

Biochemical characterization of gamma-aminobutyric acid (GABA): pyruvate transaminase during rice leaf senescenceMohammad Israil Ansari^{1,*}, Shu-Chen Grace Chen²¹Amity Institute of Biotechnology, Amity University, Lucknow, India²Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei, Taiwan

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Abstract

Senescence-associated genes (SAGs) are up-regulated during plant senescence and may have been implicated in encoding enzymes involved in metabolism of senescence tissues. The gene Osl2 (GABA:pyruvate-transaminase) which is upregulated during leaf senescence in rice (*Oryza sativa*) was functionally characterized. The recombinant Osl2 fusion protein over-expressed in *Escherichia coli* displays pyruvate-dependent gamma-aminobutyric acid (GABA) transaminase (EC 2.6.1.19) activity. Investigations of the corresponding pyruvate-dependent GABA transaminase activities in rice leaves demonstrate the up-regulation of enzyme activity during leaf senescence, with the highest activity at the S3 stage of rice leaf senescence. Here we have discussed the role of GABA:pyruvate-transaminase in the metabolism of amino acids and nitrogen recycle during rice leaf senescence.

Keywords: GABA, Leaf senescence, Senescence-associated gene, Gene expression.

INTRODUCTION

Leaf senescence represents endogenously controlled degenerative processes which ultimately lead to organ death. It is a highly regulated, ordered series of events involving the decline of photosynthetic activity, disintegration of chloroplasts, breakdown of biomolecules, loss of chlorophyll (Chl), and the recycling of valuable nutrients to other parts of the plant (Noodén, 1988; Smart, 1994, Price *et al.*, 2008). Leaf senescence progresses in an age-dependent manner but is also affected by a complex interaction of developmental stage with various internal and environmental factors (Nam, 1997). Some developmental processes such as fruit ripening and seed development also induce leaf senescence (Noodén and Guiamét, 1989). Biochemical and molecular studies over the last few decades have shown that the senescence is an active process that requires the expression of novel genes and synthesis of new proteins. Genetic studies on leaf senescence have, in general taken two different approaches. Genes with a role in the leaf senescence can be identified by the

isolation and the characterization of mutants that are defective in some aspects of senescence pathway. Identification of regulatory genes that could be manipulated to regulate the onset or progression of senescence is of key interest and importance.

Leaf senescence associated genes have been identified in Arabidopsis, maize, *Brassica napus*, potato, cucumber, asparagus, castor bean, radish, and tomato. Our group have cloned and characterized a number of SAGs cDNA in rice and sweet potato (Yap *et al.*, 2003, Chen *et al.*, 2002, Huang *et al.*, 2001, Lee *et al.*, 2001, Ansari *et al.*, 2005). Among them Osl2, is a gene encoding GABA:pyruvate-transaminase whose expression is specific to rice leaf senescence. GABA (gamma-aminobutyric acid) is a ubiquitous, four carbon nonprotein amino acid found in all prokaryotic and eukaryotic organism (Shelp *et al.*, 1999, Kinnersley and Turino 2000). It was first identified in potato tubers during 1950s. GABA is predominantly associated with neurotransmission in the mammalian brain (Varju *et al.*, 2001) but its role in plants is unknown. However, it accumulates in response to variety of biotic and abiotic stresses such as hypoxia, cold temperature and mechanical stimulation (Van Cauwenbergh *et al.*, 2002). GABA is produced in cytosol (Breitkreuz and Shelp, 1995) via the decarboxylation of glutamate in a reaction catalysed by the glutamate decarboxylase, a calcium/calmodulin-dependent enzyme (Sudden *et al.*, 1996). It has been reported that GABA-shunt is

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essential for normal plant growth (Bouche *et al.*, 2003) and GABA-transaminase is one of the important enzyme which play role in GABA-shunt. In this study we have done functional characterization of gamma-aminobutyric acid (GABA):pyruvate transaminase which play important role in metabolism of amino acid and nitrogen recycling during rice leaf senescence.

MATERIALS AND METHODS

Plant material

To study the natural leaf senescence, rice plant (*Oryza sativa* L. cv. Tainong 67) were grown in greenhouse under natural light conditions. Leaves were harvested at various developmental stages, immediately frozen in liquid nitrogen and stored at -80°C . Stages of leaf development and senescence were defined as follows: Y (young leaf roll from the one month old plant); MG (Mature Green fully expanded leaf with chlorophyll content 100%, from two month old plant); S1 (senescing leaf with chlorophyll content 80-95%, from the two and half month old plant at the stage of panicle development); S2 (senescing leaf with chlorophyll content of 60-80%, from the three month old plant at the flowering stage); S3 (senescing leaf with chlorophyll content of 45-60%, from the plant at grain filling stage); S4 (senescing leaf with chlorophyll content of 30-45% from 4 month old plant at seed maturing stage). The leaves for S1 to S4 stages were obtained from ninth to twelfth leaves on the main stem.

Expression of Osl2 protein in *Escherichia coli* and antibody production

We have cloned Osl2 gene and have reported that Osl2 gene (GenBank Accession No. AF297651) is up-regulated during leaf senescence (Ansari *et al.*, 2005). Coding region of Osl2 gene (91bp-1642bp) was amplified and cloned into expression vector pET-30 Ek/LIC (Novagen, Madison, WI, U.S.A.), resulting an in frame fusion of Osl2 with N-terminal His affinity tag sequence. Over expression of the Osl2 fusion protein in *E coli* BL-21 was induced by addition of 1 mM of isopropyl β -D-thiogalactopyranoside (IPTG). The His fusion protein was purified by a Ni NTA His bind kit (Novagen, Madison, WI, U.S.A.), washing with 20 mM of imidazole and elution of His bind protein with 400 mM of imidazole under denaturing conditions. His purified protein was further purified by preparative gel electrophoresis on an SDS-polyacrylamide gel (14%). The Osl2 protein band was excised, electroeluted, and the resultant protein solution was concentrated by Centricon 30 concentrator (Millipore Corporation, Bedford, MA). Polyclonal antibodies specific to Osl2 were raised in a rabbit with 500 μg of the purified Osl2

protein. The antiserum was subjected to ammonium sulfate precipitation at 30% saturation. The resultant antibodies were dissolved in phosphate-buffered saline (PBS, pH 7.0) and stored at -70°C . Under native condition for enzyme characterization washing of Ni NTA His bind column was done with different concentration of imidazole (20, 60, 100 mM) and protein was eluted with 400 mM of imidazole. This Osl2 purified protein was passed through the Centricon 30 to remove the imidazole and buffer was exchanged with 20 mM Tris (pH 8.0) and stored at -4°C for enzyme characterization.

DNA sequence analysis

DNA sequence was determined with an automatic DNA sequencer (ABI PRISM 377 DNA Sequencer, USA). Homology search against sequence database was performed using the BLAST program at National Center for Biotechnology Information, USA.

SDS-PAGE and western blot analysis

The expressed protein was separated by SDS-PAGE (14% acrylamide) and transferred to the nitrocellulose membrane. The membrane was blocked with 30% w/v gelatin in 1X TBS for 2 hours. The blot was subsequently washed 3 times with 1X TTBS (0.05% v/v Tween-20 in TBS), then incubated for 2 hours with His tag monoclonal antibody diluted to 1:5000 in antibody blocking solution (1%w/v gelatin in 1X TBS). Membrane was washed 2-3 times with 1X TTBS. Finally the blot was incubated for 2 hours in second antibody (Goat Anti Mouse IgG conjugate) at 1:5000 dilution. Blot was washed 2 times with 1XTTBS for 5 min each. Color reaction was carried out by developing solution. 5-Bromo-4chloro-3-indolyphosphate (BCIP) and nitroblue tetrazolium (NBT) were used to visualize bands.

Assay for GABA:pyruvate transaminase activity

The recombinant Osl2 protein was assayed for GABA transaminase activity using pyruvate or α -ketoglutarate as amino group acceptor, according to the method of Van Cauwenberghe *et al.*, (1995) with some modification. The protein sample was incubated in a reaction mixture containing 50 mM Tris-HCl buffer (pH 8.2), 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM pyridoxal 5-phosphate (PLP), 10% (v/v) glycerol, 16 mM GABA and 4 mM pyruvate or α -ketoglutarate in a final volume of 500 μl . After incubation at 30°C for 1 h, the reaction was terminated with 4mM sulphosalicylic acid. From this mixture, 100–200 μl was taken, and the amount of alanine or glutamate formed was measured by enzymatic reaction with alanine dehydrogenase (ADH, EC 1.4.1.1) or glutamate dehydrogenase (GDH, EC 1.4.1.3). ADH assay was

performed with 50 mM sodium carbonate buffer (pH 10.0), 1.5 mM NAD⁺ and 0.02 U of *Bacillus subtilis* ADH (Sigma-Aldrich Fine Chemicals, St. Louis, MO) in 1.0 ml of reaction mixture. The reactions were performed at 25°C for 10 min and the increase in absorbance at a wavelength of 340 nm was monitored with a Beckman- Coulter DU640B spectrophotometer (Beckman-Coulter, Inc., Fullerton, CA). GDH assay was performed using a glutamate/glutamine determination kit from Sigma- Aldrich Fine Chemicals. The reaction was performed with 50 mM Tris-EDTA buffer (pH 9.0) containing 1.6% (w/v) hydrazine hydrate, 1.5 mM NAD⁺, 0.5 mM ADP and 12 U of GDH in a volume of 1.0 ml. The increase in absorbance at a wavelength of 340 nm was recorded after incubation at 25°C for 40 min. To measure the pyruvate-dependent GABA transaminase activity in rice leaves at different developmental and senescence stages, leaf tissue was ground with five volumes of cold Tris-HCl buffer (50 mM, pH8.2) con containing 3 mM DTT, 0.5m M PLP, 1.25 mM EDTA and 15% (v/v) glycerol, as described by Van Cauwenberghe *et al.*, (1995). All operations were conducted at 4°C. The homogenate was centrifuged at 23 000 g for 25 min, and the supernatant was collected for GABA:pyruvate transaminase assay as described above.

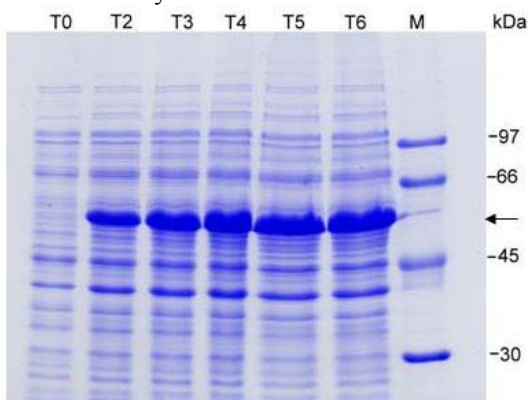


Figure 1: Time course of Osl2 protein expression in total cell protein of *E. coli* strain BL-21 (DE-3) containing recombinant pET-30 Ek/LIC construct. T0 corresponds to the time before induction of IPTG and T2-T6 corresponds to the period of induction (in hours). M- Protein Marker. Arrow indicates the expressed protein at 61 kDa.

RESULTS

Expression of Osl2 protein in *E. coli*

To express Osl2 protein in *E. coli*, IPTG induction of recombinant pET-30 Ek/LIC was carried out. His tag monoclonal antibody was used to detect the fused His tag GABA:pyruvate-transaminase protein. Very strong protein band became visible by SDS-PAGE after IPTG induction (Fig.1). Media fraction of culture was also tested and no target protein was found to be secreted from the cells. Before IPTG induction, no fusion

protein could be detected. After 1 hour of IPTG induction very intense band was visible and its intensity increases with time course (Fig.1). The detected protein has apparent molecular weight of around 61 kDa which corresponds to the predicted molecular mass of Osl2 protein (56 kDa) plus the fused His tag (5.0 kDa).

The expressed protein was further analyzed for the cellular localization and solubility. There was no Osl2 protein present in periplasmic and soluble cytoplasmic fractions, only present in the insoluble fraction when incubated at 37°C (Fig. 2). To increase the solubility of the expressed protein, culture was grown at 16°C overnight and samples were taken at different time period. The solubility of Osl2 protein increases with the time but later decreases, 19 hours was optimal for induction (Fig. 3).

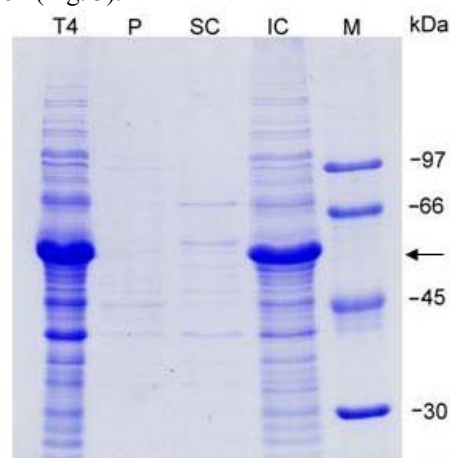


Figure 2: Cellular localization of the expressed Osl2 protein in *E. coli* strain BL-21 (DE-3) with recombinant pET-30 Ek/LIC construct after 4 hours of IPTG induction. T4 – Total Cell Protein; P- Peri-Plasmic Protein; SC- Soluble Cytoplasmic Protein; IC- Insoluble Cytoplasmic Protein; M- Protein Marker. Arrow indicates the expressed protein at 61 kDa.

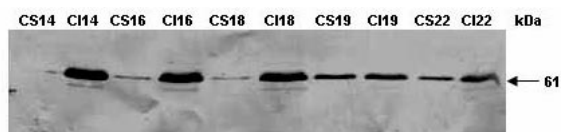


Figure 3: Western blot analysis for the solubility of expressed Osl2 protein in *E. coli* strain BL-21 (DE-3) with recombinant pET-30 Ek/LIC construct. CS- Cytoplasmic Soluble; CI-Cytoplasmic Insoluble; and CS14-CS22 and CI14-CI22 correspond to induction (in hours) with IPTG at 16° C. Arrow indicates the expressed protein at 61 kDa.

Purification of expressed Osl2 fusion protein

Osl2 was first expressed in *E. coli* in large quantity to generate the specific antibodies. Coding sequence of Osl2 full-length was cloned into the expression vector pET-30 Ek/LIC to over express His-Osl2 fusion protein (61.0 kDa) in *E. coli*. The His-Osl2 fusion protein was purified by Ni NTA His bind column. Polyclonal antibodies against the purified Osl2 protein were then

raised in rabbit. Under native condition for enzyme characterization washing of Ni NTA His bind column was done with different concentration of imidazole (20, 60, 100 mM) containing .5 M NaCl, 40 mM Tris-HCl pH 7.9 and protein was eluted with 400 mM of imidazole containing 0.5 M NaCl, 40 mM Tris-HCl pH 7.9. To increase the solubility of the expressed protein for enzyme characterization culture was grown at 16°C for 19 h. The protein extract from *E. coli* strain BL-21 (without vector), Osl2 recombinant pET-30 Ek/LIC, BL-21 and His purified proteins were separated by SDS-polyacrylamide gel (14%), a prominent band in recombinant pET-30 Ek/LIC, BL-21 and in His purified was detected with expected molecular weight of about 61.0 kDa which corresponds to the predicted molecular mass of Osl2 protein (56.0 kDa) plus the fused His tag (5.0 kDa) but it was absent in BL-21 which contain no vector.

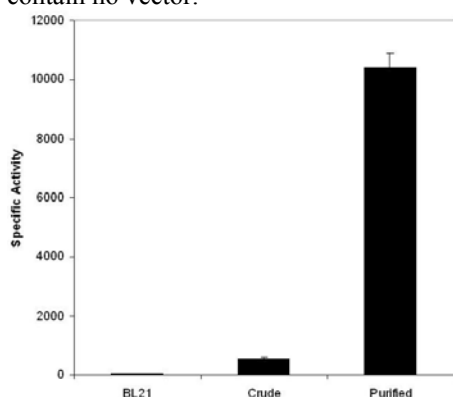


Figure 4: Specific activities of pyruvate-dependent gamma-aminobutyric acid (GABA) transaminase for the recombinant Osl2 protein produced in *E. coli* transformed with pET-30 Ek/LIC, carrying the Osl2 coding region, was grown at 16°C for 19 h after IPTG induction. The soluble cytoplasmic fraction and the purified recombinant Osl2 protein were assayed for GABA:pyruvate transaminase activity. BL21- *E. coli* strain without vector, Crude-Osl2 recombinant pET-30 Ek/LIC, Purified- His purified Osl2 recombinant pET-30 Ek/LIC. Specific Activity is represented by the number of micromoles of alanine produced per milligram of protein per minute.

Determination of the GABA:pyruvate-transaminase activity of Osl2 protein

To test whether the isolated Osl2 cDNA encodes the GABA:pyruvate transaminase, protein was isolated from Osl2 recombinant pET-30 Ek/LIC, BL-21 and assayed for the GABA:pyruvate-transaminase activity. GABA-transaminase activity with both crude and His purified enzyme was tried with pyruvate and α -ketoglutarate. There was no activity with pyruvate and α -ketoglutarate. Crude extract from both, Osl2 recombinant pET-30 Ek/LIC, BL-21 and BL-21 (without vector) was used as source of enzyme to determine the specific activity of pyruvate. With pyruvate, alanine formation in BL-21 (without vector) was not significantly affected by GABA, however, its activity was very high with recombinant pET-30 Ek/LIC, BL-21 (Fig.4). This indicate that this Osl2 is GABA:pyruvate-transaminase.

The specific activity of Osl2 His purified was compared with crude enzyme, it was almost 20 fold higher than the crude enzyme (Fig. 4). We have also tried pyruvate and α -ketoglutarate as amino group acceptor with all 22 different amino acids. There was no significant activity with any one of the reactions.

The enzymatic activity of GABA:pyruvate-transaminase was much affected by both pH and temperature. Among the temperature points tested 30° C showed the highest activity and optimum pH was 8.0. The thermal stability of the Osl2 His purified enzyme was also studied at different temperature. The enzymatic activity was almost 90% when incubated at 40° C for 30 min but 50° C incubation for 10 min only, lost its activity almost 80%.

Determination of the GABA:pyruvate-transaminase activity during leaf development and leaf senescence

GABA:pyruvate-transaminase activity was assayed and compared during leaf development and leaf senescence. The specific activity of this enzyme was very low in Y stage, this activity goes up and reached maximum at S3 stage and then declined at S4 stage of senescence. In S3 stage the activity was almost 2.5 fold higher compared to the MG stage of leaf (Fig. 5).

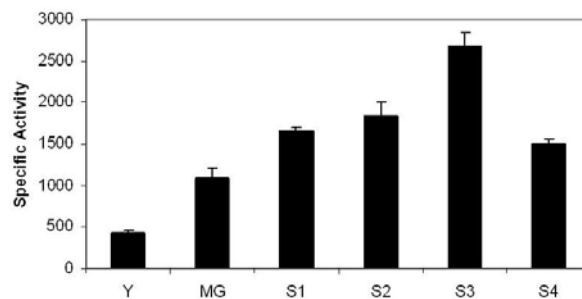


Figure 5: Pyruvate-dependent gamma-aminobutyric acid (GABA) transaminase activity in rice leaves at different stages of development and leaf senescence stage. Total soluble proteins were extracted from leaf tissues at different developmental stages and assayed for GABA:pyruvate transaminase activity. Specific Activity is represented by the number of nanomoles of alanine produced per milligram fresh weight per minute.

DISCUSSION

Leaf senescence is an important phase in the plant life cycle, it involves degradation of macromolecules and mobilization of nutrients from the senescing leaf to the growing parts of the plant (Noodén, 1988, Sykorova, *et al.*, 2008). In a variety of plants, it has been studied that the initiation and progression of leaf senescence require the co-ordinate activation of a suit of specific SAGs (Quirino *et al.*, 2000). We have been studying the molecular mechanism of leaf senescence and have

cloned and characterized a number of SAGs in rice and sweet potato (Yap *et al.*, 2003, Chen *et al.*, 2002, Huang *et al.*, 2001, Lee *et al.*, 2001, Ansari *et al.*, 2005).

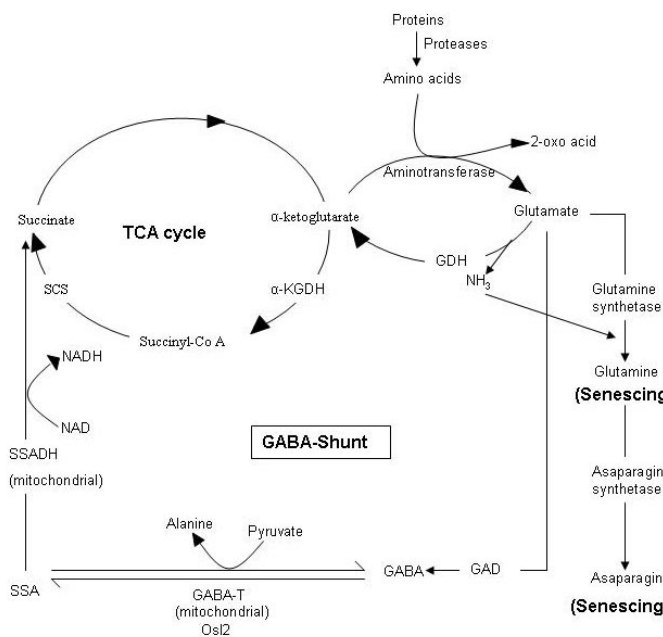


Figure 6: Possible metabolism of proteins and amino acids during rice leaf senescence and role of GABA:pyruvate-transaminase during this process (Osl2 is GABA:pyruvate-transaminase in GABA-Shunt). GDH : Glutamate dehydrogenase, GAD : Glutamate decarboxylase, α -KGDH : α -ketoglutarate dehydrogenase, GABA-T : Gamma-aminobutyric acid-transaminase, SSA : Succinic semialdehyde, SSADH : Succinic semialdehyde dehydrogenase, SCS : Succinyl CoA synthetase, TCA : Tricarboxylic Acid. The arrow indicates the direction of the reaction during senescence.

Osl2 (GABA:pyruvate transaminase) was first expressed in *E. coli* in large quantity to generate the specific antibodies. Recombinant pET-30 Ek/LIC, BL-21 and in His purified was detected with expected molecular weight of about 61.0 kDa which corresponds to the predicted molecular mass of Osl2 protein (56.0 kDa) plus the fused His tag (5.0 kDa) but it was absent in BL-21 which contain no vector. The Osl2 enzyme, GABA:pyruvate-transaminase activity was assayed at the different leaf developmental and senescence stages. In S3 stage the activity of GABA:pyruvate-transaminase was almost 2.5 fold higher compared to the MG stage of leaf. Earlier higher level of GABA accumulation has been reported in stress conditions such as hypoxia, high salt, drought, temperature and mechanical stimulation (Van Cauwenbergh *et al.*, 2002, Kinnersley and Turano 2000).

During senescence, degradation of macromolecules such as proteins, nucleic acids and lipids took place and these are mobilized to the developing area of plants (Buchanan-Wallaston 1997). The nitrogen which is present in proteins and nucleic acids molecules is converted to amino acids, particularly glutamine and asparagine, which are the predominant amino acid

transported through the phloem from the senescence leaves to the developing parts (Feller and Fischer 1994). Transaminase plays a role for converting α -amino groups from a variety of amino acids into ammonia and this ammonia can be converted into glutamine by glutamine synthetase (Mifflin and Habash 2002). This glutamine is transported from senescing leaves to other growing parts (Fig.6). It has been reported that conversion of glutamate to GABA is enhanced when there is inhibition of glutamine synthesis, reduced protein synthesis or enhanced protein degradation (Satyanarayan and Nair 1990). GABA is already known to stimulate ethylene biosynthesis (Kathiresan *et al.*, 1997) which is a triggering factor in senescing process (Smart 1994). Probably this GABA is temporary storage of nitrogen during mobilization of nitrogen during senescence. The metabolism of GABA is directly related with glutamate metabolism (Bown and Shelp 1997). So during senescence part of the glutamate converted to the GABA by enzyme glutamate decarboxylase (GAD), which is already reported that GABA synthesis from glutamate in controlled by glutamate decarboxylase (Sunedden *et al.*, 1996). This GABA is converted to succinate into mitochondria by the enzyme succinic semialdehyde dehydrogenase (SSADH) as shown in Fig. 6. The pathway that converts glutamate to succinate via GABA is called GABA shunt (Shelp *et al.*, 1999). The presence of GABA-T and succinic semialdehyde dehydrogenase (SSADH) in mitochondria has been reported in developing soybean cotyledons (Breitkreuz and Shelp 1995). Further the fate of GABA nitrogen depends on the transamination reaction, both pyruvate and α -ketoglutarate can act as amino group acceptors producing alanine and glutamate (Van Cauwenbergh and Shelp 1999). Van Cauwenbergh *et al.*, (2002) identified *Arabidopsis* GABA:pyruvate-T cDNA and functionally expressed in *E. coli*. This recombinant protein has specificity for pyruvate not with 2-oxoglutarate. Our results are consistent with these findings. Bouche *et al.* (2003) analyzed the *Arabidopsis*-T DNA knockout mutant of succinic semialdehyde dehydrogenase (SSADH), which is ultimate enzyme of GABA shunt and concluded that GABA shunt is preventing the accumulation of reactive oxygen intermediate and cell death, which is essential for plant defense against environmental stress. So GABA-shunt is essential for normal plant growth. Now it is need of the time for generation of GABA-transaminase mutant to determine the role of GABA nitrogen and its physiological function in plant. Recently (Ludewig *et al.* 2008) have reported that *Arabidopsis* mutant of GABA Transaminase (POP2) suppress the phenotype of succinic semialdehyde dehydrogenase.

In summary the Osl2 enzyme, GABA:pyruvate-transaminase play a key role in nitrogen metabolism

during rice leaf senescence. During senescence amide group from most of the amino acid can be transferred to α -ketoglutarate and this α -ketoglutarate by action of transaminase converted to glutamate. Part of this glutamate by enzyme glutamine synthetase changed into glutamine and part of glutamate goes into the GABA shunt. During GABA shunt glutamate with the help of glutamate decarboxylase (GAD) changed into GABA. Now GABA:pyruvate-transaminase catalyses the reversible conversion of GABA into succinic semialdehyde (SSA), this SSA with the help of enzyme succinic semialdehyde dehydrogenase (SSADH) changed into succinate and goes into TCA cycle (Fig. 6).

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