TIGRINA d, required for regulating the biosynthesis of tetrapyrroles in barley, is an ortholog of the *FLU* gene of *Arabidopsis thaliana*[1]

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Received 25 July 2003; accepted 25 August 2003

First published online 11 September 2003

Edited by Ulf-Ingo Flügge

Abstract Regulation of tetrapyrrole biosynthesis in higher plants has been attributed to negative feedback control of steps prior to δ-aminolevulinic acid (ALA) formation. One of the first mutants with a defect in this control had been identified in barley. The *tigrina* (*tig*) *d* mutant accumulates 10–15-fold higher amounts of protochlorophyllide than wild type, when grown in the dark. The identity of the TIGRINA d protein and its mode of action are not known yet. Initially this protein had been proposed to act as a repressor of genes that encode enzymes involved in early steps of ALA formation, but subsequent attempts to confirm this experimentally failed. Here we demonstrate that the *TIGRINA d* gene of barley is an ortholog of the *FLU* gene of *Arabidopsis thaliana*. The FLU protein is a nuclear-encoded plastid protein that plays a key role in negative feedback control of chlorophyll biosynthesis in higher plants. Sequencing of the *FLU* gene of barley revealed a frame shift mutation in the *FLU* gene of the *tig d* mutant that results in the loss of two tetratricopeptide repeats that in the FLU protein of *Arabidopsis* are essential for its biological activity. This mutation cosegregates strictly with the *tigrina* phenotype within the F1 population of a heterozygous *tig d* mutant, thus providing additional support for the *flu* gene being responsible for the *tigrina* phenotype of barley.
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Key words: FLU; Protochlorophyllide; Feedback control; *Tigrina* mutant; *Arabidopsis*

1. Introduction

In higher plants at least three distinct classes of tetrapyrroles can be distinguished, Mg^{2+} porphyrins [chlorophylls (Chls)], $Fe^{2+/3+}$ porphyrins (hemes) and phycobilins (phytochromobilin) [1]. All of them strongly absorb light and may act as photosensitizers [2,3]. Chls, hemes and phycobilins are bound to proteins and in this state may use various quenching mechanisms to dissipate absorbed light energy. Their biosynthetic precursors, however, occur mostly in a free form and are potentially much more destructive when illuminated [3–5]. In order to avoid photooxidative damage, plants had to evolve highly efficient strategies to prevent the accumulation of intermediates during tetrapyrrole biosynthesis [6]. Angiosperms control the metabolic flow of tetrapyrrole intermediates at steps prior to δ-aminolevulinic acid (ALA) formation: In the dark, the Chl synthesis pathway leads only to the formation of protochlorophyllide (Pchlide), the immediate precursor of chlorophyllide (Chlide). Once a critical level of Pchlide has been reached, ALA synthesis slows down. Only after illumination, when Pchlide has been photoreduced to Chlide by the NADPH-Pchlide-oxidoreductase (POR), does Chl biosynthesis resume [7]. This regulation of Chl biosynthesis has been attributed to feedback control of ALA synthesis [1,8]. In analogy to its regulatory role in animals and yeast [9,10], heme has also been implicated as an effector of this feedback control in higher plants [11–14]. However, it seems unlikely that heme alone is sufficient to coordinate and adjust all activities of the various branches of tetrapyrrole biosynthesis. Genetic approaches were used to identify additional factors involved in the control of tetrapyrrole biosynthesis. Mutants with a defect in the genetic control of Chl biosynthesis of higher plants were first identified in barley [15]. Four different *tigrina* mutants were described that in the dark accumulate higher levels of Pchlide than wild-type plants [15,16]. Three of these mutants, *tig b*, *tig n* and *tig o*, displayed pleiotropic effects that included not only higher levels of Pchlide but also differences in the composition of carotenoids and the fine structure of etioplast membranes [17]. In the *tig d* mutant, however, only the control of Pchlide accumulation seemed to be affected [17]. Etiolated seedlings of the *tig d* mutant accumulate 10–15-fold higher amounts of Pchlide [16]. When these mutant plants are exposed to light, their leaves bleach and often develop necrotic areas. In mutants kept under continuous illumination, however, Pchlide is immediately photoreduced and chloroplast development proceeds normally as in wild-type seedlings. Up to now the identity of the TIGRINA d protein and its mode of action is not known. Initially, the TIGRINA d protein had been proposed to act as a repressor of genes that encode enzymes involved in the early steps of tetrapyrrole biosynthesis prior to ALA formation [18]. However, attempts to confirm this proposed function have failed [19].

Recently, we have isolated the *flu* mutant of *Arabidopsis thaliana* that in several ways resembles the *tig d* mutant of barley [20]. Etiolated seedlings of *flu* contain an up to 15-fold higher level of Pchlide than wild type. When these

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[1] This paper is dedicated to Diter von Wettstein.
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Abbreviations: ALA, δ-aminolevulinic acid; Chl, chlorophyll; Chlide, chlorophyllide; Glu-TR, glutamyl-tRNA reductase; GSA-AT, glutamate-1-semialdehyde-2-1-aminotransferase; Pchlide, protochlorophyllide; POR, NADPH-protochlorophyllide oxidoreductase; TPR, tetratricopeptide repeat

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doi:10.1016/S0014-5793(03)00983-9

seedlings are transferred from the dark to the light, they rapidly bleach and die. The homozygous *flu* mutant can be rescued, however, by germinating it under constant light. The identity of the FLU protein was established by map-based cloning and sequencing of the *FLU* gene [20]. FLU is a nuclear-encoded plastid protein that after import and processing becomes tightly associated with plastid membranes. It is unrelated to known repressor proteins or any of the enzymes known to be involved in tetrapyrrole biosynthesis. Its predicted features suggest that FLU mediates its regulatory effect through interaction with enzymes involved in Chl biosynthesis. In yeast, FLU was shown to interact with the Glu tRNA-reductase (Glu-TR) of *Arabidopsis*, the first enzyme committed exclusively to the biosynthesis of tetrapyrroles [21]. Collectively, these results suggest that FLU forms part of a negative feedback loop that operates independently of heme [20].

The reported similarities between the phenotypes of the *flu* and *tig d* mutants suggest that in both mutants the mutated genes may be identical. In the present work we have tested this proposed homology of the two genes experimentally. To this end we have identified and sequenced the *FLU* gene of barley. The comparison of *FLU* sequences of barley wild type and the *tig d* mutant showed that within the *FLU* gene of the *tig d* mutant there is a frame shift mutation that results in the loss of two tetratricopeptide repeat (TPR) domains in the C-terminal part of the FLU protein. These domains have been shown previously to be essential for the interaction of the FLU protein with the Glu-TR [21]. The strict cosegregation of the mutation in the *FLU* gene and the *tig d* locus in the F1 population of a heterozygous *tig d* mutant provides additional support for the *flu* gene being responsible for the *tigrina* phenotype in barley.

2. Materials and methods

2.1. Plant material

Wild-type barley (*Hordeum vulgare* L. cv. Svalöf's Bonus) and homozygous *tigrina-d*^{12} mutants [17] were grown on moist vermiculite at 23°C for 6 days in continuous darkness. Seedlings of the segregating F1 population of heterozygous *tig d* mutants were classified by removing the apical 1 cm segments of the primary leaves under green safe light and analyzing these segments for their greening capacity during a 12 h light period or by measuring the Pchlide fluorescence. For the fluorescene measurements, leaf segments of etiolated seedlings were illuminated with blue light and examined under the Leica UZ12 fluorescence microscope with a Leica FU blue 10146146 filter. Homozygous *tig d* mutants could be rescued and propagated by growing the plants under continuous light until their seeds could be harvested.

2.2. Isolation of RNA and DNA and RT-PCR

Total RNA was isolated from wild-type and *tigrina* mutants by using the RNeasy Plant mini kit (Qiagen). First-strand cDNA was synthesized from 1 μg of RNA for 1 h at 42°C using an Improm-II reverse transcription system (Promega) and the gene-specific primer 5′-TCA GTC ATT CTC CA-3′ containing a stop codon.

PCRs were carried out using the 'Expand High Fidelity'-enzyme (Roche Applied Science, Germany) with a set of oligonucleotide primers 5′-ATG GCA CGA GGG GAG ATT TTC CT-3′ (forward) and 5′-TCA GTC ATT CTC CAA TCT TG-3′ (reverse) obtained from a barley EST database (DNA Data Bank of Japan, DDBJ). The PCR condition was as follows: pre-denaturation at 94°C for 2 min; 35 cycles of 20 s at 94°C, 45 s at 56°C and 1 min at 72°C; last extension 7 min at 72°C; hold at 10°C. Genomic DNA of barley was isolated according to Chen and Ronald [22].

2.3. Nested PCRs

For the identification of amplified PCR products, nested PCRs were carried out with two different forward primers, 5′-CTC GTC CTC AAC TGA TCC-3′ and 5′-GTG CTA CAG TTG GAG GGC-3′, deduced from the predicted *FLU* sequence of barley that was obtained from the EST database. The amplified products were separated in 1% agarose gels.

2.4. Computer-assisted analysis of the deduced amino acid sequence

The amino acid sequence of the FLU protein of barley was deduced from the cDNA sequence using NCBI (http://www.ncbi.nlm.nik.gov/BLAST/) and DDBJ (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). For multiple sequence alignment, Multalin version 5.4.1 (http://probes.toulouse.inra.fr/multalin/multalin.html) was used. For the prediction of subcellular localization and structure of protein, SignalP, ChloroP 1.1, BCM (secondary structure prediction) and Expasy proteomics tools were used.

2.5. Extraction and measurement of Pchlide

For the isolation of tetrapyrroles from the wild-type barley and the *tigrina* mutant, leaf tips of 6 day old plants were used. The leaf material was ground in 1 ml of 80% acetone supplemented with ammonia to a final concentration of 0.0083 (v/v). The relative amounts of Pchlide were determined by measuring the fluorescence emission spectrum between 600 and 700 nm using an LS50 luminescence spectrophotometer (Perkin Elmer, Rotkreuz, Switzerland). The excitation wavelength was 433 nm.

3. Results

When grown in the dark, seedlings of the *tig d* mutant and wild type can easily be distinguished by the strong Pchlide

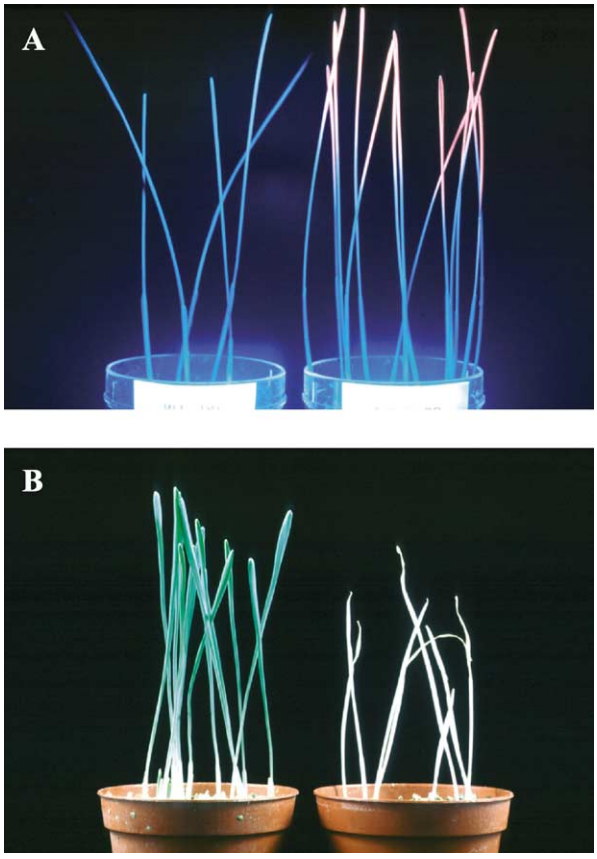

Fig. 1. A comparison of barley wild-type and the *tig d* mutant seedlings grown in the dark (A) and after transfer to the light (B). Etiolated seedlings were grown in the dark for 6 days. Mutant seedlings (right) can be distinguished from wild-type seedlings (left) by the strong red fluorescence of Pchlide that they emit after they have been exposed to blue light (A). When etiolated *tig d* seedlings are transferred from the dark to the light, they rapidly bleach and die (B, right), whereas wild-type seedlings green normally (B, left).

fluorescence that etiolated mutant seedlings emit, after they have been exposed to blue light (Fig. 1A). Similarly, etiolated seedlings of the *flu* mutant of *Arabidopsis* also emit a strong Pchlide fluorescence, when illuminated with blue light [20]. When etiolated seedlings of these mutants are transferred from the dark to the light, they rapidly bleach and die [20] (Fig. 1B). Pchlide that accumulates in these etiolated seedlings as a free pigment acts as a potent photosensitizer and upon illumination is capable of generating the non-radical reactive oxygen species singlet oxygen that is known to cause extensive photooxidative damage [23,24]. The homozygous *flu* and *tig d* mutants of *Arabidopsis* and barley, respectively, can be rescued by germinating the seedlings under constant light. Under these growth conditions mutant plants mature and produce seeds just like wild-type plants and no obvious differences between mutant and wild type can be observed.

The striking similarities between the *flu* and the *tig d* mutant prompted us to investigate the possible relationship between the *flu* gene and the *tigrina d* locus. The *tig d* mutant of barley had been characterized extensively genetically, but as yet the identity of the *tigrina d* gene is not known. In contrast, the *flu* gene of *Arabidopsis* has recently been identified by map-based cloning and shown to encode a regulator of tetrapyrrole biosynthesis that apparently mediates its regulatory effect through interactions with enzymes involved in tetrapyrrole biosynthesis [25].

In a first step, the known sequence of the *FLU* cDNA of *Arabidopsis* was used to identify the homolog of *FLU* in rice (NCBI database, gi 16904672). A comparison of the *FLU* sequences of *Arabidopsis* and rice revealed a high degree of similarity between these two genes. Using this sequence information we searched for *FLU*-related ESTs within the publicly available collections of EST clones of barley. Several such EST clones were identified that enabled us to reconstruct the complete *FLU* cDNA sequence of barley. Primer sequences were deduced from this predicted cDNA sequence and were used to synthesize the complete barley-specific *FLU* cDNA. Total RNA was isolated from etiolated seedlings of wild-type barley and the *tig d* mutant. A single band of 963 bp could be amplified from both RNA samples (Fig. 2A). The identification could be confirmed by nested PCR using the cDNA of both wild type and the *tig d* mutant (Fig. 2B). In case the *FLU* gene of barley represents the *TIGRINA d* locus one would expect to find differences between the nucleotide sequences of the *FLU* cDNAs of wild type and the mutant that should lead to the inactivation of the FLU protein in the *tigrina d* mutant. Both cDNAs were sequenced. The wild-type cDNA encodes a protein that is highly homologous to the

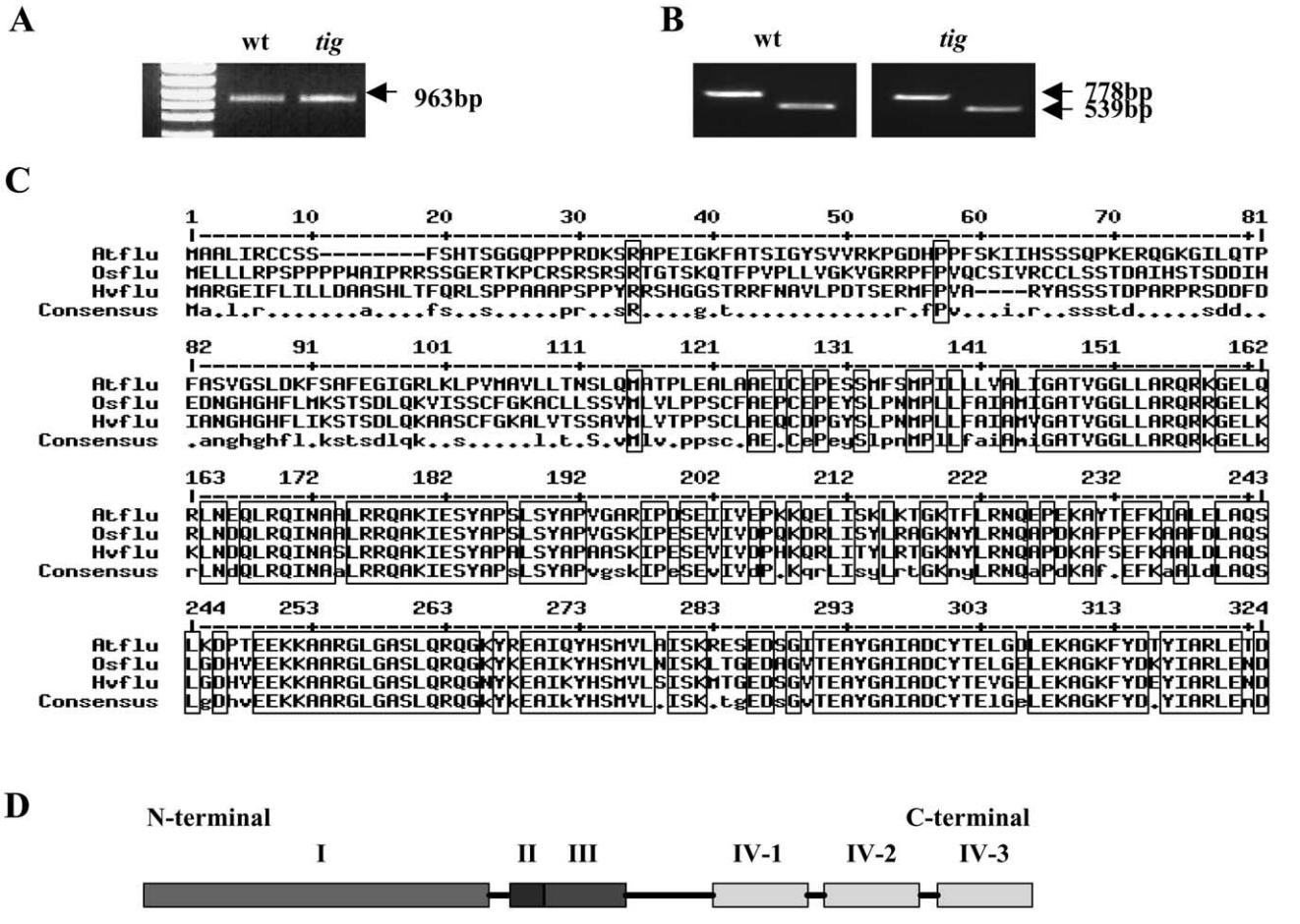

Fig. 2. Isolation and identification of the *FLU* gene of barley. A: The complete FLU cDNAs of barley wild type (wt) and the *tig d* mutant (*tig*) were amplified with gene-specific primers. B: The isolation of the FLU cDNAs was confirmed by nested PCRs using internal gene-specific reverse primers. C: A multiple alignment of the deduced amino acid sequences of the FLU proteins of *A. thaliana* (Atflu), rice (Osflu) and barley (Hvflu). D: A schematic presentation of the FLU protein of barley with four domains (I–IV): I, a putative chloroplast signal sequence; II, a hydrophobic region; III, a coiled coil motif; and IV, a TPR domain with three TPR motives.

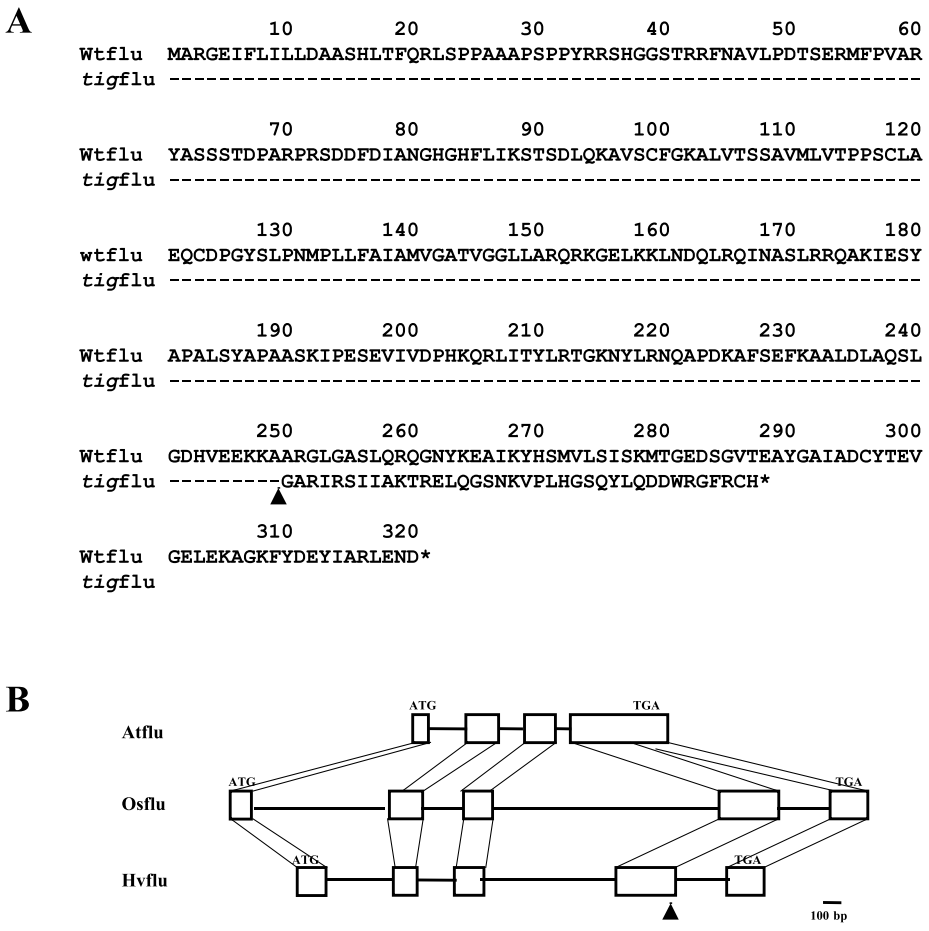

Fig. 3. The identification of the frame shift mutation in the *FLU* gene of the *tig d* mutant of barley. A: An alignment of the deduced amino acid sequences of the FLU proteins of wild type (Wtflu) and the *tig d* mutant (*tig*flu). The arrowhead marks the position of the frame shift mutation that leads to a change in amino acid sequence and erases the last two TPR repeats at the C-terminus of the FLU protein of the *tig d* mutant. B: A comparison of the gene structure of *FLU* of *A. thaliana* (Atflu), rice (Osflu) and barley (Hvflu). Open boxes show the regions encoding the open reading frame of FLU. Initiation and termination signals are indicated on the top of each gene. The arrowhead marks the position of the frame shift mutation at position +307 of the fourth exon of the barley gene.

FLU proteins of *A. thaliana* and rice (Fig. 2C). The predicted mature protein contains a hydrophobic region at its N-terminus that is followed by the hydrophilic C-terminal half with a coiled coil domain adjacent to the hydrophobic part and TPRs at the very end of the C-terminus (Fig. 2D). In the FLU protein of *Arabidopsis* only two TPR domains were found, whereas three TPR domains were predicted for the barley protein. Based on structural studies, the minimum number of TPR motives required for protein–protein interactions had previously been proposed to be three [25].

When the *FLU* cDNA sequence of wild type was compared with that of the *tig d* mutant, a difference in the nucleotide sequences could be depicted that leads to a frame shift mutation of the *FLU* cDNA of the *tig d* mutant (Fig. 3A). At position 747 of the *FLU* cDNA an additional C base was inserted. Through this mutation the C-terminal part of the predicted FLU protein containing the last two TPR domains has been erased in the *tig d* mutant (Fig. 3A).

The predicted change within the flu protein of the *tig d* mutant was confirmed by also sequencing the genomic DNAs of the *FLU* gene of wild type and the *tig d* mutant. The open reading frame of the *FLU* gene is interrupted by four introns (Fig. 3B). The *FLU* gene of barley encompasses a DNA stretch of 2541 bp. A comparison of the genomic sequences of wild type and the *tig d* mutant confirmed the frame shift mutation that affects the C-terminus of the FLU protein. The insertion of the additional C base could be found at position +307 of the fourth exon.

The similarity of phenotypes of the *flu* and *tig d* mutants and the fact that the *tig d* mutant carries a mutation within its *FLU* gene that would be expected to inactivate the FLU protein provide strong support to the notion that the *tig d* locus of barley resides within the *FLU* gene. If this suggestion is correct, the mutation within the *flu* gene of the *tig d* mutant and the *tigrina* phenotype should strictly cosegregate within an F1 population of a heterozygous *tig d* barley plant. Primer sequences were designed for DNA amplification that included the additional C base of the *flu* gene of the *tig d* mutant. PCR reactions were carried out with *flu*-specific primer pairs at different annealing temperatures, ranging from 56.6 to 61°C. With wild-type DNA as a template, stable PCR products could be detected only at temperatures below 59.3°C, whereas such PCR products could be amplified from genomic DNA of the *tig d* mutant at higher temperatures (Fig. 4A). The different stabilities of wild-type- and *tig d*-derived PCR products at 61°C could be exploited for the identification of the *flu* mutation in different descendants of a heterozygous *tig d* mutant (tig-F, Fig. 4B,C). As a control, also primer sequences were

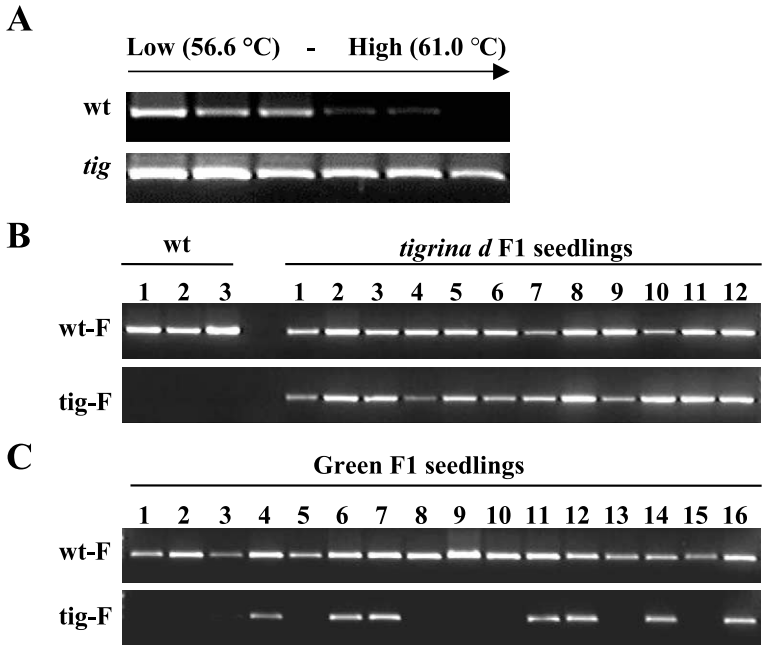

Fig. 4. Cosegregation analysis of the *tigrina* phenotype and the frame shift mutation of the *FLU* gene of the *tig d* mutant. A: A temperature gradient PCR was performed with total DNA of the *tig d* mutant (*tig*) and wild type (wt) at different annealing temperatures ranging from 56.6 to 61°C that revealed different thermal stabilities of hetero- and homoduplices of PCR products. For the PCR reactions primer sequences were used that included the additional C base of the *flu* gene of the *tig d* mutant. This difference in thermal stability could be exploited for tracing the mutated *flu* gene in different plants. B: Cosegregation of the *tigrina* phenotype and the *flu* mutation in 12 homozygous *tig d* mutants selected from a segregating F1 population of a heterozygous *tig d* mutant. For the PCR analysis of these plants two different primer sets were used, wt-F: 5′-TCA TGT TGA AGA GAA GAA GGC-3′ (21mer) that gave rise to PCR products from wild type and *tig d* DNA with identical thermal stabilities at 61°C, and tig-F: 5′-TCA TGT TGA AGA GAA GAA GGC C-3′ (22 mer) that gave rise to hetero- and homoduplices with different thermal stabilities at 61°C. Both primers were used together with identical reverse gene-specific primers. Etiolated homozygous *tig d* mutants were identified by measuring the Pchlide fluorescence of cut leaf tips of individual seedlings. Each of the *tig d* mutants contained the mutated *flu* gene, whereas in wild type controls none of the samples showed the *flu*-specific PCR product. C: 12 h after transferring the F1 seedlings from the dark to the light, DNA was extracted from 16 plants that greened and the presence or absence of the *flu* gene was analyzed by PCR at 61°C using the same *flu*-specific (tig-F) and non-specific (wt-F) primers as described above. Seven out of the 16 selected plants were *tig d*/*TIG d* heterozygotes.

used that lacked the additional C base of the mutated *flu* gene (wt-F, Fig. 4B,C). These primer sequences interacted equally well with genomic DNAs of the *tig d* mutant and wild type and no differences in the thermal stabilities of the resulting PCR products were observed. Within the segregating F1 population homozygous *tig d* mutants could easily be identified phenotypically by the red fluorescence of Pchlide that was emitted by etiolated seedlings exposed to blue light. Parts of the primary leaves of these seedlings were harvested and the DNA was extracted and used for a PCR analysis. The identification of the genotype of these plants was confirmed by transferring them from the dark to the light, where homozygous *tig d* plants would soon bleach and die, whereas heterozygous and wild-type seedlings would start to green. All of the selected homozygous F1 mutant plants contained the mutated form of the *FLU* gene (Fig. 4B). When the same PCR analysis was carried out with DNA from the F1 plants that greened in the light, heterozygous *tig d* plants could easily be distinguished from wild-type plants by the presence or absence of the *flu*-specific PCR product, respectively (Fig. 4C). When using the wild type-specific primer pairs, stable PCR products could be seen in all samples (Fig. 4B,C).

4. Discussion

In higher plants the metabolic flow of tetrapyrrole biosynthesis is regulated at the step of ALA formation [8]. Two different mechanisms have been considered to explain this regulation: light-induced changes in the synthesis of enzymes required for the formation of ALA [26,27] and the removal of an inhibitor affecting the activity of the enzymes [1,28]. One way to unravel the molecular mechanism underlying this regulation has been the isolation of mutants with a defect in the control of Chl biosynthesis. Such mutants were first described in barley [15]. Four different *tigrina* mutants were described that in the dark accumulate higher amounts of Pchlide. Only one of them, the *tig d* mutant, was considered to be affected directly in the control mechanism [17,19], whereas overaccumulation of Pchlide in the three other mutants was explained as a secondary result of mutations that only indirectly interfere with the regulation of tetrapyrrole biosynthesis [16,17]. In the *tig d* mutant the rate of ALA formation is three- to fourfold higher than in wild-type plants [19]. This increase in ALA synthesis could be due to higher steady state levels of enzymes that one could expect to occur in the mutant, if – as proposed previously [18] – the product of the wild-type *TIGRINA d* gene represses the transcription of genes involved in ALA formation. There are two enzymes committed exclusively to the synthesis of ALA, Glu-TR and glutamate-1-semialdehyde-2-1-aminotransferase (GSA-AT). The amounts of both enzymes did not increase in the *tig d* mutant and neither were the corresponding mRNA levels for these proteins upregulated [19]. Also, in the *flu* mutant of *Arabidopsis* that closely resembles the *tig d* mutant of barley the mRNA levels for

these two enzymes were not affected [20]. These results suggest that in both mutants deregulation of tetrapyrrole biosynthesis cannot be attributed to changes in the synthesis of enzymes required for ALA synthesis, but instead may reflect the disruption of an alternative control mechanism, direct metabolic feedback inhibition of tetrapyrrole biosynthesis.

This suggestion has been supported by our present finding that the *TIGRINA d* locus is identical with the *FLU* gene of barley. This identification was based on several lines of evidence. The phenotypes of the *flu* mutant of *Arabidopsis* and the *tigrina d* mutant of barley show striking similarities. Both mutants overaccumulate Pchlide in the dark and after transfer to the light suffer from severe photooxidative damage. Under continuous light, however, they behave like wild-type plants. The frame shift mutation within the *FLU* gene of the *tig d* mutant results in the loss of two TPR domains at the C-terminal end of the FLU protein that in *Arabidopsis* had previously been shown to be essential for the biological activity of the FLU protein [21]. This mutation within the *FLU* gene strictly cosegregates with the *tigrina* mutant phenotype.

In *Arabidopsis* four *flu* mutants had been initially identified that later were shown to represent four different allelic lines of the same *flu* locus [20]. Similarly, also in barley only one *tigrina* locus was found during an extensive mutant screen that affects directly the metabolic feedback control of tetrapyrrole biosynthesis [15,17]. Thus far, FLU is the only known protein involved in regulating Chl biosynthesis and appears to play a key role in suppressing Pchlide accumulation in the dark. Glu-TR represents a likely target of this control in vivo, but there may still be other proteins that interact with FLU. Identification of these proteins may be expected to further improve our knowledge of how FLU helps to coordinate and adjust the activities of the various branches of tetrapyrrole biosynthesis in higher plants.

Acknowledgements: We thank D. von Wettstein for providing seeds of the *tigrina* mutants of barley, A. Imboden for selecting and propagating homozygous *tig d* mutants, D. Rubli for photography and M. Geier-Bächtold for editorial work. This work was supported by grants from the Swiss National Science Foundation and the ETH (K.A.) and the Dongguk University (D.L.).

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