BIOLOGIA PLANTARUM 54 (2): 279-284, 2010

The lack of plastidal transit sequence cannot override the targeting capacity of *Bradyrhizobium japonicum* δ-aminolevulinic acid synthase in transgenic rice

K. BACK[1] and S. JUNG[2]*

Agricultural Plant Stress Research Center, Biotechnology Research Institute, Chonnam National University, Gwangju 500-757, Korea[1]
School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Korea[2]

Abstract

The δ-aminolevulinic acid synthase (ALA-S) is an enzyme which catalyzes the synthesis of δ-aminolevulinic acid (ALA). The *Bradyrhizobium japonicum* ALA-S coding sequence lacking plastidal transit sequence was introduced into the rice genome (C line). The transgenic lines, C4 and C5, were compared with the transgenic lines expressing TALA-S gene with plastidal transit sequence (P line) to investigate whether the plastidal sequence affects the targeting capacity of *B. japonicum* ALA-S gene and the ALA-synthesizing capacity in rice plants. The *B. japonicum* ALA-S mRNA was expressed efficiently in C lines and the protein was localized in the stroma of chloroplasts regardless of the transit sequence as in P lines. The resulting transgenic plants, C line, had similar levels of ALA-S activity, ALA, protoporphyrin IX and chlorophylls, compared to those of P lines. In response to irradiance of 350 μmol m^{-2} s^{-1}, transgenic lines C4 and C5 displayed the characteristic phenotypes of photodynamic damage, *i.e.*, decreases in photosynthetic parameter F_v/F_m, as in P5 and P14 lines, whereas wild type did not. These results indicate that the lack of the plastidal transit sequence influences neither chloroplast translocation of *B. japonicum* ALA-S nor ALA-synthesizing capacity in the transgenic rice.

Additional key words: chlorophyll, chlorophyll *a* fluorescence, chloroplast translocation, porphyrin.

Introduction

Porphyrin compounds play vital roles in various biological processes, including photosynthesis and respiration (Beale 1978, Papenbrock *et al*. 1999). δ-aminolevulinic acid (ALA) is a key precursor in the biosynthesis of porphyrins, such as chlorophyll and heme. Higher plants synthesize ALA from the five-carbon skeleton of glutamate in a process known as the C_5 pathway, in which glutamyl-tRNA synthetase, glutamyl-tRNA hydrogenase, and glutamate-1-semi-aldehyde aminotransferase carry out three sequential enzymatic reactions to produce ALA from glutamate (Beale 1978, Kruse *et al*. 1997, Tanaka and Tanaka 2007). Eight ALA molecules are fused into porphyrins which are then modified to protoporphyrin IX (Proto IX). The chelation of Proto IX with Mg^{2+} or Fe^{2+} and subsequent modifications lead to chlorophyll or heme formation.

When plants are treated with high concentrations of ALA, excess tetrapyrroles absorb energy that is utilized in photochemical reactions leading to production of singlet oxygen ($^1O_2$) (Hopf and Whitten 1978, Tripathy and Chakraborty 1991). The $^1O_2$ oxidizes unsaturated membrane lipids, generating free radicals that damage the membrane system, leading to the death of the plant. Rebeiz *et al*. (1984) reported that ALA can be utilized as a selective and biodegradable herbicide. However, ALA has some beneficial effects on crops at low concentrations, such as improvements in dry matter yield, promotive effects on photosynthetic activity and inhibitory effects on respiration. The treatment with low concentrations of ALA was found to increase the growth and yield of radish, kidney bean, barley, potato, and

Received 1 March 2008, *accepted* 15 January 2009.
Abbreviations: ALA - δ-aminolevulinic acid; ALA-S - δ-aminolevulinic acid synthase without additional plastidal transit sequence; Proto IX - protoporphyrin IX; TALA-S - δ-aminolevulinic acid synthase with additional plastidal transit sequence.
Acknowledgments: This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-531-F00004).
* Corresponding author; fax: (+82) 53 943 6925, e-mail: sjung@knu.ac.kr

garlic by 10 - 60 % (Hotta *et al*. 1997). The ALA-forming activity correlates well with the steady-state levels of mRNAs that encode light-harvesting chlorophyll-binding proteins, LHC (Kruse *et al*. 1997). ALA also has a variety of other agricultural applications, including inducing salt and cold tolerance in plants (Hotta and Watanabe 1999, Watanabe *et al*. 2000). These studies suggest that a low concentration of ALA has a potential of increasing yield and multi-stress tolerance of crops.

In *Bradyrhizobium japonicum*, ALA is formed by δ-aminolevulinic acid synthase (ALA-S), which catalyzes the first step in heme synthesis, the condensation of glycine and succinyl-CoA, releasing carbon dioxide and CoA. The gene that encodes the ALA-S of *B. japonicum*, *hemA*, encodes a 409-amino-acid protein with a molecular mass of 45 kDa (McClung *et al*. 1987). The ALA-S is firstly synthesized in the cytosol as pre-enzyme which is rapidly imported into the mitochondria and processed to give the mature form (Urban-Grimal *et al*. 1986). As part of our search for alternative ways of replacing the exogenous supply of costly ALA, we generated the rice plants expressing *B. japonicum* TALA-S (P line), in which additional plastidal transit sequence was used to import *B. japonicum* ALA-S gene into plastids (Jung *et al*. 2004). The transgenic plants showed the localization of TALA-S in stroma of chloroplasts with increases in ALA and chlorophylls, and were more susceptible to photoinhibition compared with wild-type plants.

To obtain the optimal expression of *B. japonicum* ALA-S gene in rice to avoid the photobleaching symptom, the *B. japonicum* ALA-S coding sequence lacking the plastidal transit sequence, under the control of the ubiquitin promoter, was introduced into the rice genome. We investigated whether the lack of plastidal sequence could influence the translocation of *B. japonicum* ALA-S, enhancing photosynthetic efficiency and growth through synthesizing ALA to optimum level in new transgenic rice, C line. The levels of ALA-S activity and ALA as well as the subcellular localization of the transgene were compared between the transgenic rice, P and C lines, and wild-type rice. This study will contribute to an understanding of how the flow and the synthesis of ALA molecules are regulated at appropriate levels by genetic modification.

Materials and methods

Plant transformation and regeneration: Scutellum-derived calli of rice (*Oryza sativa* cv. Dongjin) were co-cultured with *Agrobacterium tumefaciens* LBA4404 harboring either the binary vector pGA1611:TALA-S (with additional plastidal transit sequence; which was designated P line) (Jung *et al.* 2004) or pGA1611:ALA-S (without plastidal transit sequence; C line) (Fig. 1). DNA encoding the plastidal transit sequence for a tobacco plastidal protoporphyrinogen oxidase (Lee *et al*. 2000) was used to import *Bradyrhizobium japonicum* δ-aminolevulinic acid synthase gene into plastids of rice (Jung *et al*. 2004). In the present study, the *B. japonicum* ALA-S coding sequence lacking the plastidal transit sequence was introduced into rice to obtain ALA-S protein expression in cytosol. Cloning of the *B. japonicum* ALA-S gene by PCR has been described previously (Ha *et al*. 2002) and amplification was made using the primers 5′-ATCAAGCTTATGGATTA CAGCCAGTTCTT-3′ (*Hind*III site underlined) and 5′-ATTGGGTACCAAGAAAAACCTACTCCGCCGCC AGCGA-3′ (*Kpn*I site underlined). The PCR product was digested with *Hind*III and *Kpn*I and gel-purified. The DNA encoding the ALA-S was ligated into pBluescript-SK (*Stratagene*, La Jolla, CA, USA) predigested with *Hind*III and *Kpn*I. After verifying the sequence integrity, the *Hind*III-*Kpn*I fragments of ALA-S were cut and religated into the same restriction sites of vector pGA1611, in-between the maize ubiquitin promoter and the nos 3′ terminator.

A. tumefaciens LBA4404 harboring pGA1611:ALA-S was grown overnight at 28 ºC in YEP medium supplemented with 5 μg cm^{-3} tetracyclin and 40 μg cm^{-3} hygromycin. The cultures were spun down, and pellets were resuspended in an equal volume of AA medium containing 100 μM acetosyringone. Calli were induced from scutellum of rice seeds on N6 media. The calli were transferred to a co-culture medium and cultured for 2 - 3 d in darkness at 25 ºC. After hygromycin selection for 3 to 4 weeks, the calli were transferred to regeneration medium for shoot and root development. After the roots had sufficiently developed, the transgenic plants were transferred to a greenhouse and grown to maturity.

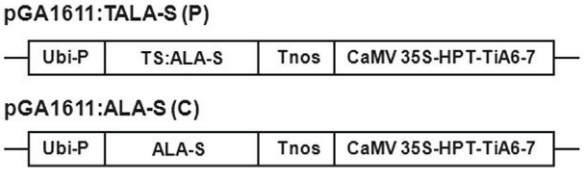

Fig. 1. Schematic diagram of T-DNA of the binary vectors used for transformation. Ubi-P - maize ubiquitin promoter; TS - tobacco plastidal transit sequence; ALA-S - *Bradyrhizobium japonicum* δ-aminolevulinic acid synthase; Tnos - nopaline synthase terminator; CaMV 35S - cauliflower mosaic virus 35S promoter; HPT - hygromycin phosphotransferase; TiA6-7 - TiA6-7 terminator.

Growth of transgenic lines and effect of irradiance: The T_2 generation of homozygous transgenic rice lines including P5, P14, C2, C4, C5 and C6 was used for experiments. Seeds of untransformed and transgenic lines were put for 3 d at 25 ºC in darkness on Murashige and Skoog (MS) medium, and then exposed to low or high

irradiance (150 or 350 μmol m^{-2} s^{-1}, respectively) with 16-h photoperiod for 7 d.

Isolation and analysis of RNA: Total RNA (10 μg) was isolated from leaf tissues of wild-type or transgenic rice plants using TRI (total RNA isolation) reagent (*Sigma*, St. Louis, MO, USA) and was fractionated on a 1 % agarose gel containing formaldehyde, using 20 mM 3-(*N*-morpholino)propanesulfuric acid (pH 7.0) as a running buffer. RNA samples were stained with ethidium bromide prior to blotting to a nylon membrane, and the blots were hybridized with a ^{32}P-labeled gene-specific probe for the *B. japonicum* ALA-S cDNA clone at 60 ºC in 250 mM sodium phosphate buffer (pH 7.5), 7 % sodium dodecyl sulfate (SDS), 1 % bovine serum albumin (BSA), and 1 mM ethylenediamine tetrachloroacetic acid (EDTA). After hybridization, the RNA blot was washed twice with 2× sodium chloride-sodium citrate (SSC)/0.1 % SDS and twice with 0.2× SSC/0.1 % SDS at 55 ºC. The radioactive signals of hybridized membranes were detected with a phosphor imaging system (*Fuji*, Tokyo, Japan).

Protein gel blot analysis: Leaves were homogenized for 10 s in homogenization buffer [330 mM sorbitol, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM EDTA, 0.1 % BSA, 4 mM dithiothreitol (DTT), pH 8.0] and filtered through Miracloth. The plastids were collected by centrifugation at 6 000 *g* for 2 min. Intact chloroplasts were separated on a *Percoll*® gradient and pelleted for *B. japonicum* ALA-S protein gel blot analysis. Purified chloroplasts were lysed in 1 cm^3 of 240 mM NaCl and incubated for 10 min on ice. Stroma and thylakoid protein fractions were obtained by centrifugation at 15 000 *g*. For cytosol fraction, an aliquot of the homogenate was centrifuged twice at 4 000 *g* for 5 min and then at 12 000 *g* for 15 min. The supernatant was saved for cytosol fraction. The protein was separated using 11 % SDS-PAGE for *B. japonicum* ALA-S, respectively, and electroblotted to polyvinylidene fluoride membranes. Immunodetection was performed using standard procedures (*Boehringer*, Mannheim, Germany). The polyclonal mouse antiserum was raised against purified *B. japonicum* ALA-S protein (Ha *et al*. 2002).

ALA-S enzyme assay and ALA measurement: Plastids were prepared as described by Zavgorodnyaya *et al*. (1997). Crude plastid preparations were resuspended in 20 mM phosphate buffer (pH 7.6). For measurements of ALA-S activity, aliquots of the suspension were added to 0.025 cm^3 of an assay mixture containing 100 mM glycine, 25 μM pyridoxal phosphate, 1 mM DTT, 10 mM levulinate, and 150 μM succinyl CoA. Reactions were carried out for 30 min at 30 ºC and stopped by the addition of 0.006 cm^3 of 100 % trichloroacetic acid. Modified Ehrlich's reagent was added, and the chromophore was detected at 553 nm by spectrophotometer (Urata and Granick 1963).

ALA-synthesizing capacity was measured as described by Papenbrock *et al*. (1999). Leaf disks were harvested for each sample, incubated in 20 mM phosphate buffer containing 40 mM levulinic acid in the light for 6 h, and frozen in liquid nitrogen. Samples were homogenized, resuspended in 1 cm^3 of 20 mM K_2HPO_4/KH_2PO_4 (pH 6.9), and centrifuged at 10 000 *g*. The 0.5 cm^3 supernatant was mixed with 0.1 cm^3 ethylacetoacetate, boiled for 10 min, and cooled for 5 min. ALA was quantified as described above.

Determination of Proto IX: To measure the Proto IX content, plant tissue (0.1 g) was ground in 2 cm^3 of methanol:acetone:0.1 M NaOH (9:10:1, v/v/v), and the homogenate was centrifuged at 10 000 *g* for 10 min to remove cell debris and proteins. Porphyrins were separated by HPLC, using a *Novapak* C_{18} column (*Waters*, Milford, MA, USA) at a flow rate of 1 cm^3 min^{-1}. Porphyrins were eluted using a solvent system of 0.1 M ammonium phosphate (pH 5.8) and methanol. The column eluate was monitored using a fluorescence detector (474, *Waters*) at excitation and emission wavelengths of 400 and 630 nm, respectively.

Photosynthetic activity: Chlorophyll *a* fluorescence was measured *in vivo*, using a pulse amplitude modulation fluorometer (*Handy PEA*, *Hansatech Instruments*, Norfolk, England), after dark adaptation for 10 min. The initial fluorescence yield, F_0, was obtained upon excitation with a weak irradiance from a pulse light-emitting diode. Maximal fluorescence yield, F_m, was obtained by application of 1-s pulse of 3 000 μmol m^{-2} s^{-1} when all reaction centers were transiently close (Krause *et al*. 1983). The ratio of F_v to F_m, representing the activity of photosystem 2, was used to assess the functional damage to plants.

Results and discussion

Gene expression and activities of the rate-determining ALA synthesis are controlled to synthesize tetrapyrrole and to balance the metabolic flow with the cellular requirements (Alawady and Grimm 2005). Rice plants expressing *B. japonicum* TALA-S gene comprising the plastidal transit sequence (P line) showed the greater contents of ALA and chlorophylls than wild-type rice plants, but no difference in seedling growth (Jung *et al*. 2004). Under irradiance of 350 μmol m^{-2} s^{-1}, however, the photobleaching of shoot was observed only in the transgenic plants (Jung *et al*. 2004, 2008). In the present study, the *B. japonicum* ALA-S synthase gene lacking the plastidal transit sequence was introduced into rice genome to examine whether the absence of the transit sequence in the ALA-S coding sequence affects a subcellular translocation of ALA-S, obtaining the optimal

increase of ALA in transgenic rice. ALA has a variety of agricultural applications not only as an herbicide and growth promoting factor, but also based on its ability to confer salt and cold temperature tolerance in plants (Sasaki *et al*. 1998, Tanaka and Kuramochi 2001). New transgenic rice plants expressing the ALA-S gene were generated according to the *Agrobacterium*-mediated transformation using the binary vector pGA1611:ALA-S (C line) (Fig. 1) to transform wild-type rice. We regenerated a total of eleven independent transgenic lines (T_0). All the transgenic lines (T_1 seeds) tested with hygromycin exhibited a single genetic locus showing a 3:1 (hygromycin resistant versus susceptible) segregation ratio (data not shown). The ALA-S gene was successfully transmitted to T_2 rice plants, as indicated by high levels of the ALA-S mRNA in all C lines examined, but no hybridizing band was detected in wild-type line (Fig. 2).

Fig. 2. RNA gel blot analysis of *B. japonicum* ALA-S. Total RNA (10 μg) was blotted onto a nylon membrane as described in the Materials and methods. WT - wild type; C2, C4, C5 and C6 - T_2 generation of homozygous transgenic rice lines expressing *B. japonicum ALA-S* gene. EtBr - ethidium bromide staining of RNA samples.

In the P lines expressing *B. japonicum* TALA-S, the ALA-S protein was expressed in the stroma of chloroplasts (Jung *et al*. 2004). As the ALA-S gene did not have the plastidal transit peptide in C line (Fig. 1), we expected that ALA-S protein would fail to translocate into chloroplasts and be expressed in cytosol of transgenic rice. To verify the subcellular localization of ALA-S protein in C lines, the location of ALA-S protein was investigated by probing immunoblots of stroma, thylakoid and cytosol fractions of transgenic lines C4 and C5. Unlike our expectation, ALA-S proteins translated from both forms of ALA-S, with (P14) or without the plastidal transit sequence (C4 and C5), were equally localized at the stroma of chloroplasts in the transgenic lines, but the protein was not detected on blots of wild-type line (Fig. 3). Neither cytosol fraction nor thylakoid fraction detected the ALA-S protein in all lines examined (Fig. 3). These results show that transgenic plants expressing a native bacterial ALA-S gene lacking the plastidal transit sequence leads to its proper import into plastids and ultimately gives rise to a functionally active enzyme, regardless of the plastidal transit sequence. The mature protein of ALA-S itself might have the information to be targeted into the chloroplasts. In addition, ALA-S activity was compared in the crude plastids from P5, P14, C4, C5 and wild-type lines. Similar ALA-S activities were observed in both types of TALA-S or ALA-S-expressing rice lines, but no activity was detected in wild-type line (Table 1). ALA, an early precursor of the tetrapyrrole pathway, increased approximately by 2-fold in C lines as well as P lines, as compared to wild type (Table 1). The increased contents of ALA and chlorophylls were consistent with the detection of plastidal ALA-S activity in transgenic lines C4 and C5. Proto IX, which is known as a photo-sensitizer, also increased in all transgenic lines in comparison to wild-type line, with the greatest increase in P14 line (Table 1).

Under irradiance of 350 μmol m^{-2} s^{-1}, transgenic lines C4 and C5 displayed a decrease in F_v/F_m ratio (Fig. 4), photobleaching of shoots and retarded shoot growth (data not shown) similar as in P lines (Jung *et al*. 2004). The transgenic line P14 suffered the greatest photoinhibition, as indicated by the greatest decline in $F_v/F_{m,}$ the parameter associated with the current status of photochemical processes (Pomar and Barceló 2007) and

Table 1. Effect of ALA-S expression on ALA-synthase activity [nmol mg^{-1}(protein) h^{-1}], and contens of ALA [μmol g^{-1}(f.m.)], Proto IX [nmol g^{-1}(f.m.)] and chlorophyll [μg g^{-1}(f.m.]. Seeds of untransformed wild type and transgenic lines were grown onto MS media in darkness at 25 ºC for 3 d and then exposed to PPFD of 150 μmol m^{-2} s^{-1} for 7 d. WT - wild type; P and C - T_2 generation of homozygous transgenic rice lines expressing *B. japonicum TALA-S* gene (P5 and P14) or *ALA-S* gene (C4 and C5); n.d. - not detected. Data represent the mean ± SE of three samples.

Plants	ALA-S activity	ALA	Proto IX	Chlorophyll
WT	n.d.	2.04 ± 0.13	1.51 ± 0.19	1833 ± 48
P5	9.74 ± 1.06	3.58 ± 0.42	2.80 ± 0.54	2002 ± 50
P14	11.41 ± 1.25	4.25 ± 0.33	4.09 ± 0.71	2108 ± 86
C4	9.28 ± 0.46	3.76 ± 0.34	2.47 ± 0.36	1971 ± 22
C5	11.08 ± 1.31	3.72 ± 0.24	2.93 ± 0.40	2026 ± 73

Fig. 3. Protein gel blot analysis of *B. japonicum* ALA-S. Proteins (10 μg) from purified stroma, thylakoid and cytosol fractions were separated on 11 % PAGE gel containing SDS and transferred onto a PVDF membrane. Immunodetection was performed with specific antibody raised against purified *B. japonicum* ALA-S protein. WT - wild type; P and C - T_2 generation of homozygous transgenic rice lines expressing *B. japonicum TALA-S* gene (P14) or *ALA-S* gene (C4 and C5).

the greatest increase in Proto IX (Table 1, Fig. 4). In plants treated with ALA at higher concentrations, *i.e.* more than 10 mM, ALA induced accumulation of chlorophyll synthesis intermediates acting as a producers of $^1O_2$, triggering photodamage of ALA-treated plants (Rebeiz *et al*. 1984). However, under low irradiance of 150 μmol m^{-2} s^{-1}, the increases of ALA did not change photosynthetic efficiency of both transgenic lines, resulting in the same F_v/F_m as in wild type (Fig. 4). Low

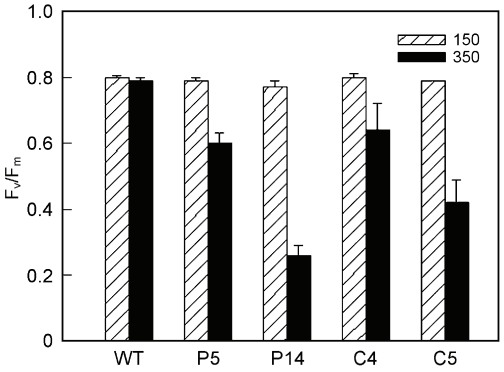

Fig. 4. Effect of ALA-S expression on variable to maximum fluorescence ratio (F_v/F_m). Seeds of untransformed wild type and transgenic lines were grown onto MS media in darkness at 25 ºC for 3 d and then exposed to irradiance either of 150 or 350 μmol m^{-2} s^{-1} for 7 d. WT - wild type; P and C - T_2 generation of homozygous transgenic rice lines expressing *B. japonicum TALA-S* gene (P5 and P14) or *ALA-S* gene (C4 and C5). Data represent the mean ± SE of three samples.

concentrations of exogenously-applied ALA increase the photosynthesis and accelerate the growth of plants (Tanaka *et al*. 1992, Sasaki *et al*. 1995, Roy and Vivekanandan 1998, Sasaki *et al*. 1998). The non-optimum level of endogenous ALA in the transgenic rice plants might not lead to promotion of photosynthesis and growth. However, application of low concentration of ALA increased salt tolerance of young cotton seedlings (Tanaka and Kuramochi 2001) and rice plants (Hotta and Watanabe 1999). Gain of additional ALA in transgenic plants through the optimum expression of ALA-S will be a useful tool for increasing growth of crops.

The *B. japonicum* ALA-S protein lacking the plastidal transit peptide was able to be imported into the stroma of chloroplasts in transgenic rice, implying that the native bacterial ALA-S polypeptide itself contains a plastidal targeting signal. Both transgenic rice lines expressing *B. japonicum* ALA-S with or without plastidal transit sequence increased ALA-S activity and ALA to a similar extent. They were more susceptible to photoinhibitory stress, as indicated by the decline in F_v/F_m, when compared to wild type. Taken together, the lack of the transit sequence did not influence chloroplast translocation ability of *B. japonicum* ALA-S and ALA-synthesizing capacity in the transgenic rice. Further investigation focusing on the optimum expression of ALA-S may provide us with information regarding how plants regulate their endogenous ALA to optimum level for possibly increasing biomass production.

References

Alawady, A.E., Grimm, B.: Tobacco Mg protoporphyrin IX methyltransferase is involved in inverse activation of Mg porphyrin and protoheme synthesis. - Plant J. **41**: 282-290, 2005.

Beale, S.I.: δ-Aminolevulinic acid in plants: its biosynthesis, regulation, and role in plastid development. - Annu. Rev. Plant Physiol. **29**: 95-120, 1978.

Ha, S.B., Lee, D.E., Back, K.: Extracellular production of 5-aminolevulinic acid and affinity purification of *Bradyrhizobium japonicum* 5-aminolevulinic acid synthase in *Escherichia coli*. - Agr. Chem. Biotechnol. **45**: 160-163, 2002.

Hopf, F.R., Whitten, D.G.: Chemical transformations involving photoexcited porphyrins and metalloporphyrins. - In: Dolphin, D. (ed.): The Porphyrins. Vol. 2. Pp. 191-195. Academic Press, New York 1978.

Hotta, Y., Tanaka, T., Takaoka, H., Takeuchi, Y., Konnai, M.: Promotive effects of 5-aminolevulinic acid on the yield of several crops. - Plant Growth Regul. **22**: 109-114, 1997.

Hotta, Y., Watanabe, K.: Plant growth-regulating activities of 5-aminolevulinic acid. - Syokubutu-no-Kagaku-Tyousetu **34**: 85-96, 1999.

Jung, S., Back, K., Yang, K., Kuk, Y.I., Chon, S.-U.: Defence response produced during photodynamic damage in transgenic rice overexpressing 5-aminolevulinic acid synthase. - Photosynthetica **46**: 3-9, 2008.

Jung, S., Yang, K., Lee, D.-E., Back, K.: Expression of *Bradyrhizobium japonicum* 5-aminolevulinic acid synthase induces severe photodynamic damage in transgenic rice. - Plant Sci. **167**: 789-795, 2004.

Krause, G.H., Briantais, T.M., Vernotte, C.: Characterization of chlorophyll fluorescence spectroscopy at 77 K. I. ΔpH-dependent quenching. - Biochim. biophys. Acta: **723**: 169-175, 1983.

Kruse, E., Grimm, B., Beator, J., Kloppstech, K.: Developmental and circadian control of the capacity for δ-aminolevulinic acid synthesis in green barley. - Planta **202**: 235-241, 1997.

Lee, H.J., Lee, S.B., Chung, J.S., Han, S.U., Han, O., Guh, J.O., Jeon, J.S., An, G., Back, K.: Transgenic rice plants expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene are resistant to diphenyl ether herbicide oxyfluorfen. - Plant Cell Physiol. **41**: 743-749, 2000.

McClung, C.R., Somerville, J.E., Guerinot, M.L., Chelm, B.K.: Structure of the *Bradyrhizobium japonicum* gene hemA encoding 5-aminolevulinic acid synthase. - Gene **54**: 133-139, 1987.

Papenbrock, J., Mock, H.P., Kruse, E., Grimm, B.: Expression studies in tetrapyrrole biosynthesis: inverse maxima of magnesium chelatase and ferrochelatase activity during cyclic photoperiods. - Planta **208**: 264-273, 1999.

Pomar, F., Barceló, A.R.: Are red leaves photosynthetically active? - Biol. Plant. **51**: 799-800, 2007.

Rebeiz, C.A., Montazer-Zouhoor, A., Hopen, H.J., Wu, S.M.:

Photodynamic herbicides: concept and phenomenology. - Enzyme Microbiol. Technol. **6**: 390-401, 1984.

Roy, C.B., Vivekanandan, M.: Role of aminolevulinic acid in improving biomass production in *Vigna catjung*, *V. mungo*, and *V. radiate*. - Biol. Plant. **41**: 211-215, 1998.

Sasaki, K., Marquez, F.J., Nishio, N., Nagai, S.: Promotive effect of 5-aminolevulinic acid on the growth and photosynthesis of *Spirulina platensis*. - J. Ferment Bioeng. **79**: 453-457, 1995.

Sasaki, K., Tanaka, T., Nagai, S.: Use of photosynthetic bacteria for the production of SCP and chemicals from organic wastes. - In: Martin, A.M. (ed.): Bioconversion of Waste Materials to Industrial Products. Second Edition. Pp. 247-291. Blackie Academic and Professional, London 1998.

Tanaka, R., Tanaka, A.: Tetrapyrrole biosynthesis in higher plants. - Annu. Rev. Plant Biol. **58**: 321-346, 2007.

Tanaka, T., Kuramochi, H.: 5-Aminolevulinic acid improves salt tolerance. - Regul. Plant Growth Dev. **36**: 190-197, 2001.

Tanaka, T., Takahashi, K., Hotta, T., Takeuchi, Y., Konnai, M.: Promotive effects of 5-aminolevulinic acid on yield of several crops. - In: Proceedings of the 19th Annual Meeting of Plant Growth Regulator Society of America. Pp. 237-241. Plant Growth Regulator Society of America, Washington 1992.

Tripathy, B.C., Chakraborty, N.: 5-Aminolevulinic acid induced photodynamic damage of the photosynthetic electron transport chain of cucumber (*Cucumis sativus* L.) cotyledons. - Plant Physiol. **96**: 761-767, 1991.

Urata, G., Granick, S.: Biosynthesis of α-aminoketones and the metabolism of aminoacetone. - J. biol. Chem. **238**: 811-820, 1963.

Urban-Grimal, D., Volland, C., Garnier, T., Dehoux, P., Labbe-Bois, R.: The nucleotide sequence of the *hem1* gene and evidence for a precursor form of the mitochondrial 5-aminolevulinic synthase in *Saccharomyces cerevisae*. - Eur. J. Biochem. **156**: 511-519, 1986.

Watanabe, K., Tanaka, T., Hotta, Y., Kuramochi, H., Takeuchi, Y.: Improving salt tolerance of cotton seedlings with 5-aminolevulinic acid. - Plant Growth. Regul. **32**: 99-103, 2000.

Zavgorodnyaya, A., Papenbrock, J., Grimm, B.: Yeast 5-aminolevulinate synthase provides additional chlorophyll precursor in transgenic tobacco. - Plant J. **12**: 169-178, 1997.