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Research Article

Stereo-Specific Transcript Regulation of the Polyamine Biosynthesis Genes by Enantiomers of Ornithine in Tobacco Cell Culture

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Background: Ornithine (Orn) plays an essential role in the metabolism of plant cells through incorporation in polyamines biosynthesis, the urea cycle and nitrogen metabolism. Physiological response of the plant cells to its two enantiomers have not been widely investigated yet.
Objectives: This study aimed to evaluate effect of ornithine enantiomers on expression of certain polyamine (PAs) biosynthetic genes in tobacco cells.
Materials and Methods: Suspension-cultured tobacco cells were treated with different concentrations of L- and D- Orn for 24 h. Cell viability was assayed by Evans Blue and hydrogen peroxide content. The changes of gene expression were analyzed by semi-quantitative RT-PCR.
Results: Exogenous D-Orn resulted in enhancement of expression of genes involved in Orn, arginine and S-adenosyl methionine metabolism. Additionally, exogenous D-Orn treatment resulted in sustained viability of cultured tobacco cells and normal levels of hydrogen peroxide were maintained. Supplied L-Orn increased the hydrogen peroxide level and lowered viability of cells. Treatment with L-Orn had a negative effect on the transcript levels for most analyzed PA-related genes. It was also illustrated that transcription of putrescine methyl transferase, key enzyme for nicotine production, was highly upregulated by L-Orn.
Conclusions: Based on the results, D-Orn was shown to have a stereo-selective function in regulation of the PAs-related genes.

Keywords: Arginine decarboxylase, D-amino acids, *Nicotiana tabacum*, Ornithine decarboxylase.

1. Background

Polyamines (PAs), such as putrescine (Put), spermidine (Spd), and spermine (Spm), are generally present in all organisms and have essential roles in certain physiological processes (1, 2). PAs are present in all parts of the plant cells and are mostly known for involvement in the regulation of genome activity, cell division and expansion, and plant growth. PAs have important antioxidant functions, growth regulatory and essential interactions with components of the cell wall (3-5). Because of PAs participation in cell division processes and regulation of genome transcription, they are widely studied in cancer of animal cells (6).

Generally, Put is synthesized from Orn decarboxylation via ornithine decarboxylase (ODC; EC 4.1.1.19), and is then further aminopropylated by *S*-adenosyl methionine, spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22) to form Spd and Spm, respectively (7). In plants, Put can also be produced by arginine decarboxylase (ADC; EC 4.1.1.17) action (1). Methylation of Put by putrescine methyl transferase (PMT) and *S*-adenosyl methionine forms methyl putrescine (mPut), which provides the pyrrolidine ring of nicotine (7). Orn decarboxylation is known to be the main step in the PAs production. Transcription and enzyme activity of ODC are shown to be dominantly

regulated in many processes of living cells (8-10). ODC is shown to be present in all living organisms and necessary for cell division, transcription process and growth under normal condition. ADC is considered for taking role under stress conditions for the biosynthesis of PAs (1).

L-Orn is a non-protein amino acid and has an essential role in PAs biosynthesis (11). It also has main roles in arginine (Arg) and urea production (12). In addition, Orn has important function in the production of proline (Pro), and nicotinic alkaloids (7).

The content of PAs in plants are associated with several physiological processes, such as N:C ratio and stress responses. PAs catabolism/anabolism, transport and conjugation define the PAs hemostasis (13).

It was previously shown that the "uncommon enantiomer", D-Orn, can cause upregulation of free and conjugated PAs, as opposed to its enantiomer (14). In addition, D-Orn potently induces production of Spd and Spm. While D-Orn has no effect on nicotine levels, L-Orn enhances nicotine production *in vivo* (14). Results of that study were the first observations suggesting a Amino Acid (D-AA) may play a critical role in plant metabolism and development. Generally, plants produce D-AAs due to microbial infection and racemization of L-AAs. Once compared to animals, plants assume to lack the D-AAs metabolizing enzymes that lead to toxicity effects of certain D-AAs in plants (15, 16). Mostly, D-AAs are thought to be toxic or futile compounds for plant metabolism, growth and development (17). D-Serine and D-Alanine are of those D-AAs, which strongly inhibit plants growth at 3 mM concentration (15).

It has long been accepted that only L-AAs can effectively enter biosynthetic pathways and be incorporated with secondary metabolites and proteins structures of cells. Although the functions of L-AAs on plant metabolism are well known, there are few reports on effects of D-AAs.

2. Objectives

The effects of L- and D-Orn on PAs biosynthetic related genes were evaluated. Following our previous report, here we planned to determine the role(s) of Orn enantiomers in suspension-culture of *Nicotiana tabacum*.

3. Materials and Methods

3.1. Suspension Culture and Treatments

Tobacco (*Nicotiana tabacum*) cells were cultivated in a modified MS medium (18). The 5-day-old cells were treated with 0, 1, 5, 10 mM of L- and D- Orn (Sigma-Aldrich) for 24 h according to (19, 20). Cells were harvested and frozen for further analysis. Evans Blue was used to assay the cells viability (18).

3.2. Hydrogen Peroxide (H_2O_2) Content Determination

H_2O_2 concentrations were assayed according to Velikova *et al*. (21). Briefly, 0.2 g fresh cells were crushed with trichloroacetic acid (0.1% w/v), followed by centrifugation at 12,000 ×g for 10 min. The supernatant (0.5 mL) was mixed with 1 mL of 1 M potassium iodide and 0.5 mL of 10 mM potassium phosphate buffer. Absorbance measurement was carried out at 390 nm.

3.3. RNA Extraction and Semi Quantitative Reverse Transcriptase-PCR Analysis

Gene expression changes were measured by semi-quantitative RT-PCR. Total RNA was isolated from cells through RNX™⁺- Plus kit (Cinna Gen Inc). The integrity and quantity of RNA was estimated using gel electrophoresis and spectrophotometer. Total RNA (3 µg) was reverse-transcribed by First-Strand cDNA synthesis Kit (Fermentas, Canada). The primer sequences corresponding to the genes under study were shown in **Table 1**. *Actin* expression was used as an internal control. PCR reaction contained 0.5 U Taq DNA polymerase, 0.3 µL of cDNA, 200 µM of deoxynucleoside triphosphates (dNTPs), 50 mM KCl, 1.5 mM $MgCl_2$ and 4 µM of forward and reverse primers in a total volume of 20 µL. The PCR conditions were as follows: initial denaturation at 94 ˚C for 1 min and 30 cycles of 94 ˚C: 30 sec; 52 to 58 ˚C (depending on gene type): 25 sec; and extension for 10 min at 72 ˚C. The products were electrophoresed in 1.2% (w/v) agarose gels, stained with ethidium bromide under ultraviolet light. The band intensity was measured

Table 1. Sequences of primers used in this study.

Genes	Sequences
ACT	F- primer 5′-GCAGGGATCCACGAGACCACC-3′ R- primer 5′-CCCACCACTGAGCA CAATGTTCC-3′
ODC	F- primer 5′-TTCCAGAAGAAGTCGACCCGCTG-3′ R- primer 5′-CA GCTCCGGTAACTGGTAATCCC-3′
ADC	F- primer 5′-ATCTGTCTTCTGGTGGCCTCC AATC-3′ R- primer 5′-CCACCAATGAACTTATCAACCTTCC-3′
PAO	F- primer 5′-GACT CGGCAATTCAGAAACTCAG-3′ R- primer 5′-ACTCCTTCTCAGGTTCACAAGGC-3′
CAT	F- primer 5′-GTTTCTCACCTCACCTGTGCCG-3′ R- primer 5′-CAGCGGCAATCGA AT CGTACAG-3′
SAMDC	F- primer 5′- TTGGTAGCAACATCAGCATGCA-3′ R- primer 5′-TGACCCTGTTTACACTCTTGAG-3′

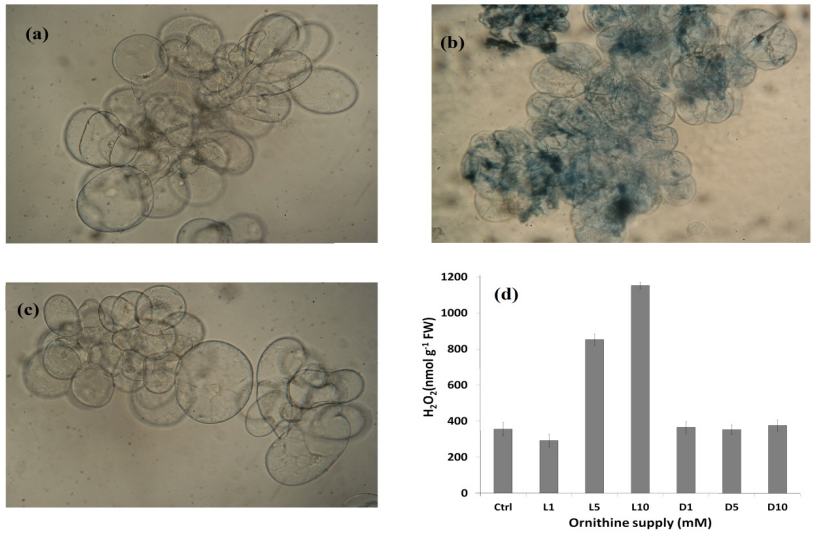

Figure 1. The viability and H_2O_2 levels of tobacco cells. a) control condition, b) L-Orn treated cells (5 mM), c) D-Orn treated cells (5 mM), d) H_2O_2 levels of cells after treatment with different concentrations of L- and D-Orn. Dead cells were stained blue due to loss of cell membrane integrity and insertion of Evans blue agent. Data represent average values from 3 separate experiments ± SD.

by UV Documentation Luminescent Image Analysis software (England). The band quantity was determined with Image Guage software.

4. Results

4.1. Effect of Orn on Cell Viability, Growth and H_2O_2 Content of Tobacco Cells

No changes in cell viability were observed in D-Orn treated cells compared to the control (**Figs. 1a and c**). Conversely, treatments of cells with different concentrations of L-Orn inhibited cell viability (**Fig. 1 b**). Effect of 1 mM concentration of L- and D-Orn on cell growth was evaluated. The results showed that cell growth was increased by L- and D-Orn (**Table 2**). Concurrent measurement of H_2O_2 levels showed that H_2O_2 concentration in the D-Orn treated cells is similar to the control condition, while L-Orn led to increasing H_2O_2 accumulation. H_2O_2 increment led to cell viability reduction (**Fig. 1d**). The key observation from D-Orn treated cells (up to 10 mM) was that these cells did not suffer from any damage. Moreover, they did not accumulate H_2O_2 or showed any lowered viability. Whereas 5 mM L-Orn resulted in eleveated H_2O_2 that consequently lowered cell viability.

Table 2. Effect of D-Orn and L-Orn on growth of tobacco cells.

	Growth (g)
Control	11 ± 0.5
D-Orn	18 ± 0.4
L-Orn	16 ± 0.3

4.2. Effect of Orn on PA Biosynthetic Gene Expression

D-Orn showed a promotional effect on the expression of *ODC* and *ADC* genes, in a concentration dependent manner (**Figs. 2 and 3**). Conversely, 5 and 10 mM L-Orn treatment did not result in considerable changes of *ODC* expression (**Fig. 3**). In comparison with L-Orn, which highly decreased *ADC* and *SAMDC* transcript expressions, D-Orn enhanced *ADC* and *SAMDC* transcript levels. Biosynthesis of nicotine from Put competes with the production of Spd in tobacco. Results showed that the *PMT* transcript, representing the key enzyme involved in mPut and nicotine biosynthesis, is highly expressed in L-Orn treated cells (**Fig. 3**).

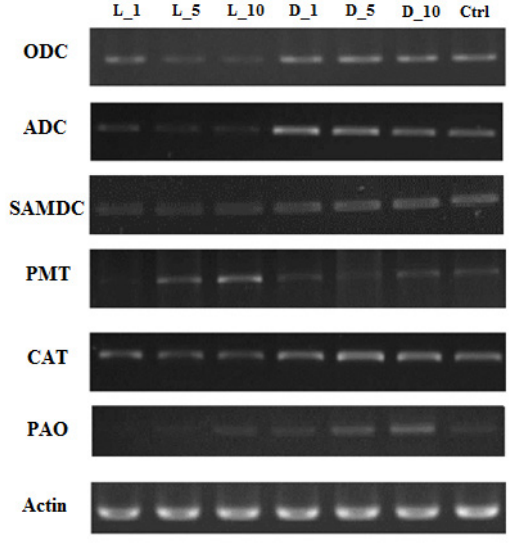

Figure 2. Expression patterns of *ODC*, *ADC*, *SAMDC* , *PMT, CAT*, *PAO* and Actin in tobacco cells after L-Orn and D-Orn treatments with 0, 1, 5 and 10 mM concentrations.

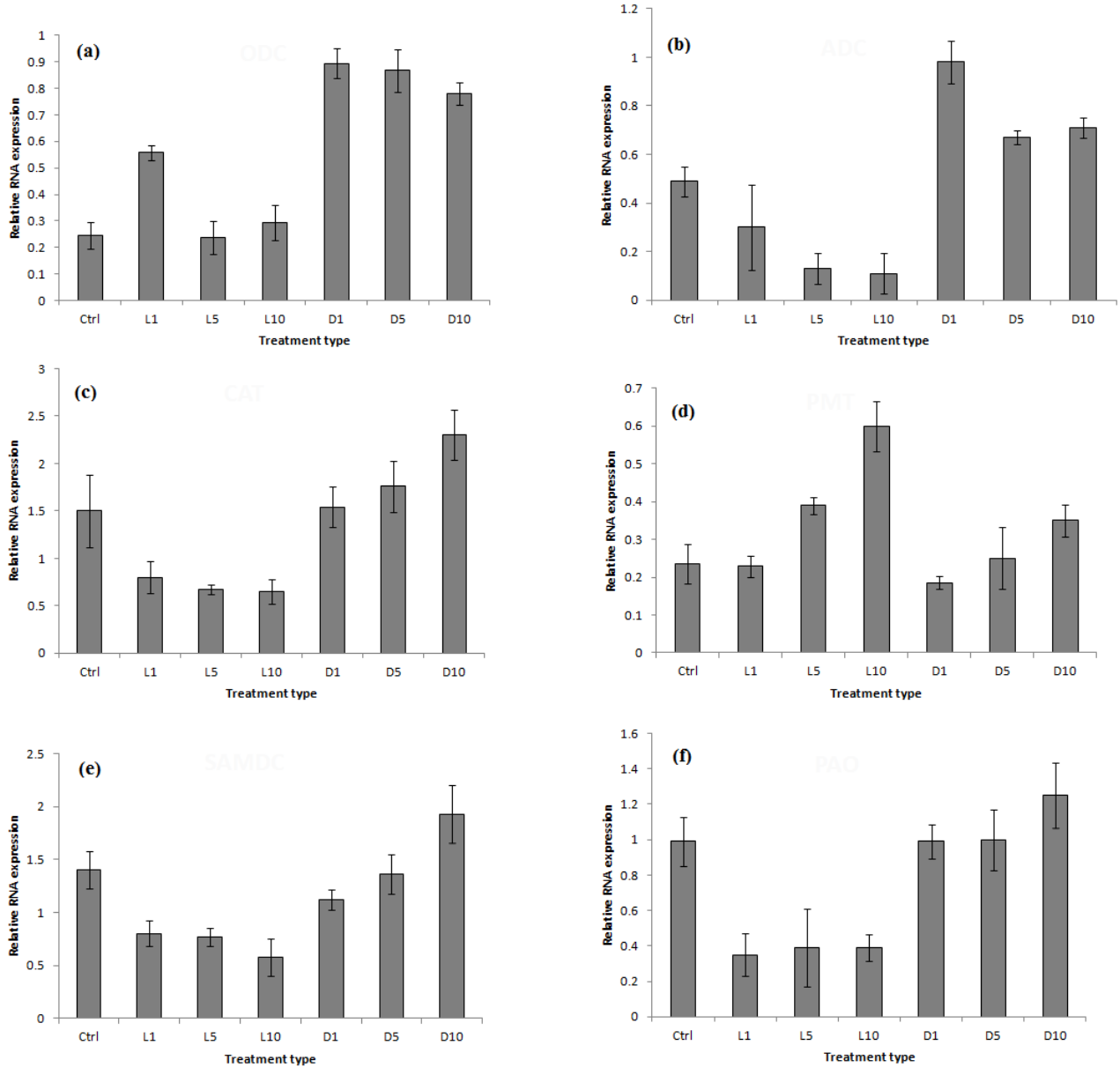

Figure 3. Expression level of the gene encoding for a) ODC, b) ADC, c) CAT, d) PMT, e) SAMDC, f) PAO in tobacco cell cultures after L-Orn and D-Orn treatments with 0, 1, 5 and 10 mM concentrations. Data represent average values from 3 separate experiments ± SD.

5. Discussion

Our results showed that the application of D-Orn caused a sustained viability. Moreover, H_2O_2 content of cells were quite similar to the cells under normal condition. Therefore, it can be speculated that the observed upregulated levels of genes are not a sign of any stress induced by the D-Orn treatments. While certain D-AAs, *e.g*. D-Ala and D-Ser (at 3 mM) were shown to be very toxic and limit plant growth (15, 16), D-Orn showed no negative effect on cell suspension culture of tobacco, even at 10 mM. Based on our results, both L-Orn and D-Orn might be involved in the regulation of respective pathways. Upregulation of all the necessary genes for Orn metabolism and PAs biosynthesis by D-Orn revealed its unique effects; the same effects have not been reported for L-Orn or any other D-AAs. While D-Orn emerged as a compound with stimulatory effects on transcript expressions of most associated genes, results for L-Orn implied a suppressive effect on their expression. In our previous paper, it was shown that L-Orn decreased Spd and Spn while increased nicotine. (14). This is in agreement with our result that

L-Orn enhanced transcript of *PMT* as the key enzyme of nicotine biosynthesis. This observation shows that each enantiomer of Orn is different, if not opposing effects on the same pathways actually. Oxidation of PAs by diamine oxidase (DAO) and polyamine oxidases (PAO) produces H_2O_2. Apparently, these oxidizing enzymes act in opposite direction to catalase (CAT), as the main H_2O_2 scavenger. *PAO* expression depicts a higher activity in the presence of D-Orn, which might be a reason to enhance the expression of *CAT*. The transcript level of *CAT* was highly enhanced in the presence of D-Orn while the L-enantiomer showed no positive effect on *CAT* transcription level.

D-ornithine inhibits ODC in yeast (22). In addition, ODC activity in tobacco cells was shown to be inhibited by putrescine and other PAs (23). We have previously reported that the application of D-orn enhanced the levels of spermine and spermidine, but not putrescine (14). It seems that D-ornithine may increase the levels of spermidine and spermine through the activation of ADC and probably other genes including *SAMDC*, *SPDS* and *SPMS*. The upregulated levels of *ODC* and *ADC* suggests that D-Orn has a potential effect on the transcript of all the related genes to PAs metabolism. Higher enhancement of *PAO* transcript, implies that PAs catabolism should be considered in addition to their production via ODC/ADC action. When examining different AAs for cell growth in suspension cultures, Behrend and Mateles (20) used a racemic mixture of Orn (at similar concentrations as in this study) and found that it acts as nitrogen source. An investigation into the effects of L-Orn on plant cell viability was reported in *Aechmea fasciata*. It was shown that L-Orn has inhibitory effects on growth of cultured pollen tubes (24). This data was in parallel to ours. Another notable study showed that L-Orn feeding to transgenic tobacco cell cultures (overexpressing an ODC) did not result in the expected accumulation of Put in cells, suggesting that Put production was limited partly by L-Orn availability (25). The results demonstrated that increasing capacity of cells to decarboxylate L-Orn increased the content of free or conjugated PAs.

Qu *et al*. showed that in comparison with the D-enantiomer, the L-enantiomer, is a more effective inhibitor of ODC (26). It has also been shown that ODCs extracted from different animal cells can be inhibited by both enantiomers. However, D-Orn is a very weak inhibitor of ODC (27), while L-Orn is a strong type. Ercal *et al*. showed that, treatments with most L-AAs are associated with a production of bulk of H_2O_2, which was not observed upon treatment with D-AAs. This is due to interconversion of AAs to each other through various cycles and catabolic reactions (28).

As expected, L-Orn can enter the amino acid cycles and pathways: resulting in increased H_2O_2 and other AAs while the D-enantiomer cannot. It can be safely argued that the constant level of H_2O_2 in D-Orn treated cells is a result of upregulation of CAT, which is the main scavenger of H_2O_2.

CAT and PAO, H_2O_2 producers via catabolism of PAs, are acting on opposite directions. Moreover, D-Orn is shown to have a stimulatory effect on the transcripts of both producing and recycling of PA enzymes. Results of our study suggest that a combination of accumulation of H_2O_2, driven from L-AAs conversions, and inhibition of gene transcripts determine the observed effects of L-Orn on tobacco cells. Current study indicates that stereospecific regulation of PAs biosynthesis might be a novel approach to enhance plant cells' metabolism, growth and development. To fully determine the role of D-Orn will demand further metabolic examination such as stable isotope labeling.

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