

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 *L. truncatula* (*MtAMYI*) and a mixed function triterpene synthase from *L. truncatula* (*MtAMYI*) and a mixed function triterpene synthase from *L. taponicus* (*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.

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

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*).

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 KNO3 (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* by *viciae* NLV8. Stress treatments were applied by subjecting three-week old plants to complete darkness or by watering the plants with 10 mM KNO₃ 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 μ l 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 dT₁₅ 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 PCRamplify 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) \times 30 cycles, 72 °C 10 min. The reaction mixture contained 300 nM of each primer, 200 μ M dNTPs, 2 μ l 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 μ l). PCR products were cloned into the pGEM-T Easy vector (Promega) and transformed into DH5 α 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. Secondstrand 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 $[\alpha^{-32}P]$ dCTP using the Megaprime labelling kit (Amersham). Probes for Southern blot analysis were generated as follows. The probe for P. sativum was PCRamplified 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 (α AS), β -amyrin synthase (β AS), 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 $2 \times$ SSC, $1 \times$ SSC, $0.5 \times$ SSC and then $0.1 \times$ 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

Ps-mAMY	MAKINGDEE	KORNINSIONA	FVGROTWE	DACTSONKA	OVERAGEOHEY	DRIGHEVRAUS	DILLWRINGTILR	BRAINCETES	80
Ps-AMY	MARINALAECC	NDPYLESTAN	FVGRQTWEYD	PEACSEDBRA	OVERARRINGY	NINRIGEVICE	DIBRARAGOVINE	SINNER(OF GG	80
Mt-AMY1	MARINAIGECK	NERVILESTAN	FVGRQTWEYD	DEACSEDBRA	<u>OVERARKNEY</u>	DINRIFRWKPCG	DININGRATOVING	BRADMONTOG	80
3g-AMY	MARINE IAE CC	RDDVIYSTRIN	FVGRQTWEYD	DGGTPINNEL	OVDAARLHEV	NEEDOVRPCC	DIALWRITOTHR	SINISTROTTAS	80
Lj-AMY1		PYIFSTNN	FVGROTWEYD	PDAGIPEERA	QVEEARODEY	NERYKVEPCC	DIGLWRISOVIDR	SARISKOTTIPS	68
Lj-AMY2	MAKUKVADGG	KNEWDESTAN	FVGRQTWEYD	DAGTENSIO	QVEEARODEY	NERYKVETCE	DRIWRINGVMR	ERINDEROPTIPS	80
Ps-CS	MORING VAE CC	-TEWLRTLAN	H <u>VGROV</u> GDED	HSESPODLD	DIDTARNAH	DARIOTHCHSD	DIMENSIOFAR	PMNEVLPK	80
Mt-LU									
		885	u and a state of the					NERCONSTRUCTOR	
Ps-mAMY	VKUKOESSUS	EGNVALMER	AVELESTLOS	NDGHWPALNA	GPLFYFPPLV	FCMYVTGHID	SIFPYEYRKE	ILRYIYCHQN	160
Ps-AMY	VKIEDSEEIT	YEKATTTIRR	GTHHATLOT	SDGHWPAQIA	GPLEEMPPLV	FCVYITGHID	SVEPPEHRKE	ILRYIYCHON	160
Mt-AMY1	VKIEDGEEIT	YEKATTILRR	GTHHDAALOT	SDGHWPAQIA	GPLFFMPPLV	FCVYIIIGHID	SVEPREHRKE	ILRYIYCHQN	160
Gg-AMY	VKIGDGEEIT	YEKATTAVER	AAHHSALOT	SDGHWPAQIA	GPLEFLPPLV	FCMYITCHLD	SVEPSEYRKE	ILRYIY <mark>Y</mark> HQN	160
Lj-AMY1	VKIEDGEEIT	YOKARUTIKR	AA	SDGHWPAQIA	GPLFFOPPLV	FCMYITGHIIN	SVEPEEYRKE	TERVIN	148
Lj-AMY2	VKLEDG⊡KVA	YDKVTTTVRR	AAHHAGLOT	SDGHWPAQIA	GPLIFTPPLI	FCMYITGHID	SVEDEVYRKE	TERVITYVHON	160
Ps-CS	VKVKDV⊡DVA	EDAVATUMER	GLNFYSTICS	HDGHWEGDYG	GEMELMPGIN	ITLSVICAUN	AVLTOPHRE	MRRVINHON	160
Mt-LU									
Ps-mAMY	BIDGGWGLIHVE	GHSIMFCIVL	NYICMRILGE	GENGEKEDAC	ARARIOTHDH	CSVIII:IVSSWC	KIWLSVLGIF	DWCASNDWPP	240
Ps-AMY	EDGGWGLHIE	GHSTMFCTAL	NYICMRILGE	GPDGGEDNAC	VRARNALROH	GGVTHIPSWG	KTWLSILGVF	DWIGSNPMPP	240
Mt-AMY1	EDGGWGLHIE	c):ISMMICEPAL	NYICMRILGE	GEDIGGODNAC	AGARN (HRAI)	GGVTMIPSWG	KTWLSILGIF	DWIIGSNPMPP	240
Gg-AMY	EDGGWGLHIE	GHSIMFCTAL	NYICMRILGE	GPDGGCDNAC	ARARENTHDE	GGVTHILPSWG	KTWILSTILGVE	DWeGSNPMPP	240
Lj-AMY1	EDGGWGLHIE	GRSTMFCTAL	NYICMRMLGE	GPDGGODNAC	ARARKALLDE	GGVTHIPSWG	KTWLSILGIE	DWRGSNPMPP	228
Lj-AMY2	EDGGWGLHIE	GHSHWIGCH VII	NYICMRILGE	GPDGGODNAC	ARARKATHDE	eleann: Aisivie	RTWISTIGHE	DWSGINPMPP	240
Ps-CS	KDICCWCUHUD	CPISHIOGSVI	CUALTRINGS	HIND GEGDM-	RECEIVED WITH BEEL	SCALLY TRAVE	REAL PROPERTY IN COMPANY	BWS CNNHWEE	240
Mt-LU									
		新学校书 文	*						
Ps-mAMY	EFWMLPSFLL	KHPAKMLCYC	RIVYMPMSYL	YGKRFVGPIT	DALEN MUSICAL	LTOPYDKVNW	KKTRHICAKE	DLYYPHPLIQ	320
Ps-AMY	EFWILPSFLP	MHPAKMWCYC	RIVYMPMSYL	YGKRFVGPIT	PLILQLREEL	HTEPYEKINW	TKTRHICAKE	DIYYPHPLIQ	320
Mt-AMY1	EFWILPSFLP	MHPAKMWCYC	RIVYMPMSYL	YGKRFVGPIT	BUIDÖRBER	HTOPYEKINW	TKSREI/CAKE	DHAJBHBFIÖ	320
Gg-AMY	EFWILPSFLP	MHPARMWCYC	RLVYMPMSYL	YGKRFVGPIT	PLILQLREEL	PTOPYDRVNW	KKARHOCAKE	DTAAbHbrid	320
Lj AMY1	EFWILPSFLP	MHPAKMWCYC	RIVYMPMSYL	YGKRFVGPIT	PLILQLREEL	FTOPYEKVNW	KKARHOCAKE	DHAISHBFIÖ	308
Lj-AMY2	EFWILPSFLP	MHPARMWCYC	RLVYMPMSYL	YGKRFVGPIT	PLILQLREEL	FTQPYEKVNW	RKARHOCAKE	DIAAbhbrið	320
Ps-CS	EIWELPYALP	VHPGRMWCHC	RYVYDPMSYL	YGKRFVGPIT	PTVISTRKET	FUVENHDIDA	NOARNLCAKE	DITABHBINO	320
Mt-LU						<u>OPV</u> DQINN	NGARNTIAKE	DIAMEHEWIO	320
Ps-mAMY	DELADSHALLS	VIDDAMENDO	NUMBER OF COMPANY	TVOCCONTRACTO	ENSRYITIGC	VERVICILAC	WVEDPNGDAF	KKHLARDPDY	400
Ps-AMY	DLINDSLYRE	TEPLITRWPF	NKLVRKRALE	VIMENTHYED	ENSRY	VEKVLCMLAC	WVEDPNGDAF	KKHHARVPDY	400
Mt-AMY1	DITEDSITY	NEISTATATIKKEISE	NEW VRERATE	VTMKHIHYED	ENSRYTTIGC	VEKVLCMLAC	WVEDPNGDAY	KKHLARVODY	400
Go-AMY	DET//DSDYL/F	THE PROPERTY AND P	NKLVREKALO	VTMKHIHYED	EASRYITIGC	VEKVLCMLAC	WVEDPNGDAF	KKHLARVPDY	400
Lj-AMY1	DIMADSIATE	TEPLLTRWPF	NKLVREKALE	VTMKHTHYED	ENSRYITIGC	VEKVLCMLAC	WVEDPNGDAF	KKHLARVPDY	388
Lj-AMY2	DLMWDSLYLF	TEPTLTRWPF	NKLIRERALO	VUNKHUHMAD	HNSRYITTIGC	VEKVLCMLAC	WVEDPNGLAF	KRHLARVPDY	400
Ps-CS	DIL ATOHKS	VERVEMNERG	KKL-RISKAIK	TAIE HINGS	ENTRYICICP	VNKVLNMLCC	WVEDPNSEAF	KLHEPRIYDY	400
Mt-LU	ML∴GFUMHV	GUEFUNCTED	TKL-ROKALE	IAIN	ENSRYLCIGS	VEKVLCILLAR	WW SDIN SEAY	KIHLARIPDY	400
De many	AND PROPERTY.	TRE-COOK	STATISTICS.	WARTED VOID		SOVEDNESCO	DESMARTSK	CSICHIGSIDK DE	480
PS-MAMI	LWVSEDGUT	OST-COTIND	AGEAVOALLA	THE TROPIC A	LAKCHDETKK	SOVUENESCO	TKSMERHTSK	GSWTESDODH	480
PS-AMI		QSI-GSQEND	AGENVONTIA	AVAIDUTEDA	TAKCHDETYK	SOWERNESCO	TROMPOSITOR	CSWTFSDODK	480
C- NO	TWO DO CM	Q32-G3QEWD	AGRAVOALLA	UNINTED TAUT	LAKCHDETKK	SOVEDNESCO	FROMVERTOR	GSWTFSDODH	480
GG-ANI T-A-AMV1	TWVSEDGMCM		AGFAVOALLA		LAKGHDETKK	SOVEDNESCO	FKNMERHTSK	GSWTFSDODH	468
LJ-AMY2	TWO STODENCY	OSE-CSOEND	AGTAVOALLS	UNUT DOIL ODA	DAKGHDETKN	SOVEDNESCO	FKSMURHTSK	CATESDODH	480
DJ-AMIZ	MANA COLORAD	OFWIGSOUND	TADAAOATTS	INTERPORT	TREATION	SOVSEDGEGD	LSKWY	CAMPESTADE	480
FS-CS M+_TIT	TOT ACHINES INT	OSIN-COOMOD	NATOTON	ONSPORT	INVERTING AND INCOME.	SOVVENESGE	DEATYREECE	GSW DD/SMHD O	480
MC-D0	E MENDER VICE	200 642410		0.1.00.210141					

Ps-mAMY	GWQVSDCTAE	SLKCCLLLSM	LPPEIVGERM	EPEMLYDSVN	THUSHOEKKE	GLPAWEPSEA	VISIONALISI	SHEELVVER	560
Ps-AMY	GWQVSDCTAE	GIRCCIDIIS	LEPETVGEKM	EPERLEDSVN	LINISIOSKKG	GLAAWEPAGA	QEWLELINPT	EFFADIVVEH	560
Mt-AMY1	GWQVSDCTAE	GLKCCLLLSM	INDERVGEKM	EPERLYDSVN	VILLSILQSKKG	GLAAWEPAGA	QEWIELLNPT	SITE AD I VVDE	560
Gg-AMY	GWQVSDCTAE	GLKCCLLLSM	IL PEPETVGERM	EPERLYDSVN	VITUSINOSIS	GLSAWEPAGA	OFWLELLNPT	EFFADIVVEH	560
Lj-AMY1	GWQVSDCTAE	GLKCCLLLSM	LEEDIVGERM	EPECLADSVN	LINISLOSKKG	GLAAWEPAGA	QEWLELLNPT	EFRADIVUER	548
Lj-AMY2	GWQVSDCTAE	GERCCLLLSM	TEPPETVGERI	SPERI GDTVN	LILISLQSKKG	GITAV (IDIPACIA	OPATION	EFFED VIER	560
Ps-CS	GWPISDCHAD	CHANNAN	IAUSHIVESPL	DSKRIMDAVA	VINSILENENS	CHATTOLTRS	TTWISTINGA	STIGOLVIDC	560
MC-TO	GWQV/SDI00/AD	STRAAD THE SK	APSD140981	S S S S S S S S S S S S S S S S S S S	THEIRESING	20 F 10 1 10 20 10	- OUT OF BUILDING	CONTRACTOR NO.	200
Ps-mAMY	EYVECTSSAI	QALVLFKRDY	PEHRKKE	DIANAVRELD	YKOTSDESWY	GNWGICETYG	SWEATNGLVA	AGKTYDNCAA	640
Ps-AMY	EYVECTGSAI	QALVLFKKLY	PGHRKKEIEN	ETENAVRELD	DIQUEDGSWY	GNWGVCFTYG	SWEALGGLAA	AGKTYINCAA	640
Mt-AMY1	EYVECTGSAT	QAINTERKIN	PGHRKKELEN	ELSEAVEFIC	DIOTADIGSWY	GNWGVCFTYG	SINVIALIGGILAA	AGKINTINCAA	640
Gg-AMY	EYVECTGSAL	OMANIETKKIN	RECEIPTON	DUANAVRELO	DICTORADICSWIZ	GRINGVCHUYG	SWEVEDGELAA	ALC: ALC: ALC: ALC: ALC: ALC: ALC: ALC:	640
Lj-AMY1	EYVECTGSAT	GANVINGGAN	PGERKKELIEN	EDSEAWROLD	DTOHADICSWI	GRWGVCETYG	SWIGHTIGGERAM	AGRUNZARICATA	628
L)-AMY2	DEVECTGSAT	GANNING (NH)	PEHRKKEIDED	CHAN WAY	DITOMADESWI	GARLOPING	TWINAINCICHEA	ACCOUNTRACTORY	640
PS-CS	PTAVECHSA 40	SIGNATING (CTV	PICHINGRIDID I QC	CLERVE VAISTIC	ALCASUESWA	GONGVOETIYG	1 TABLE CONTRACT	COVATIVE SCIELS	640
Mt-LU	orrander (eSA	NARA NARANA	CALCULAR COLUMN	CLSKAINYIC	NTONPOIGSWM	COMCTON/046	1.134100.0111	CCAMPBUSES	040
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Ps-mAMY Ps-AMY	IRKGVEFILL IRK <mark>GVKFILL</mark>	TQREDGGWGE TQREDGGWGE	STLSSSKKIY SYLSSPKKIY	VPLERSOSNI VPLEGNRSNV	VOTSWAINCH	IHAGQMERDP IHAGQSERDP	TPLHRAAKLL	INSQLEOGDW	720
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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, Glycvrrhiza 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; β AM, β -amyrin; Cy, cycloartenol; α AM, 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, *Bg*/II, *SacI* or *XbaI* 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 μ g 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 μ g 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 sevenweek old nodules) (Fig. 9C). When three-week old nodules were induced to senesce by treatment of plants with 10 mM KNO₃ 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 μ g 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* by *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 KNO₃ (3N) or maintained in complete darkness (3D). Each lane contains 10 μ g 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. trun*catula (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 KNO3 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. trun*catula 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 -Amyrinderived 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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