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RESEARCH ARTICLE

# Expression study of an Amino Acid Permease-like gene in *Phaseolus vulgaris* L.

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**KEYWORDS**

Alanine; Amino acid permease-like gene; Glycine; Proline; *P. vulgaris*.

**ABBREVIATION**

AAP, amino acid permease; RT-qPCR, real time quantitative polymerase chain reaction; IDE, insulin degrading enzyme

**ABSTRACT**

Amino acid permease-like (*AAP-like*) gene plays a critical role in absorbing amino acids through roots in plants. A number of studies have been done on amino acids uptake in plants but till date there is no report about the expression of *AAP* gene in *Phaseolus* under field allied condition. The aim of this study is to measure the expression of *AAP-like* gene on alanine, glycine and proline amino acid uptake capacity in *Phaseolus vulgaris* at field relevant concentrations. Amongst three amino acids, a drastic significant increase of 63.15 fold in expression of *AAP-like* gene is observed in 50 µM alanine at 2 hr. At 50 µM of proline and 25 µM of alanine, *AAP-like* gene expression also shows high expression of 43.71 fold at 2 hr and 42.50 fold at 1 hr respectively. This study elucidated the dose dependent relationship of glycine, alanine and proline with the expression of *AAP-like* gene in amino acid transport in natural conditions in roots of *P. vulgaris*. Additionally, this research is also useful in identification of plants needing less surplus nitrogen additions and helpful in optimizing fertilizers by tailoring *AAP* gene expression to match plant uptake capacities in agriculture.

## Introduction

Plant growth and development are dependent on the attainment and distribution of nitrogenous compounds throughout the plant body. Application of nitrogen fertilizers seems to be the only solution despite of being costly and non-ecofriendly, making this an important issue. Several compounds essential to plant development that includes nucleotides, hormones, chlorophyll and secondary metabolites are synthesized from amino acids (1). Amino acids are also the building block elements for enzymes and proteins that make the skeleton and give fuel through metabolism to the plant. Plants absorb amino acids right from the soil in the form of nitrate and ammonium and thereby assimilate them to amino acids (2, 3). Most of the amino acids are synthesized in plastids, cytosol, mitochondria and peroxisomes of roots and leaves and available immediately to metabolic processes. Compartmentalization and proper channelization of the amino acids throughout plant body are performed by several transporters present in the membrane (4–6).

A number of transporters are already well-known and has been grouped into different families. Amino acid permease1 (AAP1/NAT2) was identified first in plant a long ago in *Arabidopsis* (7–9). Near about 6500 transmembrane proteins were identified in *Arabidopsis* using bioinformatic tools and programming (10). Amino acid transporters are mainly belonging to amino acid permease (AAP), lysine/histidine-type transporter (LHT), proline/compatible solute transporter (ProT), aromatic-neutral amino acid transporter (ANT1), γ-aminobutyric acid transporter (GAT) and cationic amino acid transporter (CAT) families. Transporters generally differ in substrate selectivity and affinity when analyzed in yeast or *Xenopus* oocytes, and in tissue or cellular localization. LHT1, AAP1, AAP5, ProT2 and CAT6 transporters are involved in amino acid uptake into root cells whereas LHT, AAP8, AAP1, AAP6 and AAP2 imports amino acids into mesophyll



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cells, endosperm, embryo, xylem parenchyma and transport phloem respectively. Cellular influx of glutamine and histidine, and uptake of aspartate and glutamate is performed by SIAR1 while glutamate/malate exchange across chloroplast membrane is done by DT2.1 transporter. Yet, studies have been restricted to the physiological role that includes absorbance through root, water conducting system, mesophyll cells of leaves and seeds (6, 9, 11, 12).

*Phaseolus vulgaris* is well-known for high nutritive value and also for increasing soil fertility (13, 14) but very limited study has been conducted to characterize *AAP* gene in *Phaseolus* (15). In this present study, we have amplified an amino acid permease-like gene (*AAP-like*) (NCBI accession number MH704902) from *Phaseolus vulgaris* L 'Seville' and analyze its expression pattern at different concentration of amino acids, glycine, alanine and proline under different incubation period at transcriptome level using RT-qPCR.

## Materials and Methods

### *Plant material and growth condition*

Seeds were surface sterilized using $HgCl_2$ (0.1%) and sown on sterile vertical agar plates comprising nitrogen free Murashige and Skoog (MS) medium (16), 3 mM $NO_3^-$, 1% (w/v) agar and 0.5% (w/v) sucrose and set to pH 5.8 by 7.7 mM MES. After seed germination, they were incubated in the growth chamber maintaining 16/8 photoperiod at 24±2 °C temperature and 78% humidity. All plants were allowed to grow for seven days.

### *Amino acid uptake*

Seven days old seedlings were removed from the agar plate and the root of the seedlings were blotted gently with tissue paper and then directly immersed into three aliquots of 0.5 mM $CaCl_2$ to preserve membrane integrity. The solution in excess was blotted with tissue paper after dipping third time and right away the roots of intact plants were allowed to submerge in 25 ml of a solution of glycine, alanine and proline at a 25 µM and 50 µM concentration separately for 2 hr in 50 ml vials. The roots of untreated plants submerged with water for 2 hr were treated as control.

### *RNA isolation and cDNA synthesis*

Total RNA was extracted from ~800 mg of roots by Total RNA isolation kit (Macherey-Nagel) following manufacturer instruction at 30 min, 1 hr and 2 hr from the time of submerging in amino acid solution. Integrity was checked on 1% formaldehyde agarose gel and purity were checked in Nanodrop spectrophotometer (JENWAY). First strand cDNA synthesis was performed following the instructions directed in Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific).

### *Primer designing*

Using ExPASy tool (17), the *AAP-like* gene sequence was translated to amino acid sequence. RT-qPCR primers were designed using PRIMER3 (18) from the long stretch of open reading frame obtained from the translation of the amino acid sequence. The primers for Insulin degrading enzyme (IDE) was as per standard (19).

| Gene | Forward primer | Reverse primer |
|---|---|---|
| Amino acid permease-like gene | GCTTCTACAACCCATACT | CACTGTCTGGGAATCTAC |
| Insulin degrading enzyme | GCAACCAACCTTTCATCAGC | AGAAATGCCTCAACCCTTTG |

### *Quantification of AAP gene by RT-qPCR*

RT-qPCR reactions were standardized with IDE as an internal control (19) using Bio-Rad iQ SYBR Green Supermix in a Bio-Rad CFX96 Real-Time PCR system. Standard curve was done with five different concentrations of cDNA in triplicates with a twofold dilution. cDNA of 100 ng concentration was found to give least $C_T$ value. The reaction mixture contains 1X SYBR Green Supermix, 3.2 µM of each gene specific forward and reverse primers and 100 ng of cDNA. It was then incubated at 95 °C for 2 min for initial denaturation which was then followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 20 s. Each amplicon specificity was checked on analyzing melt curve. Each reaction was performed in triplicate and the occurrence of a single peak in melt curve, specify the specificity of the amplicon being tested. Expression pattern of *AAP-like* gene at different concentrations of three amino acids namely alanine, glycine and proline at different incubation time, 30 min, 1 hr and 2 hr, was quantitated taking IDE as normalizer.

### *Statistical analysis*

The data obtained are presented as means ± SD. Analysis of variance (ANOVA) was done using SAS software 9.4 Copyright 2002–2012 by SAS institute Inc. to analyze the data considering each variable at particular treatment and incubation time at the 5% level of significance.

## Results

### *Total RNA isolation and cDNA synthesis*

Total RNA was isolated and visualized on 1% formaldehyde agarose gel. Distinct bands of 28S and 18S rRNA were found that confirmed its integrity. The purity was checked in Nanodrop Spectrophotometer (JENWAY). The ratio of 260/280 of total RNA was found 2.0 and 260/230 between 2–2.2. A smear of cDNA was found on 2% agarose gel.

### *Relative quantification of AAP gene*

Relative quantification study was performed by RT-qPCR. Specificity of the designed primers was set by analyzing melt curve. RT-qPCR was done for *AAP-like* gene and expressions were analyzed using IDE as a normalizer. The relative quantity of *AAP-like* gene was expressed in percentage and was carried out using the formula $2^{-\Delta CT}$ at 25 µM and 50 µM of glycine, alanine, proline and in control (20) (Fig. 1–4). For this study, three incubation time points, i.e., 30 min, 1 hr and 2 hr were taken to check expression level of *AAP-like* gene in presence of three amino acids glycine,

alanine and proline separately. At 25 µM and 50 µM of glycine, proline and alanine, there is significant increase ($p \leq 0.05$) in *AAP-like* gene expression with the progression of time till 2 hr, except at 25 µM of alanine where a gradual decrease of expression has been noticed. The highest *AAP-like* gene expression of 63.15 fold was found at 50 µM of alanine and high expression of 43.71 fold was found at 25 µM of proline at 2 hr. Additionally, the *AAP-like* gene expression was high in presence of 50 µM of glycine and alanine at 2 hr than at 25 µM concentration except in presence of proline where the *AAP-like* gene expression was high at 25 µM than at 50 µM (Fig. 1). The *AAP-like* gene expression was 1.86 fold and 1.65 fold low at 25 µM and 50 µM of glycine at 2 hr respectively than in control (Fig. 2A, 2B). While on comparing the expression of *AAP-like* gene, there was no significant difference between 25 µM and 50 µM of glycine ($p \leq 0.05$) at 2 hr (Fig. 2C). In the presence of alanine, the expression of *AAP-like* gene was 4.28 fold low at 25 µM and 3.84 fold high at 50 µM at 2 hr than in control (Fig. 3A, 3B) but showing highest expression of *AAP-like* gene at 1 hr and 2 hr at both concentrations (Fig. 3C). The *AAP-like* gene expression was different in presence of proline from both glycine and alanine. The *AAP-like* gene expression was 2.66 fold high at 25 µM and 2.89 fold low at 50 µM of proline at 2 hr than in control (Fig. 4A, 4B) whereas the expression of *AAP-like* gene was found to be highest at 25 µM at 1 hr and 2 hr (Fig. 4C).

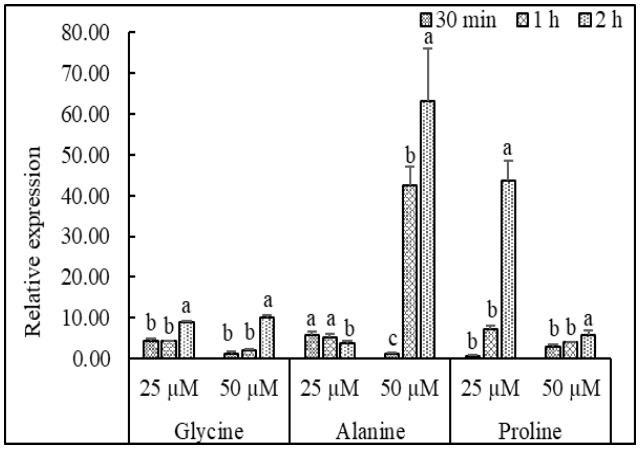


**Fig. 1.** Relative quantification of *AAP-like* gene after glycine, alanine and proline addition at 30 min, 1 hr and 2 hr in 25 µM and 50 µM concentration of each separately using IDE as internal control. Each column is the average of the three independent measure ± SD. Small alphabets notation on each error bars indicated significant differences and same alphabet notation indicated no significant differences at the 5% level of significance in between incubation time of a particular treatment. *AAP-like*- amino acid permease-like gene; IDE- Insulin degrading enzyme.

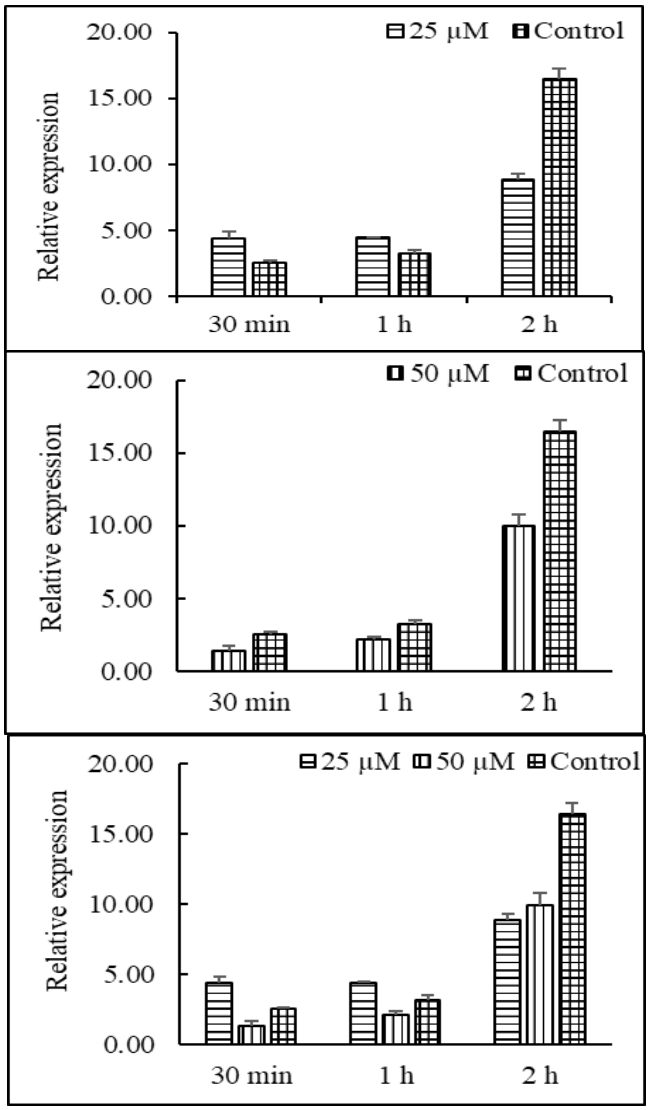


**Fig. 2.** Relative quantification of *AAP-like* gene after Glycine addition. A: 25 µM; B: 50 µM; C: Comparison between 25 µM, 50 µM and control using IDE as internal control. Each column is the average of the three independent measure ± SD. Small alphabets notation on each error bars indicated significant differences and same alphabet notation indicated no significant differences at the 5% level of significance in between treatment and control of a particular incubation time. *AAP-like*- amino acid permease-like gene; IDE- Insulin degrading enzyme.

## Discussion

A different variety of organic nitrogenous compounds including amino acids may be present in agricultural land. Amino acids amount to an important nitrogen supply to plants. The amino acids in the soil in general vary from 0.1 to 60 µM that make sum total of soluble nitrogen up to 10–40% (11, 21, 22). Different kinds of ecosystem have different amino acid concentration in the soil based on their structure. In an alpine region the free amino acids are present in range of 13–158 µM (23) and in boreal forests it is 57–73 µM (24). In grasslands overall soil amino acid varies between 20–60 µM whereas individual amino acids are in the range of 0.3–10 µM (21). Despite of the occurrence of very low micro molar concentrations of amino acid in soil, majority of the studies have been done at significantly high concentrations (23, 25–27). Very limited studies have been reported close to 0.1–10 µM amino acid concentrations present in the soil (28–30).

Amino acids are the important fraction of nitrogen being absorbed by plants in terrestrial ecosystems, particularly in low nitrogen concentration (31–34). There are many amino acid transporters reported that uptake amino acid directly from soil in plants (35). Three amino acid transporters namely AAP1, AAP5 and LHT1 already have been reported playing a major role in amino acid absorption from *Arabidopsis* roots (12).

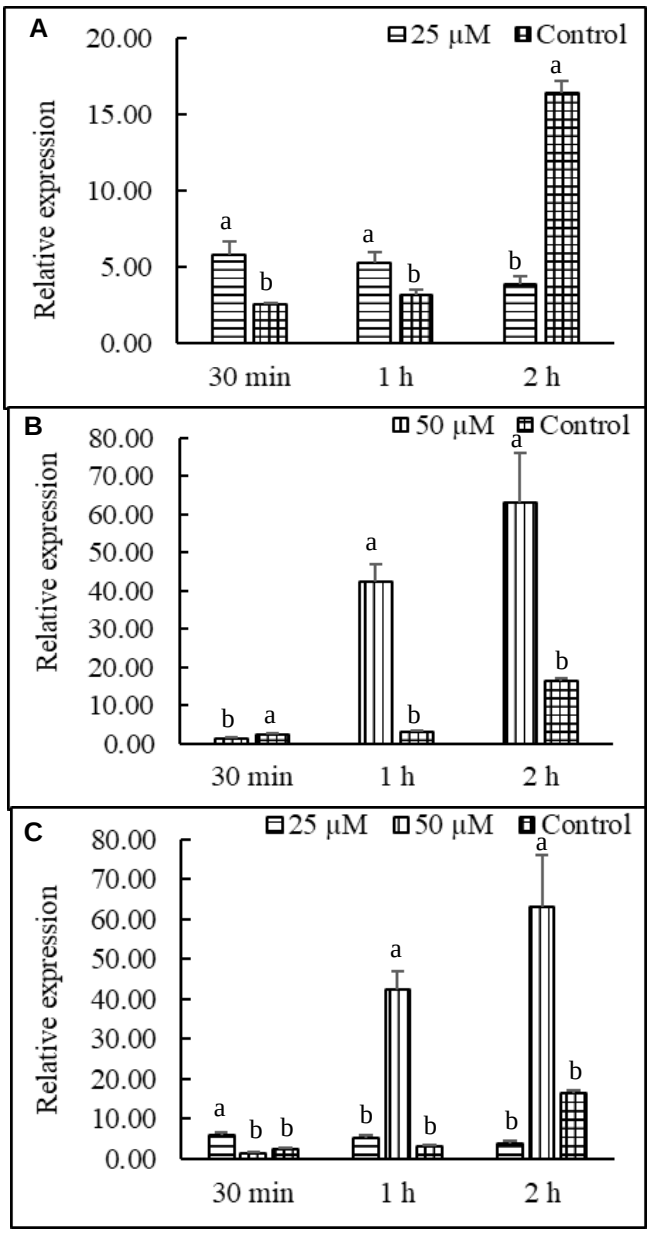


**Fig. 3.** Relative quantification of AAP-like gene after Alanine addition. A: 25 µM; B: 50 µM; C: 25 µM, 50 µM and control using IDE as internal control. Each column is the average of the three independent measure ± SD. Small alphabets notation on each error bars indicated significant differences and same alphabet notation indicated no significant differences at the 5% level of significance in between treatment and controlof a particular incubation time. AAP-like- amino acid permease-like gene; IDE- Insulin degrading enzyme.

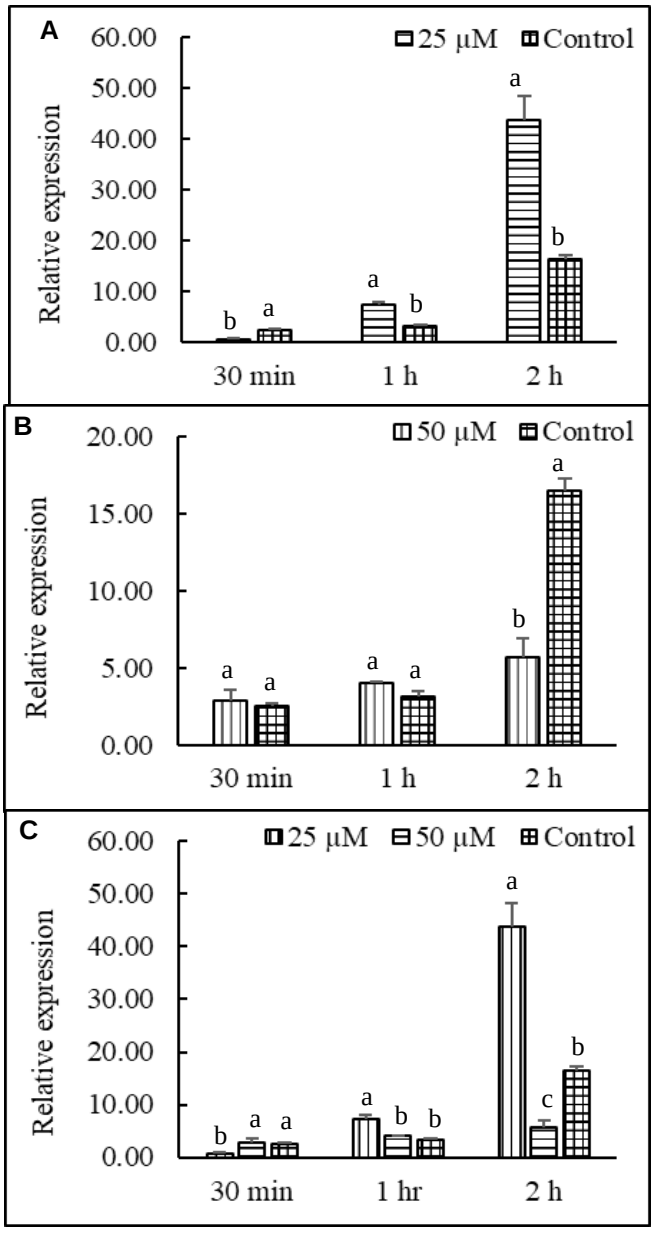


**Fig. 4.** Relative quantification of AAP-like gene after Proline addition. A: 25 µM; B: 50 µM; C: Comparison between 25 µM, 50 µM and control using IDE as internal control.Each column is the average of the three independent measure ± SD. Small alphabets notation on each error bars indicated significant differences and same alphabet notation indicated no significant differences at the 5% level of significance in between treatment and control of a particular incubation time. AAP-like- amino acid permease-like gene; IDE- Insulin degrading enzyme.

Based on the great significance of *AAP* gene in the agriculture, localization along with functional characterization has been done (15). Very recently it has been cloned and its protein is structurally characterized in *Phaseolus vulgaris* (36). Additionally, *AAP6* has also been explored to play a key role in export of nitrogen and its fixation in nodule in pea (37). Therefore, it's imperative to check on AAP transporters present naturally in other vital plants.

We addressed question in our study whether, plants can obtain amino acids through AAP transporters in the presence of amino acids at or close to field allied concentrations for roots. The high relative expression of *AAP-like* gene in 50 µM of alanine, glycine and proline but at 1 hr and 2 hr for alanine; 2 hr for glycine and 30 min for proline (Fig. 1). From this observation it is obvious to say, more the concentration of amino acid, more will be the expression of *AAP-like* gene. However, 7.68 fold *AAP-like* gene expression in 25 µM proline then in 50 µM of proline indicates that the *AAP-like* gene expression is dependent on the presence of specific amino acid. Another important observation is that the *AAP-like* gene expression gradually increases as time passes from 30 min to 2 hr, regardless of the concentrations of amino acids (Fig. 2–4).

## Conclusion

This is the first study showing the involvement of *AAP* transporter in acquiring amino acids, glycine,

alanine and proline and establish a dose dependent relationship with the expression of *AAP* at field allied concentrations for root. Additionally, the necessity of more information on the twinning of uptake, stimulus, metabolic pathways, soil properties and plant growth in the presence of other amino acids along with organic nitrogenous compounds are required. Based on this knowledge, we can stop the wasting of excess nitrogen fertilizer applied in the field that will halt eutrophication along with leaching through roots in the surrounding environment. The identification and breeding of plants showing high ability in absorbing nitrogen could also bring a breakthrough in agricultural system leading to low fertilizer usage in the field.

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## Authors contributions

NP, BC and JB designed the research. NP and BC carried out the experiment. NP, BC and JB contributed manuscript preparation and finalized the manuscript.

## Conflict of interest

The authors declare no conflict of interest.

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