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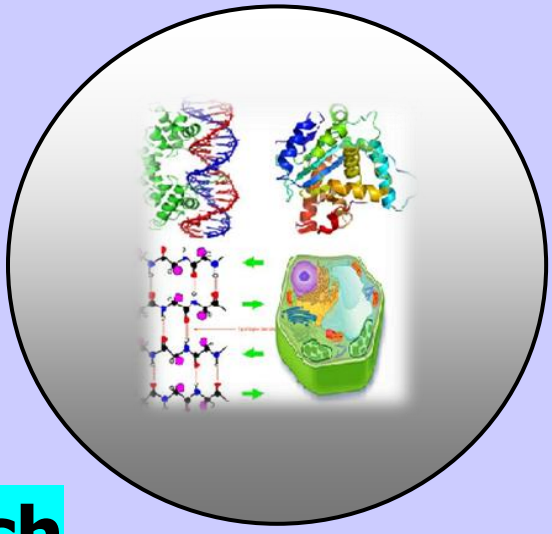
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66
Index Copernicus International Value
IC Value of Journal 47.86 Poland, Europe

J. Biol. Chem. Research
Volume 33 (2) 2016 Pages No. 909-917



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 33, No. 2: 909-917, 2016
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 33/2/46/2016

ISSN 0970-4973 (Print)
ISSN 2319-3077 (Online/Electronic)

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RESEARCH PAPER

Received: 01/08/2016 Revised: 25/11/2016 Accepted: 26/11/2016

Screening of *Arabidopsis thaliana* GABA – Transaminase Mutant and its Root Pattern Analysis

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ABSTRACT

γ-aminobutyric acid (GABA) accumulates by key signalling metabolic GABA shunt pathway that consist of three enzymes glutamate dehydrogenase (GAD) that convert glutamate into GABA that further catalysed by GABA transaminase (GABA-T) into succinic semialdehyde, which is oxidized into succinate by succinic semialdehyde dehydrogenase (SSADH) and thus enters into tricarboxylic acid cycle. GABA-transaminase plays a critical role in carbon and nitrogen metabolism during the plant development process. We have characterized GABA transaminase knock out mutant pop 2-1 that is transition mutant contains G to A transition in 3' splice site at exon 7 on chromosome 3 and pop 2-3 is T-DNA insertion mutant of Arabidopsis thaliana. Here we have screened pop 2-1 homozygous plant of GABA-T mutants by derived cleaved amplified polymorphic sequence (dCAPS) method and pop 2-3 designing primer from flanking region. Root growth pattern comparison in wild and GABA-T mutant of Arabidopsis thaliana was examined. GABA transaminase mutant are deficient root hairs where as wild type plants roots hairs are more in number and larger than pop 2-1 mutant, on the other hand pop 2-3 plants shows wavy and branched root hairs.

Key words: γ-aminobutyric acid (GABA), GABA Transaminase, GABA Shunt and Root.

INTRODUCTION

GABA shunt play key role in carbon/ nitrogen metabolism during leaf senescence and it is also important for plant growth and development (Bouché and Fromm, 2004; Fait et al., 2008Ansari et al, 2014). γ-aminobutyric acid (GABA) is one of the integral components of GABA shunt and it act as inhibitory neurotransmitter in mammalian central nervous system (Reimer et al, 2001). GABA play role as metabolites as well as signalling molecule in many plant mechanism, it accumulate during various stress conditions in plant cell including leaf senescence process (Kinnersley, 2000., Boucheand Fromm, 2004., Ansari and Chen, 2009). It has been studied that GABA shunt pathway under abiotic stress can potentially regulate GABA metabolism (Al-Quraan and Al-Share, 2016).

GABA shunt in plants has been discovered for the first time more than half a century ago in potato tuber. GABA shunt is a metabolic pathway that converts glutamate to succinate via GABA is important for normal plant growth as well as for plant defence against environmental stress (Bouche et al. 2003). GABA transaminase which converts GABA to succinic semialdehyde (SAA), is one of the important enzyme in GABA shunt (Clark et al.2009).

GABA is a non-protein amino acids found in unicellular eukaryotes, prokaryotes `and in plants (Satyanarayan and Nair 1990, Shelp et al. 1999). GABA is produced in the cytosol (Breitbrenz and Shelp, 1995) via the decarboxylation of glutamate in reaction catalysed by glutamate decarmoxylase a calcium dependent enzyme (Baum et al, 1993).The production of GABA in plants is significantly enhanced in response to various biotic and abiotic stresses such as hypoxia, low temperature and mechanical stimulation (Bown and Shelp 1997; Kinnersley and Turano 2000; Wallace et al. 1984, γ radiation, low pH (Lane and Stiller 1970) and darkness.

The role of GABA in plants has not been established but it is mostly considered to be a metabolite involved in pH regulation, nitrogen storage, development, reproduction and pathogen defence (Shelp et al. 1999). GABA has been reported to stimulate ethylene biosynthesis in sunflower (Kathiresan et al. 1997) and have been suggested that GABA acts as an intercellular signal molecule (Bouche´ et al. 2003b; Palanivelu et al. 2003). Bouche´ et al (2003a) reported that *Arabidopsis* T-DNA knockout mutants of SSADH show a rapid increase in the levels of hydrogen peroxide and enhanced cell death in response to ultraviolet radiation and heat stress. This study establishes a role for the GABA shunt in preventing the accumulation of reactive oxygen intermediates and cell death, which is essential for plant defence against environmental stress.

Palanivelu et al. (2003) reported that an *Arabidopsis* mutant of GABAT (*pop2*) displays aberrant pollen tube growth and infertility. Ansari et al. (2005) demonstrated that this GABA-T gene is up regulated during rice leaf senescence and suggested that the role of the GABA shunt within mitochondria may be to provide carbon skeletons to replenish the carboxylic acids of the TCA cycle. GABA shunt is a metabolic pathway that converts glutamate to succinate via GABA, is important for normal plant growth as well as for plant defence against environmental stress (Bouche et al. 2003).

Roots hairs are cylindrical shaped outgrowth of root emerge from epidermal cells that are important for uptake of water, nutrients, microbe interactions, and plant anchorage (Foreman J and Dolan L, 2001). The study of root hair biology in *Arabidopsis* has provided a model cell type for insights into many aspects of plant growth and development.

In this study we have analysed the *Arabidopsis thaliana* knock-out mutant plants *pop 2-1, pop 2-3* and wild type plant screening of homozygous plants and its root growth pattern comparison with wild and mutant of *Arabidopsis thaliana.*

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana Landsberg *erecta* ecotype (L *er*) was use as wild type. Seeds of the *pop2-1 and pop2-3* mutants of GABA-transaminase were kindly provided by Arabidopsis Biological Resource Centre (ABRC), Ohio State University, Columbia, USA. Plants were grown at 22^{o} C for long day condition (16 h light / 8 h dark cycle) aseptically or on soil. For soil growth, seeds were sown in Bio-Mix Potting Substratum, Soilrite (Keltech Energies Ltd. India.) and placed at 4^{o} C for 4 days in dark to break residual dormancy and later transferred to normal growth conditions. For aseptic growth condition, seeds were treated with 70% ethanol for 5 min and then with 30% household bleach for 15 min, washed 10 times with sterile double distilled water and plated on MS medium solidified with 0.8% agar. MS medium was supplemented with 2% sucrose.

Extraction of Genomic DNA

1.0g leaf sample were ground in liquid nitrogen and transferred to 10 ml polypropylene tube and add 3ml of pre warmed extraction buffer (2% CTAB, 1% PVP, 100 mM Tris HCl (pH 8.0), 25 mM EDTA pH 8.0, and 2 M NaCl) kept at 60^{o}C for 1.5 hour. Add 3 ml of chloroform: isoamyl alcohol (24:1) and mix by inversion to form emulsion. Centrifuge at 10,000 rpm for 10 min at 25^{o}C, carefully transfer the aqueous phase to fresh tube containing 1.5 ml of 5M NaCl and mix properly. Add 0.6 volume of isopropanol and let the mixture stand at room temperature for 1 hour, centrifuge at 10,000 rpm for 5 min at room temperature. Discard the supernatant and wash the pallet with 80% ethanol. Dry the pallet for 15 min and dissolve it in of TE buffer. Yield and quality of genomic DNA was checked on 0.8% agrose gel.

PCR Analysis

Total genomic DNA was extracted from the leaves of the individual plants using CTAB method. Quality of the genomic DNA was analysed by electrophoresis on 0.8% agarose gel**.** Homozygous mutants were screened by PCR by using200 ng DNA, 1U Taq DNA Polymerase, 0.55 µM gene specific forward and reverse primers (Forward primer 5' GAGTTGTTTATGAATTTCCTGTATACCTAATGC 3' and Reverse primer 5'CCAATAAATGAGGGCAATCTGTGTGT 3') 0.2 mM dNTPs, and 1X PCR buffer in a final volume of 20 µl which was performed on Bio Rad thermo cycler for 35 cycler at 94 ^{0}C for 1 min, 94^{0}C for 10 sec, 55^{0}C for 30 sec and 72^{0}C for 2 min, 72 ^{0}C for 5 min. Amplified fragments were visualized by ethidium bromide in agarose gels. *Pop 2-1* were analysed by dCAPS (Michaels and Amasino 1998) method and *pop 2-3* with T-DNA insertion was analysed by designing primers from flanking region.

Analysis of *Arabidopsis thaliana* knock-out mutants of GABA transaminase

Homology search against sequence database was performed using the BLAST programme at National Centre for Biotechnology Information, USA.

Analysis of root variation of GABA transaminase mutant and wild type plants

For visual screening of *pop 2-1*, *pop 2-3* and wild type plant the root hair phenotype, seeds were plated on MS medium solidified with 0.8% agar. MS medium was supplemented with 2% sucrose and after fifteen days visualised by Leica stereomicroscope MZ 125 (Leica, Glattbrugg, Switzerland).

Salt stress treatment on GABA transaminase mutant and wild type plant

To check *pop2-1*, *pop2-3* and wild type plant for salt stress using sodium chloride (NaCl) on MS agar medium control and experimental with different concentration of NaCl50, 100 and 150 mM. Seeds of mutant and wild type were grown on agar medium supplemented with different concentration of salts and without salts or control by placing the petriplate at 25^{o}C.After ten days root length of plantlets were measured and effect of salt concentration on root was observed.

RESULT AND DISCUSSIONS

***Arabidopsis thaliana* knock-out mutants of GABA transaminase**

GABA transaminase *Arabidobsis thaliana* knock-out mutant plants *pop 2-1* mutant has transition G→A in 3' splice site at exon 7 on chromosome 3 and *pop2-3* is T-DNA insertion mutant of *Arabidopsis thaliana*, The Osl2 (AF297651) gene has very high homology (75%) with *Arabidobsis thaliana* GABA transaminase (AF351125) and this is a single copy gene both in *Arabidobsis* as well as in rice plant. *Pop 2* closely related genes are also present in, *Capsicum* and tomato (Palanivelu et al. 2003). As we have work before on Osl 2 gene in rice plant so to characterize this gene further we decided to work on *Arabidopsis thaliana.*

Isolation of homozygous mutants and genotype characterization

Total number of 100 plants were taken and subjected to PCR analysis. The genomic DNA was isolated from the leaf tissues of selected plants and PCR was performed to isolate the homozygous mutant. Agarose gel profile representing the PCR products showed amplified fragment of 395 bp. The results of PCR confirmed the homozygous plants, out of 100, 71 plants were homozygous, 14 plants were heterozygous and 4 were wild type as shown in Fig. 1.

Variation in root morphology of GABA-transaminase knockout mutant and wild type plant

The root hairs of wild type plants are more in number and larger than *pop2-1*, on the other hand *pop 2-3* plants shows wavy, branch, and undergo random changes in the growth direction. In addition, the root hair base is frequently larger than in wild type.

Mutant plants develop root hairs that are very short. Often, swollen bases develop and the hair proper is almost absent. Similarly, root hairs of wild type plants are large and frequently have enlarged root hair bases. *Pop 2-3* plants develop a few root hairs that appear wild type like in length but often branch at the enlarged bases, whereas many root hairs develop into short stumps as shown in Fig. 2.

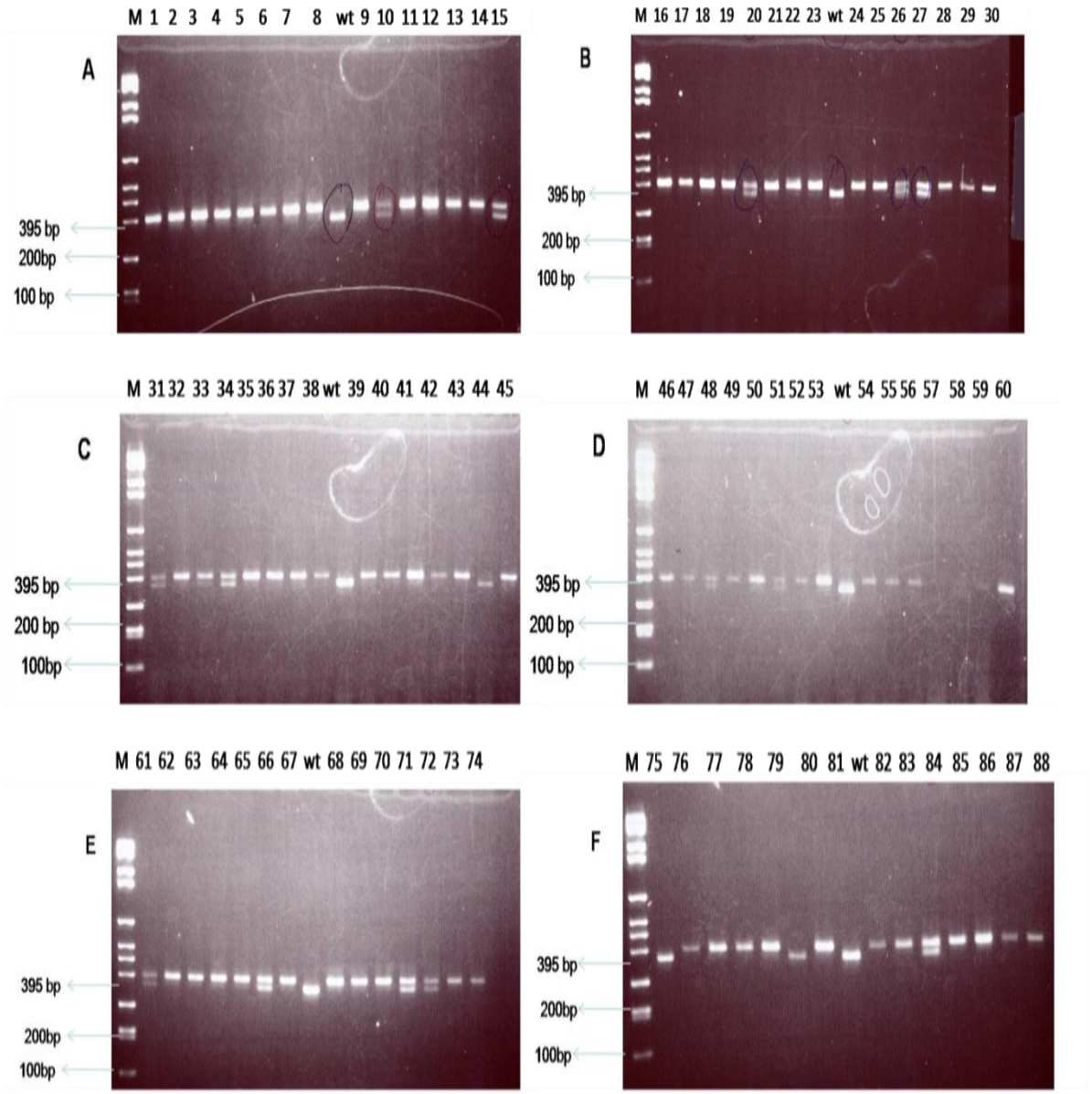

Fig. 1 (A) M-1Kb marker, lane 1,2,3,4,5,6,7,8,9,13,14,16 are homozygous and lane 10 and 15 are heterozygous and wt is wild type (B) lane 16,17, 18,19, 21, 22, 23, 24, 25, 28, 29and 30 are homozygous and lane 20, 26 and 27 are heterozygous (C) lane 32,33,35,36,37,38,39,40,41,42, 43 and 45 are homozygous and lane 31, 34 are heterozygous and wt as well as 44 are wild type (D) lane 46,47,49,50,52,53,54,55,56,57,59,60 are homozygous and lane 48, 51 are heterozygous and wt is wild type (E) lane 62,63,64,65,67,68,69,70,73 and 74 are homozygous and lane 61,66,71 and 72 are heterozygous and wt is a wild type (F) lane 76,77,78,79,81,82,83,85,86,87 and 88 are homozygous and lane 84 is heterozygous and lane 75,80 are wild type.

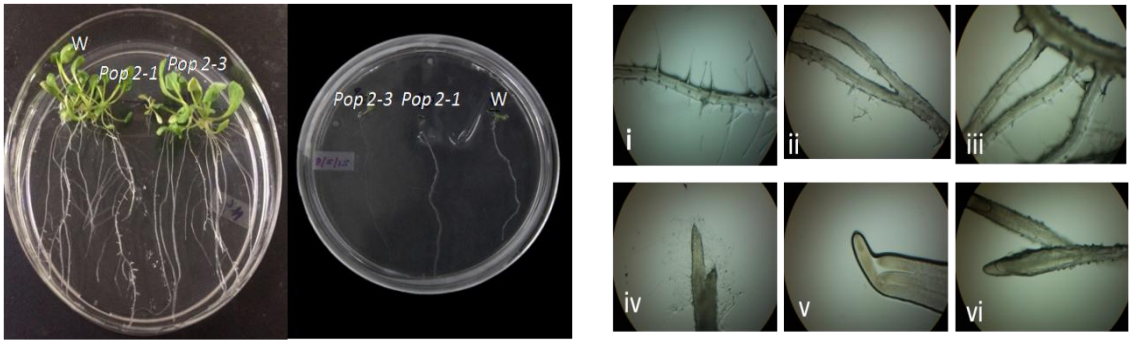

Fig. 2. Variation in root of mutant and wild type plant.(A) Variation in root morphology on MS media. (B) Microscopic analysis of wild type and mutant root i. Middle part of wild type plant root ii. Middle part of *pop 2-1* root iii. Middle part of *pop 2-3* root iv. Root tip of wild type plant v. Root tip of pop 2-1 vi. Root tip of *pop2-3*.

Root hairs are essential for plant growth and development and are convenient to study since they are on the exterior of the root (Foreman J and Dolan L, 2001).The simplicity of the patterning and the range of mutants with defects in hair pattern and morphology make the *Arabidopsis* root hair a useful model for the study of plant cell growth and for tip growth in particular. (Grierson et al. 2014).

The *pop 2-1 and pop 2-3* mutants are strongly affected in root hair development and barely develop root hairs rather than wild type. The comparison of the *GABA-T* mutation with the wild type plants suggests that the *GABA-T* mutation, which causes intolerants with stress conditions it may be due to the barely develops root hairs of GABA-T mutants.

Various studies has been done on root hair study of different mutants, as four recessive *enl* mutants were analyzed, three of which define new genetic loci involved in root hair development. (Diet et al. 2004). The *enl 1* mutant fits in the same group as *cen 1*, *cen 2*, and *cen3*, which have short root hairs combined with additional deformations (Parker et al. 2000). Mutants in the *rhd 3* locus, which encodes a GTP-binding protein, develop root hairs similar to the *enl 1* phenotype (Wang et al. 1997, Jones et al. 2002).

The GABA-T deficient mutant pop2-1 show over sensitivity to NaCl

The sensitivity to NaCl of GABA-T deficient *pop 2-1*, *pop 2-3* mutant and wild type on agar medium has been tested. In this case, NaCl treatment induced severe phenotype in the mutant, even death on agar medium supplemented with 150 mM NaCl, whereas no obvious difference occurred under control conditions between the mutant and wild type as shown in Fig. 3. *Pop 2-1* over sensitivity to NaCl, comparison to wild type on agar media supplemented with various salts concentration. Unlike to wild type mutant root growth was indeed sharply reduced at 50 mM NaCl and decreased linearly as NaCl concentration increased in the medium as shown in Fig. 3 and Table 1.

Furthermore, seedlings of *pop 2-1* showed yellowing of leaves on 50 mM NaCl concentrations and pop 2-3 seedlings yellowing of leaves on 100 mM NaCl concentration rather than wild type sidling showed no clear symptoms below 150 mM. These observations indicate that *pop 2-1* mutant is oversensitive to ionic stress. 10-day-old plantlets when treated with 150 mM NaCl for 4 days induced a greater growth inhibition in *pop 2-1* than wild type. GABA accumulation also increased in the shoot and root of A*rabidopsis* plants after salt treatment (Renault et al., 2010).

Table 1. Effect of salt stress on root length of GABA-T mutants and wild type plants.

Salt (NaCl) concentration	**Root Length (Mean ± SD) cm**		
	Wild type	***Pop 2-1***	***Pop 2-3***
Control	4.33±0.15	3.64±0.02	3.82±0.03
50mM	4.04±0.01	2.24±0.015	3.21±0.025
100mM	2.8±0.1	1.39±0.025	1.69±0.011
150mM	1.48±0.02	0.23±0.015	0.81±0.19

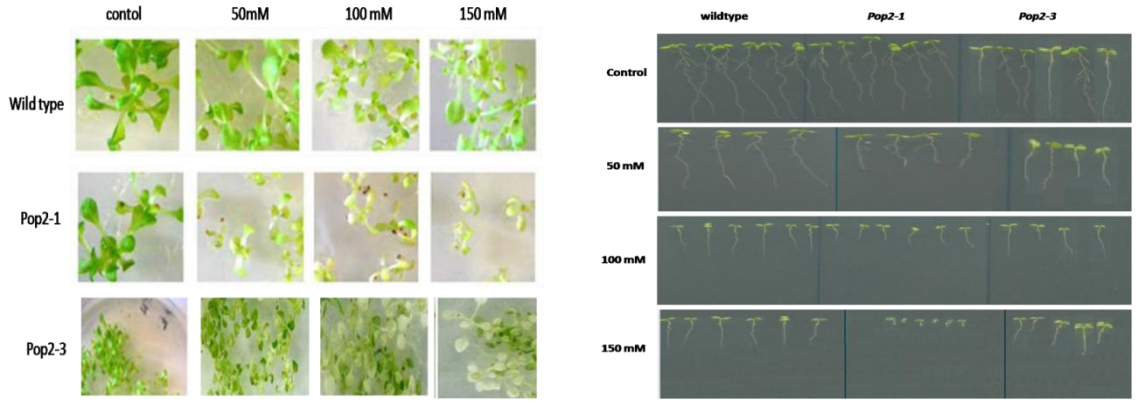

Fig. 3. Effect of NaCl treatment on seedlings and root length of wild type and mutants plants.

CONCLUSION

In plants role of γ-aminobutyric acid (GABA) is not well defined, but this is known as inhibitory neurotransmitter in mammalian central nervous system. It is reported that GABA accumulates during various stress responses. We conclude that GABA transamination *via* GABA-TA is an enzymatic step that can potentially regulate GABA metabolism in *Arabidopsis* seedlings. In this study, we have characterized GABA-transaminase knock out mutant *pop 2-1* that is transition mutant and *pop 2-3* is T-DNA insertion mutant of *Arabidopsis thaliana*. Analysis of *pop 2-1* and *pop 2-3* has revealed *pop 2-1* knock-out mutant does not show an aberrant phenotype, the *pop 2-1 and pop 2-3* mutants are strongly affected in root hair development, and barely develops root hairs.

We also suggest that wild type plant have a greater tolerance against salt stress, as compared to GABA-T deficient *pop 2-1* and *pop 2-3* mutant plants. Interestingly, the comparison of the *GABA-T* mutation with the wild type plants suggests that the *GABA-T* mutation, which causes intolerants with stress conditions it may be due to the barely develops root hairs of GABA-T mutants. Further to understand the role of GABA transaminase has tremendous potential in the field of agricultural crop productivity.

ACKNOWLEDGEMENTS

The authors are thankful to Arabidopsis Biological Resource Centre (ABRC), Ohio State University of Columbia, USA for mutant seeds. This research was financially supported by Science and Engineering Research Board (SERB), Department of Science and Technology Govt. of India, New Delhi (Grant No.SB/SO/BB-002/2012).

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