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Identification of photosynthetic mutants of *Arabidopsis* by automatic screening for altered effective quantum yield of photosystem 2

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Abstract

Quantification of chlorophyll (Chl) fluorescence is a versatile tool for analysing the photosynthetic performance of plants in a non-intrusive manner. A pulse-amplitude modulated fluorometer was combined with a CNC router for the automated measurement of the effective quantum yield of photosystem 2 (Φ_2) of *Arabidopsis thaliana* plants. About 90 000 individual plants representing 7 500 lines derived from *En*-transposon and T-DNA mutagenised *Arabidopsis* populations were screened for mutants with altered Φ_2. Forty-eight recessive Φ_2 mutations were identified of which most exhibit also altered pigmentation and increased photosensitivity. For three Φ_2 mutants the corresponding mutated genes were identified that code all for chloroplast-located proteins. Comparison of the Φ_2 mutant screen with other screening methods based on the measurement of Chl fluorescence shows that the Φ_2 mutants identified are different to mutants identified by high Chl fluorescence. Some Φ_2 mutants, on the contrary, are common to mutants identified by screens based on non-photochemical quenching.

Additional key words: chlorophyll fluorescence; electron transport; gene tagging.

Introduction

Chloroplasts descend from an original cyanobacterial endosymbiont and contain in *Arabidopsis thaliana* about 10 % of the total nucleus-encoded proteins (Abdallah *et al.* 2000). Thus, photosynthesis is embedded into a highly complex protein network and, consequently, can be influenced by several other metabolic processes. Therefore, any mutation of a gene encoding a chloroplast protein with a structural or regulatory function, or affecting protein turn-over can interfere with chloroplast biogenesis or function and, finally, with photosynthesis (Wettstein *et al.* 1971, Mascia and Robertson 1978, Rochaix 1992).

The identification of mutants with altered photosynthetic performance is the approach of choice for the genetic dissection of photosynthesis. Several strategies for the identification of those mutants are available (Somerville 1986), of which the selection of mutants with altered pigmentation or Chl fluorescence has received a wide attention. These two classes of mutants are not mutually exclusive, as pigmentation mutants may also show altered Chl fluorescence (*e.g.*, *tha4*, Walker *et al.* 1999; *crp1*, Fisk *et al.* 1999) and *vice versa*. The concept that any lesion of the photosynthetic electron transport chain affects the re-emission of absorbed energy as Chl fluorescence was utilised for the identification of high Chl fluorescence (*hcf*) mutants (Bennoun and Levine 1967) in *Chlamydomonas reinhardtii*, maize, barley, and *A. thaliana* (Miles 1980, Simpson and Wettstein 1980, Bennoun and Delepelaire 1982, Dinkins *et al.* 1994, Meurer *et al.* 1996). The *hcf* mutants reveal defects in PS1, PS2, photophosphorylation, CO_2 fixation, and in other functions associated with photosynthesis. In most of these mutants the photosynthetic electron transport is severely affected, often resulting in seedling lethality under photoautotrophic conditions. This is the major limitation of *hcf* mutants that can only be maintained

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by growing heterozygous plants. Few *hcf* genes have been cloned (*HCF106*, Martienssen *et al.* 1989; *HCF136*, Meurer *et al.* 1998; *CRP1/HCF111*, Fisk *et al.* 1999) leading to the discovery of functions necessary for the stability or assembly of thylakoids. A similar strategy has permitted the isolation of genes with mutant pigmentation phenotype; those code for components of the chloroplast protein import and targeting machinery or for other factors necessary for chloroplast development (*ALBINO3*, Sundberg *et al.* 1997; *CLA1*, Mandel *et al.* 1996; *CAO*, Klimyuk *et al.* 1999; *THA1*, Voelker *et al.* 1997; *THA4*, Walker *et al.* 1999; *CSY1*, Roy and Barkan 1998; *FFC*, Amin *et al.* 1999).

However, mutations affecting photosynthesis must not necessarily cause severe phenotypes as *hcf* or altered pigmentation. This supports a screening strategy considering mutants with relatively small alterations of their photosynthetic performance. For this class of mutants a basic Chl fluorescence parameter, the effective quantum yield of PS2 (Φ_2, Genty *et al.* 1989) is to be used. For automated measurement of Φ_2 we have combined a Pulse Amplitude Modulation (*PAM*) fluorometer (Schreiber *et al.* 1986) with a Computerised Numerical Control (CNC) router driven by an appropriate software. In this paper we describe the application of this system for a systematic and automated screen for individuals with altered Φ_2 within mutagenised populations of *A. thaliana*.

Materials and methods

Plant propagation and growth measurement: The *En*-mutagenised *A. thaliana* (ecotype Columbia 0) population comprising 8 000 lines with 48 000 *En*-insertions has been described by Wisman *et al.* (1998). Additional 8 000 lines mutagenised by T-DNA insertions were generated recently (Bernd Reiss, unpublished). Seeds of *A. thaliana* ecotype Col-0 were sown in plastic trays with *Minitray* soil (*Gebr. Patzer*, Sinntal-Jossa, Germany) and incubated for 3 d at 2-5 °C in the dark to break dormancy. Plants were grown in a greenhouse under long day (with additional irradiation for a total day length of at least 16 h). Fertilisation with *Osmocote Plus* (15 % N, 11 % P_2O_5, 13 % K_2O, 2 % MgO; *Scotts Deutschland*, Nordhorn, Germany) was performed according to manufacturer's instructions. For the determination of growth rate, seeds were sown in pots and 1 week after germination individual plants of the same size were trans-planted into trays.

For the mutant screen, plant trays were transferred 3-4 weeks after germination into a climate chamber under short-day conditions (day period of 10.5 h with 20 °C and constant PAR of 200 µmol s^{-1} m^{-2}; night period of 13.5 h with 15 °C) and maintained for at least 2 d before measuring Φ_2, the effective quantum yield of PS2.

Oligonucleotides and adapter sequences: For the isolation of transposon-flanking regions, the following adapters and primers (5'-3' orientation) were used: APL1632 (LR32 + APL16), APL1732 (LR32 + APL17), LR32 (ACTCGATTCTCAACCCGAAAGTATAGAT CCCA), APL16 (*P*-TATGGGATCACATTAA-NH_2), APL17 (*P*-CGTGGGATCACATTAA-NH_2), LR26 (ACTCGATTC TCAACCCGAAAGTATAG); for En 3': En8130s (GAG CGTCGGTCCCCACACTTCTATAC) and En8153s (TA CGAATAAGAGCGTCCATTTTAGAGTG); for En 5': En249as (GGCAGGGAGAAAGGAGAGAA) and En230as (AGAAGCACGACGGCTGTAGAATAGGA). For the isolation of T-DNA flanking regions, adapters APL1632 and APL1732 and following primers were used. Left border: T9750as (ATAATAACGCTGCGG ACATCTACATTTT) and T9697as (CTCTTTCTTTTTC TCCATATTGACCAT); right border: T4496s (CAGGG TACCCGGGGATCAGATTGTC) and T4554s (GATCA GATTGTCGTTTCCCGCCTTCAGTTT).

Isolation of *En*- and T-DNA-flanking sequences: Isolation of *Arabidopsis* genomic DNA was performed as described by Liu *et al.* (1995). Sequences flanking the ends of *En* and T-DNA were isolated by PCR amplification of restricted and adapter-ligated plant genomic DNA similar to the procedure described by Frey *et al.* (1998). Briefly, 100 ng of genomic DNA were digested with *Csp*6I (*Hin*6I) and ligated overnight at 16 °C to 12.5 pmol of adapter APL1632 (APL1732). 4 mm^3 of the ligation were used in a linear PCR with primer En8130s (En249as), and subsequently a 1 mm^3 aliquot of the linear PCR was used as the template for an exponential PCR with primers En8153s (En230as) and LR26. For the amplification of T-DNA flanking regions the linear PCR was performed with primer T9750as (T4496s) and the exponential PCR with primers T9697as (T4554s) and LR26. All amplifications were performed using the *Advantage® 2* PCR Kit (*Clontech*) and the following cycling conditions: initial denaturation for 2 min at 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 60 s annealing at 64 °C, and 90 s elongation at 73 °C. Products of exponential PCR were separated by electrophoresis on 4.5 % (m/v) polyacrylamide gels. The bands were visualised by silver staining. After excision of the candidate bands, PCR products were eluted in 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.1 % *Triton X-100*, re-amplified and directly sequenced, after gel-purification, using an *ABI* prism *377* sequencer.

Sequence analysis was done with the *Wisconsin Package* version 10.0, *Genetics Computer Group*, Madison, Wisconsin (GCG) (Devereux et al. 1984). Chloroplast import sequences prediction was performed using the

ChloroP program (version 1.0; http://www.cbs.dtu.dk/services/ChloroP/#submission; Emanuelson *et al.* 1999).

Chl fluorescence measurements: The screening for mutants with altered effective quantum yield of PS2 [$\Phi_2 = (F_{M'} - F_0)/F_{M'} = \Delta F/F_{M'}$, Genty parameter, Genty *et al.* 1989] was performed by using an automatic pulse amplitude modulation fluorometer system (*J. Kolbowski*, Schweinfurt, Germany, Fig. 1). A Computerised Numerical Control (CNC) router [controller *C116-4* and flat-bed machine *FB1* (1100×750); *ISEL Automation*, Eiterfeld, Germany) was combined with a Pulse Amplitude Modulation (PAM) fluorometer (one-channel version of *Phyto-PAM*, *Walz*, Effeltrich; Schreiber *et al.* 1986). F_S was measured under PAR of 200 μmol m^{-2} s^{-1} and 500-ms pulses of "white" saturating radiation (3 000 μmol m^{-2} s^{-1}) were used to determine $F_{M'}$ and the ratio = $\Delta F/F_{M'}$ The sensor which provides excitation and measurement of fluorescence was modified to be movable and be positioned within plant tray dimensions by an automatic steering device. The automatic PAM fluorometer system measured Φ_2 of *A. thaliana* plants one after the other in a pre-defined pattern, whereby individual leaves were identified automatically (auto-focus mode) by their optimal F_S ($100<F_S<250$). The auto-focus mode comprised a pre-defined pattern used by the sensor to identify leaves for optimal measurement (centre→all 4 corners→centre). The sensor was positioned at a distance of less than 2 cm from leaves measured to avoid cross-irradiation. Screening was performed in a climate chamber under low irradiance (200 μmol s^{-1} m^{-2}) in the middle of the day period (3-7 h after the start of the day period). Up to 1 536 plants grown in trays (128 lines with 12 individuals) were screened per week. To obtain irradiance saturation curves, Φ_2 was measured using the fluorometer *PAM101/103* (*Walz*, Effeltrich). Plants were irradiated for 10 min with "white" actinic radiation [from 6 to 1049 μmol(photon) m^{-2} s^{-1}]. For analysis of irradiation stress dependency of the effective quantum yield of PS2, entire plant trays were exposed to "white" actinic radiation of 2 575±335 μmol(photon) m^{-2} s^{-1} generated by two 1 000 W greenhouse bulbs (*Osram, HQI-T 1000/D*) for 30 min. Φ_2 was subsequently measured 1 h after the end of the irradiation stress.

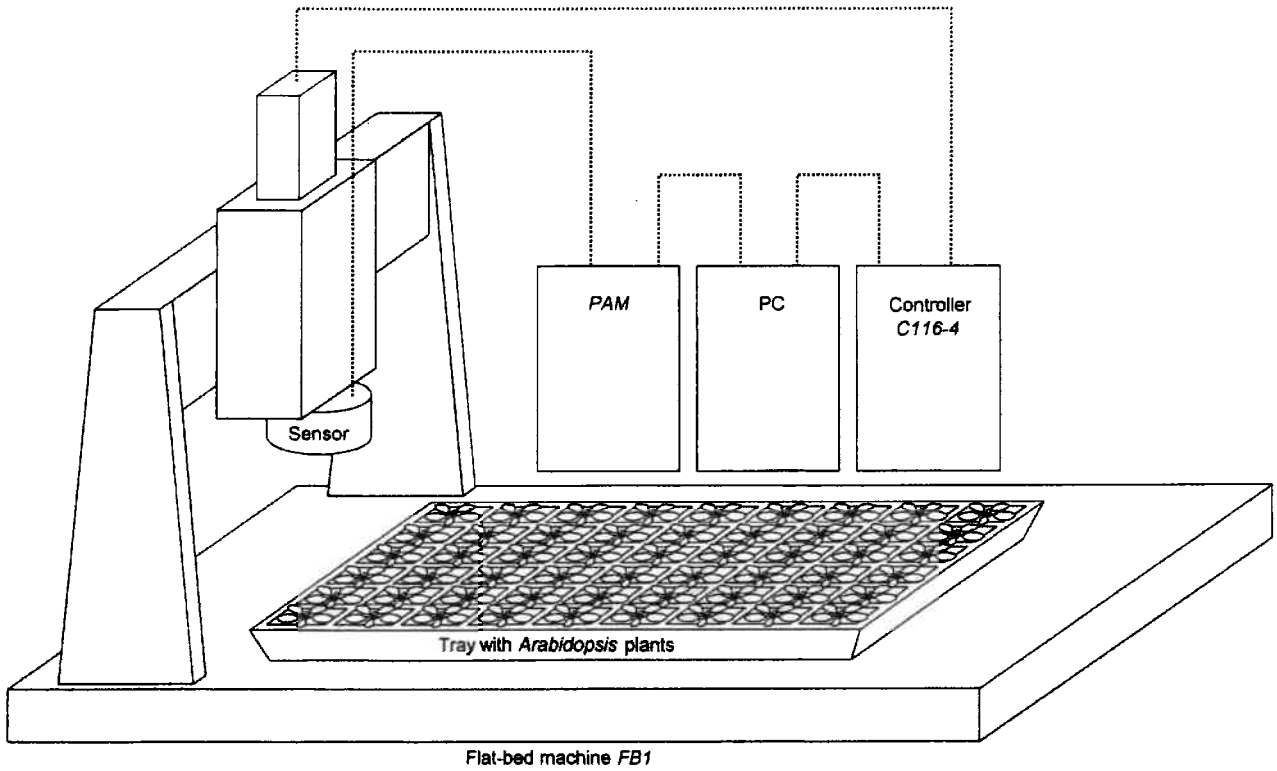

Fig. 1. Design of the automatic device used in the screening of Φ_2 mutants.

Results

An automated *PAM* fluorometer facilitates high-throughput screening of photosynthetic mutants: The measurement of Φ_2 of individual plants required less than 4 s while 10-15 s are required for moving the emitter/detector unit to the next plant. Consequently, measurement of Φ_2 in trays containing 54 or 96 *A. thaliana* plants required about 20 min or 30 min per tray, respectively. The accuracy and reproducibility of Φ_2

measurement was tested by analysing 24 wild type plants: an average value of 0.77 with a standard deviation of 0.01 was found (Fig. 2). For 90 % of plants, repeated measurements resulted in essentially identical Φ_2 values, while for 10 % of them the maximum deviations were ± 0.01.

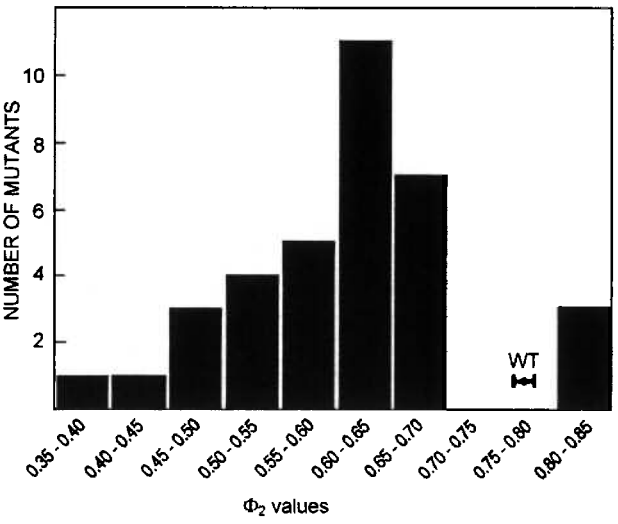

Fig. 2. Classification of mutants according to their Φ_2 values. Standard deviations of wildtype (WT) values are indicated by bars.

Twelve sibling plants for each of the 7 500 lines derived from two different mutagenised populations of *A. thaliana* (Table 1) were analysed for the presence of Φ_2 mutants. For lines with a minimum of two out of 12 individuals having non-wild type Φ_2, 30 additional siblings were analysed to confirm the mutant phenotype and to study its segregation. A total of forty-eight lines segregating recessive mutations were found with Φ_2 values between 0.40 and 0.83, with the majority of the mutants exhibiting Φ_2 values between 0.60 and 0.70 (Fig. 2). Thirteen Φ_2 mutants (0.6 %) were identified among the 2 700 *En* lines, whereas 35 Φ_2 mutants were found (0.75 %) among the 4 700 T-DNA lines (Table 1).

Classification of Φ_2 mutants: Mutants were classified based on their visual pigmentation phenotype. Thirty-five (about 70 %) of the Φ_2 mutants showed altered pigmentation (yellowish or pale green). Of the remaining thirteen mutants with wild-type pigmentation, ten mutants were affected in growth rates (Table 1) as determined by digital video image analysis (values not shown, Leister *et al.* 1999). Furthermore, mutants were analysed for their sensitivity to photoinhibition by measuring Φ_2 before and after irradiation stress generated by 2 600 µmol(photon) m^{-2} s^{-1} (Fig. 3). As measurements directly after irradiation stress revealed extensive variation of Φ_2 even for wild type plants, post-irradiation stress measurements were performed 1 h after the end of irradiation stress with a tolerable variation of Φ_2. While most mutants showed a significant reduction of Φ_2 after irradiation stress, few mutants exhibited nearly no irradiation stress-induced Φ_2 reduction. Nevertheless, the Φ_2 value of those mutants after irradiation stress was still below the one of wild-type plants. For further examination of photoinhibitory effects, Φ_2 of mutant plants for photosynthetically active radiation (PAR) from 6 to 1 049 µmol m^{-2} s^{-1} was determined ("irradiation saturation curves"). Some irradiation saturation curves are shown in Fig. 4.

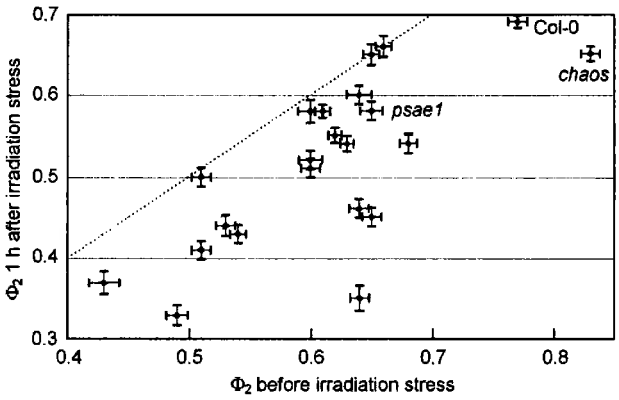

Fig. 3. Effective quantum yield of photosystem 2 before and after irradiation stress of a group of 21 Φ_2 mutants. Bars indicate standard deviations. The *dotted line* indicates no change of Φ_2.

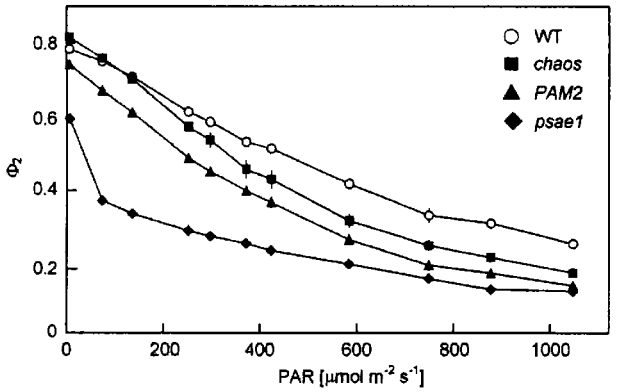

Fig. 4. Effective quantum yield of photosystem 2 for PAR from 6 to 1049 µmol m^{-2} s^{-1} (irradiation saturation curves) of three mutant lines and WT plants. Φ_2 was determined for photosynthetically active radiation (PAR) ranging from 6 to 1049 µmol m^{-2} s^{-1} for 3 mutant lines and wildtype plants. The *chaos* and *psae1* mutants are described in the text, while *PAM2* (*PAM*: "photosynthesis affected mutant") is a Φ_2 mutant for which the mutated gene is not known. 5 WT plants and 5 plants for each mutant line were measured after incubation for 20 h in the dark followed by 1 h irradiation with 160 µmol(photon) m^{-2} s^{-1}. For every PAR, Φ_2 was measured after irradiating for 10 min with the appropriate irradiance. Error bars indicate standard deviations.

Isolation of insertion sites: A prerequisite for the identification of genes inactivated in the mutants is the isolation of DNA regions flanking the insertion sites. Flanking sequences of T-DNA and *En* insertions were obtained by a PCR-based approach (Table 2). For *En*-lines that harbour on average 6 transposon insertions in their genome (Wisman *et al.* 1998), additional outcrossing and selfing steps were introduced before the isolation of DNA sequences flanking the transposon insertion. To identify which insertion caused the mutation resulting in the Φ_2-altered phenotype, flanking sequences of sibling plants were displayed on polyacrylamide gels (Fig. 5). PCR bands co-segregating with mutant phenotypes were excised, extracted from the gel, and sequenced after re-amplification. Specific primers complementary to insertion flanking regions were designed and combined with *En* or T-DNA specific primers for segregation analyses. For a total of 25 Φ_2 mutants the insertion sites were isolated and sequenced. For 13 mutants, *En* or T-DNA insertions genetically co-segregating with Φ_2 mutation were found. We conclude that about 50 % of Φ_2 mutants are tagged, *i.e.*, that the identified mutations are caused by either *En* or T-DNA insertions in specific genes.

Identified genes: Of the 13 mutants having *En*- or T-DNA insertions linked to the Φ_2 mutant phenotype, three insertions (two *En* and one T-DNA) were identified that inactivate specific genes. All three genes code for proteins with a putative N-terminal transit peptide for chloroplast import. The *chaos* mutation is caused by an *En*-insertion within the *CAO* gene that was identified and characterised by Klimyuk *et al.* (1999). The second *En*-mutation identified interrupts the *psaE1* gene encoding for the subunit E of PS1 (Varotto *et al.* 2000). The third gene identified contains a T-DNA insertion and encodes a heme oxygenase-like protein (HO1) causing the *hy1* mutation (Davis *et al.* 1999, Muramoto *et al.* 1999). The other 10 genes corresponding to tagged mutants are being characterised.

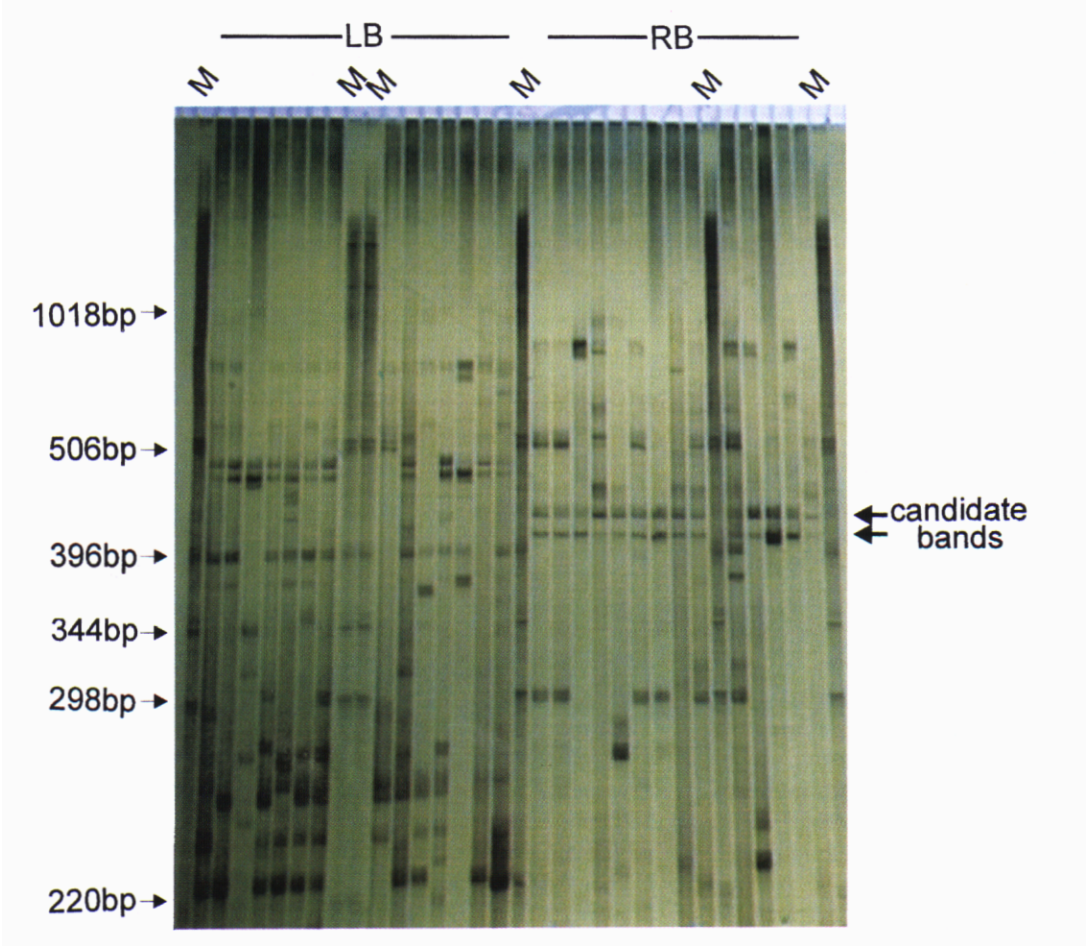

Fig. 5. Amplification and display of transposon insertion sites. Flanking regions of *En*-transposon insertions were amplified after *Csp*6I digestion and adapter ligation in mutant and wild-type plants from a population segregating for the *chaos* mutation. Two bands were identified co-segregating with all mutant plants ("candidate bands"). After DNA sequencing of those bands they were identified as part of the CAO gene. M: 1 kb ladder; LB: right border, RB: right border.

Discussion

A novel screening for identifying photosynthetic mutants: An automated screening system for the identification of mutants affected in photosynthesis by their altered effective quantum yield of PS2 (Φ_2) has been established. This procedure allows to screen for non-lethal mutants with a throughput of several thousand plants per week. Among 7 500 insertionally mutagenised lines (corresponding to 90 000 plants screened), 48 Φ_2 mutants were identified, 35 of them with altered pigmentation and 13 without pigmentation defects but with a reduction in growth rate as detectable by digital image analysis (Leister *et al.* 1999). Few other screening procedures leading to the isolation of photosynthetic mutants of *Arabidopsis*, and based on Chl fluorescence measurements, have been described (Table 3). Thirty-four mutants were identified by Meurer *et al.* (1996) selecting *hcf* phenotypes, of which most were lethal at the seedling stage. Those mutants were isolated preferentially based on photochemical quenching and show a severe reduction of photosynthetic electron transport activity.

Table 1. Overview of identified Φ_2 mutants.

Type of mutant	*En*-population 2 700 families	T-DNA population 4 800 families	Total 7 500 families
Normal pigmentation/normal growth	2	1	3
Normal pigmentation/reduced growth	2	8	10
Altered pigmentation	9	26	35
Total	13	35	48

Table 2. Steps involved in the isolation of flanking regions of En or T-DNA insertions.

1	Digestion of genomic DNA with suitable restriction enzyme (Csp*61* or Hin*61*)
2	Ligation of DNA fragments to compatible adaptors (APL1632 or APL1732)
3	Linear PCR with insertion-homologous primers
4	Re-amplification with nested insertion primer and adaptor-homologous primer
5	Denaturation and separation on polyacrylamide gel
6	Excision, re-amplification, and sequencing of candidate PCR products

Table 3. Overview of mutant screen for Chl fluorescence mutants of Arabidopsis.

Mutant phenotype	Population	Mutagen	Number of mutants	Mutation frequency	Reference
hcf	7 700 M2-families	EMS	34	1/230	Meurer *et al.* 1996
NPQ	30 000 M2-individuals	EMS	13	1/2300	Niyogi *et al.* 1998
NPQ	21 000 M2-individuals	EMS	37	1/570	Shikanai *et al.* 1999
Φ_2	7 500 families	*En*/T-DNA	48	1/160	This study

Two other laboratories have identified *Arabidopsis* mutants (13 and 37, respectively) altered in non-photochemical quenching (NPQ; Niyogi *et al.* 1998, Shikanai *et al.* 1999). Plants were cultured either on a medium lacking sucrose (Niyogi *et al.* 1998) or on soil (Shikanai *et al.* 1999), thus eliminating seedling-lethal mutations. Niyogi and co-workers reported that 3 of the 13 NPQ mutants were defective in the xanthophyll cycle. Based on a similar method, Shikanai *et al.* isolated mutants with a primary defect in NPQ, of which the largest class (19 mutants out of 37) showed a reduced quantum yield for both photosystems. In some cases their pigmentation was different from wild-type plants.

Our results show that the spectrum of mutants identified by the Φ_2 and *hcf* mutant screenings—the last one performed as by Meurer and co-workers—is different. It remains to show how much overlap exists between mutants isolated by the Φ_2 and the NPQ screen. The extent of this overlap will become clear when either a comprehensive list of mutated genes isolated by the two approaches will be available, or when reciprocal tests of NPQ mutants with the Φ_2 screening and *vice versa* will be performed. Shikanai *et al.* (1999) observed, for about half of their NPQ mutants, significant changes of Φ_2 that will probably make them detectable by a direct Φ_2 mutation screen.

Systematic identification of photosynthesis-limiting genes: A total of 48 Φ_2 mutants were identified among the *En*- and T-DNA mutant populations. For three Φ_2

mutants the corresponding genes were identified all coding for proteins located in chloroplasts. Only one of those proteins (PsaE) is directly involved in photosynthesis, while the other two are involved in phytochrome biosynthesis (HO1) and protein import into chloroplasts (CAO). This finding indicates that non-photosynthetic functions of the chloroplasts, such as synthesis of plant hormones, fatty acids and lipids, amino acids, purine and pyrimidine nucleotides, and secondary metabolites can be impaired by mutant induction and identified by their (secondary) effect on photosynthesis.

The frequency of Φ_2 mutations observed in the T-DNA mutant population was significantly higher than in the *En*-population. This is rather unexpected, because the average number of insertions in *En*-lines (about six, Wisman *et al.* 1998) is three times higher compared to T-DNA lines (about 1.5, Bernd Reiss, personal communication). There are two explanations for this apparent contradiction. First, the extrapolated 48 000 insertions present in the 8 000 *En*-lines may not evenly cover the *Arabidopsis* genome. This would decrease the effective number of insertions per genome. Second, the *En*-tagged lines have been generated following a single-seed descent strategy for more than 12 generations (Wisman *et al.* 1998). This procedure may result in a selection against less-vital mutants leading to a decreased representation of mutations causing severe phenotypes. Some Φ_2 mutants show significant growth retardation. Because a lower fraction of albino-lethal mutants are present within the *En*-population compared to the T-DNA mutant population (unpublished results of our laboratory), the second explanation can be realistic.

The slightly decreased frequency of Φ_2 mutations within the *En*-population is compensated by some positive attributes of the transposon-tagged population. Mutations caused by transposons are easily confirmed by analysing somatic or germinal revertants before gene isolation (Walbot 1992). Furthermore, complementation by *Agrobacterium*-based transformation is necessary for T-DNA mutants but not for those due to transposon insertion that can be unequivocally defined by the analysis of revertants of the specific mutated gene.

Independent from the efficiency of the two tagging systems, in our Φ_2 mutant screen only knock-out populations were used, while both, the *hcf* and NPQ screens, utilised chemical or physical mutagens. Although the map-based cloning of chemically or physically induced mutants of *Arabidopsis* can be accomplished nowadays in a relatively straightforward way, insertion tagging in appropriate mutant populations is the method of choice for fast and systematic gene isolation.

Future screening systems for photosynthetic mutants by Chl fluorescence measurements: Significant drawbacks of current screening strategies performed in plant trays are their demand for extensive greenhouse space and the difficulty to apply stress before fluorescence measurement. These drawbacks can be overcome by adopting kinetic imaging fluorometer systems that use modulated-radiation excitation (Nedbal *et al.* 2000). Current imaging systems are suitable for the efficient analysis of areas of about 0.01 m^2, a size feasible to study objects with dimensions of Petri dishes. This has been demonstrated by Niyogi *et al.* (1997, 1998) who applied a custom-made imaging device to screen for NPQ mutants of *Chlamydomonas* and *Arabidopsis*. Imaging systems are not restricted to NPQ measurements but can also be applied to measure a range of Chl fluorescence induction parameters. The major advantage of imaging system based on relatively small-sized culture devices is the possibility to analyse various kinds of stresses to the plants or to simulate specific environments. This should enable the development of screening strategies leading to the isolation of conditional mutants identified after specific stress or environmental treatments.

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