



Review

Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants

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ABSTRACT

The multitude of terpene carbon skeletons in plants is formed by enzymes known as terpene synthases. This review covers the monoterpene and sesquiterpene synthases presenting an up-to-date list of enzymes reported and evidence for their ability to form multiple products. The reaction mechanisms of these enzyme classes are described, and information on how terpene synthase proteins mediate catalysis is summarized. Correlations between specific amino acid motifs and terpene synthase function are described, including an analysis of the relationships between active site sequence and cyclization type and a discussion of whether specific protein features might facilitate multiple product formation.

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1. Introduction

Terpenes are not only the largest group of plant natural products, comprising at least 30,000 compounds (Connolly and Hill, 1991), but also contain the widest assortment of structural types. Hundreds of different monoterpene (C10) (Dewick, 1999), sesquiterpene (C15) (Fraga, 2006), diterpene (C20) (Hanson, 2000) and triterpene (C30) (Connolly and Hill, 2005) carbon skeletons are known. Natural products chemists have long marveled at the

structural diversity of terpenes and speculated on its biosynthetic basis.

The wealth of terpene carbon skeletons can be attributed to an enzyme class known as the terpene synthases. These catalysts convert the acyclic prenyl diphosphates and squalene into a multitude of cyclic and acyclic forms. The chief causes of terpene diversity are the large number of different terpene synthases and the fact that some terpene synthases produce multiple products. This review discusses the role of terpene synthases in creating terpene diversity. We first present a current list of reported terpene synthases and describe the evidence for their ability to form multiple products. Next, we summarize current knowledge of the reaction

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Table 1
Monoterpene synthase genes isolated to date.

Gene bank accession no.	Main product(s) ^a (%)	Designation ^b	Species	Cyclization type ^c	References
AAB71084	Myrcene (~100)	Ag2	<i>Abies grandis</i>	None	Bohlmann et al. (1997)
AAB71085	(-)-β-Pinene (58)	Ag3	<i>Abies grandis</i>	1-6, 2-7	Bohlmann et al. (1997)
AAB70707	(-)-Camphene (54)	Ag6	<i>Abies grandis</i>	1-6, 3-7, H	Bohlmann et al. (1999)
AAF61453	(-)-(4S)-β-Phellandrene (52)	Ag8	<i>Abies grandis</i>	1-6, H	Bohlmann et al. (1999)
AAF61454	Terpinolene (42)	Ag9	<i>Abies grandis</i>	1-6, H	Bohlmann et al. (1999)
AAB70907	(-)-(4S)-Limonene (~70)	Ag10	<i>Abies grandis</i>	1-6	Bohlmann et al. (1997)
AAF61455	(-)-(4S)-Limonene (35)	Ag11	<i>Abies grandis</i>	1-6	Bohlmann et al. (1999)
AAK83564	nd	Similar to Ag3	<i>Abies grandis</i>		Trapp and Croteau (2001)
AAK83565	nd	Similar to Ag10	<i>Abies grandis</i>		Trapp and Croteau (2001)
AAL17636	(+)-(4R)-Limonene (~100)	ArLMS	<i>Agastache rugosa</i>	1-6	Maruyama et al. (2002)
AAO41727	Myrcene (100)	Ama1e20	<i>Antirrhinum majus</i>	None	Dudareva et al. (2003)
AAO41726	Myrcene (100)	Ama0C15	<i>Antirrhinum majus</i>	None	Dudareva et al. (2003)
AAO42614	(E)-β-Ocimene (97)	Ama0a23	<i>Antirrhinum majus</i>	None	Dudareva et al. (2003)
ABR24418	(+)-(3S)-Linalool (100) #	AmNES/LIS-2	<i>Antirrhinum majus</i>	None, \$	Nagegowda et al. (2008)
AAO85533	(+)-(3S)-Linalool (100)	At1g61680	<i>Arabidopsis thaliana</i>	None, \$	Chen et al. (2003)
NP_189209	β-Myrcene/(E)-β-Ocimene	At3g25810	<i>Arabidopsis thaliana</i>	None	Chen et al. (2003)
AAN65379	(E)-β-Ocimene (94)	AtTPS03	<i>Arabidopsis thaliana</i>	None	Fäldt et al. (2003b)
AAG09310	Myrcene (56)	AtTPS10	<i>Arabidopsis thaliana</i>	None	Bohlmann et al. (2000)
AAU01970	1,8-Cineole (52)	AtTPS-Cin	<i>Arabidopsis thaliana</i>	1-6, 3-0-7, \$	Chen et al. (2004)
AAF13357	(-)-(3R)-Linalool (100)	QH1	<i>Artemisia annua</i>	None, \$	Jia et al. (1999)
AAF13356	(-)-(3R)-Linalool (100)	QH5	<i>Artemisia annua</i>	None, \$	Jia et al. (1999)
AAK58723	(-)-β-Pinene (94)	QH6	<i>Artemisia annua</i>	1-6, 2-7	Lu et al. (2002)
ABI21837	(-)-(4S)-Limonene (~90)	CsTPS1	<i>Cannabis sativa</i>	1-6	Günnewich et al. (2007)
ABI21838	(+)-α-Pinene (~85)	CsTPS2	<i>Cannabis sativa</i>	1-6, 2-7	Günnewich et al. (2007)
CAD29734	Geraniol (100)	CtGES	<i>Cinnamomum tenuipilum</i>	None, \$	Yang et al. (2005)
AAM53945	(-)-β-Pinene (81.4)	Cl(-)βPINS	<i>Citrus limon</i>	1-6, 2-7	Lucker et al. (2002)
AAM53944	(+)-(4R)-Limonene (99.1)	Cl(+)-LIMS1	<i>Citrus limon</i>	1-6	Lucker et al. (2002)
AAM53946	(+)-(4R)-Limonene (99.1)	Cl(+)-LIMS2	<i>Citrus limon</i>	1-6	Lucker et al. (2002)
AAM53943	γ-Terpinene (71.4)	ClγTS	<i>Citrus limon</i>	1-6, H	Lucker et al. (2002)
BAD91045	1,8-Cineole (>97.2)	CitMTSL1	<i>Citrus unshiu</i>	1-6, 3-0-7, \$	Shimada et al. (2005a,b)
BAD91046	(E)-β-Ocimene (>97.2)	CitMTSL4	<i>Citrus unshiu</i>	None	Shimada et al. (2005a,b)
BAD27260	β-Pinene (82.4)	CitMTSL62	<i>Citrus unshiu</i>	1-6, 2-7	Shimada et al. (2004)
BAD27256	(+)-(4R)-Limonene (97)	CitMTSE1	<i>Citrus unshiu</i>	1-6	Shimada et al. (2004)
BAD27257	(+)-(4R)-Limonene (100)	CitMTSE2	<i>Citrus unshiu</i>	1-6	Shimada et al. (2005a)
BAD27258	γ-Terpinene (85.4)	CitMTSL3	<i>Citrus unshiu</i>	1-6, H	Shimada et al. (2004)
BAD27259	γ-Terpinene (78.4)	CitMTSL61	<i>Citrus unshiu</i>	1-6, H	Shimada et al. (2004)
AAC49395	(+)-(3S)-Linalool (~100) ^o	LIS	<i>Clarkia brewerii</i>	None, \$	Dudareva et al. (1996)
AAD19840	nd	LIS2	<i>Clarkia brewerii</i>		Cseke et al., 1998
AAD19839	nd	LIS	<i>Clarkia concinna</i>		Cseke et al. (1998)
CAD57081	(+)-(3S)-Linalool (100) #	FaNES1	<i>Fragaria ananassa</i>	None, \$	Aharoni et al. (2004)
CAD57106	(+)-(3S)-Linalool (100) #	FaNES2	<i>Fragaria ananassa</i>	None, \$	Aharoni et al. (2004)
CAD57092	α-Pinene (~80)	FvPINS	<i>Fragaria vesca</i>	1-6, 2-7	Aharoni et al. (2004)
ABB73044	(+)-(4R)-Limonene (~39)	LaLIMS	<i>Lavandula angustifolia</i>	1-6	Landmann et al. (2007)
ABB73045	(-)-(3R)-Linalool (100)	LaLINS	<i>Lavandula angustifolia</i>	None, \$	Landmann et al. (2007)
AAT86042	(E)-β-Ocimene (98)	LjEPOS	<i>Lotus japonicus</i>	-	Arimura et al. (2004)
AAX69063	(-)-(3R)-Linalool (100)	LeMTS1	<i>Lycopersicon esculentum</i>	None, \$	van Schie et al. (2007)
AAX69064	β-Myrcene (~50)	LeMTS2	<i>Lycopersicon esculentum</i>	None	van Schie et al. (2007)
ACC66282	α-Terpineol (100)	Mg17	<i>Magnolia grandiflora</i>	1-6, \$	Lee and Chappell (2008)
AAP40638	Inactive	-	<i>Melaleuca alternifolia</i>		Shelton et al. (2004)
AAL99381	(-)-(3R)-Linalool (96)	-	<i>Mentha citrata</i>	None, \$	Crowell et al. (2002)
AAC37366	(-)-(4S)-Limonene (94)	LC5.2	<i>Mentha spicata</i>	1-6	Colby et al. (1993)
ABP88782	1,8-Cineole (~50)	CIN	<i>Nicotiana suaveolens</i>	1-6, 3-0-7, \$	Roeder et al. (2007)
AAR11765	Geraniol (100)	GES	<i>Ocimum basilicum</i>	None, \$	Iijima et al. (2004a)
AAV63789	(-)-(3R)-Linalool (100)	LIS	<i>Ocimum basilicum</i>	None, \$	Iijima et al. (2004b)
AAV63792	Terpinolene (~50)	TES	<i>Ocimum basilicum</i>	1-6, H	Iijima et al. (2004b)
AAV63790	Fenchol (~50)	FES	<i>Ocimum basilicum</i>	1-6, 2-7, H, \$	Iijima et al. (2004b)
AAV63791	Myrcene (100)	MYS	<i>Ocimum basilicum</i>	None	Iijima et al. (2004b)
EU596453	(+)-(3S)-Linalool (100)	Os02g02930	<i>Oryza sativa</i>	None, \$	Yuan et al. (2008)
AAV88965	Geraniol (100)	PcTPS-C	<i>Perilla citriodora</i>	None, \$	Ito and Honda (2007)
AAF65545	nd	-	<i>Perilla citriodora</i>		Ito et al. (2000)
AAG31438	(-)-(4S)-Limonene (~100)	PFLC1	<i>Perilla frutescens</i>	1-6	Yuba et al. (1996)
AAF76186	Myrcene (~54)	PTS-5526	<i>Perilla frutescens</i>	None	Hosoi et al. (2004)
ABB30218	Geraniol (100)	PfTPS-PL	<i>Perilla frutescens</i>	None	Ito and Honda (2007)
ABY65110	(E)-β-Ocimene (98)	PIOS	<i>Phaseolus lunatus</i>	None	Arimura et al. (2008b)
AAO73863	(+)-3-Carene (78)	PaJF67	<i>Picea abies</i>	1-6, 5-7	Fäldt et al. (2003)
AAS47694	(-)-(4S)-Limonene (88)	PaTPS-Lim	<i>Picea abies</i>	1-6	Martin et al. (2004)
AAS47696	Myrcene (100)	PaTPS-Myr	<i>Picea abies</i>	None	Martin et al. (2004)
AAS47693	(-)-(3R)-Linalool (97)	PaTPS-Lin	<i>Picea abies</i>	None, \$	Martin et al. (2004)
AAS47692	(-)-β-Pinene (57)	PaTPS-Pin	<i>Picea abies</i>	1-6, 2-7	Martin et al. (2004)
AAV72020	(-)-α-Pinene (62.5)	PsTPS2	<i>Picea sitchensis</i>	1-6, 2-7	McKay et al. (2003)
ABA86248	(-)-(4S)-Limonene (100)	PsTPS-Lim	<i>Picea sitchensis</i>	1-6	Byun-McKay et al. (2006)
ABA86247	nd	PsTPS-Linl	<i>Picea sitchensis</i>		Byun-McKay et al. (2006)
AAO61225	(-)-α-Pinene (79)	Pt1	<i>Pinus taeda</i>	1-6, 2-7	Phillips et al. (2003)
AAO61227	α-Terpineol (57.3)	Pt10	<i>Pinus taeda</i>	1-6, \$	Phillips et al. (2003)
AAO61228	(+)-α-Pinene (97)	Pt30	<i>Pinus taeda</i>	1-6, 2-7	Phillips et al. (2003)
AAO61229	Inactive	Pt42	<i>Pinus taeda</i>		Phillips et al. (2003)

Table 1 (continued)

Gene bank accession no.	Main product(s) ^a (%)	Designation ^b	Species	Cyclization type ^c	References
AAX07267	(-)- α -Pinene (~40)	PmeTPS1	<i>Pseudotsuga menziesii</i>	1-6, 2-7	Huber et al. (2005)
AAX07264	Terpinolene (~40)	PmeTPS2	<i>Pseudotsuga menziesii</i>	1-6, H	Huber et al. (2005)
CAC41012	Myrcene (~99)	MyrS	<i>Quercus ilex</i>	None	Fischbach et al. (2001)
ABH07677	1,8-Cineole (72)	SF-CinS1	<i>Salvia fruticosa</i>	1-6, 3-0-7, \$	Kampranis et al. (2007)
AAC26018	(+)-Sabinene (63)	SSS	<i>Salvia officinalis</i>	1-6, 2-6, H	Wise et al. (1998)
AAC26017	(+)-Bornyl diphosphate (75)	SBS	<i>Salvia officinalis</i>	1-6, 3-7, \$	Wise et al. (1998)
AAC26016	1,8-Cineole (79)	SCS	<i>Salvia officinalis</i>	1-6, 3-0-7, \$	Wise et al. (1998)
ABH07678	Sabinene (~98)	Sp-SabS1	<i>Salvia pomifera</i>	1-6, 2-6, H	Kampranis et al. (2007)
AAM89254	(+)-3-Carene (73)	-	<i>Salvia stenophylla</i>	1-6, 5-7	Hoelscher et al. (2003)
ACF24767	α -Terpineol (~44)	SamonoTPS1	<i>Santalum album</i>	1-6, \$	Jones et al. (2008)
AAG01140	(+)-(4R)-Limonene (~75)	dLMS	<i>Schizonepeta tenuifolia</i>	1-6	Maruyama et al. (2001a)
AAS79351	(-)- α -Terpineol (50.1)	VvTPS1891	<i>Vitis vinifera</i>	1-6, \$	Martin and Bohlmann (2004)
AAS79352	(-)- α -Terpineol (50.1)	VvTPS4568	<i>Vitis vinifera</i>	1-6, \$	Martin and Bohlmann (2004)
AAL59230	(-)- α -Terpineol (~40)	STC1-B73	<i>Zea mays</i>	1-6, \$	Lin et al. (2008)
ABR09292	(-)- α -Terpineol (~60)	TPS26-B73	<i>Zea mays</i>	1-6, \$	Lin et al. (2008)

The symbols indicate the following steps in the reaction mechanism: \$, carbocation capture by water; §, phosphorylation of carbocation; H, hydride shift; #, FaNES1, FaNES2, and AmNES/LIS2 also produce the sesquiterpene nerolidol; nd, the enzymes were not expressed and characterized; °, the enzyme was not expressed, but the purified protein was characterized by Pichersky et al. (1995).

^a The percentage refers to the approximate amount of major product in the total blend of terpenes produced by the enzyme.

^b The designation refers to the name in the original publication. These names have occasionally been changed in later publications.

^c The numbers refer to the carbon atoms involved in ring formation using the nomenclature of the geranyl skeleton (Fig. 1).

mechanism of these enzymes. Later sections are devoted to the terpene synthase proteins and their role in mediating terpene skeleton formation.

To keep this review to a manageable size, we limit our coverage to monoterpene and sesquiterpene synthases. Triterpene synthases and the formation of the triterpene carbon skeleton have been very authoritatively reviewed in several recent articles (Xu et al., 2004; Phillips et al., 2006). Monoterpene and sesquiterpene biosynthesis in general were covered in comprehensive fashion in two outstanding chapters written in 1999 (Wise and Croteau, 1999; Cane, 1999) and terpene synthases in three reviews (Davis and Croteau, 2000; Tholl, 2006; Christianson, 2006).

2. Monoterpene and sesquiterpene synthases in plants

The wide scope of plant terpene synthase activities was first revealed by studies with crude plant extracts and purified preparation of native enzymes (reviewed in Wise and Croteau, 1999). More recently the isolation of terpene synthase genes and their heterologous expression has provided the best evidence for the size of this enzyme family and the breadth of its catalytic prowess. A comprehensive list of all plant monoterpene and sesquiterpene synthases cloned up to and including 2008 is given in Tables 1 and 2, ordered by the genus of the taxon from which the gene was isolated. Also given are the major products of the enzyme and notes on the reaction type. Until now research has focused on a few species chosen because of their economic importance or designation as model taxa. Obviously many more terpene synthases remain to be described. The size of this enzyme class is a principal reason for terpene diversity.

Terpene skeletal diversity arises not only from the number of terpene synthases, but also from the ability of these catalysts to form multiple products from a single substrate. In addition to their main product, nearly half of all characterized monoterpene and sesquiterpene synthases also form significant amounts of additional products (defined as at least 10% of the total) when the expressed protein is assayed *in vitro* (Tables 1 and 2). This property was first recognized during investigation of terpene synthases in plant extracts when it was realized that individual activities persisted in producing multiple products in the same consistent proportions during sequential purification steps (Gambliel and Croteau, 1984). As confirmation of these observations, Croteau and co-workers demonstrated that their preparations exhibited

isotopically sensitive branching when offered certain deuterium-labeled substrates (Croteau, 1987; Wagschal et al., 1991). A reduction in the rate of formation of one or more products due to deuterium substitution was correlated with an increase in the rate of formation of others, indicating that these enzyme products are derived from a common intermediate of a single enzyme (rather than different enzymes). The most definitive proof for the capacity of a single terpene synthase to produce multiple products comes from studies with terpene synthase genes. Expression in heterologous hosts has made it clear that a single terpene synthase protein has the ability to form several terpenes from a single substrate. For example, one of the first cloned monoterpene synthases, (+)-sabinene synthase from *Salvia officinalis*, produces 63% (+)-sabinene, but also 21% γ -terpinene, 7.0% terpinolene, 6.5% limonene and 2.5% myrcene in *in vitro* assays (Wise et al., 1998). These additional products or their immediate metabolites are also present in the monoterpene-rich essential oil of the plant.

3. Reaction mechanisms

3.1. Monoterpene synthases

Research over many years has established a common carbocationic reaction mechanism for all monoterpene synthases initiated by the divalent metal ion-dependent ionization of the substrate (Fig. 1A and B). The resulting cationic intermediate undergoes a series of cyclizations, hydride shifts or other rearrangements until the reaction is terminated by proton loss or the addition of a nucleophile. This mechanism was elucidated largely by Croteau and co-workers by studies with substrate analogs, inhibitors, intermediates and analogs and native enzymes (Croteau, 1987; Wise and Croteau, 1999). For example, the participation of carbocationic intermediates was established by the inhibitory effect of fluorinated (Croteau, 1986) and sulfonium (Croteau et al., 1986) substrate analogs coupled with previous investigations of model chemical reactions.

Of the characterized monoterpene synthases in Table 1, approximately one-third convert the substrate geranyl diphosphate (GPP) to acyclic products (Fig. 1B). The mechanism of these reactions proceeds by ionization to the extended geranyl cation followed by proton loss to form (*E*)- β -ocimene (Dudareva et al., 2003; Chen et al., 2003; Fäldt et al., 2003a; Shimada et al., 2005a; Arimura et al., 2004) and myrcene (Dudareva et al., 2003; Chen et al.,

Table 2
Sesquiterpene synthase genes isolated to date.

Gene bank accession no.	Main product(s) ^a (%)	Designation ^b	Species	Cyclization type ^c	References
AAC24192	(E)- α -Bisabolene (~100)	Ag1	<i>Abies grandis</i>	1–6	Bohlmann et al. (1998)
AAC05727	δ -Selinene (~25) ^o	Ag4	<i>Abies grandis</i>	1–10, 2–7, H, P	Steele et al. (1998)
AAC05728	γ -Humulene (~29)	Ag5	<i>Abies grandis</i>	1–11	Steele et al. (1998)
AAK83562	nd	(similar to Ag1)	<i>Abies grandis</i>		Trapp and Croteau (2001)
AAK83561	nd	(similar to Ag4)	<i>Abies grandis</i>		Trapp and Croteau (2001)
ABR24417	(3S)-(E)-Nerolidol (100)	AmNES/LIS-1	<i>Antirrhinum majus</i>	None, \$	Nagegowda et al. (2008)
AAO85539	(-)-(E)- β -Caryophyllene (~80)	At5g23960	<i>Arabidopsis thaliana</i>	1–11, 2–10	Chen et al. (2003)
AAX59990	(+)- α -Barbatene (~27)	At5g44630	<i>Arabidopsis thaliana</i>	1–6, 7–11, 2–11, *, H	Tholl et al. (2005) Wu et al. (2005)
NP_193064	(Z)- γ -Bisabolene (~90)	At4g13280	<i>Arabidopsis thaliana</i>	1–6	Ro et al. (2006)
NP_193066	(Z)- γ -Bisabolene (~90)	At4g13300	<i>Arabidopsis thaliana</i>	1–6	Ro et al. (2006)
CAB94691	Amorpha-4,11-diene	KCS12	<i>Artemisia annua</i>	1–10, 1–6, H	Chang et al. (2000)
AAF61439	Amorpha-4,11-diene (~89)	NAC	<i>Artemisia annua</i>	1–10, 1–6, H	Mercke et al. (2000)
AAF98444	Amorpha-4,11-diene (~89)	-	<i>Artemisia annua</i>	1–10, 1–6, H	Wallaart et al. (2001)
AAL79181	(E)- β -Caryophyllene	QHS1	<i>Artemisia annua</i>	1–11, 2–10	Cai et al. (2002)
CAC12732	nd	ASC34	<i>Artemisia annua</i>		van Geldre et al. (2000)
CAC12731	nd	ASC125	<i>Artemisia annua</i>		van Geldre et al. (2000)
AAF80333	8- <i>epi</i> -Cedrol (~94)	-	<i>Artemisia annua</i>	1–6, 6–10, 2–11, \$, H	Hua and Matsuda (1999)
CAC08805	8- <i>epi</i> -Cedrol (~94) [^]	-	<i>Artemisia annua</i>	1–6, 6–10, 2–11, \$, H	Mercke et al. (1999)
AAX39387	(E)- β -Farnesene (100)	β -FS	<i>Artemisia annua</i>	None	Picaud et al. (2005)
ABE03980	Germacrene A (100)	AaGAS	<i>Artemisia annua</i>	1–10	Berthea et al. (2006)
CAA06614	nd	PEAS1	<i>Capsicum annuum</i>		Zavala-Paramo et al. (2000)
AAC61260	5- <i>epi</i> -Aristolochene	PEAS	<i>Capsicum annuum</i>	1–10, 2–7, *, H, P	Back et al. (1998)
AAF21053	Inactive	CASC2	<i>Capsicum annuum</i>		Back et al. (2000)
AAM21658	(+)-Germacrene A (~100)	CiGASlo	<i>Cichorium intybus</i>	1–10	Bouwmeester et al. (2002)
AAM21659	(+)-Germacrene A (~100)	CiGASsh	<i>Cichorium intybus</i>	1–10	Bouwmeester et al. (2002)
AAK54279	(E)- β -Farnesene (~100)	CjFS	<i>Citrus junos</i>	None	Maruyama et al. (2001a)
AAQ04608	Valencene (~100)	CsTPS1	<i>Citrus sinensis</i>	1–10, 2–7, *, H, P	Sharon-Asa et al. (2003)
ABX83200	δ -Cadinene (~70)	CmTPSNY	<i>Cucumis melo</i>	1–10, 1–6, H	Portnoy et al. (2008)
ABX83201	α -Farnesene (100)	CmTPSDul	<i>Cucumis melo</i>	None	Portnoy et al. (2008)
AAU05951	(E,E)- α -Farnesene (~100)	Cs α FS	<i>Cucumis sativus</i>	None	Mercke et al. (2004)
AAU05952	(E)- β -Caryophyllene (~100)	Cs β CS	<i>Cucumis sativus</i>	1–11, 2–10	Mercke et al. (2004)
AAC31570	nd	-	<i>Elaeis oleifera</i>		Shah and Cha (2000)
AAA93064	(+)- δ -Cadinene (~100)	CAD1-C1	<i>Gossypium arboreum</i>	1–10, 1–6, H	Chen et al. (1995)
CAA76223	nd	CAD1-C2	<i>Gossypium arboreum</i>		Meng et al. (1999)
AAD51718	nd	CAD1-C3	<i>Gossypium arboreum</i>		Tan et al. (2000)
AAA93065	(+)- δ -Cadinene (~100)	CAD1-C14	<i>Gossypium arboreum</i>	1–10, 1–6, H	Chen et al. (1995)
CAA65289	(+)- δ -Cadinene (~100)	CAD1-A	<i>Gossypium arboreum</i>	1–10, 1–6, H	Chen et al. (1996)
AAC12784	(+)- δ -Cadinene	CDN1-C	<i>Gossypium hirsutum</i>	1–10, 1–6, H	Davis et al. (1996)
AAF74977	(+)- δ -Cadinene	CDN1-C4	<i>Gossypium hirsutum</i>	1–10, 1–6, H	Townsend et al. (2005)
AAA86337	Vetispiradiene (>93)	VS1	<i>Hyoscyamus muticus</i>	1–10, 2–7, **, P	Back and Chappell (1995)
AAL92481	Germacrene A	IdGAS	<i>Ilex dentata</i>	1–10	Kim et al. (2005)
AAM11626	Germacrene A	LTC1	<i>Lactuca sativa</i>	1–10	Bennett et al. (2002)
AAM11627	Germacrene A	LTC2	<i>Lactuca sativa</i>	1–10	Bennett et al. (2002)
ABB73046	(E)- α -Bergamotene (~74)	LaBERS	<i>Lavandula angustifolia</i>	1–6, 2–7	Landmann et al. (2007)
AAG41889	δ -Elemene (~50) #	SSTLE1	<i>Lycopersicon esculentum</i>	1–10, H	van der Hoeven et al. (2000)
AAG41890	δ -Elemene (~75) #	SSTLE2	<i>Lycopersicon esculentum</i>	1–10, H	van der Hoeven et al. (2000)
AAC39432	Germacrene C (64)	-	<i>Lycopersicon esculentum</i>	1–10, H	Colby et al. (1998)
AAG41891	Germacrene B (~100)	SSTLH1	<i>Lycopersicon hirsutum</i>	1–10	van der Hoeven et al. (2000)
AAG41892	Germacrene D (~70)	SSTLH2	<i>Lycopersicon hirsutum</i>	1–10, H	van der Hoeven et al. (2000)
ACC66281	β -Cubebene (~24)	Mg25	<i>Magnolia grandiflora</i>	1–10, 1–6, 6–2, H	Lee and Chappell (2008)
AAO22848	Inactive (E,E)- α -Farnesene (~98)	Mg11 AFS1	<i>Magnolia grandiflora</i> <i>Malus x domestica</i>	None	Lee and Chappell (2008) Pechous and Whitaker (2004)
AAV36464	(E)- β -Caryophyllene (~92)	MtTPS1	<i>Medicago truncatula</i>	1–11, 2–10	Arimura et al. (2008a)
AAV36466	(3S)-(E)-Nerolidol (100)	MtTPS3	<i>Medicago truncatula</i>	None, \$	Arimura et al. (2008a)
ABB01625	(-)-Cubebol (30)	MtTPS5	<i>Medicago truncatula</i>	1–10, 1–6, 2–6, H, \$	Arimura et al. (2008a)
AAB95209	(E)- β -Farnesene (~85)	TSPA11	<i>Mentha x piperita</i>	None	Crock et al. (1997)
CAH10288	(Z)-Muurola-3,5-diene (45)	MxpSS1	<i>Mentha x piperita</i>	1–10, 1–6, H	Prosser et al. (2006)
CAH10289	Inactive	MxpSS2	<i>Mentha x piperita</i>		Prosser et al. (2006)
AAA19216	5- <i>epi</i> -Aristolochene (~79)	EAS3/EAS4	<i>Nicotiana tabacum</i>	1–10, 2–7, *, H, P	Facchini and Chappell (1992) Back et al. (1994) O'Maille et al. (2006)
AAP79448	5- <i>epi</i> -Aristolochene (100)	g110	<i>Nicotiana tabacum</i>	1–10, 2–7, *, H, P	Wu et al. (2005)
AAP05760	5- <i>epi</i> -Aristolochene (~100)	NaEAS12	<i>Nicotiana attenuata</i>	1–10, 2–7, *, H, P	Bohlmann et al. (2002)
AAP05761	5- <i>epi</i> -Aristolochene (~100)	NaEAS34	<i>Nicotiana attenuata</i>	1–10, 2–7, *, H, P	Bohlmann et al. (2002)
AAP05762	5- <i>epi</i> -Aristolochene (~100)	NaEAS37	<i>Nicotiana attenuata</i>	1–10, 2–7, *, H, P	Bohlmann et al. (2002)
AAV63787	γ -Cadinene (~30)	CDS	<i>Ocimum basilicum</i>	1–6, 1–10, H	Iijima et al. (2004a)
AAV63785	β -Selinene (~30)	SES	<i>Ocimum basilicum</i>	1–10, 2–7, P	Iijima et al. (2004a)
AAV63788	α -Zingiberene (~40)	ZIS	<i>Ocimum basilicum</i>	1–6, H	Iijima et al. (2004a)
AAV63786	Germacrene D (100)	GDS	<i>Ocimum basilicum</i>	1–10, H	Iijima et al. (2004a)
EU596452	Zingiberene (~25)	Os08g07100	<i>Oryza sativa</i>	1–6, H	Yuan et al. (2008)
EU596454	(E)- β -Caryophyllene (~47)	Os08g04500	<i>Oryza sativa</i>	1–11, 2–10	Yuan et al. (2008)
ABJ16553	(E)- β -Caryophyllene (~46)	OsTPS3	<i>Oryza sativa</i>	1–11, 2–10	Cheng et al. (2007)
ABJ16554	(E,E)-Farnesol (~84)	OsTPS13	<i>Oryza sativa</i>	None, \$	Cheng et al. (2007)

Table 2 (continued)

Gene bank accession no.	Main product(s) ^a (%)	Designation ^b	Species	Cyclization type ^c	References
AAS47695	Longifolene (61)	PaTPS-Lon	<i>Picea abies</i>	1–11, 1–6, 3–7, **, H	Martin et al. (2004)
AAS47697	(<i>E,E</i>)- α -Farnesene (100)	PaTPS-Far	<i>Picea abies</i>	None	Martin et al. (2004)
AAS47689	(<i>E</i>)- α -Bisabolene (100)	PaTPS-Bis	<i>Picea abies</i>	1–6	Martin et al. (2004)
ABA86249	nd	PsTPS-Sell	<i>Picea sitchensis</i>		Byun-McKay et al. (2006)
AAO61226	(<i>E,E</i>)- α -Farnesene (~100)	Pt5	<i>Pinus taeda</i>	None	Phillips et al. (2003)
AAS86319	γ -Curcumene (~90)	PatTpsA	<i>Pogostemon cablin</i>	1–6, H	Deguerry et al. (2006)
AAS86320	(–)-Germacrene D (100)	PatTpsBF2	<i>Pogostemon cablin</i>	1–10, H	Deguerry et al. (2006)
AAS86321	(+)-Germacrene A (~90)	PatTpsCF2	<i>Pogostemon cablin</i>	1–10	Deguerry et al. (2006)
AAS86322	(–)-Germacrene D (~60)	PatTpsB15	<i>Pogostemon cablin</i>	1–10, H	Deguerry et al. (2006)
AAS86323	(–)-Patchoulol (~50)	PatTPS177	<i>Pogostemon cablin</i>	1–10, 7–11, 2–6, **, \$, H	Deguerry et al. (2006)
AAR99061	(–)-Germacrene D (~79)	PtdTPS1	<i>Populus trichocarpa x deltoides</i>	1–10, H	Arimura et al. (2004)
AAX07266	(<i>E</i>)- γ -Bisabolene (100)	PmeTPS3	<i>Pseudotsuga menziesii</i>	1,6	Huber et al. (2005)
AAX07265	(<i>E</i>)- β -Farnesene (100)	PmeTPS4	<i>Pseudotsuga menziesii</i>	None	Huber et al. (2005)
–	Germacrene D (~100)	FC0592	<i>Rosa hybrida</i>	1–10, H	Guterman et al. (2002)
ACF24768	Germacrene D-4-ol (~39)	SasesquiTPS1	<i>Santalum album</i>	1–10, H, \$	Jones et al. (2008)
BAA82109	nd	PVS3	<i>Solanum tuberosum</i>		Yoshioka et al. (1999)
BAA82108	nd	PVS2	<i>Solanum tuberosum</i>		Yoshioka et al. (1999)
BAA82029	nd	PVS1	<i>Solanum tuberosum</i>		Yoshioka et al. (1999)
AAR31144	(+)-Germacrene D (~90)	(+)GDS, Sc11	<i>Solidago canadensis</i>	1–10, H	Prosser et al. (2004)
AAR31145	(–)-Germacrene D (~90)	(–)GDS, Sc19	<i>Solidago canadensis</i>	1–10, H	Prosser et al. (2004)
CAC36896	(+)-Germacrene A (~98)	Sc1	<i>Solidago canadensis</i>	1–10	Prosser et al. (2002)
AAS66358	(+)-Valencene (50)	VvVal	<i>Vitis vinifera</i>	1–10, 2–7, *	Lucker et al. (2004)
AAS66357	(–)-Germacrene D (92)	VvGerD	<i>Vitis vinifera</i>	1–10, H	Lucker et al. (2004)
AAO18435	(<i>E,E</i>)-Farnesol (~45)	TPS1-B73	<i>Zea mays</i>	None; \$	Schnee et al. (2002)
AAS88571	(<i>S</i>)- β -Bisabolene (~29)	TPS4-B73	<i>Zea mays</i>	1–6, 2–6	Köllner et al. (2004)
AAS88572	Inactive	TPS5-B73	<i>Zea mays</i>	None	Köllner et al. (2004)
AAS88574	Sesquithujene (~28)	TPS5-Del1	<i>Zea mays</i>	1–6, 2–6, H	Köllner et al. (2004)
AAS88575	Inactive	TPS5-Del2	<i>Zea mays</i>		Köllner et al. (2004)
AAX99146	(<i>E</i>)- β -Farnesene (~50)	TPS10-B73	<i>Zea mays</i>	None	Schnee et al. (2006)
AAS88576	(–)- β -Macrocarpene (~95)	TPS6-B73	<i>Zea mays</i>	1–6, 11–14, P	Köllner et al. (2008a)
AAT70085	(–)- β -Macrocarpene (~95)	TPS6-Ban	<i>Zea mays</i>	1–6, 11–14, P	Köllner et al. (2008a)
ACF58240	(–)- β -Macrocarpene (~95)	TPS11-B73	<i>Zea mays</i>	1–6, 11–14, P	Köllner et al. (2008a)
ABY79206	(<i>E</i>)- β -Caryophyllene (~90)	TPS23-Del1	<i>Zea mays</i>	1–11, 2–10	Köllner et al. (2008b)
ABY79209	(<i>E</i>)- β -Caryophyllene (~90)	TPS23-dip	<i>Zea diploperennis</i>	1–11, 2–10	Köllner et al. (2008b)
ABY79210	(<i>E</i>)- β -Caryophyllene (~90)	TPS23-hue	<i>Zea m. huehuetenangensis</i>	1–11, 2–10	Köllner et al. (2008b)
ABY79211	(<i>E</i>)- β -Caryophyllene (~90)	TPS23-lux	<i>Zea luxurians</i>	1–11, 2–10	Köllner et al. (2008b)
ABY79212	(<i>E</i>)- β -Caryophyllene (~90)	TPS23-mex	<i>Zea mays mexicana</i>	1–11, 2–10	Köllner et al. (2008b)
ABY79213	(<i>E</i>)- β -Caryophyllene (~90)	TPS23-par	<i>Zea mays parviglumis</i>	1–11, 2–10	Köllner et al. (2008b)
ABY79214	(<i>E</i>)- β -Caryophyllene (~90)	TPS23-per	<i>Zea perennis</i>	1–11, 2–10	Köllner et al. (2008b)
AAX40665	(+)-Germacrene D (~50)	–	<i>Zingiber officinale</i>	1–10, H	Picaud et al. (2006)
BAG12020	α -Humulene (~95)	ZSS1	<i>Zingiber zerumbet</i>	1–11	Yu et al. (2008a)
BAG12021	β -Eudesmol (63)	ZSS2	<i>Zingiber zerumbet</i>	1–10, 2–7, H	Yu et al. (2008b)

The symbols indicate the following steps in the reaction mechanism: *, methyl shift; **, ring contraction; \$, carbocation capture by water; H, hydride shift; P, protonation of a reaction intermediate. #, δ -elemene could have been formed from germacrene C in the GC injector at the 250 °C temperature used; °, the purified enzyme produced germacrene B (33%) as main product (Little and Croteau, 2002); nd, the enzymes were not expressed and characterized; ^, identical to AAF80333.

^a The approximate percentage refers to the amount of the major product in the total blend of the terpenes produced by the enzyme.

^b The designation refers to the name in the original publication. These names have occasionally been changed in later publications.

^c The numbers refer to the carbon atoms involved in ring formation using nomenclature of the farnesyl skeleton (Fig. 2).

2003; Fischbach et al., 2001; Martin et al., 2004; Hosoi et al., 2004; Iijima et al., 2004a; Bohlmann et al., 1997, 2000) or addition of water to form geraniol (Yang et al., 2005; Iijima et al., 2004b) or linalool (Chen et al., 2003; Martin et al., 2004; Iijima et al., 2004a; Jia et al., 1999; Dudareva et al., 1996; Aharoni et al., 2004; Crowell et al., 2002) (Fig. 1B). It is also conceivable that linalool, myrcene and (*E*)- β -ocimene are derived from the linalyl cation that is the result of a previous isomerization (Fig. 1B). The formation of cyclic products is impeded by the (*E*)-geometry of the 2,3-double bond of the geranyl cation. However, preliminary conversion of the geranyl cation to the tertiary linalyl cation facilitates cyclization to a six-membered ring. Studies with LPP as an alternative substrate in combination with GPP indicated the formation of a linalyl diphosphate (LPP) (Fig. 1A) as an intermediate in terpene synthase catalysis (Croteau et al., 1980). These observations were supported by investigations with tritium-labeled GPP (Croteau and Felton, 1981) and non-cyclizable GPP analogs (Wheeler and Croteau, 1986, 1987). The elucidation of the three-dimensional structure of *Mentha* limonene synthase cocrystallized with the substrate analog 2-fluorogeranyl diphosphate and 2-fluorolinalyl diphosphate provided further evidence for the

intermediacy of LPP in the isomerization reaction (Hyatt et al., 2007).

From the cisoid, anti-endo conformer of the linalyl cation, electrophilic attack of C1 on the C6–C7 double bond gives the cyclic α -terpinyl cation, a critical branchpoint intermediate in the formation of all cyclic monoterpenes. From the α -terpinyl cation, terpene synthase-catalyzed proton loss leads to limonene (Rajonarivony et al., 1992; Martin et al., 2004; Bohlmann et al., 1997, 1999; Maruyama et al., 2001b, 2002; Lucker et al., 2002; Shimada et al., 2004, 2005b; Colby et al., 1993; Yuba et al., 1996; Byun-McKay et al., 2006), or terpinolene (Iijima et al., 2004a; Bohlmann et al., 1999; Huber et al., 2005) while water capture gives α -terpineol (Croteau et al., 1994; Martin and Bohlmann, 2004). Terpene synthase-mediated 1,2- or 1,3-hydride shifts of the α -terpinyl cation followed by proton losses yield the isomeric terpinene and phellandrene products (LaFever and Croteau, 1993; Shimada et al., 2004; Bohlmann et al., 1999). Formation of 1,8-cineole is also thought to proceed from the α -terpinyl cation via an α -terpineol intermediate which undergoes internal additional cyclization of the alcoholic oxygen (Croteau et al., 1994; Wise et al., 1998; Chen et al., 2004).

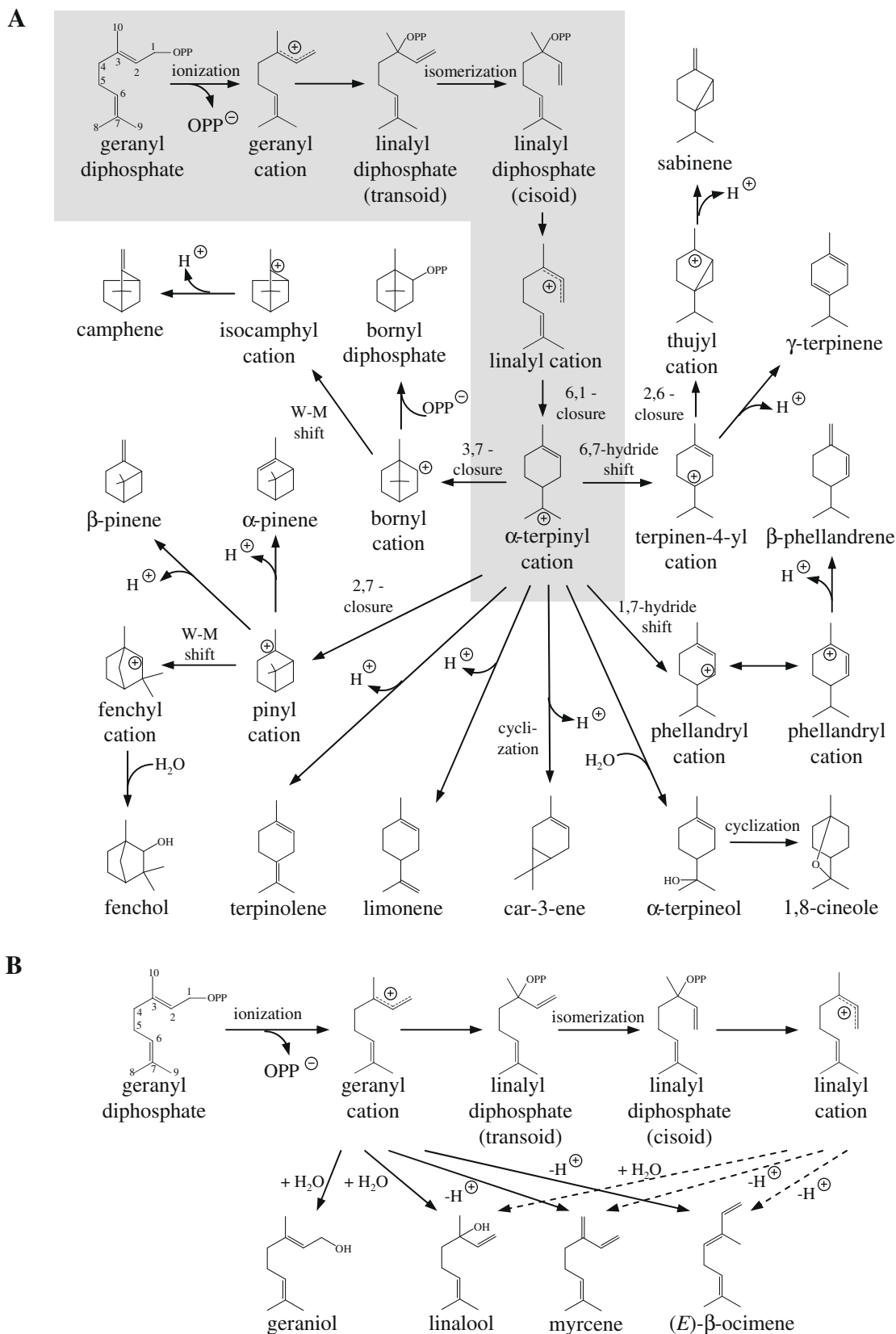


Fig. 1. The reaction mechanisms of all monoterpene synthases start with the ionization of the geranyl diphosphate substrate. The resulting carbocation can undergo a range of cyclizations, hydride shifts and rearrangements before reaction is terminated by deprotonation or water capture. The mechanisms of cyclic (A) and acyclic (B) monoterpene synthases are depicted separately. The formation of cyclic monoterpenes requires the preliminary isomerization of the geranyl cation to a linalyl intermediate capable of cyclization. The production of the initial cyclic species, the α -terpinyl cation, opens the door to secondary cyclizations. The route to the central α -terpinyl intermediate is highlighted in grey and the reaction pathways to the major products of all cloned monoterpene synthases are shown. The formation of the acyclic monoterpenes linalool, myrcene and (*E*)- β -ocimene might proceed either via the geranyl cation or via the linalyl cation. The numbering of carbon atoms of intermediates and products refers to that for GPP.

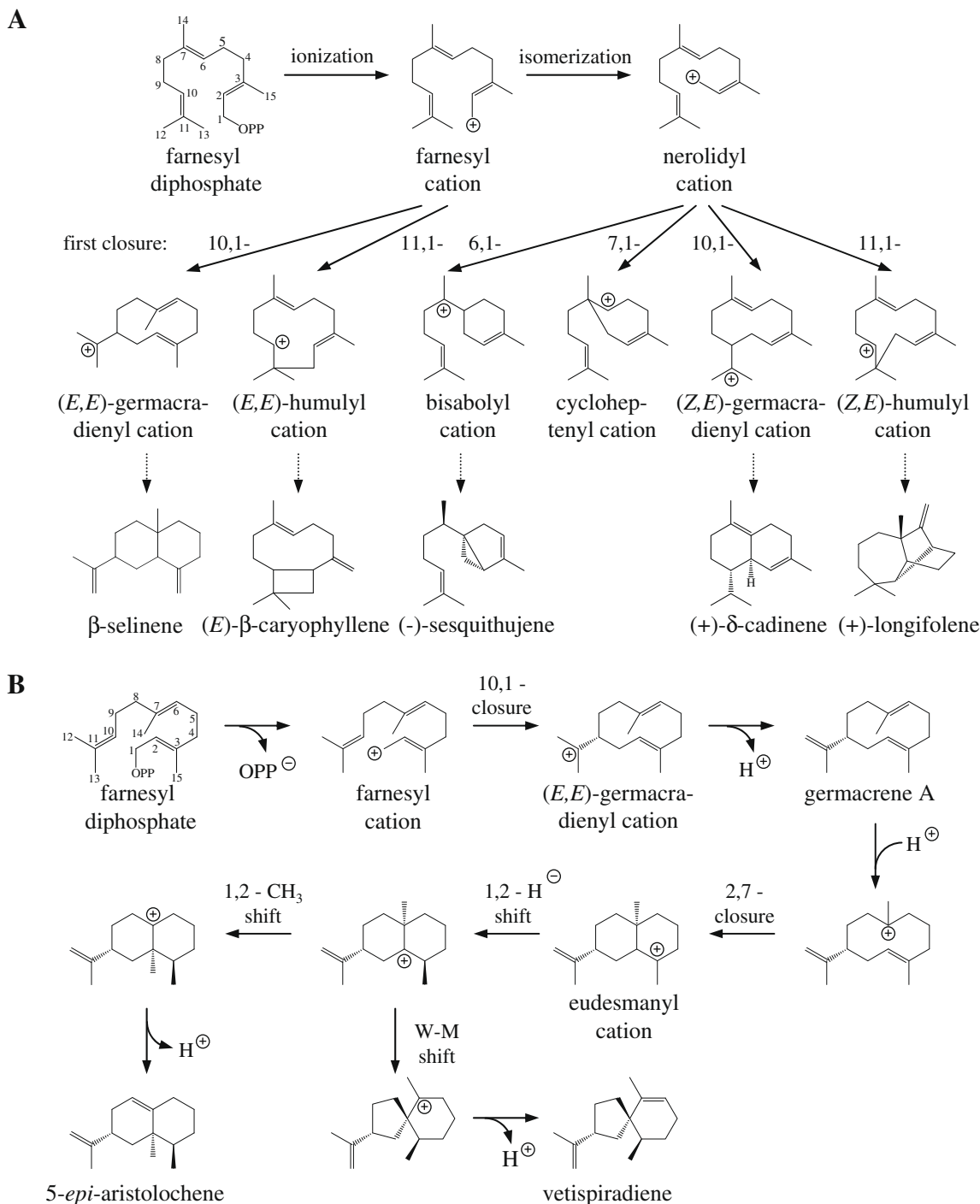


Fig. 2. The reaction mechanism of all sesquiterpene synthases start with the ionization of FPP. The resulting carbocation undergoes a range of cyclizations, some of which are preceded by isomerization to a nerolidyl intermediate (A). Steps following the initial cyclization, which include secondary cyclization, deprotonation to a neutral intermediate, hydride shifts and Wagner–Meerwein rearrangements, are not shown except for the 5-*epi*-aristolochene synthase of *Nicotiana tabacum*. The reaction mechanism of this enzyme proceeds via a neutral intermediate, germacrene A, that is re-protonated to form the bicyclic reaction products (B). The numbering of carbon atoms of intermediates and products refers to that for FPP.

The α -terpinyl cation can also undergo additional cyclizations resulting from electrophilic attack of the carbocationic center on one of the carbon atoms of the remaining double bond. A Markovnikov addition (2,7-cyclization) generates the pinyll cation, which can undergo proton loss to yield α -pinene (Croteau et al., 1989; Aharoni et al., 2004; McKay et al., 2003) or β -pinene (Croteau et al., 1989; Martin et al., 2004; Bohlmann et al., 1997; Lucker et al., 2002; Shimada et al., 2004; Lu et al., 2002), the main prod-

ucts of many terpene synthases especially in conifers. Anti-Markovnikov addition (3,7-cyclization) generates the bornyl cation (Croteau et al., 1990). The major monoterpenes with a bornyl carbon skeleton, borneol and camphor, are formed via a bornyl diphosphate intermediate (Croteau and Karp, 1977). In this process, a terpene synthase catalyzes bornyl diphosphate formation from the bornyl cation via internal return of the diphosphate moiety from the original geranyl diphosphate substrate (Whittington

et al., 2002a). The product is then hydrolyzed and oxidized to borneol and camphor, respectively (Croteau and Karp, 1979; Dehal and Croteau, 1987). The formation of two other bicyclic monoterpene skeletons are thought to involve terpene synthase-mediated Wagner–Meerwein rearrangements forming the fenchyl skeleton (precursor of fenchol) from the pinyl cation (Croteau et al., 1989; Iijima et al., 2004a) and the isocamphyl skeleton (precursor of camphene) from the bornyl cation (Croteau et al., 1990; Bohlmann et al., 1999). Another cyclization from the α -terpinyl cation is 5,7-closure to form a cyclopropyl ring leading to 3-carene (Savage and Croteau, 1993; Hoelscher et al., 2003). Terpene synthases form bicyclic monoterpenes with a cyclopropyl ring via another route in which a 1,2-hydride shift of the α -terpinyl cation is followed by 2,6-closure to give the sabinyl cation, precursor of sabinene and related monoterpenes (Hallahan and Croteau, 1989; Wise et al., 1998).

The highly reticulate general reaction mechanism of monoterpene synthases suggests a rationale for the ability of these enzymes to make multiple products. Given that an individual intermediate may have multiple fates, the enzyme may simply exploit this property. For example, a terpene synthase could allow one carbocation to be converted to a mixture of others by facilitating various cyclizations, hydride shifts or rearrangements (Lucker et al., 2002; Shimada et al., 2004; Huber et al., 2005; Martin and Bohlmann, 2004). Or it could mediate different types of termination reactions (deprotonation, water capture) on a single cation (McKay et al., 2003; Peters and Croteau, 2003). In some cases, the minor products of the enzyme have fewer cyclizations than the main product, suggesting that the minor products are formed from reaction intermediates subjected to premature termination (Iijima et al., 2004a). Whatever the explanation for multiple product formation, the occurrence of a broad spectrum of products has often proved valuable in tracing the reaction mechanism.

3.2. Sesquiterpene synthases

The formation of sesquiterpenes from farnesyl diphosphate catalyzed by sesquiterpene synthases employs similar carbocationic-based reaction mechanisms as those of monoterpene synthases. However, the larger carbon skeleton of farnesyl diphosphate (FPP) and the presence of three, instead of two, double bonds greatly increases structural diversity of the products. Thus we have space only to describe the major steps of these mechanisms. As for monoterpene synthases, the basic principles of sesquiterpene synthase mechanisms were derived from studies with native enzymes, although microbial enzymes played a much larger role here than they did for monoterpene synthases. Since this review is dedicated to plant terpene synthases, only few of the thorough studies on the reaction mechanisms of fungal and bacterial terpene synthases can be mentioned here.

The initial cyclization reactions can be divided into two types (Fig. 2A and B). One type involves cyclization of the initially-formed farnesyl cation to yield 10-membered ((*E,E*)-germacradienyl cation) or 11-membered ((*E*)-humulyl cation) rings. Given the large size of these rings, the (*E*)-geometry of the C2–C3 double bond is no barrier to cyclization. The second type of cyclization proceeds after preliminary isomerization of the C2–C3 double bond to the tertiary nerolidyl cation, in direct analogy with the isomerization of GPP to the linalyl cation in the mechanism of the monoterpene synthases. Evidence for the intermediacy of the nerolidyl intermediate in the sesquiterpene synthase mechanism comes from detailed studies with multiply-labeled FPP and nerolidyl diphosphate substrates (Cane and Iyengar, 1979; Cane et al., 1981; Cane and Yang, 1994; Cane and Tandon, 1995; Alchanati et al., 1998). The cisoid conformer of the nerolidyl cation can undergo cyclization to either the central or distal double bond form-

ing 1,6-(bisabolyl cation), 1,7-(cycloheptanyl cation), 1,10-((*Z,E*)-germacradienyl cation) or 1,11-((*Z*)-humulyl cation) products.

Subsequent cyclizations can involve carbocationic attack on either of the two remaining double bonds giving a wide range of different carbon skeletons. With so many possibilities, it is often difficult to determine the sequence of ring closures. For example, δ -cadinene which has a naphthalene-type ring skeleton could arise from initial formation of a 10-membered ring followed by partitioning into two six-membered rings, or from sequential formation of six-membered rings. Characterization of a cotton δ -cadinene synthase suggests that 1,10-cyclization is the first step followed by 1,6-closure to give ring partitioning (Chen et al., 1995; Alchanati et al., 1998; Benedict et al., 2001). However, the sequence of cyclizations may differ for sesquiterpene synthases forming other naphthalene-type skeletons. Another example of ring closure sequence is that catalyzed by 2-*epi*-cedrol synthases from *Artemisia annua* in which initial 1,6-ring formation is followed by 6,10- and then 2,11-closure (Hua and Matsuda, 1999; Mercke et al., 1999). In addition to cyclization, sesquiterpene synthase mechanisms include a range of different reaction types similar to that found in monoterpene synthase mechanisms, including 1,2-, 1,3- and 1,4-hydride shifts. Skeletal rearrangements observed include ring contractions (Martin et al., 2004; Back and Chappell, 1995) and other Wagner–Meerwein rearrangements as well as 1,2-methyl shifts (Tholl et al., 2005).

Unique to the formation of certain sesquiterpenes is the intermediacy of neutral sesquiterpene species. For example, the mechanism of 5-*epi*-aristolochene synthase proceeds via an initial 1,10-cyclization forming the (*E,E*)-germacradienyl cation to germacrene A. This neutral olefin is then protonated on the C6–C7 double bond in Markovnikov orientation to reform a cation, which then undergoes 2,7-cyclization to form a eudesmane cation (Rising et al., 2000). An analogous protonation of a neutral germacrene intermediate is part of the mechanistic sequence of vetispiradiene synthase (Back and Chappell, 1995), valencene synthase (Sharon-Asa et al., 2003) and β -selinene synthase (Iijima et al., 2004a). A different neutral intermediate, (*S*)- β -bisabolene, is involved in the biosynthesis of (*S*)- β -macrocarpene by the maize terpene synthases TPS6 and TPS11. In both enzymes, the proton required for the reprotonation of the (*S*)- β -bisabolene intermediate is abstracted from water (Köllner et al., 2008b).

4. Structure–function correlations in terpene synthases

Despite our detailed knowledge of monoterpene and sesquiterpene synthase mechanisms, comparatively little information is available about how various structural features of the proteins themselves mediate substrate binding and catalysis. Even though there is a substantial degree of amino acid sequence similarity among plant monoterpene and sesquiterpene synthases, similarity is based more on taxonomic affinities of the plant species from which the gene was isolated rather than the type of products formed (Bohlmann et al., 1998b). Nevertheless a number of common structural elements has been recognized.

4.1. General sequence and domains

In considering the entire protein, plant monoterpene synthases with 600–650 amino acids are longer than sesquiterpene synthases (550–580 amino acids) due to an N-terminal signal peptide that targets the initial translation product towards the plastids (Turner et al., 1999). These signal peptides characteristically contain a high frequency of serine and threonine residues and low amounts of acidic amino acids, but no common sequence elements have been identified (Bohlmann et al., 1997). Analysis of the

three-dimensional structures of four plant terpene synthases, a sesquiterpene synthase from *Nicotiana tabacum* (Starks et al., 1997) and three monoterpene synthases, from *Salvia officinalis* (Whittington et al., 2002b), *Mentha spicata* (Hyatt et al., 2007) and *Salvia fruticosa* (Kampranis et al., 2007), respectively, shows extensive similarities despite differences in reaction mechanism. These proteins all have a tertiary structure consisting entirely of α -helices and short connecting loops and turns and are organized into two structural domains, of which the C-terminal domain contains the active site. This domain possesses a structure similar to those of fungal and bacterial terpene synthases (Caruthers et al., 2000; Rynkiewicz et al., 2001; Shishova et al., 2007; Lesburg et al., 1997; Christianson, 2006) despite the lack of sequence similarity, suggesting the existence of a general 'terpene synthase fold' (Lesburg et al., 1997). The enzyme active site is a hydrophobic pocket of the C-terminal domain that is formed by six α -helices and closed off towards the outside by two loops which are located on the protein surface. Substrate binding in the active site of tobacco 5-*epi*-aristolochene synthase (TEAS), as determined by co-crystallization with the non-hydrolyzable FPP analogs, farnesyl hydroxyphosphonate and trifluorofarnesyl diphosphate, demonstrated that the hydrophobic moiety of FPP reaches into the active cavity while the diphosphate function interacts with magnesium ions at the entrance of the active site.

The N-terminal domain of plant terpene synthases has structural similarity with some glycosylhydrolases (Dudareva et al., 1996) but few functional elements have been identified. Evidence from mutational analyses indicates that this domain acts as a scaffold facilitating proper folding of the catalytically active C-terminal domain (Köllner et al., 2004). Experiments in which domains were exchanged between the sesquiterpene synthases TEAS and vetispiradiene synthase from *Hyoscyamus muticus* demonstrated that vetispiradiene synthase was fully functional with the N-terminal domain of TEAS, but the reverse combination resulted in a hybrid with strongly reduced activity (Back and Chappell, 1996). An unusual element of approximately 210 amino acid residues in the N-terminal domain appears in a sesquiterpene synthase, the (*E*)- α -bisabolene synthase from *Abies grandis* (Bohlmann et al., 1998a), a monoterpene synthase, the (*S*)-linalool synthase of *Clarkia breweri* (Dudareva et al., 1996), and in most isolated diterpene synthases (Bohlmann et al., 1998b). No function has yet been described for this element, although it may constitute an ancestral feature of plant terpene synthases that points to the common origin of this enzyme class (Bohlmann et al., 1998b).

The best known structural motif of the terpene synthase family is an aspartate-rich region, DDxxD, found in virtually all isolated plant terpene synthases as well as in isoprenyl diphosphate synthases and microbial terpene synthases. Site-directed mutagenesis as well as X-ray structural analysis revealed that this region is involved in binding divalent metal ions which in turn interact with the diphosphate moiety of the substrate (Starks et al., 1997; Lesburg et al., 1997; Tarshis et al., 1994, 1996; Cane et al., 1996a,b). The location of the DDxxD motif at the entrance of the catalytic site appears to be critical in positioning the substrate for catalysis. Mutations in this region frequently lead to decreased catalytic activity and the appearance of abnormal products which can be attributed to altered substrate binding (Cane et al., 1996a,b; Rynkiewicz et al., 2002; Seemann et al., 2002; Little and Croteau, 2002; Prosser et al., 2004). However, a naturally occurring variant of the DDxxD motif, a NDxxD sequence in the fully active (+) germacrene D synthase from goldenrod, has no impact on catalytic activity demonstrating that the highly conserved DDxxD motif is not as necessary for catalytic activity in farnesyl diphosphate cyclization as previously assumed (Prosser et al., 2004). X-ray structural analysis of terpene synthases revealed an additional metal cofactor binding motif located on the opposite site of the active site entry

(Christianson, 2006). This motif, designated NSE/DTE motif, has apparently evolved from a second aspartate-rich motif conserved in prenyl transferases to form a consensus sequence of (L,V)(V,L,A)-(N,D)D(L,I,V)x(S,T)xxxE (Cane and Kang, 2000; Christianson, 2006). Both the DDxxD motif and the NSE/DTE motif bind a trinuclear magnesium cluster involved in fixation of the pyrophosphate substrate. Whereas the DDxxD motif is highly conserved throughout almost all plant terpene synthases, the NSE/DTE motif appears to be less well conserved. Recently, mutational investigation of the NSE/DTE motif in abietadiene synthase from *Abies grandis* led to the speculation that a water molecule could substitute for the hydroxyl side chain of the central serine/threonine in terpene synthases when a glycine is located at this position (Zhou and Peters, 2009). In some sesquiterpene synthases, the NSE/DTE motif is replaced by a second DDxxD motif (Steele et al., 1998) which was also shown to be involved in catalysis (Little and Croteau, 2002).

Several terpene synthases from gymnosperms (Bohlmann et al., 1998b) as well as an apple α -farnesene synthase (Green et al., 2007) require potassium for enzyme activity. Recently, the H- α 1 loop, which is located in direct proximity to the NSE/DTE motif, was identified as the potassium binding region in these enzymes. It is assumed that potassium ions stabilize this loop region for optimal substrate binding (Green et al., 2009).

About 35 amino acids upstream of the DDxxD motif is a highly conserved RxR motif that was implicated in the complexation of the diphosphate function after ionization of the substrate preventing nucleophilic attack on any of the carbocationic intermediates (Starks et al., 1997).

A second arginine-rich motif found approximately 60 residues from the N-terminus of many monoterpene synthases is a tandem arginine (RR). Deletion studies on the limonene synthase of *Mentha spicata* demonstrated that all amino acids N-terminal to this point were dispensable for enzyme activity (Williams et al., 1998). However, deletion of the tandem arginine motif rendered the limonene synthase unable to accept geranyl diphosphate as a substrate. Since the enzyme was still able to convert linalyl diphosphate to limonene, this suggested that the RR motif might participate in the isomerization of GPP to a cyclizable intermediate, such as the linalyl cation (Williams et al., 1998). In keeping with this suggestion, the RR motif can be absent in monoterpene synthases producing only acyclic compounds, which do not require isomerization.

Additional amino acids have been implicated in the catalytic function of specific terpene synthases. For example, in TEAS the formation of 5-*epi*-aristolochene proceeds via the enzyme-bound, neutral intermediate, germacrene A (Cane, 1990). The protonation of this intermediate at C6 is catalyzed by a triad of amino acids consisting of Y520, D444 and D52565 as demonstrated by mutational analysis of the tyrosine residue (Rising et al., 2000). The N-terminus of the enzyme folds over the entrance of the active site after substrate binding and so might contribute to shielding the active site from the outer aqueous medium (Starks et al., 1997). However, N-terminal deletions of TEAS65 or the limonene synthase from *M. spicata* (Davis and Croteau, 2000) did not result in an increased proportion of hydroxylated products as might be expected after increased exposure of carbocationic intermediates to water.

4.2. Other structure–function correlations

A variety of structure–function correlations have been reported for other terpene synthases. In a monoterpene synthase, (–)- α -pinene synthase from *A. grandis*, the amino acids serine 485 and cysteine 480 were implicated as terminal proton acceptors in the final deprotonation of the pinyll cation to form α - and β -pinene, while cysteine 372 and phenylalanine 579 were found to influence the ratio of α - to β -pinene (Hyatt and Croteau, 2005). Domain

swapping experiments indicated that the product specificity of this α -pinene synthase and a related (–)-limonene synthase from *A. grandis* are determined by amino acids in the helices D–F which contribute to the active site cavity (Katoh et al., 2004). A similar region was found to be catalytically important in domain swapping experiments with monoterpene synthases from *Citrus limon* (El Tamer et al., 2003). Differences in termination chemistry between a 1,8-cineole synthase and sabinene synthase from *S. officinalis* were traced to a 73 amino acid region including helices C and D of the C-terminal domains (Peters and Croteau, 2003). Structural elucidation of a 1,8-cineole synthase from *S. fruticosa* identified five amino acid residues involved in the final step of the reaction, the addition of water to an α -terpinyl intermediate. Conversion of these five residues to those found in sabinene synthases of the closely related species *S. officinalis* and *Salvia pomifera* abolished the formation of 1,8-cineole in favor of sabinene (Kampranis et al., 2007).

Among sesquiterpene synthases, a mutational analysis of a germacrene A synthase from *Ixeris dentata* which has a reaction mechanism similar to the early steps of the TEAS mechanism revealed that a threonine in position 401 is important for intermediate stabilization and the formation of the eudesmane skeleton (Chang et al., 2005). Comparison of two closely related sesquiterpene synthases in maize, TPS4 and TPS5, identified a four amino acid motif that determines the stereospecificity of these multi-product enzymes (Köllner et al., 2004). The motif is located on helix G which divides the active site into two pockets with different catalytic functions (Köllner et al., 2006). In a δ -cadinene synthase from cotton, the alteration of two amino acids in the active site affected the cyclization and termination reactions resulting in the formation of a germacrene alcohol as main product (Yoshikuni et al., 2006a).

Sesquiterpene synthases have also been the subject of several large-scale structure–function studies. For example, saturating mutagenesis of four amino acid residues in the active center of γ -humulene synthase from *A. grandis* (Yoshikuni et al., 2006b) showed that the effect of mutations in these residues was additive. Seven enzymes were engineered with different combinations of mutations each of which produced a different terpene blend. Another systematic approach to identify the residues responsible for terpene synthase product specificity compared the closely related sesquiterpene synthases, TEAS from *N. tabacum* and vetispiradiene synthase from *H. muticus*. A mutational analysis of amino acid differences with different distances from the active center demonstrated that the nine amino acid residues most important in determining product specificity include residues lining the active site, and also those forming points of contact with the surrounding alpha helices. These latter residues seem more likely to function by altering the shape of the active site cavity rather than by providing functional groups for catalysis (Greenhagen et al., 2006). Study of a library of all possible combinations of these nine residues in TEAS revealed that no one single residue is more important than the others in controlling product distribution and that successive mutations usually had additive effects (O'Maille et al., 2008). Mutation of TEAS usually increased the number of minor products suggesting that extant terpene synthases have evolved from more promiscuous ancestors.

4.3. Correlation of active site residues and mechanistic features—a meta-analysis

Many of the mutational studies cited above demonstrate that the product specificity of terpene synthases is both dependent on the amino acid residues forming the active site cavity and the special constraints of the active site. Hence it is conceivable that the active site residues are conserved in enzymes with similar reaction mechanisms. The compilations of terpene synthases in Tables 1 and 2 of this review provide a simple means for an approximate

test of this hypothesis. First, we identified the residues forming the active sites of bornyl diphosphate synthase (for monoterpene synthases) and TEAS (for sesquiterpene synthases) using the structural information available for these enzymes (Back and Chappell, 1996; Greenhagen et al., 2006; O'Maille et al., 2008; Rising et al., 2000; Starks et al., 1997; Whittington et al., 2002a,b). Then, we carried out an alignment of all monoterpene synthases or sesquiterpene synthases with the program package DNASTAR utilizing a Clustal W algorithm (matrix: PAM250, gap penalty: 10, gap length: 0.2, delay divergent sequence: 20, DNA transition weight: 0.5) to identify the amino acids most likely to form the active sites in each enzyme based on its correspondence with the active site sequences of bornyl diphosphate synthase or TEAS. Finally, we created dendrograms comparing the deduced active site amino acid residues of each of the enzymes to search for correlations with specific properties of the reactions they catalyze. These graphs were made using the TREECON 1.3b software package (Van de Peer and De Wachter, 1994) employing a neighbor-joining algorithm with bootstrap values from 1000 trials (Figs. 3 and 4).

For monoterpene synthases, the resulting dendrogram of active site residues shows some correlation with reaction type. Based on similarities among the 47 residues identified as part of the active site, several enzyme clusters are evident whose members lack any cyclization mechanism, that is they make only acyclic products. For example, in the middle of the dendrogram there is a well-defined clade containing six enzymes from *Fragaria ananassa*, *Arabidopsis thaliana* and *Antirrhinum majus* that produce (*E*)- β -ocimene, myrcene and linalool. Immediately above it is a subclade of three (*E*)- β -ocimene synthases from *A. thaliana*, *Lotus japonicus* and *Citrus unshiu*, while just below is a subclade of three enzymes, two of which (from *Ocimum basilicum*) produce the acyclic products, geraniol and linalool. The existence of these clusters suggests that the production of acyclic monoterpenes may involve certain sets of conserved features in the active site. The remaining areas of the dendrogram contain principally monoterpene synthases producing cyclic products. Since all of these cyclases mediate 1,6-cyclizations to form the α -terpinyl cation, there appear to be some conserved features in the active site for this component of the mechanism.

Beyond these trends, little correlation can be noted between active site sequences of monoterpene synthases and cyclization type. And, there is no relationship between the incidence of multiple product formation and active site sequence. Instead, the major split in the dendrogram is between a clade of angiosperm monoterpene synthases (Fig. 3) and a clade of gymnosperm monoterpene synthases (below). Such sharp differences between the sequences of angiosperms and gymnosperm terpene synthases genes were previously noted (Bohlmann et al., 1998b), suggesting independent evolution of these two groups. The fact that there is a large overlap between angiosperm and gymnosperm monoterpene synthase products [e.g., myrcene, (–)-linalool, (–)-limonene, terpinolene, (–)- β -pinene, 3-carene] illustrates convergence of enzyme function using different active site constructions. Enzyme specificity may be determined to a large degree by the spatial proportions of the active center defined by the overall structure of the enzyme rather than the precise residues present (Greenhagen et al., 2006). At lower taxonomic levels, terpene synthases from the same genus often show a close similarity in active site sequence despite functional differences. However, the monoterpene synthases of *Citrus* sp. and *A. thaliana* appear in three distant subclades within the angiosperms. Clearly, different evolutionary lines of monoterpene synthases exist within a single species, a trend which may become much more evident once more terpene synthase sequences are available.

Analysis of the 45 residues forming the active site of sesquiterpene synthases also showed some correlations between enzyme

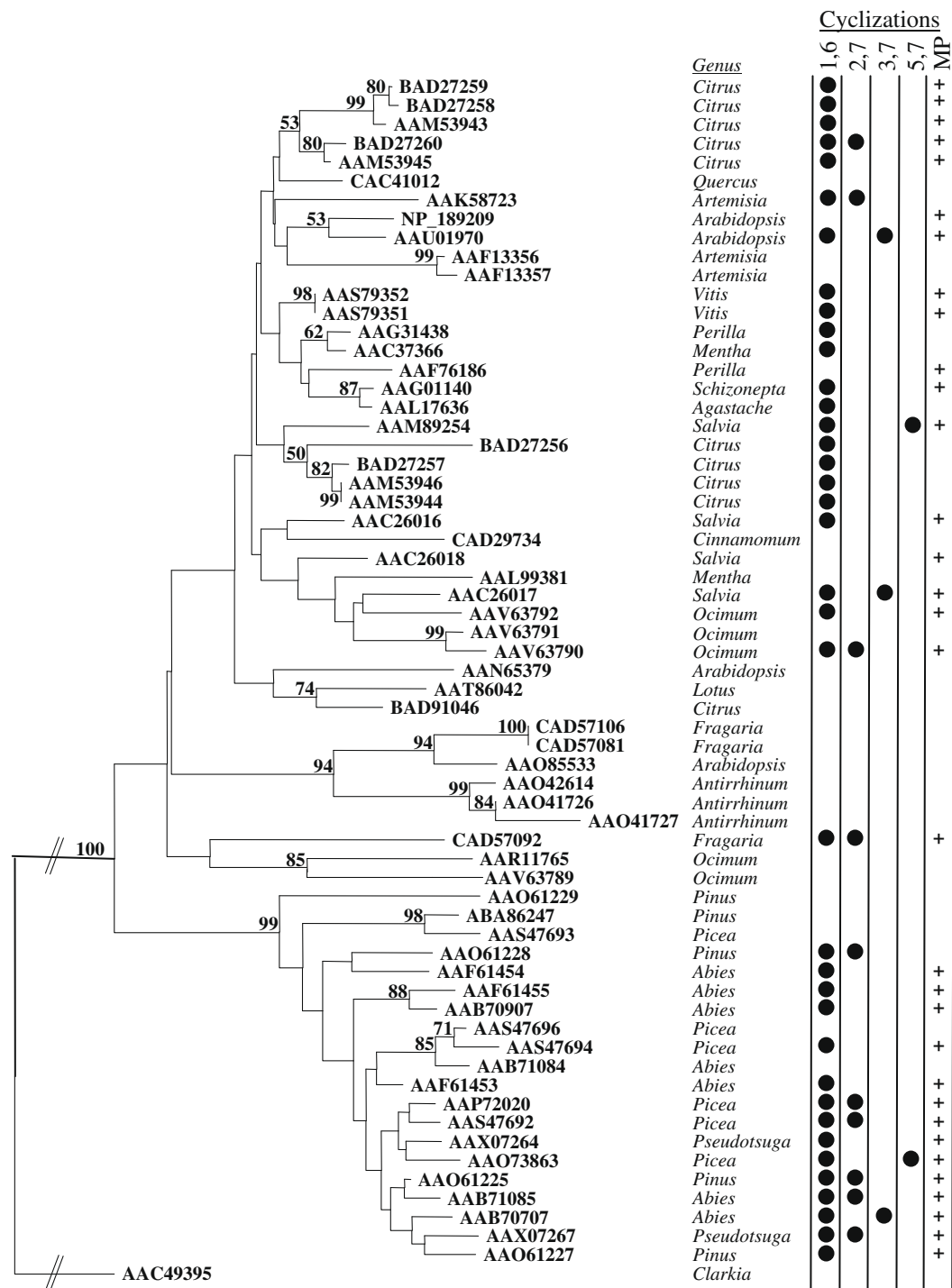


Fig. 3. Correlation of monoterpene synthase active site sequences and features of the reaction mechanism. The 47 amino acids that make up the active site of each isolated monoterpene synthase were identified by threading the sequence onto the structure of bornyl diphosphate synthase and then subjected to dendrogram analysis (Neighbor joining method with a bootstrap sample of 1000). Bootstrap values for all branches >50% are listed. Some mechanistic features of each enzyme are given in the middle columns, including the types of cyclizations present and the incidence of multiple product formation (MP).

structure and reaction mechanism. Nearly all of the top 28 sequences in the dendrogram (Fig. 4) employ a 1,10-cyclization mechanism. Included in this group are both direct 1,10-cyclizations of FPP forming the (*E,E*)-germacradienyl cation and 1,10-cyclizations following isomerization to the nerolidyl cation which form the (*Z,E*)-germacradienyl cation. The presence of both mechanism types in this sequence group indicates that similar protein

features may mediate these cyclizations, regardless of stereochemistry.

Acyclic sesquiterpene synthases are scattered over the dendrogram suggesting multiple evolutionary origins for this enzyme class. A clade of 13 sequences in the lower middle part of the dendrogram (Fig. 4) has a high proportion of enzymes making acyclic products, but also a subclade of enzymes from Asteraceae taxa

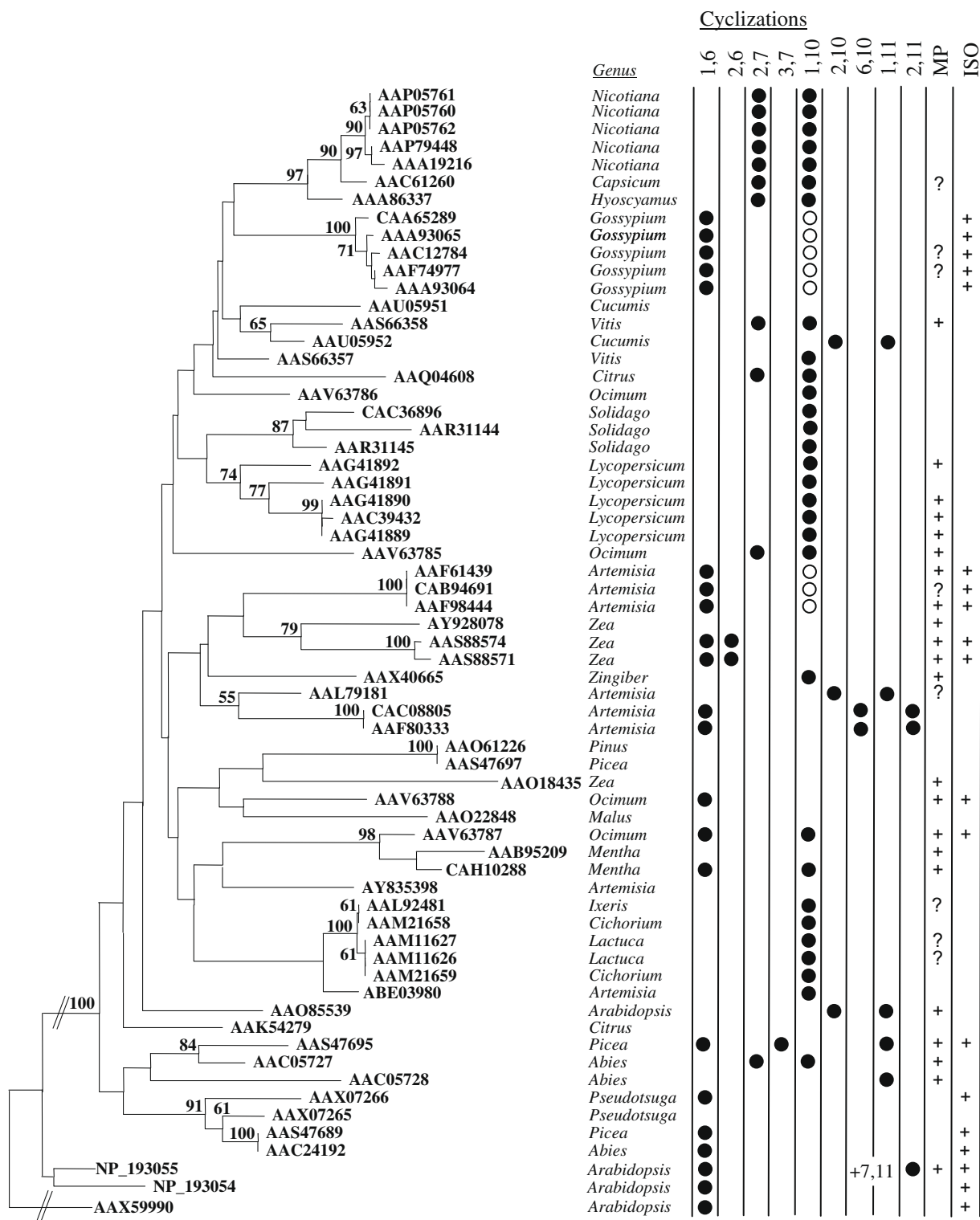


Fig. 4. Correlation of sesquiterpene synthase active site sequences and features of the reaction mechanism. The 45 amino acids that make up the active site were identified by threading the sequence onto the structure of 5-*epi*-aristolochene synthase of *Nicotiana tabacum* and then subjected to dendrogram analysis (Neighbor joining method with a bootstrap sample of 1000). Bootstrap values for all branches >50% are listed. Some mechanistic features of each enzyme are given in the middle column, including the types of cyclizations present, the incidence of multiple product formation (MP), and the catalysis of (*E,E*)-FPP to (*E,Z*)-FPP.

employing a 1,10-cyclization in their reaction mechanisms. Over the entire figure, there is no relationship between the sequence of the active site and the tendency to form multiple products, suggesting no universal feature of terpene synthases exists that distinguishes multiple- from single-product enzymes.

These conclusions about sesquiterpene synthase active site structure and mechanism are necessarily premature since the enzymes characterized to date represent only a small portion of those present in nature. Since most recent sesquiterpene synthase genes have been isolated by homology-based PCR approaches, according

to their similarity to known gene sequences, divergent types of genes might have been missed. In addition, the analysis might be refined by considering not just active site residues, but other residues adjacent to the catalytic center.

The dendrogram of sesquiterpene synthase active site sequences, unlike that for monoterpene synthases, does not give an absolute split between gymnosperm and angiosperm enzymes. In addition to the tight cluster of 7 gymnosperm sequences at the bottom of the dendrogram, two other gymnosperm sequences making acyclic products cluster with angiosperm enzymes. This may indicate that more than one type of sesquiterpene synthase existed before the evolutionary divide between gymnosperms and angiosperms. At lower taxonomic levels, there is a strong tendency for sequences from the same genus to cluster together. However, as with monoterpene synthases, sequences for certain genera are widely scattered in the dendrogram, highlighting the diversity of evolutionary lines within sesquiterpene synthases. As the terpene synthase families of genera besides *Arabidopsis* (Aubourg et al., 2002) become better known, the outline of sesquiterpene synthase evolution should become clearer, and the structural elements required for specific reactions should be better revealed.

4.4. Terpene synthase structure and multiple products

The ability of terpene synthases to convert a prenyl diphosphate substrate to diverse products during different reaction cycles is one of the most unique traits of this enzyme class. As described above, this property is found in nearly half of all characterized monoterpene and sesquiterpene synthases and may be attributed to the fact that the various reactive carbocationic intermediates can be stabilized in more than one way. However, since half of all monoterpene and sesquiterpene synthases channel their carbocationic-based catalysis into a single product, it is clear that some feature of the protein, rather than the mechanism itself, is responsible for multiple product formation. However, our simplistic analysis of the active site sequences of all currently-known enzymes (Figs. 3 and 4) did not reveal any such general feature.

Individual terpene synthases may have specific ways of generating multiple products. For example, the γ -humulene synthase of *A. grandis*, which generates 52 different sesquiterpenes, has two DDxxD motifs located on opposite sides of the active site cleft, which led to the suggestion that substrate binding in two different conformations results in different sets of products (Steele et al., 1998). Indeed, inactivation of one of the DDxxD motifs by mutagenesis resulted in enzymes that produce a lower number of products (Steele et al., 1998). The formation of multiple products might also be enhanced by the NSE/DTE motif that is situated at the position of the second DDxxD motif in some terpene synthases.

The relationship between active site architecture and multiple product formation has been studied with the terpene synthase TPS4 of *Zea mays* which forms the sesquiterpenes 7-*epi*-sesquithujene, β -bisabolene and 12 other olefins in minor amounts (Köllner et al., 2004). To understand the complex reaction mechanism of TPS4, the active site cavity was modeled and docking simulations with the substrate farnesyl diphosphate, with several predicted carbocation intermediates and with the final reaction products were conducted (Köllner et al., 2006). The results suggested that discrete steps of the reaction sequence occur in two different active site pockets, with a conformational change in the bisabolylyl cation intermediate causing a shift from one pocket to the other. Site-directed mutagenesis and measurements of mutant enzyme activity with both (*E,E*)- and (*Z,E*)-farnesyl diphosphate were employed to support this model. Amino acid alterations in pocket I indicated that early steps of the catalytic process are localized in this compartment up to the formation of the monocyclic bisabolylyl cation. Mutations in pocket II primarily inhibited the formation of bicyclic

compounds, suggesting that secondary cyclizations of the bisabolylyl cation are catalyzed in pocket II (Köllner et al., 2006).

The formation of multiple products could also be a consequence of more conformational flexibility in the active center allowing the formation of more reaction intermediates and thus more products. However, the short reaction times required by cationic cyclizations might not provide sufficient time for larger conformational changes of the intermediates (Vedula et al., 2005). Also, structural elucidation of complexes consisting of trichodiene synthase and an analog of the intermediate suggested the intermediates are present in thermodynamically preferred conformations rather than those expected from the course of the reaction mechanism (Christianson, 2006). Therefore, terpene synthase reactions are more likely to be controlled by kinetic rather than thermodynamic processes (Vedula et al., 2005). From this perspective, the formation of multiple products is dependent on differences in substrate conformation occurring at the very beginning of the reaction.

Further studies of terpene synthase structure and function are clearly necessary to understand how these enzymes catalyze the formation of multiple products. The availability of additional terpene synthase structures will no doubt speed progress in this area and facilitate further correlations between structural elements and properties of the reaction. In this regard, identification of the amino acid residues that interact with the termination steps of the cyclic cascade would be particularly helpful. The increased appreciation of theoretical methods would also be a welcome development. Knowledge of the free energies of reaction intermediates, for example, should provide new insights on multiple product formation.

The literature review for this article was completed in 2008. However, in 2009 two reports appeared that require revision of some basic assumptions about the universality of geranyl diphosphate (GPP) as a substrate for monoterpene synthases and the universality of farnesyl diphosphate (FPP = *E,E*-FPP) as a substrate for sesquiterpene synthases. A tomato monoterpene synthase was described that uses neryl diphosphate, the *Z*-isomer of GPP, as a substrate instead of GPP (Schilmiller AL, Schauvinhold I, Larson M, Xu R, Charbonneau AL, Schmidt A, Wilkerson C, Last R, Pichersky E (2009) Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. *Proc. Natl. Acad. Sci. USA* 106, 10865–10870), while a tomato sesquiterpene synthase was reported that uses *Z,Z*-FPP instead of the usual *E,E*-FPP (Sallaud C, Ronstein D, Onillon S, Jabès F, Duffé, Giagalone C, Thoraval S, Escoffer C, Herbette G, Leonhardt N, Causse M, Tissier A (2009) A novel pathway for sesquiterpene biosynthesis from *Z,Z*-farnesyl pyrophosphate in the wild tomato *Solanum habrochaites*. *Plant Cell* 21, 301–317). These and other isomeric prenyl diphosphates may prove to be more widespread substrates for the terpene synthase class.

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