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Characterization of the Ferredoxin-Gogat gene (OsGog2 clone) expression in rice

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Abstract

Ferredoxin-dependent glutamate synthase (Fd-Gogat; EC 1.4.7.1) in leaf and root plastids is the last enzyme involved in the pathway of nitrate assimilation in higher plants. *Arabidopsis thaliana* expresses two different genes: the first, light regulated, specific of green tissues and the second expressed in other tissues. In this work, we investigated whether in our clone, OsGog2 AC Y12595, this gene is up-regulated by light or it is expressed under darkness. Fd-Gogat specific activity, protein and mRNA increased after light treatment in rice shoots. In roots, the activity and the protein content remained constant, whereas the mRNA is repressed by light treatment. The results obtained using a specific probe, situated in the 3' untranslated region of the *OsGog2* cDNA, indicated that *OsGog2* gene is up-regulated by light and that its expression is tissue specific and suggested that a dark expressed Fd-Gogat gene could be present in rice similarly as in *Arabidopsis*.

Additional key words: light regulation, nitrate assimilation, *Oryza sativa*, tissue specificity.

Introduction

In higher plants inorganic nitrogen, in the form of ammonia, is assimilated via the glutamate synthase cycle or GS-Gogat pathway. This assimilation requires cofactors, reducing equivalents and carbon skeletons generated during photosynthesis. Thus, the assimilation of inorganic nitrogen occurs predominantly in leaf chloroplasts where these components are readily available; however plants are able to transport photosynthates to roots where nitrogen assimilation also occur (Oaks 1992).

In plant cells, the glutamate synthase enzyme exists in two distinct isoforms according to the electron donor: ferredoxin-dependent (Fd-Gogat, EC 1.4.7.1) and NADH-dependent (NADH-Gogat, EC 1.4.1.14). Fd-Gogat is the major isoform located in the chloroplast (Suzuki *et al.* 2001) whereas NADH-Gogat is also plastidial, but active mainly in non-photosynthetic tissues (Coschigano *et al.* 1998). Although the Fd-Gogat enzyme uses ferredoxin as a cofactor, it is found in etiolated tissues and root plastids. In *Arabidopsis thaliana*, it has been demonstrated that the Fd-dependent enzyme is the predominant form of glutamate synthase, accounting for up to 96 % of the total Gogat activity in leaves and 68 % in roots (Somerville and Ogren 1980, Suzuki and Rothstein 1997). In particular, in *Arabidopsis*, two expressed genes for Fd-Gogat are present with a different pattern of expression: one predominantly expressed in light induced leaves and the other constitutively expressed and involved in primary nitrogen assimilation in roots (Coschigano *et al.* 1998). The situation is different in maize where the Fd-enzyme seems to be encoded by a single gene (Sakakibara *et al.* 1991). Even in anaerobic rice coleoptile, a non-green tissue, Fd-Gogat represents the most active isoform since no activity of NADH-Gogat has been detected (Mattana *et al.* 1993, 1996). The presence of Fd-Gogat in roots and dark grown tissues is supported by the finding of novel forms of ferredoxin and ferredoxin-$NADP^+$reductase in roots (Morigasaki *et al.* 1990, Bowsher *et al.* 1992, Mattana *et al.* 1997). Moreover, it has been reported by Suzuki *et al.* (1982),

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Abbreviations: Fd - ferredoxin; GS - glutamine synthetase; Gogat - glutamate synthase.

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that the Fd-Gogat in root tissue is immunologically distinct from the leaf enzyme, suggesting the presence of two different proteins.

In this work, we analysed the expression of the *OsGog2* gene, a Fd-Gogat gene previously isolated in our laboratory (accession No. Y12595) in different organs of germinating rice seedlings at the mRNA, protein and enzyme activity levels, using a barley antiserum recognizing rice Fd-Gogat and the *OsGog2* cDNA.

Materials and methods

Plants and growth conditions: Seeds of rice (*Oryza sativa* L. cv. Arborio, Ente Nazionale Risi, Castel D'Agogna, Italy) were sterilized and germinated at 30 °C for 3 d in the dark as previously reported by Reggiani *et al*. (1993). For the light treatments, the 3-d-old seedlings were grown under continuous light for 3, 24 or 48 h with a photon flux density at the plant level of 270 µmol m^{-2} s^{-1}. Control seedlings were maintained in the dark for the same lengths of time.

Preparation of extract, assay of enzyme activity and Western blot analysis: The rice coleoptiles, roots and leaves were harvested, quickly frozen in liquid nitrogen and kept at -70 °C until used. The preparation of extracts and the Gogat assay were performed as previously described by Mattana *et al*. (1996). Methyl viologen (MV) was used as electron donor for the enzyme reaction and glutamate formed was estimated by HPLC by the method of Unnithan *et al*. (1984).

For immunoblot analysis, 30 µg of total protein extracted from each sample were separated by SDS-PAGE [8 % (m/v)] according to Laemmli (1970). The blotting was performed as previously described by Mattana *et al*. (1994). The antiserum raised against barley Fd-Gogat were used diluted 1:2000. The reacted polypeptides were visualized by the *ECL* Western-blot system (*Amersham Italia*, Milano, Italy) using as the second antiserum the peroxidase labelled anti-mouse antibody diluted 1:4000.

Northern blot analysis: Total RNAs were isolated as previously described by Mattana *et al*. (1994). Fifteen micrograms of total RNA were separated on denaturing agarose gel (1.5 % m/v) and blotted to a *Hybond-N^+* membrane (*Amersham*) according to Sambrook *et al*. (1989). For the experiments reported in Fig. 2 the hybridisation was performed using the 4120 bp cDNA clone (OsGog2) obtained from rice coleoptiles in our laboratory. For the experiment described in Fig. 3 a specific probe placed in the 3' untranslated region was used; it consisted of a 450 bp fragment, obtained from the above cDNA digested with ScaI and SphI. The probes were labelled with the fluorescein-11-dUTP using the Gene images random prime labelling module supplied by *Amersham Biosciences*, following the manufacturer instructions. The detection relies on an anti-fluorescein antibody conjugated to alkaline phosphatase which reaction produces a chemiluminescent signal captured on film (*Amersham Italia*, Milano, Italy). For BLAST analysis the *BLASTN 2.2.4* program was used (Altshul *et al*. 1997).

Southern blot analysis: Rice genomic DNA was extracted using the *Plant DNazol* reagent (*Invitrogen Life Technologies*, Milano, Italy), following the manufacturer instructions. Genomic DNA (15 µg) was digested with the indicated restriction enzymes and fractionated in a 0.8 % (m/v) agarose gel. The DNA was then transferred overnight by capillary to *Hybond-N^+* membrane (*Amersham Italia*). The membranes were hybri-dised with two different probes: a 340 bp *BamHI-HhaI* restricted OsGog2 generic probe (named probe 1) and a specific probe at the 3' untranslated region of 450 bp (ScaI-SphI-restricted OsGog2; named probe2). The labelling and the detection system was the *Gene Images* module supplied by *Amersham*. The blots were done in duplicate and washed under high (0.2 × SSC) and low stringency (2 × SSC) conditions. For BLAST analysis the *BLASTN 2.2.4* program was used.

Results

Regulation of Fd-Gogat activity and protein in germinating rice seedlings by light: During the first 3 d of dark germination the shoot of the rice seedling consists of a white coleoptile containing the first etiolated leaf. After 24 h of light treatment the coleoptile opens and dies and the leaf becomes green. At the 4th day of dark germination the coleoptile remains closed and etiolated. Therefore, the tissues analysed after 24 h in the dark (coleoptile) or 24 h under light (leaf) are different. After 48 h in the dark both leaf and coleoptile are present and alive, so we examined the two etiolated tissues. All the experiments were performed at least in double. Considering the tissues after 3 d of dark germination, the root enzyme activity, expressed on tissue basis, was about the 25 - 30 % of that measured in shoots (data not shown); while, due to the lower amount of soluble root

Table 1. Fd-Gogat specific activity measured in rice coleoptile, leaf and root extracts. The control corresponds to the seedlings germinated in the dark for three days; the 3, 24 and 48 h is time of treatment (light or dark). Means of three independent experiments ± SD.

Time [h]	Coleoptile light	dark	Leaf light	dark	Root light	dark
Control	-	169.59 ± 1.77	-	-	-	182.27 ± 1.53
3	164.13 ± 1.36	168.45 ± 0.81	-	-	178.81 ± 1.17	179.71 ± 1.34
24	-	168.98 ± 1.90	311.69 ± 0.94	-	182.08 ± 0.98	179.99 ± 1.08
48	-	163.59 ± 0.85	358.15 ± 1.65	166.58 ± 1.22	191.01 ± 0.87	184.26 ± 1.62

protein, the specific activity (expressed per mg of protein) was very similar in the two tissues (Table 1). The shoot and root Fd-Gogat specific activities were fairly constant during the germination in the dark (Table 1).

After treating the 3-d-old seedlings for 24 h under continuous light, the leaves specific activity showed an increase of 100 % with respect to the control (3-d-old coleoptiles). A further slight increase (about 15 %) was observed after 48 h of continuous light, indicating that the maximal induction was at 24 h. A similar trend for the activity was observed on a tissue basis (data not shown). Fd-Gogat specific activities found in extracts from 48 h dark grown leaves and coleoptiles are very closed. As far as the root activity is concerned, no light effect was observed both on tissue basis or as specific activity. The slight increase (about 10 %) found in roots specific activity after 48 h of light treatment was due to the decrease in total protein content.

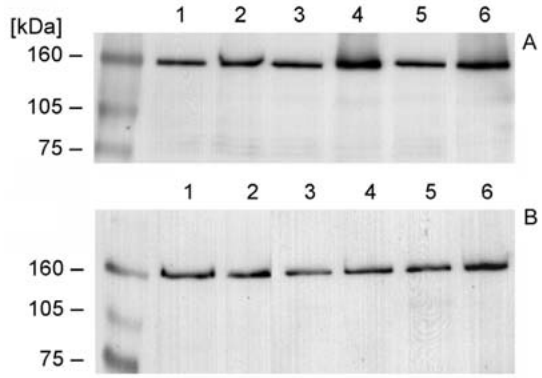

Fig. 1. Western-blot analysis of Fd-Gogat enzyme. Protein molecular mass markers are indicated on the left. *A* - Protein blot from coleoptiles and leaves. *Lane 1* is the control, corresponding to the coleoptiles extracted after 3 d of dark germination. The seedlings were then transferred for 3, 24, or 48 h to either continuous light (*lanes 2, 4, 6*) or for 24 or 48 h to continuous dark (*lanes 3, 5*). *B* - Protein blot from roots. Each lane corresponds to the same dark/light treatment described for the shoots.

The changes observed in enzyme activity were reflected by similar changes at the protein content, as determined by Western blot (Fig. 1*A*). The increase in Fd-Gogat protein reached the maximum in the leaf tissue after 24 h of continuous light. In the root tissue, the content of the enzyme seemed to be fairly constant and independent on light treatment.

Effect of light on *OsGog2* mRNA content: In an attempt to understand how light influences the expression of Fd-Gogat in rice roots and shoots, we examined the content of mRNA in the 3-d-old seedlings using the 4120 specific cDNA probe (OsGog2 clone) isolated in our laboratory. In the Northern blot a single band of the expected size (5.3 kb) was detected in each lane and its intensity increased already after 3 h of light, reaching a peak after 24 h, whereas the intensity of the band remained constant for the dark grown tissues (Fig. 2*A*). We also examined the expression of Fd-Gogat mRNA in the root tissue subjected to the same light treatments (Fig. 2*B*). The *Fd-Gogat* gene was expressed in the dark,

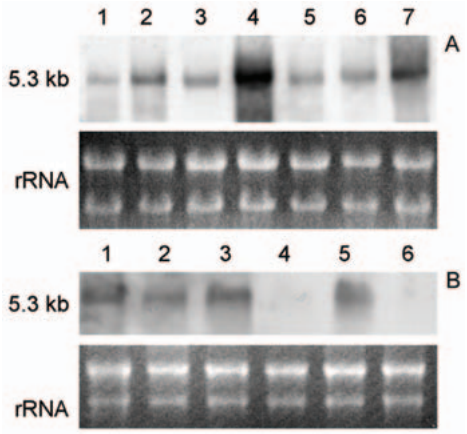

Fig. 2. Fd-Gogat mRNA accumulation in rice coleoptiles, leaves and roots after dark and light treatments. *A* - mRNA was extracted from coleoptiles of 3-d dark grown seedlings (*lane 1*). Then the plants were transferred to continuous light for 3, 24, or 48 h (*lanes 2, 4, 7*), or maintained in the dark for 24 h (*lane 3*) or 48 h (*lanes 5, 6*). *Lanes 1, 2, 3* and *5* correspond to coleoptile RNA; *lanes 4, 6* and *7* correspond to leaves RNA. The probe used was the 4.120 bp cDNA OsGog2. rRNAs are shown by ethidium bromide staining. *B* - Analogous Northern-blot was performed using the mRNA from roots tissue. *Lane 1* represents the mRNA from roots of 3 d dark grown plants; *lanes 2, 4* and *6* light treated roots for 3, 24 and 48 h; *lanes 3* and *5* dark grown roots for 24 and 48 h. rRNAs are shown by ethidium bromide staining.

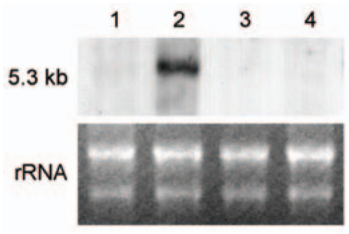

Fig. 3. Analysis of Fd-Gogat transcript from leaves and roots after 24 h treatment of continuous light or dark, using a specific probe at the 3' untranslated region of OsGog2. *Lanes 1* and *2* represent the mRNA from 24 h dark and light treated leaves, respectively; *lanes 3* and *4* represent the mRNA from 24 h dark and light roots, respectively. 15 µg of total RNA were loaded in each lane. rRNAs are shown by ethidium bromide staining.

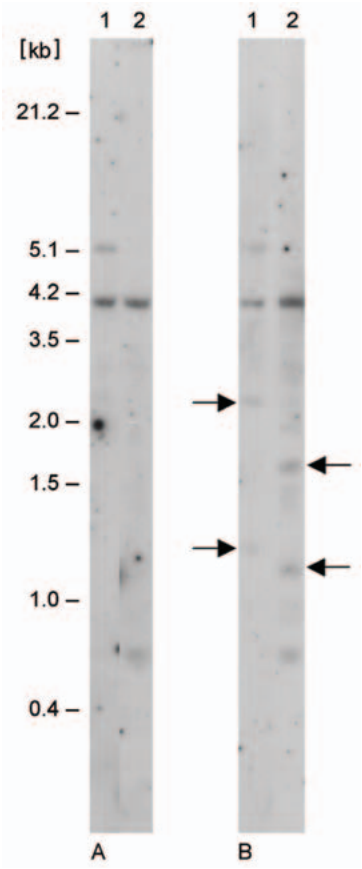

Fig. 4. Genomic Southern-blot analysis of Fd-Gogat. 15 µg of rice genomic DNA were digested with ScaI (*first lane*), ScaI and SphI (*second lane*). The two filters were hybridised with the 340 bp BamHI-HhaI restricted OsGog2 generic probe (probe 1). Molecular size markers are indicated on the left. *A* - The blot was washed under high stringency conditions: 0.2X SSC. *B* - The blot was washed under low stringency conditions: 2X SSC. *Arrows* indicates the bands present only at low stringency washes.

but its mRNA was nearly undetectable in light treated seedlings for 24 and 48 h. Considering that the presence of two *Fd-Gogat* genes differently regulated by light has

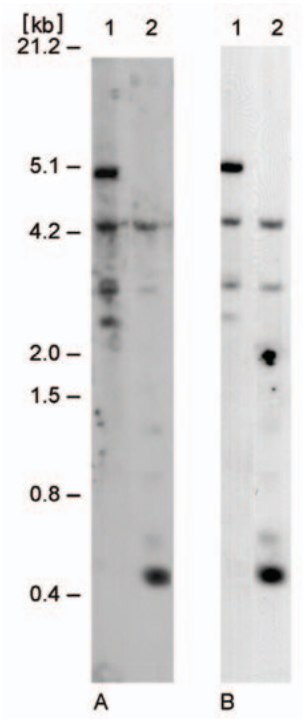

Fig. 5. Genomic Southern-blot analysis of Fd-Gogat. 15 µg of rice genomic DNA were digested with ScaI (*lane 1*), ScaI and SphI (*lane 2*). The two filters were hybridised with the 450 bp ScaI-SpHI restricted OsGog2 specific probe (probe 2). Molecular size markers are indicated on the left. *A* - The blot was washed under high stringency conditions: 0.2X SSC. *B* - The blot was washed under low stringency conditions: 2X SSC.

been reported for *Arabidopsis thaliana* and that these genes differ in the 5' and 3' untranslated regions (Coschigano *et al*. 1998), we prepared a specific probe at the 3' untranslated region to verify if this increased expression actually corresponded to an increase in the *OsGog2*. The hybridisation was performed with shoots and roots mRNA extracted from dark grown and 24 h light treated plants. The hybridisation occurred with the mRNA from 24 h light-treated leaves only (Fig. 3). The comparison between the Northern blot (Fig. 2; *Fd-Gogat* generic probe) and that of Fig. 3 (*OsGog2* specific probe) indicates that the gene isolated in our laboratory (*OsGog2*) is tissue specific and induced by light.

Genomic analysis of rice *Fd-Gogat* gene: In the case of the generic probe the pattern of hybridization depended on the washing conditions: at high stringency a simple pattern was evident, suggesting that *OsGog2* is a single copy gene, whereas at low stringency some new bands appeared, putatively corresponding to the other(s) *Fd-Gogat* gene(s) (Fig. 4*A*,*B*). The results obtained with the probe 2 were independent of the washing conditions, as expected for the specific hybridisation with *OsGoG2* (Fig. 5*A*,*B*).

Discussion

Recently, we isolated an incomplete cDNA coding for Fd-Gogat from rice coleoptiles. It has been reported that *Arabidopsis* expresses two Fd-Gogat genes differentially regulated by light (Coschigano *et al*. 1998). Uptill now single genes for Fd-Gogat have been reported for plant species with larger genomes such as maize, tobacco, barley, spinach and Scots pine (Sakakibara *et al*. 1991, Zehnacker *et al*. 1992, Avila *et al*. 1993, Nalbantoglu *et al*. 1994, Garcia-Gutierrez *et al*. 1995). However, Coschigano *et al*. (1998) suggested that other species could contain a second gene for Fd-Gogat that may have been missed in other cDNA screens because of its low expression level (Coschigano *et al*. 1998). Even though in rice the only registered cDNA for Fd-Gogat is *OsGog2*, it has been found that the protein from root is immunologically distinct from the leaf enzyme (Suzuki *et al*. 1982).

To better characterise our cDNA clone we monitored the level of Fd-Gogat enzyme activity and protein in rice root and shoot grown in the dark or irradiated for 3, 24, and 48 h. The activity increased in shoots after 24 an 48 h of light treatment, whereas no increase was observed in roots. The slight increase in specific activity observed in the light-treated roots (after 48 h) was mainly due to the decrease in the amount of total protein in this tissue when exposed to light for so long. Actually, this increase was not found when expressing the activity on a tissue basis. The changes in enzyme activity were reflected by a similar trend at the protein level as determined by Western blot analysis (Fig. 1). This kind of regulation by light is in agreement with that reported in literature for Fd-Gogat extracted from leaf of other species such as barley and maize (Pajuelo *et al*. 1997, Suzuki *et al*. 2001).

The influence of light on mRNA content correlated very well with the results concerning protein content and enzymatic activity. In fact the mRNA in shoots was induced after 3 h of light treatment reaching a peak after 24 h (Fig. 2*A*). This increase in mRNA expression is due to the light treatment and not to the different tissues examined; in fact the level found in the dark grown coleoptiles and leaves were very closed (Fig. 2*A*). On the contrary, in roots after 24 and 48 h of light treatment the mRNA was almost undetectable (Fig. 2*B*). The lack of signal observed in light treated roots mRNA hybridised with the generic probe (4120 bp *OsGog2*), may be due to a tissue specific light mediated repression and/or degradation of the *Fd-Gogat* gene mRNA. However Coschigano *et al*. (1998), reported that the *Glu2* mRNA was present in *Arabidopsis* roots of 25-d-old plants switched for 48 h to either continuous darkness or light. This discrepancy may depend on the difference of plant material used for the experiments or on a different regulation by light of the mRNA expression in the two analysed species. We do not know whether this difference is due to a general mechanism acting on the total mRNA population or on the *Glu2* specific mRNA. Based on the reported high homology of the coding region between the two *Arabidopsis Fd-Gogat* genes and on their difference at the 3' and 5' untranslated region, we used the *OsGog2 3'* untranslated region as specific probe. The results obtained from this experiment showed that the hybridisation was tissue specific occurring with the mRNA from leaves only (Fig. 3). A pale band was present in 24 h dark treated leaves and a stronger one in 24 h light-treated leaves (Fig. 3). This observation is in agreement with the pattern of expression of the *Glu1* mRNA described by Coschigano *et al*. (1998). They showed that the *Glu1* mRNA was absent in both *Arabidopsis* treated and untreated roots whereas in etiolated seedlings and plants its expression, absent in the dark, was light induced. So the specific hybridisation we found (Fig. 3), confirms the light inducibility of our *Fd-Gogat* gene and its tissue specific expression. In fact the mRNA is detectable in green tissue and nearly undetectable in roots.

Moreover these results suggest the presence of two rice *Fd-Gogat* genes as in *Arabidopsis*: one corresponding to *OsGog2* (light induced) and the other homologous but not identical (dark expressed). Data obtained by Southern analyses performed with the generic and the specific probe (Figs. 4 and 5), agree with the hypothesis that also in rice two *Fd-Gogat* genes are present.

After a search in the *Oryza sativa* gene bank we found only a cDNA clone (AK068130) identical to our sequence and a putative *Fd-Gogat* mRNA (NM_185989) derived from an annotated genomic sequence. This last putative mRNA showed some differences with our clone probably due to the inexact predicted splicing with respect to the one occurring *in vivo*; whereas the homology between our clone and the corresponding genomic DNA resulted in a 100 % identity.

However, we found a rice EST sequence which similarity to *OsGog2* dropped after 97 bp of the 3' untranslated region and it might correspond to another Fd-Gogat gene.

Further studies, beyond the aim of this paper, will be required to verify the hypothesis of the presence of a dark regulated *Fd-Gogat* gene in rice.

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