Isolation of genes involved in the production of neurotoxin in vetch, *Vicia sativa* L.

Annabelle U. Novero, Paul W.J. Taylor and Rebecca Ford

Biomarka, Institute of Land and Food Resources, University of Melbourne, Parkville, VIC 3010. Email a.novero@pgrad.unimelb.edu.au

Abstract

β-cyanoalanine synthase (β -CAS) is an enzyme that catalyzes the conversion of cysteine and cyanide to produce β-cyanoalanine and sulphide. β-cyanoalanine is a neurotoxin found in vetch seeds rendering them unfit for animal or human consumption. This compound can be readily metabolized into asparagine but this route seems to be blocked in vetch, leading to neurotoxin buildup. Vetch is an important fodder crop in Australia because of its ability to grow under poor conditions such as low rainfall and alkaline soils. Cysteine synthase (CS), which catalyses the formation of cysteine from O-acetyl-L-serine (OAS) and sulphide has been observed to exhibit CAS activity in some plants. Likewise, CAS has been shown to exhibit CS activity. Using genomic DNA, regions encoding CAS and CS in vetch were amplified. Primers were designed based on highly-conserved regions of available CAS and CS gene sequences from each member of the β-substituted alanine synthase family. One CAS gene had been isolated and analyzed previously the Biomarker Lab. In this current study, six CS genes were cloned and three were sequenced and analyzed. Molecular and biochemical characterization of CAS and CS genes is underway in order to: 1) determine genetic relatedness of vetch-specific genes to other known gene family members, and; 2) establish functional expression of the genes. Homologous sequence regions will be targeted for use in post-translational gene silencing.

Media summary

Isolation and characterization of genes involved in the production of β -cyanoalanine in vetch will give better opportunities to produce new lines without neurotoxin content.

Key Words

vetch, β -cyanoalanine, cysteine synthase, cyanoalanine synthase, neurotoxin

Introduction

Common vetch (*Vicia sativa* L.), a legume crop grown for grain, grazing, green manure and hay production, is well adapted to low rainfall areas of southern Australia (Chowdhury et al. 2001). Vetch seeds are potential quality feed for sheep and cattle because of high protein content of about 28-32% (Enneking, 1994). Sowings of vetch in Victoria and South Australia exceeded 200,000 ha after decline in popularity in mid-1990s (GRDC,2002). Due to their toxic effects on animals, vetch seeds are not recommended fit for human consumption. One particular cultivar, Blanche Fleur, has been widely utilized earlier as a lentil substitute because of the seeds' striking similarity with red lentil especially when lightly coated with vegetable oil (Tate, 1996). In the Mallee region of Victoria/South Australia, where it has been dry since February 2003, farmers consider dry sowing of vetch only as a break crop in winter wheat production. Moreover, the feed industry is cautious about using vetch grain in livestock diets (NRE, 2003). This is because β-cyanoalanine is present in vetch seeds (Ressler, 1962). It is a potent neurotoxin in monogastric animals and highly abundant in many species of *Vicia*. Of 3,000 accessions of vetch plants screened, all were found to contain the toxin while 20 lines have low levels of β-cyanoalanine (Chowdhury et al. 2001).

Development of vetch lines with little or no β-cyanoalanine content will most likely improve the value of vetch grains. If this is to be attempted using biotechnology tools such as anti-sense RNA technology, a comprehensive understanding of the mechanism of β-cyanoalanine production and the functionality of CAS and CS genes present in vetch need to be elucidated. This is because CS has the ability to catalyze

the same reaction that enables CAS to produce β-cyanoalanine. It is further complicated by the fact that CAS is able to function as a CS as well. In *Arabidopsis*, this is attributed to an evolutionary event that might have created a CAS gene via intrachromosomal duplication owing to its close position to CS (OAS C) on chromosome 3 (Hatzfeld *et al* 2000). This study is an initial phase of a project whose final aim is improving the quality of vetch grains.

Methods

To isolate CS genes, degenerate primers were designed, from sequence databases, to the conserved regions of each member of the β-subsituted alanine synthase gene family, which includes CS genes. The use of degenerate primers is a powerful tool in identifying novel members of a gene family (Buck and Axel 1991).

Homologues were PCR-isolated from total genomic DNA extracted from cv. sativa ssp. Blanche Fleur seeds. When genomic DNA is used as a template for PCR, it offers the advantage of making all members of a gene family available in equimolar concentrations. DNA amplifications were performed in a thermal cycler using initial denaturation at 94^{o}C for 2 min, denaturation at 94^{o}C for 30 sec followed by 35 cycles of 30 sec at 43.2^{o}C, extension for 45 sec at 72^{o}C for Bsas 1 and 2 genes (30 sec at 45.5^{o}C and 1 min 30 sec at 72^{o}C for Bsas 4, 5, and 6 genes). One additional extension cycle was performed for 5min at 72^{o}C.

Amplified fragments were ligated into pGEM-T vector and transformed into E. coli strain JM109. The nucleotide sequences of the PCR products were determined using the Big Dye Terminator cycle sequencing kit and sequenced in an Applied Biosystems 1377 sequencer. Nucleotide sequence data were analyzed using alignment programs available in ANGIS (Australian National Genome Information Service).

Results

Five vetch gene fragments designated as VCS1, VCS2, VCS4, VCS5 and VCS6 were sequenced and aligned with members of the Bsas protein family. A BLAST search of the nucleotide sequences revealed significant alignments for VCS5 and VCSS6. Fragment VCS5 produced significant alignment with *Oryza sativa* cysteine synthase (rcs4) mRNA (AF 073698) while fragment CVCS6 produced significant alignments with two genes from Arabidopsis, cs26 (AB003041) and At3g03630, a putative O-acetylserine thiol-lyase (cysteine synthase) cDNA (AY099573). Homology in sequence between VCS5 and At3g03630 were found in five different locations. The inferred relationship of the putative VCS genes with other members of the Bsas protein family was calculated using the UPGMA method and the tree drawn via the Phylip package in ANGIS (Australian National Genomic Information Service; Fig 1).

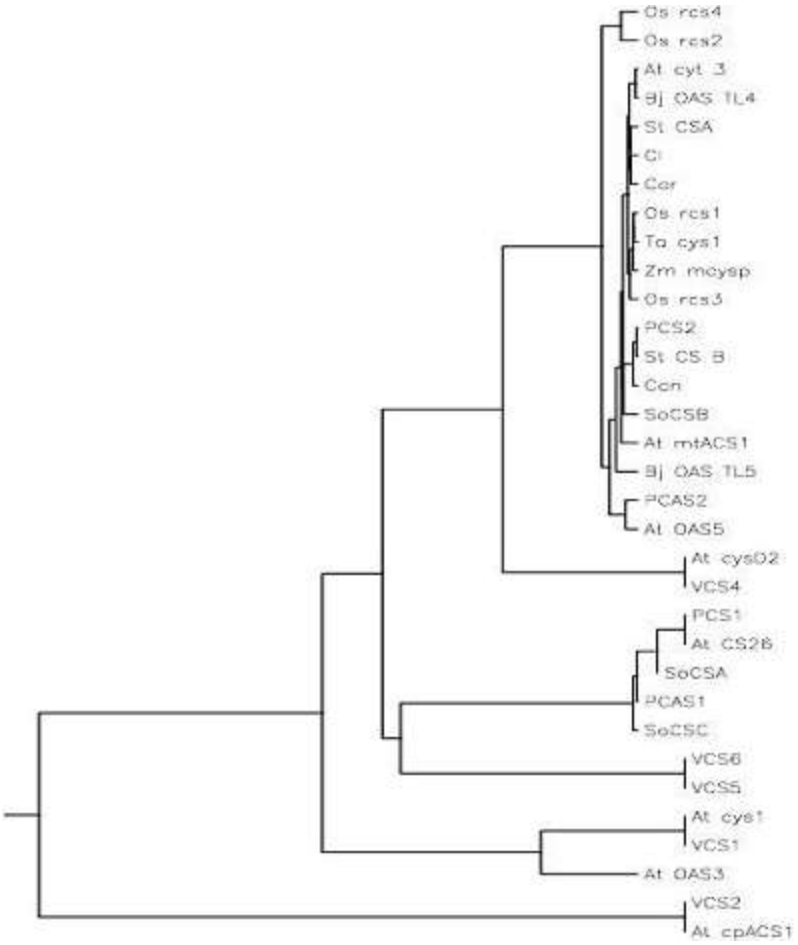

Figure 1. Phylogenetic relationship of predicted amino acid sequences of VCS1, VCS2, VCS4, VCS 5 and VCS6 isolated from *Vicia sativa* and β-substituted alanine synthase (Bsas) protein subfamily reported in plants. Predicted amino acid sequences were aligned using Clustal W. The phylogenetic tree was calculated by the UPGMA method and drawn using the Phylip package. At- *Arabidopsis thaliana* (thale cress), Bj- *Brassica juncea* (Indian mustard), Can- *Capsicum anuum* (pepper), Car- *Cicer arietenum* (chickpea), Cl- *Citrullus vulgaris* (watermelon), Os- *Oryza sativa* (rice), PCAS-potato CAS, So- *Spinacea oleracea* (spinach), St- *Solanum tuberosum* (potato), Ta- *Triticum aestivum* (wheat), Zm- *Zea mays* (maize).

Conclusion

Sequence analysis data for CS gene fragments isolated from *Vicia sativa* cv. sativa ssp. Blanche Fleur showed good levels of similarity with genes already classified under the B-substituted alanine synthase protein family. The use of degenerate primers enabled the isolation of these putative new gene members from *Vicia sativa.*

References

Buck L and Axel R (1991). A novel gene family may encode odorant receptors: A molecular basis for odor recognition. Cell 65, 175-187.

Chowdhury D, Tate ME, McDonald GK abd Hughes R (2001). Progress towards reducing seed toxin levels in common vetch (*Vicia sativa* L.). Proc. 10th Australian Agro. Conf. Hobart.

Enneking D (1994). The toxicity of Vicia species and their utilization as grain legumes. Doctoral Dissertation. Univ. of Adelaide, South Australia.

GRDC (2002). he versatility of vetch. www.grdc.com.au/growers/cd/south/S_22_04_02.htm.

Hatzfeld Y, Maruyama A, Schmidt, Noji M, Ishizawa K. and Saito K (2000). β -cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis. Plant Physiol 123, 1163-1171.

NRE (2003). Farming and agriculture drought update, May 2003. www.dpi.vic.gov.au/web/root/domino/cm_da/nrecfa.nsf

Ressler C (1962). Isolation and identification from common vetch of the neurotoxin β-cyano-L-alanine, a possible factor in neurolathyrism. J Biol Chem 237, 733-735.

Tate ME (1996). Vetches: feed or food? Chemistry in Australia 63, 549-550.