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Review

# Molecular activities, biosynthesis and evolution of triterpenoid saponins

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ABSTRACT

Saponins are bioactive compounds generally considered to be produced by plants to counteract pathogens and herbivores. Besides their role in plant defense, saponins are of growing interest for drug research as they are active constituents of several folk medicines and provide valuable pharmacological properties. Accordingly, much effort has been put into unraveling the modes of action of saponins, as well as in exploration of their potential for industrial processes and pharmacology. However, the exploitation of saponins for bioengineering crop plants with improved resistances against pests as well as circumvention of laborious and uneconomical extraction procedures for industrial production from plants is hampered by the lack of knowledge and availability of genes in saponin biosynthesis. Although the ability to produce saponins is rather widespread among plants, a complete synthetic pathway has not been elucidated in any single species. Current conceptions consider saponins to be derived from intermediates of the phytosterol pathway, and predominantly enzymes belonging to the multigene families of oxidosqualene cyclases (OSCs), cytochromes P450 (P450s) and family 1 UDP-glycosyltransferases (UGTs) are thought to be involved in their biosynthesis. Formation of unique structural features involves additional biosynthetical enzymes of diverse phylogenetic background. As an example of this, a serine carboxypeptidase-like acyltransferase (SCPL) was recently found to be involved in synthesis of triterpenoid saponins in oats. However, the total number of identified genes in saponin biosynthesis remains low as the complexity and diversity of these multigene families impede gene discovery based on sequence analysis and phylogeny.

This review summarizes current knowledge of triterpenoid saponin biosynthesis in plants, molecular activities, evolutionary aspects and perspectives for further gene discovery.



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## 1. Introduction

The term 'saponin' defines a group of natural compounds that consist of an isoprenoidal-derived aglycone, designated genin or sapogenin, covalently linked to one or more sugar moieties. The name is deduced from the Latin word *sapo* (Engl.: soap) reflecting their wide spread ability to form stable soap-like foams in aqueous solutions. In fact, many plant extracts (e.g. from soapwort – *Saponaria officinalis*, soapbark – *Quillaja saponaria*, etc., Fig. 1) previously used for their soap-resembling properties often gained this ability due to saponins (Hostettmann and Marston, 1995). This characteristic trait is caused by the amphiphilic nature of saponins due to linkage of the lipophilic sapogenin to hydrophilic saccharide side chains.

Most known saponins are plant-derived secondary metabolites, though several saponins are also found in marine animals such as sea cucumbers (Holothuroidea) (e.g. Van Dyck et al., 2010) and starfish (Asteroidea) (e.g. Liu et al., 2008). The ability to synthesize saponins is rather widespread among plants belonging to the division of Magnoliophyta, covering both dicotyledons and monocotyledons. However, the majority of saponin-producing species has been found within dicotyledons (Vincken et al., 2007).

The biological role of saponins is not completely understood. In plants, they are generally considered to be part of defense systems due to anti-microbial, fungicidal, allelopathic, insecticidal and molluscicidal, etc. activities (reviewed in Francis et al., 2002; Sparg et al., 2004).

Saponin-producing plants generally accumulate saponins as part of their normal development. However, saponin accumulation is also known to be influenced by several environmental factors such as nutrient and water availability, light irradiation or combined effects (reviewed in Szakiel et al., in press). In addition, saponin distribution has been found to vary greatly in individual plant organs or tissues during ontogenesis or to show seasonal fluctuations. Variations in saponin distribution and levels have been suggested to represent varying needs for protection and to target specific herbivores and pests, respectively. For example, Ndamba et al. (1994) propose that maximal saponin accumulation in early stages of *Phytolacca dodecandra* (soapberry) berry development is to prevent fruit loss and to assure seed maturation. Lin et al. (2009) discuss that the highest saponin accumulation among all organs of *Dioscorea pseudojaponica* Yamamoto (yam) is in tubers to provide protection for this reproductive organ. Lastly, specific accumulation of saponins in the root epidermis of *Avenae* spp. (oats) has been demonstrated to counteract soil-borne fungi (Papadopoulou et al., 1999). Saponin levels are often increased in response to treatment with elicitors such as yeast extract or jasmonate derivatives (reviewed in Yendo et al., 2010). As particularly jasmonates are well known for triggering plant defense responses to herbivory (reviewed in Howe and Jander, 2008) this further corroborates the assumption of saponins being involved in plant defense.

Due to their chemical properties and abilities as foaming agents, saponins or saponin-containing plant extracts are exploited by industry as additives to foods and cosmetics. They have the potential to be used for further industrial applications as, for instance, as preservatives, flavor modifiers and agents for removal of cholesterol from dairy products (reviewed in Güçlü-Üstündağ and Mazza, 2007; San Martín and Briones, 1999). Saponin-rich byproducts from tea oil production have also been explored as botanical vermicide for selective managing of earthworm casts on golf courses and sport fields (Potter et al., 2010). Saponins are also known to be major constituents of many traditional folk medicines (e.g. extracts of liquorice – *Glycyrrhiza* sp. or ginseng – *Panax* sp.). Although health beneficial effects of these herbal extracts have not unequivocally been confirmed in clinical studies (*Glycyrrhiza*

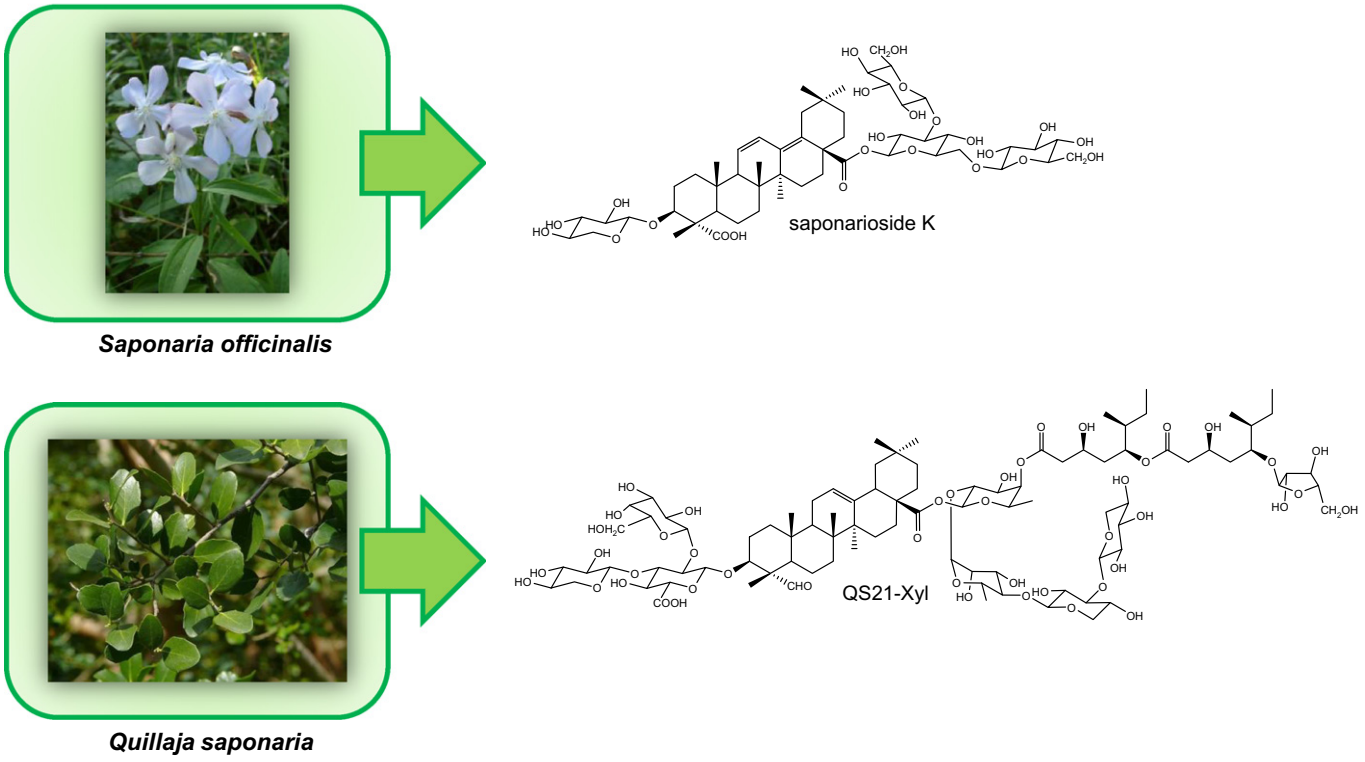


**Fig. 1.** Examples for saponins from *Saponaria officinalis* (soapwort) and *Quilllaja saponaria* (soapbark). Both plants produce complex mixtures of triterpenoid saponins, with at least 13 compounds in *S. officinalis* (Koike et al., 1999) and up to around 70 structurally discrete saponins in *Q. saponaria* (Bankefors et al., 2008).

sp.: reviewed in Asl and Hosseinzadeh, 2008; *Panax ginseng*: reviewed in Xiang et al., 2008), several of the saponins exhibit pharmacological activities and thus, attract attention as target for drug discovery. For example, several sapogenins (oleanolic acid: Sultana and Ata, 2008) and saponins are considered to possess activities such as anti-inflammatory (e.g. Sun et al., 2010b; Tapondjou et al., 2008), anti-cancerogenic (e.g. Musende et al., 2009; further reviewed in Man et al., 2010), anti-bacterial (e.g. De Leo et al., 2006; further reviewed in Saleem et al., 2010), anti-fungal (e.g. Coleman et al., 2010; Zhang et al., 2005) and anti-viral (e.g. Cinatl et al., 2003; Rattanathongkom et al., 2009; Zhao et al., 2008) effects. Saponins are also of interest as valuable adjuvants and the first saponin-based vaccines are introduced commercially (reviewed in Sun et al., 2009).

Present availability of saponins depends on their extractability from plants. Extraction procedures may be effective for saponins that occur in high concentrations in easily cultivable plants, and for applications that do not demand absolute purity. However, a steady supply of sufficient amounts of specific saponins from plants that accumulate mixtures of structurally related compounds is not feasible (Adams et al., 2010). Synthetic chemistry mainly attempts to address these issues by chemically linking desired side chains to extracted sapogenins (reviewed in Yu and Sun, 2009). However, the availability of the corresponding sapogenins, which may demand sophisticated modifications to derive from more common precursors, still constitutes bottlenecks. Biotechnological production of either complete saponins, or of saponin pathway intermediates that are not readily accessible, may circumvent the limitation of natural saponin availability. Bioengineering of crop plants with improved resistance towards specific pests as well as saponin reduction in other plants for increased food and feed quality (Dixon and Sumner, 2003) are additional motivations for elucidation of saponin biosynthesis.

Substantial efforts have been made to unravel saponin biosynthesis in plants. Although a general consensus on the basic organization of pathways and enzyme classes involved in saponin anabolism has been reached, discovery of individual genes in saponin biosynthesis has so far occurred rather sporadically. However, considerable progress has been accomplished since triterpenoid saponin biosynthesis was last reviewed (Haralampidis et al., 2002).

The aim of this review is to summarize this progress and provide an updated overview on the current knowledge of the enzymes involved in triterpenoid saponin biosynthesis. In addition, general aspects of molecular activities of saponins, with particular focus on their mode of action towards membranes, are outlined. Finally, the phylogeny of known enzymes in saponin pathways is reviewed and conclusions about evolution of saponin biosynthesis and consequences for further gene discovery are discussed.

## 2. Structural aspects and nomenclature

The commonly used nomenclature for saponins distinguishes between triterpenoid (*also:* triterpene) and steroidal saponins

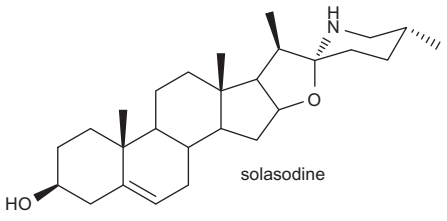


**Fig. 3.** Example of a steroidal glycoalkaloid aglycone skeleton (solasodine).

(Fig. 2). This differentiation is based on the structure and biochemical background of their aglycones. Both sapogenin types are thought to derive from 2,3-oxidosqualene, a central metabolite in sterol biosynthesis. In phytosterol anabolism, 2,3-oxidosqualene is mainly cyclized into cycloartenol. Triterpenoid sapogenins branch off the phytosterol pathway by alternative cyclization of 2,3-oxidosqualene, while steroidal sapogenins are thought to derive from intermediates in the phytosterol pathway downstream of cycloartenol formation (Haralampidis et al., 2002; Kalinowska et al., 2005, Fig. 7). A more detailed classification of saponins based on sapogenin structure with 11 main classes and 16 subclasses has been proposed by Vincken et al. (2007).

Steroidal glycoalkaloids (Fig. 3) are sometimes considered a third type of saponins (Haralampidis et al., 2002; Hostettmann and Marston, 1995; Kalinowska et al., 2005). This may be tempting as the structural characteristics suggest a similar biosynthetic origin and biological activities as proposed for steroidal saponins. However, since neither the biosynthesis of steroidal saponins nor steroidal glycoalkaloids has been fully clarified, a common biosynthetic background cannot be confirmed. From a structural point of view, Vincken et al. (2007) suggested to omit steroidal glycoalkaloids from the class of saponins due to the occurrence of the characteristic nitrogen atom in the aglycone backbone. In this review, molecular activities of steroidal glycoalkaloids towards membranes are discussed together with saponins even though the alterable protonation state of the nitrogen enhances the effect of pH on the biological activities of steroidal glycoalkaloids as compared to triterpenoid and steroidal saponins.

Saponins are also classified based on the number of attached saccharide side chains. Most known saponins are monodesmosidic saponins which means that only one position of the aglycone is glycosylated (Fig. 4A). In most monodesmosidic saponins this saccharide chain is attached by an ether linkage to the C3 hydroxy group present in the majority of sapogenins (Vincken et al., 2007). These saccharide side groups can be branched and in most cases comprise 2–5 monosaccharide units, which may increase up to 11 units (Hostettmann and Marston, 1995). Saponins with two saccharide chains are designated bidesmosidic (Fig. 4B). In triterpenoid saponins such second chains are most often attached by an ester linkage to the C28 carboxy group. Finally, in the rather rare tridesmosidic saponins, three saccharide chains are attached to the aglycone at different positions (Schwarz, 2000; Vincken et al., 2007).

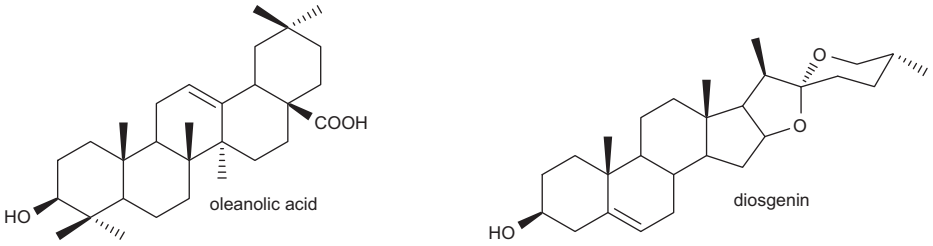


**Fig. 2.** Two representative triterpenoid (oleanolic acid) and steroidal (diosgenin) sapogenin skeletons.

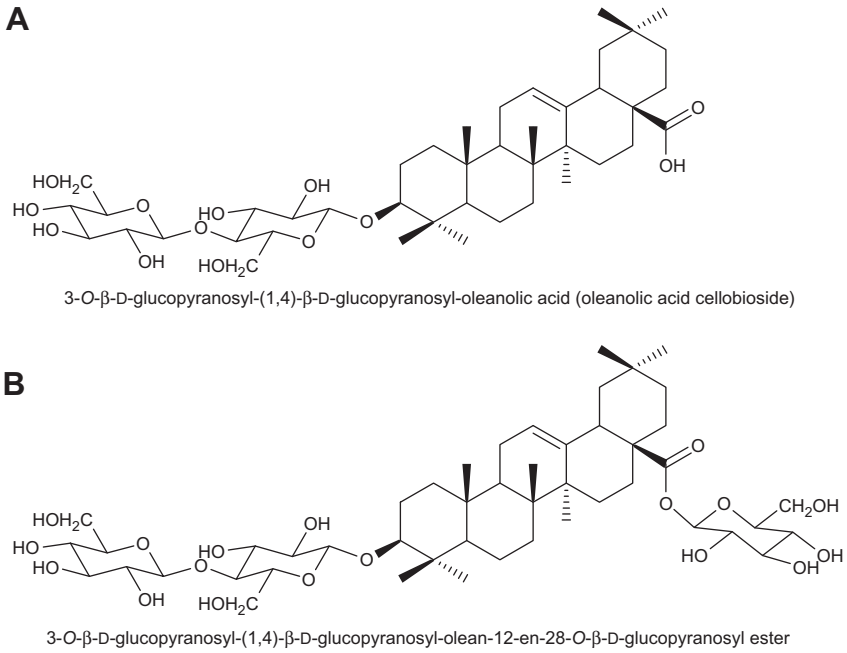


**Fig. 4.** Monodesmosidic (3-*O*-β-D-glucopyranosyl-(1,4)-β-D-glucopyranosyl-oleanolic acid; A) and bidesmosidic (3-*O*-β-D-glucopyranosyl-(1,4)-β-D-glucopyranosyl-olean-12-en-28-*O*-β-D-glucopyranosyl ester; B) saponins.

In addition to sugars, other substituents such as small aliphatic and aromatic acids, monoterpenoidal derived compounds and acyl groups are occasionally attached to sapogenins (see Vincken et al., 2007 for more details).

## 3. Molecular activities on membranes

Plant-derived saponins are mainly considered to be part of plant defense systems against pathogens and herbivores. Numerous reports emphasize the fungicidal (Lee et al., 2001; Morrissey and Osbourn, 1999; Saha et al., 2010; Sung and Lee, 2008a), anti-microbial (Avato et al., 2006; Sung and Lee, 2008b), allelopathic (Waller et al., 1993), insecticidal (Sandermann and Funke, 1970; Shinoda et al., 2002; Kuzina et al., 2009; Nielsen et al., 2010a) and molluscicidal (Aladesanmi, 2007; Gopalsamy et al., 1990; Hostettmann, 1980; Huang et al., 2003) activity of various saponins. However, the molecular mechanisms behind these deterrent and toxic effects are not completely elucidated.

The most intensively studied effect of several saponins and many steroidal glycoalkaloids is their ability to cause membrane perturbation. This property is often referred to as the 'hemolytic activity of saponins', as it is thought to be the molecular basis for the often observed ability of saponins to cause lysis of mammalian erythrocytes (Baumann et al., 2000; Chwalek et al., 2006; Seeman et al., 1973). This effect towards membranes was first described by Dourmashkin et al. (1962) in their study on saponin-inactivated Rous sarcoma virus particles by electron microscopy. The observed formation of pores (or pits) in the viral membrane coat as a consequence of the saponin treatment inspired Bangham and Horne (1962) and Glauert et al. (1962) to pursue additional experiments in this direction. Both groups concurrently reported the occurrence of cholesterol in the target membrane to be essential for saponin-induced pore formation. Since then, several research groups further elucidated the molecular basis behind the membrane penetration activity by studying various sets of saponins or steroidal glycoalkaloids on natural occurring or artificial membrane systems. The results of these studies confirmed the influence of membrane composition, especially in regards to concentration levels as well as the structure of incorporated membrane sterols, on the ability of saponins and steroidal glycoalkaloids to cause membrane perturbation (Armah et al., 1999; Gögelein and Hüby, 1984; Keukens et al., 1992, 1995; Nishikawa et al., 1984; Rosenqvist et al., 1980; Steel and Drysdale, 1988; Stine et al., 2006; Walker et al., 2008; Yu and Jo, 1984). In addition, it became obvious that the overall hemolytic potential of saponins and steroidal glycoalkaloids is affected by virtually all characteristics of their chemical composition, including structure of the aglycone (Gauthier et al., 2009a,b; Oda et al., 2000; Takechi et al., 2003; Ullah et al., 2000; Voutquenne et al., 2002; Yu and Jo, 1984), number of saccharide side chains (Hase et al., 1981; Hu et al., 1996; Voutquenne et al., 2002; Woldemichael and Wink, 2001), length of these side chains (Armah et al., 1999; Chwalek et al., 2006; Keukens et al., 1995; Nishikawa et al., 1984; Oda et al., 2000; Plé et al., 2004; Seebacher et al., 1999b; Voutquenne et al., 2002) as well as the types and linkage variants of the incorporated sugar units (Chwalek et al., 2006; Hu et al., 1996; Seebacher et al., 1999a,b, 2000; Takechi et al., 2003). Combinatory effects when applying mixtures of different steroidal glycoalkaloids or saponins were also reported by Keukens et al. (1995), Smith et al. (2001 and references herein) and Voutquenne et al. (2002). However, due to the high number of structural characteristics found to modulate the hemolytic activity, the diversity in experimental setups and the partially conflicting conclusions, an exhaustive relationship between saponin structure and their membrane perturbation activity has not been established. Consequently, the predictability of the hemolytic potential of uncharacterized saponins often remains low.

Based on their observations, Glauert et al. (1962) proposed the first model of saponin action towards membranes. According to this model, spontaneous formation of complexes between saponins and cholesterol in membranes is followed by association of these complexes into 'two-dimensional micellar-type structures' within the membrane. The hydrophilic sugar chains of the saponins, which are thought to be centrally orientated in the micellar-like complex, lead to formation of an aqueous pore. Such pores would cause an increase in membrane permeability enabling

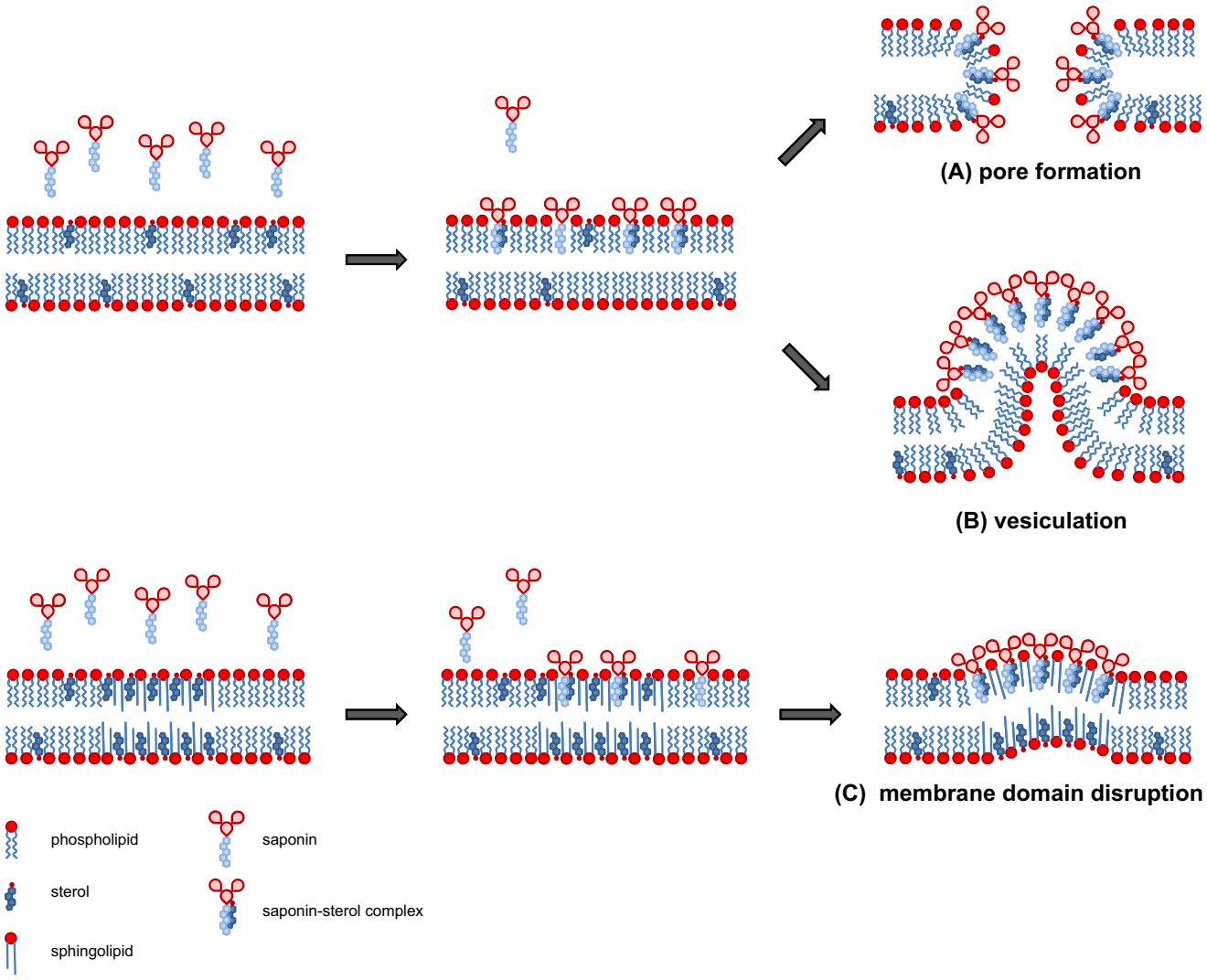


**Fig. 5.** Schematic models of the molecular mechanisms of saponin activities towards membranes. Saponins integrate with their hydrophobic part (sapogenin) into the membrane. Within the membrane they form complexes with sterols, which subsequently, driven by interaction of their extra-membranous orientated saccharide residues, accumulate into plaques. Sterical interference of these saccharide moieties causes membrane curvature subsequently leading to (A) pore formation in the membrane (Armah et al., 1999) or (B) hemitubular protuberances resulting in sterol extraction via vesiculation (Keukens et al., 1995). Alternatively, after membrane integration saponins may migrate towards sphingolipid/sterol enriched membrane domains (C) prior to complex formation with the incorporated sterols, thereby interfering with specific domain functionalities (Lin and Wang, 2010). Similarly to (B), accumulation of saponins in confined membrane domains has further been suggested to cause deconstructive membrane curvature in a dose-dependent manner.

ions and macromolecules up to proteins to pass the membrane bilayer. More recent models of the molecular mechanism behind the membrane permeabilizing activity of saponins and steroidal glycoalkaloids (Armah et al., 1999; Keukens et al., 1995; Nishikawa et al., 1984; Fig. 5) expand this initial hypothesis. The first step in these modes of action is the incorporation of saponins or steroidal glycoalkaloids into the facing membrane monolayer. This step occurs spontaneously, driven by the lipophilic character of the aglycone as it is expected to favor the hydrophobic conditions of membrane layers over aqueous surroundings. Within the membranes, saponins and steroidal glycoalkaloids then assemble into 1:1-complexes with membrane sterols as, e.g., cholesterol in mammalian cell membranes. Subsequently, the saponin–sterol or glycoalkaloid–sterol complexes accumulate into matrices or plaques by processes whose driving force is not currently understood. Armah et al. (1999) and Keukens et al. (1995) proposed interactions between the sugar moieties of the incorporated saponins/glycoalkaloids to cause these phase-separation phenomena. Finally, as a consequence of such accumulation, the sterical properties of saponins and steroidal glycoalkaloids may cause membrane curvature as a final step in the membrane perturbation mechanism. This curvature could result in the formation of either pores (Armah et al., 1999; Fig. 5A) within these saponin/sterol plaques, or hemitubular protuberances that may eventually lead to sterol extraction via vesiculation (Keukens et al., 1995; Fig. 5B). The observation of pore-like structures and holes in electron microscopy images of membranes and cells treated with saponins (Bangham and Horne, 1962; Baumann et al., 2000; Dourmashkin et al., 1962; Glauert et al., 1962; Mazzucchelli et al., 2008; Seeman et al., 1973) supports the concept of pore formation. In addition, formation of pores may provide the simplest explanation for the observed changes in ion conductivity (e.g. Armah et al., 1999; Gögelein and Hüby, 1984) and protein mobility through the membrane (e.g. Krawczyk et al., 2010; Mazzucchelli et al., 2008). However, microscopy studies based on membranes treated with steroidal glycoalkaloids did not reveal similar pores (Keukens et al., 1992, 1995; Stine et al., 2006; Walker et al., 2008). Absence of pore-resembling structures was also reported by Dourmashkin

et al. (1962) when membranes were treated with the steroidal saponin digitonin instead of the saponin extract previously found to cause pore formation. Moreover, pretreatment with digitonin in this study appeared to even prevent formation of pores during subsequent exposure to pore-inducing saponins. Further studies on the activity of digitonin towards membranes (Elias et al., 1978; Miller, 1984) reported hemitubular alterations similar to the observation made by Keukens et al. (1992, 1995) for glycoalkaloids. Finally, computational simulations based on the steroidal saponin dioscin (Lin and Wang, 2010) also suggest membrane curvature, resembling the formation of hemitubular alterations, as a consequence of increasing accumulation of saponin molecules in confined areas of the membrane. Pore formation and tubular alteration/vesiculation may therefore exist in parallel, and the chemical properties of the saponin or glycoalkaloid determine which perturbation type predominates. Such diversity in the ability to cause membrane permeabilization by different saponins was recently demonstrated by Krawczyk et al. (2010). In this study, digitonin only permeabilized plasma membranes, while a saponin extract from *Q. saponaria*, which accumulates a vast array of diverse triterpenoid saponins (Fig. 1), caused additional perturbation of intra-cellular membrane systems.

An alternative model of saponin activity towards membranes originates from coarse-grained molecular dynamics simulations (Lin and Wang, 2010; Fig. 5C). In this model, saponins migrate in the membrane to sphingomyelin and cholesterol enriched membrane domains, the so-called lipid rafts (Brown and London, 2000; Fantini et al., 2002), and complex with the cholesterols therein. Such saponin interaction with specific membrane domains is supported by recent studies on saponin-induced lipid raft disruption (Xu et al., 2009; Yi et al., 2009; Zhuang et al., 2002) and microdomain alteration (Naruse et al., 2010). Consequently, the earlier indicated plaque formation may originate from complex formation following preexisting phase separation rather than resulting from membrane reorganization processes triggered by saponin–sterol complex accumulation.

There is a general lack of knowledge on intracellular transport and subcellular storage of saponins and steroidal glycoalkaloids as well as how plants prevent disruption of endogenous membranes. Cell fractionation studies by Kesselmeier and Urban (1983) and Urban et al. (1983) on steroidal avenacosides from areal tissues of oat (*Avena* spp.), and confocal microscopy by Mylona et al. (2008) on oat root triterpenoid avenacins, suggest that saponins are mainly stored in vacuoles. That steriodal glycoalkaloids in tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) mainly occur in the soluble phase after cell fractionation further indicates that steroidal glycoalkaloids are stored in a solubilized form rather than by incorporation into host cell membranes (Roddick, 1977). Plants have developed different strategies to protect themselves against their own saponins or steroidal glycoalkaloids. An example of this is that avenacosides are known to be stored in an inactive bidesmosidic form, and are transformed into the biologically active monodesmosides by specific β-glucosidases upon tissue disruption (reviewed in Morant et al., 2008). A similar mechanism has been described for bidesmosidic saponins in *P. dodecandra* berries (Parkhurst et al., 1989). However, most saponins are presumably stored in biologically active forms and little is known about how plants protect the integrity of their cell membranes against them. Steel and Drysdale (1988) proposed that the increased resistance of steroidal glycoalkaloid-producing plants towards their own defense compounds results from reduced amounts of sterols in cell membranes or their substitution with sterol derivatives with reduced affinity to steroidal glycoalkaloids. Consistent with this, recently reported resistance of membranes of the amoeba *Dictyostelium discoideum* towards permeabilization with *Q. saponaria* saponin extracts (Mercanti and Cosson, 2010) has been suggested to be due to their plant-resembling sterol composition.

Although interference with the integrity of biological membranes is probably the most abundant effect of saponins, their activities are not limited to this type of action, and not all saponins show significant hemolytic activity (e.g. Chwalek et al., 2006; Gauthier et al., 2009a,b; Nakamura et al., 1979). Several structure–activity relation studies further emphasized that the ability to cause hemolysis often does not correlate with other known activities of saponins such as their anti-fungal and cytotoxic properties or their applicability as adjuvants (Adams et al., 2010; Chwalek et al., 2006; Gauthier et al., 2009a,b; Oda et al., 2000; Santos et al., 1997; Takechi et al., 2003). Induction of cytotoxicity may in some cases also involve disruption of lipid rafts (Xu et al., 2009; Yi et al., 2009). In addition, some saponins have been shown to influence the properties of different types of membrane proteins such as $Ca^{2+}$ channels and $Na^{+}$–$K^{+}$ ATPases (Chen et al., 2009; Choi et al., 2001; Haruna et al., 1995; Takechi et al., 2003). However, it is not yet clear, whether these effects are caused by agonistic or antagonistic binding to effector sites and changes in protein topology or as a result from changes in fluidity of the surrounding membrane layer. Another intriguing mode of action for some saponins is the ability to bind to glucocorticoid receptors. Glucocorticoids represent a class of steroidal hormones known to be involved in regulation of a manifold of physiological processes. In mammals, they play an important role in regulation of development, metabolism, neurobiology and apoptosis, etc. (reviewed in Yudt and Cidlowski, 2002). Due to the structural resemblance of saponin aglycones and steroids, it is therefore not surprising that several pharmacological activities of saponins such as anti-inflammatory (Giner et al., 2000) and neuroprotective (Zhang et al., 2008) effects as well as induction of adipogenesis (Niu et al., 2009) or apoptosis (Jia et al., 2004), are linked to interaction with receptors of glucocorticoid hormones.

An intriguing reversion of steroidal glycoalkaloid based plant defense mechanisms by plant pathogens has been reported for α-tomatine from *S. lycopersicum* (tomato). Thus, fungal tomato pests such as *Septoria lycopersici* and *Fusarium oxysporum* have been found to produce extracellular enzymes that hydrolyze different glycosidic bonds within the saccharide chain of α-tomatine. Surprisingly, the hydrolyzed products not only exhibit reduced antifungal activity but also cause suppression of induced plant defense mechanisms such as hypersensitive response and oxidative burst (Bouarab et al., 2002; Ito et al., 2004). Yet, the mechanism behind this phenomenon is unclear and similar effects have not been reported for other steroidal glycoalkaloids or saponins.

Taken together, saponin activities are as multifarious as their chemical compositions, and solely based on structure their activities are often unpredictable. As many saponin activities and in particular the ability to cause membrane permeabilization, are considered to depend on their three-dimensional shape, computational modeling combined with experimental observations may provide a key to gain further insights into the corresponding molecular backgrounds.

## 4. Biosynthesis of triterpenoid saponins

### *4.1. From acetyl-CoA to 2,3-oxidosqualene – common biosynthetic origin with phytosterols*

Current ideas of saponin biosynthesis in plants, consider them to be derived from metabolites of phytosterol anabolism. This assumption is supported by the reported concurrent upregulation of sterols and saponins in ginseng plants (*P. ginseng*) over-expressing squalene synthase (Lee et al., 2004), an enzyme catalyzing a step

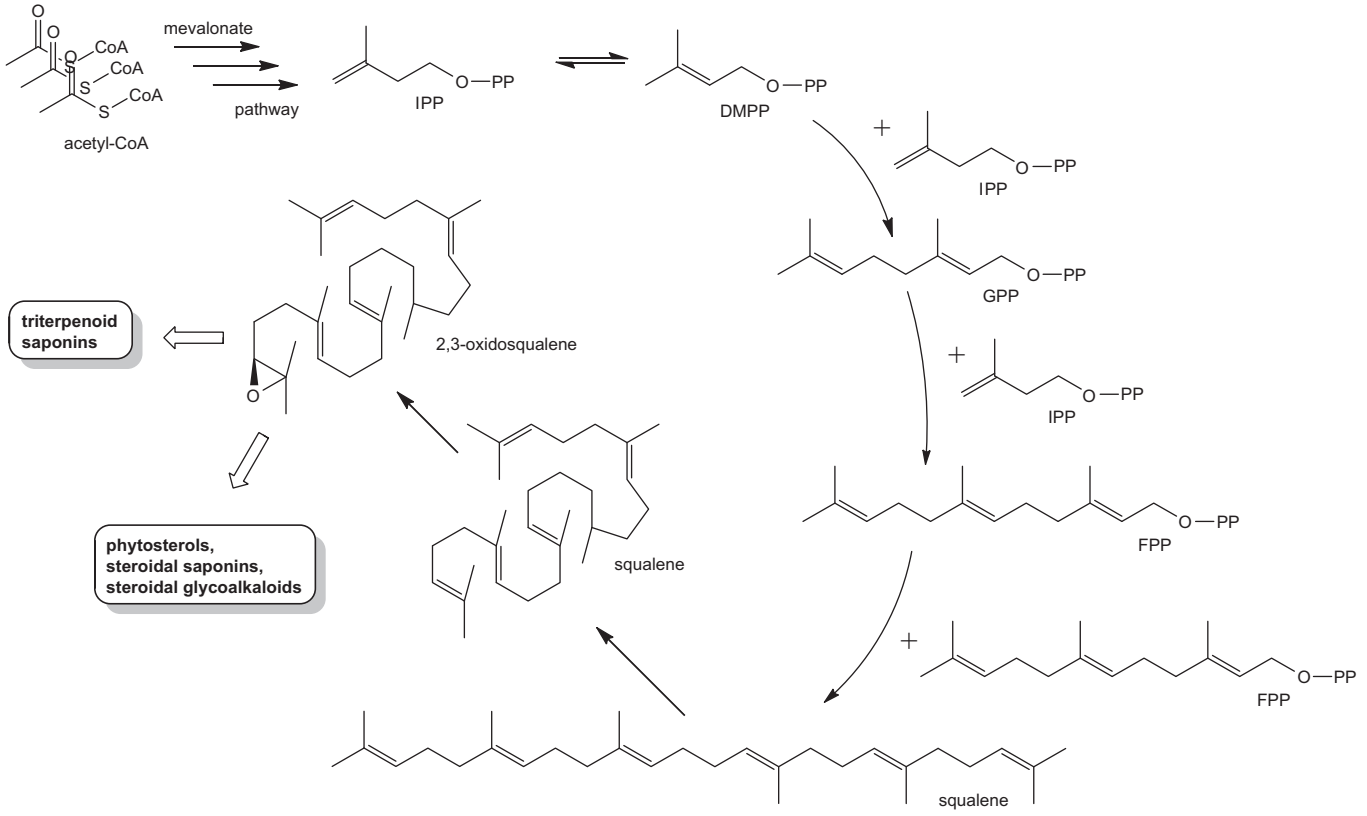


**Fig. 6.** Early steps in biosynthesis of phytosterols and triterpenoid saponins leading to the common precursor 2,3-oxidosqualene. *IPP* – Isopentenyl pyrophosphate, *DMPP* – dimethylallyl pyrophosphate, *GPP* – geranyl pyrophosphate, *FPP* – farnesyl pyrophosphate.

prior to the proposed branchpoint of the two pathways. Increased phytosterol levels in oat (*Avena strigosa*) *sad*1 (*saponin-deficient*) mutants (Qin et al., 2010) and increase of saponin aglycones and concomitant decrease of phytosterols in cell suspension cultures of bramble (*Rubus fruticosus*) and liquorice (*Glycyrrhiza glabra*) treated with cycloartenol synthase specific inhibitors (Ayabe et al., 1990; Taton et al., 1986, 1992) also support that saponins are derived from phytosterol biosynthesis.

The part of the triterpenoid saponin pathways shared with synthesis of plant sterols is outlined in Fig. 6. All terpenoids derive from condensation of five-carbon building blocks designated IPP (3-isopentenyl pyrophosphate, C5) and DMAPP (dimethylallyl pyrophosphate, C5). In plants IPP and DMAPP either derive from condensation of acetyl-CoA in the cytosolic mevalonate pathway or from pyruvate and phosphoglyceraldehyde in the plastidial MEP (*also:* DXP) pathway. Terpenoid biosynthesis in plants is extensively compartmentalized and triterpenes such as steroids and saponins are mainly synthesized in the cytosol utilizing IPP from the mevalonate pathway (Chappell, 2002; Kirby and Keasling, 2009; Rohmer, 1999; Trojanowska et al., 2000). In accordance with this conception, both phytosterol and ursolic/oleanolic acid biosynthesis have been found strongly reduced in *Uncaria tomentosa* (cat's claw) cell suspension cultures in response to treatment with an inhibitor of HMG-CoA reductase, a central enzyme of the mevalonate pathway (Flores-Sánchez et al., 2002). IPP and DMAPP undergo condensation to GPP (geranyl pyrophosphate, C10), and addition of a second IPP unit leads to FPP (farnesyl pyrophosphate, C15), the common precursor of the vast array of sesquiterpenes produced by plants. Linkage of two FPP units leads to formation of squalene (C30), which subsequently is epoxygenated to 2,3-oxidosqualene (C30). 2,3-Oxidosqualene is considered the last common precursor of triterpenoid saponins, of phytosterols and steroidal saponins (e.g. Kalinowska et al., 2005; Phillips et al., 2006; Vincken et al., 2007). The steps at which steroidal saponin and phytosterol biosynthesis diverge have not been elucidated, although cholesterol has been suggested as a precursor of steroidal saponins (Kalinowska et al., 2005; Vincken et al., 2007).

### 4.2. Cyclization of 2,3-oxidosqualene – emergence of sapogenin heterogeneity

The first committed step in the biosynthesis of triterpenoid saponins and phytosterols is the cyclization of 2,3-oxidosqualene. During this process internal bonds are introduced into the oxidosqualene backbone, resulting in formation of predominantly polycyclic molecules containing varying numbers of 5- and 6-membered rings. The high number of possibilities for establishing different internal linkages during cyclization gives rise to a vast array of diverse structures and more than 100 different triterpene skeletons (Xu et al., 2004) have been found in nature. However, from this diversity only a limited number of possible cyclization products appear to be utilized in saponin biosynthesis (Vincken et al., 2007; Fig. 7).

Enzymes catalyzing these cyclizations are designated oxidosqualene cyclases (OSCs, EC 5.4.99.x). Current knowledge of the catalytic mechanisms behind OSC activity has recently been reviewed by Abe (2007). Accordingly, oxidosqualene cyclase catalysis largely proceeds according to the biogenetic isoprene rule as originally formulated by Ruzicka (1953). Thus, OSCs fulfill three major prerequisites for catalyzing the cyclization process: (1) a catalytic acid that initiates the cyclization process by protonating 2,3-oxidosqualene, (2) a specialized catalytic cavity that by primarily spatial constraints of the active site guides the cyclizing oxidosqualene backbone through defined intermediate stages that eventually lead to formation of specific cyclization end products, and (3) shielding of reactive intermediates during the cyclization in order

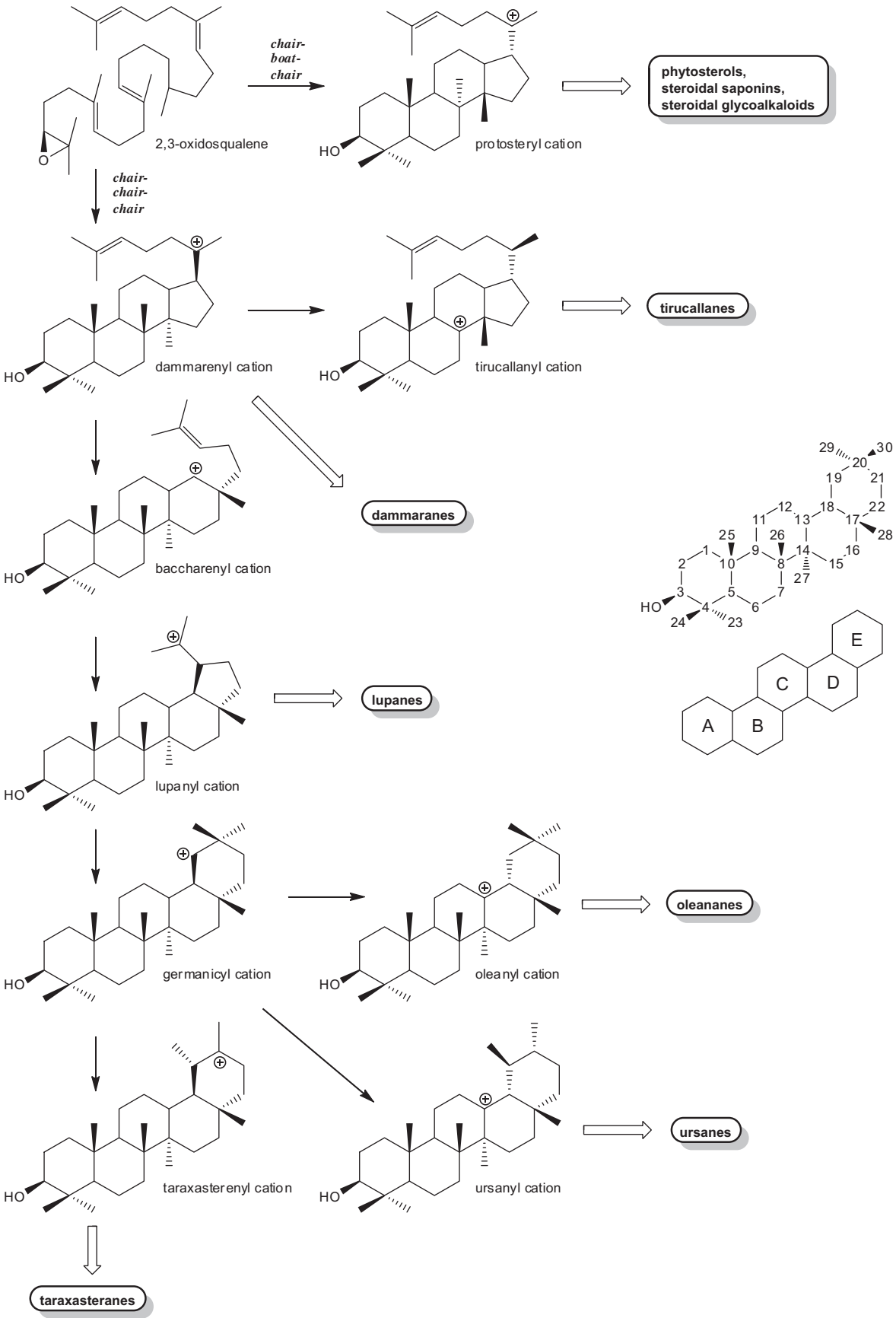


**Fig. 7.** Oxidosqualene cyclase (OSC) catalyzed cyclization cascades of 2,3-oxidosqualene into different triterpenoid sapogenin skeletons.

to prevent interfering side reactions. Termination of the cyclization process requires reconstitution of the neutral charge of the cationic intermediate. Most often, this quenching is presumably a result of deprotonation by polar amino acid residues acting as a catalytic base or due to water molecules located in the active center. As a consequence, cyclization products arising from this type of quenching usually retain a characteristic double bond at varying positions. Alternatively, some OSCs were found to quench progressing cyclization by stereospecific addition of water, giving rise to saturated, dienolic cyclization products (Kolesnikova et al., 2007a). The idea of 'guidance' of the cyclizing backbone throughout distinct intermediate steps as well as performance of position-specific deprotonation has recently been challenged, as the increased sensitivity of detection techniques revealed the tendency of some OSCs to give rise to several minor products in addition to a few major products. For example, Lodeiro et al. (2007) identified 22 byproducts co-occurring with the main cyclization product baruol (~90%) of the *Arabidopsis thaliana* OSC BARS1/At4g15370. The diverse cyclization cascades that these byproducts are thought to originate from, contradict the assumption of specialized stabilizing centers and unique proton acceptors in the active site of OSCs. Quantum mechanical calculations on cyclization intermediates (Matsuda et al., 2006) have revealed relatively low energetic barriers during cyclization, and thus there may be a subordinated demand for OSCs to reduce activation energies during cyclization. Consequently, it has been suggested that OSCs imperfectly aim to prevent alternative ways of cyclization rather than stabilize distinct intermediates on their path to predetermined end products – a concept also known as 'negative catalysis' (Rétey, 1990). Therefore, the observation of numerous minor byproducts accompanying formation of specific major products of OSCs may become a general observation with increasingly sensitive detection methods. Lodeiro et al. (2007) also refined the classification of OSCs according to their product accuracy. Enzymes such as BARS1, which give rise to several cyclization products of which one is clearly predominant, hardly fit into the previously prevalent categories of 'accurate' and 'multifunctional'. These classifications only discriminated between OSCs that either exclusively catalyze cyclization of 2,3-oxidosqualene into one single product or synthesize several structural distinct cyclization products in comparable amounts. As a result, the rough separation of an assumed 'continuum of cyclase accuracy' into 'highly accurate', 'moderately accurate' and 'multifunctional' was proposed. The ratio between the two most abundant products or the proportion of the primary product to the sum of remaining products has been suggested as means for distinction.

The characteristic trait of cyclization cascades catalyzed by OSCs involved in triterpenoid sapogenin biosynthesis (Fig. 7) is the initial direction of the 2,3-oxidosqualene backbone into a *chair–chair–chair*-conformation in contrast to the *chair–boat–chair*-conformation during catalysis of steroidal OSCs such as cycloartenol and lanosterol synthases. Thus, instead of the protosteryl cation in sterol biosynthesis, the first intermediate of the cyclization process of all triterpenoid sapogenins is the dammarenyl cation (Haralampidis et al., 2002; Phillips et al., 2006; Vincken et al., 2007; Xiong et al., 2005). Immediate deprotonation of this cation gives rise to sapogenins of the *dammarane type.* Alternatively, this cation may undergo further rearrangements leading to either the tirucallanyl (→*tirucallane type sapogenins*) or the baccharenyl cation. Proceeding annulation of the baccharenyl cation results in the pentacyclic lupanyl cation – the backbone commonly found in sapogenins of the *lupane type*. Reopening and expansion of the 5-membered E-ring of the lupanyl cation into a six carbon ring, leads to an intermediate cyclization product designated as germanicyl cation. The germanicyl cation can be further transformed into the oleanyl (→*oleanane type sapogenins*), the taraxasterenyl (→*taraxasterane type sapogenins*) or the ursanyl cation (→*ursane type sapogenins*) (Lodeiro et al., 2007; Vincken et al., 2007).

The abundance and distribution of individual sapogenin types in different plant orders is highly variable and most sapogenins are neither restricted to distinct plant orders nor do their distributions clearly follow the phylogeny of plants (overview in Vincken et al., 2007). By far the most abundant type of triterpenoid sapogenins in nature seem to be the oleananes derived from the 2,3-oxidosqualene cyclization product β-amyrin. It has not been determined whether this is the result of specific functionality conferred by the oleanane backbone or reflects thermodynamic preference of 2,3-oxidosqualene cyclization into β-amyrin over alternative cyclization end products.

Interestingly, the ability to produce precursors of triterpenoidal sapogenins was shown to be more widespread among plants than the reported distribution of the corresponding saponins. For example, several plants in the Brassicaceae family have been reported to accumulate pentacyclic triterpenoids such as β-amyrin, α-amyrin and lupeol (*A. thaliana*: Husselstein-Muller et al., 2001; Shan et al., 2008; *Brassica oleracea* cultivars: Baker and Holloway, 1975; Eigenbrode and Pillai, 1998; Martelanc et al., 2007). In fact, the ability to synthesize β-amyrin in *A. thaliana* appears to be strongly redundant since two (ATLUP2/At1g78960 and AtBAS/At1g78950) out of 13 OSCs have been reported to produce β-amyrin as one of their major products and three others (BARS1/At4g15370, CAMS1/At1g78955 and LUP1/At1g78970) also give rise to this compound as a minor byproduct (Fig. 8, Table 1). Despite this wide abundance of sapogenin precursors among the Brassicaceae, *Barbarea vulgaris* is the only Brassicacea reported to synthesize triterpenoid saponins (Agerbirk et al., 2003; Nielsen et al., 2010b; Shinoda et al., 2002). In addition to members of the Brassicaceae family, other plants of diverse phylogenetic origin such as olive (*Olea europaea*) (Allouche et al., 2009), grape (*Vitis vinifera*) (Rivero-Cruz et al., 2008), castor bean (*Ricinus communis*) (Guhling et al., 2006) and tomato (*S. lycopersicum*) (Mahjoub et al., 2009) are known to accumulate oleanane, ursane and lupane type aglycones. Lastly, a multifunctional OSC isolated from *Costus speciosus* (CsOSC2) was found to catalyze 2,3-oxidosqualene cyclization into various triterpenoids such as β-amyrin, although, this monocotyledonous plant is only known to produce steroidal saponins (Kawano et al., 2002). It has not been clarified if triterpenoid sapogenins and their precursors, particularly β-amyrin, exhibit biological functionality themselves. The occurrence of pentacyclic triterpenoids in cuticular waxes within the Brassicaceae as well as for other plants, e.g., castor bean and tomato and the correlation of the cuticular β-amyrin/α-amyrin concentration with resistance towards the Brassicaceae pest *Plutella xylostella* (Eigenbrode and Pillai, 1998) do indicate such a specific role. However, oat (*A. strigosa*) *sad1* mutants lacking β-amyrin synthase have not been reported to exhibit a distinct phenotype that is not related to the lack of avenacins (Haralampidis et al., 2001; Mylona et al., 2008).

The number of identified and biochemically characterized OSCs has significantly increased during the last decade (Table 1). Most of them have been identified by use of degenerative primers based on highly conserved motives and subsequent reconstitution of the full-length ORF by RACE (rapid amplification of cDNA ends), while others were isolated based on screening of cDNA libraries. However, analysis of the *A. thaliana* genome revealed 13 OSC genes in total, suggesting several more OSCs occur in plants than have been found by the sequence similarity-based approaches applied so far.

Not surprisingly, biochemical characterization revealed most of the identified OSCs to be cycloartenol and β-amyrin synthases, respectively. Within plant OSC phylogenetic trees (e.g. Fig. 8) cycloartenol synthases form a well-defined clade. This supports that the ability to synthesize the phytosterol precursor cycloartenol has

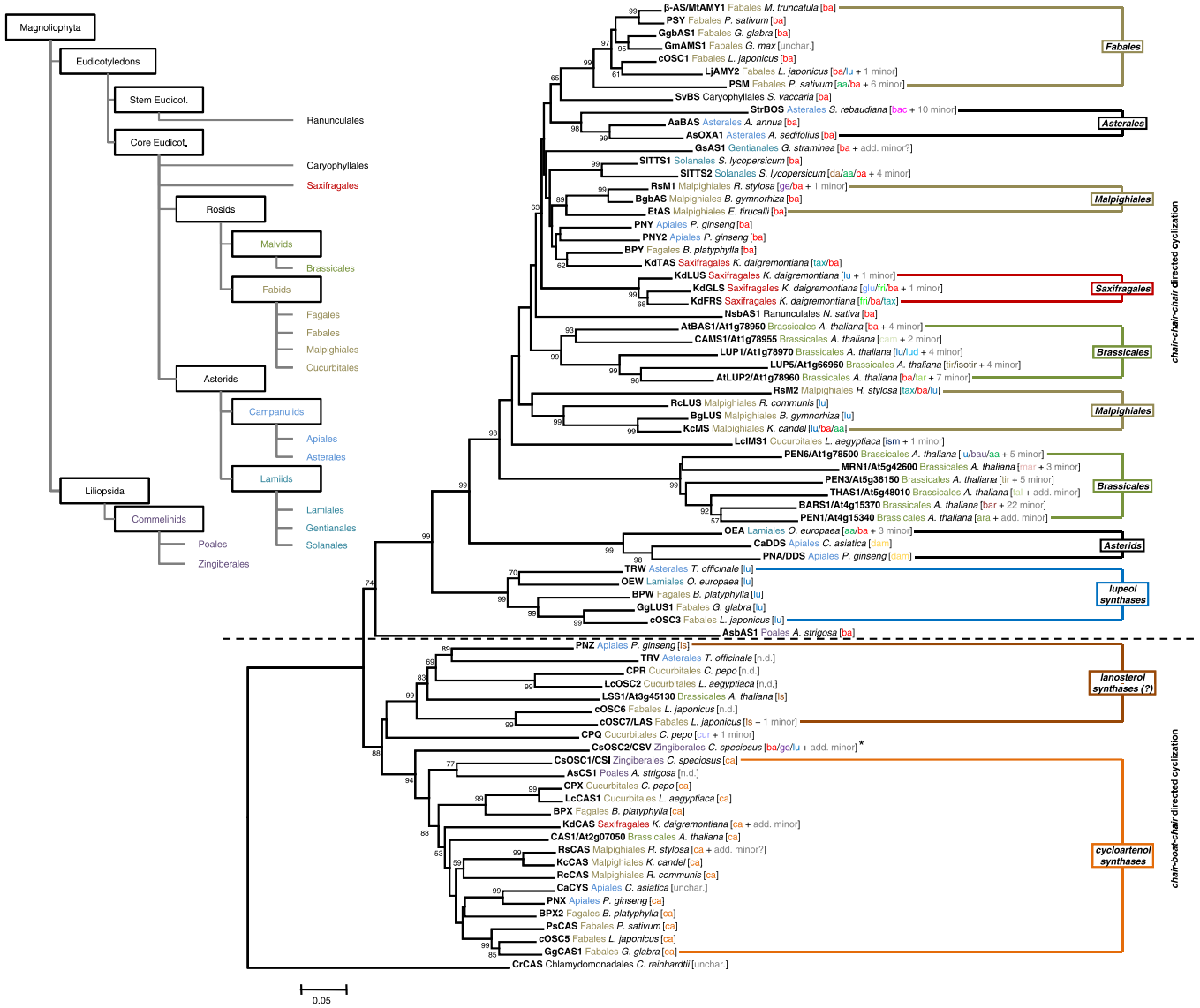


**Fig. 8.** Bootstrapped neighbor joining tree of known OSCs. CLUSTAL W alignment and bootstrap analysis were performed with MEGA4 (version: 4.0.2) using default parameters (Tamura et al., 2007) and with the putative *Chlamydomonas reinhardtii* cycloartenol synthase as outgroup (GenBank ID: XP_001689874). The given scale represents 0.05 amino acid substitutions per site. Bootstrap values below 50 are not shown. The name of individual enzymes is followed by the plant order (color coded according to their taxonomic subclass [taxonomy according to http://www.uniprot.org/taxonomy/]) as well as the plant species they originate from. 2,3-Oxidosqualene cyclization products identified to emerge from the activity of the corresponding OSC are indicated in squared brackets: *aa* – α-amyrin, *ara* – arabidiol, *ba* – β-amyrin, *bac* – baccharis oxide, *bar* – baruol, *bau* – baurenol, *ca* – cycloartenol, *cam* – camelliol C, *cur* – cucurbitadienol, *da* – δ-amyrin, *dam* – dammarenediol, *fri* – friedelin, *ge* – germanicol, *glu* – glutinol, *ism* – isomultiflorenol, *isotir* – isotirucallol, *ls* – lanosterol, *lu* – lupeol, *lud* – lupane-3β,20-diol, *mar* – marneral, *tal* – thalianol, *tar* – taraxasterol, *tax* – taraxerol, *tir* – tirucalla-7,24-dien-3β-ol, *n.d.* – no activity observed, *unchar.* – not biochemically characterized, *minor* – additional byproducts either reported to be of minor appearance or to represent <10% of the observed products. *Although CsOSC2 groups with *chair–boat–chair* directing OSCs, its products derive from direction of 2,3-oxidosqualene into *chair–chair–chair* formation. The underlying sequences in FASTA format as well as the alignment and the phylogentic tree are also available at http://www.p450.kvl.dk/OSC-sequences.fasta, http://www.p450.kvl.dk/OSC-alignment.pdf and http://www.p450.kvl.dk/OSC-phyltree.pdf, respectively.

emerged prior to speciation of plants and that cycloartenol synthases from different plant species are orthologous (e.g. Phillips et al., 2006). An exception is the multifunctional CsOSC2 within the cycloartenol synthase clade, which indicates this gene has arisen due to a recent evolutionary event. Based on mechanistic criteria, Xiong et al. (2005) proposed all additional plant OSCs to be derived from duplication events of cycloartenol synthase genes, which is supported by the phylogenetic analysis shown in Fig. 8. This also includes plant lanosterol synthases, which were thought for long time to be restricted to fungi and animals, but have recently been discovered in plants as well (Kolesnikova et al., 2006; Suzuki et al., 2006). Tracer experiments confirmed the involvement of *A. thaliana* lanosterol synthase (LSS1/At3g78500) in phytosterol biosynthesis (Ohyama et al., 2009) and led to the suggestion that the lanosterol pathway also contributes to the production of steroid-derived secondary metabolites. Other gene duplicates of cycloartenol synthases diversified widely before they acquired new functionalities in secondary metabolism. This development is reflected by the heterogeneity of OSCs that direct the 2,3-oxidosqualene backbone into a *chair–chair–chair*-conformation (Fig. 8). In contrast to the functional grouping found for cycloartenol synthases, occurrence of a similar well-defined β-amyrin synthase clade is less evident. Instead, although most accurate β-amyrin synthases roughly group together, they are significantly less conserved. In addition, several multifunctional OSCs with diverse phylogenetic origin have also been found to give rise to β-amyrin. The finding of an accurate β-amyrin synthase from *A. strigosa* (AsbAS1), which shows significantly lower relationship to all other

**Table 1**
Overview of OSCs reported in the literature. 2,3-Oxidosqualene cyclization products identified to emerge from the activity of the corresponding OSC are indicated in squared brackets: *aa* – α-amyrin, *ara* – arabidiol, *ba* – β-amyrin, *bac* – baccharis oxide, *bar* – baruol, *bau* – baurenol, *ca* – cycloartenol, *cam* – camelliol C, *cur* – cucurbitadienol, *da* – δ-amyrin, *dam* – dammarenediol, *fri* – friedelin, *ge* – germanicol, *glu* – glutinol, *ism* – isomultiflorenol, *isotir* – isotirucallol, *ls* – lanosterol, *lu* – lupeol, *lud* – lupane-3β,20-diol, *mar* – marneral, *tal* – thalianol, *tar* – taraxasterol, *tax* – taraxerol, *tir* – tirucalla-7,24-dien-3β-ol, *n.d.* – no activity observed, *unchar.* – not biochemically characterized, *minor* – additional byproducts either reported to be of minor appearance or to represent <10% of the observed products.

| Name[a] | GenBank ID | Plant species | Gene identification method(s) | Reference(s) | |
|---|---|---|---|---|---|
| *Accurate (and putative) β-amyrin synthases* | | | | | |
| AaBAS | ACA13386 | *A. annua* | Deg. PCR/RACE | Kirby et al. (2008) | [ba] |
| AsOXA1 | AAX14716 | *A. sedifolius* | Deg. PCR/RACE | Cammareri et al. (2008) | [ba] |
| AsbAS1 | CAC84558 | *A. strigosa* | cDNA-lib. (sequencing/probing with candidate seq.) | Haralampidis et al. (2001) | [ba] |
| BgbAS | BAF80443 | *B. gymnorhiza* | Deg. PCR/RACE | Basyuni et al. (2007b) | [ba] |
| BPY | BAB83088 | *B. platyphylla* | Deg. PCR/RACE | Zhang et al. (2003) | [ba] |
| EtAS | BAE43642 | *E. tirucalli* | Deg. PCR/RACE | Kajikawa et al. (2005) | [ba] |
| GgbAS1 | BAA89815 | *G. glabra* | cDNA-lib. (probing with LUP1) | Hayashi et al. (2001a) | [ba] |
| GmAMS1[b] | AAM23264 | *G. max* | SSH/RACE | Chung et al. (2007) | [unchar.] |
| GsAS1 | ACO24697 | *G. straminea* | Deg. PCR/RACE | Liu et al. (2009) | [ba + add. minor?] |
| cOSC1 | BAE53429 | *L. japonicus* | Deg. PCR/RACE | Sawai et al. (2006a) | [ba] |
| MtAMY1 = β-AS | AAO33578 | *M. truncatula* | Deg. PCR/RACE | Iturbe-Ormaetxe et al. (2003) | [ba] |
| β-AS = MtAMY1 | CAD23247 | *M. truncatula* | EST sequence analysis | Suzuki et al. (2002) | [ba] |
| NsβAS1 | ACH88048 | *N. sativa* | Deg. PCR/RACE/cDNA-lib. (probing with candidate seq.) | Scholz et al. (2009) | [ba] |
| PNY | BAA33461 | *P. ginseng* | Deg. PCR/RACE | Kushiro et al. (1998a) | [ba] |
| PNY2 | BAA33722 | *P. ginseng* | Deg. PCR/RACE | Kushiro et al. (1998b) | [ba] |
| PSY | BAA97558 | *P. sativum* | Deg. PCR/RACE | Morita et al. (2000) | [ba] |
| SlTTS1 | ADU52574 | *S. lycopersicum* | Deg. PCR/RACE | Wang et al. (2011) | [ba] |
| SvBS | ABK76265 | *S. vaccaria* | Deg. PCR/RACE | Meesapyodsuk et al. (2007) | [ba] |
| *Accurate lupeol synthases* | | | | | |
| BgLUS | BAF80444 | *B. gymnorhiza* | Deg. PCR/RACE | Basyuni et al. (2007b) | [lu] |
| BPW | BAB83087 | *B. platyphylla* | Deg. PCR/RACE | Zhang et al. (2003) | [lu] |
| GgLUS1 | BAD08587 | *G. glabra* | cDNA-lib. (probing with OEW) | Hayashi et al. (2004) | [lu] |
| cOSC3 | BAE53430 | *L. japonicus* | Deg. PCR/RACE | Sawai et al. (2006a) | [lu] |
| OEW | BAA86930 | *O. europaea* | Deg. PCR/RACE | Shibuya et al. (1999) | [lu] |
| RcLUS | ABB76766 | *R. communis* | Deg. PCR/RACE | Guhling et al. (2006) | [lu] |
| TRW | BAA86932 | *T. officinale* | Deg. PCR/RACE | Shibuya et al. (1999) | [lu] |
| *Accurate dammarenediol synthases* | | | | | |
| CaDDS | AAS01523 | *C. asiatica* | Deg. PCR/RACE | Kim et al. (2009) | [dam] |
| PNA = DDS | BAF33291 | *P. ginseng* | Deg. PCR/RACE | Tansakul et al. (2006) | [dam] |
| DDS = PNA | ACZ71036 | *P. ginseng* | cDNA-lib. (sequencing) | Han et al. (2006) | [dam] |
| *Accurate (and putative) cycloartenol synthases* | | | | | |
| AsCS1[b] | CAC84559 | *A. strigosa* | cDNA-lib. (sequencing) | Haralampidis et al. (2001) | [n.d.] |
| CAS1/At2g07050 | NP_178722 | *A. thaliana* | cDNA-lib. (activity screen) | Corey et al. (1993) | [ca] |
| BPX | BAB83085 | *B. platyphylla* | Deg. PCR/RACE | Zhang et al. (2003) | [ca] |
| BPX2 | BAB83086 | *B. platyphylla* | Deg. PCR/RACE | Zhang et al. (2003) | [ca] |
| CaCYS[b] | AAS01524 | *C. asiatica* | Deg. PCR/RACE | Kim et al. (2005) | [unchar.] |
| CPX | BAD34644 | *C. pepo* | Deg. PCR/RACE | Shibuya et al. (2004) | [ca] |
| CsOSC1/CSI | BAB83253 | *C. speciosus* | Deg. PCR/RACE/cDNA-lib. (probing with candidate seq.) | Kawano et al. (2002) | [ca] |
| GgCAS1 | BAA76902 | *G. glabra* | cDNA-lib. (probing with *P. sativum* CAS) | Hayashi et al. (2000) | [ca] |
| KcCAS | BAF73930 | *K. candel* | Deg. PCR/RACE | Basyuni et al. (2007a) | [ca] |
| LcCAS1 | BAA85266 | *L. aegyptiaca* | cDNA-lib. (probing with *P. sativum* CAS) | Hayashi et al. (2001b) | [ca] |
| cOSC5 | BAE53431 | *L. japonicus* | Deg. PCR/RACE | Sawai et al. (2006a) | [ca] |
| PNX | BAA33460 | *P. ginseng* | Deg. PCR/RACE | Kushiro et al. (1998a) | [ca] |
| PsCAS[f] | BAA23533 | *P. sativum* | Deg. PCR/RACE | Morita et al. (1997) | [ca] |
| RsCAS | BAF73929 | *R. stylosa* | Deg. PCR/RACE | Basyuni et al. (2007a) | [ca + add. minor?] |
| RcCAS | ABB76767 | *R. communis* | Deg. PCR/RACE | Guhling et al. (2006) | [ca] |
| *Accurate (and putative) lanosterol synthases* | | | | | |
| LSS1/At3g45130 | NP_190099 | *A. thaliana* | (1, 2) Genome sequence analysis | (1) Kolesnikova et al. (2006) and (2) Suzuki et al. (2006) | [ls] |
| CPR[b] | BAD34646 | *C. pepo* | Deg. PCR/RACE | Shibuya et al. (2004) | [n.d.] |
| LcOSC2[b] | BAA85267 | *L. aegyptiaca* | cDNA-lib. (probing with *P. sativum* CAS) | Hayashi et al. (2001b) | [n.d.] |
| cOSC6[b] | BAE95409 | *L. japonicus* | *No information available* | Sawai et al. (2006b) | [n.d.] |
| cOSC7/LAS | BAE95410 | *L. japonicus* | *No information available* | Sawai et al. (2006b) | [ls + 1 minor] |
| PNZ[e] | BAA33462 | *P. ginseng* | *No information available* | Suzuki et al. (2006) | [ls] |
| TRV[b] | BAA86933 | *T. officinale* | Deg. PCR/RACE | Shibuya et al. (1999) | [n.d.] |
| *Moderate accurate OSCs* | | | | | |
| CAMS1/At1g78955 | NP_683508 | *A. thaliana* | Genome sequence analysis | Kolesnikova et al. (2007b) | [cam + 2 minor] |
| AtBAS1/At1g78950 | NP_178016 | *A. thaliana* | Genome sequence analysis | Shibuya et al. (2009) | [ba + 4 minor] |
| PEN1/At4g15340 | NP_567462 | *A. thaliana* | (1) cDNA-lib. (probing with gDNA seq.) | (1) Husselstein-Muller et al. (2001) | [ara + add. minor] |
| | | | (2, 3) Genome sequence analysis | (2) Xiang et al. (2006) and (3) Kolesnikova et al. (2007a) | |
| BARS1/At4g15370 | NP_193272 | *A. thaliana* | Genome sequence analysis | Lodeiro et al. (2007) | [bar + 22 minor] |
| PEN3/At5g36150 | NP_198464 | *A. thaliana* | Genome sequence analysis | Morlacchi et al. (2009) | [tir + 5 minor] |

(*continued on next page*)

**Table 1** (*continued*)

| Name[a] | GenBank ID | Plant species | Gene identification method(s) | Reference(s) | |
|---|---|---|---|---|---|
| THAS1/At5g48010 | NP_199612 | *A. thaliana* | Genome sequence analysis | Fazio et al. (2004) and Field and Osbourn (2008)[d] | [tal + add. minor] |
| MRN1/At5g42600 | NP_199074 | *A. thaliana* | Genome sequence analysis | Xiong et al. (2006) | [mar + 3 minor] |
| CPQ | BAD34645 | *C. pepo* | Deg. PCR/RACE | Shibuya et al. (2004) | [cur + 1 minor] |
| KdLUS | ADK35126 | *K. daigremontiana* | Deg. PCR/RACE | Wang et al. (2010) | [lu + 1 minor] |
| KdCAS | ADK35127 | *K. daigremontiana* | Deg. PCR/RACE | Wang et al. (2010) | [ca + add. minor] |
| LcIMS1 | BAB68529 | *L. aegyptiaca* | cDNA-lib. (probing with GgbAS1) | Hayashi et al. (2001b) | [ism + 1 minor] |
| StrBOS | BAH23676 | *S. rebaudiana* | Deg. PCR/RACE | Shibuya et al. (2008) | [bac + 10 minor] |
| *Multifunctional OSCs* | | | | | |
| LUP1/At1g78970 | NP_178018 | *A. thaliana* | (1, 2[c]) cDNA-lib. (probing with EST seq.) | (1) Herrera et al. (1998) and (2) Segura et al. (2000) | [lu/lud + 4 minor] |
| | | | (3) cDNA-lib. (probing with EST seq.) | (3) Husselstein-Muller et al. (2001) | |
| AtLUP2/At1g78960 | NP_178017 | *A. thaliana* | (1) cDNA-lib. (probing with LUP1) | (1) Husselstein-Muller et al. (2001) | [ba/tar + 7 minor] |
| | | | (2) YAC sequence analysis | (2) Kushiro et al. (2000b) | |
| LUP5/At1g66960 | NP_176868 | *A. thaliana* | Genome sequence analysis | Ebizuka et al. (2003) | [tir/isotir + 4 minor] |
| PEN6/At1g78500 | NP_177971 | *A. thaliana* | (1, 2) Genome sequence analysis | (1) Ebizuka et al. (2003) and (2) Shibuya et al. (2007) | [lu/bau/aa + 5 minor] |
| CsOSC2/CSV | BAB83254 | *C. speciosus* | Deg. PCR/RACE | Kawano et al. (2002) | [ba/ge/lu + add. minor] |
| LjAMY2 | AAO33580 | *L. japonicus* | Deg. PCR/RACE | Iturbe-Ormaetxe et al. (2003) | [ba/lu + 1 minor] |
| KcMS | BAF35580 | *K. candel* | Deg. PCR/RACE | Basyuni et al. (2006) | [lu/ba/aa] |
| KdTAS | ADK35123 | *K. daigremontiana* | Deg. PCR/RACE | Wang et al. (2010) | [tax/ba] |
| KdGLS | ADK35124 | *K. daigremontiana* | Deg. PCR/RACE | Wang et al. (2010) | [glu/fri/ba + 1 minor] |
| KdFRS | ADK35125 | *K. daigremontiana* | Deg. PCR/RACE | Wang et al. (2010) | [fri/ba/tax] |
| OEA | BAF63702 | *O. europaea* | Deg. PCR/RACE | Saimaru et al. (2007) | [aa/ba + 3 minor] |
| PSM | BAA97559 | *P. sativum* | Deg. PCR/RACE | Morita et al. (2000) | [aa/ba + 6 minor] |
| RsM2 | BAF80442 | *R. stylosa* | Deg. PCR/RACE | Basyuni et al. (2007b) | [tax/ba/lu] |
| RsM1 | BAF80441 | *R. stylosa* | Deg. PCR/RACE | Basyuni et al. (2007b) | [ge/ba + 1 minor] |
| SlTTS2 | ADU52575 | *S. lycopersicum* | Deg. PCR/RACE | Wang et al. (2011) | [da/aa/ba + 4 minor] |

[a] Name assigned by cited reference(s).
[b] Not biochemically characterized or inconclusive characterization study.
[c] Study of Segura et al. (2000) was based on the sequence identified by Herrera et al. (1998).
[d] *In planta* functionality study.
[e] Functionality shown by complementation of lanosterol synthase-deficient yeast strain.
[f] No name assigned by Morita et al. (1997).

*chair–chair–chair*-directing OSCs (Fig. 8), supports that the ability to synthesize β-amyrin seems to have evolved independently several times during plant evolution (Haralampidis et al., 2001; Qi et al., 2004). In fact, the tendency of *chair–chair–chair*-directing OSCs to group according to enzymatic activity in general, seems remarkably lower as compared to *chair–boat–chair*-directing cyclases. An exception appears to be a lupeol synthase clade, which comprises OSCs derived from both rosids and asterids. However, in most cases clades of *chair–chair–chair*-directing OSCs display stronger tendency to group according to plant phylogeny rather than to functionality. For instance, the 11 multifunctional or moderately accurate *A. thaliana* OSCs, which occur besides cycloartenol and lanosterol synthase, group into two separate, well-defined clades. However, the product spectrum of some OSCs appears to resemble the spectra of OSCs of different phylogenetic origin more than that of their closest homologs within these clades (Fig. 8). Thus, the heterogeneity of OSCs that catalyze *chair–chair–chair*-directed cyclization seems to result not only from divergent but also convergent evolution. The observation that only a few amino acid substitutions can significantly change the product spectrum of OSCs (e.g. Kushiro et al., 2000a; Lodeiro et al., 2005; Matsuda et al., 2000; Segura et al., 2003) further underlines the high potential of OSCs in shifting activity during evolution. Accordingly, multifunctional OSCs may represent evolutionary intermediates (Phillips et al., 2006) rather than enzymes optimized to provide specific sets of cyclization products. For identification of OSCs involved in saponin biosynthesis, these findings are also crucial since they further indicate that deduction of the function of biochemically uncharacterized OSCs from sequence similarity alone might be misleading.

### 4.3. Decoration of the cyclization product – elaboration of sapogenin diversity

Following formation of basal sapogenin backbone structures mediated by OSCs, these common precursors usually undergo various modifications prior to glycosylation. The types and extent of modifications greatly vary between different saponin-producing plant species (reviewed in Vincken et al., 2007), and thus sapogenin decoration contributes considerably in increasing the structural diversity of saponins. Decoration of similar cyclization products is also found to vary among organs and tissues of individual plants (Huhman et al., 2005), as well as between intact plants and derived cell cultures (Hayashi et al., 1990, 1992). This further indicates that regulation of sapogenin modification is an additional possibility for plants to maintain specialized pools of saponins with discrete properties.

The most common sapogenin modifications are small functional groups such as hydroxyl-, keto-, aldehyde- and carboxyl-moieties at various positions of the backbone. In some cases, these primary

modifications enable further diversification. Such secondary decoration steps may involve linkage of more complex side groups such as 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) and *N*-methyl anthranilic acid, found in saponins isolated from legumes (Fabaceae; Heng et al., 2006) and oats (*Avena* spp.; Osbourn, 2003), respectively. Formation of intramolecular bridges between adjacent functional groups via ether, ester or acetal linkages represents another type of secondary modification. Lastly, post-cyclization modification of sapogenin skeletons may also involve saturation as well as desaturation events, as indicated by variation in number and position of double bonds between sapogenins of similar cyclization types (Vincken et al., 2007).

Although, this last type of modification could partially be derived from the variability in OSC-mediated cyclization termination, changes in backbone saturation may also involve activity of enzymes downstream 2,3-oxidosqualene cyclization. The introduction of intramolecular bridges as well as the addition of more complex side groups following primary oxidation steps is expected to involve enzymes of diverse biochemical background. For instance, a serine carboxypeptidase-like protein (SCPL1/Sad7) from oat (*A. strigosa*) has recently been shown to catalyze coupling of *N*-methyl anthranilic and benzoic acid to sapogenins in avenacin biosynthesis (Mugford et al., 2009). However, most sapogenin modifications are expected to be introduced by enzymes belonging to the class of cytochromes P450 (P450s, EC 1.14.x.x).

P450s are heme–thiolate monooxygenases characterized by their ability to catalyze oxidative attacks of molecular oxygen towards non-activated hydrocarbons (Werck-Reichhart and Feyereisen, 2000). In addition to hydroxylations and epoxidations P450-catalyzed reactions can be more complex including, e.g., dealkylations, isomerisations, decarboxylations, ring extensions and dimerizations (summarized in Ehlting et al., 2006; Morant et al., 2003). In plants, P450s are involved in numerous metabolic processes of primary and secondary metabolism such as anabolism and catabolism of phytohormones, fatty acids, detoxification of xenobiotics and biosynthesis of a vast array of secondary metabolites (Schuler and Werck-Reichhart, 2003). In view of this broad functional diversity, it is not surprising that P450s represent one of the largest and most diverse gene families in plants constituting up to 1% of all protein encoding sequences (Paquette et al., 2009; Wortman et al., 2003). For example, the *A. thaliana* genome contains 272 P450 genes (including 26 pseudogenes) (Werck-Reichhart et al., 2002) and the *Oryza sativa* genome at least 455 (including 99 pseudogenes) (Nelson et al., 2004).

P450s are named according to a canonical nomenclature by the P450 nomenclature committee (Nelson et al., 2004; Nelson, 2009), based on amino acid sequence identity. The root symbol 'CYP', designates a P450 and is followed by a numeral identifier of the family, a letter representing the subfamily and the number of the gene within this subfamily (e.g. CYP93E1). Sequences belonging to the same family generally exhibit >40% amino acid sequence identity among each other, whereas genes belonging to the same subfamily are at least 55% identical. Based on phylogeny, the different P450 families can be grouped into discrete clans (or clades). Individual clans are monophyletic and named after the family containing the lowest numeric identifier. Thus, *A. thaliana* P450s group into 10 different clans. Monocotyledonous plants possess an additional clan, increasing the current number of plant P450 clans to 11.

It is the large number of P450 genes and the wide diversity within the P450 multigene family that hampers identification of individual genes responsible for specific reactions based on simple homology. To date only three publications are available that report the identification of P450s involved in biosynthesis of triterpenoid saponins (Table 2). The first sapogenin-modifying P450, CYP93E1, was identified by Shibuya et al. (2006) in soybean (*Glycine max*). CYP93E1 catalyzes the hydroxylation of β-amyrin and its 22-hydroxy derivate sophoradiol at position C24 (Fig. 9), suggesting that CYP93E1 is involved in biosynthesis of soyasaponins, which are oleanane saponins found in several legumes (Fabaceae). Qi et al. (2006) reported the identification of CYP51H10 (Sad2) after sequencing of a molecular marker segregating with the *Sad* gene cluster in oat (*A. strigosa*). This operon-like gene cluster is of major interest for elucidation of saponin anabolism, as it is expected to contain most of the genes involved in avenacin biosynthesis (Osbourn and Field, 2009; Qi et al., 2004). Accumulation of mainly β-amyrin and low amounts of 23-hydroxy-β-amyrin in *sad2* mutants (Qi et al., 2006; Qin et al., 2010) indicates that CYP51H10 catalyzes an early step in avenacin sapogenin biosynthesis. However, the precise biochemical reaction mediated by CYP51H10 still remains to be elucidated experimentally. Lastly, Seki et al. (2008) identified CYP88D6 as the P450 in liquorice (*Glycyrrhiza uralensis*) responsible for C11 oxygenation of β-amyrin in the biosynthetic pathway of glycyrrhetinic acid. In this study, putative P450 ESTs were selected based on correlation of *in planta* expression with the distribution of saponins in different plant tissues. The corresponding P450 candidates were subsequently heterologeously expressed and biochemically characterized. In addition to CYP88D6, Seki et al. (2008) identified CYP93E3 as a second sapogenin-modifying P450 from liquorice, which like CYP93E1 in soybean, catalyzes 24-hydroxylation of β-amyrin. The high sequence identity (82% at the amino acid level) of CYP93E1 and CYP93E3, as well as their similar catalytic activity indicates that they are close homologs or even orthologs. In addition to the P450s mentioned so far that hydroxylate either β-amyrin or one of its derivatives, CYP705A5 and CYP708A2 are involved in the modification of the tricyclic 2,3-oxidosqualene cyclization product thalianol in *A. thaliana* (Field and Osbourn, 2008). However, no saponins derived from thalianol have been described in the literature.

The three, considering CYP93E1 and CYP93E3 as orthologs, known P450s in saponin biosynthesis originate from distantly related phylogenetically families and clans, and the individual subfamilies seem to be restricted to specific plant families. The CYP51H subfamily (CYP51 clan) has only been reported in cereals such as oat (*Avena* spp.) and rice (*O. sativa*) within the monocotyledonous Poaceae (Nelson et al., 2008; Qi et al., 2006). Similarly, the CYP88D subfamily (CYP85 clan) appears to be restricted to Fabaceae (Seki et al., 2008). Lastly, BLAST searches of CYP93E1 and CYP93E3 (CYP71 clan) against the current NCBI (nr) database suggest that the CYP93E subfamily is also Fabaceae specific. The only known close homolog of CYP93E1 and CYP93E3 is the yet biochem-

**Table 2**
Overview of so far identified sapogenin-modifying P450s.

| Name | GenBank ID | Plant species | Gene identification method(s) | Reference(s) |
|---|---|---|---|---|
| CYP51H10[a] | ABG88961 | *A. strigosa* | RFLP marker sequencing | Qi et al. (2006) |
| CYP93E1 | BAE94181 | *G. max* | EST screening (homology search for P450s of the CYP93 family)[b] | Shibuya et al. (2006) |
| CYP93E3 | BAG68930 | *G. uralensis* | Deg. PCR/RACE (primer based on CYP93E1/CYP93E2) | Seki et al. (2008) |
| CYP88D6 | BAG68929 | *G. uralensis* | EST screening (homology search for P450s)/coexpression to product localisation | Seki et al. (2008) |

[a] Not biochemically characterized.
[b] Sequence identified by Steele et al. (1999).

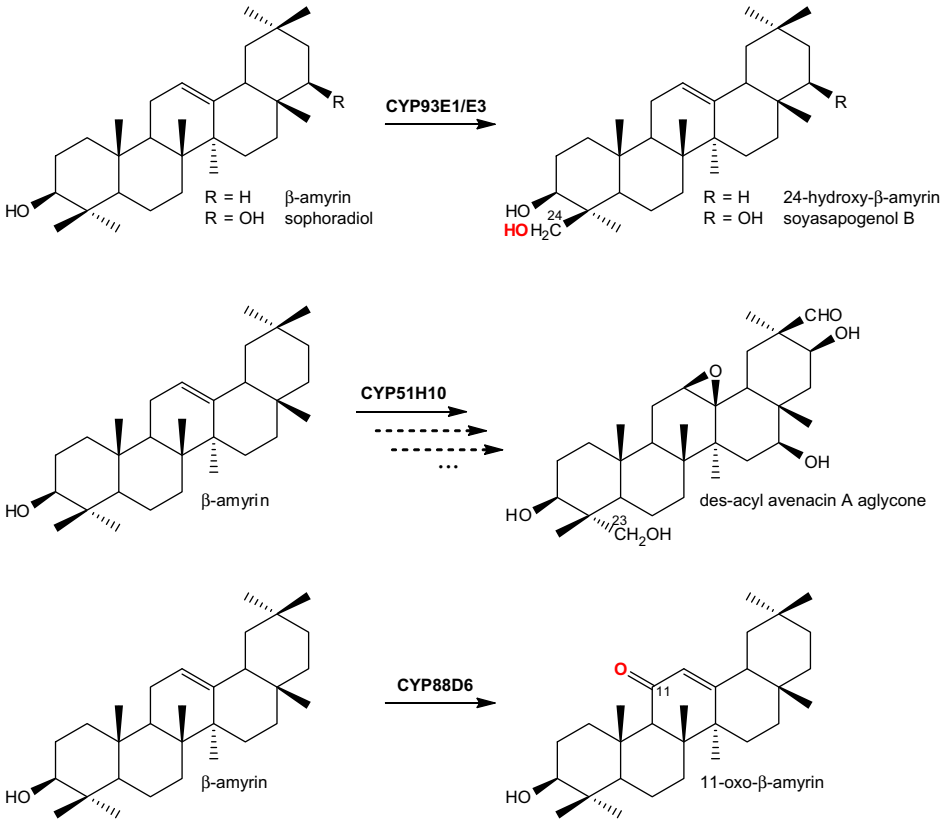


**Fig. 9.** Catalytic activities of P450s involved in saponin biosynthesis. The function of CYP93E3 has only been assayed with β-amyrin, and the specific reaction catalyzed by CYP51H10 in avenacin biosynthesis has not been elucidated. Accumulation of mainly β-amyrin and low amounts of 23-hydroxy-β-amyrin in *CYP51H10* mutant lines indicates involvement in an early biosynthetical step (biochemical activities are according to Seki et al., 2008; Shibuya et al., 2006; Qi et al., 2006).

ically uncharacterized CYP93E2 from *Medicago truncatula*. A large-scale co-expression study, based on inducibilty of gene expression concurrent with saponin biosynthesis elicitation in *M. truncatula* root cell suspension cultures, recently indicated that *CYP93E2* is also involved in saponin biosynthesis (Naoumkina et al., 2010). This suggests that CYP93Es may perform C24-hydroxylation as part of soyasaponin biosynthesis throughout the Fabaceae.

In conclusion, considering the low degree of relatedness of the currently known sapogenin-metabolizing P450s, enzyme recruitment for triterpenoid saponin biosynthesis appears to have occurred multiple times during evolution and is not restricted to a specific phylogenetic origin. Based on the known P450s in saponin pathways, the common denominator of gene recruitment seems to be the ability to process substrates that share some of their sterical properties and metabolic origin with sapogenins. For instance, P450s of the CYP51 family catalyze the essential 14α-demethylation in sterol biosynthesis (Bak et al., 1997; Lepesheva and Waterman, 2007). Other known CYP88s are involved in biosynthesis of gibberellins, which are diterpenoid compounds involved in plant regulatory processes (Davidson et al., 2003; Helliwell et al., 2001). Furthermore, other P450 families, which as CYP88s belong to the CYP85 clan, are also known for their role in the metabolic pathways of the sterol-derived brassinosteroids (Werck-Reichhart et al., 2002). Lastly, P450s from other CYP93 subfamilies have mainly been found to be involved in (iso)flavonoid biosynthesis (Shibuya et al., 2006; Steele et al., 1999). However, due to the limited number of identified P450s in saponin pathways the current conclusions about P450 evolution in saponin biosynthesis are rather preliminary. Moreover, the modifications catalyzed by these enzymes such as 24-hydroxylation and 11-oxidation are relatively rare among saponins (Vincken et al., 2007). Hence, future research will clarify if more common modifications as, for instance, 23-hydroxylation and 28-carboxylation of the oleanane backbone, are catalyzed by more conserved P450s throughout sapogenin-producing plants.

### 4.4. Sapogenin glycosylation – conferring of biological activity

Glycosylation patterns of saponins are often considered crucial for their biological activities. The introduction of saccharide side chains to the sapogenin backbone usually succeeds P450-catalyzed introduction of hydroxy- and carboxy-moieties, and consequently finalizes saponin biosynthesis. However, this is not necessarily always the case as, e.g., shown for biosynthesis of the cardiac glycoside digoxin in *Digitalis lantana*, which structurally resembles steroidal saponins, where the P450-mediated 12β-hydroxylation of the aglycone backbone succeeds glycosylation of the oxidosqualene-derived C3 hydroxy group (Petersen and Seitz, 1985, 1988). Similar observations have not been reported for triterpenoid saponins, but could be envisioned.

Typical triterpenoid saponin glycosylation patterns consist of oligomeric sugar chains, 2–5 monosaccharide units, which are most often linked at positions C3 and/or C28. Less often 1–2 monosaccharide units have been reported to occur at the positions C4, C16, C20, C21, C22 and/or C23. Glucose, galactose, glucoronic acid, rhamnose, xylose and arabinose are the most abundant hexoses and pentoses in the saccharide chains. In more rare cases also fucose, quinovose, ribose and apiose may be incorporated (Hostettmann and Marston, 1995; Tava et al., in press; Vincken et al., 2007).

Saponin glycosylation presumably involves sequential activity of different enzymes belonging to the multigene family of family 1 uridin diphosphate glycosyltransferases (UGTs, EC 2.4.1.x). UGTs

catalyze the transfer of activated glycosyl residues from uridine sugar nucleotides to a vast array of low molecular weight acceptor molecules (Ross et al., 2001; Vogt and Jones, 2000). Glycosylation of metabolites and xenobiotics is generally thought to modulate stability, biological activity, solubility, and signaling for storage or intra- and intercellular transport. Accordingly, UGTs are involved in regulatory and metabolic pathways by activating, deactivating or solubility-modifying a diverse set of compounds.

The diverse role of UGTs in plants is reflected by the large number of genes belonging to this multigene family with, e.g., 120 genes (including eight pseudogenes) in *A. thaliana* (Paquette et al., 2003). The nomenclature for UGTs (Mackenzie et al., 1997, 2005) widely resembles the nomenclature applied for P450s, where UGTs with higher than 40% amino acid identity belong to the same family, but with a subfamily cut-off at 60%.

As for P450s, the large number of UGT genes hampers identification of individual members involved in specific pathways by homology only. Early approaches to investigate UGT activity in saponin biosynthesis were based on characterization of cellular subfractions or chromatography-based protein purification (reviewed in Kalinowska et al., 2005). Interestingly, some of these studies indicated sapogenin glycosylation to be catalyzed by membrane-bound enzymes (e.g. Kurosawa et al., 2002; Wojciechowski, 1975) although plant UGTs, with the exception of UDP–glucose:sterol glucosyltransferases and monogalactosyldiacylglycerol synthases, are generally considered to be soluble cytosolic proteins.

The first sequences of triterpenoid sapogenin-glycosylating UGTs, UGT71G1 and UGT73K1, were cloned by Achnine et al. (2005). Both genes were identified by screening *M. truncatula* EST collections for UGT-encoding sequences that were co-regulated with the corresponding β-amyrin synthase (β-AS/MtAMY1). *In vitro* studies revealed UGT71G1 and UGT73K1 to catalyze the transfer of glucosyl residues to different oleanane type sapogenins found in *Medicago* species such as medicagenic acid (UGT71G1), hederagenin (UGT73K1, UGT71G1) and soyasapogenol E and B (UGT73K1) (Fig. 10). However, the position of the attached glucosyl moieties was not determined. The gene expression profile in response to different elicitors for both these UGTs is consistent with a role in biosynthesis of triterpenoid saponins rather than in glycosylation of (iso)flavonoids. However, *in vitro* assays of heterologously expressed UGT71G1 showed significantly higher activity towards (iso)flavonoids than towards sapogenins.

Another sapogenin-modifying glycosyltransferase, UGT74M1, was identified by screening for UGT sequences among ESTs derived from saponin-producing *Saponaria vaccaria* tissue (Meesapyodsuk et al., 2007). UGT74M1 was found to glucosylate different sapogenins that occur in distinct tissues and in the seeds. More detailed studies revealed that UGT74M1 catalyzes linkage of glucosyl-moieties to the C28-carboxy group of preferably oleanane type sapogenins through an ester bond. Interestingly, although the expression pattern of UGT74M1 showed substantial co-regulation with a simultaneously identified β-amyrin synthase (SvBS), the UTG74M1 transcript was not detected in all tissues where SvBS was expressed. Such observations indicate that plants can regulate downstream processes in saponin biosynthesis to generate different pools of saponins in distinct organs and tissues.

Two recent publications report the identification of further UGTs involved in saponin biosynthesis. *M. truncatula* UGT73F3 catalyzes glucosylation of hederagenin and other oleanane sapogenins at the C28-carboxy group (Naoumkina et al., 2010), similar to UGT74M1 in *S. vaccaria*. The observed decrease of saponins glucosylated at the C28 position in roots of *UGT73F3* knockout lines strongly supports a role of UGT73F3 in saponin biosynthesis *in planta*. Retarded plant development accompanied the modified chemotype of UGT73F3-deficient plants, which further indicates the importance of coordinated UGT activity to protect plants from potentially toxic intermediates of saponin biosynthesis. The observation of impaired root development related to accumulation of monodeglucosyl avenacin A-1 in *A. strigosa sad3* and *sad4* mutants supports this idea (Mylona et al., 2008). However, functionality of Sad3 and Sad4 has not yet been elucidated at the molecular level, and the reason for the accumulation of incompletely glycosylated saponins in *sad3* and *sad4* mutants therefore remains unknown. The microarray-based gene expression study by Naoumkina et al. (2010) preceding identification of UGT73F3, also indicated UGT73P3, UGT91H5 and UGT91H6 as potentially involved in saponin biosynthesis. Contravening this, no activity was observed for UGT73P3, UGT91H5 and UGT91H6 towards different sapogenins in *in vitro* studies. However, in parallel Shibuya et al. (2010) identified two close soybean (*G. max*) homologs, UGT73P2 and UGT91H4, involved in assembling the saccharide chain of soyasaponin I (Fig. 11). Thus, UGT73P2 extends the C3 saccharide moiety of the monoglycosylated oleanane aglycone soyasapogenol B with a galactosyl residue added to the previously bound glucuronyl moiety. Subsequently, UGT91H4 catalyzes the third glycosylation step in soyasaponin I biosynthesis by transferring rhamnosyl to the galactosyl residue of the diglycosylated soyasaponin III. Since both *M. truncatula* and *G. max* belong to the Fabaceae and partially produce identical saponins, including soyasaponin I, similar functionality of the *M. truncatula* glycosyltransferases UGT73P3, UGT91H5 and UGT91H6 seems plausible.

A phylogenetic analysis of plant UGT sequences showed UGTs to cluster into discrete groups, denoted from A to L, some of which emerged prior to bifurcation of monocotyledons and dicotyledons (Bowles et al., 2005; Li et al., 2001). It further appeared that distinct functionality such as transfer of rhamnosyl moieties to prior bound glucose residues and formation of ester bonds may be characteristic traits for specific monophylogenetic groups, as in this case group A and L, respectively. In agreement with this assumption UGT91H4, which extends a saccharide side chain by addition of rhamnosyl, belongs to group A and the ester linkage forming UGT74M1 clusters to group L. However, UGT73F3, which exhibits similar catalytic activity as UGT74M1, clearly does not belong to group L. Thus, the functionality of UGT73F3 seems to have emerged by convergent evolution. In fact, most of the identified sapogenin-modifying UGTs appear to belong to the UGT73 family (Table 3), which solely forms the phylogenetic group D. Other characterized UGTs of this family such as UGT73C5, UGT73C6, UGT73C8, UGT73F1 and UGT73P1 have been reported to be involved in glycosylation of brassinosteroids (Poppenberger et al., 2005) and (iso)flavonoids (Jones et al., 2003; Modolo et al., 2007 and cited references herein; Nagashima et al., 2004), respectively. Therefore, like for P450s, recruitment of UGTs for saponin biosynthesis appears to originate from enzymes processing substrates with comparable chemical and sterical properties regardless of their phylogenetic background. The apparent low correlation between catalytic activity and phylogeny of UGTs was also pointed out by Modolo et al. (2007) as a result of comparing the phylogeny of different *M. truncatula*-derived (iso)flavonoid UGTs. Moreover, a comprehensive *in vitro* screen, comprising 91 out of 112 *A. thaliana* UGTs, showed glycosylation activity towards the flavonol quercetin to be rather widespread among the assayed glycosyltransferases (Lim et al., 2004). Accordingly, 29 of them, belonging to different phylogentic groups, were found to catalyze glucosylation of quercetin, although with varying efficiency and position specificity. Consequently, it is to be expected that sapogenin processing UGTs will also be spread over different phylogenetic groups. However, plant UGTs are often remarkably promiscuous, thus accepting several glycosyl acceptor substrates rather than being restricted to one highly specific structure and therefore, activities shown in *in vitro* assays do not necessarily reflect *in planta* functionality (Hansen et al., 2003; Kramer et al., 2003; Modolo et al., 2007; Vogt

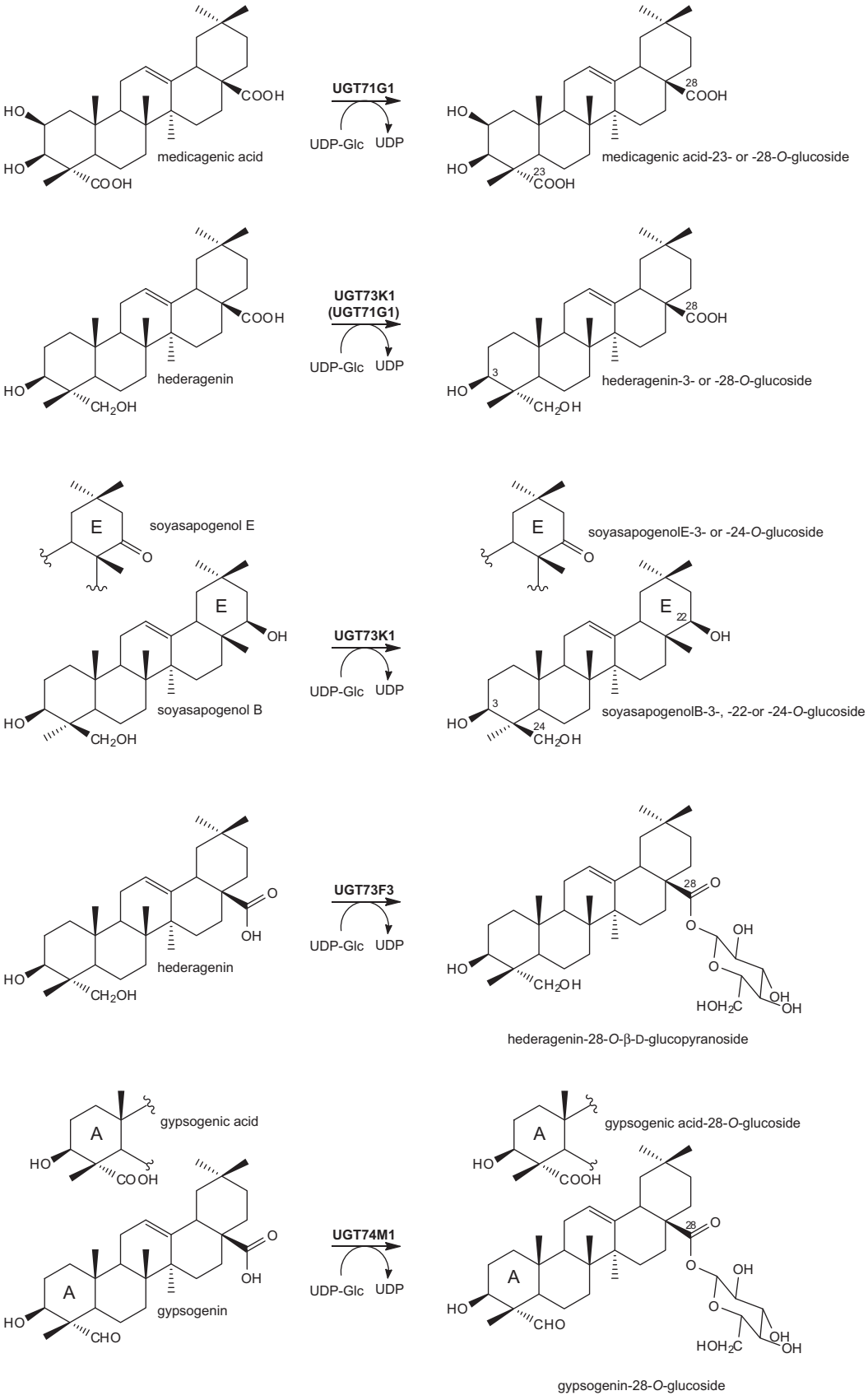


**Fig. 10.** Glycosylation of oleanane type sapogenins by UGTs in triterpenoid saponin biosynthesis. The position specificity of UGT71G1 and UGT73K1 has not been elucidated. Putative glycosylation positions suggested by the authors are indicated by numbering (biochemical activities are according to Achnine et al., 2005; Meesapyodsuk et al., 2007; Naoumkina et al., 2010).

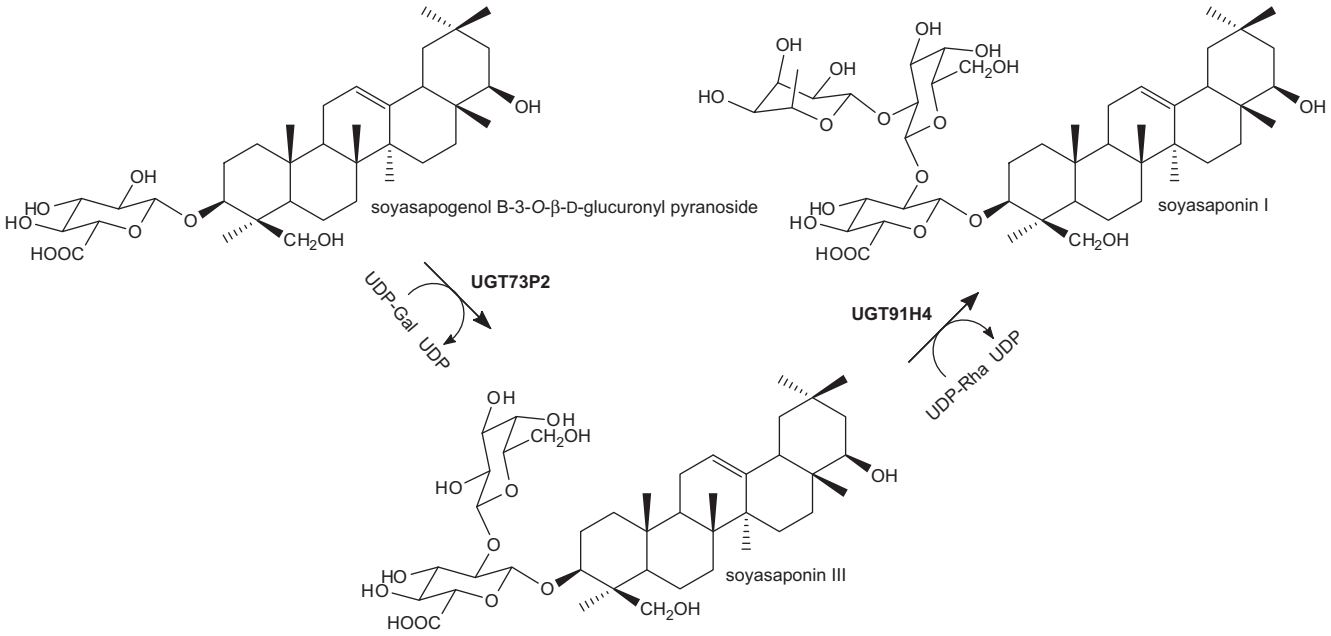


**Fig. 11.** Saccharide chain elongation during soyasaponin I biosynthesis in *Glycine max* catalyzed by UGT73P2 and UGT91H4 (Shibuya et al., 2010).

**Table 3**
Overview of known UGTs involved in triterpenoid saponin biosynthesis.

| Name | GenBank ID | Plant species | Gene identification method(s) | Reference(s) |
|---|---|---|---|---|
| UGT73P2 | BAI99584 | *G. max* | EST screening (abundance + occurrence of *M. trunc.* homologs) | Shibuya et al. (2010) |
| UGT91H4 | BAI99585 | *G. max* | EST screening (abundance + occurrence of *M. trunc.* homologs) | Shibuya et al. (2010) |
| UGT71G1[a] | AAW56092 | *M. truncatula* | EST screening (elicited expression modulation + coexpression with β-AS) | Achnine et al. (2005) |
| UGT73K1[a] | AAW56091 | *M. truncatula* | EST screening (elicited expression modulation + coexpression with β-AS) | Achnine et al. (2005) |
| UGT73F3 | ACT34898 | *M. truncatula* | Gene expression analysis (elicited expression modulation) | Naoumkina et al. (2010) |
| UGT74M1 | ABK76266 | *S. vaccaria* | EST screening (abundance in saponin producing tissue) | Meesapyodsuk et al. (2007) |

[a] Position specificity not elucidated.

and Jones, 2000). The biotechnologically motivated study by Lim et al. (2004) was performed with un-physiologically high acceptor substrate concentrations of 500 μM and consequently, the majority of quercetin-glucosylating UGTs identified in this study may not necessarily be involved in flavonol glycosylation *in planta*. In a similar manner, glycosylation activity towards sapogenins observed in *in vitro* studies may be the result of substrate promiscuity. To conclusively establish the involvement of specific UGTs in saponin biosynthesis *in planta* additional approaches are needed.

From an evolutionary perspective, substrate promiscuity of UGTs has been proposed to enable flexibility of plants to cope with newly arising metabolites and xenobiotics (Vogt and Jones, 2000). Considering the emergence and diversification of sapogenins to be caused by arbitrary changes in the activities of OSCs and P450s, initially promiscuous UGTs can be imagined to have been capable of glycosylating such newly evolved saponin precursors. Subsequently, gradual adaptation of gene regulation and substrate specificity of such promiscuous UGTs were favored during evolution finally resulting in acquiring a new role in saponin biosynthesis. Enzymes, such as UGT71G1, that exhibit *in vitro* higher activity towards (iso)flavonoids than towards sapogenins but where gene expression studies suggest the involvement in saponin biosynthesis, may indicate their recruitment for saponin pathways as a result of comparably recent evolutionary events.

## 5. Conclusions

Enzymes belonging to the multigene families of oxidosqualene cyclases, cytochromes P450 and UDP-glycosyltransferases are key players in biosynthesis of plant triterpenoid saponins. The number of identified enzymes involved in saponin pathways derived from these gene families has increased considerably over the last decade, which now allows a first assessment of the evolution of saponin biosynthesis. Thus, despite the fact that members of these three multigene families presumably perform comparable reactions throughout all saponin producing plants within the division of Magnoliophyta, it seems unlikely that saponin pathways have diverged from an ancient pathway prior to the division of dicotyledons and monocotyledons. In fact, although closely related plants within the same order (e.g. the Fabaceae) appear to employ homologous genes for the synthesis of structurally closely related or identical saponins, in particular the known P450s and UGTs in saponin pathways often show a rather diverse phylogenetic origin. In conclusion, based on the current knowledge, biosynthesis of triterpenoid saponins seems to have evolved independently several times during plant evolution.

The phylogeny of OSCs suggests independent development of cyclases with similar functionalities among different plant species, and several OSCs are known to give rise to more than one cyclization product. Both findings indicate the potential of OSCs to shift their product spectrum rather rapidly during evolution and thereby provide plants with alternating types of oxidosqualene cyclization products. The presence of triterpenoids such as β-amyrin, α-amyrin and lupeol in plants that do not produce corresponding saponins further supports this assumption. Subsequently, plants may have recruited P450s and UGTs, often originating from metabolic or regulatory pathways of chemically and sterically related compounds, to introduce modifications

eventually leading to the accumulation of saponins. Advantages in plant defense towards pests and herbivores conferred by these first saponins presumably favored conservation of the corresponding pathways during evolution once they had emerged. Ongoing neo- and subfunctionalization of genes within saponin biosynthesis further allowed plants to constantly evolve new defense strategies.

The proposed convergent evolution of metabolic pathways leading to accumulation of triterpenoid saponins, and the putatively diverged phylogenetic origin of the enzymes involved, hampers prediction of yet undiscovered genes by sequence similarity. Of the three multigene families known to be involved, most knowledge has been gained for OSCs. The limited number of OSC genes within plant genomes enables homology-based gene discovery approaches such as degenerative primers, hybridization-based cDNA library screenings and mining of genomic or transcriptomic datasets. However, elucidation of OSC functionality requires heterologous expression and biochemical analysis of the enzymes encoded. This constraint results from the observation that even minor changes in the amino acid composition may, due to the negative catalysis-based mechanism of OSCs, lead to significant changes in their product profiles. In contrast to the situation for OSCs, the diversity and extensive number of UGTs and in particular P450s, is a major obstacle for gene discovery. Accordingly, identification of P450s and UGTs involved in specific pathways faces both the challenge of identifying genes belonging to these multigene families in genomes of often still unsequenced organisms and the selection of a reasonable number of candidate sequences for characterization studies. Lastly, while all OSCs share a common substrate, identification and availability of intermediates metabolized by individual P450s and UGTs in saponin pathways represent further obstacles for gene discovery. To date, putative P450s and UGTs from plants with unsequenced genomes are most often identified by screening of EST databases for homologous sequences of known members of these multigene families from other plants. Subsequent selection of candidate sequences for *in vitro* studies often involves comparison of the expression pattern with other genes involved in saponin biosynthesis (e.g. β-amyrin synthases) or correlation of transcript abundance with saponin accumulation in different plant tissues. Major improvements and broader availability of next generation sequencing techniques in recent years will presumably accelerate gene discovery from plants with unsequenced genomes (e.g. Li et al., 2010; Sun et al., 2010a). At the same time, DNA microarray based gene expression cluster analysis was proven a viable tool for large-scale identification of candidate sequences (Naoumkina et al., 2010). Alternative strategies may include a combination of classic protein purification techniques coupled to mass spectrometry based peptide sequencing (reviewed in Koomen et al., 2005) to indentify candidate sequences within EST databases based on observed activities of protein extracts. Finally, usage of genetic maps to identify genome regions responsible for saponin biosynthesis may also facilitate gene discovery by focusing candidate searches to sequences located in defined parts of the genome (Qi et al., 2004; Kuzina et al., 2011).

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