The *Arabidopsis thaliana* mutant, *cfa1*, was defective in O_2-dependent photosynthetic pathways.

シロイヌナズナ変異体 *cfa1* は酸素依存的な光合成経路に欠損を示す

2008

Master thesis

Laboratory of Signal Transduction
Department of Integrated Biosciences
Graduate School of Frontier Sciences
The University of Tokyo

66542
Kazuhiro Hananoi

Tutor: Kintake Sonoike

シロイヌナズナ変異体 *cfa1* は酸素依存的な光合成経路に欠損を示す

2008年3月修了　先端生命科学専攻　生命応答システム分野
学生証番号：66542　氏名：花野井和弘　指導教員：園池公毅　准教授

キーワード：water-water cycle、光合成電子伝達、シロイヌナズナ、クロロフィル蛍光

序論

植物にとって光は必要不可欠な要因でもあり、危険因子でもある。光化学系Ⅱ（PSⅡと略す）アンテナタンパク質に吸収された光エネルギーによって、PSⅡで水分子から引き抜かれた電子は、電子伝達鎖を経由してNADPHという還元力の形で貯えられる。NADPHは、主に二酸化炭素固定によって消費される(図1)。しかし強光などの、二酸化炭素固定が必要とする以上の電子が流入する条件下では、電子伝達鎖は過還元状態となり、過剰な電子が酸素と反応することで活性酸素種が生成され、光障害を引き起こす。

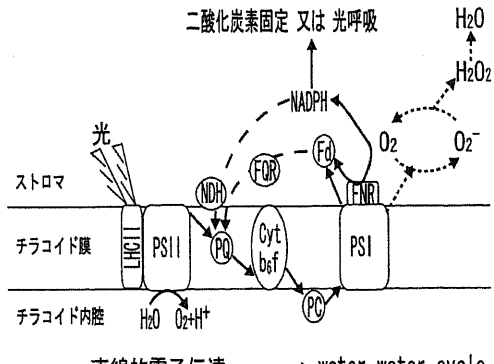

図1 様々な光合成経路の概略図

しかし、植物は電子伝達鎖の過還元状態を解消する機構を持っている。それが光呼吸、water-water cycle、光化学系Ⅰ(PSⅠと略す)循環的電子伝達である（図1)。これらの代替的な光合成経路は過還元状態を緩和すると共に、熱放散という過剰な光エネルギーを安全な熱として放出する機構も誘導する。そのため代替的な光合成経路は、非常に重要であると考えられている。

代替的な光合成経路のうち、光呼吸はルビスコのオキシゲナーゼ反応によって制御される。一方、water-water cycleやPSⅠ循環的電子伝達の制御機構に関しては明らかになっていることが少ない。

本研究室では、WTとクロロフィル蛍光挙動の異なるシロイヌナズナ変異体がいくつか単離されている。PSⅡアンテナタンパク質によって吸収された光エネルギーは、1）光合成を駆動するために使われる、2）熱放散によって放出される、のどちらかの反応によって消費される。そしてどちらの反応によっても消費されなかったエネルギーがクロロフィル蛍光として放出されることを考えると、これらの変異体は 1）又は 2）の反応に何らかの欠損を示すと考えられる。このような変異体の一つである *cfa1* 変異体は、暗条件から明条件に移した後約20-60秒でのみWTよりクロロフィル蛍光が高いという表現型を示す。このような条件下では、water-water cycleやPSⅠ循環的電子伝達が光合成を駆動するためのスターターとして機能することが示唆されていること、またルビスコなどの二酸化炭素固定に関わる酵素の光活性化に時間を要することを考え合わせると、*cfa1* 変異体はwater-water cycleかPSⅠ循環的電子伝達のどちらかに欠損があると予想される。そこで本研究では、この *cfa1* 変異体を詳細に解析した。

結果と考察

30分間の暗順応処理を行なったWTと *cfa1* 変異体のqPとNPQを生育条件と同じ光強度(80 μmol m^{-2} s^{-1}）で測定したところ、*cfa1* 変異体のqPとNPQは光照射開始後1分以内でのみWTより低かった。qPは、電子伝達鎖の因子であるプラストキノンの酸化状態を反映している。また、NPQは熱放散の程度を表している。従って、この結果から *cfa1* 変異体では電子伝達鎖がより還元的になり、熱放散が抑制されたということが示唆された。この結果はクロロフィル蛍光挙動の結果と同様に、*cfa1* 変異体はwater-water cycleかPSⅠ循環的電子伝達のどちらかの代替的な光合成経路に欠損があるという仮説を支持した。

PSⅠ循環的電子伝達には、NDH経路とFQR経路が存在する。そこでまず *cfa1* 変異体がNDH経路に欠損を持つかを調べた。NDH経路の因子であるNDH複合体に欠損がある変異体は、光照射を中止するとWTでは見られるクロロフィル蛍光の一過的上昇が見られなくなることが知られている。

そこでこの一過的上昇が *cfa1* 変異体で見られるかを調べたところ、WT も *cfa1* 変異体も光照射を中止した直後にこの一過的上昇が見られた。このことから *cfa1* 変異体は NDH 経路には欠損がないことが示された。次に FQR 経路に欠損があるかを調べた。FQR 経路の因子である PGR5 タンパク質が欠損している変異体は、*in vitro* の PS I 循環的電子伝達活性が大きく損なわれることが知られている。そこで、同様の実験法を用いて *cfa1* 変異株が FQR 経路に欠損を持つのかを調べた結果、*cfa1* 変異体の活性は WT と変わらなかった。このことから *cfa1* 変異体は FQR 経路にも欠損がないことが示唆された。

上記の結果から、*cfa1* 変異体は water-water cycle に欠損を持つのではないかと考えた。そこで、二酸化炭素固定による電子の消費の影響を取り除くため、二酸化炭素濃度を 0%にして qP と NPQ の測定を行なった。酸素依存的な光合成経路には、water-water cycle の他に光呼吸がある。そのため光呼吸が十分に働く酸素濃度条件下では、多くの電子が光呼吸を駆動するために消費され、water-water cycle 依存的な電子の消費がほとんど起こらない可能性も十分に考えられる。しかし光呼吸の酸素に対する親和性は、water-water cycle の酸素に対する親和性よりも数倍低いことが知られている。また二酸化炭素濃度が 0%かつ酸素濃度が 2%の気相条件下では光呼吸がほとんど働かないことも、光呼吸の変異体の解析から示唆されている。

そこで酸素濃度を 0、2、5、10、21%と振って、30 分間の暗順応処理を行なった WT と *cfa1* 変異体の qP と NPQ を 40 μmol m^{-2} s^{-1} の光強度下で測定した（図 2）。光照射開始 10 分後において、0%条件下においては差が見えなかった。しかし低酸素濃度(2%又は 5%)では、*cfa1* 変異体は qP と NPQ が WT より低かった。これらの結果により、*cfa1* 変異体は酸素依存的な光合成経路に欠損があることが示唆された。この WT と *cfa1* 変異体の差は酸素濃度が上がるにつれて小さくなり、10%以上の酸素濃度では差が見られなくなった。これらの結果により、*cfa1* 変異体は光呼吸がほとんど働かないような条件下でのみ、qP と NPQ が低くなることが示された。従って *cfa1* 変異体は water-water cycle に欠損があるために、低酸素条件下では酸素への電子の流れが減少し、電子伝達鎖はより還元的になり熱放散の誘導が抑制されることが示唆された。

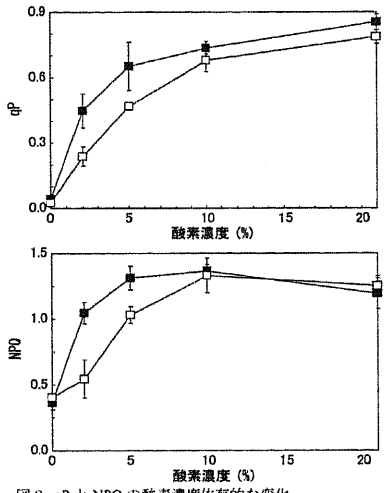

図 2 qP と NPQ の酸素濃度依存的な変化
二酸化炭素濃度は 0%。黒四角が WT, 白四角が *cfa1* 変異体を表す。

CFA1 遺伝子の機能欠損が生理学的にどのような意義を持つのかを明らかにするため、弱光条件（80 μmol m^{-2} s^{-1}）又は強光条件（200 μmol m^{-2} s^{-1}）において WT と *cfa1* 変異体の生育を比較した。弱光条件においては、*cfa1* 変異体は WT よりわずかに生育阻害を示した。一方、強光条件においては、*cfa1* 変異体は明らかな生育阻害を示し、いくつかの葉では著しい退色を示した。これらの結果から、*cfa1* 変異体は強光に順化できず、光ストレスに対する感受性が高くなったということが示された。

結論

cfa1 変異体を詳細に解析した結果から、*cfa1* 変異体は water-water cycle に欠損があることが示された。water-water cycle はいくつかの反応を経て、活性酸素種を急速にかつ効率的に消去する機構であるが、律速段階は O_2 を還元して O_2^- を生成する段階であると考えられている（図 1）。*in vitro* の実験において、いくつかの FAD 酵素がこの反応を促進することが示唆されている。しかしながら実際、生体内でどのような因子によって反応が促進されるのかは明らかになっていない。*cfa1* 変異体では、このような因子が欠損することで O_2 に対する親和性が下がり、そのために 0% CO_2/2% O_2 条件下や暗条件から明条件に移した直後といった water-water cycle の機能が電子伝達に大きな影響を及ぼす条件下で異常を示すと考えられる。なぜ *cfa1* 変異体では光ストレスに対する感受性が高くなったのかは明らかになっていないが、*CFA1* 遺伝子の機能は光ストレスにおいても重要な役割を果たすと思われる。今後、マップベースクローニング法により *cfa1* 変異体の原因遺伝子が明らかになることで、water-water cycle の制御メカニズムが明らかになっていくだろう。

TABLE OF CONTENTS

ACKNNOWLEDGMENT

I wish to express my deepest appreciation to Prof. Kintake Sonoike for his constant supervision, excellent advice, and valuable discussion through this study. I truly respect his attitude toward science, and have learned so much for him. I am greatly honored that I have done master thesis in his laboratory.

I also express my great appreciation to Prof. Yoshikazu Ohya, who gave me great advice as a geneticist and encouraged me through this study.

I am grateful to Prof. Toshiharu Shikanai for generously gifting the seeds of *Arabidopsis thaliana* wild type (ecotype Columbia *gl1*; Col *gl1*), the *crr2-2* mutant and the *pgr5* mutant.

I would like to express my appreciation to Mari Kurosawa, Hiroyuki Usuki, Hiroshi Ozaki, Mieko Higuchi, Hanayo Sato, Ban-yuh Takahashi, Kazumi Shimada, Mio Tamori, Keiichi Takeda and Taku Mouri for supporting my study as a member of photosynthesis group. I could not accomplish study in my master course without their cooperation.

I would like to express my appreciation to Satoru Nogami, Mizuho Sekiya, Machika Watanabe, Takahiro Negishi, You Kikuchi, Shinsuke Ohnuki, Takahiro Koizumi, Yasutaka Imanaga, Shigeru Nagoya and Ryoichi Yamazaki for their giving me various important suggestions especially in my colloquium despite they are in quite different field of study from me.

I express great thanks to all the members of Laboratory of Signal Transduction, Department of Integrated Bioscience, Graduate School of Frontier Sciences, the University of Tokyo for their warm encouragement and support through this study.

Finally, I express my appreciation to my family for their financial support and warm encouragement.

LIST OF FIGURES

ABSTRACT

Plants suffer from perpetual changes of environment. Under high light condition, plants develop several mechanisms to relieve the over-reduction of the photosynthetic electron flow. Among such mechanisms, photorespiration, water-water cycle and cyclic electron flow around PSI (CEF-PSI) could serves as electron sink, and trigger the dissipation of excess light energy in PSII (i.e. the thermal dissipation). The *cfa1* mutant, which was previously isolated in our laboratory, shows interesting phenotype that the relative intensity of chlorophyll fluorescence from leaves is higher than that of WT only at early time points after the shift of light condition from dark to light. Here, I characterized the *cfa1* mutant, and obtained the following results. 1) The *cfa1* mutant and WT (Ws) did not show the difference in the measurement of NDH activity *in vivo* and of CEF-PSI activity *in vitro*. The results indicate that the *cfa1* mutant is not defective in CEF-PSI. 2) The *cfa1* mutant showed lower photochemical quenching (qP) and non-photochemical quenching (NPQ) than WT at the early time points after starting of actinic light (AL) illumination. The *cfa1* mutant also showed lower qP and NPQ than WT (Ws) under CO_2 free and low O_2 conditions. Therefore, I concluded that the *cfa1* mutant was defective in water-water cycle. *CFA1* would be involved in the regulation of affinity of PSI to O_2 *in vivo*. This is the first report for the existence of such component *in vivo*.

INTRODUCTION

Plants convert light energy into chemical energy to sustain their lives. Light energy is harvested by the antenna pigments and converted into electrochemical energy at the reaction center of two photosystems located in the chloroplast thylakoid membranes. Photosysytem II (PSII) and photosysytem I (PSI) operate in tandem to produce a linear flow of electrons from H_2O to $NADP^+$ (Fig. 1). Thereby, reducing power in the forms of reduced ferredoxin or NADPH in the stroma accumulates. This linear electron flow generates a proton gradient (ΔpH) across the thylakoid membrane, which is subsequently used to synthesize ATP. NADPH and ATP are the first stable products in photosynthesis and they are used in various metabolic reactions including CO_2 fixation (Hill and Bendall, 1960).

However, the light energy is not always a good thing because too much light may cause damage to plants. The light energy which is in excess of that required for the CO_2 assimilation results in the over-reduction of the linear electron flow and is transferred to oxygen so that harmful reactive oxygen species (ROS) are generated (Niyogi, 1999). The effects of these ROS can be the oxidation of lipids, proteins, and enzymes necessary for the proper functioning of the chloroplast and the cell as a whole (Foyer et al., 1994). The conditions of the excess light energy often and suddenly can occur, for example, sun-flex, high temperature and drought.

However plants have several mechanisms to relieve over-reduction of the linear electron flow in the short term without expressing genes, such as photorespiration, water-water cycle and cyclic electron flow around PSI (CEF-PSI) (Fig. 1). Since over-reduction is relieved by those mechanisms, electron flow can occur and subsequently generates ΔpH across the thylakoid membrane under various stress conditions. The generation of ΔpH is used not only to synthesize ATP but also to induce the thermal dissipation, which can dissipate excess light energy as harmless heat energy in PSII (Muller et al., 2001; Munekage et al., 2001). Therefore, these mechanisms are thought to be essential for high-light acclimation of plants.

Let's look at the mechanisms of such alternative photosynthetic pathways in more detail. Photorespiration requires O_2 as the mechanism involves the oxygenase reaction catalyzed by ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in chloroplast, as

well as many other reactions in peroxisomes and mitochondria (Wingler et al., 2000). The ratio of the carboxylation rate to the oxygenation rate of Rubisco is dependent on the CO_2 and O_2 concentrations (Farquhar et al., 1980). Under ambient atmospheric conditions, about 20% of the total electron flux through Rubisco is diverted to oxygen. To elucidate mechanism of photorespiration, several mutants have been isolated in *Arabidopsis* (Somerville and Ogren, 1979, , 1980; Beckmann et al., 1997; Schwarte and Bauwe, 2007). These mutants show extreme growth inhibition in normal air condition, but the growth of these mutants is indistinguishable from WT under high CO_2 condition in which photorespiration is suppressed. During the photorespiration process, ATP and NADPH are consumed. Therefore, photorespiration can relieve over-reduction of the linear electron flow, especially under stress condition such as low intercellular CO_2 concentration.

In water-water cycle, O_2 is directly reduced by PSI. The primary function of the water-water cycle is rapid and effective scavenging of O_2^- and H_2O_2, reactive intermediates of oxygen reduction, to suppress their interactions with taget molecules in chloroplast. In addition, water-water cycle can relieve over-reduction of the linear electron flow as dissipating electrons from PSII in PSI via the transfer of reducing equivalent to O_2 (Asada, 1999). The phenotypes of knockdown mutant of chloroplastic CuZn-SOD, which is a key enzyme in water-water cycle, are suppressed growth, small chloroplasts and low photosynthetic activity (Rizhsky et al., 2003). The results indicate that water-water cycle is essential for photoprotection.

CEF-PSI has two pathways, namely NAD(P)H dehydrogenase (NDH)-dependent pathway and ferredoxin quinine reductase (FQR)-dependent pathway. Since electrons are recycled from NADPH or ferredoxin to plastquinone in these pathways, CEF-PSI is a mechanism that conserves electrons. The NDH-dependent pathway was originally discovered in cyanobacteria (Mi et al., 1995) and was shown to operate also in PSI cyclic electron transport in chloroplasts (Endo et al., 1998). Although the NDH complex is a major player in PSI cyclic electron flow in cyanobacteria, its contribution would be minor in higher plants (Hashimoto et al., 2003), judged from the experiments with the *crr2-2* mutant, in which accumulation of the NDH complex is impaired. On the other hand, the FQR-dependent pathway, which was discovered in the 1950s, is thought to be a primary pathway in higher plants. Recently, it was shown that a small thylakoid protein, PGR5, is essential for

FQR-dependent pathway (Munekage et al., 2002). Although the growth rate of the *crr2-2* mutant and the *pgr5* mutant is similar to that of WT under low light condition, the growth rate of the double mutant is significantly reduced (Munekage et al., 2004). CEF-PSI seems to be essential under any light conditions.

Although photorespiration is regulated by oxygenase reaction catalyzed by Rubisco, we have few ideas on how water-water cycle and CEF-PSI are regulated. In water-water cycle, it is believed that the limiting step of water-water cycle is the photoreduction of O_2 (Asada, 1999). It is shown that stromal components accelerate the electron flow to O_2 in *in vitro* assay (Miyake et al., 1998). However, we do not know whether it is also functional *in vivo* or which component is really involved in the reaction. In NDH-dependent pathway of CEF-PSI, NDH complex is the only known component (Shikanai, 2007). Recently, it is indicated that a novel nucleous-encorded chloroplast protein, PIFI, is involved in NDH-dependent pathway, although the function of PIFI is unknown (Wang and Portis, 2007). As for FQR-dependent pathway of CEF-PSI, the only component reported so far is PGR5 protein (Munekage et al., 2002). However, the function of PGR5 protein is still totally unknown. Since PGR5 protein does not have any metal binding motifs, it is unlikely that PGR5 protein is directly involved in electron transport from ferredoxin to plastoquinone as an electron carrier (Shikanai, 2007). This suggests that some other components exist in FQR-dependent pathway for electron carriers. Thus, many points are remaining as enigma about water-water cycle and CEF-PSI, yet. To understand the regulatory mechanism, the discovery of novel components in these pathways would be necessary.

In my laboratory several *Arabidopsis* mutants, which show the different chlorophyll fluorescence kinetics from WT, has been isolated. Among them, the *cfa1* mutant shows interesting phenotype that the relative intensity of chlorophyll fluorescence from plants is higher than that of WT only at early time points after the shift of light condition from dark to light. Chlorophyll fluorescence reflects the yield of photosynthetic electron flow and of thermal dissipation, since absorbed light energy is either 1) re-emitted as chlorophyll fluorescence, 2) transferred to reaction centers and used to drive photochemistry, or 3) de-excited by the thermal dissipation. Therefore, it is likely that the *cfa1* mutant has defect in some mechanism of photosynthesis or thermal dissipation upon the shift to light condition from dark. Furthermore, it is indicated that water-water cycle functions as a starter of

photosynthesis especially upon the shift to light condition from dark by generating ΔpH across thylakoid membranes to supply ATP for CO_2 fixation and to induce NPQ (Makino et al., 2002). It is also indicated that CEF-PSI functions as a starter of photosynthesis upon the shift to light condition from dark, when water-water cycle is suppressed (Makino et al., 2002). Thus, it is possibility that the *cfa1* mutant has defect in water-water cycle or CEF-PSI.

At that time, it was suggested that the disruption of At4g03200 by T-DNA insertion was responsible for the phenotype of the *cfa1* mutant. However, the *Arabidopsis* plant, in which At4g03200 was disrupted by T-DNA insertion (obtained from the Arabidopsis Biological Resource Center) did not show the phenotype observed in the *cfa1* mutant. To obtain the *cfa1* mutant, which only has the mutation in the responsible gene for the phenotype, plants without the T-DNA insertion and with the phenotype of the *cfa1* mutant was selected from the 1F2 generation from the back cross between *cfa1* and WT (Fig. 2). Furthermore, 2F1 seeds derived from the back cross between the 1F3 *cfa1* mutants without the T-DNA insertion and with the phenotype of the *cfa1* mutant and WT (Ws), and 2F2 seeds derived from self-fertilization of 2F1 *cfa1* mutants were obtained (Kurosawa, Master thesis) (Fig. 2). In this thesis, the 2F3 *cfa1* mutant is characterized in detail to reveal the function of *CFA1*. The results obtained here demonstrate that the *cfa1* mutant is defective in water-water cycle, especially the affinity with oxygen.

MATERIALS AND METHODS

Plant material

The seeds of *Arabidopsis thaliana* wild type (ecotype Wassilewskija; Ws) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The *cfa1* mutant was isolated from Feldmann T-DNA 100s set3 of *Arabidopsis thaliana* obtained from Nottingham *Arabidopsis* Stock Center. The 1F1 seeds of the *cfa1* mutant were derived from the cross between the *cfa1* mutant and WT (Ws) and 1F2 seeds of the *cfa1* mutant were derived from self-fertilization of the 1F1 *cfa1* mutant. Among the 1F2 *cfa1* mutants, the ones without the T-DNA insertion and with the phenotype of the *cfa1* mutants were selected and self-fertilized to obtain the seed of 1F3 *cfa1* mutant (Kurosawa, master thesis). The 2F1 seeds of the *cfa1* mutant were derived from the cross between the 1F3 *cfa1* mutant and WT (Ws) and 2F2 seeds of the *cfa1* mutant were derived from self-fertilization of the 2F1 *cfa1* mutant. Among the 2F2 *cfa1* mutants, ones with the phenotype of the *cfa1* mutants were selected and self-fertilized to obtain the seed of 2F3 *cfa1* mutant (Fig. 2). The seeds of *Arabidopsis thaliana* wild type (ecotype Columbia *gl1*; Col *gl1*), the *crr2-2* mutant and the *pgr5* mutant were generous gifts from Prof. Shikanai.

Growth condition

Arabidopsis plants were grown on plates with 0.5% Gelrite (Wako, Japan) medium containing 1% sucrose, 0.46% Murashige and Skoog (MS) plant salt mixture (Wako, Japan) or on soil composed of Powersoil (Showa, Japan) and Vermiculate (Kureha, Japan) (1:1). Plants were grown at 23°C under continuous illumination (80 μmol $m^{-2}s^{-1}$).

Chlorophyll fluorescence kinetics

To monitor chlorophyll fluorescence kinetics, two-dimensional chlorophyll fluorescence video-imaging system (FluorCam; Photon Systems Instruments, Czech Republic) was used. This system consists of two sets of 325 super-bright orange light emitting diodes (LED's) that provide excitation flashes or a continuous actinic irradiance, a CCD camera that detects fluorescence emitted within the area of 10 x 13 cm, and a computer for image detection, storage, and analysis. Plants were grown on plates for 10 days. Chlorophyll

fluorescence kinetics was monitored during illumination (350 μmol $m^{-2}s^{-1}$) for 2 min after pre-illumination for 40 sec (350 μmol $m^{-2}s^{-1}$) and subsequent dark-adaptation for 1 min.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured with a pulse-amplitude modulation (PAM) chlorophyll fluorometer (Waltz, Effeltrich, Germany) equipped with emitter detector unit ED101 as described in Schreiber et al. (Schreiber, 1986). The leaves of 18-days-old plants grown on plates or 25-28-days-old plants grown on soil were dark adapted for 30 min before measurements. The minimum chlorophyll fluorescence with the open PSII center (Fo) was determined by measuring light (650 nm) at 0.06 μmol $m^{-2}s^{-1}$. A saturating pulse of white light (800 ms, 3000 μmol $m^{-2}s^{-1}$) was applied to determine the maximum chlorophyll fluorescence with the closed PSII center in the dark (Fm) or during actinic light illumination (Fm'). The steady state level of chlorophyll fluorescence (F) was recorded during actinic light (AL) illumination for 10 min. Non-photochemical quenching (NPQ), which is related to the thermal dissipation, was calculated as (Fm-Fm')/Fm'. Photochemical quenching (qP), which reflects the oxidized state of plastoquinone, was calculated as (Fm'-F)/(Fm'-Fo'). Various air conditions were set in a measurement chamber of a photosynthesis analyzer, LI6400 (Meiwa, Japan) in which concentration of CO_2 was regulated. The concentration of O_2 was regulated by mixing air with N_2 gas using a flow meter.

The measurement of NDH activity

NDH activity was monitored by transient increase of dark-level chlorophyll fluorescence with PAM chlorophyll fluorometer (Waltz, Effeltrich, Germany) after actinic light (AL) illumination (80 μmol $m^{-2}s^{-1}$ for 5 min) of the leaves of 18-days old plants grown on plates. The plants were dark adapted for 30 min before measurements. The minimum chlorophyll fluorescence with the fully open PSII center (Fo) was determined by measuring light (650 nm) at 0.1 μmol $m^{-2}s^{-1}$.

Isolation of intact chloroplasts and ruptured chloroplast

Intact chloroplasts were prepared from leaves of 21-days-old plants. The leaves were chopped with a homogenizer (Polytron, Kinematica, Lucerne, Switzerland) in the ice-cold

isolation buffer containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA (pH 8.0), 50 mM HEPES-KOH (pH 7.5), and 5 mM sodium ascorbate. The mixture was filtrated through the 20 mm nylon mesh and the filtrate was centrifuged. The pellet was washed and resuspended in the same buffer and intact chloroplasts fraction was prepared with Percoll gradient centrifugation. The intact chloroplasts were osmotically ruptured in the medium containing 7 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 30 mM KC1, 0.25 mM KH_2PO_4 and 50 mM HEPES (pH7.6).

Western blot analysis of PGR5 protein

Chloroplast proteins equivalent to 4.0 μg chlorophyll were resolved by 17% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a semidry blotting system. Immunoblot detection was performed using an enhanced chemiluminescence system (ECL; Amersham Biosciences). PGR5 protein antibody was generous gift from Prof. Shikanai.

Sequencing of *PGR5* gene

Plants' DNA was extracted by the following method. A hundred mg of chilled plants was quickly homogenized in a mortar with 400 μl of extraction buffer (0.3 M NaCl, 50 mM Tris-HCl (pH 7.5), 20 mM EDTA (pH 8.0), 0.5% SDS, 5 M urea, 10 mM melcaptoethanol, and 5% phenol). The homogenate was transferred into a microtube and mixed with 400 μl of phenol/ chloroform (1:1) by multi voltex mixer for 2 min. The mixture was centrifuged at 12,000 rpm for 5 min, and then the supernatant was transferred into a new tube. 400 μl of chloroform was added and mixed by multi voltex mixer for 2 min. After centrifugation at 12,000 rpm for 5 min, the supernatant was transferred to a new tube and 2-3 vol. of 100% ethanol was added. The mixture was centrifuged at 12,000 rpm, 4°C for 5 min, decanted and the precipitate was dried. The pellet was rinsed with 1ml of 70% ethanol, and air dried completely. After drying, it was suspended in 100 μl of TE. DNA which was isolated from Ws and the *cfa1* mutant was amplified by PCR using *PGR5* gene specific primers 5'-CAT GAG AAA CGT AAT AAG TTA AGT C-3' and 5'-TCA GCT AAG ACC TTA TTG AAC AAC-3'. PCR products were sequenced by using a dye terminator cycle sequencing kit (Applied BioSystems, USA) and ABI prism sequencer (Applied BioSystems, USA).

In vitro assay of CEF-PSI activity

The ruptured chloroplasts were diluted with the assay medium containing 7 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 30 mM KC1, 0.25 mM KH_2PO_4 and 50 mM HEPES (pH7.6) to 25 μg Chl ml^{-1} and immediately used for this assay system. The redox change of plastoquinone was monitored in term of chlorophyll fluorescence with PAM chlorophyll fluorometer (Waltz, Effeltrich, Germany). As electron donors, 5 μM spinach ferredoxin and 0.25 mM NADPH were used.

Chlorophyll quantification

To quantify chlorophyll, pigments of leaves of 16-days old plants grown on plates were extracted into *N*, *N*-dimethylformamide (DMF) for 24 h at 4°C in dark. Amounts of Chl *a* and Chl *b* were determined spectrophotometrically using the equations of Porra *et al.* (Porra et al., 1989).

RESULTS

The mutation of the *cfa1* mutant was single.

The *cfa1* mutant was originally isolated on the basis of the phenotype, in which relative intensity of chlorophyll fluorescence from plants is higher than that of WT at early time points after the shift of light condition from dark to light. At that time, it was suggested that the disruption of At4g03200 by T-DNA insertion was responsible for the phenotype of the *cfa1* mutant. However, the *Arabidopsis* plant, in which At4g03200 was disrupted by T-DNA insertion (obtained from the Arabidopsis Biological Resource Center) did not show the phenotype observed in the *cfa1* mutant. To obtain the *cfa1* mutant which only has the mutation in the responsible gene, plants without the T-DNA insertion and with the phenotype of the *cfa1* mutant was selected from the 1F2 generation from the back cross between *cfa1* and WT (Fig. 2). Furthermore, 2F1 seeds derived from the back cross between the 1F3 *cfa1* mutants without the T-DNA insertion and with the phenotype of the *cfa1* mutant and WT (Ws), and 2F2 seeds derived from self-fertilization of 2F1 *cfa1* mutants were obtained (Kurosawa, Master thesis) (Fig. 2). Therefore, 2F2 *cfa1* mutants should show the segregated phenotype.

To obtain the mutants, which show the phenotype of the *cfa1* mutant, and to examine the segregation ratio, chlorophyll fluorescence kinetics of the 2F2 *cfa1* mutants was determined (Fig. 3). According to Kurosawa, the *cfa1* phenotype was regarded as higher chlorophyll fluorescence value for 20 s to 40 s after the shift to light condition than the average + SD of WT fluorescence. Based on this criteria, 29/132 of the 2F2 *cfa1* mutants showed *cfa1* mutant phenotype (phenotype ratio = 0.22, 95% confidential interval: 0.15, 0.30). The phenotype ratio was calculated by interval estimation with binominal test. The result indicates that the segregation ratio of the phenotype is 3:1 and that the mutation of the *cfa1* plants is single and recessive homo, although the result is based on the assumption that the chlorophyll fluorescence kinetics does not show intermediate phenotype.

Subsequently, I obtained 2F3 seeds derived from self-fertilization of the 2F2 *cfa1* mutant, which had the phenotype of the *cfa1* mutant. To confirm the reproducibility of the phenotype of the *cfa1* mutants, I measured chlorophyll fluorescence kinetics of 2F3 *cfa1* mutants. They showed higher chlorophyll fluorescence kinetics from 20 s to 40 s than WT (Ws) (Fig. 4), confirming the reproducibility of the phenotype of the *cfa1* mutants. Therefore,

I used the 2F3 *cfa1* mutants as the *cfa1* mutant for all the following experiments.

The *cfa1* mutant showed lower qP and NPQ at early time points after the shift to light from dark.

To characterize the *cfa1* mutant in more detail, photosynthetic parameters were determined by Pulse Amplitude Modulation (PAM) fluorometer. PAM fluorometer facilitates quantitative analysis of the chlorophyll fluorescence signal, which can be utilized in the calculation of photosynthetic parameters (see Material and Methods for detail).

Upon the illumination by actinic light (AL) at the growth light level (80 μmol $m^{-2}s^{-1}$), the *cfa1* mutant showed lower qP and NPQ than WT (Ws) at the early time points within 1 min after starting of AL illumination (Fig. 5 a, b). The parameters of the *cfa1* mutant did not show any significant difference from WT (Ws) at the later time points (Fig. 5 a, b). When higher AL (400 μmol $m^{-2}s^{-1}$) was used, the difference between the *cfa1* mutant and WT (Ws) persists more; 4 min for qP and 2 min for NPQ (Fig. 5 c, d). qP reflects the oxidized state of Q_A, the primary electron acceptor plastoquinone in photosystem II (PSII). NPQ (non-photochemical quenching) represents the extent of thermal dissipation. NPQ is induced by the formation of ΔpH across the thylakoid membranes due to photosynthetic electron transport. Therefore, these results indicate that the electron transport chain is more reduced and the thermal dissipation is suppressed in the *cfa1* mutant compared with WT (Ws) only at the early time points after starting of AL illumination. In general, the dark-adapted leaves require a few minutes to activate the enzymes related with CO_2 fixation, for example Rubisco (Usuda, 1985; Hammond et al., 1998). Therefore, I assume that the *cfa1* mutant is defective in alternative photosynthetic pathways, such as cyclic electron flow around PSI (CEF-PSI) or water-water cycle.

The *cfa1* mutant was not defective in NDH activity of CEF-PSI.

CEF-PSI has two pathways, namely NDH-dependent pathway and FQR-dependent pathway. To determine whether the *cfa1* mutant has defect in NDH-dependent pathway of CEF-PSI, the change in the level of chlorophyll fluorescence after termination of AL illumination, a good indicator of NDH-dependent pathway, was monitored. I used WT (Col *gl1*) as well as the *crr2-2* mutant, which is impaired in the accumulation of the NDH complex,

as positive and negative control, respectively (Hashimoto et al., 2003). Fig. 6 a showed typical trace of chlorophyll fluorescence change in WT (Ws). Prior to AL illumination, the minimum chlorophyll fluorescence with the open PSII center was determined by measuring light (ML) and the maximum chlorophyll fluorescence with the closed PSII center in the dark was determined by a saturating pulse (SP). Subsequently, AL of 80 μmol $m^{-2}s^{-1}$ was turned on for 5 min to build up the stromal pool of reductants (NADPH and reduced ferredoxin). Just after turning on of the AL illumination, the chlorophyll fluorescence level was rapidly increased by the reduction of plastoquinone via PSII photochemistry, and then subsequently lowered by its oxidation by the downstream electron transport pathway. The chlorophyll fluorescence reached steady-state level after 5 min. Upon turning off of the AL illumination, the photochemical reduction of plastoquinone by PSII stopped, and the plastoquinone pool was fully oxidized in a short time. A subsequent transient increase in chlorophyll fluorescence was brought about, because of non-photochemical reduction of plastoquinone by electron accumulated in stroma during AL illumination (Asada et al., 1993). This transient increase depends on the NDH activity (Munekage et al., 2004). Figs. 6 b-e shows the change in chlorophyll fluorescence after the termination of AL illumination. As reported previously, WT (Col *gl1*) showed the transient increase of the fluorescence level, while the *crr2-2* mutant did not (Fig. 6 d, e). WT (Ws) and the *cfa1* mutant both showed typical transient increase of fluorescence (Fig. 6 b, c). These results indicate that the *cfa1* mutant is not defective in NDH-dependent pathway of CEF-PSI.

The *cfa1* mutant did not have a mutation in *PGR5* gene.

At present, the only component revealed to be involved in FQR-dependent pathway is PGR5 protein. PGR5 protein is about 10 kDa protein and absent in the *pgr5* mutant by western blot analysis (Munekage et al., 2002). I conducted western blot analysis of PGR5 protein to find whether PGR5 protein is present in the *cfa1* mutant, using the *pgr5* mutant and WT (Col *gl1*) as a negative and a positive control, respectively. The band corresponding to PGR5 protein was detected in WTs and the *cfa1* mutant except the *pgr5* mutant (Fig. 7). This result alone did not eliminate the possibility that PGR5 protein is not in normal functional state because of the mutation in the ORF of *PGR5* gene in the *cfa1* mutant. However, the result of the sequencing of *PGR5* gene revealed that the *PGR5* gene does not carry any

mutation in the *cfa1* mutant (Fig. 8). These results indicate that the *cfa1* mutant has normal PGR5 protein.

The *cfa1* mutant showed normal CEF-PSI activity determined *in vitro*.

The *pgr5* mutant was known to show obvious difference from WT in *in vitro* assay of CEF-PSI activity (Munekage et al., 2002; Munekage et al., 2004). The activity of CEF-PSI was assayed by monitoring an extent of plastoquinone (PQ) reduction upon addition of NADPH and ferredoxin (Fd) to the osmotically ruptured chloroplasts in the dark. PQ reduction was monitored as an increase in chlorophyll fluorescence during the exposure to measuring light (ML) with a very low photon flux density (0.04 μmol $m^{-2}s^{-1}$). At this photon flux density, the chlorophyll fluorescence level predominantly reflects the reduction of plastoquinone by CEF-PSI from ferredoxin (Fd), not by PSII photochemistry (Fig. 9 a). The addition of 0.25 mM NADPH to assay medium induced only a slight increase of the chlorophyll fluorescence (Fig. 9 b, c). In this assay system, NADPH is essential for electron donation to Fd via the reverse reaction of ferredoxin-$NADP^+$ reductase (FNR) (Miyake and Asada, 1994). After the addition of 5 μM Fd to the assay medium, the increase in chlorophyll fluorescence was observed in WT (Col *gl1*) thylakoid membranes, but the extent of the increase is much lower in the *pgr5* mutant (Fig. 9 b). In the case of the *cfa1* mutant, however, the observed increase of the fluorescence is indistinguishable from that of WT (Fig. 9 c). The result indicates that the *cfa1* mutant has no defect in CEF-PSI.

The *cfa1* mutant showed lower qP as well as lower NPQ than WT (Ws) only under CO_2 free and 2 or 5% O_2 condition.

The results of Fig. 6-9 indicate that the *cfa1* mutant is not defective in CEF-PSI. Therefore, I assume that the *cfa1* mutant is defective in O_2-dependent photosynthetic pathways such as photorespiration or water-water cycle. The electron flow to O_2-dependent photosynthetic pathways and CEF-PSI would be larger under CO_2 free condition, as CO_2 fixation is suppressed. To measure photosynthetic parameters in various air conditions, plants grown on plate was too small. Therefore, plants grown on soil under continuous light (80 μmol $m^{-2}s^{-1}$) were used for the following experiments. Plants were dark adapted under normal air condition for 30 min. Subsequently, the leaves of the dark adapted plants were set in the

measurement chamber filled with normal air or CO_2 free air, and the chlorophyll fluorescence measurement was immediately started using a PAM fluorometer. Fig. 10 showed steady state level of qP and NPQ after 10 min from the onset of actinic light (AL) illumination (80 μmol $m^{-2}s^{-1}$) in normal air condition or CO_2 free air condition. Both in WT (Ws) and the *cfa1* mutant, qP under CO_2 free condition was slightly lower than that under normal air condition and NPQ under CO_2 free condition was much higher than that under normal air condition. The lower qP under CO_2 free condition indicates that the electron transport chain was more reduced, and the higher NPQ under CO_2 free condition indicates that the thermal dissipation was accelerated by the formation of ΔpH across the thylakoid membranes due to O_2-dependent photosynthetic pathways or CEF-PSI under suppressed CO_2 fixation. However, the *cfa1* mutant did not show significant difference from WT (Ws) not only under normal air condition but also under CO_2 free condition.

In general, it was estimated that Km value of water-water cycle to O_2 is 8% (Miyake and Yokota, 2000) while that of photorespiration is 33% (Osmond, 1981). It was also reported that photorespiration was largely suppressed under CO_2 free and at least 2% O_2 condition (Kozaki and Takeba, 1996). Therefore, under low O_2 concentration in CO_2 free condition, qP and NPQ might show the difference between WT (Ws) and the *cfa1* mutant because of the possible suppression of CO_2 fixation and photorespiration. To examine this possibility, qP and NPQ was measured with several different O_2 concentration (0, 2, 5, 10, 21%) under CO_2 free condition. The measurement of qP and NPQ was conducted as same in Fig. 10. When AL intensity was 80 μmol $m^{-2}s^{-1}$, qP and NPQ could not be measured under CO_2 free and 0% O_2 condition (data not shown). AL intensity (80 μmol $m^{-2}s^{-1}$) was too high in this condition to detect Fm', which is required to calculate qP and NPQ. Therefore, AL intensity was reduced from 80 μmol $m^{-2}s^{-1}$ to 40 μmol $m^{-2}s^{-1}$. Fig. 11 showed steady level of qP and NPQ after 10 min from starting of actinic light (AL) illumination (40 μmol $m^{-2}s^{-1}$) under CO_2 free condition with various O_2 concentration. qP and NPQ did not show significant difference between WT (Ws) and the *cfa1* mutant under CO_2 free and 21% O_2 condition as in Fig. 10 as well as under CO_2 free and 10% O_2 condition. When O_2 condition was lowered to 2% or 5%, however, the *cfa1* mutant showed lower qP and NPQ compared with WT (Ws). The result indicates that the electron transport chain is more reduced and the thermal dissipation is suppressed in the *cfa1* mutant compared with WT (Ws) under such condition. Furthermore, under CO_2 free and 0%

O_2 condition, in which not only CO_2 fixation but also O_2-dependent photosynthetic pathway cannot function, qP and NPQ did not show significant difference between WT (Ws) and the *cfa1* mutant any more. These results indicate that the *cfa1* mutant is defective in O_2-dependent photosynthetic pathways, such as photorespiration or water-water cycle.

Effect of air condition on the induction phase of photosynthesis

Although it was reported that photorespiration was largely suppressed under CO_2 free and at least 2% O_2 condition (Kozaki and Takeba, 1996), Fig. 11 did not eliminate the possibility that the *cfa1* mutant was defective in photorespiration. In general, the dark-adapted leaves require a few minutes to activate enzymes related with CO_2 fixation, for example Rubisco (Usuda, 1985; Hammond et al., 1998). On the other hand, first step of photorespiration is the oxygenase reaction catalyzed by Rubisco. Therefore, I compared qP and NPQ of the *cfa1* mutant with those of WT (Ws) at early time points after starting AL illumination. Fig. 12 showed the time courses change in the induction of qP and NPQ under CO_2 free and 0% O_2 condition under AL illumination at 40 μmol $m^{-2}s^{-1}$. Although O_2 concentration was 0%, the *cfa1* mutant showed lower qP as well as lower NPQ than WT (Ws) at 30 s after starting of AL illumination. As shown in Fig. 11, WT and *cfa1* mutant did not show significant difference in steady state photosynthetic parameter under CO_2 free and 0% O_2 condition. I hypothesized that the apparent discrepancy would be due to the fact that the inside of leaves may not be completely changed to CO_2 free and 0% O_2 condition at the initial phase of changing gas condition in the measurement chamber. To examine this hypothesis, measuring method was changed as follows. Plants were dark adapted under normal air condition for 25 min. Subsequently, the leaves of the dark adapted plants were set in the measurement chamber filled with CO_2 free and 0% O_2 air. After 5 min, chlorophyll fluorescence measurement was started. That is to say, total dark adapted time was 30 min and leaves were adapted to CO_2 free and 0% O_2 condition for 5 min.

Under such experimental condition, the *cfa1* mutant show little difference in qP and NPQ compared with WT (Ws) at any time points after starting AL illumination (Fig. 13). The result indicates that the difference of qP and NPQ between the *cfa1* mutant and WT (Ws) at 30 s after starting of AL illumination observed in Fig. 12 is resulted from the fact that the inside of leaves was not completely changed to CO_2 free and 0% O_2 condition at the earlier

time points. It should be noted that, the later time points after 30 s were not affected by adaptation to CO_2 free and 0% O_2 condition for 5 min (Fig. 12, 13). This indicates that the steady state photosynthetic parameters shown in Fig. 12 and Fig. 13 were relevant without additional adaptation to gas phase for 5 min.

There are two possibilities for the cause of the difference between Fig. 12 and Fig. 13: Low O_2 concentration or low CO_2 concentration. To distinguish the two possibilities, qP and NPQ at 30 s after starting of AL illumination were compared under CO_2 free condition, 0% O_2 condition and CO_2 free and 0% O_2 condition after plants were dark adapted under normal air condition for 30 min without prior adaptation for gas phase before the chlorophyll fluorescence measurement. As previously shown in Fig. 12, qP and NPQ under CO_2 free and 0% O_2 condition showed significant difference between WT and the *cfa1* mutant (Fig. 14, O_2/CO_2 free column). Not under CO_2 free condition but under 0% O_2 condition, the *cfa1* mutant and WT (Ws) showed diffenrece in qP and NPQ as those under CO_2 free and 0% O_2 condition (Fig. 14). These results indicate that O_2 concentration affected qP and NPQ at early time points after starting AL illumination.

The time course change of chlorophyll fluorescence of *cfa1* mutant under CO_2 free and 2% O_2 condition.

If the initial difference of qP and NPQ observed in Fig. 12 is due to the insufficient gas exchange in leaves leading to the transient low (i.e. 2-5%) O_2 concentration at earlier time point, the difference should be also observed by the prior adaptation of leaves to CO_2 free and 2% O_2 condition. This was the case, and the *cfa1* mutant showed lower qP as well as lower NPQ than WT (Ws) at early points after starting AL illumination (Fig. 15). Furthermore, the difference was also observed at later time points under this condition. The result suggests that the *cfa1* mutant was defective in water-water cycle (see discussion).

The *cfa1* mutant was sensitive to light stress.

Fig. 16 a showed the *cfa1* mutant and WT (Ws) grown on plates under photon flux density at 80 μmol $m^{-2}s^{-1}$ (the left plate) and at 200 μmol $m^{-2}s^{-1}$ (the right plate) for 16 days. The *cfa1* mutant grew slightly slower than WT (Ws) under low light at 80 μmol $m^{-2}s^{-1}$. Under higher light condition at 200 μmol $m^{-2}s^{-1}$, however, slow growth of the *cfa1* mutant became

obvious, and some leaves of the *cfa1* mutant showed significant bleaching.

The contents of chlorophyll *a* and *b* of the *cfa1* mutant did not show significant difference when compared with those of WT (Ws), not only under low light at 80 μmol $m^{-2}s^{-1}$ but also under higher light condition at 200 μmol $m^{-2}s^{-1}$ (Fig. 16 b). Apparently, the result is not consistent with the paler color of the mutant leaves under high light condition. This may due to the difficulty in selecting "standard" leaves for chlorophyll determination from partially bleaching leaves. Leaves of the *cfa1* mutant and WT (Ws) under higher light condition at 200 μmol $m^{-2}s^{-1}$ contained much lower chlorophylls than those under low light at 80 μmol $m^{-2}s^{-1}$ (Fig.16 b).

The ratio of chlorophyll *a* and *b* of WT (Ws) was slightly higher under high light at 200 μmol $m^{-2}s^{-1}$ compared with that under low light at 80 μmol $m^{-2}s^{-1}$ (Fig. 16 c). On the other hand, the ratio of chlorophyll *a* and *b* of the *cfa1* mutant was lower, instead of higher, under higher light condition at 200 μmol $m^{-2}s^{-1}$ (Fig. 16 c). In general, the decrease in chlorophyll *a/b* ratio was resulted from the reduction of PSII core proteins, which can be caused by the photoinhibition of PSII, since PSII antenna proteins have both chlorophyll *a* and *b* while PSII core proteins have only chlorophyll *a*. On the other hand, the increase of chlorophyll *a/b* ratio was resulted from the reduction of PSII antenna proteins, which can be caused by the acclimation to high light condition. Thus, the result suggests that in the *cfa1* mutant cannot acclimate to high light, and becomes sensitive to light stress.

DISCUSSION

The *cfa1* mutant was defective in water-water cycle.

The electrons derived from H_2O by absorbed light energy reduce $NADP^+$ to NADPH. NADPH is then used by CO_2 fixation and photorespiration. There are also some other electron flows, namely, water-water cycle and cyclic electron flow around PSI (CEF-PSI) (Fig. 1). As for the CO2 fixation, several transgenic plants with suppressed photosynthesis have been reported in the past: rice plants with reduced Rubisco, the key enzyme in Calvin cycle (Makino et al., 2002), tobacco plants with reduced Rubisco (Quick et al., 1991; Ruuska et al., 2000) or with reduced phosphoribulokinase, which is also a Calvin cycle enzyme (Habash et al., 1996), and potato and tobacco plants with reduced fructosebisphosphatase, which is another Calvin cycle enzyme (Bilger et al., 1995). These plants all showed higher NPQ than WT in normal air condition. However, the *cfa1* mutant showed lower NPQ rather than higher NPQ when compared with WT (Ws) (Fig. 5). The result indicates that the *cfa1* mutant was not defective in Calvin cycle (i.e. CO_2 fixation).

Under CO_2 free and 2 or 5% O_2 condition, the *cfa1* mutant showed lower qP as well as lower NPQ than WT (Ws) (Figs. 11, 15). In contrast, under CO_2 free and 0% O_2 condition, the *cfa1* mutant and WT (Ws) did not show significant difference in photosynthetic parameters (Figs. 11, 13). These results indicate that the *cfa1* mutant was defective in O_2-dependent photosynthetic pathways, such as photorespiration or water-water cycle. It was indicated that, under 1-2% O_2 condition, photorespiration is suppressed, as the Km value for O_2 of oxygenase reaction of Rubisco was relatively high (33%) (Osmond, 1981). Furthermore, low activity of photorespiration under 1-2% O_2 condition was also supported by the study using transgenic plants (Kozaki and Takeba, 1996). The transgenic tobacco plants with reduced plastidic glutamine synthetase, which is one of the key enzymes in photorespiration, showed lower electron transport rate than WT under CO_2 free and 21% O_2 condition, as photorespiration activity was declined in the mutant. Under CO_2 free and 2% O_2 condition, however, the transgenic plants and WT did not show any difference. These reports indicated that photorespiration was largely suppressed under CO_2 free and at least 2% O_2 condition. Therefore, I concluded that the *cfa1* mutant was not defective in photorespiration but in water-water cycle. The several enzymes involved in Calvin cycle, for example Rubisco, are

known as photoactivated enzymes, which take some time to be activated upon the shift to light condition from dark condition (Usuda, 1985; Hammond et al., 1998). Furthermore, it is indicated that water-water cycle functions as a starter of photosynthesis upon the shift to light condition from dark condition by generating ΔpH across thylakoid membranes to supply ATP for CO_2 fixation and to induce NPQ (Makino et al., 2002). The *cfa1* mutant showed higher chlorophyll fluorescence and lower qP and NPQ than WT upon the shift to light condition from dark condition (Figs. 3, 4, 5, 15). These results also support the conclusion that the *cfa1* mutant was defective in water-water cycle. It is also indicated that the electron flow to water-water cycle decreases with increasing CO_2 fixation and is scarcely detected when CO_2 fixation reached the steady state level under ambient CO_2 and 2% O_2 condition (Makino et al., 2002). The *cfa1* mutant showed lower qP as well as lower NPQ than WT (Ws) at early points after starting AL illumination under CO_2 and 2% O_2 condition (Fig. 15). Furthermore, the difference was also observed at later time points under this condition. The results suggest that, when CO_2 fixation is limited, water-water cycle can function as electron sink at the starter period as well as at the later stage.

From the experiments of chlorophyll fluorescence kinetics (Figs. 3, 4) and chlorophyll fluorescence measurements (Figs. 5, 11, 15) alone, I could not eliminate the possibility that the *cfa1* mutant was defective in CEF-PSI. CEF-PSI has two pathways, namely NDH-dependent pathway and FQR-dependent pathway. NDH complex is the only known component of the NDH-dependent pathway. The tobacco plant, which has disruption in the *ndhB* gene, and the *Arabidopsis* mutants, *crr3* and *crr2-2*, which impairs accumulation of the NDH complex, did not show the transient increase of chlorophyll fluorescence after the cessation of actinic light, which is usually observed for WT plants (Shikanai et al., 1998; Hashimoto et al., 2003; Muraoka et al., 2006). The contribution of the chloroplast NDH complex in photosynthetic electron transport is minor and the *Arabidopsis* mutants specifically defective in NDH activity do not show significant difference of photosynthetic parameters in normal air condition (Hashimoto et al., 2003; Muraoka et al., 2006). The *cfa1* mutant did not show significant difference of photosynthetic parameters (Fig. 5). The results were compatible with the phenotype of the NDH complex mutants. However, the *cfa1* mutant showed the transient chlorophyll fluorescence increase just as in the case of WT (Ws) (Fig. 6). Thus, it is reasonable to assume that the *CFA1* is not involved in NDH pathway of CEF-PSI.

As for FQR-dependent pathway, the only component reported so far is PGR5 protein (Munekage et al., 2002). The *pgr5* mutant showed lower NPQ under high light condition (Munekage et al., 2002). The *cfa1* mutant did have normal amount of PGR5 protein, and did not have any mutation in *PGR5* gene (Figs. 7, 8). Furthermore, the *cfa1* mutant did not show any difference from WT (Ws) compared with photosynthetic parameters under high light condition (Fig. 5 c, d) as well as the *in vitro* assay of CEF-PSI activity (Fig. 9). In this assay, both the *pgr5* mutant and the *crr2-2* mutant showed obvious difference from WT (Munekage et al., 2004). Therefore, I concluded that the *cfa1* mutant was not defective in either pathways of CEF-PSI.

Estimation of the function of *CFA1* in water-water cycle

In water-water cycle, O_2^- and H_2O_2 were rapidly scavenged by following reactions:

$2\ H_2O \rightarrow 4\ e^- + O_2 + 4\ H^+$ (PSII) (1)

$2\ O_2 + 2\ e^- \rightarrow 2\ O_2^-$ (PSI) (2)

$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$ (SOD) (3)

$H_2O_2 + 2$ AsA $\rightarrow 2\ H_2O + 2$ MDA (APX) (4)

2 MDA + 2 redFd $\rightarrow$ 2 AsA + 2 Fd (spontaneous) (5)

2 MDA + NAD(P)H $\rightarrow$ 2 AsA + $NAD(P)^+$ (MDAR) (6)

2 MDA $\rightarrow$ AsA + DHA, DHA + 2GSH $\rightarrow$ AsA + GSSG (DHA reductase) (7)

GSSG + NADPH $\rightarrow$ 2 GSH + $NADP^+$ (Glutathione reductase; GR), 2 Fd or $NADP^+ + 2\ e^-$ $\rightarrow$ 2 redFd or NADPH (PSI) (8)

The primary product of O_2 reduction, O_2^- (Eq. 2), is disproportionated to H_2O_2 and O_2 catalyzed by superoxide dismutase (SOD; Eq. 3). The H_2O_2 generated by SOD is reduced to H_2O by ascorbate (AsA) catalyzed by ascorbate peroxidase (APX), and AsA is oxidized to monodehydroascorbate radical (MDA; Eq. 4). Subsequently, MDA is directly reduced to AsA by either reduced ferredoxin (redFd; Eq. 5) or NAD(P)H catalyzed by chloroplastic MDA reductase (MDAR; Eq. 6). If MDA fails to be reduced directly to AsA, it is spontaneously disproportionated to dehydroascorbate (DHA) and AsA. DHA is then reduced to AsA by reduced glutathione (GSH) catalyzed by DHA reductase (Eq. 7). Finally, Fd and $NADP^+$ for the regeneration of AsA (Eqs. 5-7) are reduced in PSI (Eq. 8). Thus, in any pathways of the regeneration of AsA, the half electrons derived from water in PSII (Eq. 1) are used for the

univalent reduction of oxygen (Eq. 2) and another half for the generation of reductants (Eq. 8) to reduce H_2O_2 (Eqs. 4-7) (Asada, 2006).

The *cfa1* mutants showed lower qP and NPQ than WT under CO_2 free and 2 or 5% O_2 condition (Figs. 11, 15) and lower qP and NPQ than WT at early time points after starting AL illumination under normal air condition (Fig. 5). The results indicated that the electron transport chain was more reduced and the thermal dissipation was suppressed in the *cfa1* mutant compared with WT. Thus, some electron flow is declined under such condition. I think that the electron flow to water-water cycle is declined in the *cfa1* mutant, because of the defect in any one reaction from Eq. 2 to Eq. 4. It is unlikely that the *cfa1* mutant is defective in the reactions from Eq. 5 to Eq. 7, since these reactions are redundant reactions to reduce MDA.

The key enzymes in the scavenging system of reactive oxygen species (ROS) were chloroplastic APX and chloroplastic CuZn-SOD. Phenotypes of knockdown mutant of chloroplastic CuZn-SOD are suppressed growth under low light (100 μmol $m^{-2}s^{-1}$) and low photosynthetic activity (Rizhsky et al., 2003). Therefore, it is unlikely that the *cfa1* mutant is defective in Eq. 3, as the *cfa1* mutant grew slightly slower than WT (Ws) under low light at 80 μmol $m^{-2}s^{-1}$ (Fig. 16) and the *cfa1* mutant and WT did not show the significant difference of photosynthetic parameters at steady state condition (Fig. 5). Chloroplastic APX is classified into thylakoid bound (tAPX) and stroma localized (sAPX) forms. *Arabidopsis* null mutants for sAPX and tAPX (*sapx*, *tapx*) do not show obvious phenotypes as compared to WT. These results indicate that sAPX and tAPX functions redundantly (Giacomelli et al., 2007). The mutation of the *cfa1* mutant would be single judging from the segregation ratio of the 2F2 *cfa1* mutant (Fig. 3). If the *cfa1* mutant is defective in tAPX or sAPX, MDA should be produced by another APX, resulting in intact electron flow. Therefore, it is unlikely that the *cfa1* mutant is defective in tAPX or sAPX. The defect in MDA production would be also brought about by the reduced content of AsA. Several *Arabidopsis* mutants with reduced AsA content were isolated as ozone sensitive mutants (Conklin et al., 2000). All ascorbate mutants show reduced NPQ level (Noctor et al., 2000; Smirnoff, 2000; Muller-Moule et al., 2002). However, the *cfa1* mutants did not show lower NPQ than WT at steady state condition (Fig. 5). Therefore, it is unlikely that AsA content is reduced in the *cfa1* mutant.

It is believed that the limiting step of water-water cycle is the photoreduction of O_2

(Eq. 2), but not the other steps (Asada, 1999). And the reaction of oxygen reduction (Eq. 2) can be separated into two major classes. First is the interaction of O_2 with reduced iron sulfur clusters, F_A/F_B, the terminal electron acceptor of PSI reaction center complexes. A second potential pathway is the interaction of O_2 with stromal components that accept electrons from PSI, for example MDAR, GR and ferredoxin-$NADP^+$ reductase (FNR). This possibility is shown by *in vitro* assay (Miyake et al., 1998). It is likely that the *cfa1* mutant is defective in such stromal components. As the results, in the *cfa1* mutant, electron flow to O_2 would decline and the electron transport chain was more reduced leading to the suppression of thermal dissipation. FNR catalyzes electron transfer between NADP(H) and ferredoxin. In higher plants, the leaf form of FNR exists as two isoforms, AtLFNR1 and AtLFNR2. The Arabdopsis mutant, ΔFNR1, is viable, although the size of leaves is slightly smaller and the color of leaves is pale green (Lintala et al., 2007). Interestingly, the ΔFNR1 mutant did not show obvious difference of photosynthetic properties including qP (Lintala et al., 2007). At present, the functional specificity of the isoforms still remains elusive. Whether the ΔFNR mutants has defect in water-water cycle or not is also unknown. It is also unknown whether MDAR or GR is involved in water-water cycle activity *in vivo*.

Although the mechanism is not revealed, the *cfa1* mutant showed sensitivity to high light stress (Fig. 16). As one possibility of this mechanism, I assume that the rate of reduction of O_2 by PSII and subsequent reduction of plastoquinone (Cleland and Grace, 1999) is increased by the degradation of water-water cycle by the defect of some component, which accelerate the affinity with O_2 at stroma side of PSI. ROS made by PSII and reduced plastoquinone might induce photoinhibition, since the ROS might be not scavenged rapidly and effectively as in the presence of water-water cycle.

My work presented here is first report for the mutant that shows a defect in the maintenance of affinity to O_2 *in vivo*. This study would be a breakthrough to the understanding of the regulation of water-water cycle after the mutation point of the *cfa1* mutant is revealed by map-based cloning.

REFERENCES

Asada, K. (1999). The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology **50,** 601-639.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiology **141,** 391-396.

Asada, K., Heber, U., and Schreiber, U. (1993). Electron Flow to the Intersystem Chain from Stromal Components and Cyclic Electron Flow in Maize Chloroplasts, as Detected in Intact Leaves by Monitoring Redox Change of P700 and Chlorophyll Fluorescence. Plant and Cell Physiology **34,** 39-50.

Beckmann, K., Dzuibany, C., Biehler, K., Fock, H., Hell, R., Migge, A., and Becker, T.W. (1997). Photosynthesis and fluorescence quenching, and the mRNA levels of plastidic glutamine synthetase or of mitochondrial serine hydroxymethyltransferase (SHMT) in the leaves of the wild-type and of the SHMT-deficient stm mutant of Arabidopsis thaliana in relation to the rate of photorespiration. Planta **202,** 379-386.

Bilger, W., Fisahn, J., Brummet, W., Kossmann, J., and Willmitzer, L. (1995). Violaxanthin Cycle Pigment Contents in Potato and Tobacco Plants with Genetically Reduced Photosynthetic Capacity. Plant Physiology **108,** 1479-1486.

Cleland, R.E., and Grace, S.C. (1999). Voltammetric detection of superoxide production by photosystem II. Febs Letters **457,** 348-352.

Conklin, P.L., Saracco, S.A., Norris, S.R., and Last, R.L. (2000). Identification of ascorbic acid-deficient Arabidopsis thaliana mutants. Genetics **154,** 847-856.

Endo, T., Shikanai, T., Sato, F., and Asada, K. (1998). NAD(P)H dehydrogenase-dependent, antimycin A-sensitive electron donation to plastoquinone in tobacco chloroplasts. Plant and Cell Physiology **39,** 1226-1231.

Farquhar, G.D., Caemmerer, S.V., and Berry, J.A. (1980). A Biochemical-Model of Photosynthetic Co2 Assimilation in Leaves of C-3 Species. Planta **149,** 78-90.

Foyer, C.H., Descourvieres, P., and Kunert, K.J. (1994). Protection against Oxygen Radicals - an Important Defense-Mechanism Studied in Transgenic Plants. Plant Cell and Environment **17,** 507-523.

Giacomelli, L., Masi, A., Ripoll, D.R., Lee, M.J., and van Wijk, K.J. (2007). Arabidopsis thaliana deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. Plant Molecular Biology **65,** 627-644.

Habash, D.Z., Parry, M.A.J., Parmar, S., Paul, M.J., Driscoll, S., Knight, J., Gray, J.C., and Lawlor, D.W. (1996). The regulation of component processes of photosynthesis in transgenic tobacco with decreased phosphoribulokinase activity. Photosynthesis Research **49,** 159-167.

Hammond, E.T., Andrews, T.J., and Woodrow, I.E. (1998). Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase by carbamylation and 2-carboxyarabinitol 1-phosphate in tobacco: Insights from studies of antisense plants containing reduced amounts of rubisco activase. Plant Physiology **118,** 1463-1471.

Hashimoto, M., Endo, T., Peltier, G., Tasaka, M., and Shikanai, T. (2003). A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast ndhB in Arabidopsis. Plant Journal **36,** 541-549.

Hill, R., and Bendall, F. (1960). Function of the 2 Cytochrome Components in Chloroplasts - Working Hyothesis. Nature **186,** 136-137.

Kozaki, A., and Takeba, G. (1996). Photorespiration protects C3 plants from photooxidation. Nature **384,** 557-560.

Lintala, M., Allahverdiyeva, Y., Kidron, H., Piippo, M., Battchikova, N., Suorsa, M., Rintamaki, E., Salminen, T.A., Aro, E.M., and Mulo, P. (2007). Structural and functional characterization of ferredoxin-NADP(+)-oxidoreductase using knock-out mutants of Arabidopsis. Plant Journal **49,** 1041-1052.

Makino, A., Miyake, C., and Yokota, A. (2002). Physiological functions of the water-water cycle (Mehler reaction) and the cyclic electron flow around PSI in rice leaves. Plant Cell Physiol **43,** 1017-1026.

Mi, H.L., Endo, T., Ogawa, T., and Asada, K. (1995). Thylakoid Membrane-Bound, Nadph-Specific Pyridine-Nucleotide Dehydrogenase Complex Mediates Cyclic Electron-Transport in the Cyanobacterium Synechocystis Sp Pcc-68038. Plant and Cell Physiology **36,** 661-668.

Miyake, C., and Asada, K. (1994). Ferredoxin-Dependent Photoreduction of the Monodehydroascorbate Radical in Spinach Thylakoids. Plant and Cell Physiology **35,** 539-549.

Miyake, C., and Yokota, A. (2000). Determination of the rate of photoreduction of O-2 in the water-water cycle in watermelon leaves and enhancement of the rate by limitation of photosynthesis. Plant and Cell Physiology **41,** 335-343.

Miyake, C., Schreiber, U., Hormann, H., Sano, S., and Asada, K. (1998). The FAD-enzyme monodehydroascorbate radical reductase mediates photoproduction of superoxide radicals in spinach thylakoid membranes. Plant and Cell Physiology **39,** 821-829.

Muller-Moule, P., Conklin, P.L., and Niyogi, K.K. (2002). Ascorbate deficiency can limit violaxanthin de-epoxidase activity in vivo. Plant Physiology **128,** 970-977.

Muller, P., Li, X.P., and Niyogi, K.K. (2001). Non-photochemical quenching. A response to excess light energy. Plant Physiology **125,** 1558-1566.

Munekage, Y., Takeda, S., Endo, T., Jahns, P., Hashimoto, T., and Shikanai, T. (2001). Cytochrome b(6)f mutation specifically affects thermal dissipation of absorbed light energy in Arabidopsis. Plant Journal **28,** 351-359.

Munekage, Y., Hojo, M., Meurer, J., Endo, T., Tasaka, M., and Shikanai, T. (2002). PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell **110,** 361-371.

Munekage, Y., Hashimoto, M., Miyake, C., Tomizawa, K., Endo, T., Tasaka, M., and Shikanai, T. (2004). Cyclic electron flow around photosystem I is essential for photosynthesis. Nature **429,** 579-582.

Muraoka, R., Okuda, K., Kobayashi, Y., and Shikanai, T. (2006). A eukaryotic factor required for accumulation of the chloroplast NAD(P)H dehydrogenase complex in Arabidopsis. Plant Physiology **142,** 1683-1689.

Niyogi, K.K. (1999). Photoprotection revisited: Genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology **50,** 333-359.

Noctor, G., Veljovic-Jovanovic, S., and Foyer, C.H. (2000). Peroxide processing in photosynthesis: antioxidant coupling and redox signalling. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **355,** 1465-1475.

Osmond, C.B. (1981). Photorespiration and photoinhibition: some implications for the energitics of photosynthesis. Biochimica Et Biophysica Acta **639,** 77-98.

Porra, R.J., Thompson, W.A., and Kriedemann, P.E. (1989). Determination of Accurate Extinction Coefficients and Simultaneous-Equations for Assaying Chlorophyll-a and Chlorophyll-B Extracted with 4 Different Solvents - Verification of the Concentration of Chlorophyll Standards by Atomic-Absorption Spectroscopy. Biochimica Et Biophysica Acta **975,** 384-394.

Quick, W.P., Schurr, U., Scheibe, R., Schulze, E.D., Rodermel, S.R., Bogorad, L., and Stitt, M. (1991). Decreased Ribulose-1,5-Bisphosphate Carboxylase-Oxygenase in Transgenic Tobacco Transformed with Antisense Rbcs .1. Impact on Photosynthesis in Ambient Growth-Conditions. Planta **183,** 542-554.

Rizhsky, L., Liang, H.J., and Mittler, R. (2003). The water-water cycle is essential for chloroplast protection in the absence of stress. Journal of Biological Chemistry **278,** 38921-38925.

Ruuska, S.A., von Caemmerer, S., Badger, M.R., Andrews, T.J., Price, G.D., and Robinson, S.A. (2000). Xanthophyll cycle, light energy dissipation and electron transport in transgenic tobacco with reduced carbon assimilation capacity. Australian Journal of Plant Physiology **27,** 289-300.

Schreiber, U. (1986). Detection of Rapid Induction Kinetics with a New Type of High-Frequency Modulated Chlorophyll Fluorometer. Photosynthesis Research **9,** 261-272.

Schwarte, S., and Bauwe, H. (2007). Identification of the photorespiratory 2-phosphoglycolate phosphatase, PGLP1, in Arabidopsis. Plant Physiology **144,** 1580-1586.

Shikanai, T. (2007). Cyclic electron transport around photosystem I: Genetic approaches. Annual Review of Plant Biology **58,** 199-217.

Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K., and Yokota, A. (1998). Directed disruption of the tobacco ndhB gene impairs cyclic electron flow around photosystem I. Proceedings of the National Academy of Sciences of the United States of America **95,** 9705-9709.

Smirnoff, N. (2000). Ascorbate biosynthesis and function in photoprotection. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **355,** 1455-1464.

Somerville, C.R., and Ogren, W.L. (1979). Phosphoglycolate Phosphatase-Deficient Mutant of Arabidopsis. Nature **280,** 833-836.

Somerville, C.R., and Ogren, W.L. (1980). Photo-Respiration Mutants of Arabidopsis-Thaliana Deficient in Serine-Glyoxylate Aminotransferase Activity. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **77,** 2684-2687.

Usuda, H. (1985). Changes in Levels of Intermediates of the C-4 Cycle and Reductive Pentose-Phosphate Pathway during Induction of Photosynthesis in Maize Leaves. Plant Physiology **78,** 859-864.

Wang, D., and Portis, A.R. (2007). A novel nucleus-encoded chloroplast protein, PIFI, is involved in NAD(P)H dehydrogenase complex-mediated chlororespiratory electron transport in Arabidopsis(1,2[W][OA]). Plant Physiology **144,** 1742-1752.

Wingler, A., Lea, P.J., Quick, W.P., and Leegood, R.C. (2000). Photorespiration: metabolic pathways and their role in stress protection. Philosophical Transactions of the Royal Society B-Biological Sciences **355,** 1517-1529.

FIGURES

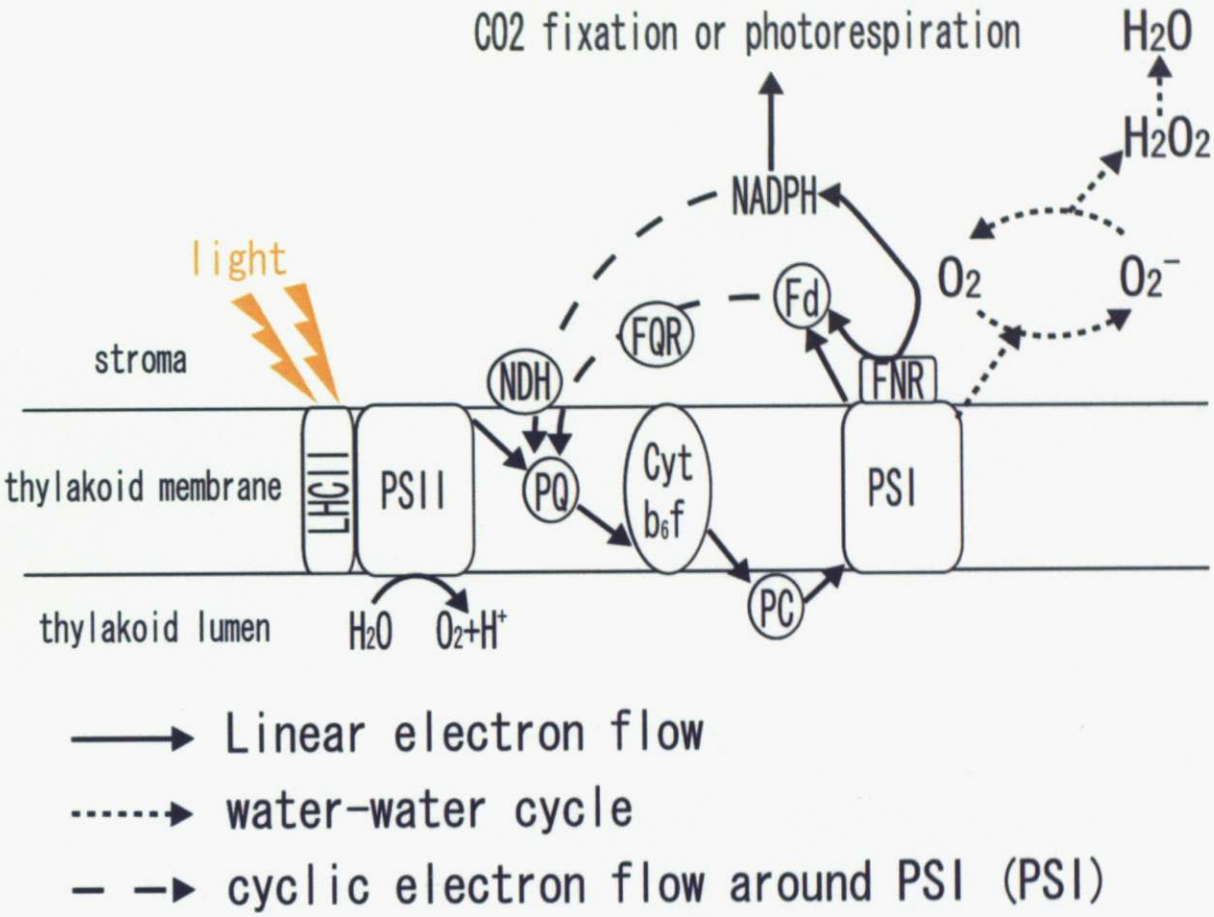

Fig. 1 Schematic model of photosynthetic electron flow

There are several electron flows involved in photosynthetic electron flow.

Abbreviations: LHCII, light-harvesting chlorophyll-protein complex II; PSII, photosystem II; PSI, photosystem I; PQ, plastoquinone; Cyt b_6f, cytochrome b_6f complex; PC, plastocyanin; Fd, ferredoxin; FQR, ferredoxin-plastoquinone reductase; NDH, NAD(P)H dehydrogenase; FNR, ferredoxin-$NADP^+$ oxidoreductase.

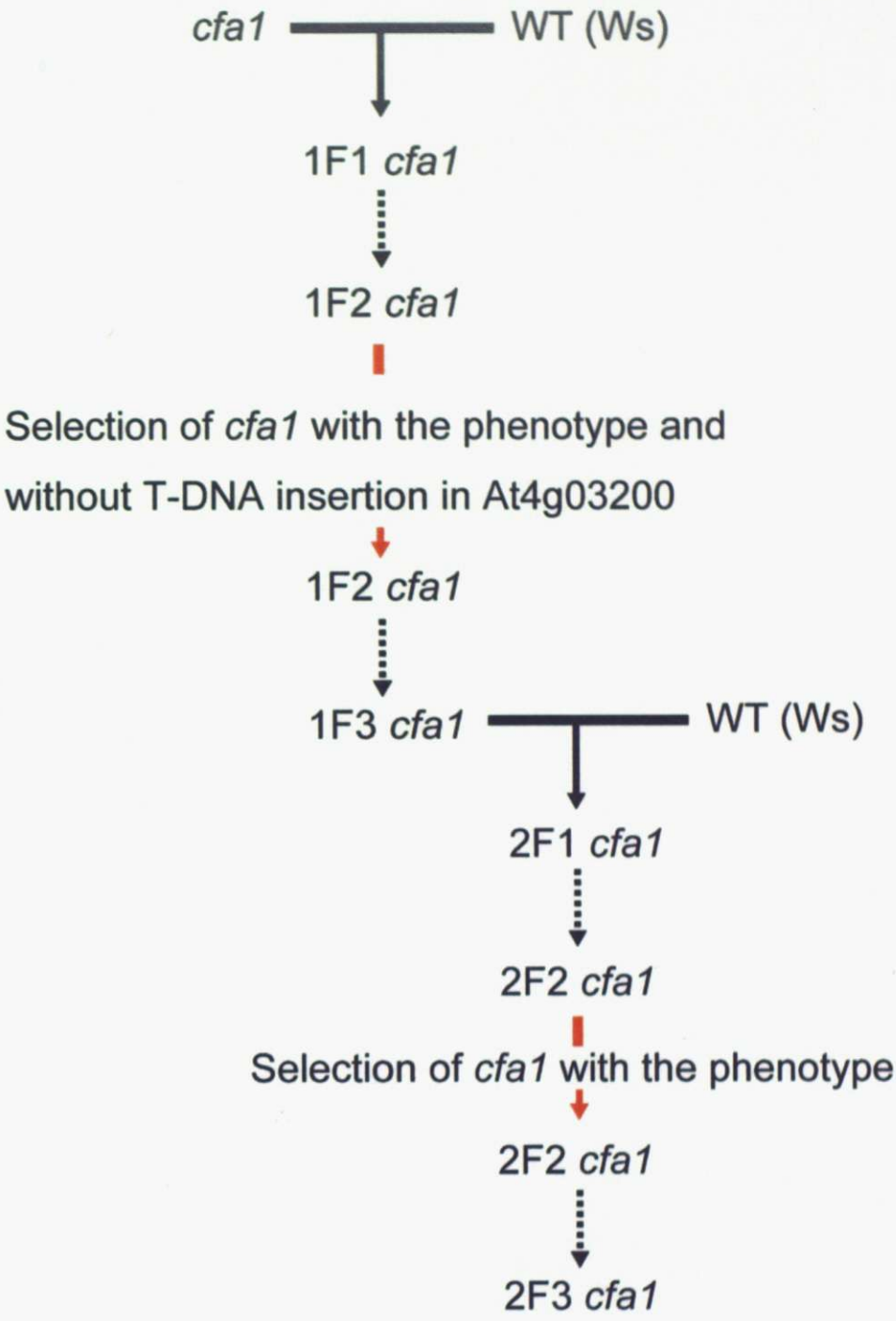

Fig. 2 The illustration of seed populations

The relationships among each seed populations are discribed and termed in this figure. Dotted line and fulled line mean self-fertilization and back cross. Red line indicated the selection. The phenotype indicated that relative intensity of chlorophyll fluorescence from plants is higher than that of WT at early time points after the shift of light condition from dark to light.

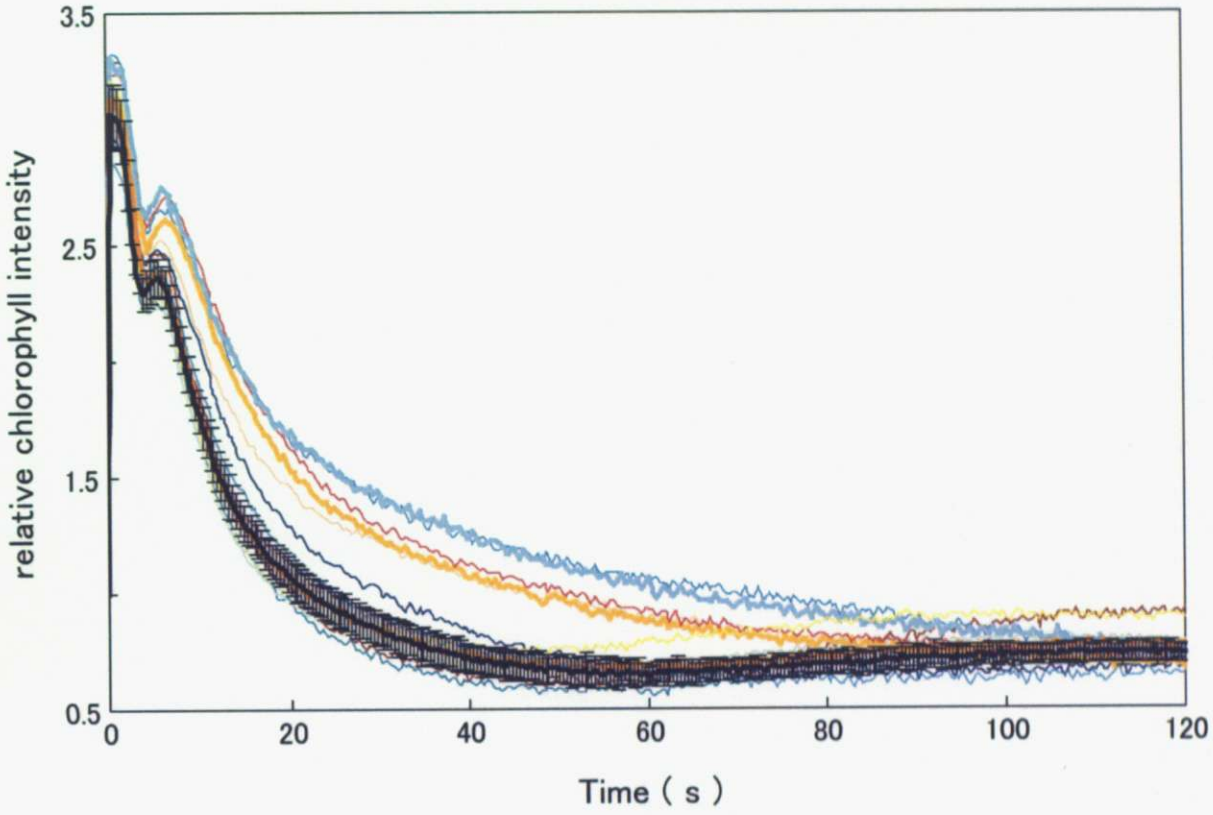

Fig. 3 Chlorophyll fluorescence kinetics of WT (Ws) and the 2F2 *cfa1* mutants

Chlorophyll fluorescence was monitored under illumination (350 μmol m^{-2} s^{-1}) for 120 s by a fluorescence video-imaging system. Black line indicates the mean ± SD of the WT (Ws) (n=4). The other color lines indicate the 2F2 *cfa1* mutant. Plants grown on plates were dark adapted for 10 min before measurement.

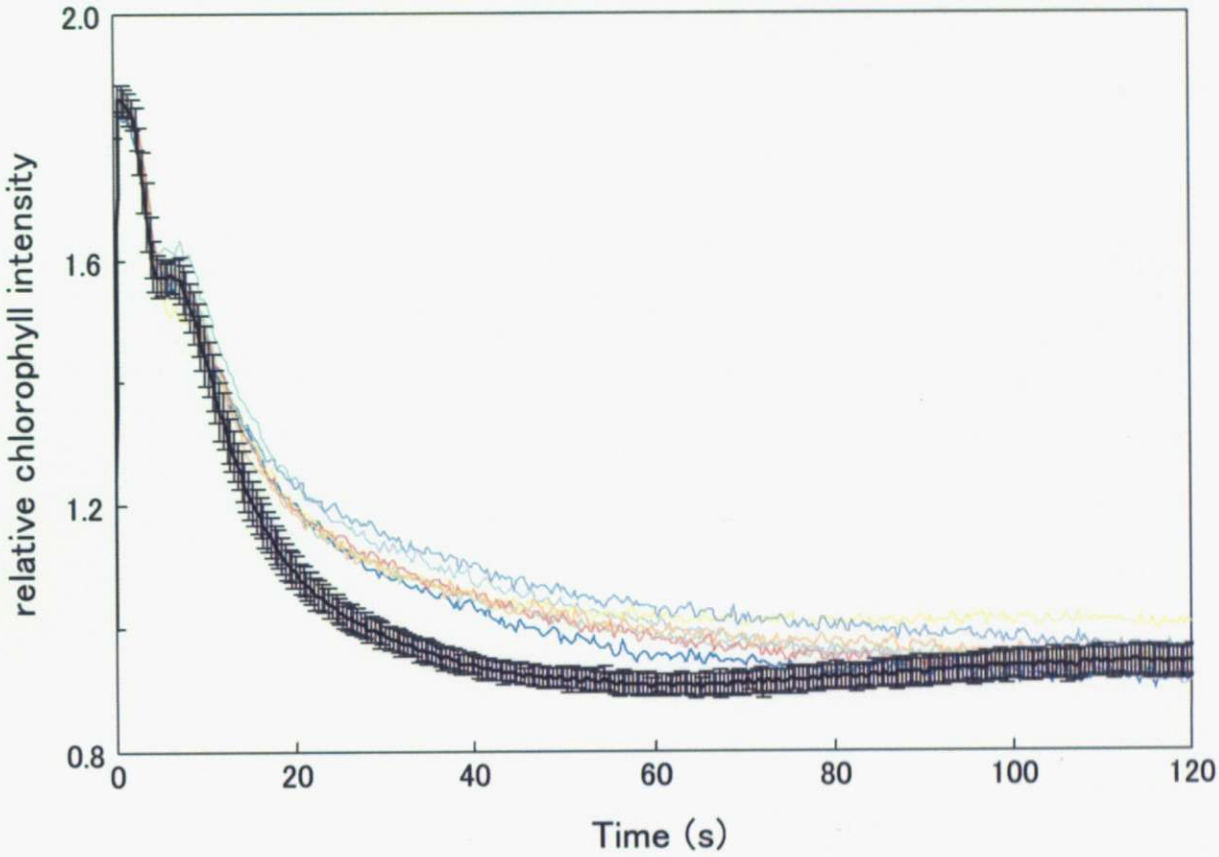

Fig. 4 Chlorophyll fluorescence kinetics of WT (Ws) and the 2F3 *cfa1* mutants

Chlorophyll fluorescence was monitored under illumination (350 μmol m^{-2} s^{-1}) for 120 s by a fluorescence video-imaging system. Black line indicates the mean ± SD of the WT (Ws) (n=8). The other color lines indicate the 2F3 *cfa1* mutant. Plants grown on plates were dark adapted for 10 min before measurement.

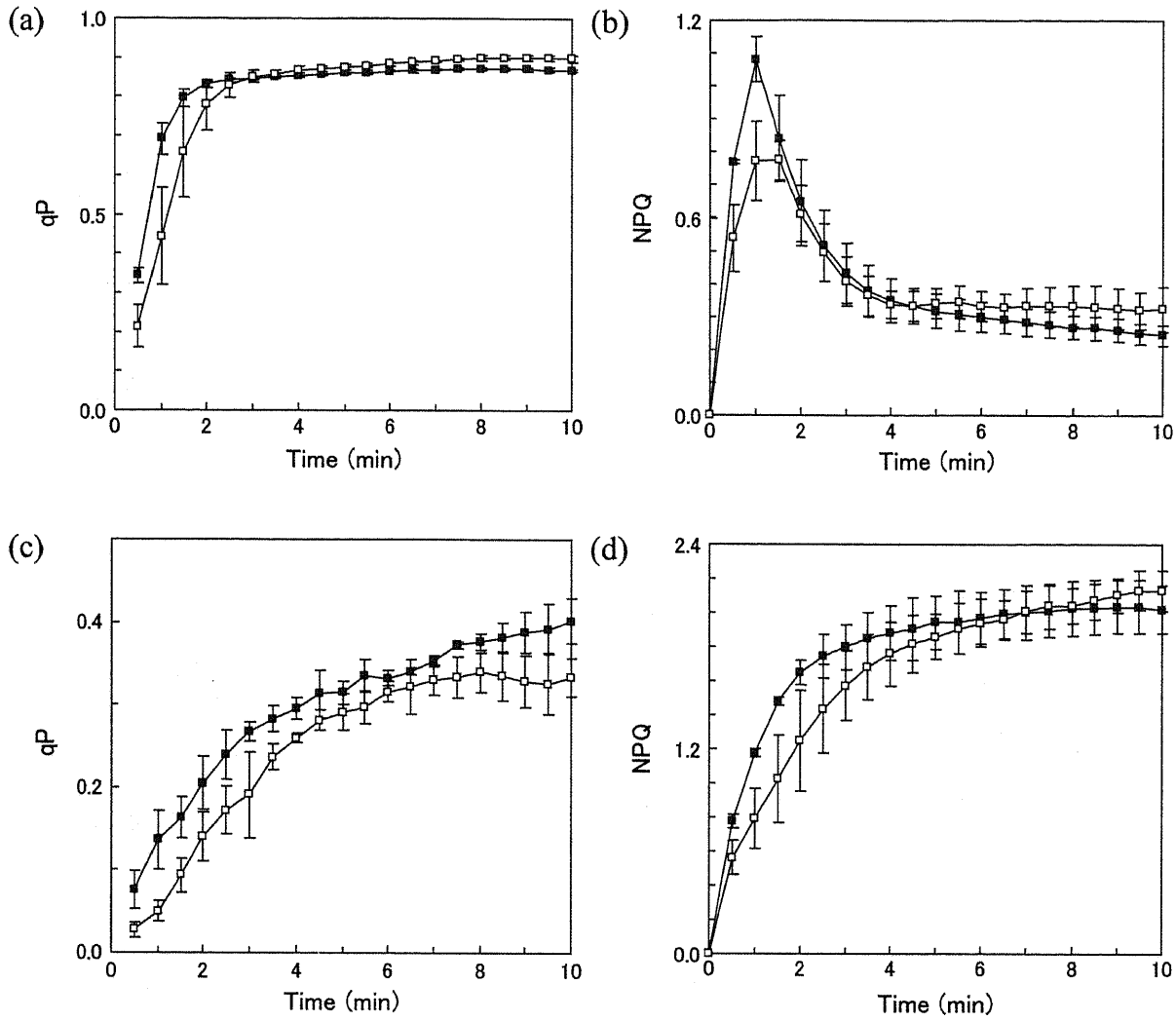

Fig. 5 Time course of qP and NPQ in WT (Ws) and the *cfa1* mutant

qP and NPQ was measured for 10 min from the onset of actinic light (AL) at 80 μmol m^{-2} s^{-1} (a) (b) or 400 μmol m^{-2} s^{-1} (c) (d). Closed and open squares indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of three samples ± SD. Plants grown on plates were dark adapted for 30 min before measurement.

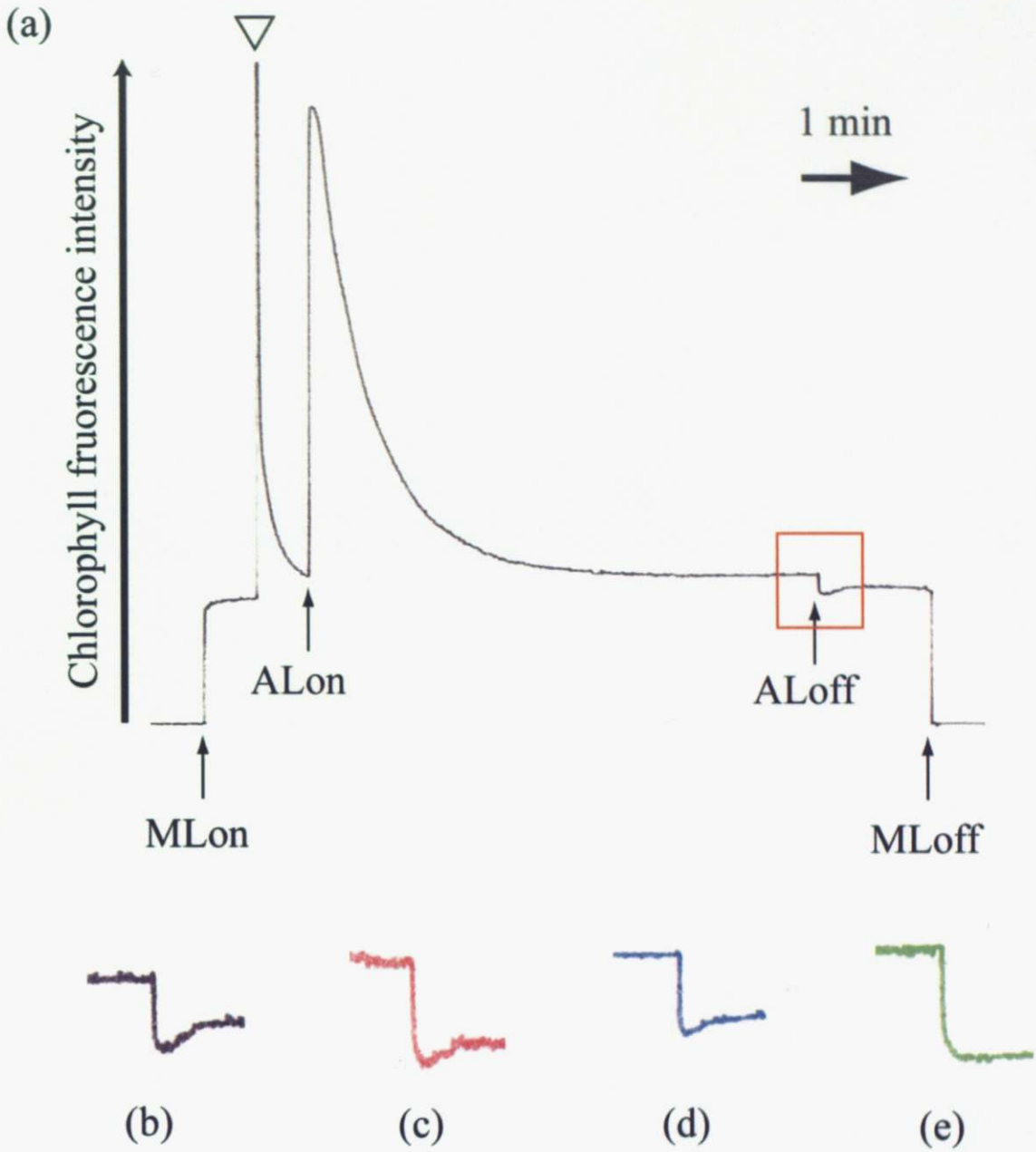

Fig. 6 Detection of NDH activity by chlorophyll fluorescence

(a) Typical trace of chlorophyll fluorescence change in WT (Ws). A red boxed region was closed up and shown to (b)-(e), (b) WT (Ws), (c) the *cfa1* mutant, (d) WT (Col *gl1*), (e) the *crr2-2* mutant. White triangle indicates applying a saturating pulse (SP). ML and AL indicate measuring light and actinic light, respectively. AL (80 μmol m^{-2} s^{-1}) was illuminated for 5 min. Plants grown on plates were dark adapted for 30 min before measurement.

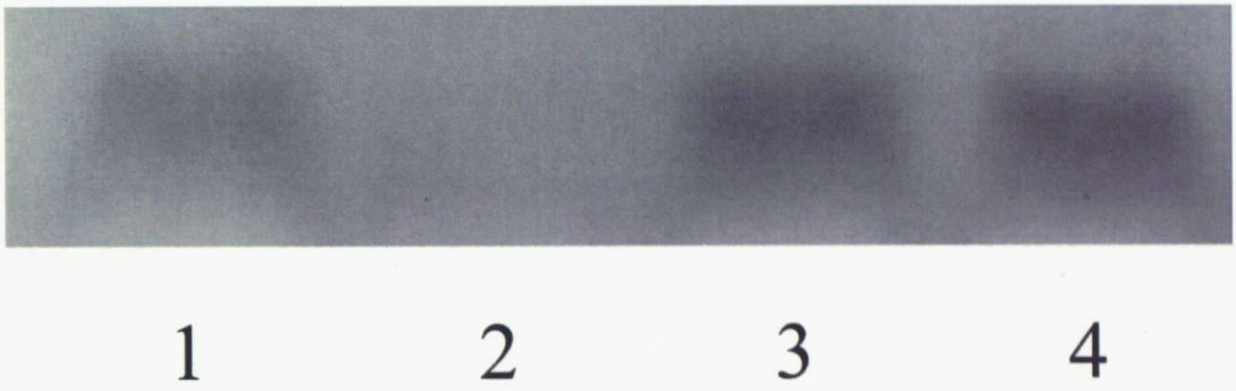

Fig. 7 Western blot analysis of PGR5 protein

Lane number 1, 2, 3, 4 indicate WT (Col *gl1*), the *pgr5* mutant, WT (Ws) and the *cfa1* mutant, respectively. Intact chloroplast was extracted from laves of plants grown on plates. Chloroplast proteins equivalent to 4.0 μg chlorophyll were resolved by 17% SDS-PAGE.

ATGGCTGCTGCTTCGATTTCTGCAATAGGATGTAATCAAACTTTGCTAGG

AACTTCCTTCTATGGAGGATGGGGAAGTTCCATCTCCGGAGAAGATTACC

AAACCATGCTCTCCAAGACAGTTGCGCCTCCGCAACAAGCCAGAGTCTCA

AGGAAAGCAATCAGAGCAGTTCCAATGATGAAGAATGTCAATGAAGGCAA

AGGCTTATTTGCACCTCTAGTTGTTGTCACACGCAACCTAGTAGGCAAGA

AGAGGTTTAATCAGCTCAGAGGAAAAGCCATTGCCTTACACTCTCAGGTG

ATCACTGAGTTTTGCAAATCGATTGGAGCAGATGCAAAACAGAGACAAGG

GCTTATCAGGCTTGCTAAGAAGAATGGAGAGAGGCTTGGTTTCCTTGCTTAG

Fig. 8 Sequencing of *PGR5* gene

Genomic DNA that was isolated from laves of plants grown on plates. ORF sequence of *PGR5* gene of WT (Ws) and *cfa1* was sequenced by using a dye terminator cycle sequencing kit and ABI prism sequencer. The sequence results for the two strains completely match with each other, so that only one sequence is presented here. The first and last under lines indicate start codon and stop codon, respectively.

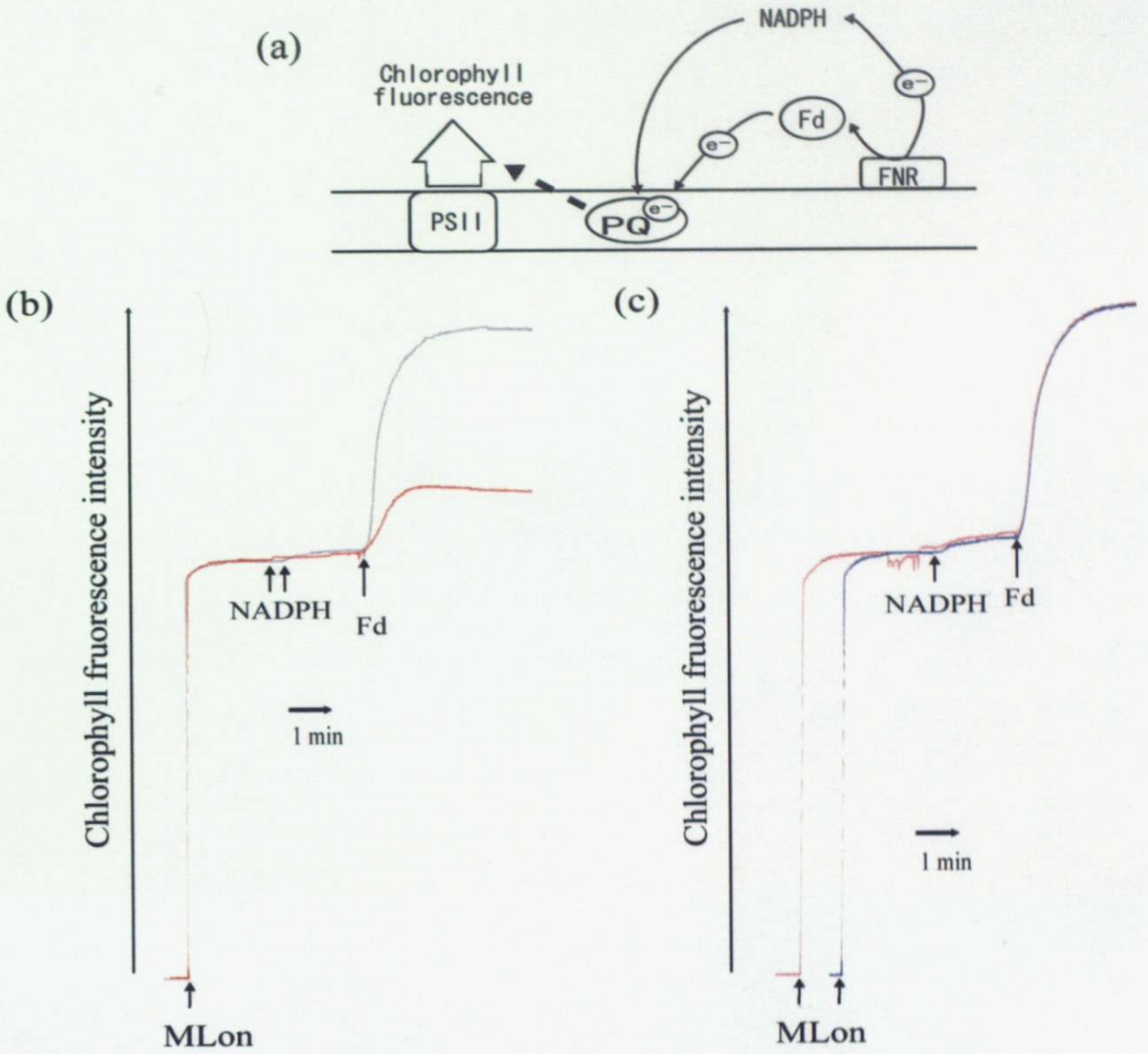

Fig. 9 ***In vitro*** **assay of CEF-PSI**

(a) Schematic model of electron flow in this assay. Dash line indicates that PQ reduction lead to the increase in chlorophyll fluorescence. (b) Blue and red lines indicate WT (Col *gl1*) and the *pgr5* mutant, respectively. (c) Blue and red lines indicate WT (Ws) and the *cfa1* mutant, respectively. The osmotically ruptured chloroplasts derived from respective plants grown on the plates were diluted with the assay medium to 25 μg Chl ml^{-1}. NADP and Fd indicate the addition of 0.25 mM NADPH and 5 μM Fd, respectively. ML indicates measureing light.

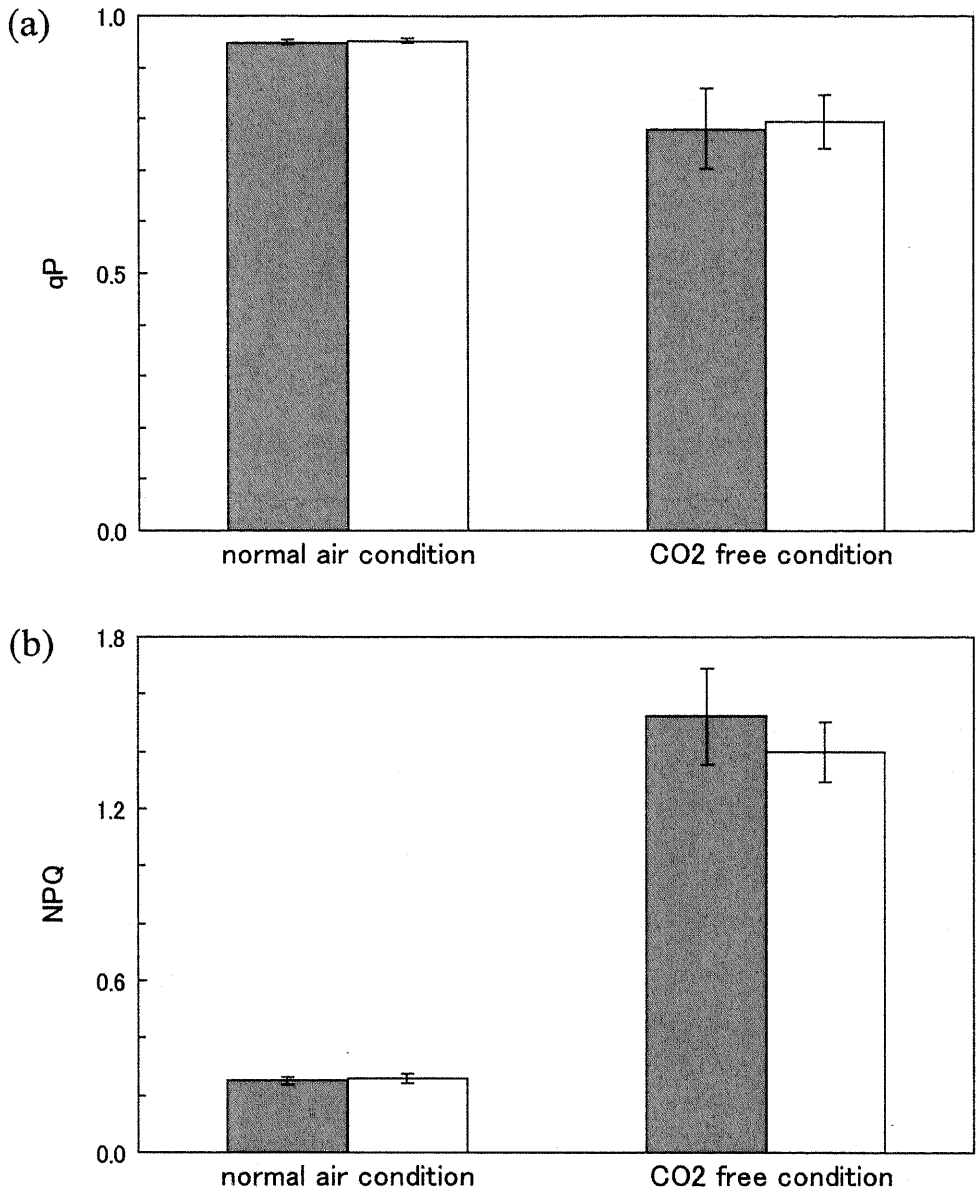

Fig. 10 Steady state qP and NPQ of WT (Ws) and the *cfa1* mutant under normal air condition or CO_2 free condition

Steady state qP (a) and NPQ (b) were measured under normal air condition or CO_2 free condition after 10 min from onset of actini light (80 μmol m^{-2} s^{-1}) illumination. Gray and white bars indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of three samples ± SD. Plants grown on soil were dark-adapted for 30 min under normal air condition. After 30 min, leaves were set in the measurement chamber filled with normal air or CO_2 free air and the measurement was immediately started.

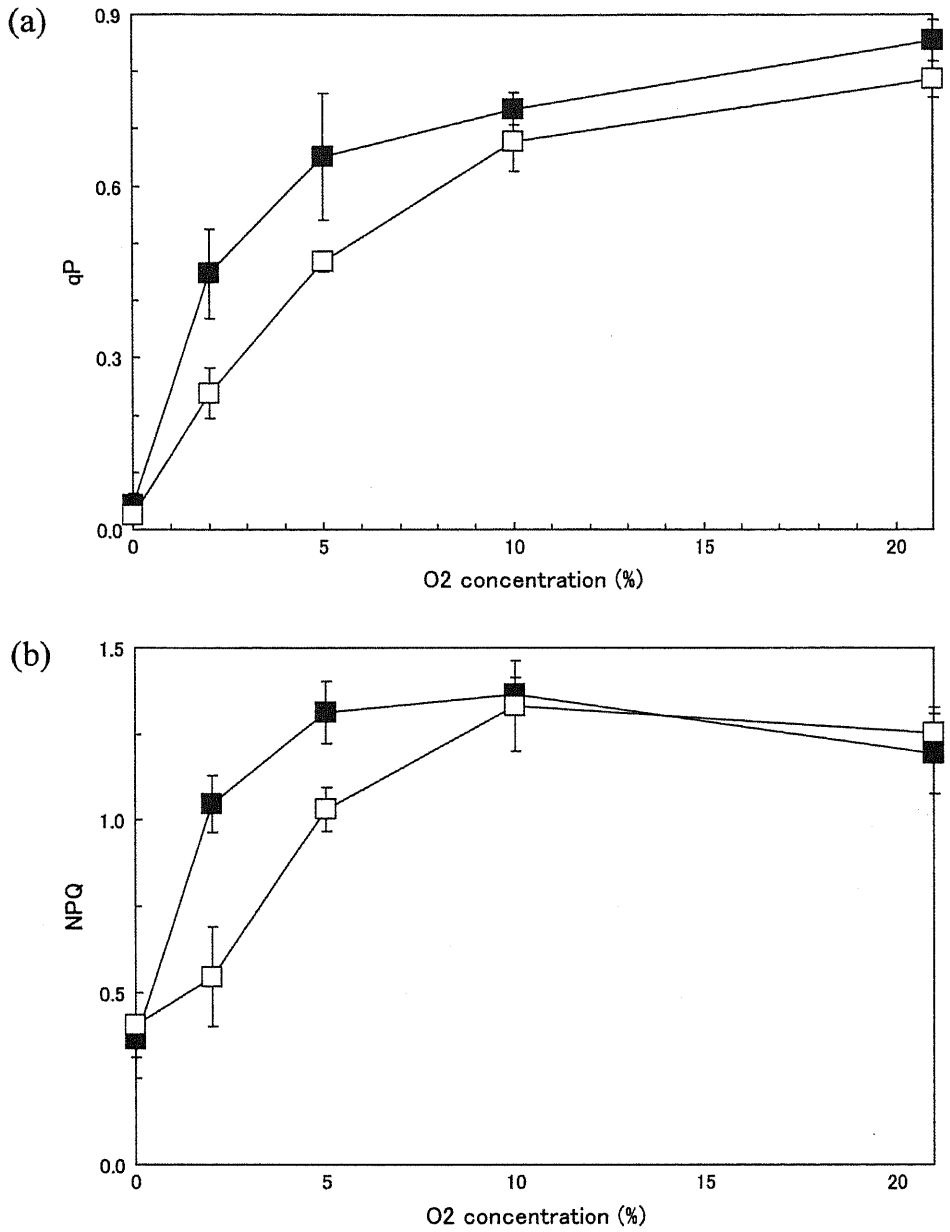

Fig. 11 O_2-dependent variation of steady state qP and NPQ of WT (Ws) and the *cfa1* mutant under CO_2 free condition

Steady state qP (a) and NPQ (b) were measured with several different O_2 concentration (0, 2, 5, 10, 21%) under CO_2 free condition after 10 min from onset of actini light (40 μmol m^{-2} s^{-1}) illumination. Closed and open squares indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of three samples ± SD. Plants grown on soil were dark-adapted for 30 min under normal air condition. After 30 min, leaves were set in the measurement chamber filled with the respective air and the measurement was immediately started.

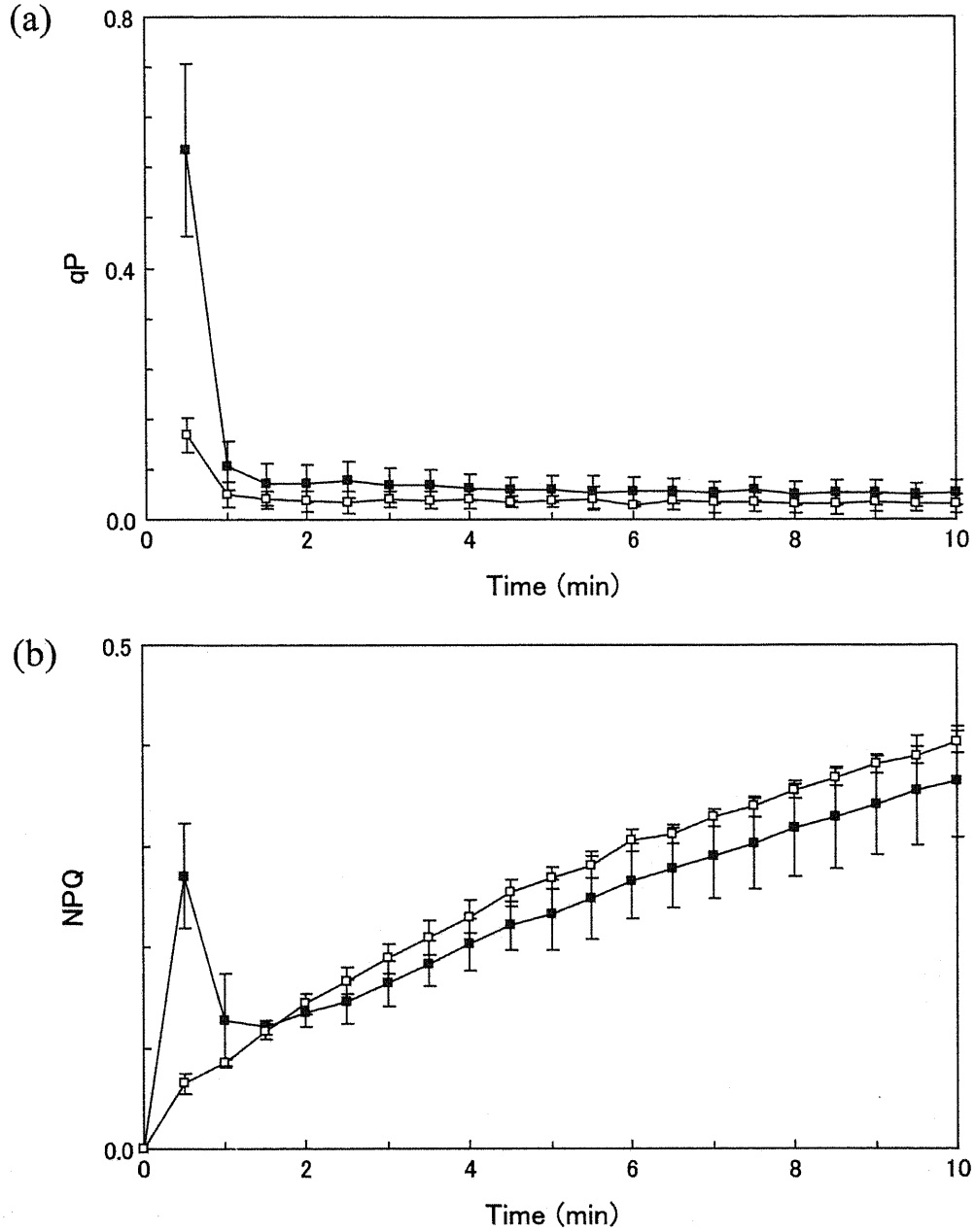

Fig. 12 Time course of qP and NPQ in WT (Ws) and the *cfa1* mutant under O_2 free and 0% O_2 condition without the adaptation to this condition

qP (a) and NPQ (b) were measured under CO_2 free and 0% O_2 condition for 10 min from onset of actini light (40 μmol m^{-2} s^{-1}) illumination. Closed and open squares indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of three samples ± SD. Plants grown on soil were dark-adapted for 30 min under normal air condition. After 30 min, leaves were set in the measurement chamber filled with CO_2 free and 0% O_2 air and the measurement was immediately started.

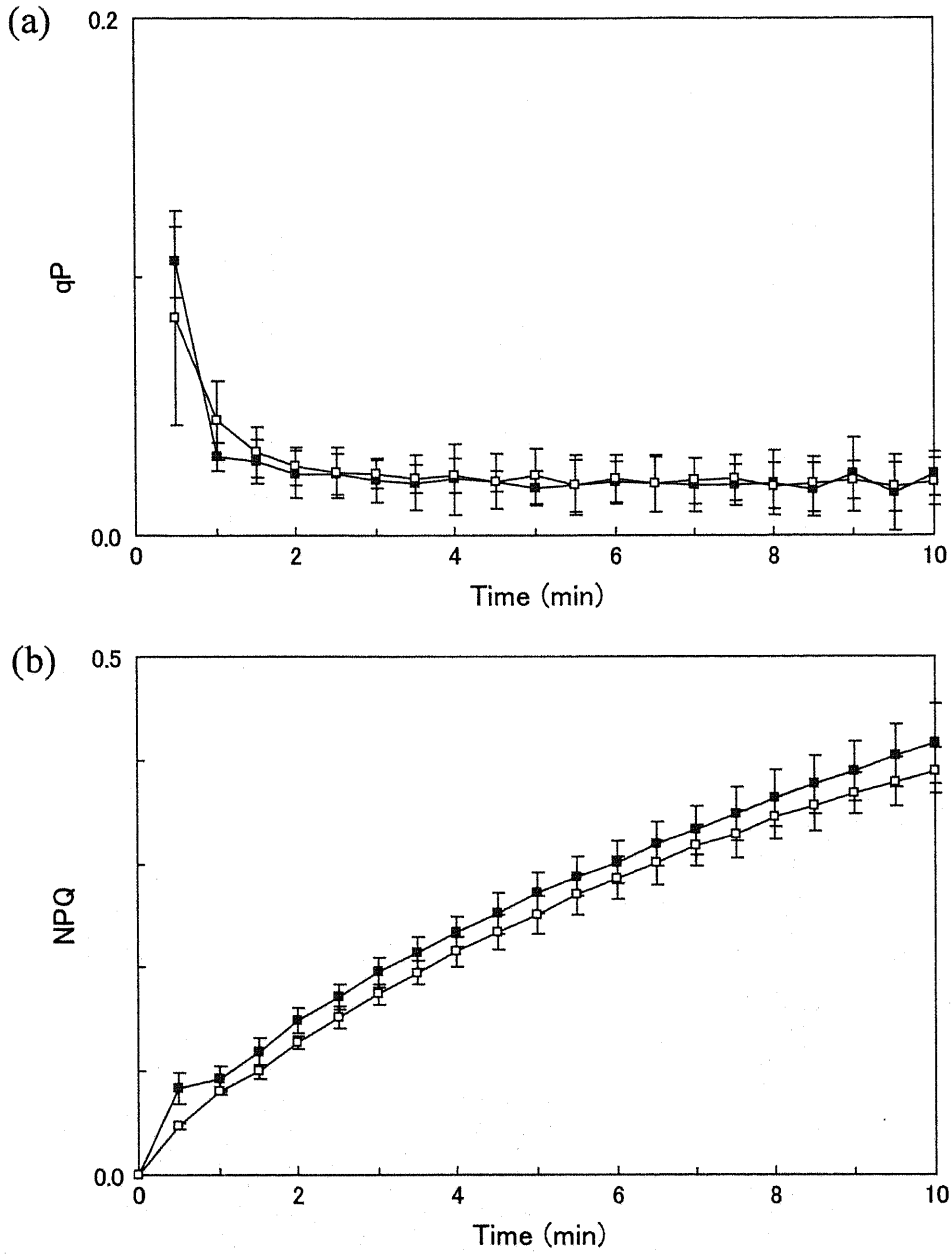

Fig. 13 Time course of qP and NPQ in WT (Ws) and the *cfa1* mutant under O_2 free and 0% O_2 condition with the adaptation to this condition for 5 min

qP (a) and NPQ (b) were measured under CO_2 free and 0% O_2 condition for 10 min from onset of actini light (40 μmol m^{-2} s^{-1}) illumination. Closed and open squares indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of three samples ± SD. Plants grown on soil were dark-adapted for 25 min under normal air condition. After 25 min, leaves were set in the measurement chamber filled with CO_2 free and 0% O_2 air. After 5 min, the measurement was started.

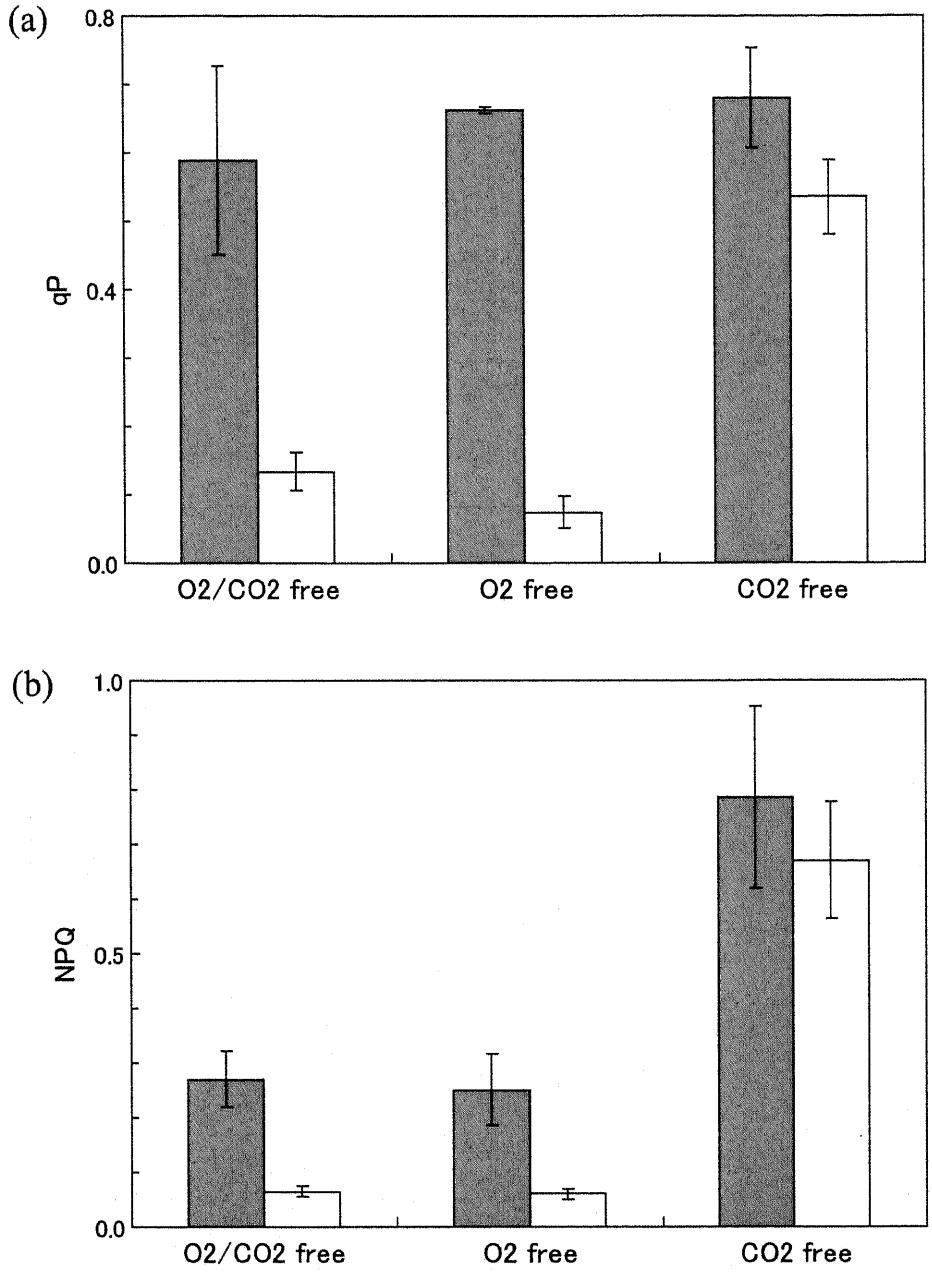

Fig. 14 Initial qP and NPQ of WT (Ws) and the *cfa1* mutant under various air conditions

Initial qP (a) and NPQ (b) were measured under CO_2 free and 0% O_2 (O_2/CO_2 free) condition, 0% O_2 (O_2 free) condition or CO_2 free condition after 30 s from onset of actini light (40 μmol m^{-2} s^{-1}) illumination. Gray and white bars indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of three samples ± SD. Plants grown on soil were dark-adapted for 30 min under normal air condition. After 30 min, leaves were set in the measurement chamber filled with the respective air and the measurement was immediately started.

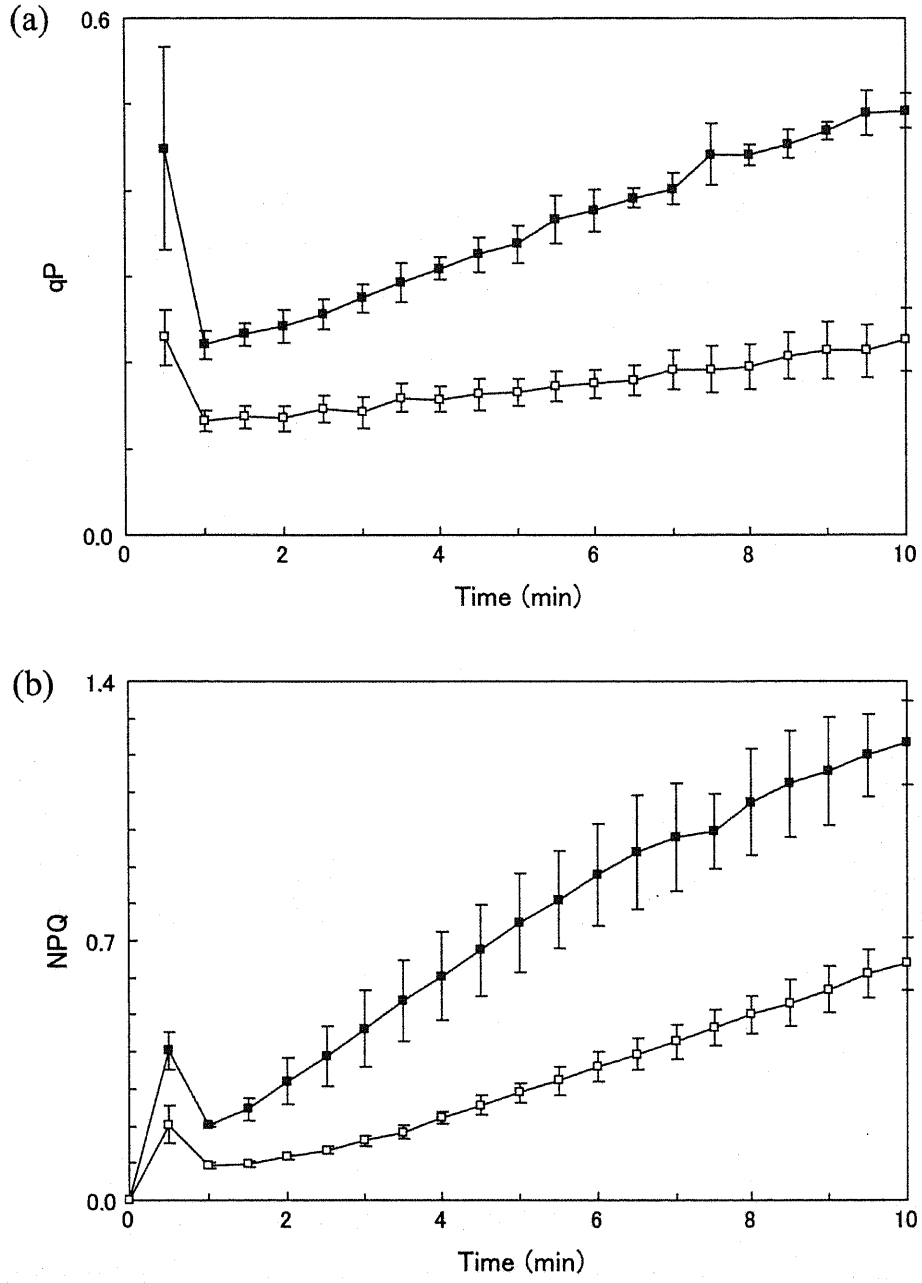

Fig. 15 Time course of qP and NPQ in WT (Ws) and the *cfa1* mutant under O_2 free and 2% O_2 condition with the adaptation to this condition for 5 min

qP (a) and NPQ (b) were measured under CO_2 free and 2% O_2 condition for 10 min from onset of actini light (40 μmol m^{-2} s^{-1}) illumination. Closed and open squares indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of three samples ± SD. Plants grown on soil were dark-adapted for 25 min under normal air condition. After 25 min, leaves were set in the measurement chamber filled with CO_2 free and 2% O_2 air. After 5 min, the measurement was started.

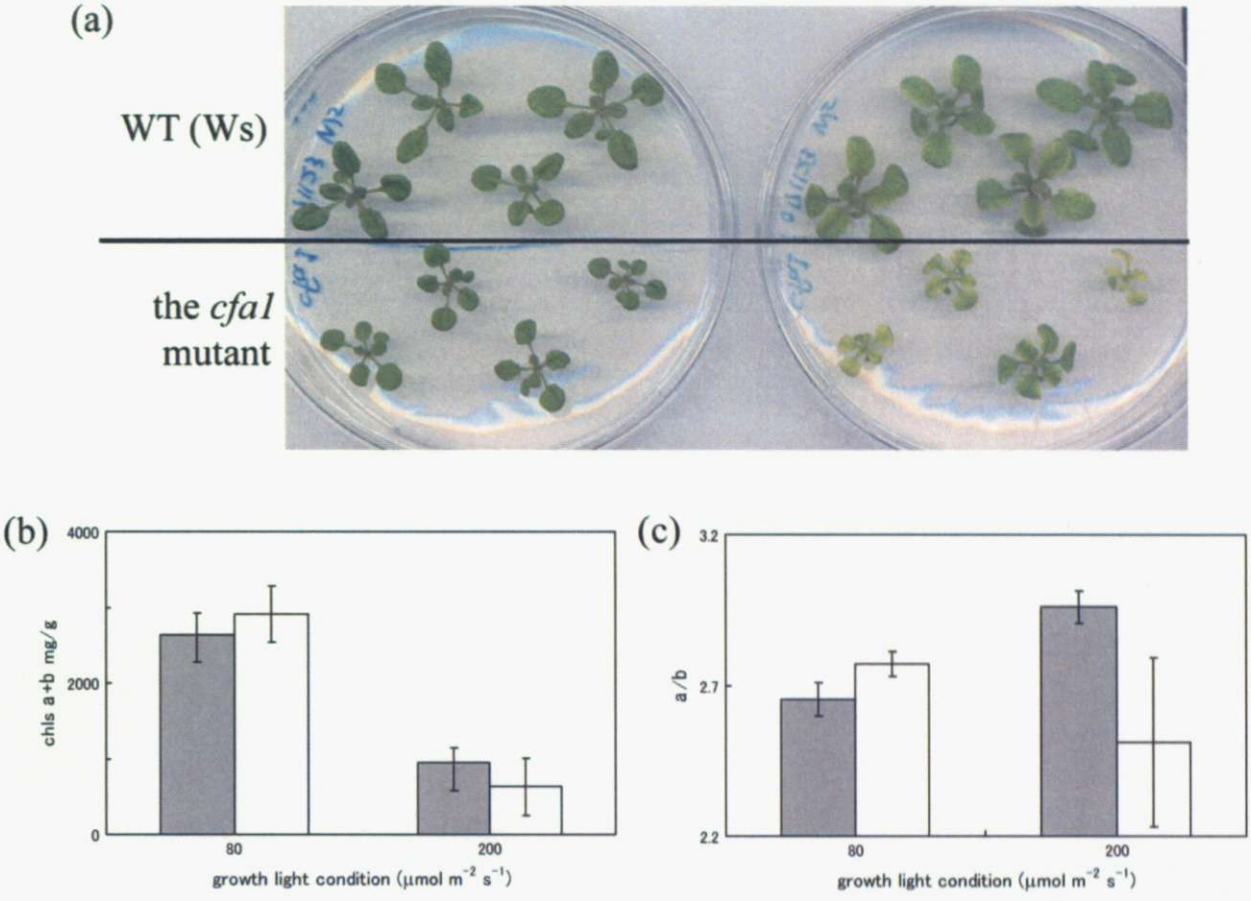

Fig. 16 Growth, total chlorophyll content and chlorophyll *a/b* ratio of WT (Ws) and the *cfa1* mutant under low growth light (80 μmol m^{-2} s^{-1}) or higher growth light (200 μmol m^{-2} s^{-1})

(a) WT (Ws) (upper half) and the *cfa1* mutant (lower half) were grown on plates under photon flux density at 80 μmol m^{-2}s^{-1} (the left plate) and at 200 μmol m^{-2}s^{-1} (the right plate) for 16 days. (b) Total chlorophyll content of leaves of (a) (in mg g^{-1} fresh weight) is shown. Gray and white bars indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of four samples ± SD. (c) Chlorophyll *a/b* ratio of leaves of (a) is shown. Gray and white bars indicate WT (Ws) and the *cfa1* mutant, respectively. Each value is the mean of four samples ± SD.