African Journal of Biotechnology Vol. 11(1), pp. 159-163, 3 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2585
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Phenylalanine ammonia-lyase (PAL) gene activity in response to proline and tyrosine in rosemary callus culture

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Accepted 4 November, 2011

Phenylalanine ammonia-lyase (PAL) catalyzes the biosynthesis of rosmarinic acid (RA), tyrosine and phenylalanine are the precursors of RA, while proline drives metabolite precursors toward Shikimate and phenylpropanoid pathway ending with the production of RA. The aim of this study was to investigate the PAL gene activity in the callus of rosemary (*Rosmarinus officinalis*) due to exogenous application of L-proline and L-tyrosine. Four different concentrations of L-proline and L-tyrosine (0, 4, 5 and 6 mM, and 0, 0.4, 0.6 and 0.8 gm/L) respectively, were added to the basal Murashige and Skoog (MS) medium. The expression of the PAL gene was investigated and compared with the callus RA production. It was found that RA production increased significantly at low proline application (4 mM), on the other hand, tyrosine application at low concentrations had no effect on RA accumulation, while high tyrosine concentration (0.8 g/L) increased RA accumulation. When comparing PAL gene activity and RA production in callus tissues, it was found that they were correlated. Proline application alone at 4 mM or tyrosine alone at 0.8 g/L enhanced PAL gene activity, and also combining both proline at 4 mM and tyrosine at 0.8 g/L enhanced PAL gene activity and produced the highest RA accumulation in callus tissues [0.047 mg/g freash weight (fw)].

Key words: Phenylalanine ammonia-lyase (PAL), gene expression, rosmarinic acid, *Rosmarinus officinalis*, tyrosine, proline.

INTRODUCTION

Research has confirmed that plant phenolic compounds have antioxidant, medicinal and antimicrobial properties. Some of the important phenolic compounds are the derivatives of the phenylpropanoid pathway (Perry and Shetty, 1999).

Proline serves as a reductant replacing the $NADH_2$ as the hydrogen donor for oxidative phosphorylation inside the mitochondria in the pentose phosphate pathway. This proline-linked redox cycle drive metabolite precursors toward Shikimate and phenylpropanoid pathway leading to the production of rosmarinic acid (RA) (Yang and Shetty, 1998).

Tyrosine changes through a series of chemical reaction to 3-4-dihydroxyphenyl-lactic acid via phenylpropanoid pathway (Mizukami and Ellis, 1991). Studies showed that the tyrosine and phenylalanine are the precursors of the 3-4-dihydroxyphenyl-lactic acid and the caffeic acid, respectively, the latter two chemical compounds when they bind they form RA (De-Eknamkul and Ellis, 1987).

Early studies on RA biosynthesis had shown that the enzyme phenylalanine ammonia-lyase (PAL) catalyzes the initial step of the phenylpropanoid pathway leading to the formation of 4-coumaroyl-CoA (De-Eknamkul and Ellis, 1987; Woodrow and Berry, 1988). Studies by Mizukami et al. (1992) have demonstrated that an increase in PAL activity in *in vitro* plant tissues coincides with an increase in RA content, indicating a role for PAL

Abbreviations: PAL, Phenylalanine ammonia-lyase; **RA,** rosmarinic acid; **MS** Murashige and Skoog

in the regulation of RA biosynthesis. The gene responsible for the production of RA is not yet known and thus cannot be studied directly.

Since proline, tyrosine and PAL enzyme activity, are linked to the synthesis of RA, this study investigated the PAL gene expression due to exogenous application of proline and tyrosine and the accumulation of RA in the callus of rosemary plant.

MATERIALS AND METHODS

This work was done in the tissue culture lab–Horticulture Department–Faculty of Agriculture-Alexandria University-Egypt.

Medium used for callus induction

Medium used was Murashige and Skoog (MS) basal salt medium with vitamins from Sigma (M5519), 4.43 g/liter medium + 3% sucrose (30 g/L sucrose) + 1.5 mg/L TDZ + 0.5 mg/L IAA + 6 g/L agar. pH was adjusted to 5.8 ± 0.02 using 1 N NaOH and 1 N HCl (Tawfik et al., 1992).

16 different media were prepared from basal MS medium supplemented with combinations of four L-proline concentrations (0, 4, 5 and 6 mM) and four L-tyrosine concentrations (0, 0.4, 0.6 and 0.8 gm/L), both L-proline and L-tyrosine were bought from Sigma-Aldrich. Media were autoclaved for 20 min at 110°C and 120 bar/cm^2.

Leaf segments about 5 mm of rosemary plant, were disinfested for 10 min in 10% bleach plus two to three drops of Tween 20. Then leaf segments were rinsed with sterile distilled water and placed on the autoclaved media.

All tubes were placed under cool white florescent light at intensity of 52 to 66 µmol/m^2/s, depending on the bulb age, for 16 hrs and 8 hrs dark at a temperature of 25°C. After two months, the second subculture was done and the callus tissues were placed on new medium containing the same basal medium and the same combinations of L-proline and L-tyrosine.

Rosmarinic acid analysis

300 mg of callus tissue from each treatment were used to detect the RA concentration; absorbance was measured at 333 ηm using spectrophotometer (UNICO 3200) as reported by Lopez-Arnaldos et al. (1995) and Komali and Shetty (1998).

The statistical design of the experiment was a split plot design with two factors. The main factor was the L-proline and the sub-factor was the L-tyrosine.

PAL gene expression

Total RNA was extracted from the callus of the 16 media combinations of the second subculture using GeneJet RNA purification kit from Fermentas.

PAL gene activity was tested using the PCR reaction to amplify the PAL sequence located on the cDNA constructed from each callus by the use of a specific designed primer. The first strand of cDNA was synthesized using reverse transcriptase enzyme (RevertAid first strand cDNA synthesis kit #K1621 form Fermentas), the sequence of the PAL gene was taken from *Agastache rugosa* (Elnaggar and Read, 2010).

The forward primer used for the PAL gene was 5'- CAG TGG CTC GGC CCT CAG AT -3' and the reverse primer sequence was 5'- GAA CTG GAG CTC GGA GCA GT -3'. Each of the forward and the reverse primers were used to amplify about 341 bp of the cDNA (El-Naggar and Read, 2010).

Master Mix was made for the PAL gene primers, the tube contained 2 µl cDNA template + 2 µl 10X PCR buffer + 0.7 µl 50 mM $MgCl_2$ + 2 µl forward and reverse primer mix + 2 µl 10 mM dNTP mix + 0.3 µl Taq DNA polymerase + water to 20 µl volume. The PCR products were loaded in 16 wells plus ladder in 1.5% agarose gel, the gel was submerged in the buffer and 100 V was used for gel running for about 1 h.

RESULTS

Using the statistical analysis software (SAS) program for the statistical analysis, it was found that the RA accumulation in callus tissues increased significantly by increasing proline concentration from 0 to 4 mM and then decreased by increasing proline concentration, reaching its lowest concentration at 6 mM proline and this occurs in all tyrosine concentrations. Tyrosine had no effect on RA accumulation at low concentrations, while the highest tyrosine concentration (0.8 gm/L) increased the RA concentration significantly as shown in Table 1 and Figure 1.

The results show bands in all callus treatments tested between 300 and 400 bp which is consistent with the 341 bp of the PAL gene primers. Based on the differences in band intensity as a measure of gene expression, it was found that there were three levels of gene expression: high gene expression in bands 2, 6, 10, 13 and 14 which represent 4 mM proline + 0 g/L tyrosine, 4 mM proline + 0.4 g/L tyrosine, 4 mM proline + 0.6 g/L tyrosine, 0 mM proline + 0.8 g/L tyrosine and 4 mM proline + 0.8 g/L tyrosine respectively, and medium gene expression in bands 15 and 16 which represent 5 mM proline + 0.8 g/L tyrosine and 6 mM proline + 0.8 g/L tyrosine and a low gene expression represented in bands 1, 3, 4, 5, 7, 8, 9, 11 and 12 which represent 0 mM proline + 0 g/L tyrosine, 5 mM proline + 0 g/L tyrosine, 6 mM proline + 0 g/L tyrosine, 0 mM proline + 0.4 g/L tyrosine, 5 mM proline + 0.4 g/lit tyrosine, 6 mM proline + 0.4 g/L tyrosine, 0 mM proline + 0.6 g/L tyrosine, 5 mM proline + 0.6 g/L tyrosine and 6 mM proline + 0.6 g/L tyrosine, respectively.

DISCUSSION

PAL is the enzyme at the entry-point of the phenyl-propanoid pathway, which yields a variety of phenolic compounds with structural and defense-related functions. PAL catalyzes the deamination of L-phenylalanine to form trans-cinnamic acid, which leads to the production of p-coumaroyl- CoA, PAL activity and the activation of PAL under stress conditions have been considered to be part

Table 1. RA concentration in mg/gm fw in callus tissue due to exogenous application of L-proline and L-tyrosine.

Proline (mM)	Tyrosine (g/L)			
	0	0.4	0.6	0.8
0	0.0207^{bc}	0.0261^{b}	0.0248^{b}	0.0339^{b}
4	0.0359^{a}	0.0372^{a}	0.0342^{a}	0.0470^{a}
5	0.0245^{b}	0.0247^{b}	0.0230^{b}	0.0334^{b}
6	0.0117^{c}	0.0132^{c}	0.0157^{c}	0.0306^{b}

Same letters in the same column means there are no significant differences between treatments at P<0.05.

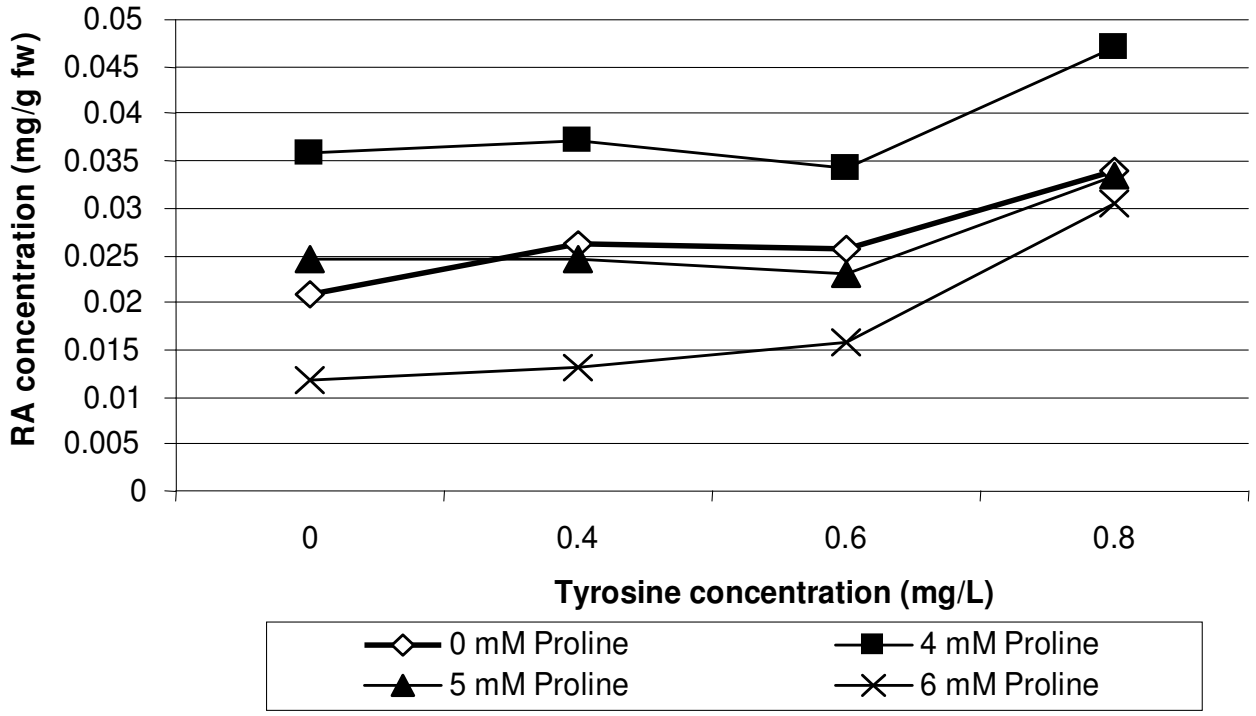

Figure 1. RA accumulation in *R. officinalis* callus tissues in 16 different combinations of L-proline and L-tyrosine.

of a defense mechanism operating in stress-afflicted cells (Dixon and Pavia, 1995; Yang and Shetty, 1998).

In this study, it was found that the concentration of RA increased due to proline application at low concentration (4 mM) and then decreased at high proline concentrations and at all tyrosine concentrations as shown in Table 1 and Figure 1.

Same results were obtained by Elnaggar and Elmokadem (2007), who found that RA concentration increased at low proline concentration and then declined at high proline concentrations at all tyrosine levels. Perassolo et al. (2007) concluded that the addition of proline at low concentration (0.25 mM) enhanced (up to 50%) the antioxidants biosynthesis in *Rubia tinctorum* suspension cultures. While Duval and Shetty (2001) found that proline at low concentrations stimulated phenolic compounds but at high concentrations, the proline analog inhibited it.

The reduction in RA at high exogenous proline levels (5 and 6 mM) might be due to the accumulation of proline which leads to the inhibition of proline production by the plant tissues or due to the limited amount of proline dehydrogenase inside the plant tissues which may not be able to oxidize the relatively large amount of proline as mentioned by Kwok and Shetty (1998).

Comparing the PAL gene expression with the RA production in the callus, it was found that both were correlated, meaning that the callus producing the highest RA concentration shows a high PAL gene activity represented in bands number 2, 6, 10, 13 and 14 (Figure

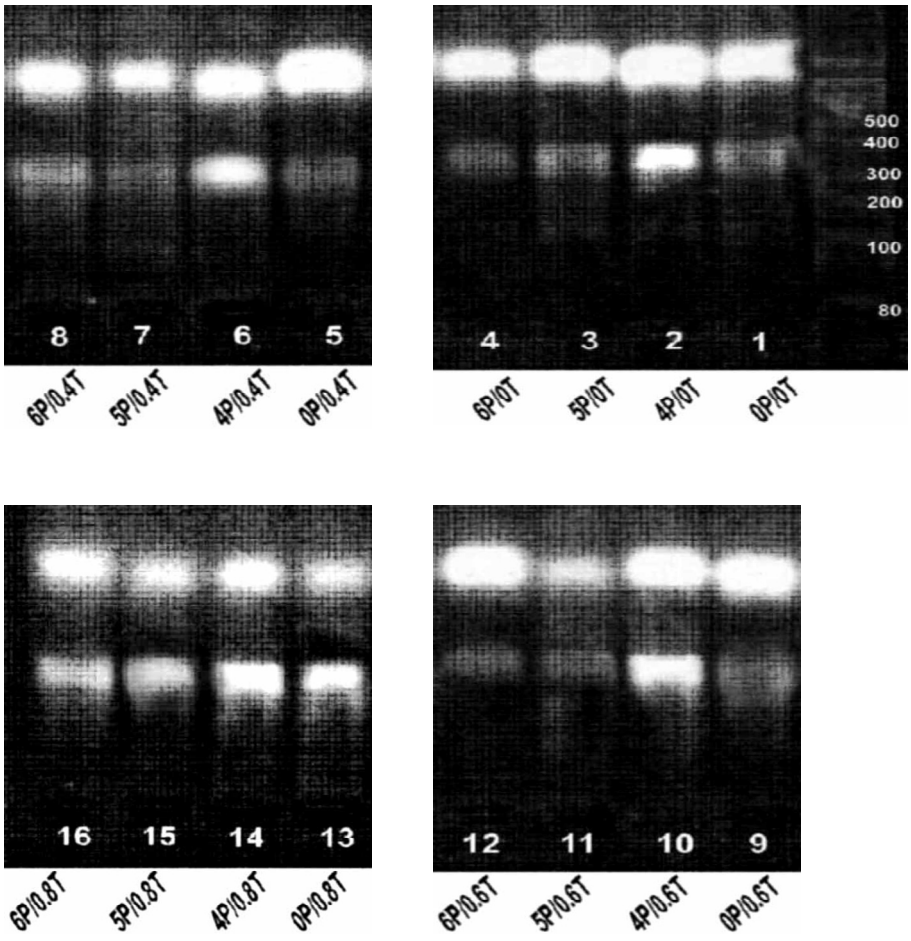

Figure 2. Gel electrophoresis of PCR reaction showing bands between 300 and 400 bp. The highest band intensity was found in bands 2, 6, 10, 13 and 14, medium band intensity was found in bands 15 and 16, while lowest band intensity was found in bands 1, 3, 4, 5, 7, 8, 9, 11 and 12. P and T represent proline and tyrosine and their concentrations, respectively.

2), their corresponding callus produced RA at 0.0359, 0.0372, 0.0342, 0.0339 and 0.047 mg/g fresh weight (fw) respectively (Table 1), while low PAL gene activity were represented in bands 1, 3, 4, 5, 7, 8, 9, 11 and 12 (Figure 2), and their corresponding callus produced RA at 0.0207, 0.0245, 0.0117, 0.0261, 0.0247, 0.0132, 0.0248, 0.023 and 0.0157 mg/g fw respectively (Table 1). Similar results were obtained by Karwatzki et al. (1989), they found that the enzymes, PAL, cinnamic acid 4-hydroxylase (CAH) and hydroxy-cinnamic acid:CoA ligase (4CL) were correlated in their activities of RA synthesis and accumulation in cell cultures of *Coleus blumei*.

De-Eknamkul and Ellis (1987) were able to demonstrate the PAL activity and the rate of RA synthesis in cell culture of *Anchusa officinalis and C. blumei* and they found that they change in a coordinated manner, as the PAL activity increases the RA level in cell culture increased.

Trond et al. (2010) mentioned that the expression of structural genes in the phenylpropanoid and flavonoid pathways, PAL, chalcone synthase (CHS), flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) increased in agreement with a corresponding increase in flavonoid and caffeoyl content.

The reduction in PAL gene expression at high proline application might be due to the competition for limiting factors such as proline dehydrogenase (Kwok and Shetty, 1998) especially at low tyrosine applications.

Elkind et al. (1990) reported that leaves of transgenic tobacco containing a cauliflower mosaic virus (CaMV) 3% promoter-bean PAL2 transgene had lower levels of endogenous tobacco PAL mRNA and PAL activity than those of normal plants, and that phenylpropanoid production was reduced. It was concluded that co-

suppression may arise from competition for a limiting transcription factor, and that accumulation of transcripts encoded by endogenous tobacco PAL genes was suppressed. It has also been shown that silencing a key enzyme of lignin synthesis increases accumulation of flavonoids, indicating competition between flavonoid and lignin synthesis for precursors (Besseau et al., 2007).

Medium PAL gene activity, bands 15 and 16 producing RA at 0.0334 and 0.0306 mg/gm fw respectively, might be due to the opposing effect of tyrosine at high concentration, which enhanced both RA accumulation and PAL gene activity, and proline at high concentrations (5 and 6 mM), which suppressed both RA accumulation and PAL gene activity, as shown in Table 1 and Figure 2. This opposite effect shows a medium expression of the PAL gene and a medium accumulation of RA.

High tyrosine concentration might activate RA production and the PAL gene activity since tyrosine and phenylalanine are the precursors of the 3-4-dihydro-xyphenyl-lactic acid and the caffeic acid respectively, which when bind together, they form the RA (De-Eknamkul and Ellis, 1987).

Conclusion

It is concluded that RA production increased at low proline application (4 mM), while high proline concentrations reduced RA accumulation in callus tissues. On the other hand, tyrosine application at low concentrations had no effect on RA accumulation, while high tyrosine concentration (0.8 g/L) increased RA accumulation. PAL gene activity and RA production in callus tissues were correlated. Proline application alone at low concentration (4 mM) or tyrosine alone at high concentration (0.8 g/L) enhanced PAL gene activity (bands 2 and 13, respectively), and also, combining both proline at 4 mM and tyrosine at 0.8 g/L enhanced PAL gene activity (band 14) and produced the highest RA accumulation in callus tissues (0.047 mg/g fw).

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