Interaction of FLU, a negative regulator of tetrapyrrole biosynthesis, with the glutamyl-tRNA reductase requires the tetratricopeptide repeat domain of FLU

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Abstract Regulation of tetrapyrrole biosynthesis in plants has been attributed to feedback control of glutamyl-tRNA reductase (GLU-TR) by heme. Recently, another negative regulator, the FLU protein, has been discovered that operates independently of heme. A truncated form of FLU that contains two domains implicated in protein–protein interaction was co-expressed in yeast with either GLU-TR or glutamate-1-semialdehyde-2-1-aminotransferase (GSA-AT), the second enzyme involved in δ-aminolevulinic acid (ALA) biosynthesis. FLU interacts strongly with GLU-TR but not with GSA-AT. Two variants of FLU that carry single amino acid exchanges within their coiled coil and tetratricopeptide repeat (TPR) domains, respectively, were also tested. Only the FLU variant with the mutated TPR motif lost the capacity to interact with GLU-TR.

Key words: Protochlorophyllide; FLU; Glutamyl-tRNA reductase; Glutamate-1-semialdehyde-2-1-amino transferase; *Arabidopsis*

1. Introduction

Photosynthetic organisms are prone to various forms of oxidative stress. Upon illumination, excited porphyrin molecules such as chlorophyll (Chl) may transfer the excitation energy directly to oxygen, thus leading to the formation of highly reactive singlet oxygen [1,2]. Most of the chlorophyll is bound to proteins and in this state may use various quenching mechanisms to dissipate absorbed light energy. Its biosynthetic precursors, however, occur in a free form and are potentially much more destructive when illuminated [3–5].

Angiosperms, the most highly evolved group of plants, use a very efficient strategy to prevent the accumulation of such intermediates by regulating the metabolic flow at the step of δ-aminolevulinic acid (ALA) synthesis [6,7]. 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 [8]. This regulation of Chl biosynthesis has been attributed to feedback control of ALA synthesis. In analogy to its regulatory role in animals and yeast [9,10], heme has been proposed to act also in plants as an effector of feedback inhibition of tetrapyrrole biosynthesis [7]. Several lines of evidence support this assumed function of heme. The activity of glutamyl-tRNA reductase (GLU-TR), the first enzyme committed to ALA synthesis and the most likely target of feedback control, has been shown to be inhibited in vitro by heme [11,12]. Inactivation of a heme oxygenase gene perturbs the breakdown of heme, attenuates the rate of ALA synthesis, and suppresses Pchlide accumulation in etiolated seedlings [13–15]. Conversely, removal of free $Fe^{2+,3+}$ by the addition of an iron chelator leads to a decline of the heme level and causes an increase in the level of Pchlide [16].

More recently, another negative regulator of tetrapyrrole biosynthesis has been discovered that seems to affect selectively the Mg^{2+} branch of tetrapyrrole biosynthesis. Mutants of *Arabidopsis* that carry a lesion in the *FLU* gene are no longer able to down-regulate ALA synthesis and overaccumulate Pchlide when grown in the dark, whereas their heme content does not differ from that of wild-type plants [17]. These results suggest that the FLU protein forms part of a control mechanism that operates separately from the heme-dependent regulation.

There are only two likely target enzymes to control the rate of ALA synthesis, GLU-TR and glutamate-1-semialdehyde-2-1-aminotransferase (GSA-AT) [18,19]. In *Arabidopsis* and in cucumber two GLU-TR genes have been described. *HEMA1* is regulated by light and expressed predominantly in leaves but also in other parts of the plant, while the expression of the second gene, *HEMA2*, has been found only in roots and flowers in a light-independent fashion [20–22]. In our current study we have analyzed only the *HEMA1* gene that has been implicated in the Chl and heme biosynthesis of photosynthetic tissues [20–22]. GLU-TR and GSA-AT are both present in the stroma fraction of chloroplasts, whereas FLU is firmly attached to chloroplast membranes [17]. Thus at present it is not known yet how FLU may control the activity of these soluble enzymes.

The presence of two different regions in the hydrophilic half of FLU that are implicated in protein–protein interactions suggests that FLU may exert its regulatory function through

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Abbreviations: ALA, δ-aminolevulinic acid; 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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physical contact with other proteins. In the present study we have analyzed the possible interaction of FLU with GLU-TR and GSA-AT using the yeast two-hybrid system. We could demonstrate that FLU interacts directly with the GLU-TR but not with GSA-AT and that this interaction requires a functional tetratricopeptide repeat (TPR) domain of FLU.

2. Materials and methods

2.1. Construction of fusion proteins

The *FLU* cDNA was amplified by polymerase chain reaction (PCR) from total cDNA, prepared from light-grown wild-type *Arabidopsis* seedlings. The *flu*1-1 and *flu*1-4 cDNAs were amplified by PCR from total cDNA, prepared from *flu*1-1 and *flu*1-4 *Arabidopsis* mutants, respectively. To amplify the *FLU*, *flu*1-1, and *flu*1-4 cDNAs, the following primers were used: 5′-GGAATTCACCGTTGGAGGGTTG-CTTGCTCGG-3′; 5′-TCCCCCGGGTCAGTCAGTCTCTAACCG-AGCAATG-3′; 5′-GGAATTCACCGTTGGAGGGTTG-CTTGTT-CGC-3′. The three cDNAs were first cloned into *Eco*RI/*Sma*I-digested Bluescript® IISK$^{+/-}$ vector, sequenced and then subcloned into *Eco*RI/*Sma*I-digested yeast vectors pGBKT7 and pGADT7 (Clontech, Basel, Switzerland).

The *GLU-TR* (*HEMA1*) and *GSA-AT* (*GSA1*) cDNAs were amplified by PCR from corresponding cDNA clones [23]. The *GLU-TR* cDNA was amplified with primers 5′-GGAATTCGCCATGGCGTC-TAATGCAGCTAGCATCTCTGCTC-3′ and 5′-GGAATTCTTAC-TTATGTTGTTGTTCCGCCATTGC-3′, cloned into *Eco*RI-digested Bluescript® IISK$^{+/-}$ vector, sequenced and subcloned into *Eco*RI-digested vectors pGBKT7 and pGADT7. The *GSA-AT* cDNA was amplified with primers 5′-TCCCCCGGGTGCC-ATGGCTGTTTCC-GTCGACGAGAAGAAG-3′ and 5′-CGGGAT-CCCTAGATCCTA-CTCAGTACCCTCTCAG-3′, cloned into *Sma*I/*Bam*HI-digested Bluescript® IISK$^{+/-}$ vector, sequenced and subcloned into *Sma*I/*Bam*HI-digested vectors pGBKT7 and pGADT7.

2.2. Yeast two-hybrid analysis

For our experiments, the Matchmaker GAL4 two-hybrid system 3 of Clontech was used. The constructs in vectors pGBKT7 and pGADT7 were co-transformed into yeast strain AH109 (*MATa*, *trp*1-901, *leu*2-3,112, *ura*3-52, *his*3-200, *gal4*, *gal80*, *LYS2*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3*, *MEL1*, *GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2*, *URA3*::*MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ*) and yeast transformants were selected according to the Clontech protocol.

3. Results

The hydrophilic half of FLU contains two different regions implicated in protein–protein interactions: A TPR domain, which consists of two TPR motifs and occupies the C-terminal part, and a short coiled coil domain that is adjacent to the hydrophobic membrane anchor of FLU (Fig. 1a). Both domains seem to be functionally important since the replacement of an alanine at a highly conserved position of the first predicted TPR motif by valine and a similar amino acid exchange within the coiled coil domain leads to the inactivation of FLU in the allelic *flu*1-1 and *flu*1-4 mutants of *Arabidopsis* [17]. We used the yeast two-hybrid system to test the possible role of the two domains during physical interaction of FLU with target proteins. A truncated version of FLU, which ranges from amino acid position 140 in the hydrophobic region to the very end of the protein, was fused in frame with the GAL4 DNA binding domain (BD; vector pGBKT7) and the GAL4 activation domain (AD; vector pGADT7)(Fig. 1b). This truncated version of FLU contains all highly conserved amino acid residues of the hydrophilic part that were identified by comparing the FLU sequence of *Arabidopsis* with those of rice (NCBI database, gi16904672) and *Chlamydomonas reinhardii* (J.D. Rochaix, personal communication). The two enzymes,

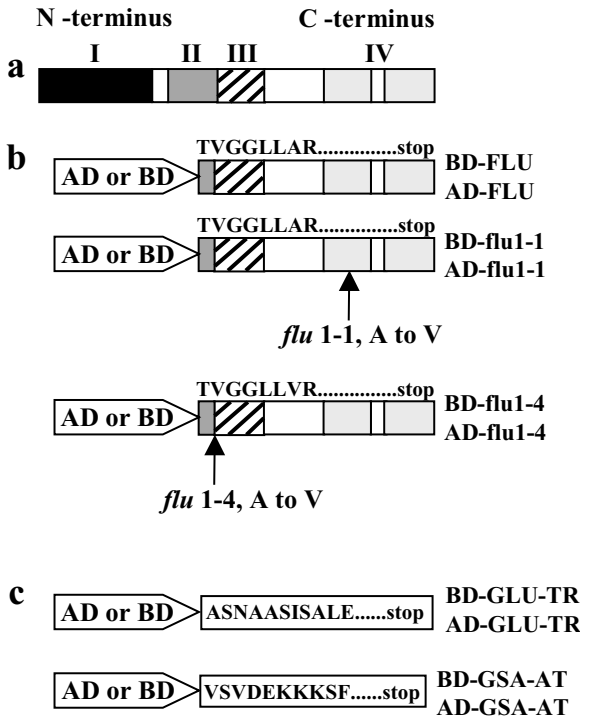

Fig. 1. a: The domain architecture of FLU. I, chloroplast transit peptide; II, hydrophobic region; III, coiled coil domain; IV, TPR domain with two predicted TPR motifs. b,c: Constructs for yeast two-hybrid analysis. The cDNAs encoding FLU, flu1-1, flu1-4, GLU-TR, and GSA-AT were cloned into yeast vectors pGADT7 (fusions with GAL4 AD) and pGBKT7 (fusions with GAL4 BD). Since all five proteins lacked their N-terminal parts, amino acids from which the corresponding protein begins in our constructs are indicated.

GLU-TR (HEMA1) and GSA-AT (GSA1), were tested for their ability to interact with FLU. Truncated enzymes, which lack their chloroplast transit peptides, were fused in-frame with BD and AD (vectors pGBKT7 and pGADT7) (Fig. 1c). BD-FLU and AD-FLU were co-expressed with AD-GLU-TR and BD-GLU-TR, respectively, as well as with AD-GSA-AT and BD-GSA-AT, in yeast. FLU interacted strongly with GLU-TR but not with GSA-AT (Fig. 2). This interaction occurred regardless of whether FLU was expressed as a fusion with the binding domain or as a fusion with the activation domain (Fig. 2). Under the same conditions FLU did not interact with itself (Fig. 2).

In order to test the physiological significance of the interaction between FLU and GLU-TR and to determine which if any of the two protein–protein interaction domains of FLU was required for recognizing the target protein, the two mutated variants of FLU were used. The flu1-1 and flu1-4 proteins carry amino acid substitutions in the first TPR motif and in the coiled coil domain, respectively (Fig. 1b). BD-flu1-1, AD-flu1-1, BD-flu1-4, and AD-flu1-4 fusions were constructed (Fig. 1b). When AD-GLU-TR and BD-flu1-4 or BD-GLU-TR and AD-flu1-4 constructs were co-expressed in yeast, the same strong interaction occurred as between GLU-TR and the wild-type FLU protein (Fig. 2). However, the flu1-1 protein with a single amino acid exchange in the TPR domain no longer interacted with the GLU-TR regardless of whether AD-flu1-1 or BD-flu1-1 was used for co-expression with the corresponding construct of GLU-TR (Fig. 2).

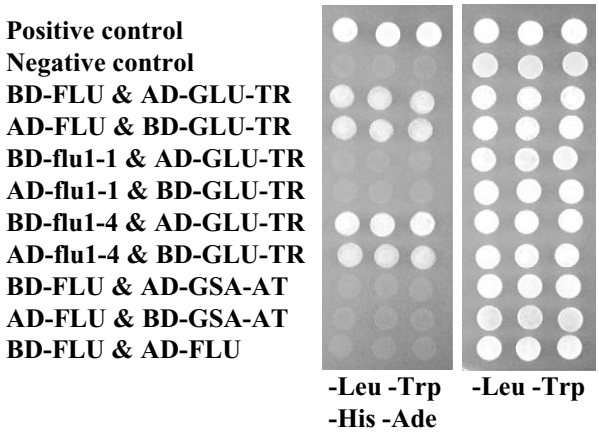

Fig. 2. Yeast two-hybrid results. The indicated construct pairs were co-transformed into yeast strain AH109 and the yeast transformants were selected on medium lacking leucine and tryptophan. On this medium, only those cells that carry both pGBKT7 and pGADT7 vectors can grow (pGBKT7 and pGADT7 carry *TRP1* and *LEU2* selectable marker genes, respectively, that complement *trp* and *leu* mutations of AH109); however, there is no selection for interaction between the test proteins. For each pair of constructs, several clones of transformants were resuspended in water and plated as spots on medium lacking leucine and tryptophan (-Leu -Trp), as well as on medium lacking leucine, tryptophan, histidine, and adenine (-Leu -Trp -His -Ade). On the latter medium only those yeast cells can grow in which the transcription of two marker genes for interaction, *HIS3* under GAL4-inducible *GAL1* promoter and *ADE2* under GAL4-inducible *GAL2* promoter, is activated. Such transcriptional activation of the marker genes occurs if the test proteins interact. Only transformants that expressed the protein pairs FLU/GLU-TR and flu1-4/GLU-TR, as well as the positive control, could grow on the -Leu -Trp -His -Ade medium. For positive control, the plasmids pGADT7-T and pGBKT7-53 (Clontech) were co-transformed, for negative control the plasmids pGADT7-T and pGBKT7-Lam (Clontech) were co-transformed.

4. Discussion

In higher plants the metabolic flow of tetrapyrrole biosynthesis is regulated at the step of ALA synthesis. Metabolic feedback inhibition is often exerted on the first enzyme of a biosynthetic route by the final product of this pathway. Among the three enzymes involved in ALA formation from glutamate, GLU-TR catalyzes the first committed step of the tetrapyrrole biosynthetic pathway and thus appears to be the primary target of feedback control [18,19]. Consistent with this proposal the two final products of the Mg^{2+} and Fe^{2+} branches of tetrapyrrole biosynthesis in dark-grown seedlings, Pchlide and heme, respectively, were implicated in metabolic feedback control [7]. However, in subsequent studies several lines of evidence indicated that only heme seemed to be directly involved in the control of tetrapyrrole biosynthesis. Firstly, GLU-TR was inhibited in vitro by heme [11,12]. Different binding sites of the GLU-TR for heme were discussed [12,24]. A truncated form of the enzyme that lacked the 30 N-terminal amino acids maintained the same specific activity as the untruncated enzyme but was no longer inhibited by heme [12]. In subsequent work, the highly conserved histidine at position 99 of the barley enzyme was implicated in heme binding [24]. This residue seems also to be essential for catalytic activity [25]. Secondly, in mutants such as *hy*1 of *Arabidopsis* and *aurea* of tomato, which seem to overaccumulate free heme due to an inactive heme oxygenase gene, ALA synthesis is down-regulated and Pchlide accumulation is suppressed in the dark [15,26], whereas a reduction of the heme level following the addition of an iron chelator in wild-type plants caused an increase in the level of Pchlide [16]. Based on these and other studies the proposed regulator role of Pchlide was abandoned and feedback control of tetrapyrrole biosynthesis was attributed to heme [18,19]. Only recently has this assumed exclusive role of heme been questioned by the discovery of a novel negative regulator of tetrapyrrole biosynthesis, the FLU protein [17]. Inactivation of this protein in the *flu* mutant led to an up-regulation of ALA synthesis and the overaccumulation of Pchlide in dark-grown plants. If FLU would be part of the heme-dependent feedback loop its inactivation should not only enhance the level of Pchlide but also the level of free heme. However, in etiolated *flu* mutants only the level of Pchlide but not that of heme was higher than in wild-type controls [17]. Furthermore, in homozygous *flu*/*hy*1 double mutants of *Arabidopsis* the rate of ALA synthesis and the level of Pchlide was lower than in the *flu* mutant, but significantly higher than in the *hy*1 mutant. These intermediate Pchlide levels and rates of ALA synthesis in the double mutant demonstrate that the *flu* mutation may in part antagonize the inhibitory effect of the *hy*1 mutation and that FLU seems to act separately from the heme-dependent feedback control (D. Goslings et al., unpublished results to be submitted elsewhere). It is not known yet, how FLU mediates the down-regulation of metabolic flow through tetrapyrrole intermediates and which of the enzymes involved in ALA synthesis may be the target of this feedback inhibition.

Pchlide would be an attractive candidate for a tetrapyrrole that operates within the second metabolic feedback control circuit. Pchlide is the final product of chlorophyll biosynthesis in dark-grown seedlings and it forms part of a photoactive ternary complex in plastids together with POR and NADPH [8]. It is localized in the hydrophobic environment of prolamellar bodies, plastid envelopes and thylakoid membranes [27,28] and thus its direct interaction with GLU-TR and GSA-AT within the hydrophilic stroma may not be feasible. The FLU protein could be necessary to bridge the gap between the membrane and the stroma and to facilitate the interaction between a tetrapyrrole intermediate that acts as an effector of feedback inhibition and hydrophilic target enzymes. Both GLU-TR and GSA-AT are dimeric enzymes [29,30] that have been proposed to form a ternary substrate enzyme complex, consisting of $tRNA^{Glu}$, GLU-TR and GSA-AT [29]. Our current work indicates that within such a complex GLU-TR might be the target of FLU-dependent feedback control. The hydrophilic half of FLU contains a predicted coiled coil domain and TPR domain both of which have been implicated in protein–protein interactions [31,32]. This truncated form of the FLU protein was expressed in yeast and its interaction with either GSA-AT or GLU-TR was tested using the yeast two-hybrid system. A strong interaction was observed only between GLU-TR and the FLU protein.

The physiological significance of this interaction was tested by using two mutated forms of FLU. Both mutations had been shown previously to disrupt the physiological activity of the FLU protein [17]. The interaction of FLU with the GLU-TR no longer occurred after replacing the highly conserved alanine residue at position 20 of the first TPR motif of FLU by valine. A similar amino acid exchange within the

coiled coil domain, however, did not have any apparent impact on the physical interaction between the two proteins, as revealed by the yeast two-hybrid assay. Thus, physical interaction between FLU and GLU-TR seems to require the intact TPR domain of the FLU protein. TPR-containing proteins are known to be able to interact with other proteins that do not carry this motif [32]. An interaction between TPR and coiled coil-containing proteins has also been described [33]. In GLU-TR a coiled coil motif has been predicted within the C-terminal end of the enzyme that seems to be unique to higher plants [23]. The physiological relevance of this motif and its possible role for protein–protein interactions is not known yet.

Our present finding opens a new way towards defining more precisely the region of GLU-TR that is involved in interacting with FLU. A final proof for the biological relevance of such an interaction, however, may come only from in planta studies. Such an analysis using the FRET method [34] has been initiated in our laboratory.

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