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**To cite this article:** Tessa Moses, Kalliope K. Papadopoulou & Anne Osbourn (2014) Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives, *Critical Reviews in Biochemistry and Molecular Biology*, 49:6, 439-462, DOI: [10.3109/10409238.2014.953628](https://doi.org/10.3109/10409238.2014.953628)

**To link to this article:** <https://doi.org/10.3109/10409238.2014.953628>



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Published online: 06 Oct 2014.



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REVIEW ARTICLE

## Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives

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### Abstract

Saponins are widely distributed plant natural products with vast structural and functional diversity. They are typically composed of a hydrophobic aglycone, which is extensively decorated with functional groups prior to the addition of hydrophilic sugar moieties, to result in surface-active amphipathic compounds. The saponins are broadly classified as triterpenoids, steroids or steroidal glycoalkaloids, based on the aglycone structure from which they are derived. The saponins and their biosynthetic intermediates display a variety of biological activities of interest to the pharmaceutical, cosmetic and food sectors. Although their relevance in industrial applications has long been recognized, their role in plants is underexplored. Recent research on modulating native pathway flux in saponin biosynthesis has demonstrated the roles of saponins and their biosynthetic intermediates in plant growth and development. Here, we review the literature on the effects of these molecules on plant physiology, which collectively implicate them in plant primary processes. The industrial uses and potential of saponins are discussed with respect to structure and activity, highlighting the undoubted value of these molecules as therapeutics.

### Keywords

Glycoalkaloid, plant development, plant growth, steroid, structure–activity relationships, triterpenoid

### History

Received 25 June 2014  
Revised 1 August 2014  
Accepted 7 August 2014  
Published online 6 October 2014

### Introduction

Saponins are naturally occurring structurally and functionally diverse phytochemicals that are widely distributed in plants. They are a complex and chemically varied group of compounds consisting of triterpenoid or steroidal aglycones linked to oligosaccharide moieties. The combination of a hydrophobic aglycone backbone and hydrophilic sugar molecules makes the saponins highly amphipathic and confers foaming and emulsifying properties. These surface-active molecules have important roles in plant ecology and are also exploited for a wide range of commercial applications in the food, cosmetic and pharmaceutical sectors. Saponins in *Quillaja saponaria* extracts are used as foaming agents in carbonated beverages and cosmetics, as emulsifiers in preparations containing lipophilic colors or flavors, as preservatives, and for removal of dietary cholesterol (Güçlü-Ustündağ & Mazza, 2007; San Martín & Briones, 1999). Likewise, licorice saponin extracts are used as flavor modifiers in baked foods, chewing gum, beverages, candies, herbs, seasonings and dietary supplements (Güçlü-Ustündağ & Mazza, 2007). Saponins are also the major constituents of traditional folk medicines such as the ginsenosides produced by *Panax* species (Qi *et al.*, 2011). A number of therapeutic properties have been ascribed to

saponins (Francis *et al.*, 2002). These molecules are potent membrane permeabilizing agents. They are also immunostimulatory, hypocholesterolemic, anti-carcinogenic, anti-inflammatory, anti-microbial, anti-protozoan, molluscicidal and have anti-oxidant properties. Furthermore, they can impair gut protein digestion, and uptake of vitamins and minerals. In general, the saponins and their biosynthetic intermediates display an array of properties that can have positive or negative effects in different animal hosts (Francis *et al.*, 2002; Sparg *et al.*, 2004).

Although the saponins are one of the largest classes of plant natural products, their biological functions are not completely understood. They are generally considered to have important roles in defense of plants against pathogens, pests and herbivores due to their antimicrobial, antifungal, antiparasitic, insecticidal and anti-feedant properties (Augustin *et al.*, 2011; Morrissey & Osbourn, 1999; Osbourn *et al.*, 2011; Sparg *et al.*, 2004). Many plants synthesize and accumulate saponins during normal growth and development. The distribution of these natural products varies greatly among plant species, individual plants, organs and tissues, during development and maturation, and shows seasonal fluctuations. Some studies have suggested that variations in saponin distribution, composition and amounts in plants may be a reflection of varying needs for plant protection. In *Phytolacca dodecandra* and *Dioscorea pseudojaponica*, maximal saponin accumulation has been noted during fruit and tuber development, and has been suggested to

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prevent fruit loss, ensure seed maturation and protect reproductive organs (Lin *et al.*, 2009; Ndamba *et al.*, 1994). However, in several plant species, the production of saponins is induced in response to biotic stress including herbivory and pathogen attack. Abiotic stress factors such as humidity, nutrient starvation, light and temperature can influence both the quality and quantity of saponin content (De Costa *et al.*, 2013; Szakiel *et al.*, 2011). Increase in saponin levels in response to stress is often mediated by the transcriptional activation of biosynthetic genes through a complex signaling cascade involving the jasmonate and salicylate hormones. Hence, the biosynthesis of these molecules can be induced using elicitors and this feature has been exploited in several plant species to improve saponin yields (Shabani *et al.*, 2009; Yendo *et al.*, 2010).

Here, we provide a brief introduction to the vast diversity of saponin structures that are synthesized and accumulated by different plant species. Emphasis is given to differences in the composition and quantity of these natural products over different phases of plant growth, development and maturation. We provide a constructive framework to suggest that these compounds, which are traditionally regarded as secondary metabolites, have important roles in plants at various stages of development, including seed germination, vegetative growth and differentiation, fruiting and nodulation. A growing number of studies have reported effects on plant growth and development due to perturbation of saponin biosynthesis, suggesting roles for these molecules and their intermediates in primary plant processes. With these data, we highlight that maintaining a delicate balance of saponin biosynthesis is essential for overall plant integrity and well-being. The industrial applications of saponins, their intermediates and derivatives are also reviewed, together with structure–activity relationship data, to provide the reader with a perspective on the importance of this class of metabolites for pharmaceutical applications.

### Biosynthesis of saponins

Most known saponins are found in angiosperms (Magnoliophyta), though some marine invertebrates such as sea cucumbers (Holothuroidea) and starfish (Asteroidea) also produce these molecules (Bordbar *et al.*, 2011; Liu *et al.*, 2008; Osbourn *et al.*, 2011; Williams & Gong, 2007). While the synthesis of saponins is widespread in plants, the majority of the producing plant species is dicotyledonous and accumulates mainly triterpenoid-type saponins. The monocotyledonous angiosperms on the other hand mostly, but not exclusively, synthesize steroidal-type saponins. This broad classification of saponin types is based on the nature of the aglycone backbone from which the saponin molecule is derived. Both triterpenoid and steroidal aglycone backbones are derived from the 30-carbon linear precursor 2,3-oxidosqualene. During the synthesis of the committed precursor, the steroidal aglycone loses three methyl groups to result in a 27-carbon backbone, whereas the triterpenoid aglycone retains all 30 carbons in its backbone. The steroidal glycoalkaloids, which are also sometimes referred to as saponins, share their biosynthetic origin with the steroidal saponins and contain a characteristic nitrogen atom

incorporated into the aglycone backbone (Augustin *et al.*, 2011; Friedman, 2006; Ginzberg *et al.*, 2009; Itkin *et al.*, 2013).

The triterpenoid and steroidal aglycone backbones are isoprenoids that are synthesized from isopentenyl pyrophosphate (IPP) units generated by the mevalonate (MVA) pathway (Figure 1). The multistep MVA/3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) pathway catalyzes the conversion of acetyl-CoA to the five-carbon terpene precursor IPP, which is then isomerized to its allylic isomer dimethylallyl pyrophosphate (DMAPP) by the enzyme isopentenyl diphosphate isomerase (IDI). The subsequent condensation of two IPP units with one DMAPP unit results in the formation of 15-carbon farnesyl pyrophosphate (FPP), the immediate prenylated precursor of the saponins. Finally, the condensation of two FPP units by squalene synthase (SQS) generates the linear 30-carbon precursor squalene, which is further epoxidized to 2,3-oxidosqualene by the enzyme squalene epoxidase (SQE). The 2,3-oxidosqualene is typically cyclized by a variety of oxidosqualene cyclases (OSCs) to polycyclic structures, which in higher plants marks the branch point between primary and specialized triterpene metabolism. The tetracyclic, primary triterpene precursor cycloartenol is generated by the cyclization of 2,3-oxidosqualene by cycloartenol synthase (CAS). In angiosperms, a mixture of phytosterols are generated from cycloartenol, including the 27-carbon cholesterol, the 28-carbon campesterol and the 29-carbon sitosterol. The steroidal saponins are synthesized by a series of oxygenations and glycosylations of the cholesterol backbone to spirostanol or furostanol derivatives with a fused *O*-heterocycle in their core aglycone structure (Thakur *et al.*, 2011). The steroidal glycoalkaloids also utilize cholesterol as the committed precursor, but incorporate an amine group through side chain modifications to generate aglycones such as solasodine, solanidine, demissidine and tomatidine (Ginzberg *et al.*, 2009; Itkin *et al.*, 2013). The aglycones are then tailored with oxidoreductases before being glycosylated with multiple sugar moieties (Friedman, 2006).

All the remaining cyclization products of 2,3-oxidosqualene are committed precursors for the synthesis of specialized triterpenes. This cyclization reaction, involving multiple carbocation rearrangement steps, generates the first level of structural diversity inherent to the triterpenoid saponin aglycones, as the single substrate 2,3-oxidosqualene can be cyclized to an array of triterpene scaffolds. As such, nine main classes of triterpene backbones have been documented to be synthesized by plants (shown in orange in Figure 1; Vincken *et al.*, 2007). The OSCs catalyzing these reactions are either specific or multifunctional in nature, and cyclize 2,3-oxidosqualene to a single product or to multiple products derived from a particular cyclization pathway in a single reaction. The triterpene aglycones are then modified, mainly oxidized, by a series of cytochrome P450-dependent monooxygenases (P450s). These modifications add a second level of complexity and expand the structural diversity of the backbone. Consecutive oxidations at different positions of the triterpene backbone increase the polarity of the scaffold and introduce reactive functional groups which are subsequently modified by an array of transferases, including UDP-dependent glycosyltransferases (UGTs), acyltransferases,

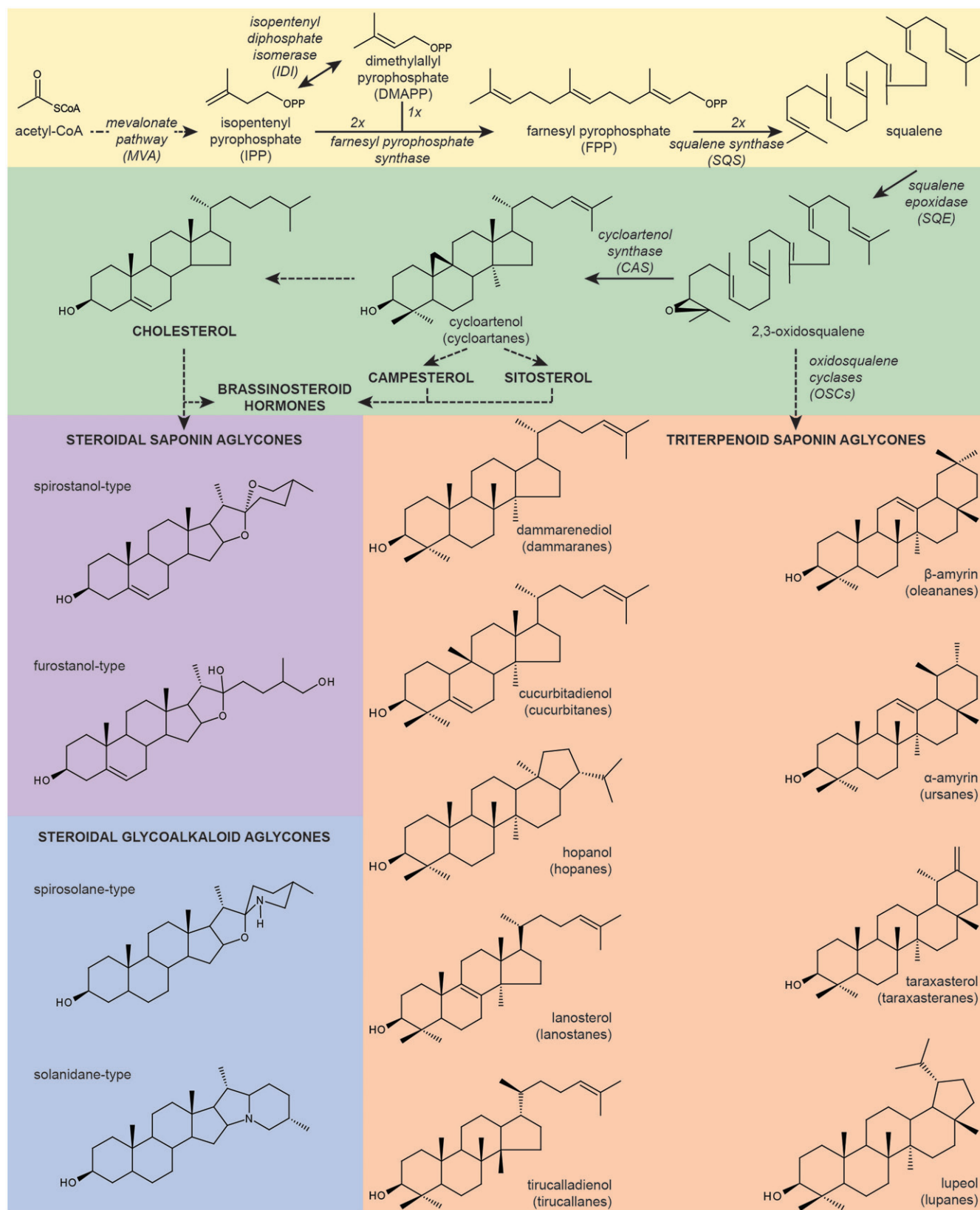


Figure 1. Overview of structural diversity in saponin aglycones. The saponin biosynthesis pathway begins with acetyl-CoA, which via the mevalonate pathway is converted to the linear 30-carbon precursor squalene (yellow). Squalene is oxidized to 2,3-oxidosqualene, which is cyclized to the cycloartane aglycone, the dedicated precursor for sterol biosynthesis in plants (green). Cholesterol is the dedicated precursor for the synthesis of steroidal saponins (violet) and steroidal glycoalkaloids (blue). The triterpenoid saponins (orange) are synthesized from 2,3-oxidosqualene that is cyclized to specialized triterpene aglycones. Enzyme and pathway names are italicized. Dashed arrows imply multiple steps in the pathway.



malonyltransferases and methyltransferases. A comprehensive overview of the biosynthesis of triterpenoid saponins can be found in Thimmappa *et al.* (2014).

The genes involved in the biosynthesis of all three types of saponins (triterpenoid, steroid and steroidal glycoalkaloid) can therefore be grouped into four main categories based on their characteristic reactions, and include the OSCs, P450s, UGTs and other tailoring enzymes (mostly encoding transferases). Recently, numerous examples of gene clusters for the biosynthesis of different classes of specialized metabolites have been discovered in a variety of plant species, including clusters for saponin biosynthesis (Boycheva *et al.*, 2014; Nützmann & Osbourn, 2014). Biosynthetic gene clusters have been identified for triterpenoid saponins and steroidal glycoalkaloids. The first triterpene gene cluster to be identified was the avenacin cluster from *Avena strigosa* (oat), which contains five biosynthetic genes encoding for an OSC, a P450, a UGT and two other tailoring enzymes (a methyltransferase and a serine carboxypeptidase-like acyltransferase) (Haralampidis *et al.*, 2001a; Mugford *et al.*, 2013; Papadopoulou *et al.*, 1999; Qi *et al.*, 2004, 2006). Other closely linked genes that form part of this cluster have been defined by genetics but not cloned (Mylona *et al.*, 2008; Papadopoulou *et al.*, 1999; Qi *et al.*, 2004). In *Arabidopsis thaliana*, two triterpenoid gene clusters have been identified for the biosynthesis of thalianol- and marneral-derived triterpenes, containing four and three genes, respectively. The thalianol cluster is comprised of genes encoding an OSC, two P450s and an acyltransferase (Field & Osbourn, 2008). Likewise, the marneral cluster encodes an OSC and two P450s (Field *et al.*, 2011). A triterpenoid gene cluster encoding an OSC and two P450s has also recently been identified in *Lotus japonicus* (Krokida *et al.*, 2013). Gene clusters for the synthesis of the steroidal glycoalkaloids  $\alpha$ -tomatine and  $\alpha$ -chaconine/ $\alpha$ -solanine have been discovered in *Solanum lycopersicum* (tomato) and *Solanum tuberosum* (potato), respectively (Itkin *et al.*, 2013). The tomato gene cluster consists of eight characterized biosynthetic genes encoding for two P450s, four UGTs and two other tailoring enzymes (a dioxygenase and a transaminase). Similarly, the potato cluster contains six characterized genes encoding for two P450s, two UGTs, a dioxygenase and a transaminase. Although once thought to be an exception, the phenomenon of physical clustering of biosynthetic pathway genes for different types of secondary metabolites in plants is becoming common (Nützmann & Osbourn, 2014). The physical clustering of genes in the genome together with co-ordinate expression of the clustered genes is proving to be a useful tool to identify biosynthetic genes involved in new pathways for the synthesis of specialized metabolites (Castillo *et al.*, 2013; Field & Osbourn, 2008; Field *et al.*, 2011; Nützmann & Osbourn, 2014).

Enzymes encoded by multigene families for OSCs, P450s, UGTs and other specialized transferases are the key actors in the biosynthesis of plant triterpenoid, steroidal and steroidal glycoalkaloid saponins. Although the number of characterized enzymes belonging to these gene families has increased over the last decade, there is still a wide gap in our understanding of saponin biosynthesis, in how the synthesis of these molecules in different plant tissues and organs is regulated

during development and in response to environmental stimuli, and in how these molecules are transported. Unraveling these molecular mechanisms will provide a better understanding of the role of saponins in plant processes, and will guide their exploitation in industrial applications.

## Occurrence and distribution in plants

Saponins are synthesized and accumulated by many different plant families (Dinda *et al.*, 2010; Sparg *et al.*, 2004; Vincken *et al.*, 2007). They occur in both wild and domesticated plants, with the triterpenoid-type saponins being predominant in cultivated crops. Some well-known triterpenoid saponin-accumulating plant families include the Leguminosae, Amaranthaceae, Apiaceae, Caryophyllaceae, Aquifoliaceae, Araliaceae, Cucurbitaceae, Berberidaceae, Chenopodiaceae, Myrsinaceae and Zygophyllaceae, among many others (Parente & Da Silva, 2009; Shi *et al.*, 2004; Sparg *et al.*, 2004). Legumes such as soybeans, beans and peas are a rich source of triterpenoid saponins. In contrast, cereals and grasses are generally deficient in saponins, with some notable exceptions such as *Avena* species (oats) which accumulate both triterpenoid and steroidal saponins (Osbourn, 2003). The steroidal saponins are typically found in members of the Agavaceae, Alliaceae, Asparagaceae, Dioscoreaceae, Liliaceae, Amaryllidaceae, Bromeliaceae, Palmae and Scrophulariaceae families and accumulate in abundance in crop plants such as yam, alliums, asparagus, fenugreek, yucca and ginseng (Hoffmann, 2003; Hostettmann & Marston, 2005). Diosgenin, the steroidal aglycone obtained by the hydrolysis of dioscin, a saponin abundant in the tubers of *Dioscorea villosa* (wild yam), is the precursor for the commercial synthesis of steroids such as cortisone, progesterone and pregnenolone (Patel *et al.*, 2012; Peng *et al.*, 2011). The steroidal glycoalkaloids are commonly found in members of the Solanaceae family including tomato, potato, aubergines and capsicum (Roddick & Melchers, 1985).

Several plants release saponins into the rhizosphere where these compounds might serve as allelopathic agents that suppress the growth of neighboring plants and prevent competition for natural resources. For instance, the germination, growth and yield of cotton was found to be lower when plants are grown on soil previously used for cultivation of *Medicago sativa* (alfalfa), compared with growth on soil after wheat cultivation or on uncultivated bare soil (Leshem & Levin, 1978). Similar effects were also observed for wheat. Alcoholic extracts of alfalfa roots suppressed germination of wheat seeds, and application of powdered alfalfa roots or compounds purified from alfalfa roots inhibited wheat seedling growth (Oleszek, 1993; Oleszek & Jurzysta, 1987). The antifungal triterpenoid saponins avenacins, which are synthesized in and released from oat roots, have also been suggested to have allelopathic potential, particularly because of the invasive nature of oat growth when compared to other cereals (Carter *et al.*, 1999; Field *et al.*, 2006; Hostettmann & Marston, 1995). A meta-transcriptomics-based analysis of bulk soil and rhizosphere of 4-week old pea, wheat and oat plants revealed major differences between their microbiomes. The relative abundance of nematodes and/or fungi was fivefold more in pea and oat rhizospheres than in the wheat

rhizosphere and bulk soil. The pea rhizosphere was enriched for fungi, unlike the oat rhizosphere, which contained antifungal avenacins. Comparison of the rhizosphere microbiomes of oat wild-type and *sad1* mutants (lacking avenacins) showed little differences in the prokaryotic community but large variation in the nematode and fungal community, suggesting a broader role for avenacins than just protecting from fungal pathogens (Turner *et al.*, 2013).

In both monocotyledonous and dicotyledonous plants, the oleananes, one of the main classes of triterpenes derived from 2,3-oxidosqualene (Figure 1), represent the most common aglycone skeleton for the synthesis of triterpenoid saponins. The oleananes are pentacyclic triterpenoids that are found in almost all plant orders except the Solanales, Juglandales, Rhamnales and Zingiberales (Vincken *et al.*, 2007). Although less abundant, the ursanes also occur in many plant families and are composed of a pentacyclic triterpene backbone that is almost identical to that of the oleanane backbone but differs in the location of one methyl group. The lupane and dammarane structures differ considerably from the oleananes and ursanes, and serve as aglycones for less common pentacyclic and tetracyclic saponins, respectively. The remaining classes of triterpenoid saponins, including the tirucallanes, hopanes, taraxasteranes, lanostanes and cucurbitanes are restricted to only a few plant families. The cycloartanes, also cyclized from 2,3-oxidosqualene, are an exception as they form the backbone for the synthesis of not only specialized metabolites (steroidal saponins and glycoalkaloids) but also phytosterols, and are present in all plants (Vincken *et al.*, 2007). Furthermore, the phytosterols cholesterol, campesterol and sitosterol are the precursors of the brassinosteroid hormones that regulate plant growth and development (Fujioka & Yokota, 2003).

OSC genes encoding  $\beta$ -amyrin synthases for the cyclization of 2,3-oxidosqualene to the oleanane backbone  $\beta$ -amyrin have been identified in a number of different plant species ([http://www.brenda-enzymes.org/php/result\\_flat.php4?ecno=5.4.99.39](http://www.brenda-enzymes.org/php/result_flat.php4?ecno=5.4.99.39)), including the model plant *A. thaliana*, which does not accumulate oleanane-derived saponins (Shibuya *et al.*, 2009). In addition to  $\beta$ -amyrin, other simple triterpenes and triterpenoids have been detected as major components of the intracuticular wax layer in many plants (Buschhaus & Jetter, 2011). The widespread presence of simple triterpenes and triterpenoids in plants when compared with the taxon-specific accumulation of specialized glycosylated saponins in particular plant species suggests a possible fundamental role for these saponin biosynthetic precursors. These molecules may form constituents of plant membranes and may further have as yet unidentified signaling roles, given their structural similarities with sterols.

Saponins accumulate in an organ- and/or tissue-specific manner and the saponin content of plants is influenced by developmental factors, indicative of differential synthesis and regulation and suggesting distinct biological functions. Different types of saponins have been isolated from roots, stem, bark, leaves, seeds, fruits and flowers, and from whole plants (Vincken *et al.*, 2007). In many plants, saponins are primarily synthesized and stored in underground organs. *Glycyrrhiza glabra* (licorice), *Panax ginseng* (ginseng) and the Chinese traditional medicine plant *Polygala tenuifolia*

accumulate glycyrrhizin (*G. glabra*), ginsenosides (*P. ginseng*) and senegenin-derived saponins (*P. tenuifolia*) in the stolon, roots and/or rhizomes (Park *et al.*, 2005; Seki *et al.*, 2008; Teng *et al.*, 2009). The roots of *Bupleurum* species and the model legume *Medicago truncatula* accumulate higher levels of saponins than the aerial organs (Huhman *et al.*, 2005; Kapusta *et al.*, 2005; Zhao *et al.*, 2013). The total saponin content of *M. truncatula* roots is estimated at 5.92  $\mu\text{g}/\text{mg}$  of dry weight, which is 5-, 10- and  $\sim 200$ -times more than the saponin content of leaves and seeds, stems and seed pods, respectively (Huhman *et al.*, 2005). Saponins can also accumulate in a tissue-specific manner in the roots of some plants. In *P. ginseng*, the ginsenosides are present in higher amounts in the phloem than the xylem, and are also found in parenchymal cells of the pith in the root (Fukuda *et al.*, 2006; Yokota *et al.*, 2011). In young *Bupleurum* roots, the saikosaponins are mainly in the pericycle and primary phloem, and in mature roots, they are distributed in the vascular cambium and secondary phloem (Tan *et al.*, 2008). In oat roots, the UV-fluorescent saponin avenacin A-1 accumulates specifically in the epidermal layer of the root tips (Osborn *et al.*, 1994; Turner, 1960). However, saponins can also preferentially accumulate in aerial organs. Examples include the avenacoside steroidal saponins which are found in oat leaves, the asiaticosides in *Centella asiatica* leaves (Kim *et al.*, 2005), the saponin tomatoside A in seeds of tomato (Moco *et al.*, 2007), and saponins in flowers, fruits and seeds of *Chenopodium quinoa* (Kuljanabagavad *et al.*, 2008).

Simple triterpenes may also accumulate in a tissue- and organ-specific manner during plant growth. For example, in legumes,  $\beta$ -amyrin and lupeol have been detected in nodule tissues (Hartmann *et al.*, 2002), and sprouting has been reported to increase both  $\beta$ -amyrin and saponin content of seedlings (Ayet *et al.*, 1997; Baisted, 1971; Guajardo-Flores *et al.*, 2012; Livingston *et al.*, 1984). Likewise, in *Solanum* species, differential tissue- and organ-specific synthesis and accumulation of steroidal glycoalkaloids has been observed (Mweetwa *et al.*, 2012). Synthesis commences during germination and peaks in the flowering period. The leaves are the first organs to attain maximum concentration, followed by unripe fruits and flowers which accumulate even higher amounts of the metabolites (Friedman, 2006). In potato tubers, the steroidal glycoalkaloids are concentrated in the peel with highest levels around the eyes constituting of the periderm, cortex and outer phloem (Friedman *et al.*, 2003). The model plant *A. thaliana* has 13 OSCs which catalyze the cyclization of 2,3-oxidosqualene to different major triterpene products. These OSC genes differ in their organ-specific expression patterns, suggesting specific functions for the different triterpene products in different organs during different stages of plant growth and development (Thimmappa *et al.*, 2014; Figure 2A).

The isoprenoids for the synthesis of both sterols and specialized triterpenes are derived from the shared mevalonate pathway, and there is evidence for inverse regulation of the biosynthetic pathways for the synthesis of phytosterols and triterpenes in plants. During the early stages of pea germination, sterol synthesis is very low and  $\beta$ -amyrin production is predominant. During development, however, sterol synthesis is prevalent. In the late fruit maturation

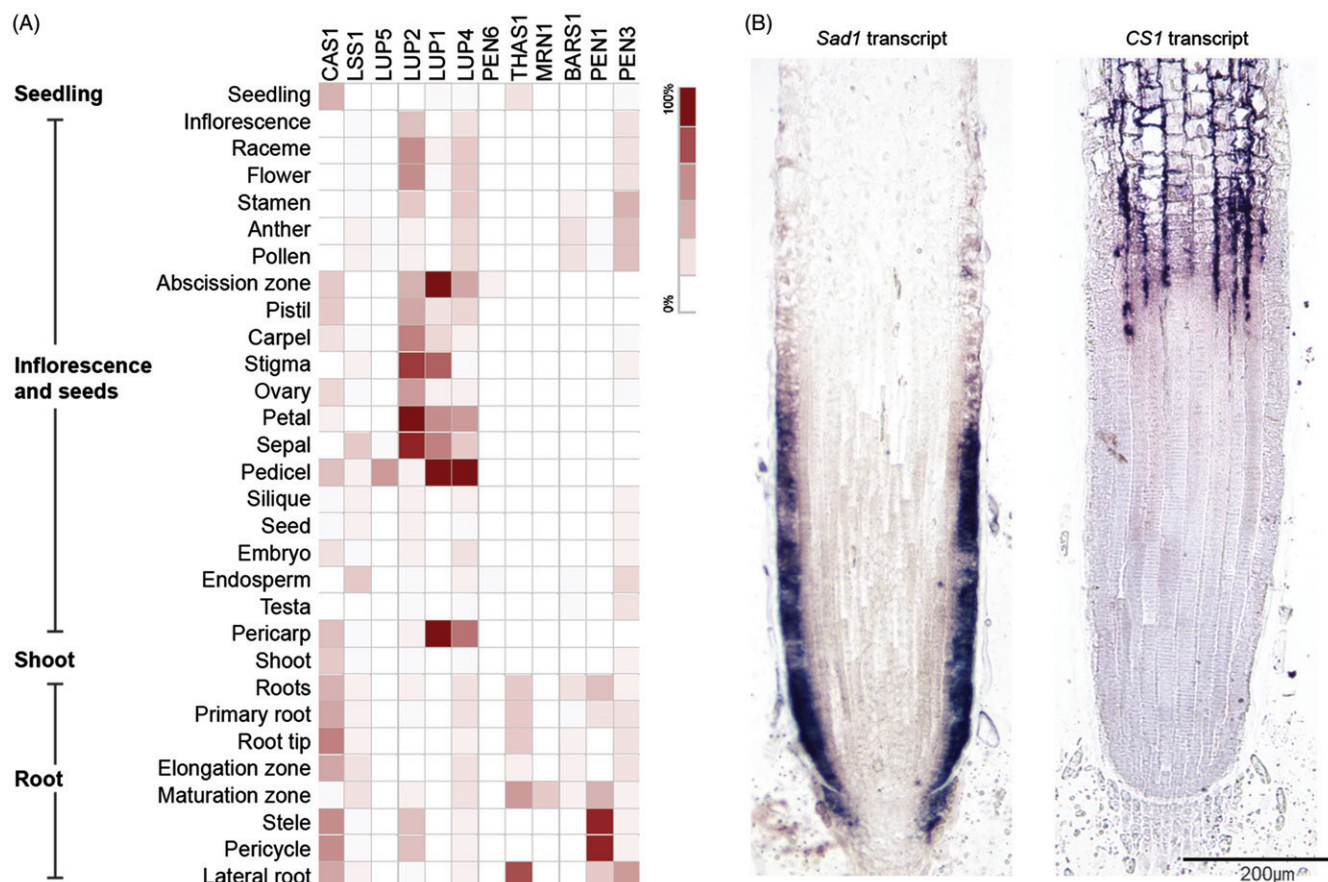


Figure 2. Organ- and tissue-specific expression of oxidosqualene cyclase genes in *Arabidopsis thaliana* and *Avena strigosa*. (A) Heat map depicting the expression profiles for 12 of the 13 *A. thaliana* OSC genes in various plant organs. Transcripts for CAMS1 (At1g78970) were not detected and hence not included. Expression data were retrieved from Genevestigator V3. BARS1, baruol synthase; CAS1, cycloartenol synthase; LSS1, lanosterol synthase; LUP1, lupeol synthase; LUP2,  $\beta$ -amyrin synthase; LUP4,  $\beta$ -amyrin synthase; LUP5, tirucalla-7,21-dien-3 $\beta$ -ol synthase; MRN1, marneral synthase; PEN1, arabidiol synthase; PEN3, tirucalla-7,24-dien-3-ol synthase; PEN6, bauerenol synthase; THAS1, thalianol synthase. From Thimmappa *et al.* (2014). (B) Distribution of  $\beta$ -amyrin synthase (*Sad1*) and cycloartenol synthase (*CS1*) gene transcripts in the root tip of *A. strigosa*. *In situ* mRNA hybridization of young root tips shows *Sad1* transcripts in the epidermis, weakly in parts of the subepidermis and some columella cells. *CS1* expression is restricted to the cortex in the elongation zone above the root tip. From Wegel *et al.* (2009).

period, sterol synthesis decreases considerably and there is significant synthesis of  $\beta$ -amyrin (Baisted, 1971). Similarly, sorghum grains accumulate  $\beta$ -amyrin early in root development, while sterol biosynthesis becomes active only later (Palmer & Bowden, 1977). The end products of sterol and triterpenoid biosynthesis also manipulate the flux through these biosynthetic pathways (Haralampidis *et al.*, 2001b). For example, feeding radiolabeled acetate to *Gypsophila paniculata* and *Saponaria officinalis* cell suspension cultures pretreated with gypsogenin 3, *O*-glucuronide (a saponin precursor in *G. paniculata*, but not *S. officinalis*) resulted in reduced incorporation of radioactivity in saponins and their precursors, but not sterols in *G. paniculata*. Compared to untreated controls the ratio of  $\beta$ -amyrin to cycloartenol reduced by  $\sim 10$ -fold in *G. paniculata* but remained unchanged in *S. officinalis* (Henry *et al.*, 1992). Also, in cell suspension cultures of *Tabernaemontana divaricate*, inhibition of the activity of cycloartenol synthase resulted in the induction of  $\beta$ - and  $\alpha$ -amyrin synthases, which in turn increased flux through the triterpenoid pathway (van der Heijden *et al.*, 1989). In many cases, there is a good correlation between the accumulation of metabolites and the expression pattern of the respective biosynthetic genes. In *Glycyrrhiza glabra*

seedlings, the expression of the  $\beta$ -amyrin synthase gene is maximal two days after germination and thereafter decreases rapidly, whereas the expression of the cycloartenol synthase gene remains relatively constant throughout germination (Hayashi *et al.*, 2004). In oat roots, a marked difference is observed in the spatial expression patterns of the cycloartenol synthase and  $\beta$ -amyrin synthase genes, which are required for synthesis of sterols and triterpenoid saponins, respectively. In young oat roots, the  $\beta$ -amyrin synthase gene *Sad1* is expressed in the epidermal cells of the root tips, in accordance with the accumulation of the  $\beta$ -amyrin derived avenacins. In contrast, expression of the cycloartenol synthase gene *CS1* is restricted to the cortex in the elongation zone above the root tip (Figure 2B; Wegel *et al.*, 2009).

Genotype- and cultivar-dependent variation in the saponin content of plants has also been reported. For instance, the grain saponin content of different cultivars of *C. quinoa* is estimated to range from 0 to  $\sim 10$  mg/g dry weight, and based on the total saponin content these cultivars are classified as bitter (high saponin) or sweet (low saponin) (Koziol, 1992). Between the two *Centella* species, *C. asiatica* and *C. glabrata*, the triterpenoid saponin asiaticoside and related compounds are major constituents in the former species and



absent in the latter (Long *et al.*, 2012). Similarly, a systematic analysis of the triterpene glycosides asiaticoside and madecassoside in sixty *C. asiatica* accessions from South India grown under identical environmental conditions revealed huge variations in the content of these saponins, with three accessions having no detectable asiaticoside/madecassoside (Thomas *et al.*, 2010). Analysis of the saponin composition of 800 cultivated (*Glycine max*) and 329 wild (*Glycine soja*) soybean seeds showed clear variety-specific differences between the accessions. These differences were attributed to variety-specific expression of biosynthetic genes that utilize soyasapogenol glycosides as substrates (Tsukamoto *et al.*, 1993). In *G. max*, the levels of group B soyasaponins represent one of the most discriminatory metabolic indicators of closely related salt-sensitive and salt-tolerant soybean genotypes (Wu *et al.*, 2008). Large variation in the seed saponin composition of 3025 *G. soja* accessions from nine regions of Korea has also been reported. Based on the saponin composition of the seed hypocotyls, the accessions were grouped into seven phenotypes (Panneerselvam *et al.*, 2013). In addition, comparison of six *Cajanus cajan* (chickpea) and five *Phaseolus vulgaris* (common bean) genotypes showed genotypic variations in their sapogenol content (Vasishtha & Srivastava, 2011, 2012).

In addition to genetic determinants of saponin content, environmental and agronomic factors may also influence the saponin composition of plants (Etebu, 2012; Schwarzbach *et al.*, 2006). For example, potato tubers show pronounced drought- and light-induced accumulation of steroidal glycoalkaloids in their outer layers when stored in the light instead of the dark (Bejarano *et al.*, 2000; Percival, 1999). In contrast, ginsenoside biosynthesis in *P. ginseng* is enhanced in the dark (Kim *et al.*, 2014). Seasonal variation in the expression of the  $\beta$ -amyrin synthase gene and the content of glycyrrhizin has also been observed in *G. glabra* plants (Hayashi *et al.*, 1998, 2004). The combination of environmental stress and agricultural factors such as water-logging or drought, fertilization and low or high temperatures influences the content of triterpenoids and saponins in plants. In the medicinal herb *Hypericum brasiliense*, betulinic acid content increases in response to drought and low or high temperatures (Nacif de Abreu & Mazzafera, 2005). Drought stress significantly increases the production of the triterpenoids rosmarinic, ursolic and oleanolic acid in spicas (reproductive parts) of *Prunella vulgaris* (Chen *et al.*, 2011). Also in *Bupleurum chinense*, mild water stress increases the saikosaponin content of roots (Zhu *et al.*, 2009).

The occurrence and distribution of saponins in plants in a tissue- and organ-specific manner at different growth and development stages suggests that these metabolites may have functions in physiological processes in plants, in addition to their traditionally accepted roles as phytoalexins and defense molecules.

### Effects on plant growth and development

The ability of different plant species to synthesize distinct secondary metabolites is generally thought to be essential for their survival. Indeed, the saponin content and composition of a plant is variable and influenced by its surrounding

environment and growth stage (Szakiel *et al.*, 2011). Stress- or developmentally regulated changes in saponin composition contribute to the total plant secondary metabolite pool where they may ensure protection of plant organs and overall plant survival. However, as described above, in many plants, saponins and their biosynthetic intermediates accumulate during normal growth and development. Manipulating the biosynthesis of these molecules causes morphological and physiological effects, suggesting additional functions (Abe *et al.*, 1988; Guhling *et al.*, 2006). In this section, we present growing evidence linking the saponins and their biosynthetic intermediates to various physiological processes during plant growth, development and maturation (Figures 3 and 4). We also highlight the impact of modulating saponin synthesis and accumulation in plants and suggest their underexplored primary role in plant well-being.

### Effects of exogenous application

The external application of saponins to plants impacts different biological processes (Geuns, 1978). Seeds of pea and corn absorb water more rapidly in the presence of saponins, leading to more rapid germination. Crude saponin extracts from *Quillaja*, *Polygala*, *Saponaria* and *Sapindus* species can double the growth rate of isolated wheat and pea embryos (Helmkamp & Bonner, 1953). Asiaticosides extracted from *Centella asiatica* stimulate growth and chlorophyll biosynthesis in radish, pea, *Lupinus* and *Linum* species (Boiteau & Ratsimamanga, 1956). Saponins from pea have been proposed to regulate gravitropism and cellulose synthesis in different plant species (Ohana *et al.*, 1998; Rahman *et al.*, 2001). Chromosaponin I, isolated from 7-day-old etiolated *Pisum sativum* seedlings, stimulates cortical cell division and elongation leading to enhanced growth of roots in *A. thaliana*, *Lactuca sativa*, *Chrysanthemum coronarium*, *Medicago sativa*, *Trifolium repens*, *Astragalus sinicus*, *Brassica juncea*, *Brassica campestris*, *Cryptotaenia japonica*, *Phleum pratense* and *Lolium multiflorum* (Rahman *et al.*, 2000; Tsurumi & Wada, 1995). In addition to enhanced elongation of roots, a reduction in root diameter was also observed in *A. thaliana* (Figure 3A) and *L. sativa*, suggesting a potential growth regulatory role for chromosaponin I, which appears to be modulated by the auxin influx carrier protein AUX1 (Rahman & Tsurumi, 2002; Rahman *et al.*, 2001). In contrast, betulin and its glycosides show growth inhibitory effects on alfalfa radicles, with the inhibitory activity tending to increase with an increase in the number of  $\beta$ -D-glucose residues. The hypocotyls were less affected than the radicles for all tested betulin derivatives, suggesting that these compounds primarily affect the roots (Figure 3B). In the absence of sugar molecules, the triterpenoid betulinic acid, with a carboxyl group at carbon position 28, showed stronger inhibitory activity than betulin, which has a hydroxyl group at the same position. Oleanolic acid, also with a carboxyl group on position 28, but having an oleanane instead of a lupane backbone (Figure 8), showed no growth effects on alfalfa radicles or hypocotyls (Ohara & Ohira, 2003).

In contrast to the effects observed with triterpenoid saponins, which mainly affect the roots, the exogenous



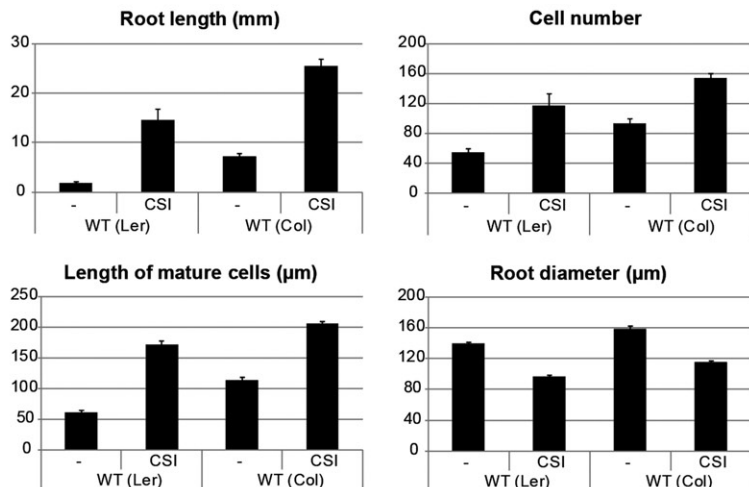
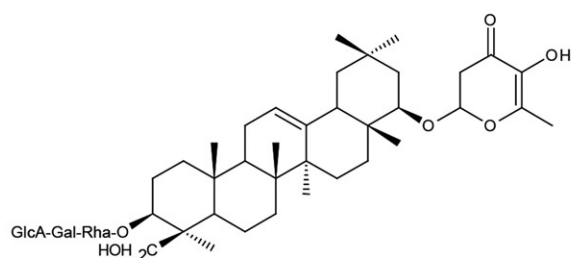
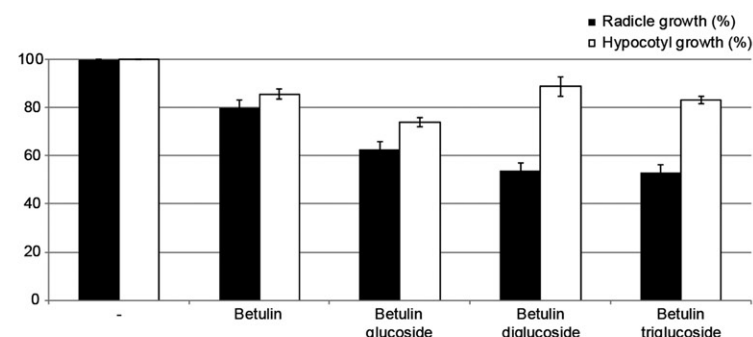
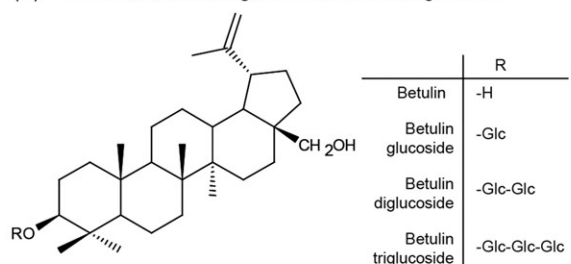
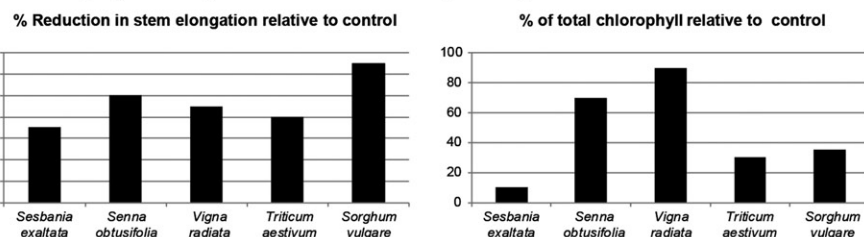
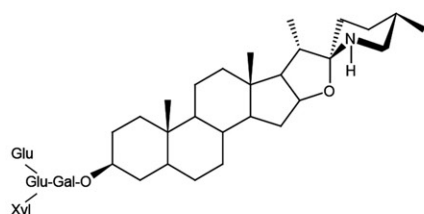
(A) Effect of chromosaponin I (CSI) on *Arabidopsis thaliana* roots(B) Effect of betulin and glucosides on *Medicago sativa*(C) Effect of  $\alpha$ -tomatine on *Sesbania exaltata*, *Senna obtusifolia*, *Vigna radiata*, *Triticum aestivum* and *Sorghum vulgare*

Figure 3. Plant growth and development effects upon external application of saponins and their intermediates. (A) Effect of application of chromosaponin I (CSI, isolated from *Pisum sativum*) on *Arabidopsis thaliana* root growth. Root length, root cell number and length of mature cells increase in both Landsberg erecta (Ler) and Columbia (Col) wild-type (WT) ecotypes upon CSI application. However, the root diameter decreases upon CSI treatment in both ecotypes. From Rahman *et al.* (2000). (B) Effect of application of 0.018% betulin or its glucosides on *Medicago sativa* radicle and hypocotyl growth. No effects were observed on hypocotyl growth, but a clear inhibitory effect was seen on radicle growth with increasing number of glucosides attached to betulin. From Ohara & Ohira (2003). (C) Inhibitory effect of application of  $\alpha$ -tomatine on stem elongation and chlorophyll accumulation in *Sesbania exaltata*, *Senna obtusifolia*, *Vigna radiata*, *Triticum aestivum* and *Sorghum vulgare*. From Hoagland (2009). The structures of the applied compounds are given. Gal, galactose, Glc, glucose, GlcA, glucuronic acid; Rha, rhamnose; Xyl, xylose.

application of the steroidal glycoalkaloid saponin  $\alpha$ -tomatine inhibits stem elongation and chlorophyll accumulation in seedlings of *Sesbania exaltata*, *Senna obtusifolia*, *Vigna radiata*, *Triticum aestivum* and *Sorghum vulgare* (Figure 3C).  $\alpha$ -Tomatine also causes electrolyte leakage from leaf discs of *Zea mays*, *Cucumis sativus*, *Nicotiana tabacum* and *Nicandra physaloides*, but not from tomato and potato tissues (Hoagland, 2009). In comparison, the aglycone tomatidine causes greater electrolyte leakage in *Z. mays*, *C. sativus*, *N. tabacum* and *N. physaloides*. Application of pure tomatidine to *A. thaliana* seedlings caused root growth effects without affecting their germination rate. The roots of seedlings grown on medium supplemented with tomatidine were shorter than those of seedlings grown on control medium or medium supplemented with  $\alpha$ -tomatine (Itkin *et al.*, 2011).

## Effects of altering the endogenous levels of simple triterpenes

### Growth and development

Modulation of the expression of genes encoding the OSCs that generate triterpene backbones has revealed that simple triterpenes can effect plant growth and development. *Arabidopsis thaliana* seedlings overexpressing thalianol synthase (*THAS*) gene, which encodes a protein for the cyclization of 2,3-oxidosqualene to the specialized triterpene thalianol, show stunted growth but have longer roots compared to wild type seedlings (Field & Osbourn, 2008). Similarly, lack or overaccumulation of marnerial and/or its metabolites incur detrimental effects on plant growth and development in *A. thaliana* (Field *et al.*, 2011; Figure 4).

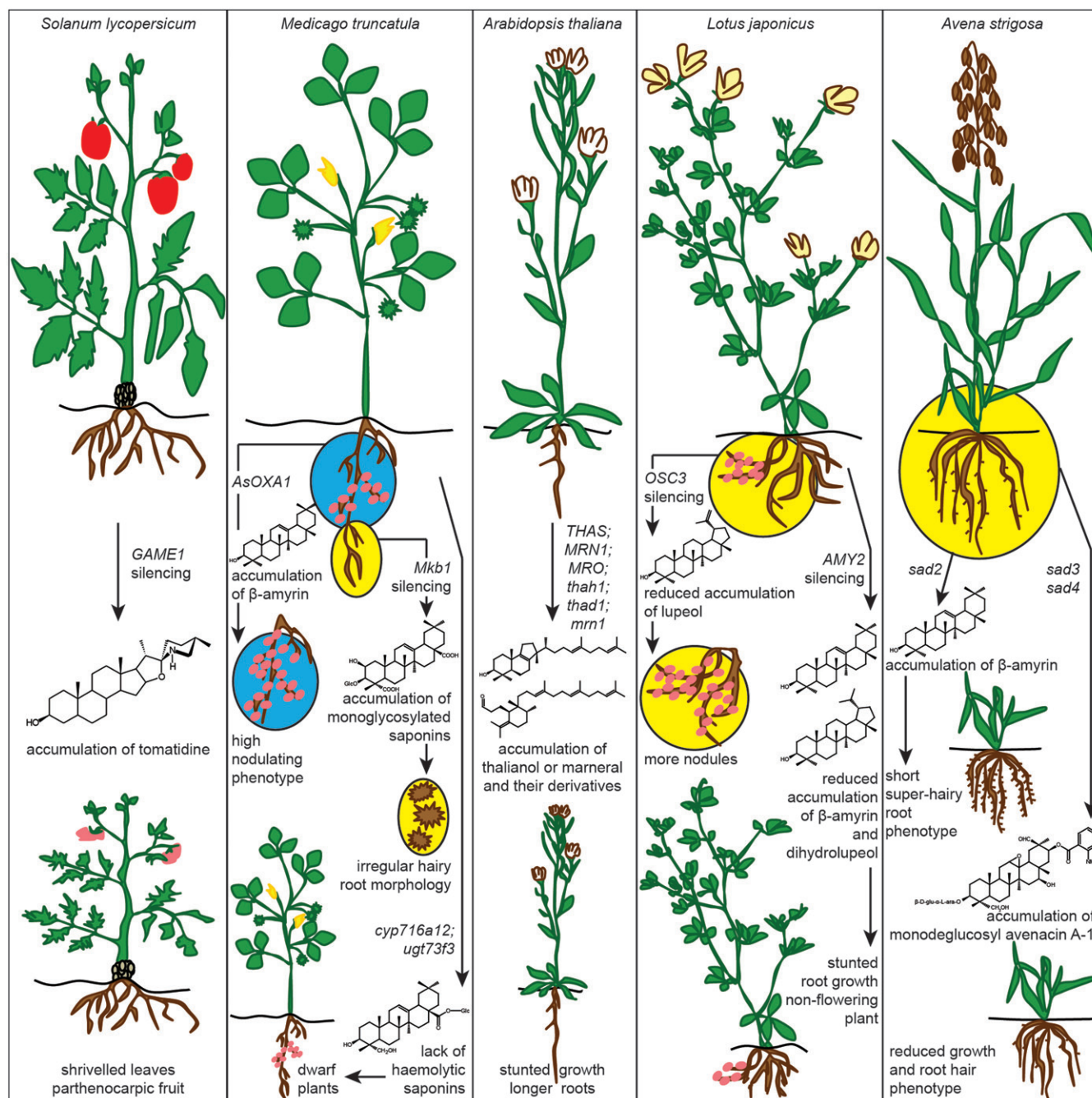


Figure 4. Plant growth and development effects resulting from altered *in planta* saponin biosynthesis. Various developmental effects resulting from the over or reduced accumulation of saponins and/or their intermediates is depicted in *Solanum lycopersicum*, *Medicago truncatula*, *Arabidopsis thaliana*, *Lotus japonicus* and *Avena strigosa*. Gene names in upper and lower cases imply gene overexpression and knock out, respectively.

Mutants for the OSC marneral synthase (MRN1), which cyclizes 2,3-oxidosqualene to marneral in *A. thaliana*, show delayed embryogenesis, late flowering, rounded leaves, aberrant seed morphology, reduced growth caused by inhibition of cell expansion and elongation and defects in membrane integrity and permeability. Interestingly, these effects could not be attributed to the altered expression of genes involved in cell expansion, while it is unknown whether marneral and its derivatives serve as membrane constituents. At the transcriptional level, these compounds have been proposed to have a negative role in flower development, since the delayed flowering phenotype in the *mrn1* knockout mutant could be attributed to increased expression of the flowering

locus C (*FLC*) gene that encodes a repressor of flowering in *A. thaliana* (Go *et al.*, 2012).

Simple triterpenoids are also essential constituents of the cuticles that cover plant aerial surfaces (Brendolise *et al.*, 2011; Guhling *et al.*, 2005; Huang *et al.*, 2012). The cuticle is composed of two main components – very long chain fatty acid-derived cutin; and waxes, which are mixtures of primary alcohols, aldehydes and triterpenoid alcohols (Jetter *et al.*, 2000). The wax is both epicuticular and intracuticular, forming a continuous layer on the top of the cutin and also being embedded in it. Triterpenoids are dominant constituents of the intracuticular wax (Buschhaus & Jetter, 2011; Buschhaus *et al.*, 2007; Van Maarseveen & Jetter, 2009).

In *A. thaliana*, the GLABROUS1 (*GLI*) gene is required for the development of specialized trichome cells. The *A. thaliana glI* mutant is trichome-less and lacks triterpenoids in its wax layer. Ectopic expression of *AtLUP4*, a  $\beta$ -amyrin synthase gene, in the *glI* mutant results in the accumulation of  $\beta$ -amyrin in the intracuticular wax layer of *A. thaliana* (Buschhaus & Jetter, 2012). This accumulation of  $\beta$ -amyrin in the *glI* mutant as a consequence of the expression of *AtLUP4* under the control of a double constitutive cauliflower mosaic virus 35S promoter caused a significant reduction in water barrier effectiveness and thereby reduced intracuticular wax resistance.

In oats, increased  $\beta$ -amyrin content in the roots has recently been shown to result in shorter roots with a super-hairy phenotype (Kemen *et al.*, 2014). *sad2* mutants of diploid oat (*A. strigosa*) are blocked in the second step in the avenacin pathway (the oxidation of the  $\beta$ -amyrin scaffold; Geisler *et al.*, 2013) and so accumulate elevated levels of this triterpene. Accumulation of high levels of  $\beta$ -amyrin triggers a change in cell fate in the root epidermis, with more cells being specified as trichoblasts (cells that give rise to root hairs; Kemen *et al.*, 2014). This is the first demonstration of a role for a simple triterpene in plant development by changing cell fate in the root. A clear link between root length and the amount of  $\beta$ -amyrin accumulating in the roots was also observed, indicating that accumulation of elevated levels of  $\beta$ -amyrin triggers changes in cell specification and root length (Figure 4).

### Nodulation

Lupane-type triterpenes have been proposed to play important roles as hydrophobic and antibiotic barriers in the development of symbiosis in legumes (Hayashi *et al.*, 2004). During the establishment of symbiotic relations with rhizobia bacteria or mycorrhizal fungi, the synthesis of the ursane- and oleanane-type triterpenes  $\alpha$ - and  $\beta$ -amyrin is also up-regulated (Baisted, 1971; Grandmougin-Ferjani *et al.*, 1999; Hernández & Cooke, 1996; Iturbe-Ormaetxe *et al.*, 2003). The expression of the native  $\beta$ -amyrin synthase is high in root nodules of *G. glabra* (Hayashi *et al.*, 2004) and pea seedlings infected with *Rhizobium leguminosarum* (Iturbe-Ormaetxe *et al.*, 2003). From an analysis of *P. sativum* nodules, Hernández & Cooke (1996) detected  $\beta$ -amyrin in peribacteroid membranes and the microsomal fraction of nodule cells.  $\beta$ -Amyrin was not detected in free-living bacteria or in the plasma membrane of the roots, suggesting that this compound is of host-plant origin (Hernández & Cooke, 1996). About 90% of the *Vicia faba* nodule outer cortex tissue is composed of triterpenoids. Of the 90%, over 82% is betulin while the remaining 8% is lupeol (Hartmann *et al.*, 2002). The ectopic expression of *AsOXA1* (a  $\beta$ -amyrin synthase from *Aster sedifolius*) in *M. truncatula* resulted in the accumulation of significantly higher levels of  $\beta$ -amyrin-derived sapogenins in the leaves, roots and nodules of the transgenic plant, which exhibited a high-nodulating phenotype (Confalonieri *et al.*, 2009; Figure 4). Together, these reports suggest a yet undefined role for specialized simple triterpenes in the establishment and progress of nodulation in several legumes. However, more recent data indicate that simple triterpenes may have more complex roles in symbiosis.

The *AMY2* gene, which encodes a multifunctional OSC that cyclizes 2,3-oxidosqualene to  $\beta$ -amyrin and dihydrolupeol, is highly expressed in the roots of *Lotus japonicus* and in nodules infected with the symbiotic bacterium *Mesorhizobium loti*. Silencing of *AMY2* caused root growth and development defects but did not have any obvious effect on nodulation. Transgenic *AMY2* silenced roots generated using *Agrobacterium rhizogenes*-mediated transformation led to severe retardation of root growth, while stably silenced plants had short stunted roots (Krokida *et al.*, 2013). Expression of the gene for another *L. japonicus* OSC, *OSC3* (a lupeol synthase) is induced upon nodulation and is localized to the vascular bundles, inner cortical cells and uninfected cells of the central tissue of developing and mature nodules upon infection with *M. loti*. The expression pattern of the *OSC3* gene may suggest a structural role for lupeol in the membrane of developing nodules and/or a role in transportation of compounds in and out of the nodules (Delis *et al.*, 2011). Silencing of *OSC3* in *L. japonicus* abolished lupeol synthesis and resulted in significantly more visible nodules compared to the control plants 20 days post inoculation with *M. loti* (Figure 4). In *M. truncatula* and *G. glabra*, the lupeol synthase homologs are also expressed during nodule formation, suggesting a general role for lupeol in nodulation across leguminous species (Hayashi *et al.*, 2004; Gamas *et al.*, 1996; Shibuya *et al.*, 1999). In the *OSC3* silenced *L. japonicus* roots, the absence of lupeol resulted in a rapid nodulation phenotype and was accompanied by the elevated expression of early nodulin 40 (*ENOD40*), an early marker gene for nodule primordia initiation and cortical cell division. Exogenous application of lupeol to *M. loti*-infected wild-type plants provided further evidence for a negative regulatory effect of lupeol on the expression of *ENOD40* (Delis *et al.*, 2011). Through a series of *in vitro* screens lupeol has also been shown to activate apoptosis in human cancer cells by decreasing the  $\beta$ -catenin protein level and inducing degradation in the  $\beta$ -catenin signaling pathway (Saleem *et al.*, 2009). Homologs of the mammalian  $\beta$ -catenin gene have been identified in plants and like in mammals the  $\beta$ -catenin protein in plants localize to the nucleus or cytosol depending on the availability of interacting proteins (Coates, 2003). Although the mode of action of the  $\beta$ -catenin proteins in plants has not been entirely characterized, associated proteins have been identified in gibberellin and brassinosteroid signaling pathways (Amador *et al.*, 2001; He *et al.*, 2002; Lee *et al.*, 2002). Also in *A. thaliana*,  $\beta$ -catenin related proteins have been identified and shown to promote lateral root development (Coates *et al.*, 2006). However, a role for  $\beta$ -catenin and related proteins in nodulation has not yet been reported.

Considering the structural similarity of triterpenes and triterpenoids with primary sterols, these specialized compounds have been proposed to function as membrane constituents. However, very little is known about the temporal and spatial embedding of these molecules in the membranes or the role that they may play during normal plant growth. Triterpenes may substitute sterols at certain developmental stages as has been suggested for marneral (Go *et al.*, 2012), or they may have functions in various physiological processes such as nodule formation and cuticle composition. It has also been suggested that isoprenoids, unidentified as yet but other



than sterols, are responsible for the activity of plant microRNAs (miRNAs) that guide ARGONAUTE (AGO) protein complexes to regulate expression of complementary RNAs. In the *A. thaliana mad3* mutant, which has a miRNA activity deficient phenotype, the functions of AGO1 are compromised due to its reduced membrane association. This membrane perturbation was suggested as the underlying mechanism for the miRNA deficiency (Brodersen *et al.*, 2012). It is intriguing to consider that the presence of specialized triterpenes in plant membranes, and possibly also the membrane permeabilizing properties of saponins, may be implicated in these phenotypes.

### Effects of accumulating other saponin pathway intermediates

Mutation of genes encoding P450s and/or UGTs involved in saponin biosynthesis in *M. truncatula* and *S. lycopersicum* has revealed detrimental effects on plant growth that may be attributable to the accumulation of toxic intermediates. The following examples highlight the need for full glycosylation of saponins in order to maintain normal plant architecture (Figure 4). *M. truncatula* synthesizes a diverse array of saponins that can be classified into two main types, the hemolytic and non-hemolytic saponins, based on their ability to lyse red blood cells. Both types of saponin are synthesized through sequential oxidation and glycosylation of the oleanane aglycone,  $\beta$ -amyrin. The nature of the P450-mediated oxidation of the aglycone determines whether the saponins are hemolytic or non-hemolytic. The multistep oxidation of carbon 28 by the P450 CYP716A12 is essential for the synthesis of hemolytic saponins. Following this oxidation event, a glucose moiety is added at this position by the glucosyltransferase UGT73F3. The *M. truncatula* loss-of-function mutants of CYP716A12 and UGT73F3 do not accumulate hemolytic saponins and have a dwarf phenotype (Carelli *et al.*, 2011; Naoumkina *et al.*, 2010). The UGT73F3 mutants flowered and generated pods with morphologically normal seeds that had a reduced germination rate (Naoumkina *et al.*, 2010). A similar, strong retarded growth phenotype is also observed in tomato plants upon silencing of the GlycoAlkaloid MEtabolism 1 (*GAME1*) gene, a galactosyltransferase involved in the synthesis of the steroidal glycoalkaloid  $\alpha$ -tomatine. The *GAME1*-silenced plants accumulate ~300-fold more tomatidine than wild-type plants, develop deformed leaves and produce many small flower buds, most of which are aborted before fertilization, resulting in the development of a small number of parthenocarpic, shriveled fruits (Itkin *et al.*, 2011).

In *A. strigosa* and *M. truncatula*, full glycosylation of root saponins has been shown to be critical to prevent deleterious effects on plant root physiology (Figure 5). Mutants of *A. strigosa* that are affected at either the *Sad3* or the *Sad4* locus (both of which are as yet uncloned) accumulate avenacin A-1 lacking the  $\beta$ -1,4-linked D-glucose moiety. Both types of mutants have reduced root growth and reduced numbers of root hairs, root epidermis-specific membrane trafficking defects, and accumulate callose (a plant polysaccharide composed of glucose residues). As callose deposition can be induced in plants upon pathogen attack or wounding, its

accumulation in the *sad3* and *sad4* mutants is likely to be a stress response caused by toxicity of the incompletely glycosylated intermediate. The *sad3* and *sad4* root phenotypes are suppressed in a *sad1* mutant background, consistent with this; *sad1/sad1 sad3/sad3* and *sad1/sad1 sad4/sad4* double mutants do not accumulate avenacin intermediates and have normal roots, confirming that the accumulation of incompletely glycosylated avenacin biosynthetic intermediates is responsible for the cytotoxic effects observed in the roots (Mylona *et al.*, 2008).

In *M. truncatula* the silencing of Makibishi 1 (*Mkb1*) gene encoding an E3 ubiquitin ligase involved in controlling the activity of HMGR (the rate-limiting enzyme of the MVA pathway) results in accumulation of monoglycosylated saponins in transgenic hairy roots. The cells in the transgenic hairy roots had an irregular shape instead of the normal cylindrical shape and lacked intercellular space, which caused severe cell enlacing rendering the tissue in the root cortex zone rigid and prone to tissue rupturing. As a result, the hairy roots were dissociated from each other and formed caltrop-like structures. *Mkb1* recruits the endoplasmic reticulum-associated degradation (ERAD) protein quality control system to regulate the levels and activity of HMGR and hence the amount of saponin precursors generated via the MVA pathway. This mechanism prevents the unrestrained biosynthesis of saponins in *M. truncatula*. Therefore, upon silencing of *Mkb1* the flux through the saponin biosynthesis pathway is disturbed, leading to an over-accumulation of monoglycosylated saponins and severe root morphology defects. The transgenic hairy root phenotype could be mimicked by the overexpression of the catalytic domain of HMGR, which does not undergo ERAD control and therefore allows unrestrained synthesis of saponins in *M. truncatula*. These transgenic hairy roots also accumulated monoglycosylated saponins and showed the caltrop-like hairy root morphology seen in *Mkb1*-silenced hairy roots, indicating that the incompletely glycosylated saponins are associated with the observed root morphology effects (Pollier *et al.*, 2013).

The *A. strigosa sad3* and *sad4* mutants and the *M. truncatula Mkb1*-silenced mutant described above accumulate incompletely glycosylated saponin biosynthetic intermediates and show strikingly similar root phenotypes (Figure 5B). In the mutants of both *A. strigosa* and *M. truncatula*, the root epidermal cell layer was incompletely formed exposing the inner root cortical cells to the growth environment. The morphological damage to the roots caused leakage of intracellular compounds and release of incompletely glycosylated saponin intermediates. In contrast to growth medium of control hairy roots that did not contain saponins, the growth medium of the *Mkb1*-silenced *M. truncatula* roots accumulated monoglycosylated saponins and its application to control hairy roots caused transient tissue loosening and mimicked the phenotype of the *Mkb1*-silenced hairy roots, suggesting toxic effects of incompletely glycosylated saponins on plant cells (Pollier *et al.*, 2013).

The severe growth and development effects observed in plants when saponin glycosyltransferases are impaired indicates that complete glycosylation of saponin intermediates is important in protecting the plant from the toxic effects of accumulation of incompletely glycosylated compounds.

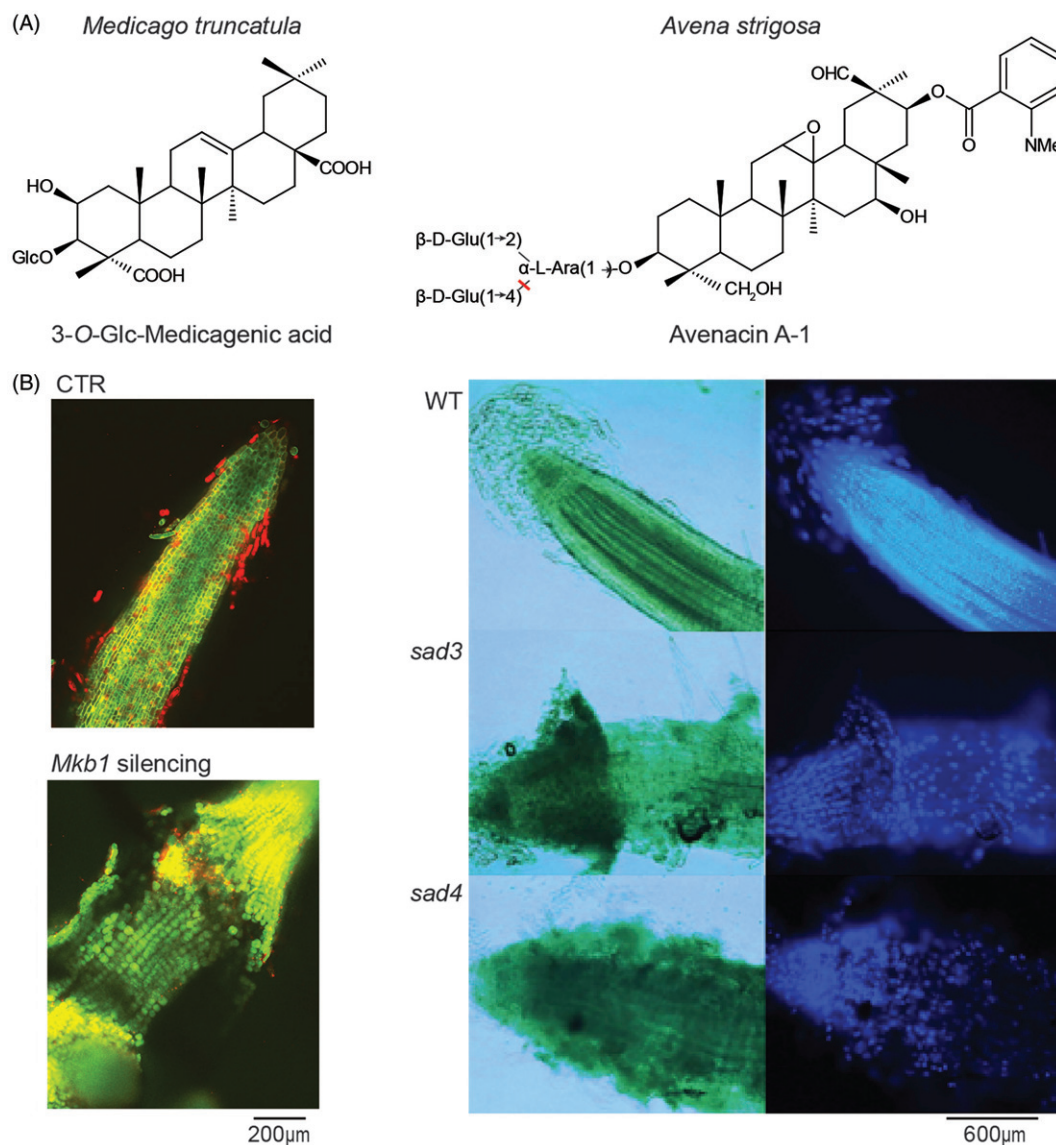


Figure 5. Phenotypic similarity of *Medicago truncatula* and *Avena strigosa* mutants accumulating incompletely glycosylated saponins. (A) Chemical structures of a representative monoglycosylated saponin, 3-O-Glc-Medicagenic acid accumulating in *Mkb1* silenced *M. truncatula* roots (left) and avenacin A-1 lacking the  $\beta$ -1,4-linked D-glucose (marked with a red line) accumulating in *sad3* and *sad4* oat mutants (right). (B) Confocal microscopy analysis (left) of control (CTR, top) and *Mkb1* silenced (bottom) *M. truncatula* hairy roots, compared to bright field (left) and UV illumination (right) microscopy of wild type (WT), *sad3* and *sad4* oat mutants (right) show similar root cap and root epidermal layer defects in both plants. From Mylona *et al.* (2008) and Pollier *et al.* (2013).

For instance, the avenacosides accumulate in a biologically inactive form in oat leaves and are activated by deglycosylation upon tissue damage or pathogen attack (Gus-Mayer *et al.*, 1994; Nisius, 1988). For other saponins, glycosylation is essential for membrane permeabilization and antimicrobial activity, which is lost upon removal of the sugar residues (Morrissey & Osbourn, 1999). For example, the ability of the fungal pathogen *Gaeumannomyces graminis* var. *avenae* to infect oat roots is dependent on its ability to produce a fungal avenacin hydrolase (Bowyer *et al.*, 1995). Similarly, the ability to degrade the tomato steroidal glycoalkaloid  $\alpha$ -tomatine contributes to the pathogenicity of various fungal pathogens of tomato (Morrissey & Osbourn, 1999; Ökmen *et al.*, 2013; Pareja-Jaime *et al.*, 2008; Sandrock & VanEtten, 2001). These  $\alpha$ -tomatine detoxification mechanisms commonly involve removal of sugars from  $\alpha$ -tomatine to partially glycosylated molecules such as  $\beta_2$ -tomatine, or to the

aglycone, tomatidine (Bednarek & Osbourn, 2009; Morrissey & Osbourn, 1999; Sandrock & Vanetten, 1998).  $\alpha$ -Tomatine degradation products have also been reported to suppress induced defense responses in plants, suggesting that these molecules may have signaling properties. The tomato pathogen *Septoria lycopersici* produces a tomatinase enzyme that hydrolyses a single sugar from the tetrasaccharide chain of  $\alpha$ -tomatine to give  $\beta_2$ -tomatine. This hydrolysis product suppresses induced plant defenses, suggesting that the host plant has subverted this fungal detoxification mechanism to bolster its own defenses (Bednarek & Osbourn, 2009; Bouarab *et al.*, 2002; Ito *et al.*, 2004). It has also been reported that the aglycone tomatidine suppresses induced defenses in tomato cell cultures (Ito *et al.*, 2004). Ökmen *et al.* (2013) tested the effects of  $\alpha$ -tomatine and tomatidine on tomato cells and did not observe suppression of induced defense responses. They did not, however, test the effects of

$\beta_2$ -tomatine. The effects of steroidal alkaloids on plant cells require further investigation. Of note, these molecules are known to interfere with regulation of sterol homeostasis in yeast (Simons *et al.*, 2006).

### Industrial potential as bioactive principles

With the broad diversity in structures and biological activities of saponins and their biosynthesis intermediates, these compounds have been sought for various applications in the pharmaceutical, cosmetic, food and agronomic industry (Augustin *et al.*, 2011; Moses *et al.*, 2013; Sparg *et al.*, 2004). Saponins from *Camellia oleifera* and *Sapindus mukorossi* are useful in detergents and cosmetics as they have excellent foaming properties, moderate detergency and anti-dermatophytic activity (Chen *et al.*, 2010; Tamura *et al.*, 2012; Yang *et al.*, 2010). Quil A, a mixture of over 25 different saponins extracted from *Quillaja saponaria*, is the most widely used saponin adjuvant in veterinary vaccines (Dalsgaard, 1978). *Yucca schidigera* and *Q. saponaria* extracts are used as livestock dietary additives for fecal odor control (Cheeke, 2009; Killeen *et al.*, 1998). Several C-27 steroidal saponins show antifungal activity (Morrissey & Osbourn, 1999; Yang *et al.*, 2006), and glycyrrhizin accumulating in licorice roots is an efficient natural sweetener (Kitagawa, 2002). The wide spectrum of biological activities of this specialized class of compounds has been attributed to the diverse array of structures synthesized naturally by plants. Although such structural diversity is beneficial, the vast spectrum of biological activities can prove to be antagonistic to their application, particularly in the pharmaceutical sector. For instance, many saponins are cytotoxic and therefore potential chemotherapeutic agents, but also have hemolytic activity hindering their application as anti-cancer drugs (Podolak *et al.*, 2010).

Several studies have hence focused on generating semi-synthetic derivatives inspired by the naturally accumulating saponins and their biosynthetic intermediates to generate a new range of candidate drugs with enhanced activity and reduced adverse effects. For example, synthetic derivatives of the pentacyclic triterpenoids oleanolic, ursolic and betulinic acid, which naturally accumulate to high concentrations in plants, have been evaluated to be strikingly more active than the parent compounds in several clinical applications (Liby & Sporn, 2012; Liby *et al.*, 2007a,b). The semi-synthetic derivatives of oleanolic acid (Figure 6), bardoxolone (CDDO) and bardoxolone methyl (CDDO-Me) were clinically evaluated as anti-cancer agents (Hong *et al.*, 2012; Tsao *et al.*, 2010). CDDO-Me was also clinically tested for anti-inflammatory activity in patients with chronic kidney disease and type 2 diabetes to improve kidney function (Pergola *et al.*, 2011). However, the worldwide phase III trial of the drug was terminated following severe adverse effects and mortality in patients administered the drug (<http://www.clinicaltrials.gov/show/NCT01351675>). It has been speculated that CDDO and its derivatives interact with multiple targets or entire networks in the cell, and therefore might be most effective in the early stages of disease progression prior to irreversible tissue damage, rather than at advanced stages of disease (Liby & Sporn, 2012; Sporn *et al.*, 2007).

The patients in the phase III trial with CDDO-Me had moderate-to-severe chronic kidney disease with type 2 diabetes. A detailed analysis of data collected during this terminated trial can provide essential insight into the relation between adverse effect, mortality and dose of CDDO-Me given to patients, which can then be utilized for future tests with other CDDO analogues (Abboud, 2013). The imidazolidine and ethylamide derivatives of bardoxolone, CDDO-Im and CDDO-EA, respectively, induce chondrogenic differentiation in organ cultures and could be used for the treatment of diseases where enhancing chondrogenesis is therapeutically useful, including the prevention or treatment of osteoarthritis (Suh *et al.*, 2012). Multiple *in vitro* and *in vivo* studies with CDDO-Me have also demonstrated the effect of this compound in the prevention and treatment of solid tumors (Wang *et al.*, 2014). The combination of CDDO-Me or CDDO-EA with vorinostat, a histone deacetylase inhibitor, effectively delayed tumorigenesis in mouse models, proving to be a promising combination for chemoprevention (Tran *et al.*, 2013). Another semi-synthetic derivative evaluated clinically in phase I and II trials is bevirimat (Figure 6). This betulinic acid derivative is a potent inhibitor of human immunodeficiency virus (HIV) maturation, which showed promising clinical outcomes (Smith *et al.*, 2007). Other derivatives of betulin, betulinic acid and dihydrobetulin also have demonstrated potent anti-HIV and anti-leishmanial activities (Sousa *et al.*, 2014; Xiong *et al.*, 2010).

Naturally, accumulating plant compounds also have biological activities of interest for clinical applications and are routinely evaluated for potential novel therapeutics. YQ2, a 6:4 (v:v) blend of partially purified *Y. schidigera* and *Q. saponaria* extracts, has been clinically studied as an anti-obesity medicine. Administration of YQ2 to 51 hypercholesterolemic patients showed a decrease in total and low-density lipoprotein cholesterol levels in blood plasma, compared to 36 patients given a placebo treatment. YQ2 also improved gastrointestinal motility in the treated patient group, thereby enhancing gastrointestinal functions (Kim *et al.*, 2003). The triterpenoid saponin escin (Figure 6), isolated from seeds of *Aesculus hippocastanum* (horse chestnut), has been clinically evaluated in gel formulations as combination therapy with diethylammonium salicylate and heparin. The gel combination of the three active principles showed positive effects on tenderness reaction, edema formation and joint function, compared to placebo. This randomized, double blind, controlled, group comparative study with 158 patients, topically treated or not with escin, concluded that the gel combination containing escin was well tolerated and led to rapid pain reduction, implicating its recommended use for the treatment of blunt impact injuries (Wetzel *et al.*, 2002). Celastrol (Figure 6), extracted from roots of the traditional Chinese herb *Tripterygium wilfordii*, has potent anti-inflammatory and anti-cancer activities, and holds great potential for the treatment of multiple myelomas and other hematological malignancies (Chiang *et al.*, 2014; Kannaiyan *et al.*, 2011). QS-21 (Figure 6), is a soluble fraction of triterpenoid saponins from *Q. saponaria* with immunological adjuvant activity. QS-21 is currently being clinically evaluated in phase I and II studies for use in human vaccines against HIV (Leroux-Roels *et al.*, 2014), *Mycobacterium*



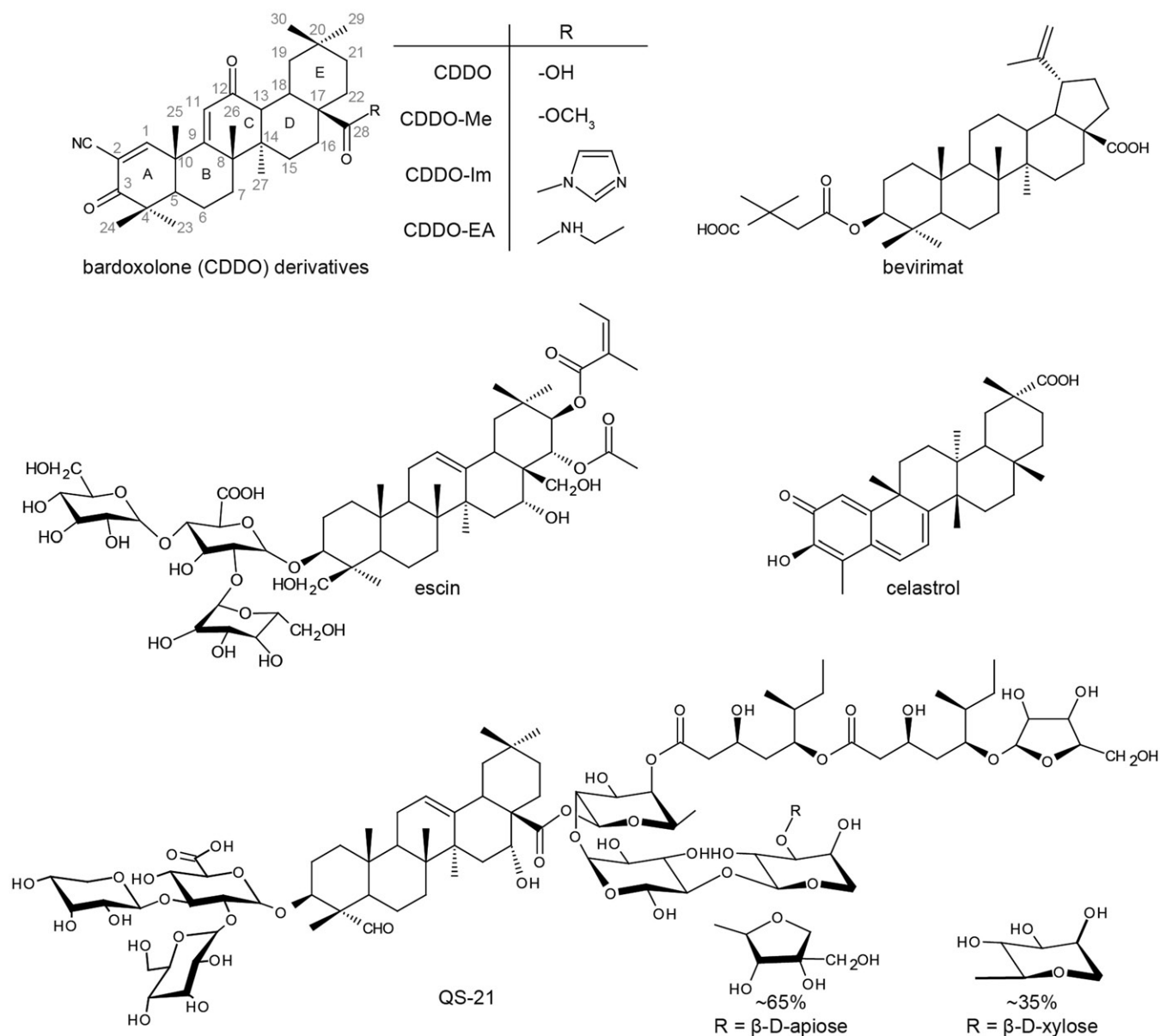


Figure 6. Overview of chemical structures of triterpenoids and saponins with industrial potential.

*tuberculosis* (Montoya *et al.*, 2013), varicella-zoster virus (Lal *et al.*, 2013), cancer (Garçon & Van Mechelen, 2011) and malaria (Ndungu *et al.*, 2012).

Despite the various potential applications of saponins, their biosynthetic intermediates and derivatives, very few compounds are currently commercially exploited. A major concern hindering their widespread application as therapeutics is the lack of information about the mechanisms of adsorption, distribution, metabolism, excretion and toxicity of these compounds in human and animal cells. Well-designed, associated, systematic *in vitro* and *in vivo* studies will play a key role in deepening our understanding of the mechanisms involved in the action of saponins, before their widespread usage as drugs in the future.

### Exploring structure–activity relationships

Saponins, their biosynthetic intermediates and derivatives have been ascribed a number of pharmacological activities (Figure 7), most notably permeabilization of cell membranes,

cytotoxic effects on cancerous cells, adjuvant properties, immunomodulatory potential and lowering of serum cholesterol (Francis *et al.*, 2002; Hostettmann & Marston, 2005; Man *et al.*, 2010; Sjölander *et al.*, 1998; Sun *et al.*, 2009). Despite their structural diversity these compounds share distinct biological properties, and therefore several studies have focused on understanding the various biological activities associated with them in relation to their structural features. Identification of effective combinations of key functional entities and aglycones responsible for specific bioactivities allows targeted synthesis of novel active principles with enhanced efficacy and reduced adverse effects. Here, we summarize some of the most investigated structure–activity relationships of these compounds.

### Effect on cell membranes

Several biological effects of saponins are attributable to their ability to permeabilize cell membranes. The hemolytic activity of saponins is a consequence of the formation of

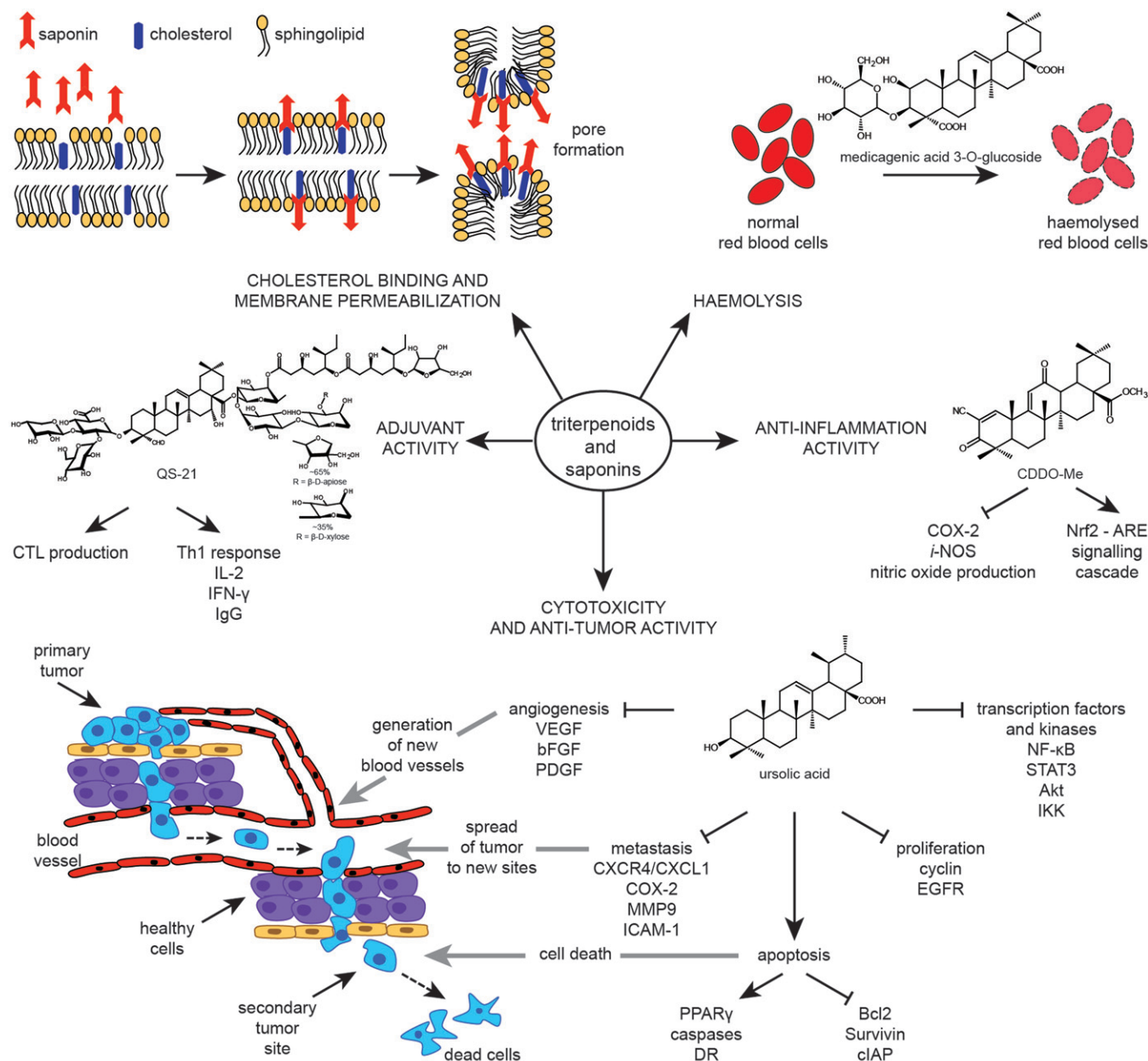


Figure 7. Overview of biological activities of triterpenoids and saponins in animals. The structures of representative compounds for each activity are given, together with the various molecular targets and affected pathways. Gene, pathway or metabolite activation and inhibition are indicated by arrow heads and blunt ends, respectively. Akt, serine/threonine-specific protein kinase; Bcl2, B-cell lymphoma 2; bFGF, basic fibroblast growth factor; cIAP, cellular inhibitor of apoptosis; COX-2, cyclooxygenase-2; CTL, cytotoxic T-lymphocyte; CXCR4/CXCL12, C-X-C chemokine receptor type 4 complex with C-X-C motif chemokine 12; DR, death receptor; EGFR, epidermal growth factor receptor; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon γ; IgG, immunoglobulin G; IKK, IκB kinase; IL-2, interleukin 2; i-NOS, inducible nitric oxide synthase; MMP9, matrix metalloproteinase 9; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2-ARE, nuclear factor (erythroid-derived 2)-like 2, antioxidant responsive element; PDGF, platelet-derived growth factor; PPARγ, peroxisome proliferator-activated receptor-γ; STAT3, signal transducer and activator of transcription 3; Th1, T helper 1; VEGF, vascular endothelial growth factor.

stable pores in the erythrocyte membrane (Seeman, 1967). The interaction of saponins with cholesterol in the lipid membrane leads to the formation of insoluble complexes, which rearranges the architecture of the cholesterol-containing lipid bilayer to generate pores and increase membrane permeability (Augustin *et al.*, 2011; Lorent *et al.*, 2014; Morrissey & Osbourn, 1999). The membrane permeabilizing activity of saponins is correlated with their amphipathic nature and affected by the aglycone structure, saccharide chain length, sugar variants in the glycosidic chain and type of aglycone-sugar and sugar-sugar linkages.

Among the various saponin structures, there is a clear association between the type of aglycone backbone and hemolytic activity. Saponins derived from the lupane aglycone do not permeabilize membranes and are only slightly hemolytic, compared to those derived from the oleanane aglycone which strongly permeabilize membranes and hemolyse red blood cells (Figure 8A; Gauthier *et al.*, 2009a,b). However, not all oleanane-type triterpenoid saponins have hemolytic properties. In *M. truncatula* two distinct groups of oleanane-derived triterpenoid saponins have been described; the non-hemolytic soyasaponins and the hemolytic

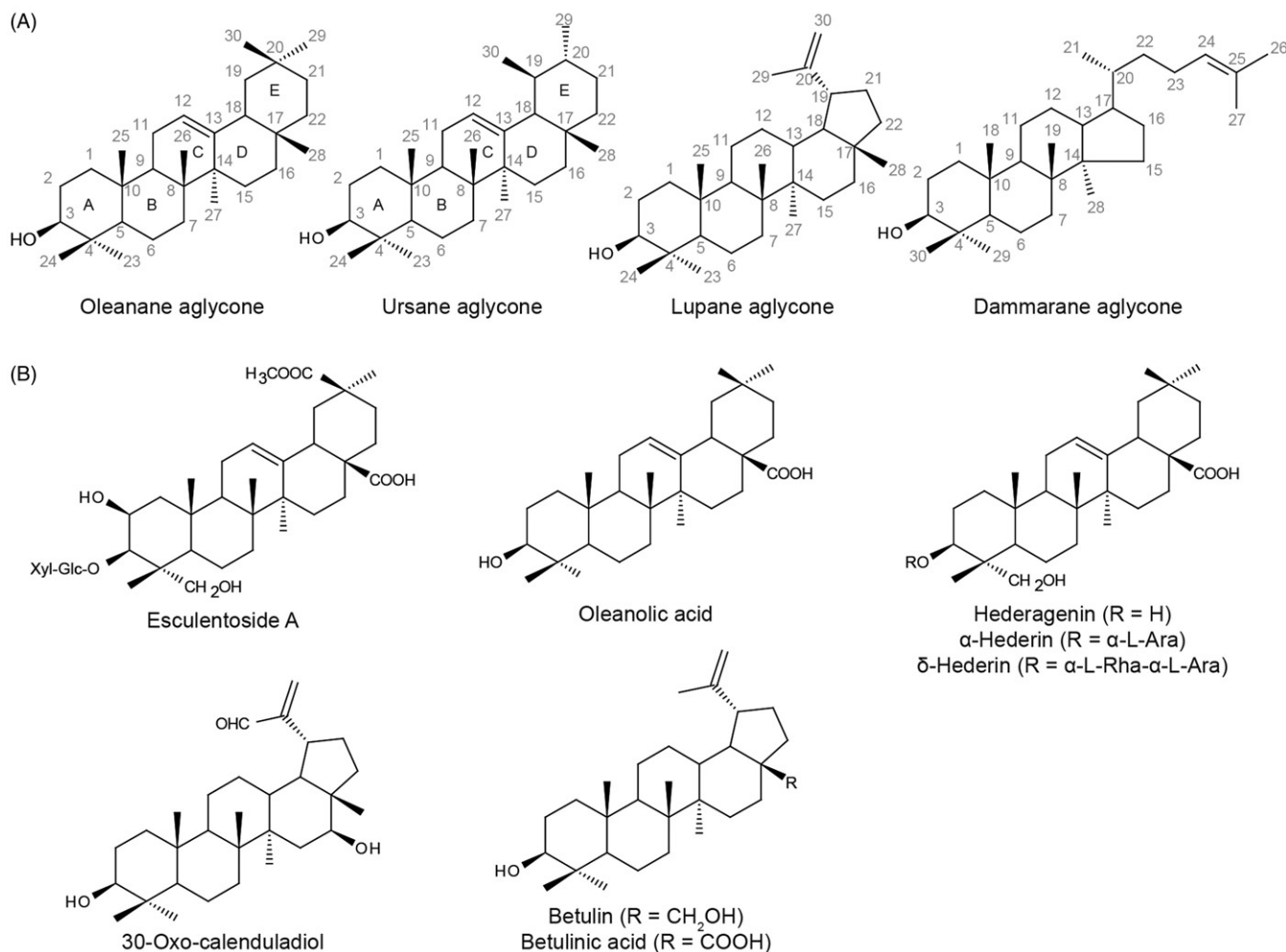


Figure 8. Overview of chemical structures. (A) Carbon and ring numbering of oleanane, ursane, lupane and dammarane aglycones. (B) Examples of triterpenoids and saponins discussed in structure-activity relationship studies.  $\alpha$ -L-Ara,  $\alpha$ -L-arabinose;  $\alpha$ -L-Rha,  $\alpha$ -L-rhamnose; Glc, glucose; Xyl, xylose.

saponins derived from oleanolic acid (Oleszek, 1996; Yoshiki *et al.*, 1998). The hemolytic saponins have a carboxyl group at position 28 of the oleanane backbone and other oxidations on carbons 2, 16 and/or 23, while the non-hemolytic soyasaponins have a hydroxyl group at carbon 24 and additional oxidations on carbons 21 and/or 22 (Figure 8A). Modification of the carboxyl group at position 28 through chemical addition of amide-containing aromatic rings significantly lowers hemolytic activity of the saponin, as demonstrated for esculentoside A (Figure 8B, an oleanane saponin isolated from roots of *Phytolacca esculenta*) compared with the non-modified parent saponin (Wu *et al.*, 2007). As described above, targeted silencing of *CYP716A12* encoding the P450 that catalyzes the C-28 carboxylation of *M. truncatula* saponins, results in transgenic plants of which the saponin extracts have lost their hemolytic activity, in contrast to wild type *M. truncatula* plant extracts which are highly hemolytic (Carelli *et al.*, 2011). It has also been suggested that increasing the polarity in ring A of oleanane saponins (Figure 8A) by addition of a hydroxyl or carboxyl group together with maintaining relative hydrophobicity in rings D or E is essential for hemolytic activity (Voutquenne *et al.*, 2002). Esterification of the carboxyl group at position 28 of hederagenin-derived saponins (Figure 8B), reduces

polarity in rings D–E and increases hemolytic activity compared to its corresponding free acid (Chwalek *et al.*, 2006).

The saccharide chain on the saponins has been extensively studied to establish correlations between chain length, sugar components and interglycosidic linkages that influence membrane-permeabilizing activity, as the sugar side chain is essential for the hemolytic activity of saponins. For both digitonin and avenacin A-1, sequential deglycosylation and sugar removal reduces hemolytic activity and decreases membrane permeability (Armah *et al.*, 1999; Nishikawa *et al.*, 1984). Comparison of two hederagenin-derived saponins  $\alpha$ -hederin and  $\delta$ -hederin (Figure 8B) has revealed that the former is more hemolytic than the latter, and this has been attributed to differences in the length of the glycosidic chain (Chwalek *et al.*, 2006);  $\delta$ -hederin has an  $\alpha$ -L-rhamnose linked to an  $\alpha$ -L-arabinose at carbon 3, while  $\alpha$ -hederin has a single  $\alpha$ -L-arabinose. The anomeric configuration of the linked sugars has also been demonstrated to influence the hemolytic activity of saponins. For instance, when the  $\alpha$ -L-rhamnose is linked to a  $\beta$ -L-anomer of arabinose instead of the  $\alpha$ -L-anomer like in  $\delta$ -hederin, the hemolytic activity is considerably reduced in comparison to  $\delta$ -hederin (Chwalek *et al.*, 2006). It has been observed that in general the monodesmosidic



saponins, which have sugar moieties linked to a single carbon on the aglycone, are more hemolytic than the bidesmosidic saponins with sugars linked at two different positions, usually carbon 3 and carbon 28, of the aglycone. Among the monodesmosidic saponins, those that are glycosylated at position 3 are often more hemolytic than those at position 28 (Voutquenne *et al.*, 2002). The type of terminal sugar and the linkage between the saccharides of the saponin can also have considerable influence on hemolytic potential. For the diglycosidic saponins derived from hederagenin, increased activity was associated with  $\beta$ -configuration of the terminal sugar and 1 $\rightarrow$ 4 linkages between the sugars when compared with 1 $\rightarrow$ 6 linkages (Chwalek *et al.*, 2006).

### Cytotoxicity and anti-tumor activity

The cytotoxic activity of numerous triterpenes and their triterpenoid saponins has been exploited for anti-tumor application, and the ability of these compounds to induce apoptosis by different cellular mechanisms has been extensively investigated (Lee *et al.*, 2011; Podolak *et al.*, 2010; Raju & Mehta, 2009; Sun *et al.*, 2006a; Thakur *et al.*, 2011). The triterpenoid aglycones oleanolic acid and ursolic acid exhibit cytotoxicity against human leukemia and lymphoma cells, with the latter being more potent (Chiang *et al.*, 2003). A series of derivatives of these triterpenes has been tested for the ability to inhibit nitric oxide production by macrophages, as potential anti-cancer and chemopreventive drugs (Favaloro *et al.*, 2002; Finlay *et al.*, 1997; Honda *et al.*, 1997, 1999, 2000a,b, 2002). Several systematic structure-activity studies for these derivatives have led to some important general observations. The oleanane derivatives were found to be more potent than the corresponding ursane derivatives (Figure 8A). In ring A, enone functionality at position 3 of the aglycone significantly increases activity. A carboxyl, methoxycarbonyl, or nitrile group at position 2 also enhances activity, contrary to a hydroxyl, aminocarbonyl, methoxy, chloride or bromide groups, which decrease activity of the derivative. However, a formyl group at position 2 makes the derivative highly cytotoxic. The methyl groups at position 23 and 24 of the aglycone are important for nitric oxide inhibition activity, as their removal significantly decreases this activity. In the C ring, modifying the naturally occurring 12(13)-ene double bond to 9(11)-en-12-one, 12(13)-en-11-one, 13(18)-en-11-one or 12-one functionalities enhanced potency of the derivative, while a 9(11)-ene did not affect potency and saturation of ring C, 11(12),13(18)-diene or 9,11-epoxide functionalities were less potent. Among all the ring C modifications, the 9(11)-en-12-one functionality enhanced potency by 10–100 times and therefore was used for further modifications in ring A of the derivative. The presence of a carboxyl or nitrile group at position 2 and an enone functionality at position 3, synergistically enhanced potency  $\sim$ 10 000 fold. Also the combination of 12(13)-en-11-one or 12(18)-en-11-one with enone at position 3 and nitrile at position 2 strongly enhanced potency. The clinically evaluated semi-synthetic derivative of oleanolic acid, CDDO (bardoxolone) was developed because of these meticulous structure-activity studies. CDDO contains the highly potent 9(11)-en-12-one functionality in ring C, a nitrile group at position 2, and an enone functionality at position 3 of

the aglycone, and shows the strongest nitric oxide inhibitory activity among all the generated oleanolic acid derivatives. Preliminary structure-activity studies also indicate that derivatization of the carboxyl group at position 28 of  $\beta$ -hederin abolishes broad-spectrum antitumor activity, but increases antitumor selectivity and cytotoxicity (Liu *et al.*, 2010).

Next to the oleananes and ursanes, the lupane- and dammarane-type triterpenoid saponins (Figure 8A) have been studied for their cytotoxic activity against various human cancer cell lines (Park *et al.*, 2005; Podolak *et al.*, 2010). Compared to hederagenin and oleanolic acid, the lupane triterpenoids betulin and betulinic acid showed higher cytotoxicity (Figure 8B; Gauthier *et al.*, 2009a,b). Among the monodesmosidic lupane-type saponins, cytotoxicity was observed when  $\alpha$ -L-rhamnose,  $\alpha$ -D-mannose or  $\alpha$ -D-arabinose sugar was linked to position 3, but not when  $\beta$ -D-glucose, or  $\beta$ -D-galactose was present at the same position. The bidesmosidic saponin of betulinic acid bearing  $\alpha$ -L-rhamnose moieties at both the carbon positions 3 and 28 was more potent than the naturally occurring saponin containing  $\alpha$ -L-arabinose at position 3 and  $\beta$ -D-glucose at position 28 (Gauthier *et al.*, 2009a,b). The lupane-type saponins also show better cytotoxicity and stronger anti-cancer activity than the germanicane-type saponins (Thibeault *et al.*, 2007). The lupane triterpenoid, dihydrobetulinic acid is a potent inhibitor of HIV replication. Structure-activity relationship studies have also suggested that the substitution at position 19 enhances the anti-HIV activity of the molecule, compared to betulinic acid (Fujioka *et al.*, 1994). Also, the non-acid semi-synthetic lupane-type triterpenoid 30-oxo-calenduladiol (Figure 8B) shows potent anti-HIV activity. The aldehyde function at position 30 was found to be essential for the anti-HIV activity, as its absence resulted in an inert compound (Barroso-González *et al.*, 2009). In contrast to most triterpenoid saponins, for the ginsenosides anti-cancer activity is inversely correlated with the number of linked sugars, as sugar moieties reduce the hydrophobicity of the compound and decrease membrane permeability (Qi *et al.*, 2010). Also, reduced anti-cancer activity is observed with ginsenosides glycosylated at position 6 (Figure 8B), compared to those with sugars linked at position 3 or 20 (Chen *et al.*, 2009; Li *et al.*, 2009; Popovich & Kitts, 2002).

A major drawback in the clinical development of anti-cancer drugs, particularly those based on triterpenoid saponins, has been the hemolytic activity associated with these compounds. The oleanane triterpenoid saponins derived from hederagenin showed a strong correlation between hemolytic activity and cytotoxicity (Chwalek *et al.*, 2006). For the steroidal saponins however, these activities are not correlated and it is suggested that, they unlike the triterpenoid saponins, execute hemolysis and cytotoxicity through distinct mechanisms (Wang *et al.*, 2007). The structure-activity relationship studies for the steroidal saponins with respect to the variation in sugar chain and steroidal aglycone are extensively reviewed in Podolak *et al.* (2010).

### Adjuvant properties

The ability of saponins to activate the mammalian immune system has led to significant interest in their potential as

vaccine adjuvants. The most promising lead candidates are Quil A and its derivative QS-21 which are actively being evaluated in both human and animal vaccine formulations against HIV, cancer, malaria, various bacterial and viral infectious agents (Sun *et al.*, 2009). However, the major drawbacks limiting their widespread application in human vaccines are the high toxicity, hemolytic properties and instability in aqueous solutions associated with saponins. Various structure–activity studies have therefore aimed at identifying the structural features that reduce undesirable effects and enhance or maintain their adjuvant activity. A comparative analysis of 47 saponins for adjuvant and hemolytic activities suggested that both the sugar chain and aglycone backbone are essential for adjuvant activity (Oda *et al.*, 2000). The aldehyde functionality at position 4 of the QS-21 saponin (Figure 6) was found to be essential for antibody stimulation and induction of an immune response, as derivatives modified at this function did not show adjuvant activity (Soltysik *et al.*, 1995). Using DS-1 a deacylated derivative of QS-21, the lipophilic acyl side chain was also shown to be essential for stimulating cytotoxic T-lymphocyte production against antigens, as the deacylated derivative only induced a weak immune response (Liu *et al.*, 2002). Re-acylation at a different position than in QS-21 restored adjuvant activity to some extent, but like its parent DS-1 failed to induced cytotoxic T-lymphocyte production.

The soyasaponins and lablabosides also show strong adjuvant activity and low hemolytic activity, and contrary to the QS-21 do not contain acyl, carboxyl or aldehyde functionalities on their structure. On the other hand, the escins have an acyl group, but show poor adjuvant activity and high hemolytic activity making them poor adjuvant candidates (Oda *et al.*, 2000). These observations highlight that the functionalities on the aglycone alone are not determinant factors for the adjuvant properties of the saponins, but the overall conformation harmoniously influence adjuvant activity. The soyasaponins with linked sugars were strong adjuvants, contrary to the corresponding soyasapogenol aglycones, which were inert. In addition, among the soyasaponins, the adjuvant activity generally increased with increase in sugar chain length (Oda *et al.*, 2003). For the protopanaxadiol and protopanaxatriol-type saponins the number, position and type of sugar linkages did not affect adjuvant activity, but significantly influenced the nature of immune response (Sun *et al.*, 2005, 2006b).

### Molecular targets identified in bioassays

The simple triterpenoids, glycoalkaloids and saponins are multifaceted in their molecular mechanisms as they modulate multiple targets, including transcription factors and kinases, in multiple pathways (Figure 7). The activation of various pro-inflammatory mediators linked with different stages of cancer progression and inflammation like the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), signal transducer and activator of transcription 3 (STAT3) and serine/threonine-specific protein kinase (Akt), are down-regulated by natural and semi-synthetic triterpenoids, alongside the triterpenoid and steroidal saponins and steroidal glycoalkaloids (Kannaiyan *et al.*, 2011; Laszczyk, 2009;

Lee *et al.*, 2011; Liby & Sporn, 2012; Pollier & Goossens, 2012; Raju & Mehta, 2009; Shanmugam *et al.*, 2013; Wu *et al.*, 2007; Yadav *et al.*, 2010). The anti-inflammatory activity of the semi-synthetic triterpenoid CDDO and its derivatives is attributed to the ability of these compounds to inhibit the *de novo* synthesis of inducible nitric oxide synthase (i-NOS) and cyclooxygenase-2 (COX-2) in macrophages (Honda *et al.*, 1997, 1999, 2000a,b, 2002). CDDO is an agonist of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), which induces growth arrest and apoptosis in a variety of tumor cell types (Lapillonne *et al.*, 2003; Tsao *et al.*, 2010; Wang *et al.*, 2000). The strongest anti-inflammatory and anti-carcinogenic activity induced by CDDO and its derivatives is the activation of the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2) – antioxidant responsive element (ARE) signaling cascade, which is a major regulator of the cellular anti-oxidant response (Liby *et al.*, 2007b; Sporn *et al.*, 2007).

The adjuvant properties of saponins to promote antibody response are mediated by their unique ability to stimulate both the T helper 1 (Th1) immune response and the production of cytotoxic T-lymphocytes (CTLs) (Sjölander *et al.*, 1998; Sun *et al.*, 2009). The simultaneous triggering of different immune responses makes them ideal for use in subunit vaccines, vaccines against intracellular pathogens and in therapeutic cancer vaccines.

Triterpenoids have also shown great promise for the treatment of HIV through their novel mechanism of action. The semi-synthetic triterpenoid bevirimat prevents the maturation of new virions and thus spread of the virus to new cells. During the Gag processing cascade, bevirimat specifically blocks the cleavage of the Gag capsid (CA) precursor CA-SP1 to mature CA protein, leading to defective core condensation and release of immature non-infectious virus particles (Li *et al.*, 2003).

It is essential to emphasize the need for robust, systematic, well-designed studies to decipher the underlying mechanisms of the therapeutic action of saponins. In most structure–activity relationship investigations, the cell lines and animal models are randomly chosen and very few studies employ the same investigative models, making data comparison across studies futile. There are also a large number of studies with contradicting outcomes when *in vitro* and *in vivo* effects are compared; often questioning the relevance of structure–activity studies. However, to our opinion, the choice of cell lines and animal models partially account for some of these disconcerted results. Another key aspect to consider is the bioavailability of saponins in animal models, which is not addressed in *in vitro* investigations employing cell lines. Therefore, more studies are certainly needed to evaluate pharmacokinetics and bioavailability of saponins, their bio-synthetic intermediates and derivatives.

### Concluding remarks

Simple and elaborated triterpenoids affect fundamental cellular processes both in plants and animals. The molecular targets that are subject to the action of these compounds have not yet been identified in plants. Nevertheless, there is a remarkable functional conservation of groups of proteins that

regulate cell proliferation, cell fate decisions and signal transduction pathways in plants and mammalian systems (Jonak & Hirt, 2002). The non-exhaustive series of proven protein targets of simple triterpenes in mammalian cell lines could be used as a navigation tool in comparative trans-kingdom studies to illustrate the physiological role of saponins in processes pertaining to multicellular organization. On the other hand, knowledge acquired by deciphering the biosynthetic pathways of saponins in plants can provide vital clues to the role of saponins and their specialized intermediates in plant well-being, as well as provide pivotal elements for the rational production of new drugs in synthetic biology approaches. The identification of enzymes responsible for the functionalization of precursors at biologically relevant positions may be exploited for structure-guided compound synthesis in heterologous microbial, plant and animal hosts. Moreover, the identification of regulatory proteins such as transcription factors, protein kinases and phosphatases involved in saponin biosynthesis can be used to augment natural product synthesis in the native plant species. Ancillary to this, systematic studies of the environmental and physiological factors and regulatory proteins that modulate the synthesis of this family of compounds are likely to reveal further as yet unidentified roles for these developmentally and industrially relevant class of compounds.

## Acknowledgements

The authors thank James Reed and Jacob Pollier for useful discussions and critically reviewing this document.

## Declaration of interest

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant “Understanding and Exploiting Plant and Microbial Secondary Metabolism” (BB/J004596/1), the John Innes Foundation, a joint Engineering and Physical Sciences Research Council/National Science Foundation award to AO as part of the Syntegron consortium (EP/K03459/1), and the EU FP7-funded programme Triterpenes for Commercialization (TriForC; A. O. and K. P.). The authors declare no conflicts of interest.

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