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Collective Calculation of Actual Values of Non-Photochemical Quenching from Their Apparent Values after Chloroplast Movement and Photoinhibition

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Abstract

Chlorophyll fluorescence parameters such as F_v/F_m, *NPQ* and Φ_{II} (Y_{II}) are widely used to estimate the fitness and photosynthetic activity of plant leaves and non-photochemical dissipation of excessive excitation energy in photosystem II. The effect of chloroplast movement on these fluorescence parameters reduces the accuracy of estimations of the size of de-excitation processes, but there is no method to calculate correct parameters from altered (fluctuated) parameters. Chloroplast movement was recently identified as the "middle" kinetic component of *NPQ*. In this paper, we devised a complex but reasonable mathematical method to remove the effect of chloroplast movement on fluorescence parameters, based on our previously reported fluorescence theory. The fraction of "*S* fluctuation" (designated as σ) was estimated from fluorescence observations and used to calculate the non-fluctuated F_s and F_m' fluorescence yields. From the σ values, the fractional change of light absorbance by a leaf caused by chloroplast movement was estimated at 70% - 100%, which varied according to the experimental conditions and plant species. The effect of photoinhibition on fluorescence parameters was also examined in this paper. The photochemical and non-photochemical de-excitation sizes during photoinhibition (measured by the parameters *qPI* and *qSlow*) changed on a single regression line. Using this correlation, *qPI* and *qSlow* can be predicted from F_v/F_m, and the non-fluctuated F_m and F_o values can be estimated from the fluctuated F_m'' and F_o'' values.

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Keywords

***Arabidopsis thaliana*, Chlorophyll Fluorescence, Non-Photochemical Quenching, Rice**

1. Introduction

Typically, 2% of the light energy absorbed by a plant's chlorophyll is emitted as red fluorescence [1]. The fluorescence intensity (or fluorescence yield) of leaf chlorophyll changes according to light conditions because of the photochemical activities of the quenching processes in photosystem II. Such changes can be relatively easily measured by the pulse amplitude modulation (PAM) method. Several fluorescence parameters such as F_v/F_m F_v/F_m, Φ_{II} (also referred to Y_{II} in some publications) and *NPQ* are used to estimate the leaf fitness, rate of photosynthesis, and non-photochemical dissipation of excessive excitation energy [2] [3]. Agricultural applications of fluorescence parameters have also been developed, including the detection of pathogen infection, estimation of stress tolerance in different cultivars, and detection of non-visible symptom of manganese deficiency [4]-[7]. We previously compared the fluorescence parameters of rice cultivars and detected differences in the size of non-photochemical quenching (*NPQ*: measured by the parameter *NPQ*) between two subclasses of Indica and Japonica [8]. Although the agricultural significance of the difference in *NPQ* size is not clear, it may explain the cold tolerance of Japonica cultivars.

Fluorescence is emitted from chlorophyll molecules bound to the photosystem II super-complex. Fluorescence is one of the de-excitation processes of chlorophyll excitation energy. The fluorescence process is classified into 'basal dissipation,' together with internal conversion and intersystem crossing. Basal dissipations are intra-molecular de-excitation processes of chlorophyll excitation energy, whereas inter-molecular de-excitation processes (quenching) are divided into two groups: photochemistry and *NPQ*. Photochemistry represents de-excitation by the photosynthetic electron transport chain, especially pheophytin and plastoquinone, and the excitation energy is eventually transferred to photochemistry and used for carbon fixation. *NPQ* is the sum of the quenching processes except photochemistry. The Lake model approximates the interaction between photosystem II super-complexes. Based on the Lake model, ratios between rate constants of the de-excitation processes can be calculated with the Stern-Volmer approach [9]. By using the simple formula of the Lake model, we previously devised a simple set of calculations to provide more detailed comparisons of rate constants [10]. The fundamental formula of our calculation is as follows:

$$k_{fid} + k_{NPQ} + k_p = S \cdot k_f \cdot F^{-1} \quad (1)$$

Because the inverse value of fluorescence yield (F) is the key in this formula, Formula (1) is referred to as the "Inverse equation" in this paper, and k_{fid}, k_p, k_{NPQ} and k_f are the rate constants for basal dissipation, photochemistry, *NPQ* and fluorescence, respectively, and S is the sensitivity factor. The S and k_f values are normally constant. Our previous report [10] outlined additional information on this formula. The mathematical relationship between rate constants and fluorescence yields were integrated into a single figure (**Figure 1(a)**), where *qS*, *qL* and *NPQ* represent ratios between the rate constants, and F_v/F_m, Φ_{II}, Φ_{NPQ} and Φ_{NO} represent quantum yields. Specific formulae for the calculation of fluorescence parameters are described in the materials and methods section. Nomenclature for specific fluorescence yields varies in the literature. In this report, we use F_o, F_m, F_s, F_m', F_o'' and F_m'' for fluorescence yields with or without illumination of a saturating pulse after dark adaptation (F_o and F_m), under actinic illumination (F_s and F_m'), and during dark relaxation after actinic illumination (F_o'' and F_m'') [10]. These fluorescence yields were written as F_0, F_M, $F(t)$, F_M', F_0'' and F_M'' in a recent review [3].

NPQ is absent in the dark and induced by illumination. The *NPQ* components are divided into three parts (fast, middle and slow kinetics) according to the time span of their relaxation in the dark. Relaxation half-lives of the fast-kinetic, middle-kinetic and slow-kinetic *NPQs* are approximately 1 min, 10 - 20 min and greater than 1 h, respectively. The fast, middle and slow-kinetic *NPQs* were originally referred to as *qE*, *qT* and qI [11]. The middle component is also referred to as *qM* [12], and this term will be used here instead of *qT* to represent the middle-kinetic *NPQ*. *qE* is the main *NPQ* component in higher plants and regulated by the PsbS protein and xanthophyll cycle [13] [14]. The molecular identity of *qI* is not clear.

The influence of chloroplast avoidance movement on the apparent *NPQ* value has been suggested. Light ab-

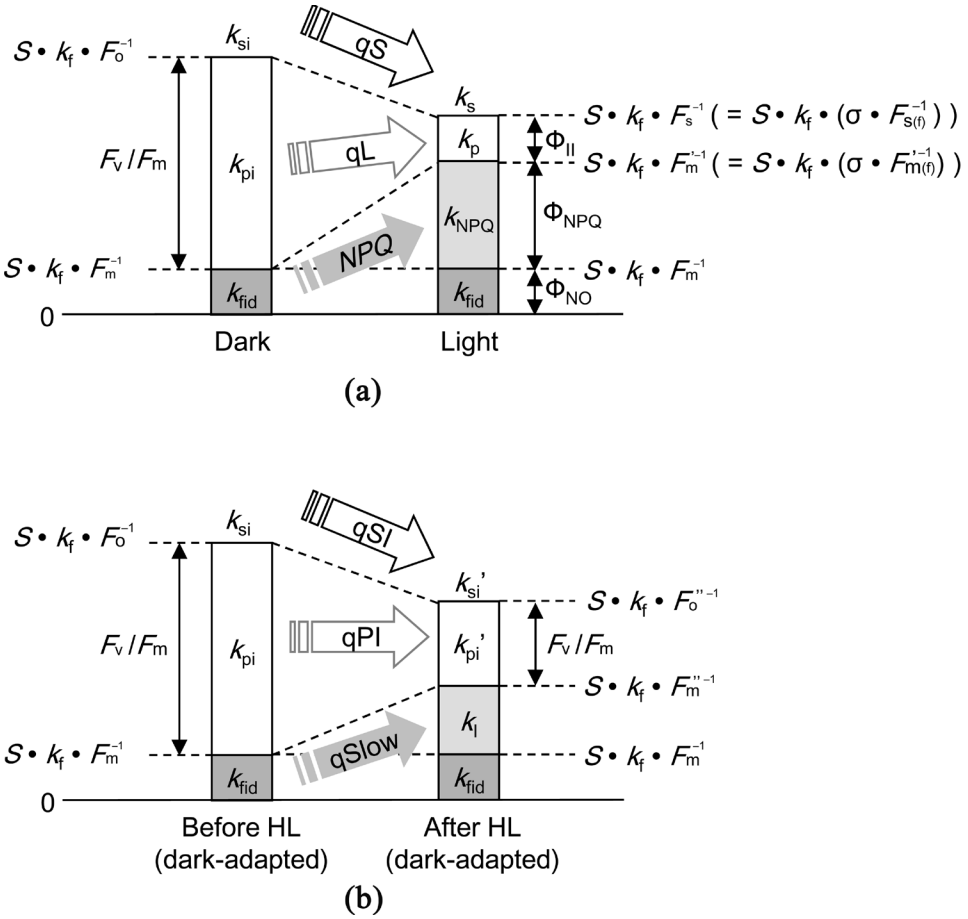

Figure 1. Overview of the relationship between fluorescence parameters, rate constants and fluorescence yields. (a) Values in standard measurements. The left-side bar represents values after dark adaptation, and the right-side bar represents values under actinic illumination. (b) Values during photoinhibition. The left-side bar represents values before photoinhibition (dark-adapted state), and the right-side bar represents values after photoinhibition (dark-adapted state). "HL" represents high light. Data modified from [10].

sorption is decreased after exposure to high light by approximately 10% in Oregon oxalis and approximately 5% in California manroot, whereas light absorption is not significantly influenced by exposure to high light in Japanese holly fern and common sunflower. Thus, chloroplast avoidance movement may increase the apparent *NPQ* value by reducing the light absorption in plant species such as Oregon oxalis [15]. Mathematically, alterations in the light absorption ratio caused by chloroplast avoidance movement change the *S* value. When light absorption is decreased by 10%, the *S* value is also decreased by 10%. Such hypothetical alterations in the *S* value are referred to as "*S* fluctuations", and the influence of *S* fluctuations on the apparent *NPQ* size has been mathematically estimated [10]. Arabidopsis (*Arabidopsis thaliana*) mutants such as *phototropin* 2 (*phot*2) and *chloroplast unusual positioning* 1 (*chup*1) are defective in chloroplast movement [16]-[18]. *phot*2 is the blue light receptor for the chloroplast avoidance response [16], and CHUP1 is a chloroplast-localized actin-binding protein [18]. Recently, a measurement using *phot*2 detected a clear effect of chloroplast avoidance movement on the apparent *NPQ* size. Light absorption was clearly decreased in the wild-type plant but not in *phot*2 mutant plants, and the decrease of light absorption was correlated with the relaxation kinetics of *qM*. *qM* is observed under white actinic light but absent under red actinic light, which is consistent with the activity of the *phot*2 photoreceptor. Thus, *qM* is caused by chloroplast avoidance movement [12] [19].

Because *qM* does not actually quench but rather increases the apparent *NPQ* size, the influence of *qM* should not be considered so that the actual *NPQ* size can be determined. The first goal of this report was to demonstrate how to eliminate such an unfavorable effect of *qM* on *NPQ* values through a calculation using the Inverse equation. We were also interested in a mutant analysis and measured the *NPQ* kinetics in the *phot*2 and *chup*1 mu-

tants, although we did not observe a clear effect of chloroplast movement on *NPQ*. This observation provides insight for an alternative method of minimizing the induction of *qM* during fluorescence measurements.

Photoinhibition also has similar but different effect on apparent *NPQ* size. The second goal of this paper was to estimate the effect of photoinhibition on the apparent *NPQ* size and demonstrate how to eliminate such effects in a series of calculation. Thus this paper collectively suggests novel mathematical processes to calculate actual values of chlorophyll fluorescence parameters from their possibly fluctuated values under various conditions.

2. Materials and Methods

2.1. *phot*2 and *chup*1 Mutants

Seeds of wild-type (accession/ecotype Col-0) and *phot*2-1 and *chup*1-2 mutants of *Arabidopsis thaliana* (L.) Heynh. were used. The plants were cultivated on Jiffy-7 (Jiffy International AS, Kristiansand, Norway), a nourished sphagnum peat pellet, for 33 d under an 8 h/16h light/dark scheme using white fluorescent light with a photosynthetic photon flux density (PPFD) of 60 μmol·m^{-2}·s^{-1} at 25˚C. Light intensity was made relatively low in order to enhance the effects of chloroplast movement.

2.2. *psbS*1 Mutant

Wild-type *Oryza sativa* (cultivar Hwayoung) and a homozygous line of *psbS*1 mutant (1C-032-61) developed by Gynheung An and obtained from the Pohang University of Science and Technology were selected in a previous report [8]. After germination in a growth chamber, the seeds were grown in nutrient-rich soil (Son-sol no. 1, Sumitomo Chemical, Tokyo, Japan) for 41 d in a partly sun-lit greenhouse. The characteristics of *psbS*1 were described by [8].

2.3. Fluorescence Measurement

To measure chloroplast movement, the expanded leaves of Col-0, *phot*2-1 and *chup*1-2 (referred to as wild-type, *phot*2 and *chup*1, respectively, in the text) were excised, placed on solid media, and dark-adapted for 1 h. After measuring the F_o and F_m, an actinic light (white LED) was illuminated. The F_s and F_m' were measured 30 min after the actinic light had been turned on (**Table 1**). The actinic light was also illuminated for 30 min for the relaxation analysis. The F_o'' and F_m'' were measured at 2, 5,10, 15, 20, 25, 30, 35, 40, 45 and 50 min after the actinic light had been turned off (**Figure 2**).

For photoinhibition, leaf discs were excised from the middle part of the leaf blades of the 6th leaves of the wild-type and *psbS*1 mutant and floated on deionized water. The leaves were dark-adapted for 2 h before measuring the F_o and F_m. The F_s and F_m' at each light intensity were measured after 5 min of actinic illumination (**Figure 3(a)** and **Figure 3(b)**), and photoinhibition was measured every 1 h. Every hour, the high actinic light was illuminated for 55 min and dark-adapted for 5 min before measuring the F_o'' and F_m''. After repeating this process 5 times, the actinic light was turned off so that the photosystem II could recover from photoinhibition (**Figure 3(c)**). Although the dark adaptation during actinic illumination was only 5 min, the effect of *qM* (chloroplast movement) would be negligible because the fluorescence was measured with a two-dimensional (2-D) PAM apparatus in rice leaves.

All of the fluorescence yields were measured with the Closed FluorCam (Photon Systems Instruments, Brno, Czech Republic), a 2-D PAM measuring apparatus. The actinic source was a red LED up to a PPFD of 200 μmol·m^{-2}·s^{-1} and white LED with a PPFD from 200 to 1500 μmol·m^{-2}·s^{-1}. Saturating pulses were supplemented for 780 ms with the white LED at a PPFD of approximately 6000 μmol·m^{-2}·s^{-1}.

Table 1. Chlorophyll fluorescence parameters of *phot*2 and *chup*1.

	F_v/F_m	Φ_{II} [*1]	*NPQ* [*1]	*NPQ* [*2]
Col-0	0.83 ± 0.00	0.30 ± 0.03	1.20 ± 0.14	1.97 ± 0.07
*phot*2	0.83 ± 0.00	0.22 ± 0.03	1.34 ± 0.08	1.94 ± 0.08
*chup*1	0.84 ± 0.00	0.27 ± 0.04	1.12 ± 0.13	1.72 ± 0.04

[*1]PPFD = 400 μmol·m^{-2}·s^{-1} for 30 min. [*2]PPFD = 1500 μmol·m^{-2}·s^{-1} for 30 min. Underlined data are significantly different from Col-0 by Student's *t*-test ($P < 0.05$). $n = 6$.

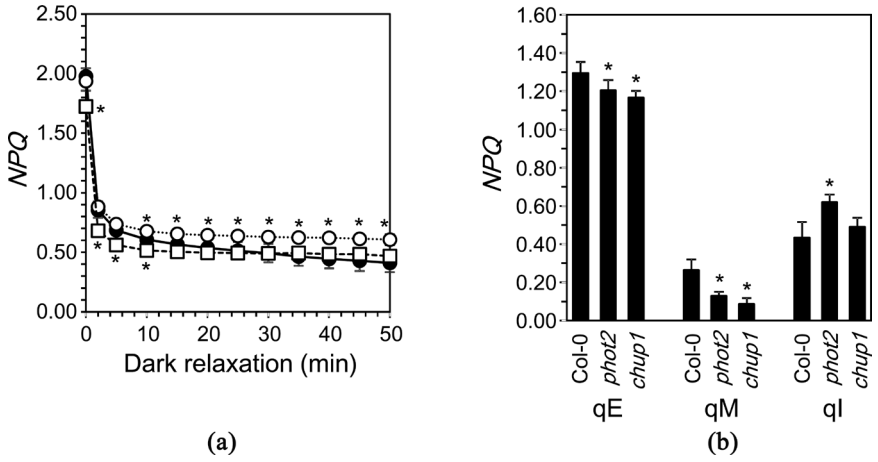

Figure 2. Fluorescence measurement during chloroplast movement. (a) *NPQ* values of Col-0 (wild-type: filled circle), *phot*2 (open circle) and *chup*1 (open square) during dark adaptation. *NPQ* was calculated from the F_m, F_m', and F_m'' values at each time point. The F_m' value and F_m'' values were measured after illumination with a white LED (PPFD = 1500 μmol·m^{-2}·s^{-1}) for 30 min. (b) *qE*, *qM* and *qI* components of *NPQ*. Data are represented by the means and SDs. Asterisks indicate significant differences from Col-0 by Student's *t*-test ($P < 0.05$). $n = 6$.

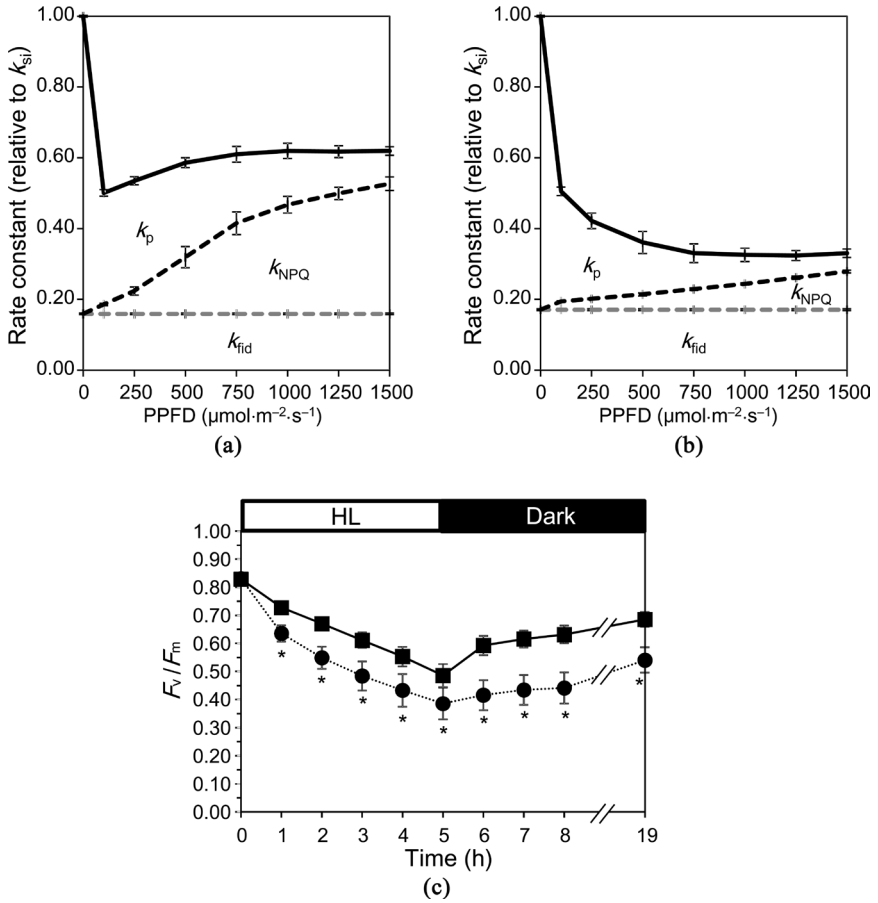

Figure 3. Fluorescence measurement during photoinhibition. (a) Relative sizes of the rate constants of basal dissipation (k_{fid}), *NPQ* (k_{NPQ}) and photochemistry (k_p) in wild-type rice at each light intensity. (b) Relative sizes of rate constants in *psbS*1. (c) F_v/F_m values of wild-type (filled square) and *psbS*1 (filled circle) during photoinhibition under high light (HL: white LED, PPFD = 1500 μmol·m^{-2}·s^{-1}) and dark recovery. Data are represented by the means and SDs. Asterisks indicate significant differences from the wild-type by Student's *t*-test ($P < 0.05$). $n = 8$.

2.4. Fluorescence Parameters

The fluorescence parameters were calculated with the following formulae [10]:

$$F_v/F_m = \left(F_m - F_o\right)/F_m$$

$$NPQ = F_m/F'_m - 1$$

$$qL = \left(F_s^{-1} - F_m'^{-1}\right)/\left(F_o^{-1} - F_m^{-1}\right)$$

$$qS = F_o/F_s$$

$$\Phi_{NO} = F_s/F_m$$

$$\Phi_{NPQ} = \left(F_m'^{-1} - F_m^{-1}\right)/F_s^{-1}$$

$$\Phi_{II} = \left(F'_m - F_s\right)/F'_m$$

$$F_v/F_m\left(\text{after photoinhibition}\right) = \left(F''_m - F''_o\right)/F''_m$$

$$qSlow = F_m/F''_m - 1$$

$$qPI = \left(F_o''^{-1} - F_m''^{-1}\right)/\left(F_o^{-1} - F_m^{-1}\right)$$

$$qSI = F_o/F''_o$$

Please refer to the text for the calculation used to determine the fluctuated values and *S* fluctuation fraction.

3. Results

3.1. Introduction of Chloroplast Movement Effects into the Inverse Equation

The first step for calculating the effect of chloroplast movement on *NPQ* size is to introduce the effect of chloroplast movement into the Inverse equation. The sensitivity factor in the Inverse equation consists of multiplying several factors. Although there may be additional factors, three factors are usually included: proportion of incident light that is absorbed by the leaf (A_{leaf}), fraction of absorbed light that is received by photosystem II ($\text{fraction}_{\text{PSII}}$) and instrumental response (Resp) [2] [9] [10]:

$$S = A_{\text{leaf}} \cdot \text{fraction}_{\text{PSII}} \cdot \text{Resp} \tag{2}$$

The A_{leaf} value is changed by chloroplast movement; thus, the *S* value changes proportionally with changes in the A_{leaf} value with chloroplast movement. Here, fluctuation-induced changes in the *S* value are represented as $S_{(f)}$ to discriminate this value from the *S* value before fluctuation. The original (non-inverse) equation of the Stern-Volmer approach without *S* fluctuation is as follows:

$$F = S \cdot k_f \Big/ \left(k_{fid} + k_{NPQ} + k_p\right) \tag{3}$$

The original equation for the fluctuated *S* value is as follows:

$$F_{(f)} = S_{(f)} \cdot k_f \Big/ \left(k_{fid} + k_{NPQ} + k_p\right) \tag{4}$$

The *F* value changes in proportion to the *S* value between Formulae (3) and Formulae (4). The inverse versions of these formulae are as follows:

$$k_{fid} + k_{NPQ} + k_p = S \cdot k_f \cdot F^{-1} \tag{1}$$

$$k_{fid} + k_{NPQ} + k_p = S_{(f)} \cdot k_f \cdot F_{(f)}^{-1} \tag{5}$$

The influence of chloroplast movement on the apparent fluorescence intensity can be estimated with the above formulae. The F_o and F_m values are not vulnerable to *S* fluctuations because these fluorescence yields are measured before illumination by actinic light:

$$k_{fid} + k_{pi} = S \cdot k_f \cdot F_o^{-1} \tag{6}$$

$$k_{fid} = S \cdot k_f \cdot F_m^{-1} \tag{7}$$

The F_s and F_m' values, however, are vulnerable to S fluctuations:

$$k_{fid} + k_{NPQ} + k_p = S_{(f)} \cdot k_f \cdot F_{s(f)}^{-1} \tag{8}$$

$$k_{fid} + k_{NPQ} = S_{(f)} \cdot k_f \cdot F_{m(f)}'^{-1} \tag{9}$$

Please note that F_o, F_m, $F_{s(f)}$ and $F_{m(f)}'$ are the actual measured fluorescence yields. $F_{s(f)}$ and $F_{m(f)}'$ are the fluctuations from the original F_s and F_m'.

3.2. Calculation of the Fraction of *S* Fluctuation

Formulae (6)-(9) cannot be directly compared because they have different *S* values (*S* and $S_{(f)}$). Therefore, the true rate constants are calculated from fluctuated *F* values by determining the fraction of *S* fluctuation (σ):

$$\sigma = S_{(f)} / S \tag{10}$$

There are several possible methods of calculating σ from fluorescence yields. Here, let us hypothesize that the non-fluctuated formulae for F_s and F_m' is as follows:

$$k_{fid} + k_{NPQ} + k_p = S \cdot k_f \cdot F_s^{-1} \tag{11}$$

$$k_{fid} + k_{NPQ} = S \cdot k_f \cdot F_m'^{-1} \tag{12}$$

Comparisons between Formulae (8) and (11) or (9) and (12) provide the σ value:

$$\sigma = F_{s(f)} / F_s = F_{m(f)}' / F_m' \tag{13}$$

The fluctuated and non-fluctuated values of F_s and F_m' can be compared with special experimental setups, such as comparisons between the wild-type and *phot*2 mutant and between white actinic light and red actinic light (as performed by [12]). In these cases, *NPQ* fluctuates in the wild-type or under white actinic light, whereas it does not fluctuate in *phot*2 or under the red actinic light. The non-fluctuated and fluctuated *NPQ* values (*NPQ* and $NPQ_{(f)}$) can also be used to calculate σ under such experimental setups. The non-fluctuated and fluctuated 1 + *NPQ* values are as follows:

$$1 + NPQ = 1 + \left(F_m'^{-1} / F_m^{-1} - 1 \right) = F_m'^{-1} / F_m^{-1} \tag{14}$$

$$1 + NPQ_{(f)} = 1 + \left(F_{m(f)}'^{-1} / F_m^{-1} - 1 \right) = F_{m(f)}'^{-1} / F_m^{-1} \tag{15}$$

Dividing Formula (14) by Formula (15) produces the following:

$$(1 + NPQ) / \left(1 + NPQ_{(f)} \right) = F_{m(f)}' / F_m' = \sigma \tag{16}$$

According to observations by [12], the *NPQ* value of the wild-type plant (fluctuated *NPQ*) is approximately 2.3 and that of the *phot*2 plant (non-fluctuated *NPQ*) is approximately 1.3 after exposure to actinic light for 1 h. Formula (16) produces an σ value of 0.70 from these data, which indicates that the light absorbance ratio and F_s and F_m' fluorescence values of the wild-type plant were decreased by as much as 30% by chloroplast avoidance movement in this experiment. Once the fraction of *S* fluctuation (σ) is calculated, the actual rate constants of the quenching processes are calculated using the following equations (note $S_{(f)} = \sigma S$):

$$k_{fid} + k_{NPQ} + k_p = S_{(f)} \cdot k_f \cdot F_{s(f)}^{-1} = S \cdot k_f \cdot \left(\sigma \cdot F_{s(f)}^{-1} \right) \tag{17}$$

$$k_{fid} + k_{NPQ} = S_{(f)} \cdot k_f \cdot F_{m(f)}'^{-1} = S \cdot k_f \cdot \left(\sigma \cdot F_{m(f)}'^{-1} \right) \tag{18}$$

Thus, the F_s^{-1} and $F_m'^{-1}$ values in the fluorescence parameter calculations are replaced by formulae including σ:

$$F_s^{-1} = \sigma \cdot F_{s(f)}^{-1} \tag{19}$$

$$F_m'^{-1} = \sigma \cdot F_{m(f)}'^{-1} \quad (20)$$

These alternative formulae for F_s^{-1} and $F_m'^{-1}$ are shown in **Figure 1(a)**. Again, σ is also proportional to the light absorption ratio (A_{leaf}), and measuring σ provides a simple method of estimating changes in the light absorption ratio with the PAM equipment.

3.3. Fluorescence Measurement in *phot*2 and *chup*1

The effect of chloroplast movement on fluorescence yield was unexpected. We also measured the fluorescence of *phot*2 and *chup*1, and **Table 1** shows the fluorescence parameters of the wild-type (Col-0), *phot*2 and *chup*1 plants. The *NPQ* values were not significantly different between the wild-type and *phot*2 plants even after exposure to actinic light (PPFD = 400 or 1500 μmol·m^{-2}·s^{-1}) for 30 min. In *chup*1, the *NPQ* value was only slightly smaller after exposure to high light (PPFD = 1500 μmol·m^{-2}·s^{-1}) but not significantly different under medium light intensity (PPFD = 400 μmol·m^{-2}·s^{-1}). Although the F_v/F_m values were high and similar between these genotypes, the Φ_{II} value was clearly smaller in the *phot*2 mutant. For unknown reasons, the growth of the *phot*2 mutant appeared somewhat defective under our growth conditions, which is not the normal growth pattern, although the cylindrical structure of palisade cells is mostly lost in *phot*2 [20].

The relaxation kinetics of *NPQ* after exposure to high light was also observed (**Figure 2(a)**). *NPQ* relaxation reached a quasi-plateau in *phot*2 and *chup*1 after 10 min in the dark, whereas the *NPQ* of the wild-type plant gradually decreased after 10 min in the dark. This small but continuous decrease of *NPQ* appears to represent chloroplast movement-induced *S* fluctuations. The *qE*, *qM* and *qI* sizes were estimated based on this relaxation analysis. *qE* is the fraction of *NPQ* relaxation within 5 min in the dark; *qM* is the fraction of *NPQ* relaxation between 5 and 50 min in the dark; and *qI* is the fraction of *NPQ* that does not relax after 50 min in the dark in this analysis (**Figure 2(b)**). The *qM* size that corresponded to chloroplast movement was slightly but significantly lower in *phot*2 and *chup*1 plants than in the wild-type plants, which is consistent with the original report [12]. *qE* was also slightly higher in the wild-type, which could have been caused by an apparent but not actual increase of *qE* by chloroplast movement. *qI* was significantly higher in *phot*2, reflecting the defective growth of this mutant. The σ value calculated by comparing the *NPQ* values of the wild-type and *chup*1 plants after exposure to high light was as high as 0.92, which is in contrast to the estimated value of 0.70 in the original report. Such a reduced induction of chloroplast avoidance movement could have been caused by differences in the PAM equipment (discussed later).

3.4. Photoinhibition in Rice *psbS*1

Along with chloroplast movement, we were interested in the effect of photoinhibition on the apparent *NPQ* size. To analyze photoinhibition, we measured a rice (*Oryza sativa*) *psbS*1 mutant that cannot induce qE [8]. **Figure 3(a)** and **Figure 3(b)** show rate constants of the photochemistry, *NPQ* and basal dissipation under various light intensities in the wild-type and *psbS*1 plants, respectively. The fractional difference of the total de-excitation capacity by illumination (*qS*) in the wild-type rice was approximately 0.6, which was similar to that of the wild-type *Arabidopsis* [10]. The *qS* was decreased to less than 0.4 in the *psbS*1 plant because of the lack of *qE* induction. The decreased de-excitation capacity of *psbS*1 caused a hyper-sensitivity to high light. The F_v/F_m values were observed every 1 h for up to 5 h under illumination by high light and then allowed to recover for an additional 14 h in dark conditions (**Figure 3(c)**). The F_v/F_m values decreased with time under illumination, and the decrease was more severe in the *psbS*1 than in the wild-type plants. The F_v/F_m values slowly recovered in the dark but did not fully recover within 14 h time period. Although excised leaf discs were used in this experiment, these results indicate that damage by photoinhibition can be carried over until the following day. The lower F_v/F_m values appear to be common in field experiments and analyses of stressed plants. The F_v/F_m values of the undamaged leaves were 0.82 - 0.85.

A reduction in F_v/F_m values by photoinhibition is usually attributed to a decreased rate constant in the dark-adapted photochemistry (k_{pi}). However, the induction of slow components of *NPQ* also reduce F_v/F_m, which is the maximum yield of the photochemistry. **Figure 1(b)** illustrates the changes in the rate constants of the de-excitation processes before and after photoinhibition [10]. The F_o and F_m values after photoinhibition are referred to as F_o'' and F_m'', respectively, because changes in the F_o and F_m values occur after photoinhibition. The rate constant of dark-adapted photochemistry changes from k_{pi} to k_{pi}' with photoinhibition. The ratio be-

tween k_{pi} and k'_{pi} (shown by the parameter *qPI*) represents the fraction of the functional photosystem II reaction center. The parameter qSlow represents the size of *qI* (the rate constant is denoted as k_I). As shown in **Figure 1(b)**, both a decrease of photochemistry and induction of *qI* are expected to cause a decrease in the F_v/F_m value.

3.5. Correlation between *qPI* and *qSlow* Values

The *qPI* and *qSlow* values were calculated for the wild-type and *psbS*1 leaves. As expected, the *qPI* value decreased and *qSlow* value increased under illumination. *qPI* was smaller in *psbS*1 plants than in the wild-type plants at the same time points, and *qSlow* was greater in *psbS*1. The *qPI* value gradually increased and *qSlow* value gradually decreased in the dark. A plot of the *qSlow* values during illumination and dark recovery against the *qPI* values (**Figure 4(a)**) shows that all of the data, including that of the wild-type and *psbS*1 plants, roughly fit along a single regression curve ($R = 0.958$ when fitted to a quadratic function):

$$qSlow = 1.70 \cdot (qPI)^2 - 4.10 \cdot (qPI) + 2.397 \quad (21)$$

If *qPI* and *qSlow* are correlated during photoinhibition, then parameters such as F_v/F_m and *qSI* are naturally correlated with *qPI* and *qSlow*.

It is nearly impossible to measure the fluorescence yield of an unstressed state when fluorescence is measured on damaged leaves of stressed plants. Alternatively, a mathematical estimation of fluorescence yield to deter-

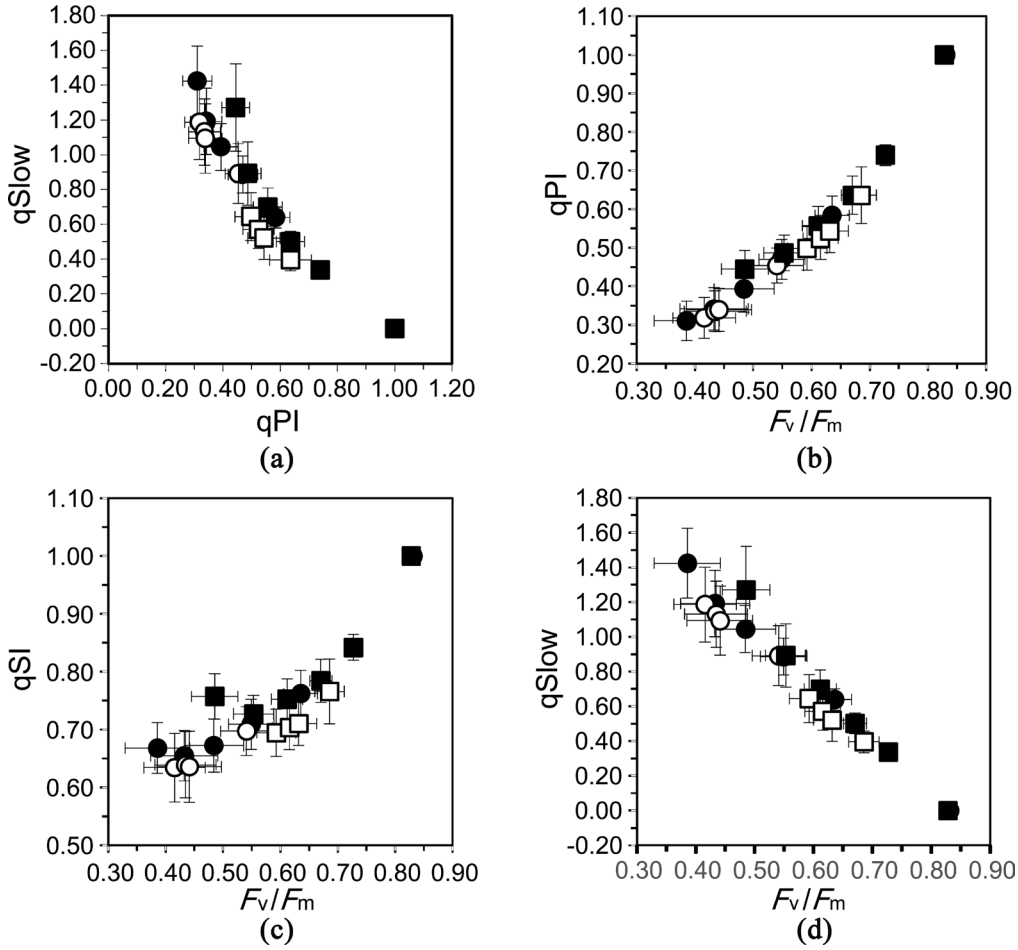

Figure 4. Relationship between parameters of photoinhibition. F_v/F_m values in **Figure 3(c)** and their derivative *qPI*, *qSI* and *qSlow* values were compared. Correlations of *qPI*-*qSlow* (a) F_v/F_m-*qPI* (b) F_v/F_m-*qSI* (c) and F_v/F_m-*qSlow* (d) were examined for the wild-type during photoinhibition (filled square), wild-type during recovery (open square), *psbS*1 during photoinhibition (filled circle) and *psbS*1 during recovery (open circle). Data are represented by means and SDs. $n = 8$.

mine the F_o and F_m of an unstressed (non-fluctuated) state is possible based on the correlation between the fluorescence parameters observed above. The correlation of F_v/F_m with the parameters *qPI*, *qSI* and *qSlow* during photoinhibition in the wild-type and *psbS*1 plants are shown in **Figures 4(b)-(d)**. The formulae of the quartic regression functions for these graphs are as follows:

$$qPI = 3.34\cdot\left(F_v/F_m\right)^4 - 1.36\cdot\left(F_v/F_m\right)^3 - 2.80\cdot\left(F_v/F_m\right)^2 + 3.16\cdot\left(F_v/F_m\right) - 0.504 \quad (22)$$

$$qSI = 5.86\cdot\left(F_v/F_m\right)^4 - 7.73\cdot\left(F_v/F_m\right)^3 + 2.67\cdot\left(F_v/F_m\right)^2 + 0.547\cdot\left(F_v/F_m\right) + 0.346 \quad (23)$$

$$qSlow = 17.1\cdot\left(F_v/F_m\right)^4 - 36.3\cdot\left(F_v/F_m\right)^3 + 27.3\cdot\left(F_v/F_m\right)^2 - 11.6\cdot\left(F_v/F_m\right) + 3.45 \quad (24)$$

The R^2 values for these regression lines were 0.993, 0.936 and 0.968. **Table 2** shows the values of *qPI*, *qSI* and *qSlow* for each F_v/F_m value (0.30 - 0.83) calculated with these formulae. For example, in a damaged leaf with an F_v/F_m value of 0.70, only 67% of the photochemistry of photosystem II is functional (*qPI* = 0.67), the total capacity of de-excitation is reduced to 79% (*qSI* = 0.79), and the relative size of *qI* compared with that of basal dissipation is 0.36 (*qSlow* = 0.36). The original F_o and F_m values can also be calculated from the fluctuated F_o'' and F_m'' values:

$$F_o = qSI\cdot F_o'' \quad (25)$$

$$F_m = \left(1+qSlow\right)\cdot F_m'' \quad (26)$$

One of the potential problems in fluorescence measurements of damaged leaves is underestimating the *NPQ* size. The actual *NPQ* is calculated as follows:

Table 2. Conversion of F_v/F_m values to parameters of photoinhibition.

F_v/F_m	0.83	0.82	0.81	0.80	0.79	0.78	0.77	0.76	0.75
qPI	0.997	0.965	0.934	0.904	0.875	0.848	0.822	0.798	0.774
qSI	1.001	0.977	0.955	0.935	0.916	0.898	0.881	0.866	0.851
qSlow	-0.012	0.011	0.035	0.061	0.087	0.115	0.143	0.173	0.203
F_v/F_m	0.74	0.73	0.72	0.71	0.70	0.69	0.68	0.67	0.66
qPI	0.752	0.730	0.710	0.690	0.671	0.654	0.637	0.620	0.605
qSI	0.838	0.825	0.814	0.803	0.793	0.784	0.775	0.767	0.760
qSlow	0.234	0.265	0.297	0.329	0.362	0.395	0.428	0.461	0.494
F_v/F_m	0.65	0.64	0.63	0.62	0.61	0.60	0.59	0.58	0.57
qPI	0.590	0.575	0.562	0.548	0.535	0.523	0.511	0.499	0.488
qSI	0.753	0.746	0.741	0.735	0.730	0.725	0.721	0.716	0.712
qSlow	0.528	0.561	0.594	0.628	0.661	0.693	0.726	0.758	0.790
F_v/F_m	0.56	0.55	0.54	0.53	0.52	0.51	0.50	0.49	0.48
qPI	0.477	0.466	0.456	0.445	0.435	0.425	0.415	0.405	0.395
qSI	0.708	0.705	0.701	0.697	0.694	0.690	0.687	0.683	0.680
qSlow	0.822	0.854	0.885	0.916	0.946	0.976	1.006	1.036	1.065
F_v/F_m	0.47	0.46	0.45	0.44	0.43	0.42	0.41	0.40	0.39
qPI	0.384	0.374	0.364	0.354	0.343	0.332	0.322	0.310	0.299
qSI	0.676	0.673	0.669	0.665	0.661	0.656	0.652	0.647	0.642
qSlow	1.094	1.123	1.152	1.180	1.208	1.236	1.265	1.293	1.321
F_v/F_m	0.38	0.37	0.36	0.35	0.34	0.33	0.32	0.31	0.30
qPI	0.287	0.276	0.263	0.251	0.238	0.225	0.211	0.197	0.182
qSI	0.637	0.632	0.627	0.621	0.615	0.609	0.603	0.596	0.589
qSlow	1.349	1.377	1.406	1.434	1.464	1.493	1.523	1.554	1.585

$$NPQ = F_m / F'_m - 1 = \left(F'^{-1}_m - F^{-1}_m\right) / F^{-1}_m = k_{NPQ} / k_{fid} \quad (27)$$

In damaged leaves, the F''_m value is smaller than that of the F_m. The fluctuated *NPQ* value with F''_m ($NPQ_{(f)}$) is calculated as follows:

$$NPQ_{(f)} = F''_m / F'^{-1}_m - 1 = \left(F'^{-1}_m - F''^{-1}_m\right) / F''^{-1}_m = \left(k_{NPQ} - k_I\right) / \left(k_{fid} + k_I\right) \quad (28)$$

The numerator of the right-most section of Formula (28) is smaller than that of Formula (27), and the denominator is larger than that of Formula (27) because of the value of k_I. Thus, *qI* decreases the apparent value of *NPQ*. The actual *NPQ* sizes can be estimated with the actual F_m calculated with Formula (26). Alternatively, the actual *NPQ* can be calculated from *qSlow* and $NPQ_{(f)}$ as follows:

$$\begin{aligned} \left(1+NPQ\right) / \left(1+NPQ_{(f)}\right) &= F_m / F''_m = 1 + qSlow \\ NPQ &= \left(1+qSlow\right) \cdot \left(1+NPQ_{(f)}\right) - 1 \end{aligned} \quad (29)$$

4. Discussion

Although a clear effect of chloroplast avoidance movement on *NPQ* was demonstrated, especially the middle-kinetic *NPQ* component *qT* (*qM*) [12], the method of removing the effect of chloroplast movement on the apparent *NPQ* size have not been understood. The key for such calculations is to determine the σ value of light absorbance caused by chloroplast movement because σ is equivalent to the fractional difference of the sensitivity factor *S*. In this report, we showed a method of calculating σ by comparing the fluctuated and non-fluctuated F_s, F'_m or *NPQ* values. In addition to the comparison of these values, a comparison of *NPQ* values during dark relaxation, *i.e.*, the *NPQ* values before and after *qM* relaxation (approximately 2 min and 45 min), may also roughly estimate the σ value. A further alternative is to compare the apparent k_{pi} values (non-fluctuated $F_o^{-1} - F_m^{-1}$ value and fluctuated $F'^{-1}_{o(f)} - F'^{-1}_{m(f)}$ value) during actinic illumination. In this case, σ is calculated as follows:

$$\sigma = k_{pi} / \left(k_{pi} / \sigma\right) = \left(F_o^{-1} - F_m^{-1}\right) / \left(F'^{-1}_{o(f)} - F'^{-1}_{m(f)}\right) \quad (30)$$

Pleaes note the F'_o values are obtained under illumination of far-red light to preferentially excite photosystem I [2]. It is recommended to calculate the σ values to determine the actual sizes of the rate constants and parameters under experimental conditions in which chloroplast avoidance movement is clearly induced, such as observations under high light intensity for a long period (30 min or more). The growth stages and plant species may also influence the degree of chloroplast movement. Notably, rice leaves did not show a decrease of light absorbance under high illumination in a previous report [21]. It is also noteworthy that the middle-kinetic component of *NPQ* (approximate size = 0.3) is observed even in the *npq*4 *phot*2 mutant, which is defective in both *qE* and *qM* [19]. The size of the middle *NPQ* in the *npq*4 *phot*2 plant is similar to that observed in the rice leaves [10], suggesting that the middle *NPQ* observed in the rice leaves in our previous report was not caused by chloroplast movement. Collectively, the *NPQ* components cannot be clearly discriminated based on relaxation kinetics; however, *qM* (chloroplast avoidance movement) and qI (unknown slow *NPQ*) overlap until 1 h of relaxation in the dark. The size of *qI* relaxing within 1 h is typically 0.3, and *qM* would be negligible in rice leaves.

A restricted effect of chloroplast movement was observed on the σ value in our experiment with Arabidopsis *phot*2 mutant, although this effect was clearly observed in the original report (**Figure 2**), and the same mutant grown under similar environments was observed under similar measurement conditions in these two experiments. A clear difference between these experiments was the PAM equipment. An ordinary type of PAM fluorometer that measures spots on the leaves (PAM 101, Heinz-Walz GmbH, Effeltrich, Germany) was used in the previous experiment, and a 2-D PAM fluorometer measuring 2-D images (Closed FluorCam, Photon Systems Instruments, Brno, Czech Republic) was used in our experiments. In the measurements with the 2-D PAM, the leaf samples were placed near the bottom of a closed box, and fluorescence was observed with a camera at the center of the ceiling of the box with illumination from LED panels at the edges of the box ceiling. Thus, the leaves are illuminated from two to four sideways angles in 2-D PAM, and this illumination most likely caused a lack of clearly observed chloroplast avoidance movement in our experiments. The σ value was reduced by only 10% in the 2-D PAM, which is approximately one-third of that observed with the ordinary PAM. The measure-

ment with the 2-D PAM is an alternative method of reducing the effect of chloroplast movement on fluorescence yields and parameters.

In addition to *NPQ*, the *qL* value can also be affected by *S* fluctuation caused by chloroplast movement. *qL* represents the "openness" of the photosystem II photochemistry, and it can be calculated by two different formulae. The first formula (qL_1) includes F_s, F_m' and F_o' in the calculation as follows [9] [10]:

$$qL_1 = \left(F_s^{-1} - F_m'^{-1}\right) \Big/ \left(F_o'^{-1} - F_m'^{-1}\right) \tag{31}$$

The second formula (qL_2) includes F_o, F_m, F_s and F_m' in the calculation as follows [10] [22]:

$$qL_2 = \left(F_s^{-1} - F_m'^{-1}\right) \Big/ \left(F_o^{-1} - F_m^{-1}\right) \tag{32}$$

qL_1 is not affected by *S* fluctuations because all of the F_s, F_m' and F_o' values are fluctuated by chloroplast movement. The apparent value of qL_2 is increased by chloroplast avoidance movement because of the $1/\sigma$-fold increase in F_s^{-1} and $F_m'^{-1}$ values.

In addition to chloroplast movement, the apparent *NPQ* size also fluctuates during photoinhibition. The correlation and formula of the regression curve between *qPI* and *qSlow* was determined (**Figure 4(a)**). To our knowledge, this is the first examination of correlations in the changes of rate constants between the photochemical and non-photochemical processes during photoinhibition. Further analyses are required to determine whether the regression curves and their formulae for rice leaves are applicable to other plant species or other conditions of photoinhibition. However, the regression curves for the F_v/F_m-*qSI* and F_v/F_m-*qSlow* correlations and their values (**Table 2**) provide a mathematical method of estimating the non-fluctuated F_o and F_m values from the fluctuated F_o'' and F_m'' values observed in damaged leaves. The *NPQ* values of damaged plants reported in other publications were small. For example, the *NPQ* of a control *Physcomitrella patens* was 2.82, and the *NPQ* was curiously decreased to 1.64 when treated with 0.8 M sorbitol [23]. The F_v/F_m values for the control and stressed plants were 0.73 and 0.32, respectively, in this report. The *qSlow* values corresponding to these F_v/F_m values were 0.265 and 1.523, respectively (**Table 2**). The fractions of the fluctuation of basal dissipation (1 + *qSlow*) were 1.265 and 2.523. The non-fluctuated *NPQ* values calculated from the above data were 3.83 for the control and 5.66 for the stressed plant. Thus, the *NPQ* was highly induced in the stressed plant; however, the apparent *NPQ* value was greatly decreased by the fluctuation of basal dissipation.

In this paper, the mathematical methods used to estimate the fluctuation of fluorescence yields and parameter values accompanying chloroplast movement and photoinhibition were derived. The fluctuation of *S* values by chloroplast movement should be calculated by a direct measurement of leaf absorbance; however, accurate measurements of light transmittance and light reflection in the leaves are difficult to perform. The alternative method of calculating *NPQ* fluctuations after photoinhibition is to perform a comparison between winter and summer leaves. The fluorescence of winter leaves are affected by photoinhibition, whereas summer leaves are not. A whole-year measurement of pine needles revealed an induction of *qSlow* ("stable *NPQ*") as large as 7 in mid-winter [3] [24] [25]. This method requires special equipment and a long time course.

5. Conclusion

A line of new methods of calculating fluctuations that accompany fluorescence measurements were suggested in this paper. Although these calculations are theoretical biology came simply from mathematical interests and difficult to understand, the calculations in this report may enable rapid estimations of fluctuations and benefit correct description of photosynthetic states of plant leaves in the future.

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