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Molecular cloning and characterization of triterpene synthases from *Medicago truncatula* and *Lotus japonicus*

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Abstract

Cloning of OSCs required for triterpene synthesis from legume species that are amenable to molecular genetics will provide tools to address the importance of triterpenes and their derivatives during normal plant growth and development and also in interactions with symbionts and pathogens. Here we report the cloning and characterization of a total of three triterpene synthases from the legume species *Medicago truncatula* and *Lotus japonicus*. These include a β-amyrin synthase from *M. truncatula* (*MtAMYI)* and a mixed function triterpene synthase from *Lotus japonicus* (*LjAMY2*). A partial cDNA predicted to encode a β-amyrin synthase (*LjAMY1*) was also isolated from *L. japonicus*. The expression patterns of *MtAMY1*, *LjAMY1* and *LjAMY2* and of additional triterpene synthases previously characterised from *M. truncatula* and pea differ in different plant tissues and during nodulation, suggesting that these enzymes may have distinct roles in plant physiology and development.

Abbreviations: OSC, Oxidosqualene Cyclase; TLC, Thin Layer Chromatography; HPLC, High Pressure Liquid Chromatography; RACE, Rapid Amplification of cDNA Ends; EST, Expressed Sequence Tag

Introduction

Triterpenes are natural compounds that are produced primarily by higher plants. Like sterols, they are synthesised from the isoprenoid pathway by the cyclization of 2,3-oxidosqualene (Abe *et al*., 1993) (Fig. 1). These cyclization events are catalysed by the oxidosqualene cyclases (OSCs) cycloartenol synthase and lanosterol synthase (for sterols) and triterpene synthases (for pentacyclic triterpenoids) (Nes and McKean, 1977; Abe *et al*., 1993). The cyclization of 2,3-oxidosqualene to sterols and triterpenes represents a branch point between primary and secondary metabolism.

The nucleotide sequence date reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF78453 (*MtAMY1*), AF78454 (*LjAMY1*) and AF78455 (*LjAMY2*).

Sterols are well known as important membrane constituents and also serve as precursors for hormone biosynthesis. The biological function of triterpenes in plants is less clear. A number of plants accumulate substantial amounts of triterpenes as glycosides (otherwise known as triterpenoid saponins), usually in the roots (Hostettmann and Marston, 1991). Many glycosylated triterpenes have antimicrobial activity, and so are likely to protect plants against attack by pathogens (Price *et al*., 1987; Hostettmann and Marston, 1991; Papadopoulou *et al*., 1999). However some of these molecules may have additional functions. For example, saponins from pea have been proposed to regulate gravitropism and cellulose synthesis in plants (Ohana *et al*., 1998; Rahman *et al*., 2001).

In pea and sorghum β-amyrin production is very active during development and just after germination while sterol biosynthesis increases several days later, suggesting that such switches in metabolism may be

a common phenomenon during development (Baisted, 1971; Palmer and Bowden, 1977; Abe *et al.*, 1988). Elevated levels of β-amyrin and other triterpenes may be an indication of active saponin biosynthesis. However triterpene aglycones may also have biological functions in plants and have been proposed to act as structural components of membranes during normal plant growth and development (Baisted, 1971; Nes and Heftmann, 1981) and during the establishment of rhizobial and mycorrhizal symbioses (Hernandez and Cooke, 1996; Grandmougin-Ferjani *et al.*, 1999).

Since OSCs are implicated in regulation of flux through the isoprenoid pathway leading to sterols and triterpenoids, manipulation of these enzyme activities may be expected to change the metabolic flow towards these two pathways (Baisted, 1971; Threlfall and Whitehead, 1990). This is supported by experiments with inhibitors, triterpenoid saponin precursors and elicitors that modulate OSC activity, where differential effects on sterol and triterpene synthesis have been observed (reviewed in Haralampidis *et al.*, 2001a). Recently a number of OSCs that catalyse the synthesis of triterpenes have been cloned and their functions confirmed by expression in yeast (Haralampidis *et al.*, 2001a). These include β-amyrin synthases from ginseng (*Panax ginseng*) (Kushiro *et al.*, 1998a, b), liquorice (*Glycyrrhiza glabra*) (Hayashi *et al.*, 2001a) and pea (*Pisum sativum*) (Morita *et al.*, 2000) and lupeol synthases from olive (*Olea europea*) (Shibuya *et al.*, 1999), dandelion (*Taraxacum offinale*) (Shibuya *et al.*, 1999) and thalecress (*Arabidopsis thaliana*) (Herrera *et al.*, 1998). In addition, mixed function triterpene synthases that generate α- and β-amyrin or these two triterpenes plus lupeol as the major products have been characterised from pea (Morita *et al.*, 2000) and *A. thaliana* (Kushiro *et al.*, 2000a; Husselstein-Muller *et al.*, 2001), respectively, and an OSC that synthesises the triterpene isomultiflorenol has been cloned from *Luffa cylindrica* (Hayashi *et al.*, 2001b). A novel monocot β-amyrin synthase that is clearly distinct from dicot β-amyrin synthases has also recently been cloned and characterised from oat (Haralampidis *et al.*, 2001b).

Cloned triterpene synthases represent attractive tools for investigating the regulation of synthesis and the physiological role of triterpenes and should also prove valuable for the manipulation of sterol and triterpene content in transgenic plants by overexpression or gene silencing (Haralampidis *et al.*, 2001a). The legume species *M. truncatula* and *L. japonicus* offer an opportunity to address these issues. These species are amenable to transformation (Cook, 1999; Stougard, 2001), form nodules and produce triterpenes and triterpene saponins that may be involved in modulating interactions with symbionts and pathogens (Jurzysta *et al.*, 1992; Ali *et al.*, 2001; Huhman and Sumner, 2002). Here we report the cloning and characterization of three triterpene synthases from these model legume species. Expression analysis of these and additional triterpene synthase genes from *M. truncatula* and pea indicates that these genes have different expression patterns in distinct plant tissues and during nodulation. This suggests that these enzymes may play a variety of different roles in plant physiology and development.

Materials and methods

Plant material, RNA extraction and cDNA synthesis

Seeds of *M. truncatula* cv Jemalong genotype J5 and *L. japonicus* cv Gifu were surface-sterilised in sulphuric acid for 10 min, followed by several washes in cold sterile water. *L. japonicus* seeds were then soaked in water for 2–3 h. Seeds were imbibed in the dark at 4 °C for two days on wet 3MM Whatman paper and then germinated at room temperature for 3 days. Healthy seedlings were transferred to pots containing a mixture of sand and potting compost *(M. truncatula)* or BioSorb (Collier Turf Care Ltd., King's Lynn, Norfolk, UK) (*L. japonicus*) and grown in a glasshouse under controlled light and temperature. The plants were watered with 1/2 strength nutrient solution containing 0.5 mM KNO_3 (Hoffmann *et al.*, 1997) and were inoculated with the appropriate Rhizobia ((*Sino*)*rhizobium meliloti* strain RCR2011 for *M. truncatula* and *Mesorhizobium loti* strain N2P2235 for *L. japonicus*) three days after transferral to pots.

Pea seeds (*Pisum sativum* L. cv Lincoln) were washed for 3 min in 70% ethanol followed by 15 min in 10% bleach, washed in sterile water, and then grown in pots containing perlite-vermiculite (2:1) in controlled environmental cabinets as described in Gogorcena *et al.* (1997). Root nodules were collected 2, 3, 5 and 7 weeks after inoculation with *Rhizobium leguminosarum* bv *viciae* NLV8. Stress treatments were applied by subjecting three-week old plants to complete darkness or by watering the plants with 10 mM KNO_3 for 4 days. The effect of early Rhizobium infection in pea roots was studied by inoculating germinated roots of three-day old seedlings with 100 μl of *Rhizobium leguminosarum* grown in

liquid TY medium up to a density of OD_{600}=0.050. Control plants were inoculated with sterile TY liquid medium and roots were collected 2 and 4 days after inoculation.

Tissues were collected, frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. Total RNA was extracted from roots of *P. sativum*, *M. truncatula* and *L. japonicus* using a hot phenol-LiCl procedure (deVries *et al.*, 1982). First-strand cDNA was synthesised using reverse transcriptase (Superscript II, Life Technologies) and an oligo dT_{15} primer (Promega) at 42 °C for 60 min.

Cloning of β-amyrin synthases from Lotus japonicus and Medicago truncatula

Primers matching conserved regions of the pea β-amyrin synthase *PSY* (GenBank accession no. AB034802; Morita *et al.*, 2000) were used to PCR-amplify related sequences from *M. truncatula* and *L. japonicus* cDNA. The forward and reverse primer sequences were 5′-ACATTGAGGGTCATAGCACCA-3′ and 5′-AGTTTCCATACCATGAACCAT-3′, respectively. Amplification was performed using Expand High Fidelity DNA Polymerase (Roche Biochemicals) with the following cycling conditions: 94 °C 2 min, (94 °C 30 s, 50 °C 30 s, 72 °C 2 min) × 30 cycles, 72 °C 10 min. The reaction mixture contained 300 nM of each primer, 200 μM dNTPs, 2 μl of cDNA and 3.5 units of Expand DNA Polymerase in a buffer containing 20 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5% Tween20 and 0.5% Nonidet P40 (final volume 100 μl). PCR products were cloned into the pGEM-T Easy vector (Promega) and transformed into DH5α *E. coli* competent cells. Sequencing reactions were performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with fluorescent sequencing (FS) AmpliTaq DNA polymerase (Perkin-Elmer) and analyzed on ABI 377 sequencing gels. Sequence similarity searches were performed using the BLASTX algorithm (Altschul *et al.*, 1997). Full-length cDNA sequences were obtained by 5′- and 3′-RACE using specific primers. Second-strand cDNA was synthesized and adaptors were ligated following standard procedures (Life Technologies). 5′- and 3′-RACE was performed using two nested primers matching the adapter sequences (PR11F: 5′-TGCGAGTAAGGATCCTCACGCAAG-3′ and PR22F: 5′-CACGCAAGGAATTCCGACCA-GACA-3′) and two nested gene-specific primers in two rounds of PCR amplifications. Conditions for the first round of amplification were 94 °C 2 min, (94 °C 2 s, 72 °C 3 min) × 7 cycles, (94 °C 2 s, 67 °C 3 min) × 32 cycles, 67 °C 4 min. The second-round PCR amplification was performed using 1/100 of the first PCR and the following cycling conditions: 94 °C 2 min, (94 °C 2 s, 72 °C 3 min) × 5 cycles, (94 °C 2 s, 67 °C 3 min) × 20 cycles, 67 °C 4 min. Resulting bands were gel-purified, cloned into the pGEM-T Easy vector and sequenced.

Expression in yeast

Full-length cDNAs were cloned into the yeast expression vector pYES2 (Invitrogen) under the control of the *GAL1* promoter. Yeast expression experiments were carried out as described (Kushiro *et al.*, 1998a). After galactose induction cells were extracted with hexane and the extracts analysed by TLC (solvent mix, 1:1 hexane/ethyl acetate). Oxidosqualene cyclisation products were detected by spraying the TLC plate with *p*-anisaldehyde:sulphuric acid:acetic acid (1:1:40 vol:vol:vol), followed by baking at 130 °C for 5 min (Hostettmann and Marston, 1991). HPLC analysis of the reaction products was carried out as described in Haralampidis *et al.*, (2001b) with α- and β-amyrin, lupeol, and cycloartenol (Apin Chemicals) as standards.

Southern and Northern blot analysis

Southern and Northern blot analysis was carried out using standard methods (Sambrook *et al.*, 1989). Genomic DNA was isolated from *P. sativum*, *M. truncatula* and *L. japonicus* leaves following standard protocols (Dellaporta *et al.*, 1983). Filters were probed at high stringency with gene specific probes that had been labelled with [α-^{32}P] dCTP using the Megaprime labelling kit (Amersham). Probes for Southern blot analysis were generated as follows. The probe for *P. sativum* was PCR-amplified from cDNA using the forward and reverse primers 5′-AAGATGTGGAGGTTGAAGATA-3′ and 5′-AAGGCCAACGAGTCAAAAGT-3′, respectively. The full-length *MtAMY1* cDNA clone was used as a probe in the *M. truncatula* Southern. The *L. japonicus LjAMY2* probe was amplified using the forward and reverse primers 5′-ATAGAGGGTCATAGCACCA-3′ and 5′-ATTTCCATACCAAGAACCATC-3′, respectively. For Northern blot analysis the *LjAMY1* and *LjAMY2* transcripts were distinguished using specific probes for the 3′-UTR region. The

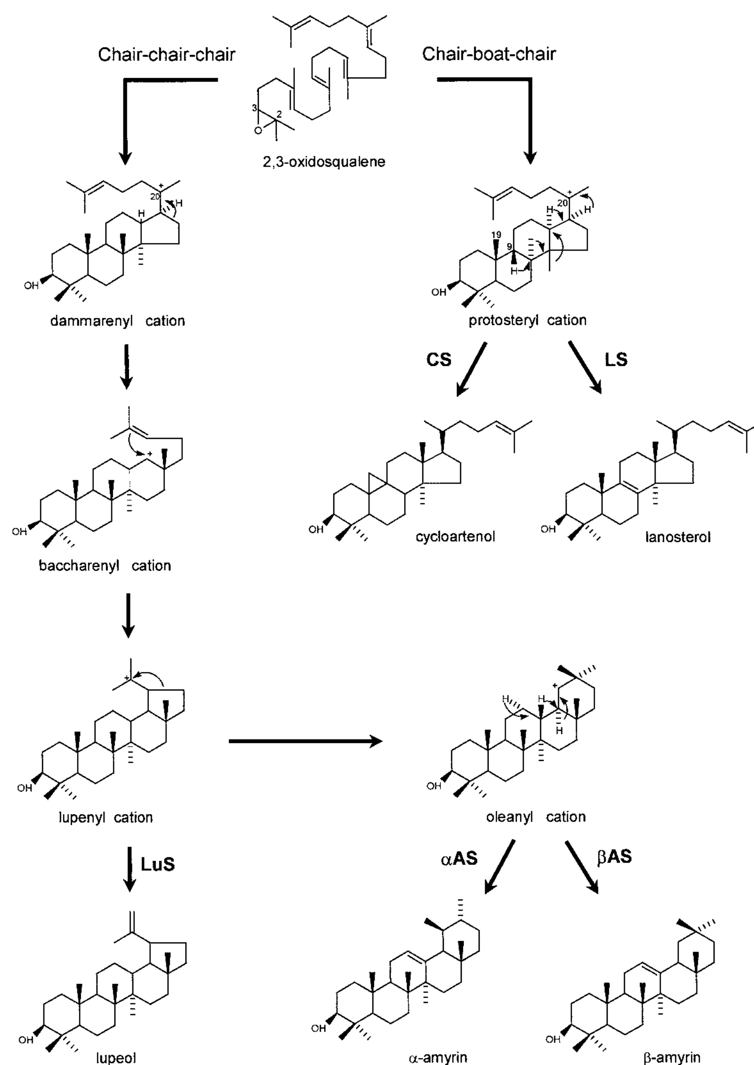

Figure 1. Cyclization of 2,3-oxidosqualene to triterpenoids (lupeol, α-amyrin and β-amyrin) and sterols (cycloartenol and lanosterol). The 2,3-oxidosqualene cyclases that catalyze the formation of the different products shown in the figure are lupeol synthase (LuS), α-amyrin synthase (αAS), β-amyrin synthase (βAS), cycloartenol synthase (CS) and lanosterol synthase (LS).

forward and reverse primers were *LjAMY1* 5′-CTTGTAAAGGTGTGAGCACAAAAAGA-3′ and 5′-ATTCCATTTGGAGTTGGACATCC-3′ for *LjAMY1* and 5′-CTTGTAAAGATAGCTGTGTGCAC-3′ and 5′-GGCAACAAACCGACACTAAATAC-3′ for *LjAMY2*. The probe for the *M. truncatula* putative lupeol synthase (Gamas *et al.*, 1996) was amplified with the following primer pair: forward 5′-GGGTTGAAGACCCAAACTCAGAGG-3′ and reverse 5′-CGATACTCTCCCAGAGCCCATATG-3′. The primers for *M. truncatula MtAMY1* were 5′-GAATTCTCGGAGAAGGACC-3′ (forward) and 5′-GGAACCCGTCTACGATACTC-3′ (reverse). Filters for Southern and Northern blot analysis were hybridised in 0.5 M sodium phosphate buffer pH 7.0, 7% SDS, 1 mM EDTA, 1% BSA at 65 °C overnight and washed under high stringency at 65 °C (15 min washes in 2× SSC, 1× SSC, 0.5× SSC and then 0.1× SSC, all with 0.1% SDS). Autoradiography was performed using standard procedures.

Results

Cloning of triterpene synthases from Medicago truncatula and Lotus japonicus. We used primers to conserved regions of characterized β-amyrin synthases

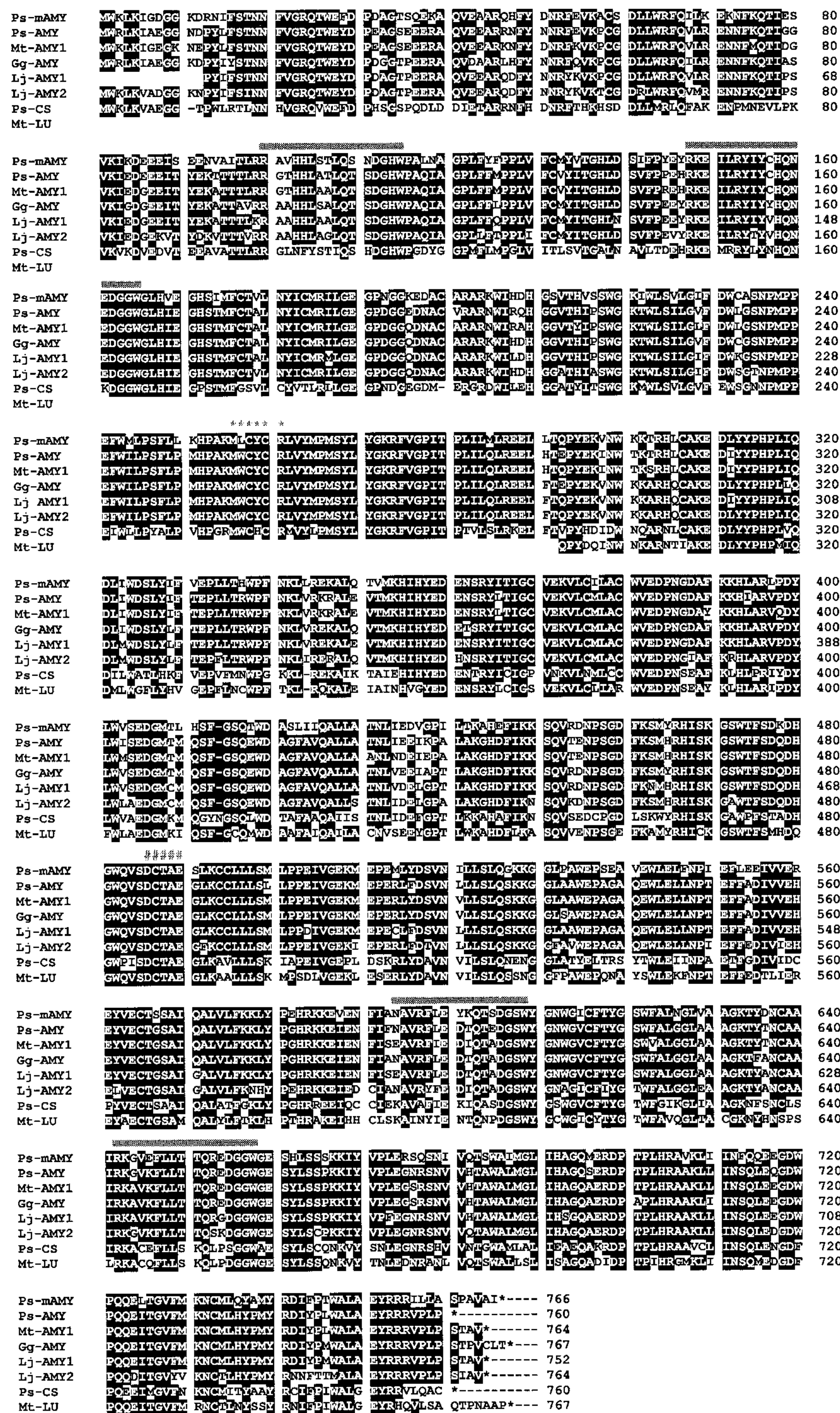

Figure 2. Alignment and sequence comparison of the deduced amino acid sequences of oxidosqualene cyclases from legumes. GenBank accession numbers: Ps-mAMY, *P. sativum* mixed-amyrin synthase *PSM* (AB034803); Ps-AMY, *P. sativum* β-amyrin synthase *PSY* (AB034802); Mt-AMY1, *M. truncatula* β-amyrin synthase (AF78453); Gg-AMY, *G. glabra* amyrin synthase *GgbAS1* (AB037203); Lj-AMY1, *L. japonicus* β-amyrin synthase 1, partial sequence (AF78454); Lj-AMY2, *L. japonicus* β-amyrin synthase 2 (AF78455); Ps-CS, *P. sativum* cycloartenol synthase (D89619); Mt-LU, *M. truncatula* putative lupeol synthase partial sequence (Y15366). Sequence alignment was performed using the PileUp program in the GCG software. Amino acid residues that are identical in at least 5 out of the 8 sequences are boxed in black. #, DCTAE amino acid motif implicated in substrate binding. *, motif associated with β-amyrin or lupeol specificity. QW motifs are indicated by solid bars.

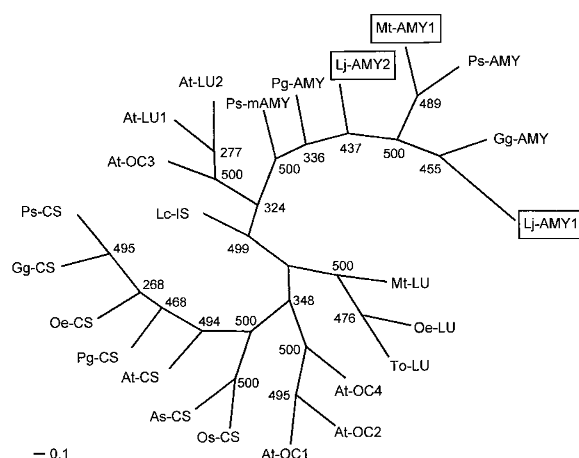

Figure 3. Amino acid sequence relatedness of MtAMY1, LjAMY1, LjAMY2 (boxed) with other members of the oxidosqualene cyclase superfamily. GenBank accession numbers are as follows. Cycloartenol synthases: Ps-CS, *Pisum sativum* (D89619); Gg-CS, *Glycyrrhiza glabra* (AB025968); Oe-CS, *Olea europaea* (AB025344); Pg-CS, *Panax ginseng* (AB009029); At-CS, *Arabidopsis thaliana* (P38605); As-CS, *Avena strigosa AsCS1* (AJ311790); Os-CS, *Oryza sativa* (AF169966); Predicted OSCs of unknown product specificity: At-OC1, *A. thaliana* (AB026657); At-OC2, *A. thaliana* (AB018112); At-OC4, *A. thaliana* (AC007260); lupeol synthases: Oe-LU, *Olea europaea* (AB025343); To-LU, *Taraxacum officinale* (AB025345); Mt-LU (predicted), *M. truncatula* (Y15366); β-amyrin synthases: Gg-AMY, *Glycyrrhiza glabra* (AB037203); Ps-AMY, *P. sativum* (AB034802); Pg-AMY, *Panax ginseng* (AB009030); multifunctional triterpene synthases: Ps-mAMY, *P. sativum* (AB034803); At-LU2, *A. thaliana* (AC002986); At-LU1, *A. thaliana* (U49919); Predicted OSC of unknown product specificity: At-OC3, *A. thaliana* (AC002986); isomultiflorenol synthase: Lc-IS, *Luffa cylindrica* (AB058643) The phylogenetic tree was constructed by using the unweighted pair group method with arithmetic mean (UPGMA) method as implemented in the "Neighbor" program of the PHYLIP package (Version 3.5c). Amino acid distances were calculated using the Dayhoff PAM matrix method of the "PROTDIST" program of PHYLIP. The numbers indicate the numbers of bootstrap replications (out of 500) in which the given branching was observed (Felsenstein, 1996). The protein parsimony method (the "PROTPARS" program of PHYLIP) produced trees with essentially identical topologies.

to PCR-amplify related sequences from cDNA from roots of *M. truncatula* and *L. japonicus*. The primers were designed based on the sequence of the pea β-amyrin synthase *PSY* (Morita *et al.*, 2000). Several PCR products were obtained and larger cDNA clones were generated by 5′- and 3′-RACE using specific primers. A total of three distinct sequences were obtained, one from *M. truncatula* (*MtAMY1*) and two from *L. japonicus* (*LjAMY1* and *LjAMY2*). *LjAMY1* was truncated based on comparison with other OSC sequences and it was not possible to obtain the 5′ end of the cDNA despite repeated attempts. The open reading frames of *MtAMY1* and *LjAMY2* both consist of 2286 bp nucleotides encoding 762-amino acid proteins of around 87 kDa. The MtAMY1, LjAMY1 and LjAMY2 sequences are highly homologous to the pea β-amyrin synthase PSY (97, 96 and 92% amino acid similarity, respectively) (Morita *et al.*, 2000) (Fig. 2). MtAMY1 shares 91 and 95% amino acid identity with LjAMY1 and LjAMY2, respectively, and the two *L. japonicus* clones share 94% identity. MtAMY1, LjAMY1 and LjAMY2 all contain the highly conserved amino acid motif DCTAE which has been implicated in substrate binding (Abe and Prestwich, 1995) and the β-strand turn QW motifs that are characteristic of the OSC superfamily (Poralla *et al.*, 1994) (Figure 2).

Comparison of amino acid sequences of MtAMY1, LjAMY1 *and* LjAMY2 *with other OSCs*

A number of OSCs that mediate the synthesis of triterpenes such as β-amyrin, α-amyrin and lupeol (Fig 1) have recently been cloned and their function confirmed by expression in yeast. Amino acid sequence comparisons indicate that these triterpene synthases are clearly distinct from cycloartenol synthases (reviewed in Haralampidis *et al.*, 2001a). A phylogenetic tree was constructed with the amino acid sequences described in this work (MtAMY1, LjAMY1 and LjAMY2) together with 21 other OSC sequences from plants (Fig. 3). These included cycloartenol synthases, lupeol synthases, β-amyrin synthases, multifunctional β-amyrin synthases, an isomultiflorenol synthase from *Luffa cylindrica* and also other uncharacterised enzymes denoted with the generic name of triterpene or oxidosqualene cyclases. As expected, MtAMY1, LjAMY1 and LjAMY2 are closely related to the *P. sativum* β-amyrin synthase PSY (Morita *et al.*, 2000; Ps-AMY in Fig. 3), and also to β-amyrin synthases from liquorice (Hayashi *et al.*, 2001a) and ginseng (Kushiro *et al.*, 1998a) (Gg-AMY and Pg-AMY in Fig. 3). Other closely related OSCs include PSM from *P. sativum*, which synthesizes α- and β-amyrin as the major products (Morita *et al.*, 2000); ATLUP1, which synthesizes primarily lupeol (Herrera *et al.*, 1998; Husselstein-Muller *et al.*, 2001; Segura *et al.*, 2000); ATLUP2, which produces β-amyrin, α- amyrin and lupeol in the ratios 55%:30%:15% (Kushiro *et al.*, 2000a; Husselstein-Muller *et al.*, 2001) and a predicted OSC from *A. thaliana* of unknown product specificity (Husselstein-Muller *et al.*, 2001) (labeled Ps-mAMY, Ar-LU1, At-LU2 and At-

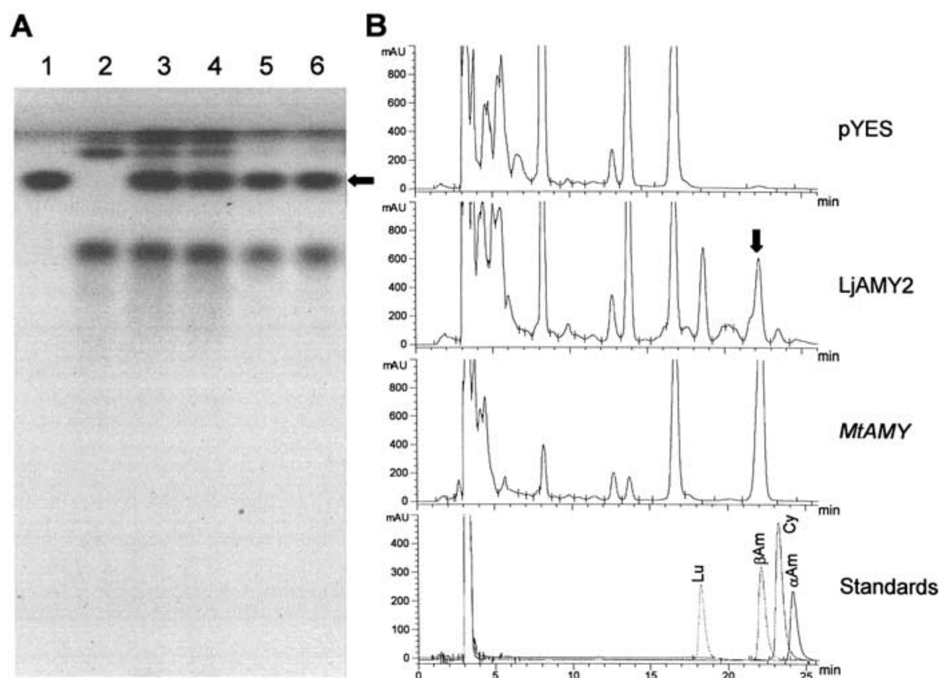

Figure 4. Analysis of extracts from yeast cells expressing the *M. truncatula MtAMY1* or the *L. japonicus LjAMY2* cDNAs. **A**: TLC separation of cell extracts followed by chromogenic detection of 2,3-oxidosqualene cyclization products. Lane 1, β-amyrin standard; lane 2, empty pYES vector; expression of two independent *LjAMY2* cDNA clones (lanes 3 and 4) and two independent *MtAMY1* cDNA clones (lanes 5 and 6). The migration position of the β-amyrin standard is indicated by an arrow. **B**: HPLC analysis yeast cell extracts. From top to bottom, empty pYES vector; *LjAMY2* cDNA; *MtAMY1* cDNA; standards (Lu, lupeol; βAM, β-amyrin; Cy, cycloartenol; αAM, α-amyrin). MtAMY1 produced β-amyrin as the sole product. Cells expressing the LjAMY2 cDNA produced β-amyrin (indicated by an arrow) and lupeol and also a number of other minor products.

OC3 in Fig. 3). The lupeol synthases OEW and TRW, from olive (*Olea europaea*) and dandelion (*Taraxacum officinale*), respectively, both produce lupeol as the sole product when expressed in yeast (Shibuya *et al*., 1999) (Oe-LU and To-LU, Fig. 3) but differ from the *A. thaliana* lupeol synthase ATLUP1 in their deprotonation mechanisms (Kushiro *et al*., 1999a; Kushiro *et al*., 1999b). These enzymes form a distinct group along with the candidate lupeol synthase from *M. truncatula* (Mt-LU, Fig. 3) (Gamas *et al*., 1996; Shibuya *et al*., 1999). Three other predicted OSCs of unknown product specificity from *A. thaliana* (At-OC1, At-OC2 and At-OC4) (Fig. 3) identify a further subgroup within the OSC superfamily (Husselstein-Muller *et al*., 2001).

Expression of MtAMY1 and LjAMY2 in yeast

The product specificity of MtAMY1 and LjAMY2 was tested by expression of the full-length cDNAs in yeast under the control of the *GAL1* promoter. Novel products were observed following TLC analysis (Fig. 4A). These products were not present in extracts of cells that had been transformed with the pYES2 vector alone and were detectable only after galactose induction. HPLC analysis indicated that the yeast cells expressing MtAMY1 accumulated a product with a retention time that was identical to that of β-amyrin (Fig. 4B), while LjAMY2 produced primarily lupeol and β-amyrin in approximately equivalent amounts and also a number of other minor products (Fig. 4B). These results were confirmed by expression of additional independent cDNA clones of MtAMY1 and LjAMY2 in yeast. The product specificity of LjAMY1 has not been assessed by expression in yeast because a full-length cDNA clone was not available.

Southern blot analysis

Genomic DNA of pea, *M. truncatula* and *L. japonicus* was digested with restriction enzymes and hybridized with the respective homologous cDNA probes for *PSY*, *MtAMY1* or *LjAMY2*. Hybridization and washes were carried out under conditions of high stringency. Additional related sequences were detected in *M. trun-*

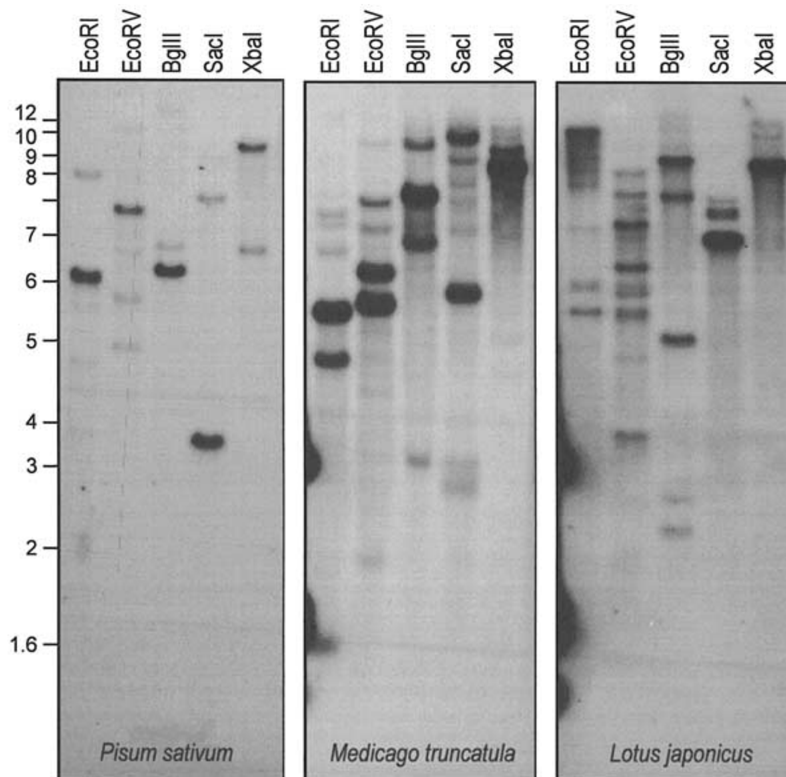

Figure 5. Southern blot analysis. Genomic DNA of *P. sativum*, *M. truncatula* and *L. japonicus* was digested with the restriction enzymes *Eco*RI, *Eco*RV, *Bgl*II, *Sac*I or *Xba*I and Southern blotted (10 μg DNA/track). Blots were hybridized to the appropriate radiolabelled homologous probes (*P. sativum PSY*; *M. truncatula MtAMY1*; *L. japonicus LjAMY2*) under high stringency conditions. The molecular weight marker (kb) is indicated on the left.

catula and *L. japonicus*, while *PSY* was probably the only sequence that was recognized in pea (Fig. 5).

Expression of triterpene synthases in different plant tissues and during nodulation

Expression of *MtAMY1, LjAMY1* and *LjAMY2* was analysed by Northern blot analysis. A partial sequence from *M. truncatula* that may encode a lupeol synthase (GenBank accession no. Y15366; Gamas *et al.*, 1996; Shibuya *et al.*, 1999) was also used as a probe. *MtAMY1* was expressed in all tissues analysed, with highest transcript levels in the shoot meristem and stem tissue (Fig. 6). In contrast, the putative lupeol synthase transcript (*MtLUP1*) was detected in leaf and root tissue and in nodules but was not detected in meristem and stem tissue. Expression of *LjAMY1* and *LjAMY2* was analysed in leaves, roots and nodules of *L. japonicus*. The *LjAMY1* and*LjAMY2* are closely related (90% nucleotide identity) and so gene-specific probes derived from the 3′-UTR regions were used. Northern blot analysis with RNA from leaves, roots and nodules revealed signals with both probes only in the roots (Fig. 7)

The expression of the pea β-amyrin synthase gene *PSY* (Morita *et al.*, 2000) was also examined under different conditions and in different tissues. Analysis of gene expression during germination indicated that *PSY* transcript levels peaked at two days and then declined (Fig. 8). This is consistent with observations that β-amyrin levels and β-amyrin synthase activity are maximal just after germination (Baisted, 1971; Abe *et al.*, 1988, 1989). We then compared the expression levels of *PSY* in leaves, stems, roots and 3-week-old nodules of pea. *PSY* transcripts were readily detectable in stem and root tissue, with only a faint signal in the leaves (Fig. 9A). *PSY* was also expressed in nodules, although transcript levels were lower than in uninoculated roots. When three-day old pea seedlings were inoculated with *R. leguminosarum* or with a control treatment of sterile TY medium and roots were collected 2 and 4 days later, *PSY* transcript levels were slightly higher in the inoculated

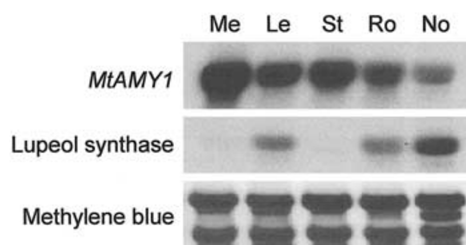

Figure 6. Northern blot analysis of expression of the β-amyrin synthase gene *MtAMY1* and the putative lupeol synthase in *Medicago truncatula*. Me, shoot meristem; Le, fully expanded leaves; St, stems; Ro, roots; No, three-week old nodules. Each lane contains 10 μg total RNA. RNA levels were monitored by staining the blot with methylene blue.

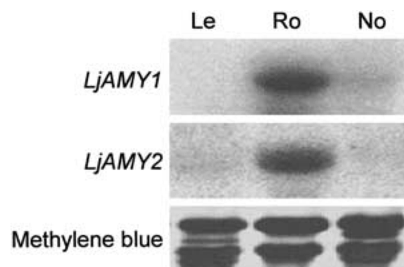

Figure 7. Northern blot analysis of expression of the predicted β-amyrin synthase gene *LjAMy1* and the mixed function triterpene synthase gene *LjAMY2* in *L. japonicus* leaves (Le), roots (Ro) and three-week old nodules (No). Each lane contains 15 μg total RNA. RNA levels were monitored by staining the blot with methylene blue.

roots compared with the control at the two-day time point (Fig. 9B). This difference was reproducible from experiment to experiment but was not evident four days after inoculation. Thus inoculation of roots of pea seedlings with *R. leguminosarum* appears to have a transient effect on *PSY* gene expression. Finally, we investigated expression of *PSY* during nodule development and senescence. Pea nodules were collected 2, 3, 5 and 7 weeks after inoculation with *R. leguminosarum*. *PSY* expression was highest in immature two-week old nodules, moderately high in three-week old nodules that were actively fixing nitrogen, and then declined as the nodules senesced (five- and seven-week old nodules) (Fig. 9C). When three-week old nodules were induced to senesce by treatment of plants with 10 mM KNO_3 or by subjecting them to continuous darkness reduced *PSY* transcript levels were observed (Figure 9C).

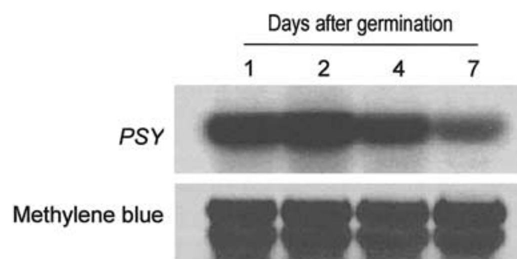

Figure 8. Northern blot analysis of the pea β-amyrin synthase gene *PSY* in pea roots 1, 2, 4 and 7 days after germination (upper panel). Each lane contains 10 μg total RNA. RNA levels were monitored by staining the blot with methylene blue (lower panel).

Discussion

Product specificity of triterpene synthases from Medicago truncatula *and* Lotus japonicus

The three triterpene synthase cDNA sequences reported here (*MtAMY1*, from *M. truncatula*, and *LjAMY1* and *LjAMY2* from *L. japonicus*) were cloned from root material using PCR primers designed to match conserved regions of β-amyrin synthases. Amino acid sequence comparisons confirmed that these three OSCs are all closely related to β-amyrin synthases from pea, liquorice and ginseng (Figs. 2 and 3). The tryptophan residue in the MWCYCR motif of the pea β-amyrin synthase (residues 256–261, Fig. 2) has been identified by site-directed mutagenesis as being important for β-amyrin formation and is conserved amongst all other published β-amyrin synthase sequences (Kushiro *et al.*, 2000b). The MtAMY1, LjAMY1 and LjAMY2 amino acid sequences all contain the MWCYCR motif (Fig. 2), suggesting that these enzymes are likely to be β-amyrin synthases. HPLC analysis of extracts from yeast cells expressing MtAMY1 indicate that this enzyme catalyses the synthesis of β-amyrin as the sole detectable product. LjAMY1 is also likely to be a β-amyrin synthase based on its high sequence similarity to other enzymes with this product specificity although this remains to be confirmed by expression in yeast.

Expression of LjAMY2 in yeast yielded products with retention times that were identical to β-amyrin and lupeol in approximately equal amounts, and also other uncharacterized minor products. Thus LjAMY2 is a mixed-function triterpene synthase. Mixed-function triterpene synthases have been characterized from other plant species. The pea OSC PSM produces α- and β-amyrin (Morita *et al.*, 2000). The *A. thaliana* OSC LUP1 synthesizes primarily lupeol

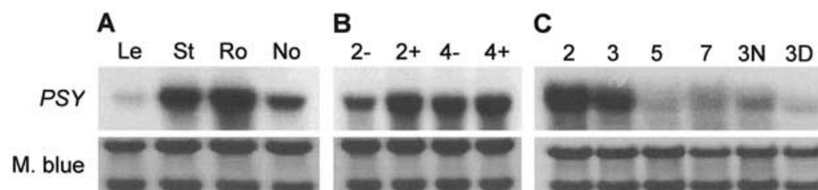

Figure 9. Expression of the β-amyrin synthase gene *PSY* in pea. **A**: Different pea tissues; leaves (Le), stems (St), roots (Ro) and three-week old nodules (No). **B**: Roots 2 and 4 days after inoculation with sterile TY medium (2−, 4−) or *Rhizobium leguminosarum* bv *viciae* (2+, 4+). **C**: Expression of *PSY* in pea nodules during nodule development (2, 3, 5 and 7 week old nodules) and three-week old nodules treated with 10 mM KNO_3 (3N) or maintained in complete darkness (3D). Each lane contains 10 μg total RNA. RNA levels were monitored by staining the blot with methylene blue.

but also other minor triterpenoid products including β-amyrin (Herrera *et al.*, 1998; Shibuya *et al.*, 1999). Another *A. thaliana* OSC ATLUP2 catalyses the production of β-amyrin, α-amyrin and lupeol in the ratio 55:30:15 (Husselstein-Muller *et al.*, 2001). These pea and *A. thaliana* enzymes (Ps-mAMY, At-LU1 and At-LU2, respectively in Fig. 3) share relatively close amino acid sequence similarity with β-amyrin synthases (Fig. 3). LjAMY2 appears to be novel in that it is the only enzyme described to date that synthesizes lupeol and β-amyrin as the two major products. Triterpene synthases that produce lupeol as the sole product have been cloned and characterized from olive and dandelion (Shibuya *et al.*, 1999). However amino acid sequence comparisons indicate that these are clearly distinct from β-amyrin synthases and mixed function triterpene synthases (Fig. 3).

Although only α- and β-amyrin and lupeol are shown in Fig 1, the triterpenes are in fact a large and structurally diverse group of molecules. A survey of natural products has identified at least 83 different triterpenes that are likely to be generated via enzymatic cyclisation of 2,3-oxidosqualene, and it is clear that more triterpene synthases with different product specificities will be cloned and characterized in future (Matsuda, 1998). In *A. thaliana* there are at least 13 predicted OSCs although the product specificity of only three of these enzymes is known (Corey *et al.*, 1993; Herrera *et al.*, 1998; Kushiro *et al.*, 2000a; Husselstein-Muller *et al.*, 2001). Southern blot analysis indicates that there are other sequences present in the *M. truncatula* and *L. japonicus* genomes that hybridize with the cloned triterpene synthases reported here (Fig. 5), and these related sequence may encode OSCs that synthesize a variety of different triterpene products.

Expression of triterpene synthases in different plant tissues and during nodulation

The β-amyrin synthase genes from pea and *M. truncatula* (PSY and MtAMY1) are expressed in the nodules of these plants (Table 1; Figs. 6 and 9). *PSY* appears to be transiently induced during early nodulation (two days post inoculation; Fig. 9B), although the difference in transcript levels is no longer evident at 4 days post-inoculation. *PSY* transcript levels apparently decrease in senescing nodules and in nodules that have been treated with KNO_3 or maintained in the dark (Fig. 9C), although this decrease may be due to general effects on plant gene expression rather than specific reduction in *PSY* expression. Hernandez and Cook (1996) were not able to detect β-amyrin in membranes of free living bacteroids and so concluded that the β-amyrin detected in the peribacteroid membrane may be synthesized by the host plant. The results presented here indicate that this is likely to be the case, at least for pea and *M. truncatula*. However, although *PSY* and *MtAMY1* transcripts were readily detected in nodules expression of both genes was higher in other plant tissues (Table 1). Neither of the OSCs cloned from *L. japonicus* (the predicted β-amyrin synthase LjAMY1 and the multifunctional triterpene synthase LjAMY2) were expressed at detectable levels in nodules although both were strongly expressed in the roots. *L. japonicus* forms determinate nodules in which the meristem becomes arrested and inactive when the nodule has reached maturity, while pea and *M. truncatula* both produce indeterminate nodules with a persistent meristem that undergoes constant division. Differences in OSC expression between these species could therefore be associated with the nature of the nodules formed.

The partial cDNA that was isolated from *M. truncatula* nodules and initially annotated as a predicted cycloartenol synthase (Gamas *et al.*, 1996) shares

greatest amino acid sequence similarity to lupeol synthases from olive and dandelion (Shibuya *et al.*, 1999; Fig. 3). This OSC (MtLUP) is therefore likely to synthesise lupeol although its function has not yet been confirmed by expression in yeast. Interestingly, *MtAMY1* and *MtLUP* show complementary expression patterns in different plant tissues. *MtAMY1* expression was highest in the shoot meristems and stems while *MtLUP* transcripts were not detectable in RNA from these tissues by Northern blot analysis and were highest in the nodules. Thus lupeol or a derivative of this triterpenoid may also have some role in nodule development.

Many plant species store glycosylated triterpene saponins in the roots, where these molecules may act as antimicrobial phytoprotectants (Hostettmann and Marston, 1991; Morrissey and Osbourn, 1999; Papadopoulou *et al.*, 1999; Haralampidis *et al.*, 2001). β-Amyrin-derived triterpenoid saponins are present in the roots of *M. truncatula* (Huhman and Sumner, 2002). The saponin content of *M. truncatula* leaves has not yet been characterized although the foliage of related species such as *Medicago sativa* does contain β-amyrin-derived saponins (Oleszek *et al.*, 1992). The expression of the β-amyrin synthase gene *MtAMY1* in the roots and leaves of *M. truncatula* is therefore consistent with a role for MtAMY1 in the synthesis of triterpenoid saponins in these tissues. β-Amyrin-derived triterpenoid saponins are also found in seeds of *Lotus* species (Jurzysta, 1973) and in the seeds and germinating seedlings of pea (Price *et al.*, 1987; Ohana *et al.*, 1998; Rahman *et al.*, 2001) although information on the saponin content of the roots and leaves of these species is scant. More detailed analysis of the saponin content of different plant tissues and also of nodules is required in order to assess whether the other OSCs in Table 1 are likely to be involved in saponin biosynthesis in different plant parts.

In summary, the cloned triterpenoid synthases reported here represent tools that can now be used to generate transgenic plants with altered levels of these enzyme activities, either by overexpression or by gene silencing. This will allow the role of triterpenoids and their saponin derivatives in the establishment of interactions between symbiotic and pathogen microbes to be addressed.

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