

## Two Novel Alliin Lyase (Alliinase) Genes from Twisted-Leaf Garlic (*Allium obliquum*) and Mountain Garlic (*Allium senescens* ssp. *montanum*)

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### Abstract

Alliinase (Alliin lyase EC 4.4.1.4), a pyridoxal phosphate-dependent lyase, represents one of the major protein components of *Allium* species. The enzyme is a homodimeric glycoprotein and catalyzes the synthesis of allicin (diallyl thiosulfinate, a biologically active compound), pyruvate, and ammonia starting from the specific non-protein sulfur-containing amino acid alliin ((+S)-allyl-L-cysteine sulfoxide). Using newly developed specific primers two new alliinase genes from *Allium obliquum* and *Allium senescens* ssp. *montanum* were amplified and sequenced, as well as their homologs, from *Allium fistulosum* and *Allium schoenoprasum*. The G+C content of the alliinase region ranges between that of other dicot plants and that reported in monocot cereal plants, in all four species. Investigations of gene expression revealed a significantly higher enzyme expression level in bulbs than in leaves in all four taxa. The deduced alliinase sequences displayed a high variability among different species, since the lowest sequence similarity was found to be 55.5% between *Allium senescens* ssp. *montanum* and *Allium cepa*, while the highest similarity is 77.5%, between *Allium senescens* ssp. *montanum* and *Allium fistulosum*. Leucine is the most common amino acid in all four alliinases, while cysteine is also more frequent than in other enzymes, suggesting a high stability of the molecules due to the possible disulfide bonds.

**Keywords:** allicin, alliinase, mountain garlic, qPCR, twisted-leaf garlic

### Introduction

In onion (*Allium cepa*), garlic (*Allium sativum*) and other related *Allium* species, the characteristic flavor is due to the alliinase enzyme (EC 4.4.1.4). Also known as alliin lyase, or alkyl Cys sulfoxide lyase, it hydrolyzes the S-alk(en)yl-L-Cys sulfoxides (alliin) to form pyruvate, ammonia, and sulfur-containing volatiles (allicin) (Lancaster and Collin, 1981; Stoll and Seebeck, 1951). Alliinase was first described in garlic, in 1947 (Stoll and Seebeck, 1947). The alliinase from garlic bulbs was the first one to be purified (Mazelis and Crews, 1968). Its activity is inhibited by hydroxylamine and cysteine, while the addition of pyridoxal phosphate, EDTA, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup> or Fe<sup>2+</sup> stimulates the reaction rate. First DNA sequences of genes encoding for alliinase were reported in *Allium sativum* (garlic), *Allium cepa* (onion), and *Allium ascalonicum* (shallot) (Van Damme *et al.*, 1992). They are highly similar both at the nucleotide and amino acid levels. In garlic, the molecular mass of the enzyme is 51451 Da (Van Damme *et al.*, 1992). Several new alliinases were further purified, displaying a variety of isoforms (Lancaster *et al.*, 2000; Manabe *et al.*, 1998). In Chinese chive (*Allium tuberosum*), the molecular mass of alliinase is 54083 Da, and the deduced amino acid sequence exhibited 66-69% identity to the reported alliinases of onion, garlic, and shallot. Two isoforms with alliinase activity were isolated from onion, and the gene

homology to other reported allium alliinase genes was 50% (Lancaster *et al.*, 2000).

In some alliinase genes, the G+C content is similar to that of dicot plants and lower than that of monocot cereal plants, although onion is a monocot (Do *et al.*, 2003). The three-dimensional structure of the garlic alliinase at 1.5 Å resolution revealed that the active enzyme is a pyridoxal-5'-phosphate-dependent homodimeric glycoprotein (Kuettner *et al.*, 2002), while another X-ray structure of the enzyme has improved the understanding of the pyridoxal phosphate binding to the active site (Shimon *et al.*, 2007). A novel heteropentameric alliinase was detected and purified from the roots of the Amazonian medicinal plant *Petiveria alliacea* (Musah *et al.*, 2009b), the same species displaying a novel lachrymatory factor synthase (LFS) (Musah *et al.*, 2009a), an enzyme active only in the presence of alliinase (Eady *et al.*, 2008). Another alliinase, purified from the bacterium *Ensifer adhaerens*, displays a novel type of substrate specificity (Yutani *et al.*, 2011). Several attempts to elucidate the conditions that may increase the enzyme activity have been carried out. Thus, some evidences suggest that the activity of alliinase might be enhanced by temperature, pH, low frequency, and mild intensity ultrasound (Musah *et al.*, 2009a; Wang *et al.*, 2011; Yutani *et al.*, 2011).

Alliinase can be immobilized through lysine residues on the surface of various matrices to produce allicin (Miron

et al., 2006). Allicin and alliin are well-studied biological compounds, since they may play an important medical role (Freeman and Kodera, 1995; Jones et al., 2004; Miron et al., 2004; Shadkchan et al., 2004). In particular, garlic is the most used alliinase-producing species in cancer therapy (Seki et al., 2008), in cardiovascular protection (Oron-Herman et al., 2005; Shimon et al., 2007), or in respiratory diseases (Appel et al., 2010; Rose et al., 2005).

In this paper the characterization of two novel bulb and leaf alliinase genes from *Allium obliquum* and *Allium senescens* ssp. *montanum* subsequent to cloning their cDNA is reported. The genes are slightly different in sequence from other alliinase genes, and the deduced proteins may have novel characteristics compared to other *Allium* alliinases. The function of these novel alliinases is discussed.

## Material and methods

### Plants

Whole fresh plants of *Allium obliquum* (Twisted-leaf Garlic), *Allium senescens* ssp. *montanum* (Mountain Garlic), *Allium fistulosum* (Welsh Onion) and *Allium schoenoprasum* (Chives) were collected from natural habitats in Transylvania, Romania, and they were stored at -80°C until further analysis.

### New primers design and use

Forty-seven alliinase sequences obtained from GenBank were aligned using CLC Workbench 6.0 (Finlandsgade 10-12, Katrinebjerg 8200 Aarhus N, Denmark). Several new primer pairs were designed based on the consensus sequence of approx. 1700 bp in the alliinase gene region, using PerlPrimer (Marshall, 2004). Moreover, two primer pairs for real-time polymerase chain reaction (qPCR) were designed, targeting a 220 bp alliinase gene fragment (Tab. 1). All primer pairs were first tested using the "in silico PCR" tool of the FastPCR 6.1 software (Kalendar et al. 2009).

### DNA and RNA isolation; real time PCR

Genomic DNA and total RNA were isolated from plant bulbs and leaves using Wizard® Genomic DNA Purification Kit and SV Total RNA Isolation System (Promega, Madison, WI, USA), following manufacturer's instructions. Two units of Turbo DNase treatment (Ambion Europe, LTD Huntingdon, Cambridgeshire, UK) were applied in order to remove the traces of residual genomic DNA. cDNA was obtained with a First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada) using random primers. Expression of the gene encoding the enzyme alliinase was probed by qPCR with a BioRad iQ5 thermocycler using the two pairs of specific primers (Tab. 1), and using the phosphoenolpyruvate carboxylase gene as reporter, by applying a mathematical model (Pfaffl, 2001).

### PCR amplification of alliinase and 18S rRNA genes

The PCR assay conditions for the alliinase gene were optimized for the newly designed primers as follows: each 50 µl reaction volume included 50 ng cDNA template, 5 µl 10X buffer with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Fermentas), dNTPs (200 µmol/l), MgCl<sub>2</sub> (1.5 mmol/l), primers (400 nmol/l) and 2.5 U DreamTaq DNA Polymerase (Fermentas). Cycling conditions were: 95°C for 2 min, followed by 33 cycles at 93°C for 12 sec, 54°C for 22 sec, 72°C for 1 min, with the final elongation of 5 min at 72°C.

The 18S rRNA gene was amplified with the CV1-CV4 primer pair (Sawayama et al., 1992) (Tab. 1). 50 ng of genomic DNA were added to 49 µl of the amplification mixture, for a final concentration of 1X DreamTaq Buffer (Fermentas), 200 µM dNTP mix, 0.4 µM of each of the forward and reverse primers, and 1.25 U/µl of DreamTaq Polymerase (Fermentas), in a final volume of 50 µl. The PCR program included 1 cycle of initial denaturation at 93°C for 5 min, followed by 33 cycles with a denaturing step of 30 s at 95°C, an annealing step of 30 s at 57°C, and an elongation step of 1 min at 72°C. The final elongation was done for 5 min at 72°C.

All PCR products were separated on 1% agarose gel in a 1x TAE running buffer, stained with ethidium bromide (1 µg/ml) and visualized by transillumination. The FastRuler™ Middle Range DNA Ladder (Fermentas) was used to measure the PCR product size. Each PCR was performed 3-5 times.

### Cloning and sequencing of a partial alliinase cDNA and 18S rRNA genes

PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) and then cloned into the pGEM-T vector using the pGEM-T vector system (Promega). Plasmid DNA was isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega) from colonies that had the correct insert size, as determined by PCR with M13 forward and reverse primers. Sequencing reactions were performed using a terminator cycle sequencing kit (ABI PRISM Big Dye kit, Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI PRISM 3100 Genetic Analyzer. All the resulted fragments were deposited in the GenBank database under accession number JN797255-JN797262.

### Bioinformatic analysis

The 18S rRNA nucleotide sequences generated in this study were aligned with other 40 similar fragments from GenBank using CLC Workbench 6.0 (Finlandsgade 10-12, Katrinebjerg 8200 Aarhus N, Denmark). The alignment was used to generate a phylogenetic tree with 31 different sequences (the multiple sequences belonging to same species were removed, so that each species was represented by one single sequence), with the 18S rRNA gene of *Lacistema aggregatum* (a dicot species) as outgroup.

Translated amino acid sequences of alliinases were generated using ExPaSy (Gasteiger *et al.*, 2003), starting from the fragments amplified with the newly designed primers. These sequences, together with other alliinase available sequences (GenBank, NCBI) were aligned using CLC Workbench 6.0, in order to identify similarities/differences between them. Moreover, all the enzyme sequences obtained from the four studied species were analyzed with regard to their isoelectric point, molecular weight, and topology, using the corresponding tools on the ExPaSy web site (Gasteiger *et al.*, 2003).

**Results**

*Analysis of alliinase and 18S rRNA genes*

A fragment of approx. 1700 bp of the alliinase gene was successfully amplified with the new primers using cDNA as template (Fig. 1a). Similarly, a 2000 bp fragment was generated with the CV1-CV4 primer pair during the PCR amplification of the 18S rRNA gene using genomic DNA as template (Fig. 1b). The length of the PCR amplified fragments matched the one expected from *in silico* amplification using FastPCR 6.1 software (1705-1731 bp) (Tab. 1).

Four different alliinase genes were amplified and sequenced from the four studied species. In each species, the gene from the bulb was identical to the one from the leaves. The length of gene sequences obtained from the four species ranged from 1728 (in *Allium obliquum*) to 1788 bp (in *Allium schoenoprasum*), close to the average length of other monocots alliinase genes.

The G+C content of the analyzed alliinase genes is: 40,9% in *Allium obliquum*, 41,7% in *Allium senescens* ssp. *montanum*, 43,5% in *Allium fistulosum*, 43,6% in *Allium schoenoprasum*, and very similar to the one found in *Allium sativum* (39,9%). Overall, the G+C content of the

four alliinase regions ranges between the 33% of the other genomic regions in dicot plants and the 45% reported in monocot cereal plants.

The phylogenetic analyses based on the 18S rRNA sequences have grouped the four species studied in the present work into a single cluster with a bootstrap value of 85 (Fig. 2). Among all the species in the tree, it seems that *Agapanthus africanus* and *Behnia reticulata* are the closest related species to the *Allium* genus. The single dicot species included in the tree, *Lacistema aggregatum*, was grouped apart from all the other species, with a maximum bootstrap value of 100.

*qPCR*

The expression of bulb and leaf alliinase genes in four species was also investigated: *Allium obliquum*, *Allium senescens* ssp. *montanum*, *Allium fistulosum* and *Allium schoenoprasum*. It was revealed that the enzyme located in the bulb is expressed at a higher level than in leaves in all four of them. Thus, the bulb:leaves expression ratio is 6.8:1 in *Allium obliquum*, 3.4:1 in *Allium senescens* ssp. *montanum*, 5:1 in *Allium fistulosum*, and 3.3:1 in *Allium schoenoprasum*.

*Analysis of the amino acid sequences*

The deduced alliinase amino acid sequence from *Allium obliquum* and *Allium senescens* ssp. *montanum* were aligned with other alliinase amino acid sequences of *Allium*. Homology with *Allium cepa* alliinase is 59.4% for *Allium obliquum* and 55.5% for *Allium senescens* ssp. *montanum*, respectively. Homology with garlic (*Allium sativum*) clove alliinase is 71.4% and 68.5% (Van Damme *et al.*, 1992). Homology with shallot (*Allium ascalonicum*) is 71.8% and 65.4%, and with chinese chives (*Allium tuberosum*) homology is 69.4% and 74.3% (Manabe *et al.*, 1998). When compared with the other two *Allium* species

Tab. 1 The primers used during this study. F2-All + R2-All and F3-ALL + R3-ALL were used to amplify the entire alliinase gene, while F1\_int-ALL and F2\_int-ALL were used as internal primers in order to sequence the gene. The FqPCR1-ALL + RqPCR1-ALL primer pair amplified an approx. 220 bp fragment of the alliinase gene for the qPCR analyses. The CV1 + CV4 primer pair was used to amplify the 18S rRNA gene and to sequence it, also using the internal primer CV2 (Sawayama *et al.*, 1992). Primers designed here are bolded

Primer name	Sequence 5'-3'	Melting temp. Tm (°C)	Amplicon expected length (bp)
<b>F2-ALL</b>	GAGGCGGTGGCTAATATAAACTG	57	1731
<b>R2-ALL</b>	TATACGTCCATTCTGGGCAGTCA	58	
<b>F3-ALL</b>	AGGCAGTGGGGGATATAAACTG	58	1705
<b>R3-ALL</b>	CGTCCATTCTGAAATGTCTGGT	56	
<b>F1_int-ALL</b>	TTGCTCATGCCCTTATTATCC	56	-
<b>F2_int-ALL</b>	TGGAGTATAGGTGGCTCAACTC	57	
<b>FqPCR1-ALL</b>	GCTCTGCTGATGTTGCCAGGT	59	221
<b>RqPCR1-ALL</b>	CCACTGCATGCATCATGTAGTG	58	
CV-1	TACCTGGTTGATCCTGCCAGTAG	59	2000
CV-4	ACCTTGTTACGACTTCTCCTTCCTC	59	
CV-2	CCAATCCCTAGTCGGCATCGT	60	

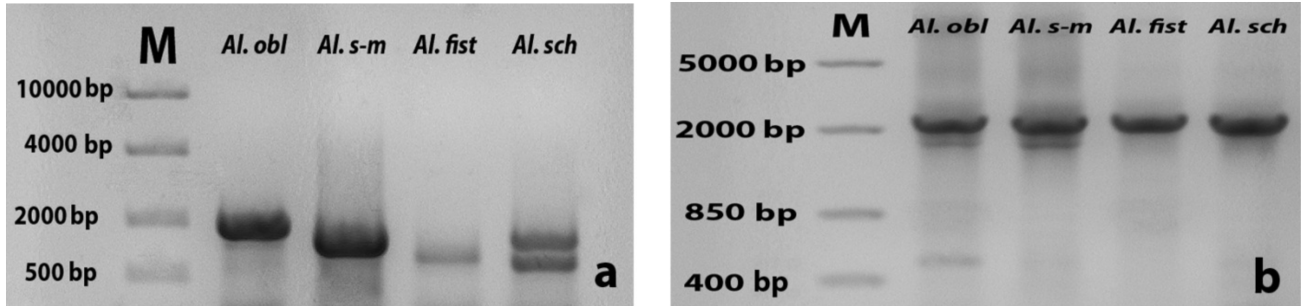


Fig. 1. PCR amplification of the alliinase (a) and 18S rRNA (b) genes. The alliinase gene was amplified with new primer pairs, designed during this study, while the 18S rRNA gene was amplified with the CV1-CV4 primer pair (Sawayama *et al.*, 1992). Al. obl = *Allium obliquum*; Al. s-m = *Allium senescens* ssp. *montanum*; Al. fist = *Allium fistulosum*; Al. sch = *Allium schoenoprasum*

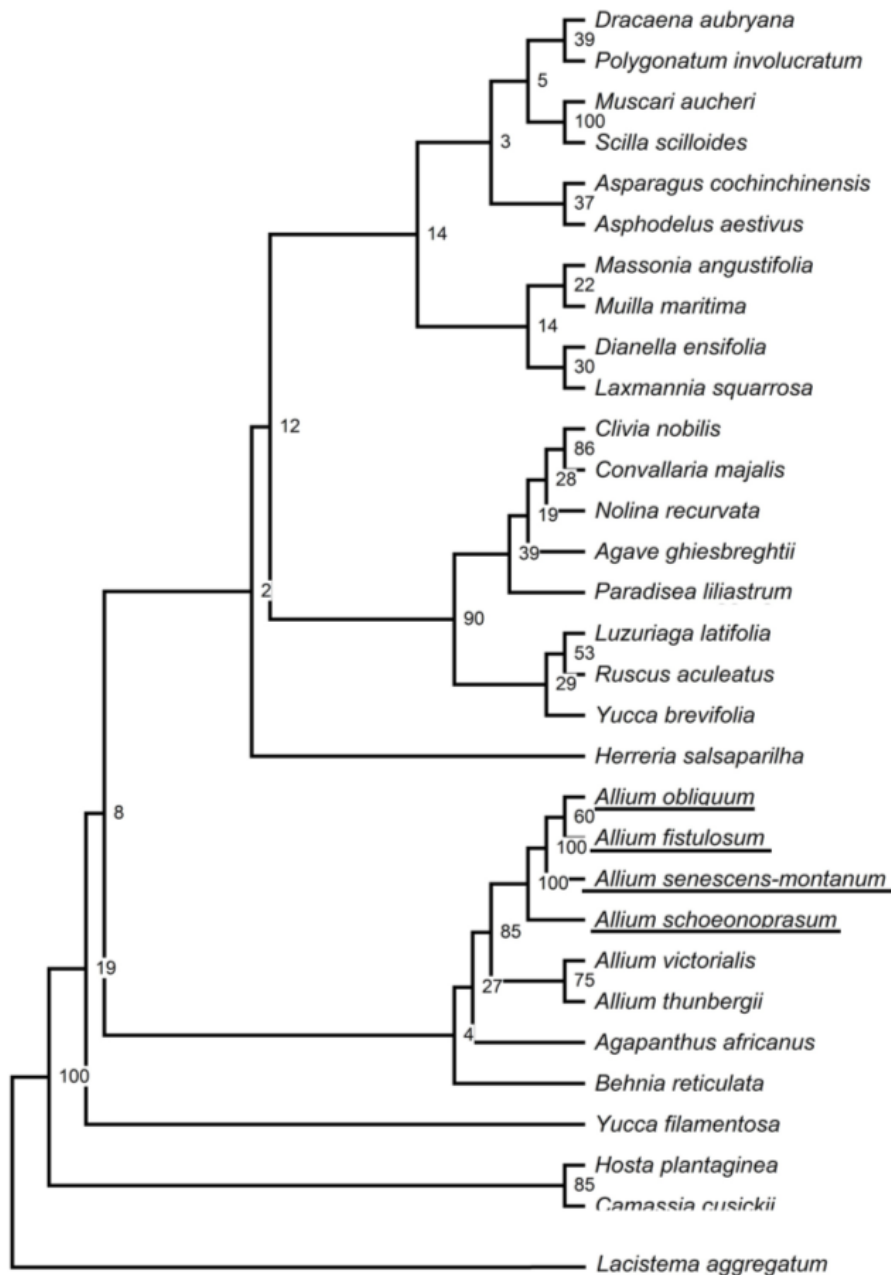


Fig. 2. The phylogenetic tree generated using the *Minimum evolution* method, with the four *Allium* species analyzed here being underlined. The bootstrap test was performed in 100 replicates

studied here, there was revealed that the homology with *Allium fistulosum* is 77,5% and 66%, while the homology with *Allium schoenoprasum* is 56,5% and 64,8%. The homology between the analyzed species, *Allium obliquum* and *Allium senescens* ssp. *montanum* is 70,5%, while between *Allium fistulosum* and *Allium schoenoprasum* the amino acid sequence homology is 67,4%.

The most frequent amino acid in the four sequenced alliinases is leucine, with about 10% of the total amino acid pool. Cysteine, one of the most important amino acid in proteins due to its role in stability, is also well represented in two of the four alliinases analyzed here: 4,5% in *Allium fistulosum* and 4,9% in *Allium schoenoprasum*. In the other two species the pool of cysteine represents 3% (in *Allium obliquum*) and 3,1% in *Allium senescens* ssp. *montanum*. However, cysteine is better represented in these species than in garlic (*Allium sativum*), where it represents only 2,5% of the total alliinase amino acid pool. The isoelectric point and molecular weight calculated for the four alliinases is: 9,28 and 58440 Da in *Allium obliquum*, 9,37 and 55123 Da in *Allium senescens* ssp. *montanum*, 8,11 and 49554 Da in *Allium fistulosum* and 8,47 and 49121 Da in *Allium schoenoprasum*.

## Conclusions

Here there are reported the sequencing and characterization of two novel alliinase genes from *Allium obliquum* (Twisted-leaf Garlic) and *Allium senescens* ssp. *montanum* (Mountain Garlic), in comparison with two other similar genes sequenced from *Allium fistulosum* (Welsh Onion) and *Allium schoenoprasum* (Chives). Eight new primers were successfully designed and used: four primers (two pairs) for the PCR amplification of the whole alliinase gene, two primers (one pair) targeting a 221-bp fragment of the alliinase gene for the qPCR analyses, and two forward primers used for sequencing the alliinase gene.

The range of G+C content among the alliinases sequenced here gives them a distinct pattern, different from monocots, although these plants are classified into this group. Horizontal gene transfer (HGT) may be responsible for this, together with genetic recombination, a lot of other genomic sequences being relocated this way in plants, even at inter-specific level.

The gene expression investigations revealed that the alliinase enzyme is expressed at a much higher level in bulbs versus leaves in all species that were analyzed here. Particularly in *Allium obliquum*, the gene expression in bulbs is almost seven-fold higher than in leaves. This conclusion is consistent with the expression pattern of other alliums reported in the literature. The reason why these genes are differentially expressed in related species is still unclear, but it can be speculated that this is in connection with the number/density of the animals that can consume the plants, as long as the lachrymatory factor (synthesized to avoid the plants to be eaten) is active only if the alliinase

is also present. However, further studies of gene expression under different growth conditions (variations in soil composition, temperature, light) will probably contribute to the understanding of this expression pattern.

The deduced amino acid sequences of the alliinases described here display a wide range of similarity with other known alliinases. These findings are in accordance with other results from various papers, showing that the alliinase gene displays a high variability among different species. Thus, it can't be used as a phylogenetic marker, but it could easily discriminate between closely related species.

Among the amino acids, leucine is the most frequent one, thus probably increasing the hydrophobicity of the enzyme. Moreover, cysteine is another amino acid whose frequency is higher than in garlic (*Allium sativum*-2,5% cysteine) in all four analyzed species. It can be speculated that the alliinases studied here have an enhanced stability due to the disulfide bonds that may form between cysteines. Moreover, the activity of the enzymes could be higher, as long as many metal cofactors in enzymes are bound to the cysteine residues.

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