Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Molecular cloning and biotic elicitation response of phenylalanine ammonia-lyase gene of *Astragalus chrysochlorus*

Neslihan Turgut-Kara*, Özgür Çakır, Burcu Arıkan, Şule Arı

Istanbul University, Faculty of Science, Department of Molecular Biology and Genetics, Istanbul, Turkey

Correspondence to: neslihantk@istanbul.edu.tr

Received November 9, 2017; **Accepted** April 20, 2018; **Published** April 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.5.17

Abstract: Phenylalanine ammonia lyase (PAL) is the first enzyme of the phenylpropanoid pathway, and it is necessary to upregulate flavonoid biosynthesis in most of the plant species. In this study, we have cloned PAL gene from endemic *Astragalus chrysochlorus* which is a producer of phenolic nicotiflorin (kaempferol-3-O-rutinoside). The cDNA encoding PAL was cloned from *A. chrysochlorus* using RT-PCR (reverse transcription-polymerase chain reaction) with conserved primer pairs. Amino acid sequence alignments showed that AcPAL (2160 bp, Accession number: KM189182) has more than 95% amino acid identity with their homologues in other *Astragalus* species. The coding sequence for the protein of AcPAL is 720 amino acids with a calculated molecular weight of 78.53 kDa. Full length *AcPAL* was cloned and expressed in *Escherichia coli*. qPCR (quantitative real-time PCR) analysis of the expression of PAL gene of *A. chrysochlorus* suggested that maximum transcript level was observed in 3 h yeast extract elicited suspension cells. Our findings suggest that AcPAL plays role in early response for yeast extract treatment. The isolation of *AcPAL* gene could be result in further studies for overproduction of secondary metabolite, nicotiflorin.

Key words: PAL; *Astragalus*; Kaempferol 3-O-rutinoside; Flavonoid; Yeast extract.

Introduction

Majority of plant phenolic compounds are derived from trans-cinnamic acid. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is the first key enzyme in the phenylpropanoid pathway and catalyzes the conversion of L-phenylalanine to trans-cinnamic acid. PAL is one of the most studied enzyme in plants and it is the subject of those studies in various ways. It has been reported in many studies that its expression is in correlation with phenolic compounds produced by the induction of abiotic and biotic stressors (1,2).

Astragalus is one of the widely used traditional Chinese medicine. It possesses many biological activities, and has been used by Chinese medicine for centuries. It has also been proven to be effective for clinical treatment of nephritis, cardiovascular diseases, hypertension, diabetes, and cancers (3,4). The best known components of *Astragalus membranaceus* are flavonoid, triterpene saponins, and polysaccharides. This phenylpropanoid derivated flavonoids are associated with its antioxidant and cytotoxic activities of the plants (5,6). Numerous studies have done on *Astragalus* species and their components, but there is still a need for further studies to learn more about this species using genetic or molecular biology methods.

Astragalus chrysochlorus Boiss. and Kotschy is a Turkish endemic, it grows in 32-36° meridian in Anatolia (7). In our earlier works we studied *A. chrysochlorus* in various ways such as we determined antioxidant, cytotoxic, phagocytic effects and selenium accumulation capacity (8,9,10). The keystone of increasing the bioactive phenolics in *A. chrysochlorus*, is the studying of the key enzymes of important pathways. Therefore, we focused on the very first enzyme of phenylpropanoid metabolism.

Bioassays showed that nicotiflorin has been reported as neuroprotective in neuronal cultures, and in permanent focal cerebral ischemia (11). It also exhibits rat lens aldose reductase inhibitory activity *in vitro* (12). It showed hACAT1 (human Acyl CoA: cholesterol transferase 1) inhibitory activity (13). It was also found to have a significant *in vitro* immunomodulatory effect (14). Nicotiflorin also significantly inhibits the decrease of blood flow during the anaphylactic responses (15). Abreu and his colleagues (16) reported that kaempferol 3-O-rutinoside isolated from *Ebenus pinnata* showed significant antioxidant activity in the DPPH, and TEAC, reducing power assays. Also, potential *in vitro* antiglycation activity was exhibitied by nicotiflorin or kaempferol-3-O-rutinoside which was isolated from the aerial parts of *Osyris wightiana* Wall. ex Wight (17).

NMR (nuclear magnetic resonance) analysis demonstrated that a natural flavonoid, a type of bioactive flavonol glucosides, nicotiflorin accumulated in callus tissues of *A. chrysochlorus* which were the starting material of cell suspension cultures (18). In this study, we have cloned and examined well studied enzyme phenylalanine ammonia-lyase (PAL) belonging to phenylpropanoid pathway, is necessary to upregulate flavonoid biosynthesis in most of the plant species. Also complete sequence of PAL gene from *A. chrysochlorus* was determined. Transcript levels of *AcPAL* was analysed for *A. chrysochlorus* cell suspension cultures after yeast extract treatment in different period of time to evaluate stress response.

Materials and Methods

Plant material and callus induction

Astragalus chrysochlorus seeds were collected from Sertavul, Karaman, Turkey, and classified by Prof. Dr. Zeki Aytaç (Gazi University, Ankara, Turkey), and a voucher specimen was deposited in the herbarium at İstanbul University, Faculty of Science Herbarium (ISTF). Long-term (13 years) *in vitro* cultured *Astragalus chrysochlorus* calli tissues were used as described in Turgut-Kara and Kahraman (19).

Cell suspension culture and yeast extract treatment

Long term *in vitro* cultured *A. chrysochlorus* callus derived primary cell suspension cultures were propagated in the dark as described by Çakır and Arı (20). Five ml culture (with 1 ml packet cell volume) from the primary cell suspension culture was subcultured in 25 ml fresh MS medium (21) consisted of 3% sucrose and 1 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) in 100 ml Erlenmeyer flask. Yeast extract was prepared according to Chen and Chen (22) by ethanol precipitation. It was added to cell cultures as 10 g/l concentration on the 13th day which was the first day of log phase (20). Water was used as control and equal volume of water added to the control cultures. Samples were collected from cultures at 0, 3, 6 and 18 hours for gene expression analysis.

RNA extraction and quantitative Real-Time PCR analysis

For qPCR analysis, total RNA was isolated using the Favorgen Plant RNA isolation kit (FAPGK001, Favorgen Biotech Corp., Kaohsiung, Taiwan, ROC), with the application of DNaseI (EN0521, Thermo Scientific, Fermentas Inc, Glen Burnie, MD, USA) treatment during the isolation process. cDNA synthesis kit (K1612, Thermo Scientific, Fermentas Inc, Glen Burnie, MD, USA) was used for the synthesis of cDNA, which served as a template for qPCR. It was carried out with the DyNAmoe HS SYBR Green qPCR Kit (F410L, Thermo Scientific, USA) following the manufacturer's instructions. Forward and reverse qPCR primers for PAL and actin were as follows: PAL, forward primer: 5'-TGACTTGAGGCATTTGGAGG-3' and reverse primer: 5'-GAGTTCTCCATTGAC-3'; actin, forward primer: 5'-TCAAGACGAAGGATG-3' and reverse primer: 5'-TTGGATTCTGGTGAT-3'. Reaction mixtures consisting of 12.5 ml 2x SYBR Green master mix, 0.2 mM each of forward and reverse primers and 5 µl cDNA were brought to 25 µl with ultra pure nuclease-free water. qPCR was done accordingly: 2 min at 50°C/ min (Uracil-DNA glycosylase incubation), 15 min at 95°C (pre-incubation), 40 cycles of 10 s at 94°C, 30 s at 60°C and 30 s at 72°C. A gradual temperature step was added to determine melting curve data from 55°C to 95°C at the rate of 1°C/10 s. A non-template control was run along side, and serial dilutions (1, 1:5, 1:25 and 1:125) of the reference (actin) and the target genes were included with every assay. Amplification specificity of each reaction was verified by melting curve analysis. Actin was used as the reference gene. The software used to calculate gene Expression was the MX3000 Stratagene software (Agilent Technologies, Inc., Santa Clara,USA). qPCR experiments were repeated in triplicates for samples.

Isolation of cDNA encoding complete *AcPAL*

PAL gene sequences of phylogenetically close species were compared with alignment to obtain a list of degenerate primer pairs. Using this approach, primer pairs could be designed for PCR amplification of cDNAs of species which have not got any available sequence information. To identify the unknown genes, the PCR based strategy could be applied by aligning the sequences of conserved and variable parts of known genes (23). Therefore, we performed multiple alignments between PAL cDNA sequences of legumes from databases. By using that data we designed PAL specific primer pairs: PAL, forward primer: 5'-ATGGAGGGAGAAGGA-3' and reverse primer: 5'-TAAGAAATTGGAAGAGGAGC-3'. This primer pairs make it possible to obtain full length cDNA of AcPAL. Reaction mixtures consisting of 2 µl 10X buffer, 1 µl 10 mM dNTP, 1 µl 20 mM $MgCl_2$, 0.5 µl 10 mM each of forward and reverse primers, 1 µl 100 ng cDNA and 0.125 µl 5U/ µl Taq polymerase were brought to 20 µl with ultra pure nuclease-free water. The PCR cycling parameters were set as follows: 94 ºC for 5 min, 1 cycle; 94 ºC for 30 s, 51 ºC for 30 s, 72 ºC for 2:30 min, 35 cycles; and 72 ºC for 10 min, 1 cycle. The band of proper size was purified from 1.2 % agarose gel, and cloned into the pCR®4-TOPO® vector for sequencing.

Homology searches were performed on NCBI BLAST webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conceptual translation of nucleotide sequence, prediction of molecular mass were carried out using ExPASy proteomics server (http://expasy.org/tools/). Sequence alignments were carried out using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/).

Expression of *AcPAL* gene in *E. coli*

First of all, full length *AcPAL* product was amplified with *Pfu*nds-DNA Polymerase (M3004.0250, Genaxxon Bioscience GmbH), and was cloned to plasmid pET100D (K100-01, Champion™ pET100 Directional TOPO® Expression Kit, Invitrogen). Then, pET100DAcPAL was cloned to *E. coli* strain BL21Star (DE3). It was aimed that the PAL gene is overexpressed constitutively under T7 promoter. Cells were grown in 20 ml of LB medium containing 50 µg/ml ampicillin at 37°C and maximal aeration and grown until the cell reach a density of A_{600}= 0.5. Overexpression of PAL was begun by adding the 1 mM as final volume of isopropylthiogalactoside (IPTG, Sigma Cat no. I6758). 0.5 ml sample were collected at 0, 2, 4, 6 h.

Results

Expression analysis of elicited cell cultures

As described in Turgut-Kara and Uhra Kahraman (19), thirteen years old, actively growing, green, friable, and well adapted callus cultures were used as starting material for establishing cell suspension cultures. For this reason as a first step, 21th day subcultured calli were used to establish cell suspension cultures. Therefore the convenient culture system was obtained to study the reaction of *A. chrysochlorus* cells against yeast extract. Cell suspension cultures were propagated as described

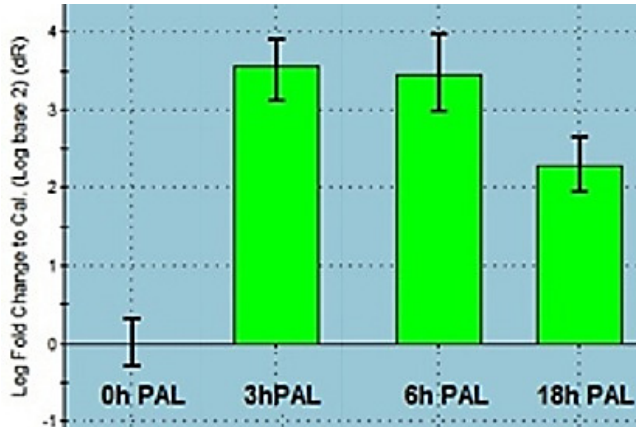

Figure 1. Relative expression levels of *AcPAL* under 0, 3, 6 and 18 hours yeast extract stress conditions. The expression levels were calibrated according to 0h yeast extract treatment, and normalized according to reference gene actin. The values and error bars represent the mean ± standard error from three samples.

(20). 13th day (first day of log phase) of cell culture 10 g/L concentration of yeast extract was added to the cell suspensions according to (20). To test the response of *PAL* gene expression after yeast extract elicitation, cultures were treated with yeast extract for 0, 3, 6 and 18 hours.

We analyzed that the differential expression patterns of *AcPAL* under 0, 3, 6 and 18 hours yeast extract stress conditions. In general, *AcPAL* transcripts increased rapidly in the first 3 hours of treatment but then decreased gradually to the initial level 18h later. Three hours elicitor treatment caused 3.56-fold higher accumulation of the *AcPAL* transcripts in cell cultures according to zero point to enhance the accumulation of total phenylpropanoids (Figure 1).

Isolation and characterization of *A. chrysochlorus* full-length PAL

A. chrysochlorus PAL cDNA fragment was obtained by RT-PCR using the conserved primers designed according to knowledge of other *Astragalus* PAL sequences [*A. membranaceus* (EF567076), *A. membranaceus* var. mongholicus (AY986506, EF110924, EF110925)], and was named as *AcPAL* (Accession number: KM189182). *AcPAL* contained an open reading frame of 2160 bp and encodes 720 amino acid residues with a molecular mass of approximately 78.53 kDa. Deduced amino acid sequences of the BLAST analysis are shown in (Figure 2). So far, multigene family members, PAL1, PAL2 and PAL3 sequences of *Astragalus membranaceus* and *Astragalus membranaceus* var. *mongholicus* have been registered to the gene bank. According to our results, AcPAL shares the highest protein identity with *Astragalus mongholicus* PAL1 mRNA (Accession number: AY986506) (96%), *Astragalus membranaceus* PAL mRNA (Accession number: EF567076) (95%), and *Trifolium pratense* phenylalanine ammonia lyase PAL1 mRNA (Accession number: DQ073809) (87%).

To confirm the full length protein encoded by PAL from *A. chrysochlorus*, the gene was cloned into plasmid pET100D-TOPO to be expressed in *E.coli*. Overexpression of transferred gene in recombinant *E.coli* led to insoluble protein (Figure 3). The amount of PAL protein expression increased when time prolonged. Molecular mass of PAL is about 78.53 kDa as validated by SDS-PAGE, which is basically in agreement with an expected size of 81.53 kDa (78.53 kDa derived from the gene

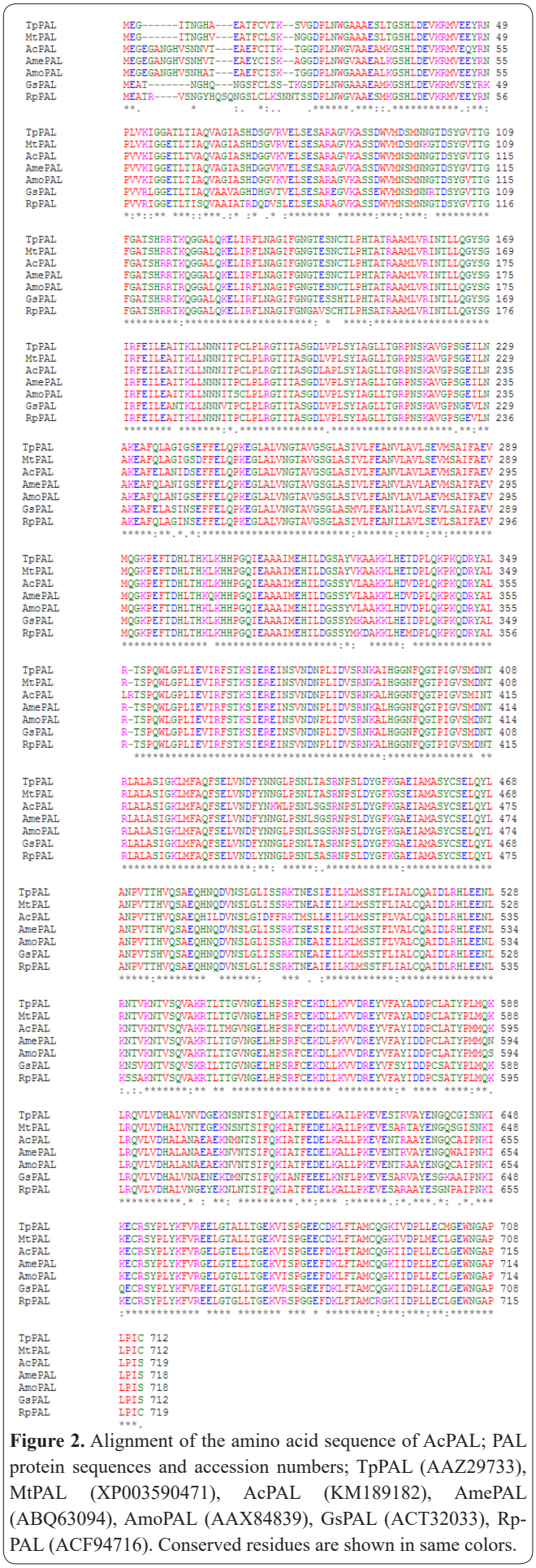

Figure 2. Alignment of the amino acid sequence of AcPAL; PAL protein sequences and accession numbers; TpPAL (AAZ29733), MtPAL (XP003590471), AcPAL (KM189182), AmePAL (ABQ63094), AmoPAL (AAX84839), GsPAL (ACT32033), RpPAL (ACF94716). Conserved residues are shown in same colors.

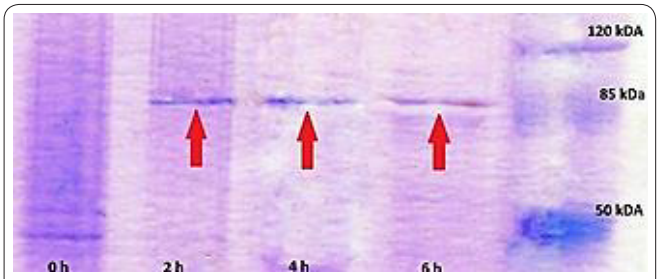

Figure 3. SDS-PAGE analysis of expressed PAL proteins derived from *Astragalus chrysochlorus* PAL gene in *E. coli* BL21 Star (DE3) at 0, 2, 4, and 6 h IPTG induction.

sequence and 3 kDa belongs to plasmid).

Discussion

In our previous study, we showed that a bioactive flavonol glucosides, nicotiflorin, accumulated in callus tissues of *A. chrysochlorus* which were the starting material of cell suspension cultures in this study (18). PAL is the first enzyme in biosynthesis of phenylpropanoids, that is also the key of secondary phenylpropanoid metabolism in plants (1). Because of this knowledge, to enhance the production of economically important phenylpropanoids, many strategies have focused on PAL enzyme so far. In this study, we have cloned and examined well studied enzyme PAL, is necessary to upregulate flavonoid biosynthesis in most of the plant species. Transcript levels of *AcPAL* was analysed for *A. chrysochlorus* cell suspension cultures after yeast extract treatment in different period of time to evaluate stress response. Because, a variety of biotic or abiotic stresses factors can induce PAL gene expression (24). *AcPAL* transcripts increased in the first 3 hours of treatment but then decreased gradually to the initial level 18h later. Three hours elicitor treatment caused about 3.5-fold higher accumulation of the *AcPAL* transcripts in cell cultures according to zero point to enhance the accumulation of total phenylpropanoids. According to our gene expression data, AcPAL was induced by yeast extract stress, and this signify that it could be also considered as a potential target gene to improve stress tolerance.

Previous studies showed that the metabolites of phenylpropanoid pathway markedly accumulated upon biotic and abiotic stress, which is a result of increase in expression of PAL and other phenylpropanoid enzymes. Nevertheless, PAL gene from diverse origins may have different responsive capacities to stress factors (24, 25, 26). It was achieved to trigger the coumarins and phenylpropanoids accumulation by adding crude cell wall fractions from *Phytophtora sojae* to *A. majus* cell suspensions, and butenyl and hydroxybutenyl ethers of umbelliferone accumulated in the culture fluid and reached to maximum concentrations after 35h (27). After the elicitation, at 12h, the activities of dimethylallyldiphosphate:umbelliferone 6-C- and 7-O-dimethylallyltransferases reached a first maximum (27). It is thought that maximum transcript abundances occured within the first 6h.

A. chrysochlorus PAL cDNA fragment was obtained by RT-PCR using the conserved primers designed according to knowledge of other PAL sequences of *Astragalus* spp. Open reading frame of *AcPAL* (Accession number: KM189182) constitutes 2160 bp and encodes 720 amino acid residues with a molecular mass of approximately 78.53 kDa. Multigene family members, PAL1, PAL2 and PAL3 sequences of *Astragalus membranaceus* and *Astragalus membranaceus* var. *mongholicus* have been registered to the gene bank. AcPAL shares the highest protein identity with *A. mongholicus* PAL1 (Accession number: AY986506) (96%), *A. membranaceus* PAL (Accession number: EF567076) (95%), and *Trifolium pratense* PAL1 (Accession number: DQ073809) (87%).

To confirm the full length protein encoded by PAL from *A. chrysochlorus*, the gene was cloned into plasmid pET100D-TOPO to be expressed in *E.coli*. Molecular mass of PAL is about 78.53 kDa as validated by SDS-PAGE. Putative amino acid sequence alignments which were done by using the ClustalW2 program demonstrated that Ac*PAL* was most closely related to other Astragalus species, among the surveyed plant species. Multiple alignments of amino acid sequences between AcPAL and others showed that there are many well-conserved motifs.

Due to the metabolical roles of phenylpropanoids, it is important to understand this pathway in all plants including the medicinal ones. The enzymes which are encoded by key genes of pathways have to be cloned and characterized (28). This is the keystone to reveal the regulatory mechanisms wich rule the phenylpropanoids in *Astragalus*. It will be helpful in discovering new strategy for efficient genetic modification of *Astragalus* to increase the nicotiflorin content for nutritional or medical purpose as well as stress tolerance.

Acknowledgements

This study was financially supported by the Research Fund of the Istanbul University (Project numbers 3640 and 16173).

Conflict of interest

The authors declare that there are no conflicts of interest.

References

1. Hahlbrock K, Scheel D. Physiology and molecular-biology of phenylpropanoid metabolism. Annu Rev Plant Phys 1989; 40: 347-369.

2. Boudet AM. Evolution and current status of research in phenolic compounds. Phytochemistry 2007; 68: 2722-2735.

3. Miller AL. Botanical influences on cardiovascular disease. Altern Med Rev 1998; 3, 422-431.

4. Sinclair S. Chinese herbs: A clinical review of Astragalus, Ligusticum, and Schizandrae. Altern Med Rev 1998; 3: 338-344.

5. Ma XQ, Shi Q, Duan JA, Dong TT, Tsim KW. Chemical analysis of radix astragali (huangqi) in china: A comparison with its adulterants and seasonal variations. J Agric Food Chem 2002; 50: 4861-4866.

6. Xiao HB, Krucker M, Albert K, Liang XM. Determination and identification of isoflavonoids in radix astragali by matrix solid-phase dispersion extraction and high-performance liquid chromatography with photodiode array and mass spectrometric detection. J Chromatogr A 2004; 1032: 117-124.

7. Aytaç Z. The revision of the section Dasyphyllium bunge of the genus. Turk J Bot 1997; 31-57.

8. Hasançebi S. Studies on tissue culture and transformation of Astragalus chrysochlorus. Istanbul University, PhD Thesis, Istanbul, 2003.
9. Karagoz A, Turgut-Kara N, Cakir O, Demirgan R, Ari S. Cytotoxic activity of crude extracts from Astragalus chrysochlorus (leguminosae). Biotechnol Biotec Eq 2007; 21: 220-222.
10. Ari S, Cakir O, Turgut-Kara N. Selenium tolerance in Astragalus chrysochlorus: Identification of a cdna fragment encoding a putative selenocysteine methyltransferase. Acta Physiol Plant 2010; 32: 1085-1092.
11. Li RP, Guo ML, Zhang G, Xu XF, Li Q. Neuroprotection of nicotiflorin in permanent focal cerebral ischemia and in neuronal cultures. Biol Pharm Bull 2006; 29: 1868-1872.
12. Lim SS, Jung YJ, Hyun SK, Lee YS, Choi JS. Rat lens aldose reductase inhibitory constituents of Nelumbo nucifera stamens. Phytother Res 2006; 20; 825-830.
13. Yang H-J, Myoung-Chong S, Myun-Ho B, Jin-Hee L, In-Sik C, Youn-Hyung L, Tae-Sook J, Byoung-Mog K, Sung-Hoon K, Dae-Keun K, Mi-Hyun P, Nam-In B. Development of biologically active compounds from edible plant sources-xii. Flavonol glycosides from Trigonotis peduncularis Benth and its hacat1 inhibitory activity. J Korean Soc Appl Biol Chem 2005; 48: 98-102.
14. Akbay P, Basaran AA, Undeger U, Basaran N. In vitro immunomodulatory activity of flavonoid glycosides from Urtica dioica L. Phytother Res 2003; 17: 34-37.
15. Ishiguro K, Ohira Y, Oku H. Preventive effects of impatiens balsamina on the hen egg-white lysozyme (hel)-induced decrease in blood flow. Biol Pharm Bull 2002; 25: 505-508.
16. Abreu PM, Braham H, Ben Jannet H, Mighri Z, Matthew S. Antioxidant compounds from Ebenus pinnata. Fitoterapia 2007; 78: 32-34.
17. Shyaula SL, Abbas G, Siddiqui H, Sattar SA, Choudhary MI, Basha FZ. Synthesis and antiglycation activity of Kaempferol-3-o-rutinoside (Nicotiflorin). Med Chem 2012; 8: 415-420.
18. Turgut-Kara N, Çakır Ö, Hasançebi S, Karabey F, Arı Ş . Identification and production of phenolic nicotiflorin in Astragalus chrysochlorus callus. Farmacia 2018 (Article in press).
19. Turgut-Kara N, Kahraman BU. Effects of long-term culture of Astragalus chrysochlorus callus onmorphology, genetic structure, gene expression and metabolism. Plant Biosyst 2015; 149: 329-336.
20. Cakir O, Ari S. Defensive and secondary metabolism in Astragalus chrysochlorus cell cultures, in response to yeast extract stressor. J Environ Biol 2009; 30: 51-55.
21. Murashige T, Skoog FA (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 1998; 15: 473-497.
22. Chen H, Chen F. Effect of yeast elicitor on the secondary metabolism of ti-transformed Salvia miltiorrhiza cell suspension cultures. Plant Cell Rep 2000; 19: 710-717.
23. Fredslund J, Schauser L, Madsen LH, Sandal N, Stougaard J. Prifi: Using a multiple alignment of related sequences to find primers for amplification of homologs. Nucleic Acids Res 2005; 33: W516-W520.
24. Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. Plant Cell 1995; 7: 1085-1097.
25. Liu RR, Xu SH, Li JL, Hu YL, Lin ZP. Expression profile of a pal gene from Astragalus membranaceus var. Mongholicus and its crucial role in flux into flavonoid biosynthesis. Plant Cell Rep 2006; 25: 705-710.
26. Tasaki Y , Hayashi S, Kato M. L-Phenylalanine supplementation increases the production of phenylalanine ammonia-lyase and methyl cinnamate in the mycelia of Tricholoma matsutake. Mycoscience 2018; 59: 8-11.
27. Hamerski D, Beier RC, Kneusel RE, Matern U, Himmelspach K. Accumulation of coumarins in elicitor-treated cell-suspension cultures of Ammi majus. Phytochemistry 1990; 29: 1137-1142.
28. Li H, Jiao X, Zhou W, Sun Y, Liu W, Lin W, Liu A, Song A, Zhu H. Enhanced production of total flavones from Inonotus baumii by multiple strategies. Preparative Biochemistry and Biotechnology 2018; 48:2, 103-112.