

# Quantitative Genetic Analysis of Thermal Dissipation in *Arabidopsis*<sup>1[W][OA]</sup>

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Feedback deexcitation is a photosynthetic regulatory mechanism that can protect plants from high light stress by harmlessly dissipating excess absorbed light energy as heat. To understand the genetic basis for intraspecies differences in thermal dissipation capacity, we investigated natural variation in *Arabidopsis* (*Arabidopsis thaliana*). We determined the variation in the amount of thermal dissipation by measuring nonphotochemical quenching (NPQ) of chlorophyll fluorescence in *Arabidopsis* accessions of diverse origins. Ll-1 and Sf-2 were selected as high NPQ *Arabidopsis* accessions, and Columbia-0 (Col-0) and Wassilewskija-2 were selected as relatively low NPQ accessions. In spite of significant differences in NPQ, previously identified NPQ factors were indistinguishable between the high and the low NPQ accessions. Intermediate levels of NPQ in Ll-1 × Col-0 F1 and Sf-2 × Col-0 F1 compared to NPQ levels in their parental lines and continuous distribution of NPQ in F2 indicated that the variation in NPQ is under the control of multiple nuclear factors. To identify genetic factors responsible for the NPQ variation, we developed a polymorphic molecular marker set for Sf-2 × Col-0 at approximately 10-centimorgan intervals. From quantitative trait locus (QTL) mapping with undistorted genotype data and NPQ measurements in an F2 mapping population, we identified two high NPQ QTLs, *HQE1* (high qE 1, for high energy-dependent quenching 1) and *HQE2*, on chromosomes 1 and 2, and the phenotype of *HQE2* was validated by analysis of near isogenic lines. Neither QTL maps to a gene that had been identified previously in extensive forward genetics screens using induced mutants, suggesting that quantitative genetics can be used to find new genes affecting thermal dissipation.

Plants require light energy, by definition, to drive photosynthesis. However, too much light causes photooxidative damage in plants (Barber and Andersson, 1992). Thus, plants have diverse defense mechanisms against high light stress (Niyogi, 1999). For example, chloroplasts can move to absorb less light energy (Kasahara et al., 2002), and light-harvesting antenna size in chloroplasts can be reduced (Anderson, 1986). Plants also can harmlessly dissipate excess absorbed light energy as heat (Müller et al., 2001), and they have alternative electron transport pathways to relieve overreduction of electron transport components under stress conditions (Niyogi, 2000; Ort and Baker, 2002).

Thermal dissipation is mediated by a mechanism called feedback deexcitation. Feedback deexcitation dissipates excess absorbed light energy as heat, thereby protecting plants from high-light stress (Horton et al., 1994; Niyogi, 1999). The amount of feedback deexcitation

can be quantified by measuring nonphotochemical quenching (NPQ) of chlorophyll fluorescence (Müller et al., 2001; Baker, 2008). NPQ is induced by appearance of high light and is relaxed following disappearance of the high light. Based on its relaxation kinetics, NPQ can be divided into at least three components: energy-dependent quenching (qE), state-transition quenching, and photoinhibitory quenching (Maxwell and Johnson, 2000; Müller et al., 2001). Among them, qE is generally the major component in plants (Maxwell and Johnson, 2000). Biochemical and molecular genetics studies have shown that a pH gradient across the thylakoid membrane (Briantais et al., 1979; Munekage et al., 2001, 2002), the xanthophyll cycle (Demmig-Adams et al., 1990; Niyogi et al., 1998), and the PsbS protein (Li et al., 2000) of PSII are important factors involved in controlling the induction and/or extent of NPQ. Based on the semidominance of loss-of-function mutations (Li et al., 2000, 2002a) and overexpression of the *psbS* gene (Li et al., 2002b) in *Arabidopsis* (*Arabidopsis thaliana*), the expression level of the PsbS protein has been suggested as an important factor in determining the qE (and total NPQ) capacity of plants.

Naturally occurring variation in NPQ capacity has been observed in different plant species (Johnson et al., 1993; Demmig-Adams and Adams, 1994; Demmig-Adams, 1998). The variation of saturated NPQ values ranges from 2.5 to 4.5 in British plant species, and plants grown in open habitats tend to have larger NPQ capacity (Johnson et al., 1993). Sun-acclimated plants contain up to four times as much NPQ capacity as low-

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light-acclimated plants of the same species (Osmond et al., 1993; Ruban et al., 1993; Brugnoli et al., 1994; Demmig-Adams and Adams, 1994; Demmig-Adams et al., 1995; Demmig-Adams, 1998; Roberts et al., 1998). In *Monstera deliciosa*, for example, sun-acclimated leaves showed higher NPQ than low-light-acclimated leaves (Demmig-Adams and Adams, 1994), and this difference in NPQ is correlated with changes in PsbS protein levels (Demmig-Adams et al., 2006). Although it has been suggested that there may be a genetic basis for the variation (Horton et al., 1994), this possibility has not yet been analyzed.

This kind of natural variation in plant traits, in most cases, shows continuous variations that are under the control of polygenic factors, and quantitative genetic studies are required to understand the genetic basis of the variation (Alonso-Blanco and Koornneef, 2000). *Arabidopsis* has become a model system for plant quantitative trait locus (QTL) mapping (Alonso-Blanco and Koornneef, 2000; Maloof, 2003; Tonsor et al., 2005) because it has considerable trait variations among accessions, advanced molecular biological tools for efficient genotyping with molecular markers, and a fully sequenced genome. In addition, development and improvement of statistical tools for QTL mapping facilitate analyses of quantitative traits (Lander and Botstein, 1989; Zeng, 1994; Sen and Churchill, 2001). Using *Arabidopsis*, a number of quantitative traits have been analyzed; however, QTL mapping is still underused for photosynthesis-related traits, in spite of advantages for quantitative genetic studies, such as simple ways for quantification of photosynthetic parameters (Krause and Weis, 1991; Laisk et al., 2002; Long and Bernacchi, 2003; Baker, 2008).

In this article, we report natural variation of NPQ among *Arabidopsis* accessions and test the hypothesis that the variation between a high NPQ accession (Sf-2) and a low NPQ accession (Columbia-0 [Col-0]) is related to the PsbS protein. We measured induction and relaxation of NPQ in *Arabidopsis* accessions and divided them into high and low NPQ accessions. Biochemical and molecular biological experiments did not associate the NPQ differences with PsbS or other previously identified NPQ factors. Genetic analyses revealed that the differences are controlled by polygenic nuclear factors. To identify these factors, we performed QTL mapping using Sf-2 × Col-0 F2 progeny as a mapping population and identified two high NPQ QTLs. The significance of NPQ variation and possible roles for these QTLs in thermal dissipation are discussed.

## RESULTS

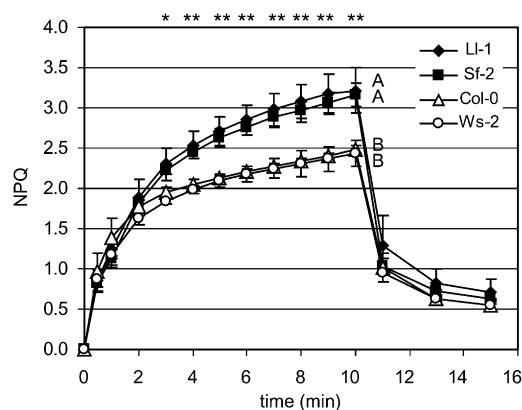
### NPQ in *Arabidopsis* Accessions

To study the natural variation of NPQ capacity, we initially measured total NPQ in 62 *Arabidopsis* accessions (Supplemental Table S1). The tested accessions originated from diverse growth conditions, and pa-

rental lines of recombinant inbred lines (RILs) that are available or will be available in the near future were also included (<http://www.inra.fr/internet/Produits/vast/RILs.htm>). To focus on the major qE component, NPQ was measured for 10 min during actinic illumination with  $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , followed by 5 min in the dark to determine its relaxation. All NPQ measurements were done between 1 and 5 PM to exclude any possible effects of circadian control of NPQ. The results showed that there are variations in NPQ capacity among *Arabidopsis* accessions (Supplemental Table S1) and that most of the NPQ is qE because it relaxed very fast in the dark (data not shown). Based on these measurements, the accessions were classified into high NPQ and low NPQ *Arabidopsis* accessions.

Ll-1, Sf-2, Ts-1, Van-0, Mz-0, Mr-0, and Kin-0 are high NPQ *Arabidopsis* accessions containing total NPQ capacity higher than 3.0, whereas Col-0, Landsberg *erecta* (Ler), and Wassilewskija-2 (Ws-2), so-called lab accessions, are low NPQ *Arabidopsis* accessions having total NPQ capacity below 2.5 (Supplemental Table S1). Interestingly, the three highest accessions, Ll-1, Sf-2, and Ts-1, all originated from Spain. Except for Ll-1, the other high NPQ accessions have been used as parental lines of RILs, but the RILs were not yet available at the time these experiments were initiated. We decided to use Ll-1 and Sf-2 as high NPQ accessions and Col-0 and Ws-2 as low NPQ accessions for further measurement and characterization.

After growing these selected accessions together in identical growth conditions, we measured their NPQ in the next generation. Ll-1 and Sf-2 showed significantly higher total NPQ than Col-0 and Ws-2 after 3 min in actinic illumination ( $P < 0.01$ ), and the NPQ differences became larger with time ( $P < 0.001$ ) (Fig. 1).



**Figure 1.** NPQ variation in high and low NPQ *Arabidopsis* accessions. NPQ values in high and low NPQ *Arabidopsis* accessions were measured on attached rosette leaves using an FMS2 fluorometer during actinic illumination with  $1,500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 10 min, followed by relaxation in the dark for 5 min. Each data point represents the mean  $\pm$  SD ( $n = 3$ ). The significance of differences was tested by ANOVA (\*,  $P < 0.01$ ; \*\*,  $P < 0.001$ ), and the grouping (A and B) was made using multiple comparisons (Duncan).

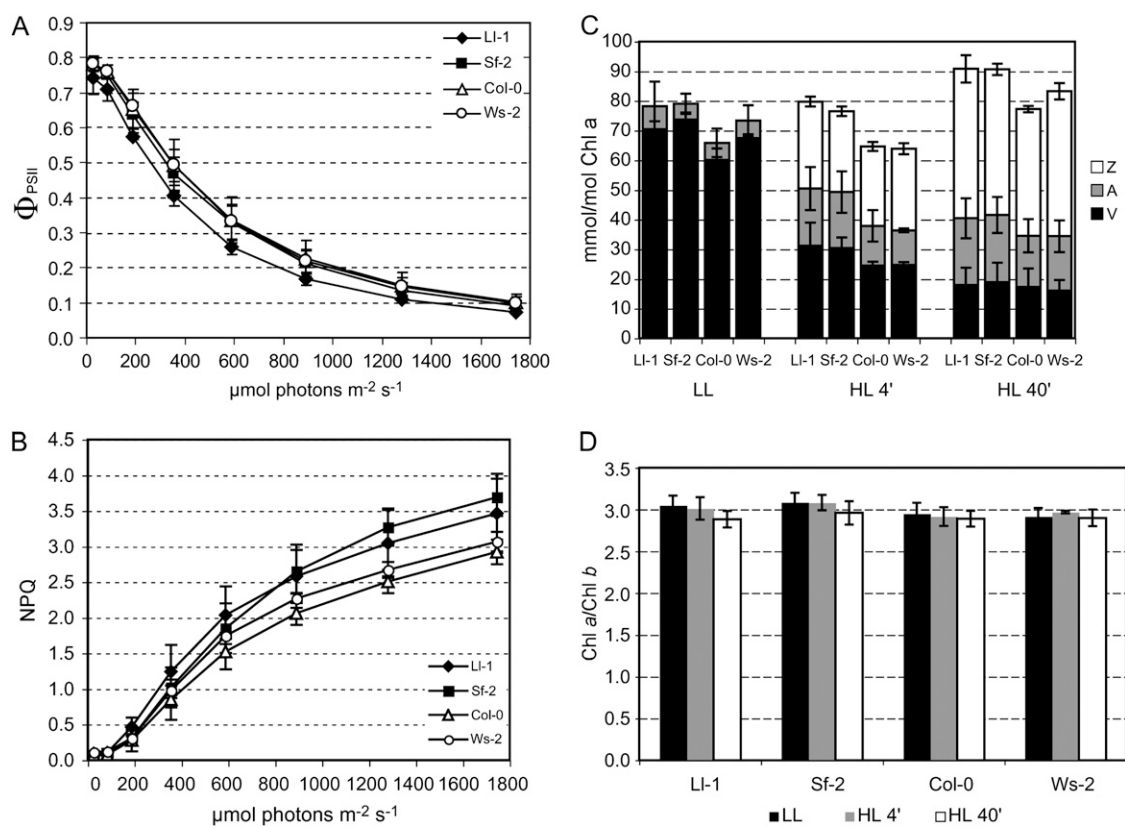
### Characterization of NPQ Factors in Selected Accessions

Photosynthetic efficiency and previously identified NPQ factors were compared between these high and low NPQ accessions. First of all, quantum yield of PSII ( $\Phi_{\text{PSII}}$ ) measuring the proportion of energy used for photochemistry (Maxwell and Johnson, 2000) was calculated (Fig. 2A). The levels of  $\Phi_{\text{PSII}}$  in these accessions were very similar to each other, although Ll-1 had a slightly lower value than the others. In addition, the maximum quantum yield of PSII (maximum photochemical efficiency of PSII in the dark-adapted state) of the accessions was also indistinguishable (data not shown). Ll-1 and Sf-2 showed higher NPQ than Col-0 and Ws-2 in increasing light intensities; however, the differences were not statistically significant up to the highest light intensity applied (Fig. 2B), possibly due to the longer measuring time and greater contributions of other NPQ components (e.g. photoinhibitory quenching).

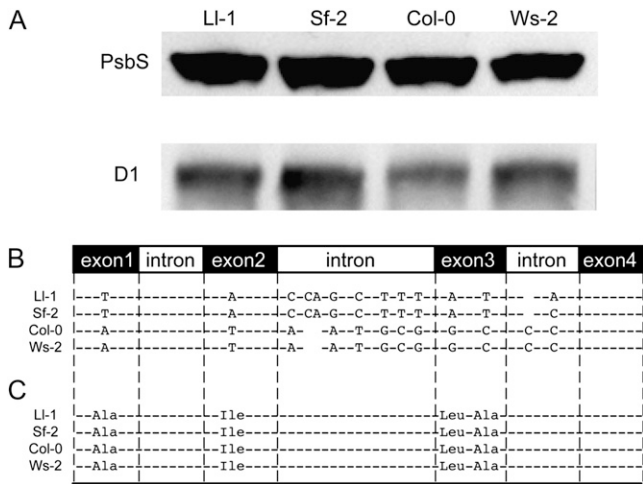
To check differences in xanthophyll cycle activity, the contents of each xanthophyll cycle pigment were determined. The deepoxidated xanthophylls, antheraxanthin (A) and zeaxanthin (Z), are generated from

violaxanthin (V) by V deepoxidase (VDE) in high light (Yamamoto et al., 1962). In low light ( $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), Ll-1, Sf-2, Col-0, and Ws-2 contained a very small proportion of deepoxidated forms (A and Z). After being exposed to high light ( $1,700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 4 and 40 min, all accessions showed very similar levels of deepoxidation (Fig. 2C). These results indicated that the high and low NPQ accessions contain functional VDE and that there are no differences in VDE activation by high light between the two groups. Additionally, the chlorophyll *a/b* ratios reflecting the PSII antenna size were similar to each other (Fig. 2D).

Third, the amount of the PsbS protein was compared. PsbS is required for qE, and qE (and total NPQ) is proportionally increased as the PsbS protein level increases (Li et al., 2000, 2002a, 2002b). To determine whether PsbS is responsible for the NPQ variation, the amount of PsbS in each accession was estimated by immunoblot analysis. In these accessions, the relative PsbS protein levels were very similar to each other, compared to D1 protein levels, although the D1 protein level in Col-0 was slightly lower than the others



**Figure 2.**  $\Phi_{\text{PSII}}$ , NPQ, xanthophyll cycle deepoxidation, and chlorophyll *a/b* ratio of high and low NPQ Arabidopsis accessions. A and B,  $\Phi_{\text{PSII}}$  and NPQ values were estimated by applying a pulse of saturating light to attached rosette leaves that had been exposed to the corresponding light intensity for 5 min. Each data point represents the mean  $\pm$  SD ( $n = 3$ ). C and D, Total xanthophyll cycle pigments (V, violaxanthin; A, antheraxanthin; Z, zeaxanthin) relative to chlorophyll (Chl) *a*, and chlorophyll *a/b* ratio were determined in low light (LL;  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and after being treated in high light (HL;  $1,700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 4 and 40 min. Each data point represents the mean  $\pm$  SD ( $n = 2$  or 3).



**Figure 3.** PsbS protein levels and PsbS genomic DNA polymorphisms in high and low NPQ Arabidopsis accessions. A, PsbS protein levels in each accession were determined by immunoblot analysis with anti-PsbS antibody (Li et al., 2002a). D1 levels were included as a control. The image in B displays PsbS genomic DNA sequences showing DNA polymorphisms among accessions, and C shows the predicted amino acid at each polymorphic site.

(Fig. 3A). We also determined the PsbS genomic DNA sequences and then compared their predicted amino acid sequences because changes in amino acid residues could affect PsbS function (Li et al., 2002c). Interestingly, we found DNA sequence polymorphisms between the high and the low NPQ accessions: LI-1 and Sf-2 have nearly identical PsbS genomic DNA sequences, and the Col-0 and Ws-2 sequences are identical to each other (Fig. 3B). However, all polymorphisms in exons are silent; therefore, it was predicted that there are no amino acid differences. Taken together, these high and low NPQ accessions contained nearly identical levels of previously identified NPQ factors, including VDE activity and PsbS, sug-

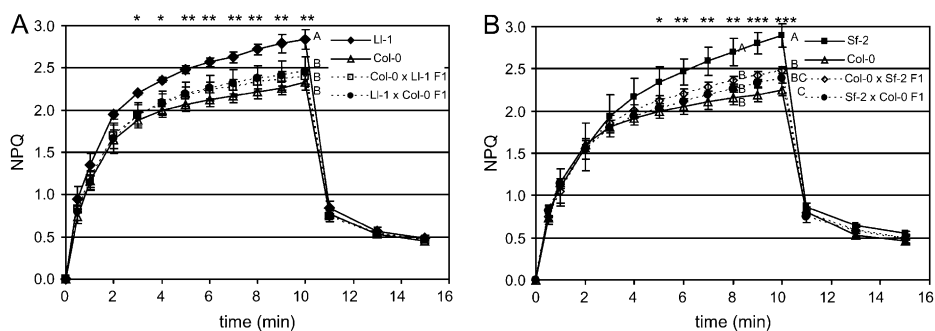
gesting that the differences in NPQ may be controlled by a novel factor or factors.

### NPQ of Sf-2 × Col-0 F1 and F2 Progenies

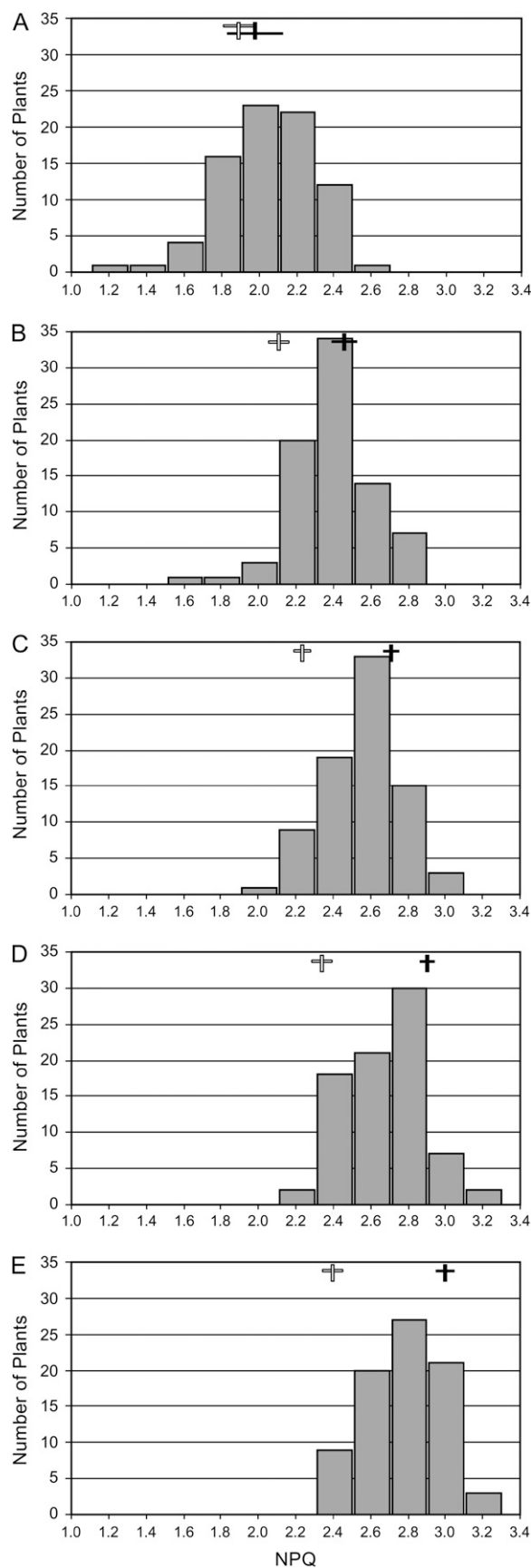
To understand the genetic basis of the NPQ variation, we crossed LI-1 and Sf-2 to Col-0 and measured induction and relaxation of NPQ in F1 and F2 progenies. Col-0 was used as a low NPQ parental line because the genomic DNA sequences of Col-0 are completely determined, and physical map positions of molecular markers are also available (Arabidopsis Genome Initiative, 2000). NPQ levels in the F1 were significantly lower than those of LI-1 and Sf-2 and were slightly higher than that of Col-0, although multiple comparison tests (Duncan) indicated that F1 from both crosses were in the same group as Col-0 (Fig. 4). F1 plants from reciprocal crosses showed similar levels of NPQ and were statistically grouped together (Fig. 4). These observations indicated that the high NPQ is controlled by nuclear rather than cytoplasmic factor(s). To determine how many nuclear factors are involved, we measured NPQ in 80 Sf-2 × Col-0 F2 progeny and analyzed the frequency distribution of NPQ every 2 min during illumination with actinic light. At each time point, NPQ levels were widely distributed (Fig. 5), and a normality test (Shapiro-Wilk) showed that NPQ levels follow a normal distribution ( $P < 0.01$ ), indicating that more than one factor is responsible for the NPQ variation. Between these two high NPQ accessions, LI-1 is very similar to LI-0 that is being used as a parental line for generation of LI-0 × *Ler* RILs. Therefore, we decided to use Sf-2 and Col-0 as high NPQ and low NPQ accessions, respectively, to locate QTLs responsible for high NPQ.

### Determination of Genotypes

To determine genotype in the F2 mapping population, we developed a polymorphic DNA marker set



**Figure 4.** NPQ in F1 plants from reciprocal crosses of LI-1 × Col-0 (A) and Sf-2 × Col-0 (B). The solid lines with closed and open symbols represent NPQ values in high and low NPQ parental lines, respectively, measured during actinic illumination with  $1,500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 10 min, followed by relaxation in the dark for 5 min. The dotted lines represent NPQ values in F1 plants. Each data point represents the mean of two or three measurements. Significant differences and groupings (A to C) were done by ANOVA (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ) followed by multiple comparisons (Duncan).



(Supplemental Table S2). Using the polymorphic markers, genotypes at each marker position were determined in 72 F2 progeny. The genotyping was almost complete, with only a few missing genotype data. From the genotype data, genotype frequencies at marker positions were calculated (Supplemental Fig. S1). The G-test (Dytham, 2003) indicated that overall, no significant segregation distortion was observed in our F2 mapping population (Supplemental Fig. S1). With the genotype data, we also confirmed the order of markers on each chromosome and estimated the distance between each two consecutive markers using MAPMAKER/EXP 3.0 (Lander et al., 1987). The reestimated marker order was the same as the marker order on the Arabidopsis physical map (Supplemental Table S2). The average distance between flanking markers was calculated as 10.7 centimorgan (cM). The longest interval was 31.9 cM between SO392 and A44575 on chromosome 1, and the shortest one was 2.2 cM between C6 and NGA126 on chromosome 3 (Supplemental Table S2).

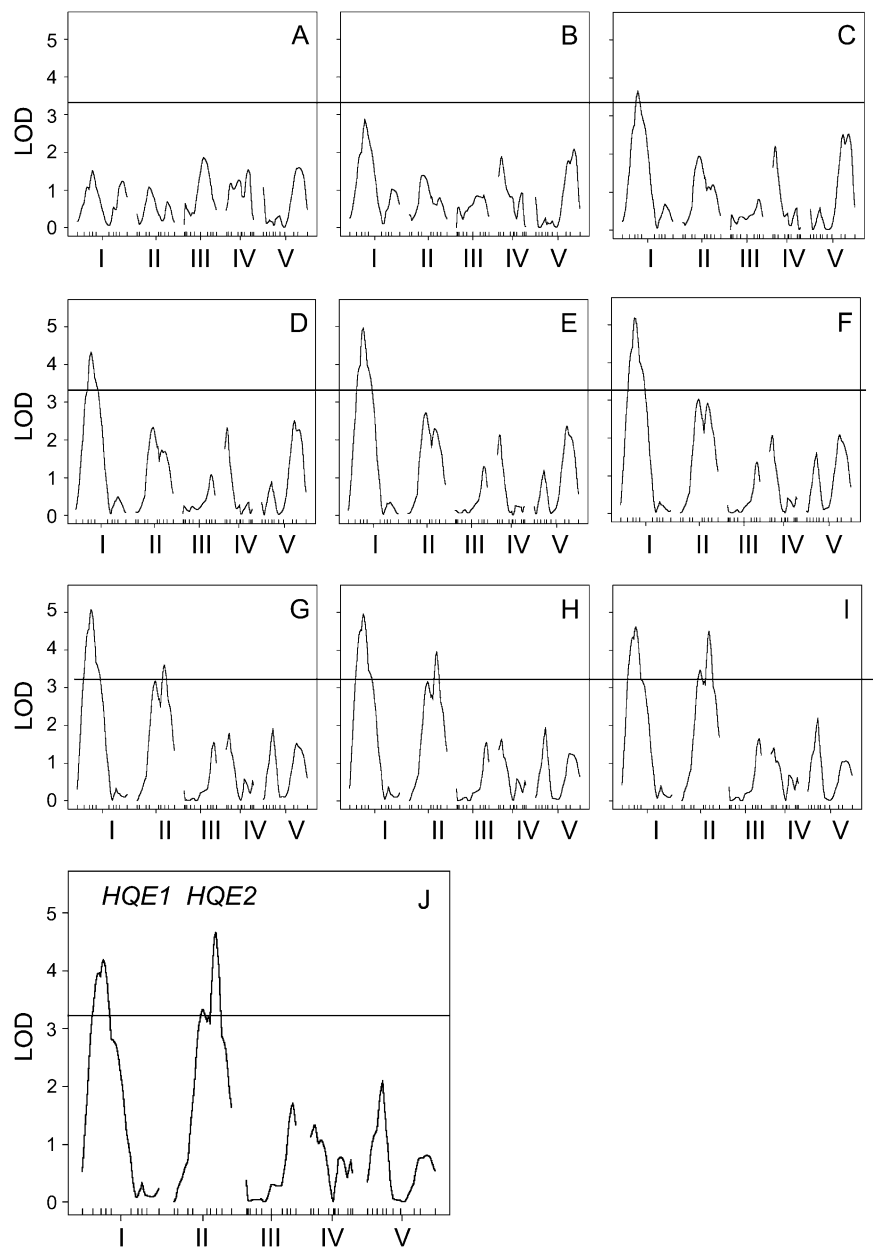
### QTL Mapping

To find chromosome regions that are responsible for the high NPQ phenotype of Sf-2, we conducted QTL mapping using the R/qtl program (Broman et al., 2003). The QTL mapping with 10 min NPQ identified two high NPQ QTLs, High qE 1 (*HQE1*) and High qE 2 (*HQE2*) (Fig. 6J). The log of the odds (LOD) scores of both QTLs were higher than the threshold LOD score of 3.2 that was estimated after 1,000 permutations with 0.05 significance (Churchill and Doerge, 1994). *HQE1* is 35 cM from the first marker on chromosome 1, and the nearest marker is NF19G9. *HQE2* is 67.5 cM from the first marker on chromosome 2, and the nearest marker is C4H (Table I). Because all three possible genotypes are represented in the F2 mapping population, we were able to determine dominance (*d*) and additive (*a*) effects of each QTL. The degree of dominance (*d/a*) indicated nearly additive gene action ( $-0.06$ ) of *HQE1* and partly dominant gene action ( $0.49$ ) of the Sf-2 allele of *HQE2* (Table I; Tanksley, 1993). The estimated 1.5 LOD support intervals (Lander and Botstein, 1989) were between 12.5 and 57.5 cM on chromosome 1 for *HQE1* and between 40.0 and 77.0 cM on chromosome 2 for *HQE2* (Table I). Among total variance, the portion of variance explained by these two QTL was 37.8%. In addition, there was little evidence supporting interaction between *HQE1* and *HQE2* (data not shown).

We also did QTL mapping using NPQ values measured at each minute and found interesting differences

**Figure 5.** Frequency distribution of NPQ in the Sf-2 × Col-0 F2 progeny. NPQ values at 2 min (A), 4 min (B), 6 min (C), 8 min (D), and 10 min (E) in actinic illumination were used to calculate the number of F2 plants in each interval. Vertical and horizontal boxes correspond to averaged NPQ values and their SD ( $n = 5$ ), respectively, of Sf-2 (closed) and Col-0 (open).

**Figure 6.** Results of high NPQ QTL mapping. QTLs for high NPQ were localized using R/qtl with NPQ values measured at 1 min (A), 2 min (B), 3 min (C), 4 min (D), 5 min (E), 6 min (F), 7 min (G), 8 min (H), 9 min (I), and 10 min (J). The horizontal line represents significance threshold ( $P < 0.05$ ) estimated from 1,000 permutations.



between *HQE1* and *HQE2* (Fig. 6, A–I). The LOD score of *HQE1* became significant after 3 min in actinic illumination (Fig. 6C); thereafter, *HQE1* remained as a significant high NPQ QTL during the 10-min measurement. At 6 min, *HQE1* alone explained 27.9% of total NPQ variance (Table I). The support interval of *HQE1* at 6 min was 23 to 47 cM, which was narrower than the interval at 10 min (Table I). In contrast, the response of *HQE2* to the actinic light was relatively slower than that of *HQE1*. *HQE2* became a significant QTL after 7 min in the light (Fig. 6G), and it remained significant until the end of the 10-min measurement. Therefore, from 3 to 6 min, only *HQE1* seems to be responsible for the high NPQ, and from 7 min in the actinic light, both *HQE1* and *HQE2* may control the high NPQ phenotype.

To confirm our NPQ QTL mapping, we generated near isogenic lines, NQE2, harboring the *Sf-2* allele of *HQE2* in the Col-0 background. F1 plants of *Sf-2* and Col-0 cross were backcrossed to Col-0 four times, and lines were selected based on genotyping. NPQ measurements indicated that NQE2 showed higher NPQ than control lines containing the Col-0 allele of *HQE2* (Fig. 7).

## DISCUSSION

In our survey of natural variation of NPQ capacity in *Arabidopsis*, we tried to find some accessions that have much less NPQ than other accessions. In such cases, it might be relatively straightforward to deter-

**Table 1.** Results of QTL mapping for NPQ in *Sf-2* × *Col-0* F2 population

Ch, chromosome; a, additive effects; d, dominance effects; d/a, degree of dominance; % Var, percentage of variance explained.

Time <sup>a</sup>	QTL	Ch	cM <sup>b</sup>	Marker <sup>c</sup>	LOD	1.5 LOD	a	d	d/a	% Var
10 min	High qE 1 (HQE1)	1	35.0	NF19G9	4.18	12.5 ~ 57.5	0.146	-0.009	-0.06	12.1
10 min	High qE 2 (HQE2)	2	67.5	C4H	4.66	40 ~ 77	0.158	0.078	0.49	15.2
10 min	HQE1 + HQE2									37.8
6 min	High qE 1 (HQE1)	1	35.0	NF19G9	5.18	23 ~ 47	0.158	0.008	0.05	27.9

<sup>a</sup>Time at which NPQ values used for QTL mapping were measured. <sup>b</sup>Position from the first marker on each chromosome. <sup>c</sup>The closest marker to the peak of corresponding QTL.

mine the genetic basis for the NPQ difference, as found for other traits (Maloo et al., 2001). However, so far, we could not find Arabidopsis accessions having very low NPQ. That might be because NPQ is an important photoprotection mechanism without which plants would suffer more oxidative stress (Li et al., 2002b) and show much lower fitness estimated by lifetime seed production (Külheim et al., 2002).

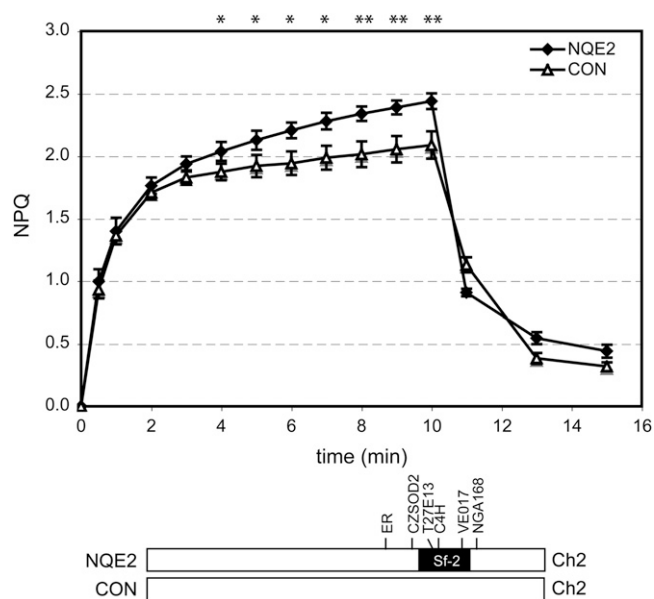
Several accessions exhibited relatively high NPQ compared to the standard lab accession, *Col-0*. Why do the high NPQ accessions have a higher capacity for thermal dissipation than other accessions? We found a significant, but very weak correlation between latitude and NPQ among European accessions (data not shown). The latitude of geographical origin has been shown to have a significant relationship with variation of other traits in Arabidopsis (Li et al., 1998; Maloo et al., 2001; Stinchcombe et al., 2004). Instead of latitude, environmental parameters in the natural habitat may be more critical for NPQ capacity because NPQ is critical to deal with sudden changes in light intensity (Müller et al., 2001; Külheim et al., 2002). To better understand the relationship between NPQ and natural habitat, habitat information needs to be quantified (Johnson et al., 1993).

Another possible explanation for the weak relationship between latitude and NPQ is that mechanisms other than NPQ may play a major role in photoprotection in some accessions (Johnson et al., 1993). Although NPQ capacity in *Cvi* and *Ler*, for example, was very similar (Supplemental Table S1), *Cvi* showed higher photooxidative stress tolerance than *Ler* as expected from *Cvi*'s origin, a tropical zone (Abarca et al., 2001). Interestingly, *Cvi* contains a different allele of chloroplastic Cu/Zn-superoxide dismutase 2 (*CSD2*) from that of *Ler* in predicted amino acid sequences and in mobility in a protein gel, and it was suggested that the *Cvi*-specific *CSD2* is one of the factors responsible for the higher tolerance of *Cvi* (Abarca et al., 2001).

Strong relationships were observed between deep-oxidation state and NPQ capacity among plant species as well as among plants of the same species grown in different light conditions (Johnson et al., 1993; Brugnoli et al., 1994; Demmig-Adams and Adams, 1994; Demmig-Adams et al., 1995; Demmig-Adams, 1998; Adams et al., 1999). In addition, it has been known that the

PsbS protein level can determine the NPQ level and that certain amino acid residues in PsbS play an important role in NPQ (Li et al., 2002b, 2002c). However, the deepoxidation states of the xanthophyll cycle in *L1-1*, *Sf-2*, *Col-0*, and *Ws-2* were similar to each other (Fig. 2C), and *L1-1* and *Sf-2* contained almost identical levels of PsbS compared to *Col-0* and *Ws-2* without any polymorphisms in predicted amino acid sequences (Fig. 3). These results suggest that the QTLs identified here, *HQE1* and *HQE2*, do not act directly or indirectly through NPQ factors that have been found previously in extensive biochemical and genetic analyses of NPQ.

The response of *HQE1* to photosynthetically active light was faster than that of *HQE2* (Fig. 6, A–J). After



**Figure 7.** NPQ levels of near isogenic lines for HQE2 (NQE2). NPQ levels were measured in near isogenic lines for HQE2 (NQE2), which contain the *Sf-2* allele of *HQE2* (black) in a *Col-0* background (white bar). The breakpoints of the *Sf-2* introgression are between the markers CZSOD2 and T27E13 on one side and between the markers VE017 and NGA168 on the other side. Segregants containing the *Col-0* allele of *HQE2* (CON) were used as control lines. The significance of differences between NQE2 and CON was tested using Student's *t* test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). Ch2, Chromosome 2.

being exposed to actinic illumination for 3 min, *HQE1* became a significant high NPQ QTL (Fig. 6C), and from 7 min, both *HQE1* and *HQE2* were responsible for the high NPQ (Fig. 6G). These results indicated that early NPQ induction might be affected by *HQE1* alone, and saturated NPQ levels might be explained by both *HQE1* and *HQE2* without any interaction between *HQE1* and *HQE2*. Therefore, it could be speculated that sudden changes in light intensity would be dealt with by NPQ controlled by *HQE1* and that, if the high light intensity continues, *HQE2* and *HQE1* together increase NPQ capacity to cope with the high light. Although *HQE1* was the only identified significant QTL between 3 and 6 min, the NPQ measured during that period time was widely distributed (Fig. 5).

In conclusion, we were able to identify high and low NPQ Arabidopsis accessions and map QTLs that influence NPQ in two of these accessions. This information provides a guideline for selection of appropriate RILs and experimental design for future NPQ QTL mapping. We have also developed DNA molecular markers showing polymorphisms between Sf-2 and Col-0 that can be used for genotyping of Sf-2 × Col-0 RILs. Although we had hypothesized that the difference in NPQ between the accessions would be related to PsbS, no differences in known NPQ factors were found. Analysis of other accessions might reveal cases in which NPQ variation is related to PsbS. Identification of the high NPQ QTLs described here will eventually make it possible to clone new genes responsible for high NPQ.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) seeds were obtained from the Arabidopsis Biological Resource Center and from the seed collection of the Niyogi laboratory. Before planting on soil, the seeds were kept in water at 4°C for 1 week. Arabidopsis plants were grown in short-day conditions (10 h light/14 h dark) for 5 to 6 weeks in a light- (150  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and temperature (22°C)-controlled growth chamber before chlorophyll fluorescence measurements and leaf tissue harvests.

### Chlorophyll Fluorescence Measurements

Plants were dark-adapted for 2 h before NPQ measurement. The measurement began at 1 PM, and all measurements were completed by 5 PM. Chlorophyll fluorescence parameters were measured on attached rosette leaves using an FMS2 fluorometer (Hansatech) during actinic illumination with 1,500  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  for 10 min, followed by darkness for 5 min. NPQ was calculated as  $(F_m - F_m')/F_m'$ , with  $F_m$  and  $F_m'$  being maximum fluorescence measured after dark adaptation and in the light-adapted condition, respectively, by applying a saturating pulse of light.  $\Phi_{\text{PSII}}$  was calculated as  $(F_m' - F_s)/F_m'$ , with  $F_m'$  being measured by applying a saturating pulse of light at the end of each 5-min illumination at a given intensity, and  $F_s$  being steady-state fluorescence during the illumination (Maxwell and Johnson, 2000).

### Pigment Analyses

Pigment analyses were done following Niyogi laboratory protocols with some modifications (Müller-Moulé et al., 2002). Leaf discs were collected with a puncher (diameter of 8 mm) from rosette leaves and floated on top of water

in petri dishes. Following incubation in low light (150  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ), the leaf discs were exposed to high light (1,700  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) for 4 and 40 min. The treated leaf discs were frozen in liquid nitrogen and ground to a fine powder. From the leaf powder, pigments were extracted twice with 200  $\mu\text{L}$  of 100% (v/v) acetone, and following centrifugation supernatants were filtered through a 0.2- $\mu\text{m}$  nylon filter and subjected to HPLC.

### Immunoblot Analysis and DNA Sequence Determination

Total proteins were extracted from plant leaves of each accession. Leaf tissue (200 mg) was frozen in liquid nitrogen and then ground to a fine powder. To the tissue powder, 200  $\mu\text{L}$  protein extraction buffer (4% [w/v] SDS, 25 mM Tris-HCl, pH 8.8, and 2.5% [v/v] glycerol) was added to the tissue powder. Following further grinding in the buffer, the protein samples were denatured in boiling water for 5 min. After centrifugation for 3 min, the supernatant was separated and then kept in a -70°C freezer. The protein concentration was determined using a Bio-Rad Protein assay kit (Bio-Rad Laboratories), and 30  $\mu\text{g}$  of total proteins were loaded in each well of a precast 10% to 20% Tris-Glycine gel (Invitrogen). The separated proteins were blotted onto a nitrocellulose membrane (Schleicher & Schuell) using a semidry transfer unit (Hofer Pharmacia Biotech). For quantification of the PsbS protein, a polyclonal antibody raised against a PsbS oligopeptide was used (Li et al., 2002a), and for the D1 protein, a polyclonal antibody raised against spinach (*Spinacia oleracea*) D1 (kindly provided by Prof. A. Melis at University of California, Berkeley, CA) was used. As the secondary antibody, horseradish peroxidase-labeled antibody was used, and the chemiluminescence signal was detected using ECL western blotting detection reagents (Amersham Biosciences).

Genomic DNA sequences were determined from PCR-amplified DNA. For PCR from genomic DNA, primers were designed using the web-based Primer3 program ([http://www.broad.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi)), and PCR products were separated on agarose gels and then purified using a gel extraction kit (Qiagen). Sequencing of purified DNA was performed using the Big-Dye Terminator v3.0 ready reaction cycle sequencing kit (Applied Biosystems) and an ABI 3100 automated DNA sequencer (Applied Biosystems).

### Marker Set Generation and Genotyping

PCR conditions for previously available markers were obtained from The Arabidopsis Information Resource, and restriction enzymes used for each marker are listed in Supplemental Table S2. For NQE2, CZSOD2 and T27E13 were used to determine genotypes between ER and C4H, and COP1 and VE017 were used for genotyping between C4H and NGA168. The newly generated insertion/deletion markers for Sf-2 × Col-0 were C42050 (forward: 5'-GCTGAGTATAGAGCAGGTTGGTG-3'; reverse: 5'-CGTCCTGTTTCAAT-TGTGTCATC-3') and D01650 (forward: 5'-CTGCTTTTAGACCGTTTCC-3'; reverse: 5'-TCCAATGCTAAGTCTGCTG-3'). PCR for C42050 was annealed at 60°C and polymerized at 72°C for 30 s. Annealing for D01650 was at 59°C, and polymerization lasted for 1 min. The PCR products of C42050 and D01650 were separated on 3% and 1.2% agarose gels, respectively. The newly developed cleaved-amplified polymorphic sequence markers were SNP397C (forward: 5'-TTTGAGCTTGTTCCTCGTG-3'; reverse: 5'-ATATCTGTGGG-GTTGCTTGG-3'), A44575 (forward: 5'-CAAACCCAAAACCAAAGCTG-3'; reverse: 5'-TGCTTACATGGGGGAAAAAG-3'), SNP299C (forward: 5'-TTG-AAGGCATGAGTTGTTGG-3'; reverse: 5'-CGCCGATTAGTACGACTACG-3'), and D12320 (forward: 5'-TGGGAGAGCCTAATGTTTCTG-3'; reverse: 5'-CCAACCCAAAATCCTTAC-3'). The PCR reactions of SNP397C and SNP299C were annealed at 58°C and polymerized for 1 min, and then the PCR products were digested with *Hpy*Ch4 IV and *Dde*I, respectively. Amplified A44575 DNA following PCR at 59°C annealing and 1 min polymerization was digested with *Hinf*I. PCR for D12320 was done at 59°C annealing and 30 s polymerization, and then *Dde*I-digested fragments were separated on 3% agarose gels.

For genotyping, genomic DNA was extracted as follows: frozen small leaves were ground in a 1.5-mL tube and then the fine powder was thawed in 10  $\mu\text{L}$  of alkali solution (0.5 N NaOH) (Klimyuk et al., 1993). Following boiling at 100°C for 30 s, 100  $\mu\text{L}$  of neutralization solution (0.2 M Tris-HCl, pH 8.0, and 1 mM EDTA) were added. For a 20- $\mu\text{L}$  PCR reaction, 2  $\mu\text{L}$  of the leaf tissue solution was used.



## Statistical Analysis and QTL Mapping

Test statistics including ANOVA, Student's *t* test and multiple comparison tests were calculated using Microsoft Excel and XLSTAT. Following the genotyping, a *G*-test was used to test whether the genotype ratio at each marker position fitted to the expected F2 segregation ratio (Supplemental Fig. S1) (Dytham, 2003). Intervals between neighboring markers were estimated using MAPMAKER/EXP 3.0 (Lander et al., 1987). The first marker on each chromosome was set at 0 cM, and each marker position was calculated from accumulated intervals between markers.

QTL mapping was performed using the R/qtl program (Broman et al., 2003). The LOD significance threshold was estimated after permutation tests that had been replicated 1,000 times (Churchill and Doerge, 1994). LOD support interval was calculated using the lodint function with 1.5 LOD unit drop to form the support interval. Additive and dominance effects of each QTL were calculated using estimations of the means of the three genotype groups that were in the output of scanone function with the Expectation Maximization method in the R/qtl program (Lander and Botstein, 1989; Tanksley, 1993). Estimation of percentage of variance explained by each QTL was obtained from a drop-one-term analysis of results in an additive model. The additive model was calculated using the fitqtl function with imputation method in the R/qtl program (Sen and Churchill, 2001).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Genotype segregation of Sf-2 × Col-0 F2 progeny at each marker position.

**Supplemental Table S1.** List of NPQ values in Arabidopsis accessions.

**Supplemental Table S2.** List of markers, positions, types, and PCR conditions.

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