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Selective inhibition of *HEMA* gene expression by photooxidation in *Arabidopsis thaliana*

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Abstract

Norflurazon (NF), a photobleaching herbicide, inhibits carotenoid biosynthesis. Lack of carotenoid pigments leads to photo-oxidative damage of chloroplasts. In this study of *Arabidopsis thaliana* we demonstrate that NF-treated photobleached plants are still able to make 5-aminolevulinic acid (ALA) the first precursor of porphyrins and tetrapyrroles. ALA is formed in the tRNA-dependent two-step C_5-pathway in the chloroplast of plants. The expression of glutamyl-tRNA reductase (GluTR), the first enzyme in the pathway, was severely inhibited by NF, while treatment with this compound did not significantly reduce the levels of the other enzyme, glutamate-l-semialdehyde aminomutase, or of $tRNA^{Glu}$, the initial metabolite of the pathway. Extracts of these plants retained the capacity, albeit reduced, to convert exogenously added glutamate to ALA. Thus, the much-reduced level of ALA formation in photobleached plants is due to selective inhibition of GluTR expression.

Keywords: Glutamyl-tRNA reductase; Aminolevulinic acid; Norflurazon; Photooxidation; *Arabidopsis thaliana*; Brassicaceae

1. Introduction

Most chloroplast proteins are nuclear encoded. However, the expression of some of these genes is believed to be regulated by the chloroplast; a 'plastidic factor' involved in such chloroplast-nucleus communications has been proposed. In previous attempts to define the role of the plastidic factor plants lacking functional chloroplasts were used (Kuhlemeier, Green & Chua, 1987). One of the ways to generate such plants is their growth in the presence of chlorosis-inducing herbicides. One such herbicide, norflurazon, blocks carotenoid biosynthesis. Since carotenoids quench singlet oxygen released during photosynthesis, inhibition of their biosynthesis by NF therefore causes photo-oxidation, resulting in massive damage of chloroplasts in the presence of light (Reiss, Bergfeld, Link, Thien & Mohr, 1983).

In addition to the absence of chlorophyll, photo-oxidized chloroplasts lack 70 S ribosomes and thus do not support plastid protein biosynthesis (Reiss et al., 1983). Continuous exposure of plants to NF in white light arrests chloroplast development at the proplastid stage. However, biological processes confined to the cytoplasm or other cellular compartments are not affected by photo-oxidative damage (Reiss et al., 1983; Feierabend, Winkelhüsener, Kemmerich & Schulz, 1982). Photo-oxidation of chloroplasts alters the expression of various chloroplastidic-nuclear genes. Rubisco expression is completely inhibited in mustard seedlings (Oelmüller & Mohr, 1986), but only reduced by 50% in pea (Sagar et al., 1988). Similarly, photo-oxidation reduces ferredoxin expression to 50% in pea. On the other hand, *Cab* mRNA was reduced to 20% upon exposure of peas to NF, while in rye a four-fold

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suppression of *Cab* and rubisco mRNA is seen (Ernst & Schefbeck, 1988).

In the present study, we examine the effect of photo-oxidation on ALA synthesis in *Arabidopsis thaliana*. ALA is formed in the tRNA-dependent two-step C_5-pathway in the chloroplast of plants (Kumar, Cszankovski & Söll, 1996). Glu-tRNA^{Glu} is reduced by glutamyl-tRNA reductase (GluTR) to glutamate-1-semialdhyde with the release of intact tRNA^{Glu}. In the next step glutamate-l-semialdehyde is converted to ALA by glutamate-l-semialdehyde-1,2-aminomutase (GSA-AM). ALA is the first precursor in the synthesis of porphyrins and tetrapyrroles (e.g. chlorophyll, heme and phytochromobilin). While the genes for GluTR (*HEMA*) and GSA-AM (*GSA*) are nuclear-encoded and chloroplast targeted, the tRNA^{Glu} gene (*trnE*) is located on the chloroplast genome (Kumar, Schaub, Söll & Ujwal, 1996). The synthesis of ALA in plants thus involves a well-coordinated interplay of nuclear and chloroplastidic components.

2. Results and discussion

2.1. Photo-oxidized plants contain lower levels of heme and ALA

Although it is known that photo-oxidation of chloroplasts results in dysfunction of chloroplastidic pathways, the effect of such damage on the C_5-pathway is known. The ability of the photo-oxidized plants to grow under the experimental conditions suggests the existence of a functional respiratory pathway. As heme, a vital component of respiration, is formed from ALA which is generated in the chloroplast in the C_5-pathway, the capacity of photodamaged chloroplasts to support this pathway was examined. Treatment with NF reduced significantly both heme and ALA levels (Fig. 1). The extent of concomitant reduction suggests a direct correlation between the heme and ALA levels; none of the enzymatic reactions leading to the formation of protoporphyrin IX (the backbone of heme) from ALA are affected by NF in white mustard seedlings (Thomsen, Oelze-Karow, Schuster & Mohr, 1993). It remains to be explored whether the lower ALA level is a consequence of NF leading to inhibition of ALA synthesis or whether the lowered demand for heme in photobleached plants causes feedback inhibition of ALA formation.

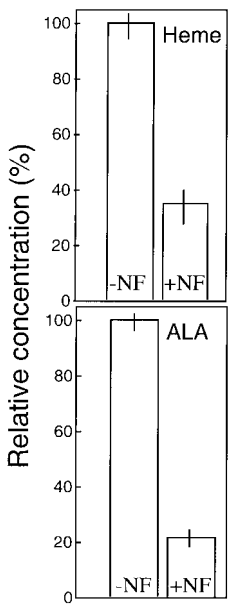

Fig. 1. Estimation of heme and ALA in NF-treated plants. The amount of heme and ALA in NF-treated plants is compared to the amounts found in untreated plants (100%).

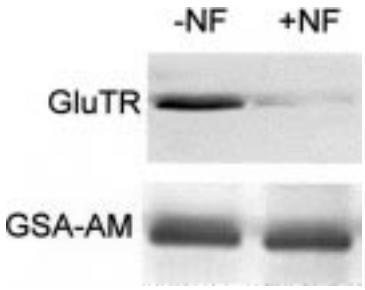

Fig. 2. Western blot analysis to detect GluTR and GSA-AM in extracts of NF-treated and untreated *Arabidopsis* plants.

2.2. Is the C_5-pathway operative in photo-oxidized chloroplasts?

Chloroplast destruction by NF or by mutations that affect chloroplast development, as observed in a barley *albina* mutant (Hess, Schendel, Rüdiger & Börner, 1992), should obliterate the C_5-pathway. However, ALA and heme were still detected under these conditions. To explore the route of ALA formation in NF-treated plants we searched for the enzymes of the C_5-pathway. Immunoblots of extracts derived from NF-treated whole plants indicated the presence of GluTR protein, albeit in lower concentration (approximately 10% of the abundance in untreated plants), while the levels of GSA-AM were unchanged (Fig. 2). Although both GluTR and GSA-AM proteins are nuclear encoded and targeted to the chloroplast, only the expression of GluTR was found to be affected by NF treatment. Possibly the expression of GluTR depends on the functional state of the chloroplast as described for certain chloroplast-targeted nuclear genes (Oelmüller & Mohr, 1986; Sagar, Horwitz, Elliott, Thompson & Briggs, 1988). A plastidic factor from the chloroplast was proposed to be involved in such organelle-nucleus communication.

2.3. Glutamyl-tRNA reductase in NF-treated plants is encoded by the HEMA1 gene

Two pools of ALA were reported to exist in cucumber cotyledons (Huang, Bonner & Castelfranco, 1990). The results of biochemical inhibition studies suggested that one of the two pools of ALA is under phytochrome control and destined for chlorophyll biosynthesis while the other pool mediates heme biosynthesis and is governed by a feedback regulation (Huang et al., 1990). Interestingly, we have identified and characterized two *HEMA* genes encoding the GluTR in *A. thaliana* (Kumar et al., 1996a). The *HEMA*1 gene is expressed in all parts of the plant and is light regulated, while *HEMA*2 gene expression is only seen in flowers and roots. Based on this tissue-specific expression pattern, it was inferred that the *HEMA*1 gene is involved in chlorophyll and heme biosynthesis, while the *HEMA*2 gene assumes an active role in heme biosynthesis probably regulated by a feedback mechanism (Kumar et al., 1996a). To examine the role of chlorophyll formation on *HEMA*2 gene expression, *HEMA*1 and *HEMA*2 mRNA levels in leaves of photobleached plants were analyzed by hybridization with gene-specific DNA probes. NF treatment drastically affected *HEMA*1 gene expression (Fig. 3). However, it did not reverse leaf *HEMA*2 gene expression (data not shown). Our inability to detect *HEMA*2 mRNA in non-green leaf casts doubt on the suggested regulatory role of chlorophyll precursors in *HEMA*2 gene expression (Kumar et al., 1996a).

2.4. Extracts from the photobleached plants convert glutamate to ALA

The data presented above reveal that GluTR and GSA-AM, the two enzymes of the C_5-pathway are present in NF-treated plants. Thus, if Glu-tRNA^{Glu} were present, the photobleached chloroplasts should be able to convert glutamate to ALA. Chloroplast tRNA^{Glu} is transcribed from a chloroplast-encoded *trnE* gene. To generate Glu-tRNA the chloroplast must be capable of transcribing the *trnE* gene and processing the tRNA^{Glu} precursor. It has been shown that NF-damaged chloroplasts do contain small amounts of 16 S rRNA, although the assembly of 70 S ribosomes is prevented by photo-oxidation (Feierabend & Berberich, 1991). To examine if photobleaching affects chloroplast tRNA^{Glu} gene expression, total RNA isolated from NF-treated plants was probed with the *Arabidopsis* chloroplast *trnE* gene (Hori, Kumar, Verkamp & Söll, 1996). As shown in Fig. 4, this gene hybridized to RNA from NF-treated and control plants. Since the chloroplast tRNA^{Glu} gene also cross-hybridized (to a lower level) with cytoplasmic tRNA^{Glu}, genomic and chloroplast DNA samples were used as controls. The tRNA^{Glu} probe produced a stronger signal with chloroplast DNA than with genomic DNA, indicating that the signal generated by the probe is chiefly due to the hybridization with the chloroplast tRNA^{Glu} and not with the cytoplasmic tRNA^{Glu} in the total RNA. From this we conclude that in NF-treated plants chloroplast tRNA^{Glu} is present albeit at a reduced level compared to the untreated control (Fig. 4).

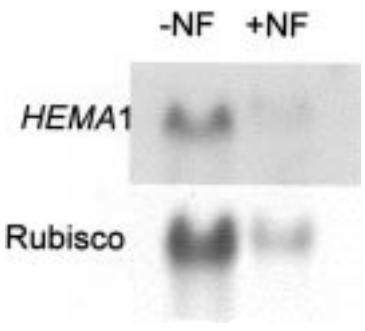

Fig. 3. Northern blot analysis of NF-treated and untreated *Arabidopsis* plants. Like the expression of *HEMA*1, Rubisco gene expression was also lowered upon exposure to NF.

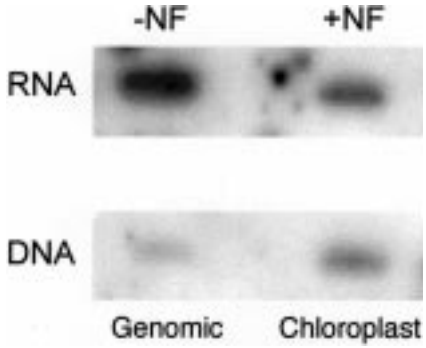

Fig. 4. Top: Detection of chloroplast-specific tRNA^{Glu} (Northern blot) in NF-treated and in untreated *Arabidopsis* plants. Bottom: Detection of the tRNA^{Glu} gene (*trnE*) in genomic and chloroplast DNA.

Glutamylation of chloroplast tRNA^{Glu} is achieved by a nuclear-encoded and chloroplast-targeted glutamyl-tRNA synthetase (GluRS). This enzyme is quite different from the cytoplasmic GluRS (Ratinaud, Thomes & Julien, 1983) and glutamylates chloroplast tRNA^{Glu} preferentially (compared with the corresponding cytoplasmic tRNA). Preliminary experiments with antibodies against *Chlamydomonas* chloroplast GluRS (Chen, Jahn, Schön, O'Neill & Söll, 1990) indicated the presence of GluRS in NF-treated plants. Thus, it was likely that Glu-tRNA^{Glu} was formed. Therefore we wanted to show the in vitro conversion of glutamate to ALA in extracts of NF-treated plants. When [^{14}C]glutamate was added to a cell extract under established conditions (Weinstein & Beale, 1985), worked up by Dowex-50 chromatography

and radiolabeled ALA was isolated (Fig. 5). The amount of ALA formed in photobleached plants was approximately a third of that synthesized in untreated plants.

3. Summary

The results obtained in this study demonstrate that the C_5-pathway is operative in a reduced capacity under photobleached conditions. Photo-oxidation predominantly affects the expression of GluTR in this pathway. Decreased ALA levels and the lowered expression of GluTR suggest that GluTR activity in NF-treated plants is the rate-limiting step in ALA biosynthesis. The low expression level of GluTR supports the concept that its nuclear-encoded *HEMA*1 gene is downregulated; thus a 'plastidic factor' could be involved in the expression of this gene.

4. Experimental

4.1. Plant growth

Surface sterilized seeds of *A. thaliana* (ecotype Columbia) were grown on sterile MS (Murashige Skoog) agar medium supplemented with 3% sucrose. To generate photo-oxidative damage of the chloroplasts, seeds were grown in the above medium containing NF (Sandoz) at 5×10^{-5} M concentration and incubated under normal growth room conditions (22°C, 60% relative humidity and with a regimen of 16 h white light [90 μE m^{-2} s^{-1}] and 8 h dark day cycle) for 2–3 weeks before processing.

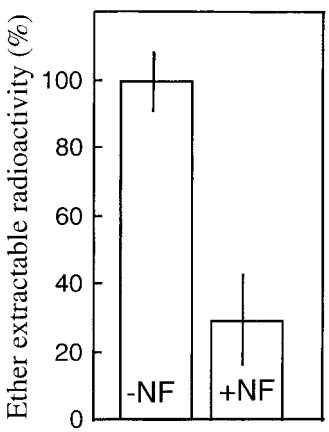

Fig. 5. Conversion of glutamate to ALA. ALA formed in the reaction was converted to ALA-pyrrole and extracted into ether as described in Experimental. The amount of ALA-pyrrole in NF-treated plants is expressed in relative terms to the ALA detected in untreated plants (100%).

4.2. Analytical methods

Total non-covalently bound hemes (protoheme and heme*a*) were extracted by the acid acetone method and estimated as described (Weinstein, Mayer & Beale, 1984). ALA in the total plant extract was determined after precipitating the proteins with trichloroacetic acid and analyzed colorimetrically (Beater & Kloppstech, 1993). To investigate the intactness of the C_5-pathway, plant extracts were incubated in the presence of [^{14}C]glutamate and the radiactive compound was purified by Dowex-50W-X8 column chromatography as described (Weinstein & Beale, 1985).

4.3. Northern blot analysis

Total RNA was isolated from two-week-old *Arabidopsis* plants according to the published protocol (Chomczynski & Sacchi, 1987). The RNA (20 μg) was heat denatured in the presence of formamide and separated on 1.2% agarose gel as described (Ausubel et al., 1987). Prehybridization, hybridization and washing conditions of the membranes were described above. To monitor the expression levels of *HEMA* genes, gene-specific DNA fragments from *HEMA*1 and *HEMA*2 (Kumar et al., 1996a) were labeled with [γ-^{32}P]dATP using random hexanucleotide priming (Ausubel et al., 1987).

To measure the $tRNA^{Glu}$, total RNA (approximately 5 μg) and to detect *trnE* gene, DNA (approximately 5 μg and 2 μg of genomic DNA and chloroplast DNA) was processed and applied on to a nitrocellulose membrane as described. The genomic DNA and choroplastidic DNAs were purified as described (Hori et al., 1996). The probe used was a *BamH*1 and *EcoR*1 fragment from pACE1 (Hori et al., 1996).

4.4. Western blot analysis

Plants were ground in liquid nitrogen and suspended in the extraction buffer (1 g/ml) containing Tris-HCl (50 mM, pH 8.0), NaCl (100 mM), 2-mercaptoethanol (50 mM), Triton X-100 (0.5%), phenymethylsufonylflouride (PMSF) (0.2 mM), leupeptine (1 μg/ml), apoprotein (1 μg/ml). Similarly, choroplast extracts were made from the purified chloroplasts (Hori et al., 1996). The suspension was spun at 13,000 *g* for 10 min and protein in the supernatant was measured (Bradford, 1976). An equal amount of protein was analyzed by a 10% SDS-polyacrylamide gel electrophoresis. The proteins were blotted to a nitrocellulose membrane as described (Ausubel et al., 1987). GluTR and GSA-AM proteins on this blot were detected using antibodies and anti-antibodies following the conditions as described (Ausubel et al., 1987).

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