Arabidopsis Mutants Define a Central Role for the Xanthophyll Cycle in the Regulation of Photosynthetic Energy Conversion

Krishna K. Niyogi, Arthur R. Grossman, and Olle Björkman
Carnegie Institution of Washington, Department of Plant Biology, 260 Panama Street, Stanford, California 94305

A conserved regulatory mechanism protects plants against the potentially damaging effects of excessive light. Nearly all photosynthetic eukaryotes are able to dissipate excess absorbed light energy in a process that involves xanthophyll pigments. To dissect the role of xanthophylls in photoprotective energy dissipation in vivo, we isolated Arabidopsis xanthophyll cycle mutants by screening for altered nonphotochemical quenching of chlorophyll fluorescence. The npq1 mutants are unable to convert violaxanthin to zeaxanthin in excessive light, whereas the npq2 mutants accumulate zeaxanthin constitutively. The npq2 mutants are new alleles of aba1, the zeaxanthin epoxidase gene. The high levels of zeaxanthin in npq2 affected the kinetics of induction and relaxation but not the extent of nonphotochemical quenching. Genetic mapping, DNA sequencing, and complementation of npq1 demonstrated that this mutation affects the structural gene encoding violaxanthin deepoxidase. The npq1 mutant exhibited greatly reduced nonphotochemical quenching, demonstrating that violaxanthin deepoxidation is required for the bulk of rapidly reversible nonphotochemical quenching in Arabidopsis. Altered regulation of photosynthetic energy conversion in npq1 was associated with increased sensitivity to photoinhibition. These results, in conjunction with the analysis of npq mutants of Chlamydomonas, suggest that the role of the xanthophyll cycle in nonphotochemical quenching has been conserved, although different photosynthetic eukaryotes rely on the xanthophyll cycle to different extents for the dissipation of excess absorbed light energy.

INTRODUCTION

Plants in nature experience variations in incident light quantity over several orders of magnitude on a daily basis. Although the reactions that convert solar energy into chemical energy are remarkably efficient, the capacity of these reactions is limited; therefore, plants often absorb more light energy than they are able to use for photosynthesis. In addition, many environmental stresses, including drought, extremes of temperature, or nutrient deprivation (Demmig-Adams and Adams, 1992), can further limit the ability of a plant to utilize light energy. Absorption of excessive light energy can lead to sustained depressions in photosynthetic efficiency (photoinhibition), often due to oxidative damage to the photosynthetic apparatus.

Photosynthetic organisms have evolved multiple mechanisms to cope with the absorption of excessive light and its consequences. Interception of incident light can be decreased by reorientation and/or movement of chloroplasts within cells (Brugnoli and Björkman, 1992) and, in some plants, by leaf movements (Björkman and Demmig-Adams, 1994). Carotenoids, including the xanthophylls, have essential photoprotective roles as quenchers of triplet chlorophyll (3Chl) and singlet oxygen (1O2) and as inhibitors of lipid peroxidation (Cogdell and Frank, 1987; Frank and Cogdell, 1993; Demmig-Adams et al., 1996). Another important lipid-soluble antioxidant is α-tocopherol (vitamin E), which can quench reactive oxygen species in the thylakoid membrane and prevent fatty acid peroxidation (Fryer, 1992). Abundant soluble antioxidants in the chloroplast include ascorbate (vitamin C) and glutathione (Foyer et al., 1994). In addition, some reactive oxygen species can be scavenged by enzymes such as superoxide dismutase (Bowler et al., 1994) and ascorbate peroxidase (Asada, 1994). Photorespiratory oxygen metabolism can maintain linear electron transport and utilization of absorbed light energy, especially under conditions of CO2 limitation (Osmond, 1981; Heber et al., 1996; Kozaki and Takeba, 1996; Park et al., 1996; Osmond et al., 1997). Nevertheless, damage to critical protein subunits and pigment cofactors of photosystem II (PSII), such as the D1 protein in particular, appears to occur as a consequence of normal reaction center function, resulting in inactivation of entire reaction centers and necessitating protein turnover and subsequent reaction center repair (Aro et al., 1993).

The dissipation of excess absorbed light energy is believed to play a key role in regulating light harvesting and
electron transport and appears to be critical for the prevention of photooxidative damage to the photosynthetic apparatus. An increase in the proton gradient (ΔpH) across the thylakoid membrane in excessive light triggers the harmless dissipation of excess energy as heat in the light-harvesting complexes (LHCs) associated with PSII (reviewed in Demmig-Adams and Adams, 1992; Björkman and Demmig-Adams, 1994; Horton et al., 1994, 1996; Gilmore, 1997). This process, which is measured (and referred to) as non-photochemical quenching of chlorophyll fluorescence, occurs in almost all photosynthetic eukaryotes. Although pH-dependent energy dissipation (also called qE) is the major component of NPQ under most conditions, decreases in PSII fluorescence due to state transitions (qT) and photoinhibition (qI) may also contribute to what is measured as NPQ (Krause and Weis, 1991). The pH-dependent part of NPQ can usually be distinguished based on the kinetics of its relaxation, which occurs rapidly in darkness because of loss of the light-induced ΔpH.

The majority of NPQ is thought to occur in the PSII antenna pigment bed (Demmig-Adams and Adams, 1992; Horton et al., 1994). A key role of the ΔpH may involve protonation of acidic amino acids on the chlorophyll a/b binding polypeptides of the PSII LHCs (reviewed in Crofts and Yerkes, 1994; Horton and Ruban, 1994; Gilmore, 1997), resulting in a conformational change that is required for NPQ. This hypothesis is supported by experiments showing that dicyclohexylcarbodiimide, an inhibitor of NPQ in vitro, binds to glutamate side chains of specific LHC polypeptides (Jahn and Junge, 1990; Walters et al., 1994, 1996; Pesaresi et al., 1997). In addition, evidence correlating the association of a conformational change with NPQ has come from measurements of a light-induced spectral absorbance change at 535 nm in intact leaves (Bilger et al., 1989; Bilger and Björkman, 1990, 1994; Ruban et al., 1993). Recently, NPQ in isolated thylakoids has been associated with the formation of a complex with a discrete, decreased lifetime of chlorophyll fluorescence (Gilmore et al., 1995, 1996).

Xanthophyll pigments in the LHCs also appear to have a critical role in NPQ. The extent of NPQ in plants is strongly correlated with the levels of zeaxanthin and antheraxanthin that are formed from violaxanthin via the xanthophyll cycle (see Figure 1) (Demmig-Adams, 1990; Pfundel and Bilger, 1994; Gilmore et al., 1995, 1996; Demmig-Adams and Adams, 1996a, 1996b; Eskling et al., 1997). In low or limiting light, the enzyme zeaxanthin epoxidase converts zeaxanthin to violaxanthin via the intermediate antheraxanthin. In excessive light, when the [H+] in the thylakoid lumen reaches a critical threshold, the enzyme violaxanthin deepoxidase is activated and converts violaxanthin back to antheraxanthin and then zeaxanthin. Experiments using DTT as an inhibitor of violaxanthin deepoxidase (Yamamoto and Kamite, 1972) in isolated thylakoids and detached leaves have shown that the xanthophyll pigments in the LHCs do not contribute significantly to NPQ. However, other studies have suggested that zeaxanthin acts to amplify NPQ within a certain ΔpH range but that the ΔpH is all that is absolutely required for NPQ (Noctor et al., 1991).

The isolation and characterization of mutants of the unicellular green alga Chlamydomonas that are blocked in the synthesis of specific xanthophylls have confirmed a role for the xanthophyll cycle in NPQ and also suggested the involvement of other xanthophylls (Niyogi et al., 1997a, 1997b). The Chlamydomonas npq1 mutant, which is unable to deepoxidize violaxanthin to zeaxanthin (see Figure 1), is only partially defective in NPQ, suggesting that not all NPQ in Chlamydomonas depends on operation of the xanthophyll cycle. Characterization of the lor1 mutant (Eichenberger et al., 1986; Chunaev et al., 1991), which lacks xanthophylls derived from α-carotene, revealed a possible role for lutein, which is the most abundant xanthophyll in the thylakoid, in NPQ (Niyogi et al., 1997b). Furthermore, an npq1 lor1 double mutant lacked almost all NPQ and was very susceptible to photooxidative bleaching in high light (Niyogi et al., 1997b), providing in vivo evidence for the importance of NPQ for photoprotection. This conclusion, however, was complicated by the roles of xanthophylls in several photoprotective processes, including quenching of 3Chl and 1O2 and inhibition of lipid peroxidation.

To what extent are the conclusions drawn from the studies of Chlamydomonas xanthophyll cycle mutants applicable to vascular plants? To address this question, we isolated Arabidopsis mutants that are defective in the xanthophyll cycle. Detailed physiological and molecular genetic analyses demonstrated that although the general features of NPQ are

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Figure 1. The Xanthophyll Cycle in the β-Carotene Branch of the Carotenoid Biosynthetic Pathway in Plants.

The reactions impaired in the npq1 and npq2 mutants are indicated.

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similar in green algae and plants, the xanthophyll cycle plays a more dominant role in NPQ in Arabidopsis.

RESULTS

Isolation of Arabidopsis Xanthophyll Cycle Mutants

Arabidopsis mutants that exhibited aberrant NPQ when exposed to excessive light were identified using a chlorophyll fluorescence video-imaging system (see Methods), which was used to isolate similar mutants of the unicellular green alga Chlamydomonas (Niyogi et al., 1997a). Approximately 30,000 Arabidopsis M₂ seeds were sown on agar medium, grown photoautotrophically for 12 days at a photon flux density (PFD) of 100 μmol photons m⁻² sec⁻¹, and screened for an alteration in the extent of rapidly reversible NPQ during exposure to 500 μmol photons m⁻² sec⁻¹ actinic light. Images of maximum chlorophyll fluorescence were captured and digitized after 4 min in high light and then again after a subsequent recovery period of 1 min in the dark. False-color images depicting reversible NPQ were generated from the fluorescence data, allowing identification of npq mutants as shown in Figure 2A. Thirteen independent npq mutants with defects in the dissipation of excess absorbed light energy were isolated for further characterization.

Pigment analysis of the npq mutants before and after treatment with high light uncovered four mutants with blocks in the xanthophyll cycle. Table 1 shows the results of the pigment analysis for two of the mutants, which were selected for further physiological characterization. The npq1-1 (not shown) and npq1-2 (Table 1) mutants did not convert violaxanthin to antheraxanthin and zeaxanthin during exposure to high light, suggesting a defect in violaxanthin de-epoxidase activity. In contrast, the npq2-1 (Table 1) and npq2-2 (data not shown) mutants accumulated high levels of zeaxanthin and contained no detectable antheraxanthin, violaxanthin, or neoxanthin; this phenotype is consistent with a block in the zeaxanthin epoxidase reaction. The previously identified zeaxanthin epoxidase aba1 mutants of Arabidopsis (Koornneef et al., 1982) exhibit the same xanthophyll profile as npq2 (Duckham et al., 1991; Rock and Zeevaart, 1991), suggesting that the npq2 mutants represent new alleles of aba1. Furthermore, in the video-imaging assay, the available aba1 mutants exhibited the same phenotype as the npq2 mutants (data not shown). The metabolic defects in the npq1 and npq2 mutants are indicated in Figure 1.

Absorbance and Fluorescence Characteristics of Xanthophyll Cycle Mutants

The xanthophyll cycle defects in npq1 and npq2 mutants were corroborated by measurements of leaf absorbance before, during, and after exposure to high light (1900 μmol
photons m\(^{-2}\) sec\(^{-1}\)). A light-induced absorbance change at 505 nm is associated with xanthophyll cycle activity and is due to differences in absorbance between violaxanthin and zeaxanthin (Yamamoto and Kamite, 1972). Figure 3 shows that both npq1 and npq2 mutants failed to exhibit a significant \(\Delta A_{505}\) in difference spectra (high light minus dark), whereas the wild-type strain exhibited a very prominent \(\Delta A_{505}\) band. The absorbance change at 535 nm, which is attributed to a conformational change within the thylakoid membrane that occurs upon protonation of the LHCs in the presence of zeaxanthin (Bilger et al., 1989; Bilger and Björkman, 1990, 1994; Ruban et al., 1993), was observed in wild-type and npq2 leaves (Figure 3). However, the \(\Delta A_{535}\) was undetectable in the npq1 mutant, which lacks zeaxanthin, confirming that zeaxanthin is necessary for this absorbance change.

The induction kinetics and extent of NPQ in the xanthophyll cycle mutants were examined by measurements of modulated fluorescence that were performed simultaneously with the absorbance change measurements. As shown in Figure 4, induction of NPQ in wild-type Arabidopsis leaves occurred during exposure to high light, reaching a maximum extent within 3 min. The \(\Delta A_{505}\) band due to zeaxanthin formation appeared at the same time as the NPQ induction in the wild type (data not shown). Upon subsequent darkening, the NPQ in wild-type leaves relaxed rapidly because of the loss of the light-induced \(\Delta p\text{H}\) across the thylakoid membrane. In contrast, the npq1 mutant exhibited very little NPQ in high light. Most of the NPQ in npq1 developed within the first minute of illumination, and this NPQ was only partially reversible within 5 min in the dark. Induction of NPQ in the npq2 mutant occurred more rapidly than it did in the wild type. A similar, rapid induction of NPQ was observed for the Arabidopsis aba1 mutant (Tardy and Havaux, 1996; Hurry et al., 1997). In addition, the NPQ of npq2 was more slowly reversible after darkening of the leaf. The slower recovery in npq2 explains why these mutants were recovered in the video-imaging screen, which targeted mutants impaired in rapidly reversible NPQ.

To examine more closely the early induction of NPQ in wild-type, npq1, and npq2 leaves, saturating pulses were applied at shorter time intervals after a transition from darkness to moderately high light (1087 \(\mu\text{mol} \text{ photons m}^{-2} \text{ sec}^{-1}\)). Figure 5 shows that the NPQ induction in wild-type leaves was biphasic at this PFD, with a rapid initial increase

### Table 1. HPLC Analysis of Pigment Composition of the Wild Type and Xanthophyll Cycle Mutants before and after Exposure to High Light\(^a\)

<table>
<thead>
<tr>
<th>Pigment(^b) (mmol/mol Chl a)</th>
<th>Wild Type</th>
<th>npq1-2</th>
<th>npq2-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before HL(^c)</td>
<td>After HL(^c)</td>
<td>Before HL</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>46.6</td>
<td>49.3</td>
<td>50.5</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>46.0</td>
<td>17.4</td>
<td>70.3</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>1.5</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>1.9</td>
<td>27.2</td>
<td>0.8</td>
</tr>
<tr>
<td>(\beta)-Carotene</td>
<td>110.5</td>
<td>105.6</td>
<td>131.3</td>
</tr>
<tr>
<td>Lutein</td>
<td>246.3</td>
<td>266.0</td>
<td>241.8</td>
</tr>
<tr>
<td>Chl b</td>
<td>373.7</td>
<td>376.2</td>
<td>370.6</td>
</tr>
<tr>
<td>(V + A + Z)</td>
<td>0.068</td>
<td>0.642</td>
<td>0.025</td>
</tr>
<tr>
<td>((A + Z))/((V + A + Z))</td>
<td>Chl a (mmol m(^{-2}))</td>
<td>257.6</td>
<td>226.8</td>
</tr>
</tbody>
</table>

\(^a\)Six leaves of each genotype were detached, floated on water, and exposed to light from a xenon lamp.

\(^b\)Pigments are expressed relative to chlorophyll a (mmol/mol Chl a), except for chlorophyll a, which is based on leaf area. Values are from a single representative experiment. A, antheraxanthin; V, violaxanthin; Z, zeaxanthin.

\(^c\)Pigment composition of leaves was determined either before or after exposure to high light (HL, 2000 \(\mu\text{mol} \text{ photons m}^{-2} \text{ sec}^{-1}\)) for 30 min.

Figure 3. Light-Induced Spectral Absorbance Changes in Leaves of the Wild Type, npq1, and npq2.

Curves show the changes caused by a 3-min exposure to a PFD of 1900 \(\mu\text{mol} \text{ photons m}^{-2} \text{ sec}^{-1}\). Each point represents the mean of two or three determinations. wt, wild type.
in NPQ followed by a slight lag and then a secondary increase. The npq1 mutant retained the rapid initial increase in NPQ (during the first 10 sec of illumination), but further increases occurred more slowly. These results suggest that induction of NPQ in the wild type involves a rapid phase that is independent of xanthophyll cycle operation followed by a xanthophyll cycle–dependent phase. The induction kinetics of NPQ in the npq2 mutant showed only a single, rapid phase, resulting overall in much faster NPQ induction than was observed in the wild type. In the presence of constitutively high levels of zeaxanthin, NPQ in the npq2 mutant is presumably driven solely by induction of the ΔpH.

Light–response curves for chlorophyll fluorescence parameters revealed altered regulation of photosynthesis in xanthophyll cycle mutants. Figure 6 shows steady state values of gross NPQ, the estimated reduction state of the first stable PSII electron acceptor QA, the efficiency of PSII photochemistry, and the relative rate of PSII photochemistry for an increasing series of PFDs. At elevated PFDs, the npq1 mutant exhibited lower steady state NPQ than did the wild type (Figure 6A). Although the final extent of NPQ at the highest PFD did reach a value of 1.1 in the npq1 mutant, approximately half of this NPQ was irreversible within 5 min (data not shown), indicating that it was not due to pH-dependent energy dissipation in the LHCs. Consistent with an impaired ability to dissipate excess absorbed light energy, the npq1 mutant exhibited a higher reduction state of QA at each PFD (Figure 6B). In contrast, the npq2 mutant had slightly elevated levels of NPQ (Figure 6A) and a lower reduction state of QA (Figure 6B), especially at moderate PFDs. However, despite the observed effects on QA reduction state, the efficiency of PSII photochemistry (Figure 6C) at each PFD and thus the relative rates of PSII photochemistry (Figure 6D) were only slightly affected in npq1 compared with the wild type and npq2.

Sensitivity of npq1 to Excessive Light

To begin to assess the effects of impaired violaxanthin de-epoxidation on growth of Arabidopsis in excessive light, we maintained wild-type and npq1 plants together in natural sunlight after germination under controlled conditions (see Methods for details). No obvious differences in the coloration, size, or fertility of well-watered wild-type and npq1 plants were observed (data not shown).

However, leaves of the npq1 mutant experienced greater photoinhibition than did wild-type leaves when exposed to full sunlight under conditions that would limit photosynthetic gas exchange. Mature plants acclimated to 450 μmol photons m⁻² sec⁻¹ in a growth chamber were moved outdoors and gradually exposed to natural sunlight during a 2-day period. Compared with plants grown at 230 μmol photons m⁻² sec⁻¹ (Table 1), leaves of these wild-type and npq1 plants had a xanthophyll cycle pool size that was approximately two times higher (data not shown). Detached leaves were floated on water and exposed to full sunlight (2000 μmol photons m⁻² sec⁻¹) for 70 min. The xanthophyll cycle pool was mostly deepoxidated (84% zeaxanthin plus antheraxanthin) in wild-type leaves, whereas only 3% of the xanthophyll cycle pool in npq1 leaves was present as zeaxanthin plus antheraxanthin. Table 2 shows that after exposure to full sunlight, the PSII efficiency at this PFD was 37% lower in npq1 leaves than in wild-type leaves. Furthermore, during recovery at very low PFD, npq1 leaves exhibited sustained depressions in PSII efficiency and maximum fluorescence compared with the wild type.
The npq1 and npq2 mutants were backcrossed to the wild type (ecotype Columbia [Col-0]) to determine the genetic basis for the mutant phenotypes. The data in Table 3 show that in the F1 generation, all progeny exhibited wild-type levels of NPQ (NpqW). Furthermore, the npq1/NPQ1 heterozygous plants were able to deepoxidate violaxanthin to zeaxanthin, and the npq2/NPQ2 heterozygotes had normal levels of all xanthophylls (data not shown). In the subsequent F2 generation, the NpqW phenotype appeared in ~25% of the progeny (Table 3 and Figure 2B), demonstrating that both npq1 and npq2 are single, recessive nuclear mutations.

Crosses between npq1-1 and npq1-2 yielded only NpqW progeny (Table 4). The lack of complementation suggests that npq1-1 and npq1-2 are independent mutations in the same gene. Similarly, a complementation test between npq2-1 and aba1-3 confirmed that npq2-1 is a new allele of the gene that was defined previously by the aba1 mutations (Koornneef et al., 1982; Table 4).

The npq1 Mutants Are Defective in the Gene Encoding Violaxanthin Deepoxidase

The genetic map position of npq1 was determined by testing the segregation of simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994) among F2 progeny of a cross between npq1-1 and a polymorphic wild-type Arabidopsis strain (ecotype Landsberg erecta [Ler-0]). Tight linkage was observed between npq1 and the nga63 marker on chromosome 1 (Figure 7A). Also located

Figure 6. Light Dependence of Steady State Chlorophyll Fluorescence Parameters in Leaves of the Wild Type, npq1, and npq2.

Each point represents the mean of two or three determinations. wt, wild type.

(A) Gross NPQ. The standard error for each point is <10% of the mean.

(B) Estimated reduction state of Qa (1 – qP). The fraction of Qa in the reduced state was estimated as (Fm – Fo′)/(Fm′ – Fo′). The standard error for each point is <15% of the mean.

(C) Efficiency of PSII photochemistry (ΦPSII). PSII efficiency was calculated as (Fm – Fo)/(Fm′) in the absence of actinic light and as (Fm′ – Fo′)/(Fm′) in the light. The standard error for each point is <10% of the mean.

(D) Relative PSII electron transport rate (ETR). The rate of electron transport through PSII was calculated as ΦPSII × PFD × α × 0.5, where α is the fraction of incident light absorbed by the leaf. In all leaves, α was in the range 0.84 to 0.86. The standard error for each point is <10% of the mean.
on chromosome 1 is the violaxanthin deepoxidase gene (Bugos and Yamamoto, 1996), which is immediately adjacent to the KNOLLE gene (Lukowitz et al., 1996) (GenBank accession number U39452). The available physical mapping data for chromosome 1 (http://cbil.humgen.upenn.edu/~atgc/physical-mapping/physmaps.html) revealed that nga63 and the violaxanthin deepoxidase gene are closely linked (they are found on a single yeast artificial chromosome clone). Therefore, these results placed the npq1-1 mutation close to the Arabidopsis violaxanthin deepoxidase gene and suggested that npq1-1 may contain a mutation in the violaxanthin deepoxidase gene.

Genomic DNA containing the violaxanthin deepoxidase gene was amplified from the wild type and npq1-1 by polymerase chain reaction (PCR). Direct sequencing of the PCR products from independent reactions revealed a single base pair difference between the wild type and npq1-1. Figure 7B shows that the G-to-A transition mutation is predicted to affect the Arabidopsis growth conditions may have been less permissive for partial loss-of-function mutations suggesting that the Arabidopsis mutants may involve a deletion or rearrangement of the violaxanthin deepoxidase gene.

The npq1-1 and npq1-2 mutations were complemented by a wild-type copy of the violaxanthin deepoxidase gene (Figure 2C). Wild-type genomic DNA containing the violaxanthin deepoxidase gene was subcloned between T-DNA borders and introduced into npq1-1 and npq1-2 plants by Agrobacterium-mediated transformation. Whereas control transgenic plants containing only the pBIN19 vector still showed the npq- mutant phenotype, npq1 plants that were transformed with the wild-type violaxanthin deepoxidase gene exhibited the wild-type, npq+ phenotype (Figure 2C).

DISCUSSION

Mutations in the Arabidopsis Genes Encoding Xanthophyll Cycle Enzymes

To isolate plants defective in NPQ and the xanthophyll cycle, we used a digital video-imaging technique that was used previously to isolate Chlamydomonas mutants (Niyogi et al., 1997a). By screening mutagenized plants growing photoautotrophically in Petri plates, ~100 Arabidopsis plants could be analyzed simultaneously, and npq mutants were recovered at a frequency of approximately one in 2000. This frequency is considerably lower than that obtained from the screening of Chlamydomonas mutants, suggesting that the Arabidopsis growth conditions may have been less permissive for partial loss-of-function mutations affecting photosynthetic electron transport. Perhaps as a result of this increased stringency, a relatively large fraction (four of 13) of the Arabidopsis npq mutants exhibited defects in the xanthophyll cycle.

The Arabidopsis npq1 and npq2 mutations affect the structural genes encoding the enzymes of the xanthophyll cycle. Several lines of evidence demonstrated that the npq1 mutants are defective in the Arabidopsis violaxanthin deepoxidase gene (Bugos and Yamamoto, 1996). The npq1-1

Table 2. Sensitivity of npq1 and Wild-Type Leaves to Photoinhibition

<table>
<thead>
<tr>
<th>Time</th>
<th>npq1-1</th>
<th>Wild Type</th>
<th>npq1-2/</th>
<th>Fm'/Fm'</th>
<th>npq1-2/</th>
<th>Fm'/Fm'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>6</td>
<td>0.812 ± 0.003</td>
<td>0.801 ± 0.002</td>
<td>1.01</td>
<td>831 ± 8</td>
<td>802 ± 1</td>
</tr>
<tr>
<td>14 min</td>
<td>6</td>
<td>0.517 ± 0.019</td>
<td>0.639 ± 0.014</td>
<td>0.81</td>
<td>323 ± 8</td>
<td>366 ± 15</td>
</tr>
<tr>
<td>34 min</td>
<td>6</td>
<td>0.641 ± 0.015</td>
<td>0.752 ± 0.006</td>
<td>0.85</td>
<td>482 ± 15</td>
<td>593 ± 6</td>
</tr>
<tr>
<td>57 min</td>
<td>6</td>
<td>0.657 ± 0.012</td>
<td>0.754 ± 0.007</td>
<td>0.87</td>
<td>532 ± 14</td>
<td>633 ± 10</td>
</tr>
<tr>
<td>68 min</td>
<td>6</td>
<td>0.672 ± 0.014</td>
<td>0.757 ± 0.007</td>
<td>0.89</td>
<td>551 ± 14</td>
<td>663 ± 9</td>
</tr>
<tr>
<td>85 min</td>
<td>6</td>
<td>0.680 ± 0.013</td>
<td>0.765 ± 0.006</td>
<td>0.89</td>
<td>570 ± 16</td>
<td>675 ± 5</td>
</tr>
<tr>
<td>115 min</td>
<td>6</td>
<td>0.702 ± 0.009</td>
<td>0.764 ± 0.005</td>
<td>0.92</td>
<td>589 ± 10</td>
<td>678 ± 12</td>
</tr>
<tr>
<td>174 min</td>
<td>6</td>
<td>0.724 ± 0.006</td>
<td>0.771 ± 0.005</td>
<td>0.94</td>
<td>626 ± 13</td>
<td>696 ± 19</td>
</tr>
<tr>
<td>237 min</td>
<td>6</td>
<td>0.730 ± 0.006</td>
<td>0.772 ± 0.005</td>
<td>0.95</td>
<td>639 ± 13</td>
<td>714 ± 19</td>
</tr>
<tr>
<td>297 min</td>
<td>6</td>
<td>0.737 ± 0.007</td>
<td>0.776 ± 0.005</td>
<td>0.95</td>
<td>654 ± 16</td>
<td>717 ± 7</td>
</tr>
</tbody>
</table>

Leaves were detached, floated on water, and exposed to natural sunlight (2000 μmol photons m⁻² sec⁻¹) for 70 min until time zero. The recovery of treated leaves was then followed for 5 hr at a PFD of 1 μmol photons m⁻² sec⁻¹. Fluorescence parameters were measured as described in Methods and expressed as means ±SE.

The efficiency of PSII photochemistry (ΦPSII) was calculated as (Fm' - Fv'Fm'). All measurements of ΦPSII were made at 1 μmol photons m⁻² sec⁻¹ except for the time zero measurement, which was made at 2000 μmol photons m⁻² sec⁻¹.

Measurements were also made the following morning after 20 hr of recovery.
Table 3. Results of Crosses between Xanthophyll Cycle Mutants and the Wild Type

<table>
<thead>
<tr>
<th>Cross</th>
<th>Type</th>
<th>Total</th>
<th>Npq⁺</th>
<th>Npq⁻</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPQ1/NPQ1 × npq1-1/npq1-1</td>
<td>F₁</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₂</td>
<td>121</td>
<td>92</td>
<td>29</td>
<td>0.069³</td>
</tr>
<tr>
<td>npq1-2/npq1-2 × NPQ1/NPQ1</td>
<td>F₁</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₂</td>
<td>48</td>
<td>33</td>
<td>13</td>
<td>0.33³</td>
</tr>
<tr>
<td>npq2-1/npq2-1 × NPQ2/NPQ2</td>
<td>F₁</td>
<td>28</td>
<td>28</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₂</td>
<td>48</td>
<td>33</td>
<td>15</td>
<td>1.0³</td>
</tr>
</tbody>
</table>

*Pollen from the male parent (listed first) was crossed onto stigmas of the female parent to generate F₁ seeds. The heterozygous F₁ plants were allowed to self-pollinate to generate F₂ seeds. The phenotypes of F₁ and F₂ plants were scored by fluorescence video imaging after 12 days of growth on agar medium.

The 3:1 segregation of the Npq phenotype in the F₂ generation is consistent with the hypothesis that npq1-1 and npq1-2 are single recessive nuclear mutations (P > 0.5).

The 3:1 segregation of the Npq phenotype in the F₂ generation is consistent with the hypothesis that npq2-1 is a single recessive nuclear mutation (P > 0.1).

Table 4. Results of Complementation Tests

<table>
<thead>
<tr>
<th>Cross</th>
<th>Type</th>
<th>Total</th>
<th>Npq⁺</th>
<th>Npq⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>npq1-2/npq1-2 × npq1-1/npq1-1</td>
<td>F₁</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>abal-1/abal-3 × npq2-1/nq2-2</td>
<td>F₁</td>
<td>21</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>

*Pollen from the male parent (listed first) was crossed onto stigmas of the female parent to generate F₁ seeds. The phenotypes of F₁ plants were scored by fluorescence video imaging after 12 days of growth on agar medium.

Mutation mapped to the same chromosomal location as the cloned violaxanthin deepoxidase gene, and DNA sequencing revealed a single base pair substitution in the gene from npq1-1 plants (Figure 7). In addition, transformation of npq1-1 and npq1-2 with the violaxanthin deepoxidase gene resulted in rescue of the mutant phenotypes (Figure 2C). The npq2-1 mutation failed to complement the aba1-3 mutation (Table 4), which defines the zeaxanthin epoxidase gene of Arabidopsis (Marin et al., 1996), suggesting that npq2-1 and npq2-2 are new mutations affecting the zeaxanthin epoxidase gene. The aba1 mutants were originally identified as abscisic acid-deficient mutants (Koornneef et al., 1982), and consistent with this finding is the observation that the npq2-2 mutant exhibited a dramatic "wilty" phenotype (data not shown). However, the npq2-1 mutant that we used for physiological and genetic analyses was not particularly susceptible to wilting, presumably because it synthesizes sufficient abscisic acid. It is likely that npq2-1 is not a complete loss-of-function mutation affecting zeaxanthin epoxidase activity, as evidenced by the very low but detectable levels of antheraxanthin, violaxanthin, and neoxanthin in npq2-1 (Table 1).

Synthesis of Zeaxanthin by the Xanthophyll Cycle Is Required for the Bulk of pH-Dependent NPQ in Arabidopsis

Induction of NPQ in excessive light was severely impaired in the npq1 mutant (Figures 4 and 5), demonstrating that de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin is necessary for most of the rapidly reversible NPQ in Arabidopsis. This finding is consistent with the results of experiments in which DTT was effective as an inhibitor of NPQ in detached leaves of many but not all plants (Bilger and Björkman, 1990; Demmig-Adams et al., 1990; Johnson et al., 1994). In Arabidopsis, providing 2 mM DTT to wild-type leaves via the petiole resulted in an exact phenocopy of the npq1 mutation, but treatment with DTT had no effect on npq1 leaves (data not shown). These results confirm that DTT affects NPQ primarily by inhibition of violaxanthin deepoxidase activity (Bilger and Björkman, 1990).

However, the npq1 mutant retained a small but significant amount of reversible NPQ that was induced very rapidly after the transition from darkness to excessive light (Figures 4 and 5), suggesting that a component of reversible NPQ in Arabidopsis is independent of the xanthophyll cycle. The residual NPQ could be due to the very low but persistent levels of antheraxanthin and zeaxanthin (Table 1) that accumulate in the npq1 mutant as intermediates in the biosynthesis of violaxanthin (see Figure 1). Recent modeling of pH-dependent xanthophyll-dependent NPQ based on chlorophyll fluorescence lifetime measurements has suggested that even small amounts of antheraxanthin and zeaxanthin can lead to significant NPQ (Gilmore, 1997). Alternatively, the reversible NPQ in the npq1 mutant may involve the xanthophyll lutein, which is derived from α-carotene. A role for lutein in NPQ was suggested by characterization of Chlamydomonas xanthophyll mutants (Niyogi et al., 1997b). The issue of whether lutein or antheraxanthin is responsible for the residual NPQ in npq1 can be addressed by using existing lutein-deficient Arabidopsis mutants (Pogson et al., 1996). A third possibility is that the remaining NPQ in npq1 mutants is pH dependent but xanthophyll independent (Noctor et al., 1993; Gilmore et al., 1995, 1996).

The npq1 mutants of Arabidopsis and Chlamydomonas have provided unequivocal molecular genetic evidence that violaxanthin deepoxidase activity is involved in NPQ, but it is apparent that the extent to which different organisms rely on the xanthophyll cycle can be very different (Johnson et al., 1994; Casper-Lindley and Björkman, 1998). When transferred from darkness to high light, reversible NPQ was impaired in the Chlamydomonas npq1 mutant by only 25% (Niyogi et al., 1997b). In contrast, 85% of the reversible NPQ was inhibited in the Arabidopsis npq1 mutant (Figure 4), suggesting that there is a greater relative contribution of xanthophyll cycle-dependent NPQ in Arabidopsis compared with Chlamydomonas. It is possible that the ΔpH has a
Why Have a Xanthophyll Cycle?

Almost all photosynthetic eukaryotes have a xanthophyll cycle (reviewed in Pfündel and Bilger, 1994; Eskling et al., 1997). The cycle involving violaxanthin, anteraxanthin, and zeaxanthin is found in green plants, green algae, and many brown algae, whereas a deepoxidation cycle involving diadinoxanthin and diatoxanthin occurs in all other eukaryotic algae except for rhodophytes and cryptophytes. All organisms that possess a xanthophyll cycle appear to have a chlorophyll-based, LHC type of major peripheral antenna associated with PSII, whereas those that lack a xanthophyll cycle have a PSII antenna composed largely of phycobiliproteins (Grossman et al., 1995; Green and Durnford, 1996). This observation is consistent with the hypothesis that the xanthophyll cycle has an important role in the regulation of photosynthetic energy conversion and the prevention of 1O2 formation. Furthermore, xanthophyll deepoxidation must have evolved relatively early in the history of photosynthetic eukaryotes, at a time before the divergence of the green algae from the other algae. It is also possible that xanthophyll deepoxidation arose independently in different lineages.

Leaves of the Arabidopsis npq1 mutant showed a greater sensitivity than did the wild type to a short-term photoinhibitory light treatment during which gas exchange was restricted (Table 2), although growth of npq1 plants in natural sunlight did not appear to be inhibited (data not shown). These initial results suggest that the xanthophyll cycle may be especially important under conditions of high light combined with additional environmental stresses.

Characterization of the aba1 mutant, which is allelic to the npq2 mutants, failed to reveal any increased susceptibility to photoinhibition (Hurry et al., 1997), showing that accumulation of zeaxanthin has no deleterious effect in terms of photoprotection. Given that an elevated ΔpH (in addition to zeaxanthin) is required for most of the NPQ in Arabidopsis, what advantage does the xanthophyll cycle provide over constitutive accumulation of zeaxanthin?

One possible disadvantage of having high levels of zeaxanthin present continuously in npq2 plants is that more energy dissipation occurs at steady state in moderate, subsaturating light (Figure 6A). In addition, the slower reversibility of NPQ in npq2 (Figure 4) means that NPQ would remain engaged for a longer period of time after a decrease in incident PFD. The presence of high levels of zeaxanthin may amplify the level of NPQ occurring at intermediate ΔpH during relaxation of a high ΔpH (Noctor et al., 1991; Gilmore and Yamamoto, 1993). This enhanced NPQ could decrease the efficiency of photosynthesis in limiting light. Indeed, retention of zeaxanthin has been associated with sustained depressions in photosynthetic efficiency in plants in the laboratory (I ahns and Miehe, 1996; Demmig-Adams et al., 1998) and in the field, especially in overwintering plants (Adams et al., 1994; Adams and Demmig-Adams, 1995; Ottander et al., 1995; Verhoeven et al., 1996). The consequences of zeaxanthin accumulation in npq2 for growth in different environments, such as fluctuating light, remain to be tested.

In addition to the photoprotective functions of xanthophylls in NPQ and in quenching of 1Chl and 1O2, xanthophylls are essential structural components of the LHCs...
(Plumley and Schmidt, 1987). Replacement of violaxanthin and neoxanthin with zeaxanthin in the abal1 mutant appears to affect the structure of the LHCs, specifically the peripheral, trimeric LHCII (Hurry et al., 1997). Perturbation of LHC structure could affect the efficiency of light harvesting in limiting light.

Although the exact mechanistic role of xanthophylls in NPQ remains unclear (Demmig-Adams and Adams, 1996a; Horton et al., 1996; Young and Frank, 1996; Gilmore, 1997), recent estimates of excited state energy levels have established the feasibility of direct deexcitation of 1Chl by xanthophylls (Horton et al., 1996; Frank et al., 1994; Owens, 1994). The lowest excited singlet states (S1) of antheraxanthin and zeaxanthin (and lutein) appear to lie at or below that of 1Chl, suggesting that downhill transfer of excitation energy from Chl to xanthophyll could occur. The excited xanthophyll could then return to ground state by dissipating the excitation energy as heat. In contrast, the S1 energy level of violaxanthin lies above 1Chl, which is consistent with a function for violaxanthin as an accessory light-harvesting pigment, absorbing light not available to Chl and then transferring the energy to Chl. The xanthophyll cycle would then be involved in interconversion of light-harvesting and energy-dissipating pigments in the LHCs, and this ability may confer a selective advantage in many natural environments. We hope that further characterization of the Arabidopsis xanthophyll cycle mutants will provide additional insights into the various roles of different xanthophylls in plants.

**METHODS**

**Strains, Growth Conditions, and Genetic Crosses**

*Arabidopsis thaliana* M2 seeds (ecotype Columbia [Col-0]) derived from mutagenesis with ethyl methanesulfonate were generously provided by M. Liscum (University of Missouri, Columbia), and M2 seeds derived from mutagenesis by fast-neutron bombardment were obtained from Lehle Seeds (Round Rock, TX). The abal-3 mutant was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The npq1-2 and npq2-1 alleles were used for all physiological analyses.

For growth in 100 × 25-mm Petri plates, seeds were surface-sterilized and sown on plant nutrient agar medium (Haughn and Somerville, 1986) lacking sucrose. The plates were wrapped with gas-permeable tape (Scotch 394; 3M, St. Paul, MN) and incubated at 22°C under continuous illumination (100 μmol photons m⁻² sec⁻¹; cool-white fluorescent). Plants were grown routinely in Pro-Mix HP potting mix (Premier, Laguna Beach, CA) in a shaded greenhouse with natural sunlight during the day (maximum of 200 μmol photons m⁻² sec⁻¹) and supplemental lighting (15 μmol photons m⁻² sec⁻¹) at night. The greenhouse temperature was 22 ± 4°C during the day and 20 ± 2°C at night. For examination of growth in natural sunlight, plants were maintained in pots in an unshaded location for several weeks during the summer in Stanford, CA.

Plants for the measurements shown in Figures 3 and 4 were grown in controlled growth chambers at the Carnegie Institution (Stanford, CA), in 7-cm-diameter plastic pots containing potting mix. Leaves used for these experiments had developed at a photon flux density (PFD) of 250 μmol photons m⁻² sec⁻¹ under a photoperiod of 11.5 hr of light at 22°C alternating with a 12.5-hr dark period at 17°C. Light was provided by a bank of fluorescent tubes (cool white; very high output) supplemented with incandescent lamps.

Plants used for the measurements shown in Table 1 and Figures 5 and 6 were grown in controlled growth chambers at the Research School of Biological Sciences, Australian National University, Canberra. They were grown in 7.5 × 7.5-cm plastic pots in a commercial potting mix obtained from a local nursery. Leaves used for these experiments had developed at a PFD of 230 μmol photons m⁻² sec⁻¹ under a photoperiod of 12 hr of light at 23°C alternating with a 12-hr dark period at 16°C. Light was provided by a bank of fluorescence-coated high-intensity discharge and incandescent lamps. Air humidity during the light was in the range of 60 to 70%.

Plants used for the photoinhibition experiments shown in Table 2 were grown initially under the same conditions as above, except that the PFD during leaf development was 450 μmol photons m⁻² sec⁻¹. Well-established potted plants were moved outdoors on November 30 (southern summer) and gradually exposed to full daylight. On December 2, plants were kept outdoors in full sunlight until 11:00 AM, when leaves were cut and floated on water. The incident PFD on the cut leaves was 2000 μmol photons m⁻² sec⁻¹ until 12:10 PM, when fluorescence parameters were determined at 1971 μmol photons m⁻² sec⁻¹. The treated leaves were then moved indoors, and recovery at a PFD of 1.0 to 1.6 μmol photons m⁻² sec⁻¹ was followed over the next 5 hr. Fluorescence parameters were also determined the next morning after recovery overnight.

Plants were hand-watered daily; periodically, a standard nutrient solution was added. All plants, including the npq2-1 mutant, grew well under these conditions with no signs of wilting. Fully expanded rosette leaves were used for all measurements.

Genetic crosses were performed according to standard procedures (Somerville and Ogren, 1982).

**Fluorescence Video Imaging**

Screening of mutants by digital video imaging of chlorophyll fluorescence was done as described previously (Niyogi et al., 1997a), with the following modifications. The light source was a 2500 W xenon arc lamp (model XBO 2500 W OFR; Osram, Van Nuys, CA) contained in a lamp housing that provided a collimated, slightly divergent light beam. A wide-band hot mirror (Optical Coating Laboratory, Inc., Palo Alto, CA) was placed normal to the exit beam of the lamp housing, and a cyan dichroic filter (Optical Coating Laboratory, Inc.) was placed normal to the exit beam. A laboratory-built pneumatic shutter system was used to provide 2.5-sec saturating light pulses (2000 μmol photons m⁻² sec⁻¹) at 1-min intervals during a 4-min illumination with actinic light (500 μmol photons m⁻² sec⁻¹). A final saturating pulse was applied after a subsequent 1-min dark period. The opening and closing of the shutters were controlled by a laboratory-built digital interface connected to a standard personal computer equipped with the Hewlett-Packard (Palo Alto, CA) Video to Photo frame grabber and software running on the Windows 95 operating system. Color video images of Fm (maximum fluorescence in the dark-adapted state) or Fm’ (maximum fluorescence in any light-adapted state) were captured during saturating pulses, and false-color images of nonphotochemical quenching (NPQ) were generated as described previously (Niyogi et al., 1997a).
Pigment Determination

HPLC analysis of carotenoids and chlorophylls was done as described previously (Niyogi et al., 1997a).

Measurements of Fluorescence and Spectral Absorbance Changes

Simultaneous measurements of light-induced changes in absorbance (450 to 650 nm) and fluorescence (Figures 3 and 4) were performed with the apparatus previously described (Brugnoli and Björkman, 1992; Bilger and Björkman, 1994). The fiber-optic arrangement was modified to accommodate the relatively small Arabidopsis leaves. To minimize apparent changes in absorbance caused by chloroplast movements (Brugnoli and Björkman, 1992), predarkened leaves were kept in yellow light (≈8 μmol photons m⁻² sec⁻¹) for 1 to 2 hr before the measurements. Also, the actinic light used to induce the absorbance and fluorescence changes was passed through a yellow dichroic filter (Optical Coating Laboratory, Inc.) that blocked radiation <500 nm.

Determination of conventional fluorescence parameters (Van Kooten and Snel, 1990) was performed with an OS-500 pulse-modulated fluorometer (Opticsciences, Tyngsboro, MA). Plants were dark-adapted for 1 to 2 hr before experiments to allow maximal reoxidation of zeaxanthin and antheraxanthin to violaxanthin and to ensure complete relaxation of the thylakoid pH gradient. An attached, fully expanded rosette leaf was placed in the leaf clip, allowing air to circulate freely on both sides of the leaf. At the start of each experiment, the leaf was exposed to 2 min of far-red illumination (2 to 4 μmol photons m⁻² sec⁻¹) for determination of F₀ (minimum fluorescence in the dark-adapted state). Saturating pulses (1 sec) of white light (8000 μmol photons m⁻² sec⁻¹) were applied to determine Fₚ or Fₚm values. Actinic light provided by a 20-W halogen lamp (EZX/FG, 8°; Philips, Somerset, NJ) was filtered through a wide-band hot mirror (Optical Coating Laboratory, Inc.) and attenuated as necessary with neutral density filters (Melles Griot, Irvine, CA). Fₚ is the fluorescence yield during actinic illumination. Fₚm (minimum fluorescence in the light-adapted state) was measured in the presence of far-red light after switching off the actinic light. Gross NPQ was calculated as (Fₚm - Fₚm)VFₚm, where Fₚm is the maximum value taken before exposure to actinic light.

Light curves for fluorescence parameters were determined as follows. The actinic light was increased in steps. At low and moderate actinic intensities, fluorescence measurements at each step were continued until a steady state was reached (usually within 10 min). These Fₚ and Fₚm values (in the light) and the Fₚm' value recorded immediately after the extinction of the actinic light were used to calculate the fluorescence parameters shown in Figure 6. At actinic light levels >1000 μmol photons m⁻² sec⁻¹, measurements were limited to 10 min at each step to minimize effects of photodamage.

Genetic Mapping and Molecular Biology

The npq1-1 mutation was mapped relative to simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994). Genomic DNA was isolated from F₂ plants derived from a cross between npq1-1/npq1-1 (Col-0) and NPQ1/NPQ1 Landsberg erecta (Ler-0) strains, and the segregation of SSLP markers was scored by polymerase chain reaction (PCR) for 50 F₂ plants. For marker nga63, all 14 Npq⁻ F₂ progeny (npq1-1/npq1-1) were homozygous for the Col-0 polymorphism, whereas all 36 Npq⁻ progeny (npq1-1/NPQ1 or NPQ1/NPQ1) were either heterozygous or homozygous for the Ler-0 polymorphism.

Genomic DNA containing the wild-type and npq1-1 alleles of the violaxanthin deepoxidase gene was amplified by PCR, using oligonucleotide primers KN75 (5'-GGGGAAGATTAGTAGTGTGAG-3') and KN76 (5'-TTACCTTATATGACCGAAC-3') and Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). Double-stranded PCR products were purified (Ausbuhl et al., 1989) and sequenced using a dye terminator cycle sequencing kit and an ABI prism model 310 genetic analyzer (Perkin-Elmer).

For complementation of the npq1 mutations, a 4-kb EcoRI-BgIII genomic DNA fragment from pSE48/B (Lukowitz, 1996) containing the wild-type violaxanthin deepoxidase gene was subcloned into vector pBIN19 digested with EcoRI and BamHI to generate pVC1. Arabidopsis npq1-1 and npq1-2 plants were transformed by vacuum infiltration (Bechtold et al., 1993) of Agrobacterium tumefaciens GV3101 containing either pVC1 or pBIN19.

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