses before visible symptoms occur. Therefore, this technique has been applied in screening for stress tolerance of many crop plants (Renger and Schreiber 1986, Smillie and Hetherington 1990) and has been also applied to problems in forest ecology (Ball *et al.* 1994). Among parameters obtained from the analysis of Chl fluorescence transients or Chl fluorescence induction curves, the rate of Chl fluorescence rise (Fr) is the most frequently used indicator for chilling-tolerance screening because of its greater sensitivity than other parameters (Smillie and Hetherington 1983, Renger and Schreiber 1986, MacRae *et al.* 1986, Hetherington and Quist 1988, Smillie and Hetherington 1990).

The measured value of Fr has been an approximated estimate, because the measurement was frequently made from a line drawn on the induction curve to fit the fluorescence rise starting from the 'dip' (D) level as shown in Smillie and Hetherington (1990) and Chun *et al.* (1993). Therefore, Fr can be defined as the maximum rate of fluorescence rise after the D level. For an accurate analysis of the fluorescence rise curve, the first derivative analysis of the Chl fluorescence transient has been developed by Lee *et al.* (1995).

In this report, we, therefore, employed the first derivative analysis for more accurate measurement of Fr and other chilling-sensitive changes in the Chl fluorescence transients. A problem raised in the measurement of Fr and suggestions for other candidates of chilling stress indicators will be mentioned.

MATERIALS AND METHODS

Plant materials, growth and chilling treatment conditions

Cucumber (*Cucumis sativus* L. cv. Ilmichungjang) and pea (*Pisum sativum* cv. Giant) seeds were germinated in moistened cloth at 25℃ for 24 h in the dark, and grown at 25℃ −28℃ under continuous light from white fluorescence tubes giving PAR of 20 mol · m^{-2} · s^{-1}. For chilling, 20~30 day-old seedlings were placed at 4℃ under the same light condition used for their growth. Plants were well-watered to avoid water stress during chilling.

Chl fluorescence measurements

Chl fluorescence transients at room temperature were measured using a pulse modulated (PAM) fluorometer (Walz, Germany) as described by Lee *et al.* (1995). Leaf discs (10mm in diameter) were excised and placed immediately in petri dishes filled with distilled water. After dark-adaptation for 20 min, Chl fluorescence from the adaxial side of the leaves was measured.

The initial fluorescence (Fo) was measured with a modulating beam (0.2 mol · m^{-2} · s^{-1} PAR) alone, and an actinic light (AL) (110 mol · m^{-2} · s^{-1} PAR) was provided by a light emitting diode (H2000, Stanley, Japan). The maximum fluorescence (Fm) was induced by a saturated light pulse (2200 mol · m^{-2} · s^{-1} PAR) provided by a halogen lamp (KL1500, Schott, Germany) for 0.8 s. The maximum variable fluorescence ((Fv)m) was obtained by subtraction of Fo from Fm.

Chl fluorescence was measured in two ways. For the measurement of the fast fluorescence rise curve, 15,000 data were collected for 3 s using a data acquisition board (DAS16G, Metrabyte, USA) installed in an IBM compatible personal computer. One min after each measurement, a saturation light pulse was given to measure Fm. For the measurement of the slow overall Chl fluorescence transients, the actinic beam was turned on for more than 5 min. At the beginning of both measurements, Fo was obtained using the modulating beam.

Fluorescence rise curves and the derivative analysis

For the analysis of the fast fluorescence rise curves of the Chl fluorescence transients, 15,000 data were collected for 3 s, which were reduced to 3,000 points by averaging and were used for plotting as described by Lee *et al.* (1995). For the derivative analysis, the data were averaged further to give 150 points and the rate of fluorescence change (dFv /dt) was calculated.

Fluorescence quenching analysis

For the quenching analysis, saturation light pulses (2200 mol · m^{-2} · s^{-1} PAR) were given for 0.8 s to measure (Fv)s (Fv with a saturation beam on) during and after the measurement of the overall slow Chl fluorescence transients. The photochemical quenching coefficient (qQ) and the non-photochemical quenching (qN) were measured at the steady state with the AL turned on. The energy-dependent quenching coefficient (qE) and the coefficient for the fluorescence quenching that is not reversed by DCMU (3 (3′,4-dichlorophenyl)-1,1-dimethylurea) (qR) were measured after the AL was turned off.

Quenching coefficients were calculated as described by Oxoborough and Horton (1988); $qQ = 1 - (Fv)/(Fv)s$, $qN = 1 - (Fv)s/(Fv)m$, $qE = 1 - (Fv)s/(Fv)_E$, and $qR = 1 - (Fv)_E/(Fv)m$, where $(Fv)_E$ is (Fv)s measured after the qE relaxation reached to a plateau with the AL turned off.

RESULTS AND DISCUSSION

Chilling induced changes in Chl fluorescence transients

Light-chilling induced alterations of Chl fluorescence transients were monitored for 24 h. The overall slow Chl fluorescence transients are shown in Fig. 1, in which saturation pulses were given to measure Fm and quenching coefficients. Parameters obtained from the analysis of the Chl fluorescence transients and quenching coefficients are listed in Table 1.

During 24 h chilling in the light, a decrease in Fm and a slow increase in Fo were observed (Fig. 1, Table 1). Fo began to increase considerably at 24 h, and was almost doubled in the subsequent recovery period, suggesting that the PSII centers are irreversibly damaged. The decrease in Fm may be due to an increase in the rate of thermal energy dissipation (Somersalo and Krause, 1988, 1989) and/or a damage in PSII centers. The increase in Fo can be explained as an increase in Chl molecules which can not transfer the excited energy to reaction centers and/or an increase in modified PSII centers due to conformational changes in the D1 proteins (Kirilovsky *et al.* 1990).

Although the decrease in Fm and the increase in Fo have been used as chilling stress indicators (Renger and Schreiber 1986), the ratio (Fv)m/Fm or (Fm - Fo)/Fm seemed to be a better indicator, because the variations in Fm and Fo among samples can be

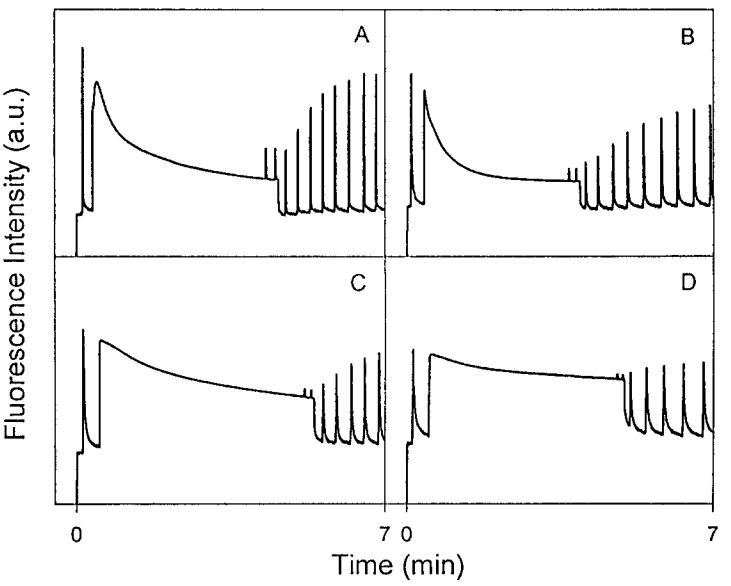

Fig. 1. Changes in the overall slow Chl fluorescence transients in cucumber leaves during light-chilling. (A) 0 h control, (B) 6 h, (C) 12 h, and (D) 24 h chilled at 4℃ in the light (20 μmol · m^{-2} · s^{-1}).

Table 1. Changes in parameters from the analysis of fast fluorescence rise curves in cucumber leaves during light-chilling

Time (h)	Fo (V)	Fmax (V)	(Fv)m/Fm	qQ	qE	qR
0	0.29	1.39	0.80	0.28	0.38	0.10
	(0.02)	(0.09)	(0.00)	(0.03)	(0.02)	(0.02)[b]
6	0.3	1.23	0.75	0.17	0.25	0.30
	(0.03)	(0.09)	(0.01)	(0.06)	(0.06)	(0.09)
12	0.32	1.07	0.71	0.06	0.19	0.24
	(0.02)	(0.09)	(0.86)	(0.02)	(0.03)	(0.09)
18	0.35	1.02	0.66	0.04	0.11	0.16
	(0.03)	(0.09)	(0.00)	(0.00)	(0.01)	(0.08)
24	0.37	0.95	0.62	0.04	0.10	0.09
	(0.02)	(0.04)	(0.02)	(0.00)	(0.02)	(0.03)
Recovery time[a] (h)						
25	0.55	0.37	0.37	0.06	0.07	0.19
	(0.06)	(0.07)	(0.02)	(0.01)	(0.01)	(0.01)
52	0.75	1.09	0.32	0.08	0.16	0.15
	(0.09)	(0.10)	(0.02)	(0.00)	(0.00)	(0.01)

[a]Recovery duration at room temperature after 24 h chilling.

[b]Values inside the parentheses are standard errors from multiple measurements.

reduced by using their ratio (Table 1) (because both Fm and Fo are relatively proportional to the Chl content), and (Fv)m/Fm has a physical meaning as the potential quantum yield of photochemical reaction (Horton and Bowyer 1990). (Fv)m/Fm decreased by 23% after 24 h chilling, and 60% reduction was observed during 52 h recovery period after 24 h chilling (Table 1). Fm/Fo has been also frequently used as a general stress indicator (Lichtenthaler 1988), which has the advantage of the reduction of variations, and the changes in Fm and Fo can be amplified by using their ratios.

Chilling-induced changes in quenching coefficients

Other remarkable changes observed during 24 h chilling in the light were an increase in Fv after peak and a decrease in qE quenching (Fig. 1, Table 1). The increase in Fv at steady-state is probably due to the hindrance in ATP synthesis and utilization by the carbon assimilation cycle (Govindjee and Satoh 1986) or due to a decrease in the electron transport activity by a damage in PSI by light-chilling (Havaux and Davaud 1994, Terashima *et al.* 1994).

The decrease in qE is due to the blockage of pH formation across the thylakoid mem-

brane (Horton and Hague 1988) and/or the hindrance of the role of xanthophyll cycle in the energy dissipation (Demmig-Adams *et al.* 1990). Peeler and Naylor (1988) reported uncoupling in cucumber thylakoids by chilling in the light, and Demmig-Adams *et al.* (1989) suggested that the formation of large amount of zeaxanthin is probably an important factor in the acclimation of plants to chilling temperatures. The photochemical quenching (qQ) also significantly decreased by light-chilling, and non-photochemical quenching remaining after the reversal of qE (qR) increased during the first 6 h light-chilling and decreased thereafter (Table 1).

Parameters showing early symptoms of chilling injury

To examine early symptoms of chilling injury in the light, changes in the fast fluorescence rise curves were monitored (Fig. 2). After 6 h, the fluorescence decrease after 'peak' (P) began to disappear, and the 'dip' (D) level became less prominent. The slowdown of the decreasing slope after P is probably due to the hindrance in reoxidation of QA- and/or other causes that result in the increase in Fv. The delay in the reoxidation of QA- can cause the less prominent D level.

First derivative analysis of the fluorescence rise curves and Fr

Time-dependent alterations of the fluorescence rise curve shown in Fig. 2 could be de-

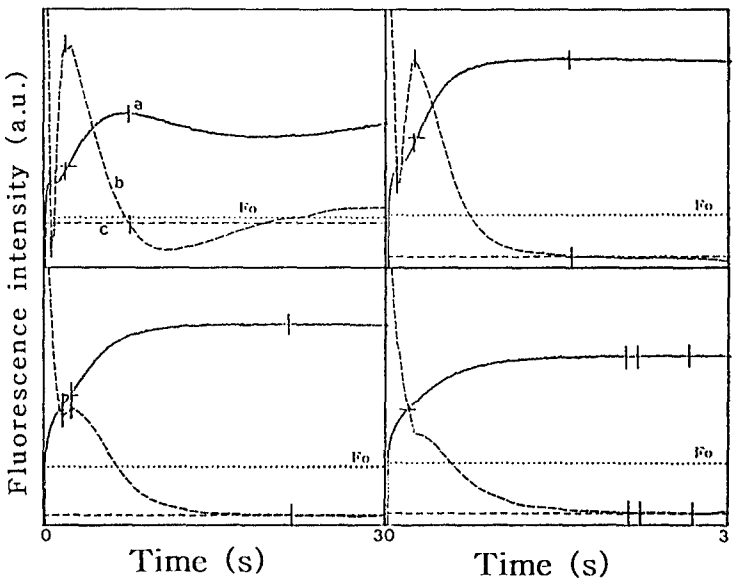

Fig. 2. Changes in the fast fluorescence rise curve and its first derivative plot in cucumber leaves during light-chilling. (A) 0 h control, (B) 6 h, (C) 12 h, and (D) 24 h chilled at 4℃ in the light (20 mol · m^{-2} · s^{-1}), where (a) is the original curve, (b) the first derivative curve, and (c) horizontal line where the value of the first derivative is equal to zero (vertical axis scale of the derivative plot is normalized).

scribed quantitatively in the first derivative (dFv /dt) plots (curve b). In the fluorescence rise curve (curve a) and in the derivative plot (curve b), three small vertical bars and one horizontal bar are marked. The first vertical bar corresponds to the D level, which is the first minimum point in the derivative plot. The third vertical bar corresponds to P level, where the derivative curve crosses the horizontal line (dFv /dt = 0, curve c).

The second vertical bar corresponds to the Fr level, which is the first maximum point following the first minimum point in the derivative plot. According to Smillie and Hetherington (1990), Fr is defined as the maximal rate of the increase in the fluorescence rise curve. By manually drawing a line following the fluorescence rise starting from D level, Smillie and Hetherington (1990) and Chun *et al.* (1993) calculated Fr. Considering this, Fr should be defined as the maximal rate of the fluorescence increase in the D-P rise curve. An accurate measurement of Fr could be possible by employing the first derivative analysis of the digitized data of the fluorescence rise curve.

Usage of HFr instead of Fr

A problem came out when the D level in the fast fluorescence rise curve became obscure (e.g. Fig. 2(D)). In the derivative analysis, Fr could not be measured without knowing the point of D level (see Fr at 24 h in Table 2). This problem occurred often at earlier times, sometimes at 6 h of light-chilling. To overcome this problem, the rate of fluorescence rise at a time, when Fv is equal to a half of Fp, was used as a new parameter, which was named as HFr (Table 2). As shown in Table 2, HFr gave very similar values to Fr. This new parameter would give a similar value to the one obtained by manual line drawing as shown in Smillie and Hetherington (1990) and Chun *et al.* (1993).

A new parameter, ΔS(D-Fr)

As mentioned previously, the D level became obscure by the progress of light-chilling stress (Fig. 1). In the derivative plot, the absolute difference in slope between at D and at Fr also decreased gradually by light-chilling. To show this change, a new parameter, ΔS(D-Fr), was defined as the absolute difference between dFv /dt at D and dFv /dt at Fr. The I-D decline reflects the equilibration time between Q_A and Q_B. The ΔS(D-Fr) reflects both the I-D decline and the D-P rise. During light-chilling, ΔS(D-Fr) decreased rapidly (Table 1). Therefore, the decrease in ΔS(D-Fr) could result from the shortening of the equilibration time between Q_A and Q_B and/or the delay in

Table 2. Changes in Fr, HFr and ΔS(D-Fr) from the analysis of fast fluorescence rise curves in cucumber leaves during light-chilling

Chilling Time (h)	Fr (V/s)	HFr (V/s)	ΔS(D-Fr) (V/s)
0	1.36	1.37	1.54
6	1.62	1.62	0.96
12	1.05	1.03	0.09
18	0.86	0.84	0.03
24	n.d.[a]	0.77	0.00

[a]not correctly determined

the reoxidation of Q_A^-, which was probably due to the chilling induced decrease in the membrane mobility resulting in the delay in the supply of Q_A from the plastoquinone pool. Kirilovsky *et al.* (1990) suggested that the chilling induced conformational changes in PSII reaction centers cause a shift in the redox potential of the Q_A/Q_B^- resulting in slowdown of the Q_A^- reoxidation.

Comparison of new parameters with Fr as early chilling stress indicators

To compare the sensitivities of the ΔS(D-Fr) with Fr and HFr, they were monitored during the first 8 h of the light-chilling (Fig. 3). Among them, ΔS(D-Fr) was the most sensitive parameter changing in the early chilling stress in the light. Therefore, this parameter would be useful for checking the short-term changes possibly in the rate of reoxidation of Q_A^- and in the thylakoid membrane mobility related to the supply of Q_A from the plastoquinone pool.

In Fig. 3, both HFr and Fr showed similar pattern, supporting that HFr could be used instead of Fr. Both of them increased during the first 2 h chilling in the light. In this period, ΔS(D-Fr) remained the same or showed a tendency of a slight increase.

Very early changes in Chl fluorescence transients

To examine the very early changes in Chl fluorescence transients, leaf discs were put in a dark aluminum chamber, and chilled for 15 min at 4℃ for dark adaptation. In the fast

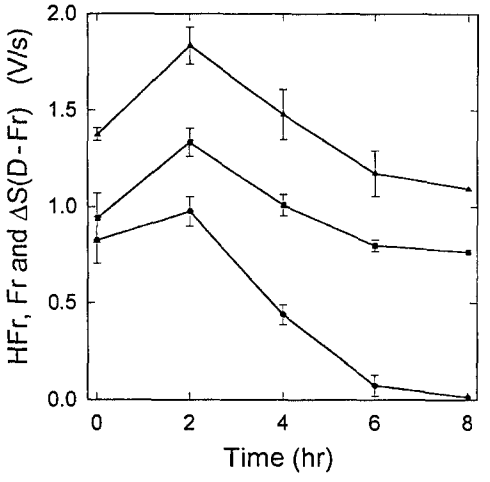

Fig. 3. Early changes in Fr, HFr and ΔS(D-Fr) from the analysis of fast fluorescence rise curves in cucumber leaves during light-chilling. (▲) HFr, (■) Fr and (●) ΔS(D-Fr). Leaves were chilled at 4℃ in the light (20 mol · m^{-2} · s^{-1}).

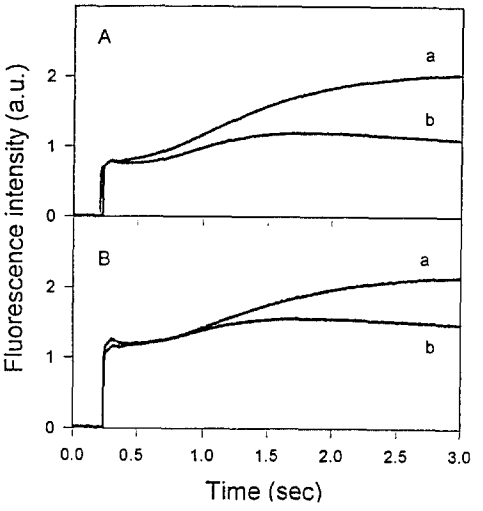

Fig. 4. Early changes in the fast fluorescence rise curves in cucumber and pea leaf discs during dark-chilling. (A) cucumber, and (B) pea leaf discs were chilled in the dark for 15 min. (a) 4℃ chilling, and (b) 25℃ control.

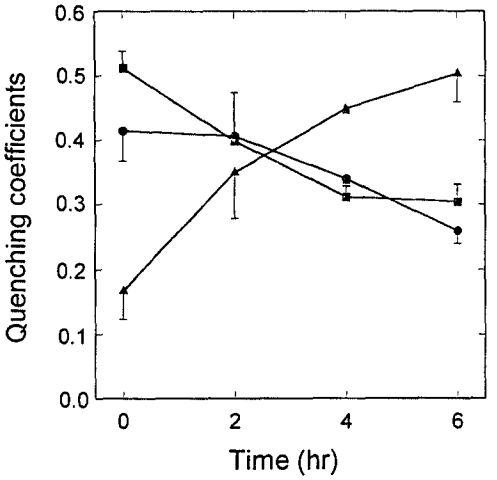

Fig. 5. Early changes in qE, qQ and qR in cucumber leaves during light chilling. (■) qE, (●) qQ and (▲) qR. Leaves were chilled at 4℃ in the light (20 mol · m^{-2} · s^{-1}).

fluorescence rise curve, the increase in Fr was observed by chilling only for 15 min in the dark (Fig. 4(B)). However, the same phenomenon was also observed in chilling-resistant pea (Fig. 4(A)), which suggests that the early increase in Fr is not an indicative symptom of chilling-sensitive plants at least in the case of cucumber when compared with pea.

Early changes in quenching coefficients

As shown in Table 1, significant changes in quenching coefficients were observed during the first 6 h light-chilling. Changes in the quenching coefficients were also monitored during the first 6 h light-chilling (Fig. 5). Among them, qE and qR showed significant changes by light-chilling for 2 h. Because the value of qR increased for 6 h, and dropped thereafter, qE can be chosen as the best indicator for light-chilling stress indicator among the three quenching coefficients. Peeler and Naylor (1988) reported uncoupling in cucumber thylakoids by light-chilling, even during the chloroplast isolation procedure on ice.

Conclusion with evaluation of the candidates for chilling stress indicators

Frequently used indicators for environmental stresses are Fm, Fo, (Fv)m/Fm, and Fm/Fo. All these parameters showed gradual changes during light-chilling in cucumber leaves (Fig. 1 and Table 1). Fo was a good indicator, especially when it was used in the recovery period. However, rice plants did not show significant changes in Fo, even in the recovery period (Lee *et al.* 1993). For several reasons mentioned above, (Fv)m/Fm or Fm/Fo can be recommended as good chilling stress indicators.

Although Fr has been frequently used as a chilling stress indicator, HFr is also recommended for some difficulties in monitoring Fr by using the first derivative analysis of Chl fluorescence transients. For monitoring early (2~6 h) effect of light-chilling, Fr, HFr and ΔS(D-Fr) were good indicators, and ΔS(D-Fr) was the most sensitive parameter changing in the early chilling stress in the light. Quenching coefficients also showed significant changes during 6 h light-chilling, and qE was the most sensitive indicator for light-chilling stress. During this period, there was no considerable decrease in (Fv)m/Fm.

For monitoring very early (less than 2 h) effect, qE was a good indicator. The changes in Fr, HFr and ΔS(D-Fr) were significant in this period, but similar changes were also

monitored in chilling-resistant pea leaves. Although these parameters can not be used as chilling stress indicators in these species, they may be useful for discriminating plants showing significant differences in thylakoid membrane lipid composition (Murata 1982, Murata 1983).

As mentioned above, it should be noted that chilling stress induced changes in some parameters are dependent on plant species under investigation. For example, Fo is not a good indicator for chilling-resistance screening among rice cultivars (Lee *et al.* 1993). Therefore, we recommend to use multiple parameters in screening stress resistant plants, and preliminary tests for choosing suitable parameters for a given stress are required, especially when the screening was performed among varieties of a species.

적 요

저온 민감성 식물인 오이(*Cucumis sativus* L. cv. Ilmichungjang)잎에서 저온 광저해에 의한 광합성 기구의 변이를 엽록소 형광유도과정의 변화를 분석하여 조사하였다. 24시간동안의 저온처리중 (Fv)m/Fm, qE, qQ의 감소와 Fo의 증가가 관찰되었다. 변화된 파라미터들의 값은 상온에서 회복되지 않았고, Fo는 저온처리후 상온에서 회복시 크게 증가하였다. 6시간 저온처리에 의하여 형광유도곡선의 'dip' (D) 수준이 불분명하여지고 극대치이후의 감소가 둔화되었다. 초기의 빠른 형광증가곡선의 변화는 시간에 대한 1차미분 (dFv/dt) 곡선을 구하여 보다 정밀하게 분석할 수 있었다. D 수준에서 극대치까지의 기울기 즉 Fr은 저온장애의 지표로 빈번히 사용되어왔으나, D 수준이 불분명하여질 때 1차미분법으로 분석이 곤란하였다. 따라서 Fv가 최대치의 1/2이 되는 시간의 형광증가율 (HFr)을 Fr 대신에 사용하였으며, 얻어진 값은 Fr과 매우 유사하였다. 이와 아울러 D 수준의 변화를 정량적으로 나타내기 위하여 새로운 지표 (ΔS(D-Fr))을 도입하였다. 이들 3가지 변수들은 저온 광저해 초기에 매우 민감하게 감소하였다. 이들 변수들은 저온처리 초기 2시간 이내에 증가하였다. 그러나, 저온 저항성 식물인 완두 잎에서도 유사한 증가현상을 보였기 때문에 초기 2시간 이내의 이들 값의 증가는, 적어도 이들 두 식물종 사이에는, 저온 광저해 지표로 사용될 수 없다. 형광소멸계수들도 저온 광저해에 의하여 매우 민감한 변이를 보였으며, 그 중에서도 qE가 좋은 지표로 생각된다. 저온 광저해 지표로서 형광유도과정의 분석에서 얻은 이들 변수들의 사용에 대하여 고찰하였다.

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Abbreviations

Chl, chlorophyll; (Fv)m, maximum Fv (Fm minus Fo); Fm, maximal level of Chl fluorescence; Fo, constant or initial Chl fluorescence; Fp, fluorescence level at 'peak' (P); Fr, maximal rate of fluorescence increase in the D-P rise curve; Fv, variable Chl fluorescence; HFr, rate of fluorescence increase at a time when Fv is a half of (Fv)m; O-I-D-P, Kautsky notation of the different points in the Chl fluorescence transients; Q_A, a primary quinone acceptor in PSII; qE, energy-dependent quenching; qQ, photochemical quenching; qR, non-photochemical quenching remaining after the reversal of qE; ΔS (D-Fr), the absolute difference between dFv /dt at D and dFv /dt at Fr.

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