Cloning and molecular analysis of two new sesquiterpene cyclases from *Artemisia annua* L.*

Els Van Geldre a, Isabel De Pauw a, Dirk Inzé b, Marc Van Montagu b, Elfride Van den Eeckhout a,*

**a** Laboratory for Pharmaceutical Biotechnology, University of Ghent, Harelbekestraat 72, 9000 Ghent, Belgium

**b** Laboratory for Genetics, University of Ghent, K. L. Ledeganckstraat 35, 9000 Ghent, Belgium

Received 5 April 2000; received in revised form 15 June 2000; accepted 15 June 2000

**Abstract**

*Artemisia annua* L. is the only source of artemisinin, a new promising antimalarial drug (Qinghaosu Antimalarial Coordinating Research Group, Chin. Med. J. 92 (1979) 811). Our efforts are focused on the overproduction of this valuable medicine by genetic engineered *A. annua* plants. Therefore, we decided to isolate the gene(s) encoding sesquiterpene cyclase(s) in *A. annua* as a first step in improving artemisinin yield. Four partial genomic clones, *gASC*21, *gASC*22, *gASC*23 and *gASC*24, were isolated through polymerase chain reaction (PCR) with degenerated primers based on homologous boxes present in sesquiterpene cyclases from divergent sources. Intronic-exonic organisation of those partial genomic clones was analysed and it was shown that *A. annua* contains a gene family for sesquiterpene cyclases. Based on *gASC*21, *gASC*22, *gASC*23 and *gASC*24 sequences, the full-length cDNA clones *cASC*34 and *cASC*125 were subsequently isolated by rapid amplification of cDNA ends PCR. The derived amino acid sequences of both full-length clones show high homology with sesquiterpene cyclases from plants. Reverse transcription-PCR analysis revealed transient and tissue specific expression patterns for *cASC*34 and *cASC*125, in contrast to the constitutively expressed 8-epicedrol synthase, a previously reported sesquiterpene cyclase from *A. annua*. Both *cASC*34 and *cASC*125 could only be detected in flowering plants when artemisinin concentration is at highest. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Terpene cyclases; Artemisinin; Malaria

**1. Introduction**

*Artemisia annua* L., a member of the Compositae family, has been used for decennies in the treatment of fever and malaria [1]. Analysis of the active compounds responsible for these pharmacological activities led to the discovery of a sesquiterpene lacton, called artemisinin or qinhaosu [1]. This molecule possesses a different mode of action in comparison with existing antimalarial drugs (e.g. quinine) and is active against chloroquine resistant forms of *Plasmodium falciparum* [2]. Based on this secondary plant metabolite, new semi-synthetic drugs for the treatment of malaria, e.g. arteether, arteether and artesunate, are produced [1]. Unfortunately, only very low amounts

---

*Abbreviations: A, adenine; aa, amino acid(s); bp, base pair(s); C, cytosine; CAD, cadinene synthase; cDNA, DNA complementary to RNA; dNTP, deoxyribonucleotide triphosphate; FPP, farnesylpyrophosphate; G, guanine; M, A:C; PCR, polymerase chain reaction; pi, isoelectric point; R, A:G; RT-PCR, reverse transcription-polymerase chain reaction; RACE-PCR, rapid amplification of cDNA ends polymerase chain reaction; S, C:G; Taq, Thermus Aquaticus Polymerase; TEAS, 5-epi-aristolochene synthase; T, thymidine; VS, vetispiradiene synthase; Y, C:T.*

*Corresponding author. Tel.: +32-9-2648053; fax: +32-9-2206688.

E-mail address: elfriede.vandeneeckhout@rug.ac.be (E. Van den Eeckhout).
of artemisinin can be isolated from the aerial parts of *A. annua* (between 0.01 and 0.6% dry weight) [3–5]. The limited supply of this valuable compound from *A. annua* L. prompted researchers to develop alternate means of production. Many attempts have been undertaken to overproduce artemisinin through cell or tissue cultures [6–9]. However, so far, only very limited success has been obtained. Also, total chemical synthesis of artemisinin was revealed to be not economically interesting due to stereochemical problems [10]. Therefore, all efforts are focused on the overproduction of this valuable compound by genetic engineered *A. annua* plants. Such plants can be obtained through genetic improvement of the biosynthetic pathway of artemisinin by sense and/or antisense techniques [11]. An *Agrobacterium tumefaciens*-mediated transformation procedure has already been established in our laboratory [11–13]. In the biosynthesis of artemisinin, very little is known about enzymes responsible for the production of the different intermediates. At one of the important branchpoints farnesylpyrophosphate (FPP) is cyclised by the action of a sesquiterpene cyclase to amorpha-4,11-diene, which leads to sequentially to artemisinic acid, dihydroartemisinic acid and artemisinin by a series of oxidation steps and nonenzymatic conversions [3,14–17] (Fig. 1). Thus, the cyclase reaction establishes an important stereochemical framework upon which all other chemical modifications take place [17]. Several publications report on the fact that this cyclisation step is a putative regulatory point, probably rate limiting [17,18]. The accumulation of artemisinic acid and dihydroartemisinic acid in absence of any intermediates en route from FPP also supports this hypothesis [14]. Farnesyl diphosphate synthase, which produces FPP, has already been cloned from *A. annua* [19], but this enzymatic reaction is probably not rate limiting for the production of artemisinin.

Therefore, we decided to isolate the gene(s) encoding sesquiterpene cyclase(s) in *A. annua* as a first step in improving artemisinin yield. In 1992, tobacco 5-epi-aristolochene synthase (TEAS) was cloned from elicitor-treated tobacco cell cultures [20]. Based on the TEAS sequence information, vetispiradiene synthase from *Hyoscyamus muticus* [17] and (+)-delta-cadinene synthase from *Gossypium arboreum* [21] were cloned. During the course of this work, the isolation of 8-epicedrol synthase from *A. annua* was reported [22,23]. The role of 8-epicedrol synthase in *A. annua* and artemisinin biosynthesis is uncertain, since 8-epicedrol cannot be detected by gas chromatography–mass spectrometry in *A. annua* extracts and the conversion of epicedrol to the putative artemisinin bioprecursor artemisinic acid would require rearrangements that do not seem plausible [22,23].

![Fig. 1. Proposed biosynthetic pathway for artemisinin in *A. annua* L. (adapted from Refs. [14,27]).](image-url)
This paper reports the isolation of two other members of the sesquiterpene cyclase gene family from *A. annua*. Both full-length isolated genes are new potential candidates involved in the rate-limiting cyclisation step of artemisinin biosynthesis, thereby enabling a molecular approach for improvement of artemisinin production.

2. Materials and methods

2.1. Materials

Restriction endonucleases were purchased from Boehringer Mannheim (Mannheim, Germany) and Biolabs (New England). Taq DNA polymerase was purchased from Perkin Elmer (Norwalk, CT, USA). ABI PRISM™ Ready Reaction Dye Deoxy™ Terminator Cycle Sequencing Kit and ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase FS were from Applied Biosystems. Dynabeads mRNA Direct Kit was from Dynal (Oslo, Norway). The SMART RACE PCR Kit was from Clontech (Palo Alto, CA, USA). Superscript II Reverse Transcriptase was from Gibco BRL (Life Technologies). Gene Images random priming labeling module and CDP-star detection module was from Amersham Life Science (Little Chalfont, Buckinghamshire, UK). *Escherichia coli* strain JM109 was used as bacterial host for pGem-T-Easy recombinant plasmids. Wizard SV DNA extraction, dNTPs, SP6 Promotor primer, Reverse Transcription System and pGem-T-Easy vector were purchased from Promega (Madison, USA). M13/pUC universal sequencing primer 17-mer was from Boehringer Mannheim.

2.2. Plant materials

Seeds of *A. annua* of West Virginia origin were kindly provided by the Walter Reed Army Institute of Research (Washington). Plants were grown in an experimental greenhouse using Hg- and Na-vapor lamps 16 h/day and at a temperature of 22°C and a relative humidity of 40%.

2.3. Isolation of nucleic acids

Genomic DNA was isolated from plant leaves according to the extraction procedure from Stacey and Isaac [24]. Plasmid DNA samples were prepared by an alkaline lysis method with the Wizard DNA Purification System (Promega) according to the manufacturer’s instructions. Total RNA was isolated with the Trizol reagent according the procedures of Life Technologies. mRNA could be isolated with Dynabeads from Dynal (Oslo, Norway).

2.4. Oligonucleotide primer design

Comparison of the amino acid sequences from sesquiterpene cyclases isolated from divergent sources *Nicotiana tabacum*, *H. muticus* and *G. arboreum*, revealed highly conserved boxes for which degenerate primers were designed (PILEUP and LINEUP from GCG package). These boxes were used to design degenerate primers by back-translation from the respective amino acid sequences. At places of three- and fourfold base redundancy, inosines were incorporated to prevent high degeneracy of the primers. One forward primer (5’-TAYCATYTGARATIGARGA-3’) and one reverse primer (5’-GARTAYTGIG-GTTCAARTAIACICC-3’) (R = A, G; Y = C, T) were thus selected with respective annealing temperatures of 62°C and 68°C calculated with [2°*(A + T) + 4°*(C + G)].

2.5. Polymerase chain reactions

The primers were subsequently used in a Hot Start polymerase chain reaction (PCR) on genomic DNA from *A. annua*. (10 mM Tris–HCl (pH 8.3), 25°C, 50 mM KCl, 200 μM dNTP, 1.2 U Taq polymerase and 20 pmol of each primer in a total volume of 50 μl; concentrations of MgCl2 ranged from 1.5 to 4.5 mM). Cycling conditions were set as follows: 94°C for 1 min, 50°C for 1 min and 72°C for 1 min for a total of 35 cycles. This PCR resulted in a predominant 900 base pair (bp) product as detected by agarose gel electrophoresis and ethidium bromide staining. This product was sequenced after TA-cloning in pGem-T-Easy Vector System (Promega) according to the manufacturer’s instructions. Recombinant plasmids were isolated after amplification in *E. coli* JM109 and analysed for insert by blue/white screening and PCR.
2.6. Nucleotide sequencing and analysis of nucleic acids

Double-stranded forms of template DNA were used in dideoxynucleotide cycle sequencing reactions. Both sense and antisense strands of the TA-cloned PCR products were sequenced with vector-specific primers (M13/pUC universal sequencing primer 17-mer or SP6 Promotor primer) or ABI PRISM™ Ready Reaction Dye Deoxy™ Terminator Cycle Sequencing Kit and ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase FS (Applied Biosystems) using an automated fluorescence-based system ABI373 or ABI310 (Applied Biosystems), respectively. DNA sequences and the deduced amino acid sequences were analyzed and aligned with related sequences using programs available through the Genetics Computer Group package (University of Wisconsin).

2.7. Southern blot

Twenty micrograms of genomic DNA were blotted onto a + charged nylon membrane (Boehringer Mannheim) according to standard procedures [25]. Prehybridisation and hybridisation were performed at 55°C. The probe, fluoresceine labeled, consisted of the partially purified cDNA sequence already isolated in \( A. \) annua (Gene Images Random priming labeling and CDP-star detection).

2.8. RNA analysis

In order to isolate corresponding cDNA sequences, new degenerate primers based on the partial genomic sequences were designed. One forward (5'-AAGCMCTTCSCTYTGGTTCMGA-3') and one reverse primer (5'-CCAAAARTAR-CMTTCAACYATTCT-5') were selected with the program OLIGO™ (M = A or C; S = C or G; Y = C or T). Dnase-treated RNA isolated from leaves and flowers harvested on different stages during plant development (from young cotyledons to flowering plants) was denatured for 2 min at 90°C and subsequently transcribed to cDNA with AMV Reverse Transcriptase during 60 min at 42°C. This reaction could be used immediately for PCR with 20 pmol of both primers and 2.5 U Taq in 10 mM Tris–HCl (pH 8.3), 25°C, 50 mM KCl and 200 μM dNTP. The reaction conditions were 94°C during 30 s, 53°C during 30 s and 72°C during 1 min for a total of 40 cycles. For detection of specific transcripts by reverse transcriptase (RT)-PCR exact primers (5'-GGCTATAAAACTTGCCTAGTAG-3' and 5'-CCGGATCCTCATGTAATGATGGCATC-3') for 8-epicedrol synthase; 5'-CCTCAACACGATG-TAGAAG-3' and 5'-AGTGGATGTGGTTGGGACATC-3' for cASC34; 5'-TCTGTATGAAGCCGCATTATG-3' and 5'-GTCTCAACACATAGAAGCTTTA-3' for cASC-C125) were designed on three isolated sequences and used for PCR with the following cycling conditions: 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 30 cycles. Repeating the experiment twice with newly isolated RNA from fresh plant samples compensated the lack of an internal constitutively expressed control.

2.9. Rapid amplification of cDNA ends PCR

Both 5' rapid amplification of cDNA ends (RACE) and 3' RACE PCR were performed with gene-specific primers designed against partial sequences obtained through RT-PCR with the SMART-RACE PCR kit (Clontech) according to the manufacturer’s instructions. Primer sequences were as follows: for cASC32, 5' RACE 5'-AGGTAGTTTGGGACATC-3' and nested primer 5'-CTCAAATATGTTGCCTCGTACAGC-3'; 3' RACE 5'-CCTCACCAACGATGTAGAAG-3'; for cASC125, the primers used for 5'-RACE were 5'-GTCTCAACACATAAGGAAGCTTTA-3' and nested primer 5'-CCCTCAAAATGCGCCGCTTC-ATACAG-3', and 3'-RACE was performed with 5'-TCTGTATGAAGCCGCATTATG-3'.

3. Results and discussion

3.1. Cloning of partial genomic clones gASC21, gASC22, gASC23 and gASC24

Molecular comparisons of plant terpene cyclases have revealed a striking level of sequence similarity [17,26,27]. After comparison of the amino acid sequences of sesquiterpene cyclases isolated from \( N. \) tabacum [20], \( G. \) arboreum [21] and \( H. \) mutficus [17], different degenerate primers were subsequently designed against conserved boxes highlighted by this comparison. RT-PCR with these primers on mRNA isolated from young \( A. \) annua
Table 1

Identities (%) and similarities (%) (in parentheses) obtained through the BLASTX/BEAUTY search for gASC21–gASC24, cASC34 and cASC125 with corresponding amino acid sequences of sesquiterpene cyclases from divergent sources a

<table>
<thead>
<tr>
<th></th>
<th>TEAS</th>
<th>CAD1A</th>
<th>VS</th>
<th>GS</th>
<th>8-epicedrol synthase</th>
<th>CAD</th>
<th>VS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>gASC21</td>
<td>N. tabacum</td>
<td>47 (63)</td>
<td>51 (68)</td>
<td>46 (62)</td>
<td>56 (74)</td>
<td>50 (77)</td>
<td>49 (65)</td>
</tr>
<tr>
<td>gASC22</td>
<td>G. arboreum</td>
<td>50 (68)</td>
<td>54 (71)</td>
<td>50 (67)</td>
<td>53 (72)</td>
<td>70 (82)</td>
<td>53 (70)</td>
</tr>
<tr>
<td>gASC23</td>
<td>H. muticus</td>
<td>40 (58)</td>
<td>40 (61)</td>
<td>40 (59)</td>
<td>40 (61)</td>
<td>53 (67)</td>
<td>39 (61)</td>
</tr>
<tr>
<td>gASC24</td>
<td>L. esculentum</td>
<td>56 (68)</td>
<td>64 (82)</td>
<td>57 (72)</td>
<td>59 (79)</td>
<td>71 (78)</td>
<td>62 (81)</td>
</tr>
<tr>
<td>cASC34</td>
<td>A. annua</td>
<td>42 (63)</td>
<td>47 (66)</td>
<td>43 (65)</td>
<td>45 (65)</td>
<td>57 (76)</td>
<td>47 (65)</td>
</tr>
<tr>
<td>cASC125</td>
<td>G. hirsutum</td>
<td>40 (60)</td>
<td>42 (61)</td>
<td>40 (61)</td>
<td>41 (62)</td>
<td>49 (68)</td>
<td>41 (61)</td>
</tr>
</tbody>
</table>

Comparison of intron–exon organisation of gASC21, gASC22, gASC23 and gASC24 with corresponding sequences of TEAS from N. tabacum and vetispiradiene synthase from H. muticus revealed that intron positions (Fig. 2) and sizes (not shown) of the A. annua clones were consistent with earlier reported results [17,20]. This correlates with the fact that plant sesquiterpene cyclases have highly conserved intron–exon organisation of genomic DNA [17]. This conservation can even be extended to the diterpene cyclases [29].

3.2. Detection of sesquiterpene cyclase transcripts

Based upon the four partial genomic sesquiterpene cyclase sequences, a new set of degenerated primers was designed for RT-PCR. mRNA isolated from leaves and flowers of A. annua L. plants at different moments of development served as a template for RT-PCR. We detected a specific product both in leaves and flowers of flowering plants (data not shown). Also, in Fragaria vesca L., a partial sequence encoding sesquiterpene cyclase was isolated along with seven other ripening-induced cDNAs by differential screening of a cDNA library [30]. The author states that the absence of a detectable signal in commercial strawberry either suggests that sesquiterpene cyclase does not play an important role in the ripening of the fruits or that the transcript levels are too low in commercial strawberries [30]. It is possible that this phenomenon also takes place in A. annua, and that sesquiterpene cyclase transcripts are only detectable in flowering plants, when expression levels are highest.

Table 2

Identities (%) for gASC21, gASC22, gASC23 and gASC24 at the nucleotide level

<table>
<thead>
<tr>
<th></th>
<th>gASC22</th>
<th>gASC23</th>
<th>gASC24</th>
</tr>
</thead>
<tbody>
<tr>
<td>gASC21</td>
<td>51</td>
<td>45</td>
<td>89</td>
</tr>
<tr>
<td>gASC22</td>
<td>43</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>gASC23</td>
<td></td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Alignment of sesquiterpene cyclases isolated from divergent plant sources (PILEUP GCG package, University of Wisconsin). cASC34 and cASC125, new sesquiterpene cyclases isolated from *A. annua*; gASC21–gASC24, corresponding AA sequences deduced from respective partial genomic sequences gASC21–gASC24; Aa, *A. annua* 8-epicedrol synthase; Hmut, *H. muticus* VS; Ntab, *N. tabacum* TEAS; Garb, *G. arboreum* CAD. Conserved amino acid sequences used for primer design are marked with a frame. ▼, Positions of introns in partial genomic sequences gASC21–gASC24; ▲, introns in TEAS and VS. The shading colors of the residues correspond to the level of similarity: black, 100% similarity; dark gray, ≥ 80% similarity; light gray, ≥ 60% similarity.
Two partial cDNA sequences were obtained (cASC34-p and cASC125-p), of 465 and 462 bp, respectively. Comparison of the deduced amino acid sequences revealed that gASC22 and cASC34-p were identical. The corresponding genomic sequence of cASC125-p, however, has not yet been isolated. cASC125-p showed only identities between 49 and 61% and similarities between 62 and 77% with the four partial genomic sequences. Identities and similarities with sesquiterpene cyclases cloned from N. tabacum, H. muticus, G. arboream, Gossypium hirsutum, S. tuberosum and Lycopersicon esculentum were comparable with those obtained for the gASC21 to gASC24 (identities ranging from 45 to 8% and 46 to 58%; similarities between 65 and 81% and 65 and 78%, for cASC34-p and cASC125-p, respectively). These values clearly indicate that cASC34-p and cASC125-p are partial cDNA sequences encoding two different members of the A. annua gene family for sesquiterpene cyclases. The fact that, for cASC125-p, the corresponding genomic sequence escaped isolation thus far can be due to the high degeneracy of the primers used for PCR on genomic DNA. Out of ten partial genomic sequences isolated and analyzed, we identified four different sesquiterpene cyclases. In addition, it could also be possible that not all members of the sesquiterpene cyclase gene family are active at the flowering time of A. annua or that some members of the gene family stay below detection limits. It is not clear at which moment of plant development the artemisinin biosynthetic pathway is activated. Studies on influences of elicitors on artemisinin production have been established, but the influence on enzymes related to this biosynthesis has not been analyzed. Analysis of artemisinin yield estimated at different stages of development reveals a positive correlation between plant age and artemisinin yields, without any peak production at any moment of the plant development [3,31–33]. This probably means that enzymes related to artemisinin biosynthesis are active during a large part of plant development, but probably stay below detection limits at mRNA level. RNA transcripts of previously isolated sesquiterpene cyclases from N. tabacum, H. muticus and G. arboream could only be detected after induction by elicitor treatment [17,20,21], and UV treatment was necessary to detect sesquiterpene cyclase protein in pepper [26].

3.3. Cloning of cASC34 and cASC125

Using cASC125-p and cASC34-p as a probe to screen a cDNA library of A. annua, we did not obtain any clone containing the corresponding full-length sequence of the respective probe used, but we cloned a full-length sequence encoding another member of the sesquiterpene cyclase family (unpublished results). By the time we were performing expression experiments with the protein encoded by this gene, two other research groups reported the isolation of 8-epicedrol synthase from A. annua [22,23], which appeared to be exactly the same sequence as our full-length clone. But this sesquiterpene cyclase is apparently not involved in artemisinin biosynthesis [22,23].

Since we were unable to clone the corresponding full length sequence of cASC34-p and cASC125-p from our cDNA library, 5’RACE and 3’RACE PCR for the full-length cloning of both genes was performed.

For cASC34, a sequence of 2026 bp was obtained containing 38 bp of the untranslated 5’ end with an ORF encoding a 549 amino acid (aa) protein of 64.2 kDa and a calculated isoelectric point (pI) of 5.28. Two AATAAT–poly-adenylation signals were found in the 338 bp untranslated 3’ sequence (positions, 1876 and 2003 bp).

The cASC125 sequence contained an ORF of 1734 bp encoding a 577 aa protein with a molecular weight of 67.4 kDa and a pI of 5.50. This sequence has 60 bp at the 5’ untranslated end and an untranslated 3’ sequence of 48 bp. Here, we found the poly-adenylation signal AATTAA at 1820 bp.

Comparison of deduced amino acid sequences from cASC34 and cASC125 with cloned sesquiterpene cyclases from divergent sources revealed high similarities and identities as shown by Table 1 and Fig. 2.

3.4. RNA analysis for three sesquiterpene cyclases from A. annua

Fig. 3 shows the expression in leaves and flowers of 8-epicedrol synthase, cASC34 and cASC125 during plant development. We were able to demonstrate that 8-epicedrol synthase is expressed during the whole plant development in leaves and flowers, but that cASC125 is only present in mRNA-extracted from flowers. cASC34, on the
Fig. 3. RNA analysis for 8-epicedrol synthase (A), cASC34 (B) and cASC125 (C).

other hand, could be detected in leaves of flowering plants and flowers. In leaves, cASC34 transcripts disappear completely when flowering stopped and seeds are formed. In flowers, both cASC34 and cASC125 transcripts are still present when flowers faded but also disappear later when seeds are formed. The fact that, in the RT-PCR with degenerated primers, transcripts were only detected in RNA isolated from flowering plants can be explained by the high degeneration of the primers. Primers designed based on gASC21-24 were probably not compatible with the 8-epicedrol synthase sequence and did not amplify the mRNA transcripts corresponding to this enzyme.

4. Conclusion

Recently, Bouwmeester et al. [14] described an amorpha-4,11-diene synthase activity in leaf extracts of A. annua. Amorpha-4,11-diene is likely to be the olefinic intermediate in the biosynthesis of artemisinin, the first step after FPP [14]. The authors partially purified and characterised the enzyme responsible for this activity, but did not isolate the corresponding gene(s). If cASC34 and/or cASC125 possess amorpha-4,11-diene synthase activity, this would mean a big step forward in the overproduction of artemisinin through genetic manipulation. Artemisinin is mainly present in leaves and flowers of A. annua L. plants, with the highest content around flowering time [31,32]. Therefore, the fact that cASC34 transcripts are present in these plant parts at flowering time could be an indication for relationship of this gene to the artemisinin biosynthesis. The enzymes related to this biosynthetic pathway are possibly more actively transcribed with as a consequence that their mRNA transcripts are detectable during this moment of plant development. Further experiments are necessary, and bacterial expression analysis of cASC34 and cASC125, in order to determine their exact enzymatic activity, will be performed in the near future. On the other hand, if cASC34 or cASC125 do not possess amorpha-4,11-diene activity, the search for the gene(s) encoding a protein with this enzymatic activity will be continued by use of the partial genomic sequences gASC21, gASC23 and gASC24 reported here.

Acknowledgements

The authors thank Prof. L. Gheysen and all members of the Nematode Group (Laboratory of Genetics, University of Ghent, Belgium) for assistance during the course of this work, Christophe Gilot (Laboratory of Genetics, University of Ghent, Belgium) for synthesis of primers, and Ardiles Diaz Wilson and Villarroel Raimundo (Laboratory of Genetics, University of Ghent, Belgium) for sequencing analysis of cASC34 and cASC125. Prof. Chappell is gratefully acknowledged for the gift of pBSK-TEAS, used in a preliminary stage of our experiments, and Dr D.L. Klayman for providing Artemisia annua L. seeds.

References


