ORIGINAL ARTICLE

Functional complementation of *pop2-3* T-DNA insertion mutant of *Arabidopsis thaliana* by overexpression of gamma-aminobutyric acid transaminase gene to reveals its role in complex pattern of leaf senescence

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Abstract

Aim: Gamma-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the mammalian central nervous system, and its important role is reducing the neuronal excitability throughout the nervous system. The GABA transaminase (GABA-T) enzyme catalyzes the transamination of GABA to form succinic semialdehyde using pyruvate as amino acid acceptor in GABA shunt pathway. The *Arabidopsis thaliana* GABA-T gene (pollen-pistil incompatibility 2 [*POP2*]; *At3g22200*) belongs to Class III pyridoxal-phosphate-dependent aminotransferase family. The full-length cDNA of the gene encodes a protein of 513 amino acids residues and located at chromosome 3. It could be demonstrated that the GABA-T is involved in the regulation of leaf senescence in *A. thaliana*. **Materials and Methods:** To ensure the role of GABA-T gene in leaf senescence, we have performed functional complementation using transformation of pPZP200GB-GABA-T construct into *pop2-3* T-DNA insertion mutant (GABA-T knockout mutant) of A. *thaliana* (GABA-T complement plant) that the mutant plant reverts back to wild-type plant. We have measured and compared the physiological characteristics associated with senescence (chlorophyll content, ion leakage and lipid peroxidation) and quantified the GABA shunt components (GABA content, GABA-T, and glutamate decarboxylase activity) of the wild-type, *pop2-3* mutant, and GABA-T complement plant of *A. thaliana*. **Results:** The result showed the low chlorophyll content, increased ion leakage, and higher level of lipid peroxidation in *pop2-3* mutant than wild-type plant after 30 days in normal growth condition. GABA-T *pop2-3* mutants act as signals that mediate early leaf senescence. GABA-T complement plant shared similar characteristics of senescence parameters with wild-type plants. **Conclusion:** These data concluded that GABA-T is involved in the regulation of leaf senescence in *A. thaliana*.

Key words: Complementation, gamma-aminobutyric acid-transaminase, senescence, T-DNA insertion mutant

INTRODUCTION

Gamma-aminobutyric acid (GABA) is a ubiquitous non-protein amino acid and found in prokaryotes and eukaryotes.[1] GABA has received increased attention in research due to its role as an inhibitory neurotransmitter in the mammalian central nervous system by binding to specific transmembrane receptors in the plasma membrane of both pre- and post-synaptic neuronal processes.[2,3] In humans, GABA is also directly responsible for the regulation of muscle tone, and it regulates many of the depressive and sedative actions in brain tissue and is critical for relaxation.[2-4] Rapid GABA accumulation has been reported in plants under biotic and abiotic stress conditions,[5] and the

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evolvement of GABA-shunt in responses to abiotic stress has been reported by Bouché and Fromm[1] and Ludewig *et al*.[6] These stresses initiate a signal transduction pathway, which increased cytosolic calmodulin-dependent activity of the glutamate decarboxylase (GAD). However, it appears that GABA is important for vegetative development. GABA is catabolized through GABA transaminase (GABA-T) to succinic semialdehyde (SSA), which in turn is oxidized through a NAD-dependent SSA dehydrogenase (SSADH) to succinate.

GABA-T (EC 2.6.1.19) is a key enzyme in GABA shunt that involved in the carbon and nitrogen metabolism in plants. The GABA-T encoding gene (*At3g22200*) is present as a single copy in the *Arabidopsis* genome and was initially termed pollen-pistil incompatibility 2 (*POP2*).[7] The GABA-T enzyme catalyzing the reversible transamination of GABA with either pyruvate or 2-oxoglutarate as an amino acceptor to form SSA and either alanine or glutamate.[8] *A. thaliana* GABA-T knockout *pop2-3* has T-DNA insertion mutation at intron 14, and T-DNA insertion plants are indistinguishable from wild types during vegetative growth.[7,9]

During the process of senescence, some protein will be degraded into α-amino acid with the assistance of proteases. The ammonia released from the transamination of α-amino acid will be used to convert it into glutamate and α-keto acids with the assistance of enzyme glutamine synthetase into glutamine. Further, this glutamine with enzyme asparagine synthetase will be converted into asparagine.[10-12] GABA is produced in cytosol through the decarboxylation of glutamate in pathway catalyzed by GAD. The process of GABA to glutamate transformation accelerates when there is restraint of protein synthesis.[12,13] The metabolism of GABA is identified with glutamate metabolism in senescence process, and then, the glutamate will go into GABA shunt. This GABA is transaminated to SSA through GABA-T; thus, SSA is oxidized to succinate through SSADH in mitochondria and enter into tricarboxylic acid cycle. Therefore, GABA-shunt pathway converts glutamate into succinate through GABA in the plant leaf senescence and nitrogen assimilation.[11,14]

We have previously published the physiological and metabolic characterization of the GABA-T *pop2-3* mutant on different stress responses. Based on screening of senescence parameters, we showed that knockout of the GABA-T causes enhanced accumulation of reactive oxygen species and cell death in response to different stress conditions.[15] In the present study, we have investigated the function of *A. thaliana* GABA-T gene in leaf senescence by functional complementation of GABA-T into *pop2-3* T-DNA insertion mutant in conjunction with GABA-shunt component analysis. We demonstrate here that *pop2-3* T-DNA insertion mutant expressed early leaf senescence than wild type, based on screening of senescence parameters. A comparative analysis in the *pop2-3* mutant, wild-type and GABA-T complement plant reveals that GABA-T controls both cellular protection activities and senescence activities and involved in the regulation of leaf senescence process.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

A. thaliana Landsberg erecta ecotype (Ler) were used as wild type, and *A. thaliana pop2-3* homozygous T-DNA insertion mutant plants for GABA-T (Stock name CS6387) obtained from Arabidopsis Biological Resource Center (Ohio State University, USA) were find out using polymerase chain reaction (PCR) with primer from the left and right border of T-DNA and primer from flanking region. Plants were grown at 22°C for long-day condition (16 h light/8 h dark cycle) on the soil. For soil growth, seeds were sown in Bio-Mix Potting Substratum (Keltech Energies Ltd. India) and placed at 4°C for 4 days in the dark to break residual dormancy and later transferred to normal growth conditions.

RNA Isolation and Reverse Transcriptional PCR (RT-PCR)

Total RNA for RT-PCR was isolated from the 3-week-old leaf of *A. thaliana* by RNA extraction mini kit (Promega, Madison, WI) according to the manufacturer's instructions. cDNA synthesis (using SuperScript III Reverse Transcriptase) was done as described by the supplier (Invitrogen, Karlsruhe, Germany). The cDNA was diluted 25 times and used to amplify the GABA-T gene using gene-specific primers as described by Miyashita and Good, Al-Quran and Share.[16,17]

Generation of Plant Transformation Construct and *Arabidopsis* Transformation for Complementation

Coding region of *A. thaliana* GABA-T gene (GenBank accession number AF351125) was cloned into binary vector pPZP200GB using *XbaI* and *SacI* restriction enzyme by replacing the β-glucuronidase fragment. This pPZP200GB with β-glucuronidase and BAR (BASTA resistance gene) cassettes was derived from pBI221 (Clontech Laboratories, Palo Alto, CA) and pSK-35S-BAR.[18] The obtained plasmid construct was named pPZP200GB-GABA-T. The pPZP200GB-GABA-T construct was transformed into *Agrobacterium tumefaciens* strain C-58 by electroporation. *A. thaliana* GABA-T T-DNA insertion mutant (Stock name CS6387) was transformed through *Agrobacterium*-mediated transformation by floral-dipping method.[19] Transgenic plants were selected by spraying seedlings at 7, 9, and 11 days after germination with a solution of 0.4% of BASTA herbicide.[20] T2 generations were selected for isolating homozygous lines.

Analysis of Leaf Senescence Parameters

All parameters of leaf senescence (total chlorophyll content, electrolyte leakage, and lipid peroxidation) were measured with the fifth rosette leaves, which were harvested at specified days from different developmental stages (10-, 20-, and 30-day-old plant of *A. thaliana*).

Measurement of Total Chlorophyll

Total chlorophyll content was isolated from the leaves of *pop2-3* mutant, wild-type, and complement plants from 10-, 20-, and 30-day-old plant and measured as per milligram fresh weight of tissue.[21]

Measurement of Membrane Lon Leakage

Leaves material of *pop2-3* mutant, wild-type, and complement plants at different developmental stages (10-, 20-, and 30-day-old plant) were used for the determination of membrane ion leakage according to Chen *et al*.[22]

Quantification of Malondialdehyde (MDA)

The extent of lipid peroxidation was estimated by determining the concentration of MDA from leaf material (FW) of *pop2-3* mutant, wild-type, and complement plants at different developmental stages (10-, 20-, and 30-day-old plant) according to Heath and Packer.[23]

Quantification of GABA Content

GABA was extracted and determined from frozen tissues of different developmental stages (10-, 20-, and 30-day-old plant) of wild-type, *pop2-3* mutants, and complement plants; the GABA content in the sample was calculated based on the difference in absorbance. The standard GABA was prepared in micromole concentration and used for constructing the calibration graph as done in the previous study by Jalil *et al.*[15]

Determination of Enzyme (GABA-T and GAD) Activities

GABA-T and GAD activities assay were performed from the protein that was extracted from the leaves of wild type, *pop2-3* mutants, and complement plants of *A. thaliana* at different stages (10-, 20-, and 30-day-old plant) as described earlier by Jalil *et al.*[15]

Statistical Analysis

Statistical analysis was carried out using Microsoft excel, and each data point was expressed as mean ± standard deviation of three independent experiments.

RESULTS

Comparative Analysis of Physiological Parameters of Leaf Senescence

To confirmed the physiological state of the complement plants, chlorophyll content, ion leakage, and lipid peroxidation of the *pop2-3* mutant, wild–type, and complement plants leaves at different developmental stages (10-, 20-, and 30-day-old plants) were measured. We first examined the chlorophyll content of wild-type, *pop2-3* mutants, and complement plants, and we observed that the chlorophyll content of *pop2-3* mutants in 10, 20, and 30 days (1.29 ± 0.01, 1.28 ± 0.01, and 0.63 ± 0.09/mg, respectively) were less than the wild-type plants (1.97 ± 0.01, 1.92 ± 0.02, and 1.79 ± 0.10/mg, respectively). Chlorophyll content of *pop2-3* mutant was stable up to 20 days but decreased from 30 days, whereas chlorophyll content in wild-type plant was almost stable up to 30 days. The chlorophyll content of complement plant was approximately similar to wild-type plant and stable up to 30 days (1.86 ± 0.1, 1.79 ± 0.1, and 1.67 ± 0.06/mg, respectively) as shown in Figure 1a.

Ion leakage in *pop2-3* mutant was stable up to 20 days from the date of emergence (10 days old 16.97 ± 0.01% and 20 days old 17.34 ± 0.001%) and began to sharply increase from 30 days (54.37 ± 0.05%). However, ion leakage in wild-type leaves stable up to 20 days (16.01 ± 0.004% and 16.94 ± 0.006%) and slightly increased from 30-day-old plant (19.36 ± 0.024%) and the ion leakage of complement plant were approximately similar to wild-type plant, and there was no increase in the ratio throughout 30 days in complement plant (16.27 ± 0.03%, 17.09 ± 0.05%, and 17.93 ± 0.03%, respectively) as shown in Figure 1b.

The MDA content in the *pop2-3* mutant was slightly greater than wild-type plant and stable up to 20 days (0.014 ± 0.001 and 0.015 ± 0.003 μM/mg, respectively) and start to increases from 30-day-old plants (0.023 ± 0.001 μM/mg). Whereas, in wild-type plants, MDA content was stable from 10 to 30 days (0.012 ± 0.002, 0.013 ± 0.001, and 0.014 ± 0.001 μM/mg, respectively). The MDA content of GABA-T complement plant was approximately similar to wild-type plant and stable up to 30 days (0.012 ± 0.003, 0.013 ± 0.001, and 0.013 ± 0.003 μM/mg, respectively) as shown in Figure 1c.

Quantification of GABA Content from Leaves of Wild-type, *pop2-3* Mutant, and GABA-T Complement Plants

A targeted analysis of GABA content in GABA-T complement, *pop2-3* mutant, and wild-type plants was performed and showed that GABA content of *pop2-3* mutant leaves was much higher than wild-type plant. GABA content in *pop2-3* mutant was stable up to 20-day-old plants (10 days 0.10 ± 0.03 and 20 days 0.11 ± 0.05 μmoles/g FW)

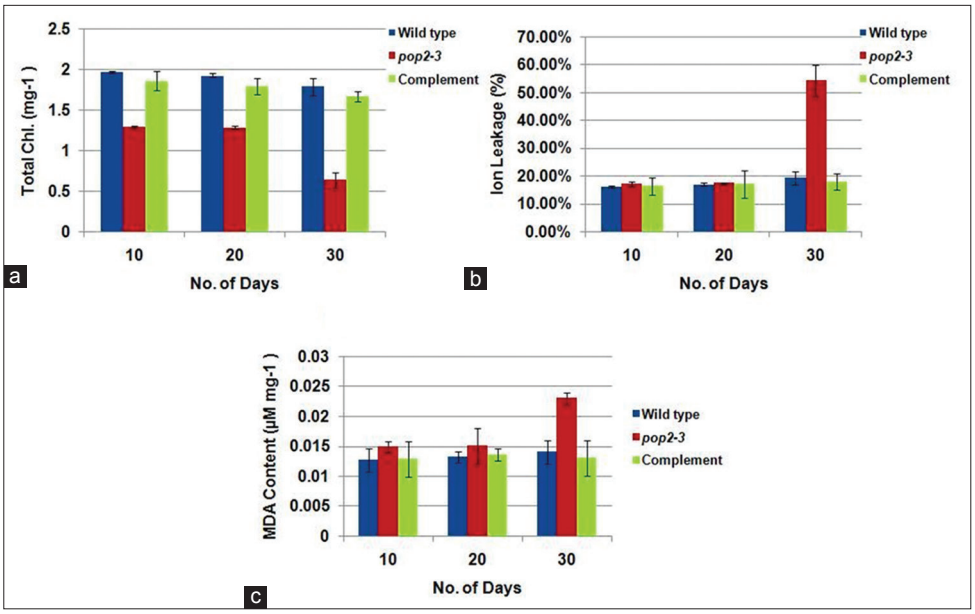

Figure 1: Physiological parameter of leaf senescence of *pop2-3* mutant, gamma-aminobutyric acid transaminase (GABA-T) complement, and wild-type plant of *Arabidopsis thaliana*. (a) Determination of chlorophyll content of wild-type, GABA-T complement, and *pop2-3* mutant leaves, (b) determination of ion leakage of wild-type, mutant, and GABA-T complement leaves, (c) Quantification of lipid peroxidation by the level of malondialdehyde wild-type, GABA-T complement, and mutant leaves. Data represent mean ± standard deviation of 3-5 biological replicates

and sharply increases from 30 days (0.46 ± 0.07 µmoles/g FW), whereas in wild type, it was approximately stable up to 30 days (0.18 ± 0.03, 0.19 ± 0.04, and 0.21 ± 0.03 µmoles/g FW, respectively). GABA content of GABA-T complement plant was approximately similar to wild-type plant and stable up to 30 days (0.19 ± 0.05, 0.22 ± 0.03, and 0.24 ± 0.01 µmoles/g FW, respectively) as shown in Figure 2.

DETERMINATION OF ENZYME ACTIVITY (GABA-T AND GAD)

Enzyme activity of GABA-T and GAD was assayed and compared during 10-, 20-, and 30-day-old plants of *A. thaliana* wild type, *pop2-3* mutant, and GABA-T complement plants [Figure 3a and b]. The enzyme activity (GABA-T and GAD) was much more in wild-type plants than in *pop2-3* mutants. The enzymes (GABA-T and GAD) activity of *pop2-3* mutant were stable till 20 days (GABA-T in 10 days 2.34 ± 0.54, 20 days 2.89 ± 0.37 nmoles/min/mg protein, and GAD in 10 days 10.09 ± 0.6, 20 days 10.27 ± 0.51 nmoles/min/mg protein), whereas it was stable till 30 days in wild-type plant (GABA-T 12.28 ± 0.36, 12.92 ± 0.57, and 13.67 ± 0.44 nmoles/min/mg protein, respectively, and GAD 20.97 ± 0.8, 21.08 ± 0.7, and 22.37 ± 0.83 nmoles/min/mg protein, respectively). The enzymes (GABA-T and GAD) activity of GABA-T complement plant was approximately similar to wild-type plant and stable up to 30 days (GABA-T 12.28 ± 0.36, 12.92 ± 0.57, and 13.76 ± 0.68 nmoles/min/mg protein, respectively, and GAD 18.86 ± 0.72, 19.32 ± 1.68, and 20.97 ± 0.91 nmoles/min/mg protein, respectively) as shown in Figure 3a and b.

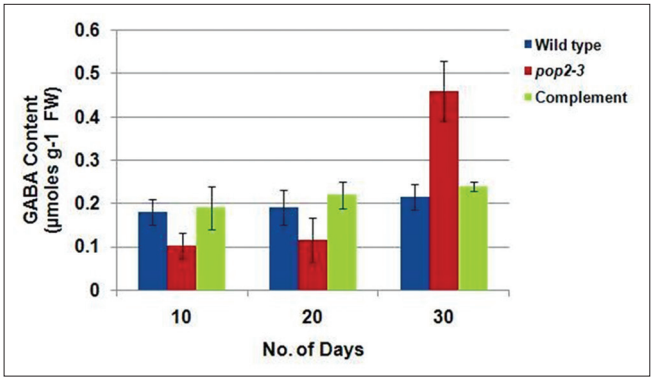

Figure 2: Determination of gamma-aminobutyric acid (GABA) content of *pop2-3* mutant, GABA transaminase complement, and wild type leaves of *Arabidopsis thaliana*. Data represent mean ± standard deviation of 3-5 biological replicates

DISCUSSION

Leaf senescence is dependent on age and developmental stage under normal conditions, and in the absence of external stress, the symptoms of senescence are normally observed in old leaves. Involvement of GABA-T gene in regulation of carbon/nitrogen metabolism and plant development has been reported by several investigators.[8,24,25] It has been also studied that wild type of *A. thaliana* is more tolerant to oxidative stress, as compared to *pop2-3* mutant of GABA-T stress, and morphology of *pop2-3* mutant might differ to wild type plants.[9,15,17,26] The oversensitivity of the *pop2-3* mutants to the abiotic and oxidative stress treatment indicates that a GABA-T defect impaired GABA accumulation that might be required for growth and implicates that GABA is involved

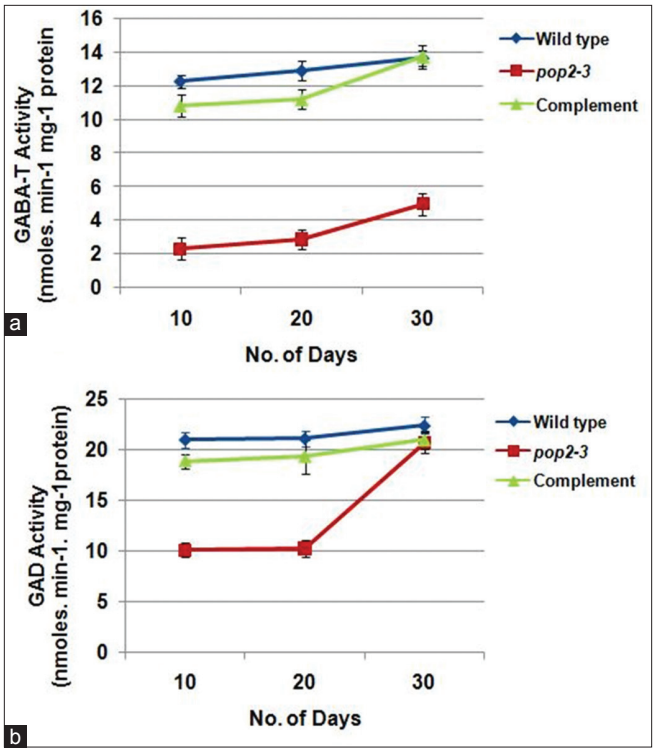

Figure 3: Determination of enzymatic activities of leaves of pop2-3 mutant, gamma-aminobutyric acid transaminase (GABA-T) complement, and wild-type plant of *Arabidopsis thaliana*, (a) enzymatic activity of GABA-T in *pop2-3* mutant, GABA-T complement, and wild type, (b) glutamate decarboxylase activity in *pop2-3* mutant, GABA-T complement, and wild-type leaves. Data represent mean ± standard deviation of 3-5 biological replicates

in intracellular signalling, especially during leaf senescence under stress conditions. T-DNA insertion *pop2-3* mutant of *A. thaliana* displays reduced seed production under self-fertilization, implying that the role of this enzyme is restricted to flower tissue.[7]

GABA-T *pop2-3* mutants act as signals that mediate early leaf senescence. Further, to confirm the role of GABA-T in leaf senescence, we have done the transformation of *A. thaliana pop2-3* T-DNA insertion mutant with pPZP200GB-GABA-T construct (GABA-T complement plant) that revert to wild-type plant. Similar experiments were performed for the complementation of *egy1* mutant to ensure the function of ethylene-dependent gravitropism-deficient and yellow-green 1 in regulation of leaf development and senescence in *A. thaliana*.[27] Several other investigators also performed the same experiment to confirm the role of a respective genes such as *TLP 18.3* gene,[12] *ASP2* gene,[28] and *CYP724A1* gene.[29]

We have previously established physiological parameters for the screening of leaf senescence that based on the determination of chlorophyll content, ion leakage, and MDA content at different leaf developmental stages.[15] We have compared the physiological characteristics associated with senescence to the wild-type, *pop2-3* mutant, and GABA-T complement plant of *A. thaliana*. Chlorophyll can provide basic information of photosynthesis, and chlorophyll degradation is an important feature of senescence. Our results showed that chlorophyll contents were low in *pop2-3* mutant than wild-type plant after 30 days in normal growth condition. Probably low chlorophyll content in mutant showed precocious leaf senescence [Figure 1a], which was similar to the reported data in *egy1* mutants,[27] *porB-1porC-1* double mutant of older seedlings,[30] and *mex1* leaves of *A. thaliana*.[31] The chlorophyll content of GABA-T complement plant was approximately similar to wild-type plant up to 30 days [Figure 1a].

During leaf senescence, membrane became fragile, leak, and released many ions in the cell. Ion leakage is an indicator of membrane integrity and important parameter to measure leaf senescence. In this study, ion leakage in *pop2-3* mutant was stable up to 20 days from date of emergence but higher than wild type plant and began to sharply increase from 30 days. However, ion leakage in wild-type leaves stable up to 20 days and slightly increased from 30-day-old plant, whereas there was no increase in the ratio throughout 30 days in GABA-T complement plant and showed approximately similar ion leakage percentage to wild-type plant [Figure 1b]. The result was as similarly found in *egy1* mutant leaf of *A. thaliana*.[27]

Determination of lipid peroxidation is another parameter to measure the leaf senescence. Our result showed that increases the level of lipid peroxidation and cell damage in *pop2-3* mutant plant in an earlier stage of leaf development as compared to wild type [Figure 1c]. Similarly, the MDA content started to increase at 40 days in wild-type plants but at 30 days in (glutathione reductase 2) *igr2-9* and *igr2-14* mutant's plant of *A. thaliana*.[32] However, GABA-T complement plant showed approximately similar lipid peroxidation level to wild-type plant up to 30 days.

GABA is an important component of GABA shunt, so we determine and compare the GABA content in *pop2-3* mutant with wild-type and GABA-T complement plant. Our result showed that GABA content of *pop2-3* mutant leaves was much higher than wild type plant and was stable up to 20 days sharply increases from 30 days, whereas in wild type, it was approximately stable up to 30 days [Figure 2]. GABA content of GABA-T complement plant was approximately similar to wild-type plant and stable up to 30 days. It has been also reported that *ataldh10A8* and *ataldh10A9* T-DNA-insertion mutants reduced GABA accumulation than wild-type plants of *A. thaliana* during salinity treatment and are more sensitive to salinity stress.[33]

GABA-T and GAD are the key enzymes in GABA-shunt pathway. Enzymes activities of these enzymes were assayed at different leaf developmental stages in wild-type and *pop2-3* mutant plant and compared with GABA-T complement plant. The enzyme activity (GABA-T and GAD) was very

less in *pop2-3* mutant than in wild-type plants. The enzyme (GABA-T and GAD) activities were stable till 20 days in *pop2-3* mutant, whereas it was stable till 30 days in wild-type plant [Figure 3a and b]. Analysis of *AtGHBDH* activity for *A. thaliana* γ-hydroxybutyrate dehydrogenase,[34] analysis of GABA-T activity in stress conditions.[9,17] The enzyme (GABA-T and GAD) activities of GABA-T complement plant were approximately similar to wild-type plant and stable up to 30 days. Taken together, these results suggested that GABA-T is involved in controlling the onset of leaf senescence in plants.

CONCLUSION

The mutation of GABA-T gene might release silencing of a subset of critical genes that regulating the leaf senescence process. It appears that GABA-T controls both cellular protection activities and senescence activities. Compare to the wild-type *A. thaliana, pop2-3* mutant showed low chlorophyll content, increased ion leakage, and increased level of lipid peroxidation than wild-type plant after 30 days in normal growth condition. Probably, these characteristics in *pop2-3* mutant showed precocious leaf senescence. *A. thaliana pop2-3* T-DNA insertion mutant after transformation with pPZP200GB-GABA-T construct (GABA-T complement plant) revert to wild-type plant and showed similar characteristics of senescence parameters with wild-type plants. Thus, this confirmed that GABA-T is an important regulator of leaf senescence in *A. thaliana* and plays crucial roles in GABA accumulation and catabolism.

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