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Plant metabolic clusters – from genetics to genomics

Hans-Wilhelm Nützmann, **Ancheng Huang**, and **Anne Osbourn**

Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK

Summary

Plant natural products are of great value for agriculture, medicine and a wide range of other industrial applications. The discovery of new plant natural product pathways is currently being revolutionized by two key developments. First, breakthroughs in sequencing technology and reduced cost of sequencing are accelerating the ability to find enzymes and pathways for the biosynthesis of new natural products by identifying the underlying genes. Second, there are now multiple examples in which the genes encoding certain natural product pathways have been found to be grouped together in biosynthetic gene clusters within plant genomes. These advances are now making it possible to develop strategies for systematically mining multiple plant genomes for the discovery of new enzymes, pathways and chemistries. Increased knowledge of the features of plant metabolic gene clusters – architecture, regulation and assembly – will be instrumental in expediting natural product discovery. This review summarizes progress in this area.

Keywords

biosynthetic gene clusters; chromatin; genome mining; natural products; operons

I. Introduction

Plant natural products have been used by humans for hundreds, and in some cases thousands, of years as medicines, drugs, dyes, pigments, flavourings, fragrances, nutraceuticals, cosmetics and agrochemicals. The plant kingdom is a fertile source of biologically active molecules. These molecules, in contrast to randomly constructed compound libraries, have been prescreened and honed by nature for stability, bioavailability and ‘useful’ biological activities. The vast majority of the chemical potential of the plant kingdom awaits discovery and is hidden away in the dark matter of plant genomes. One of the great challenges for plant biology, and for evolutionary biology more widely, lies in understanding how and why different plants make different kinds of chemicals, and how new natural product pathways are formed.

The discovery of new plant natural product pathways and chemistries is now being revolutionized by two key developments. First, breakthroughs in sequencing technology and reduced cost of sequencing are accelerating the ability to discover new natural product

Author for correspondence: Anne Osbourn, Tel: +44 (0)1603 45407, anne.osbourn@jic.ac.uk.

pathways by identifying the underlying genes, as genome sequences of numerous different plant species are becoming available. Transcriptomics in combination with metabolite analysis of different plant tissues, developmental stages and/or elicitor-treated material is also being used to great effect to identify candidate genes for the synthesis of natural products of interest and to piece together pathways (e.g. Geu-Flores *et al.*, 2012; Lau & Sattely, 2015). Second, it has emerged that the genes encoding certain natural product pathways are organized in biosynthetic gene clusters within plant genomes (Boycheva *et al.*, 2014; King *et al.*, 2014; Nützmann & Osbourn, 2014; Shang *et al.*, 2014; Boutanaev *et al.*, 2015; Schneider *et al.*, 2016). These examples include pathways for diverse classes of chemicals from a wide variety of different plant species, including eudicots and monocots (both annuals and perennials) (Table 1; Fig. 1). This clustering phenomenon is interesting because it challenges the assumption that gene ordering on plant chromosomes is more or less random. It is also beginning to allow the straightforward prediction and subsequent characterization of biosynthetic pathways from genome sequences, an approach that has proved highly successful in bacteria and fungi (Cimermancic *et al.*, 2014; Doroghazi *et al.*, 2014; Medema *et al.*, 2014; Ziemert *et al.*, 2014; Li *et al.*, 2016; Van der Lee & Medema, 2016). Until recently, much of what is known about pathways for the synthesis of natural products in plants has stemmed from examination of individual pathways. It is now becoming possible to mine multiple plant genomes for the discovery of a huge number of predicted genes and enzymes of biosynthetic pathways (Boutanaev *et al.*, 2015). The challenges now lie in developing refined search methods to streamline this discovery platform, and in making sense of and exploiting the outputs. Understanding the features of the metabolic clusters that have been reported so far will be instrumental in enabling such methods to be developed.

II. Plant metabolic gene clusters – some rules and anomalies

The natural product biosynthetic gene clusters so far reported from plants range from ~ 35 kb to several hundred kb in size and consist of three to 10 genes. They include genes for a minimum of at least three (and sometimes six or more) different types of biosynthetic enzymes (King *et al.*, 2014; Nützmann & Osbourn, 2014; Shang *et al.*, 2014; Boutanaev *et al.*, 2015). The salient features of these plant metabolic clusters will be considered here.

1. Scaffolds and networks – what constitutes the first step in a clustered pathway?

Plant metabolic clusters typically (but not always) contain the gene encoding the first committed pathway step and two or more genes encoding other downstream pathway enzymes (Fig. 2). Examples of enzymes that catalyse the first committed step include oxidosqualene cyclases (OSCs) (for the synthesis of triterpenes) (Thimmappa *et al.*, 2014), class I and II diterpene synthases (DTSs) (which together make diterpene scaffolds) (Chen *et al.*, 2011; Zi *et al.*, 2014), and the tryptophan synthase α homologue BX1 (which diverts indole-3-glycerol phosphate from tryptophan biosynthesis into indole for the synthesis of cyclic hydroxamic acids) (Frey *et al.*, 1997) (Fig. 2). These enzymes draw metabolites from primary metabolism into specialized metabolism. A type III chalcone synthase-like enzyme required for the synthesis of diketone wax aliphatics in barley has also been reported (Schneider *et al.*, 2016). While in most cases the nature of the ‘signature’ enzyme, and hence

the type of scaffold, is evident based on cursory examination of these clusters, this may not always be the case. For example, the first committed step in cyanogenic glycoside biosynthesis (the conversion of amino acids to oximes) is catalysed by cytochrome P450 (CYP) enzymes belonging to the CYP79 family (Koch *et al.*, 1995; Bak *et al.*, 1998; Andersen *et al.*, 2000; Forslund *et al.*, 2004; Olsen *et al.*, 2008; Takos *et al.*, 2011; Saito *et al.*, 2012) (e.g. the dhurrin pathway from sorghum – Fig. 2). At first glance the scaffold for the cyanogenic glycoside clusters may not be immediately obvious. However, CYP79 enzymes are unusual amongst the CYP superfamily in having amino acids as their substrates (Hamberger & Bak, 2013). These CYPs convert amino acids to oximes, thereby siphoning them into specialized metabolism. Of note, CYP79 enzymes also catalyse the first committed step in the biosynthesis of noncyanogenic plant defence compounds such as glucosinolates and camalexin (Hull *et al.*, 2000; Mikkelsen *et al.*, 2000; Forslund *et al.*, 2004; Glawischnig *et al.*, 2004; Hamberger & Bak, 2013), pathways for which the genes are not clustered.

Opium poppy (*Papaver somniferum*) produces a variety of different types of benzylisoquinoline alkaloids (BIAs), including the potential anticancer drug noscapine (Beaudoin & Facchini, 2014; Chen *et al.*, 2015) (Fig. 3). A gene cluster for the synthesis of noscapine has recently been discovered in a high noscapine-producing poppy variety (Winzer *et al.*, 2012) (Fig. 2). This cluster contains 10 genes encoding five distinct enzyme classes and includes the gene for the first committed step in the noscapine pathway – the conversion of scoulerine to tetrahydrocolumbamine by the *O*-methyltransferase PSMT1 (Fig. 3). One of the steps in the pathway has previously been shown to be carried out by the enzyme tetrahydroprotoberberine cis-*N*-methyltransferase (TNMT) (Liscombe & Facchini, 2007). The *TNMT* gene is not represented within the noscapine cluster, although open reading frames with *TNMT* homology are present in the flanking regions. Unlike the rest of the pathway genes, *TNMT* is also present and expressed in nonnoscapine-synthesizing poppy varieties and is likely to have additional functions (Winzer *et al.*, 2012).

The step that provides the branch point between primary metabolism and BIA biosynthesis lies several steps upstream of scoulerine (Fig. 3). The genome sequence of *P. somniferum* is not yet available. It therefore remains to be seen whether the noscapine cluster is a discrete group of 10 genes dedicated to this particular branch of BIA biosynthesis or whether it forms part of a larger cluster that contains additional genes required for the synthesis of the scoulerine precursor. Similarly, it is not known whether the genes for other branches of the BIA biosynthetic network are clustered or unclustered. It has recently been shown, however, that the first committed step in morphinan biosynthesis in poppy is catalysed by a P450 oxidoreductase fusion protein (STORR: (*S*)- to (*R*)-reticuline) (Winzer *et al.*, 2015) (Fig. 3). The existence of this chimaeric protein implies that extreme selection pressure has been at work to drive functional modularity in other parts of BIA biosynthesis in addition to the noscapine pathway.

Analysis of a draft genome sequence for Madagascan periwinkle (*Catharanthus roseus*) has provided evidence for partial clustering of genes for the biosynthesis of the monoterpene indole alkaloids (MIAs) vinblastine and vincristine (Kellner *et al.*, 2015). MIA biosynthesis is highly complex, involving condensation of the indole tryptamine with the iridoid

secaloganinto form strictosidine. Strictosidine is a common intermediate in the biosynthesis of thousands of MIAs, including those of vinblastine and vincristine (De Bernonville *et al.*, 2015). Thus, as for noscapine, the vinblastine/vincristine pathway is part of a much larger and more complex biosynthetic network that gives rise to a wealth of other diverse products. The MIA pathway also has one of the most complex and elaborate forms of compartmentalization known for a plant specialized metabolic pathway (Verma *et al.*, 2012; De Bernonville *et al.*, 2015). The N50 scaffold size for the draft *C. roseus* genome sequence was in the region of 26–27 kb. Although not optimal for discovery of linked genes in plant genomes because of the large intergenic distances, Kellner *et al.* (2015), aided by bacterial artificial chromosome (BAC) sequencing, were able to identify seven small clusters each of two to three genes that contained genes encoding enzymes for vinblastine/vincristine biosynthesis and also other genes that may encode missing steps in the pathway, along with a gene for a predicted multi-antimicrobial extrusion protein (MATE) transporter that could potentially be involved in the transport of pathway intermediates. The development of a higher quality genome sequence will establish whether these small clusters are dispersed throughout the genome or whether they form a larger cluster, and how these genes are distributed relative to those required for the synthesis of other types of MIAs produced by *C. roseus*. A simplistic assumption may be that clustering is a feature of dedicated linear pathways that are insulated from the rest of metabolism and that take place in the same cell types. Analysis of complex networks such as those for alkaloid biosynthesis in *P. somniferum* and *C. roseus* should help to test this hypothesis and to understand what precisely constitutes a metabolic gene cluster in the context of wider metabolism.

In summary, most of the plant metabolic gene clusters that have been reported so far contain the genes encoding the enzymes for the first committed steps in the respective pathways. These may be obvious scaffold-generating enzymes (e.g. oxidosqualene cyclases; class I and class II diterpene synthases); other enzymes that divert metabolites from primary metabolism into specialized metabolism (e.g. the tryptophan synthase α homologue BX1, which catalyses the first step in DIBOA/DIMBOA biosynthesis; CYP79 P450s, which convert amino acids into oximes); or enzymes that siphon intermediates from complex specialized metabolic networks into dedicated pathways for specific end products (e.g. the *O*-methyltransferase PSMT1, which converts scoulerine to tetrahydrocolumbamine – the first committed step in the noscapine pathway). As more metabolic gene clusters are characterized from plants, the inventory of ‘signature’ enzymes that are diagnostic of particular types of biosynthetic pathways will grow, so making pathway prediction easier. In some cases, such genes do not appear to be present. For example, the tomato steroidal glycoalkaloid α-tomatine is predicted to be synthesized from cholesterol (Eich, 2008). The genes for α-tomatine biosynthesis are located in one major cluster of six genes on chromosome 7, with a further two pathway genes adjacent to each other on chromosome 12 (Fig. 2). The *CYP72* gene *GAME7*, which is predicted to catalyse the first step in this pathway (the conversion of cholesterol to 22-hydroxycholesterol) (Itkin *et al.*, 2013), is also located on chromosome 7 but is 7880 kb away from the nearest cluster gene (Sato *et al.*, 2012; A. Aharoni, pers. comm.).

2. Tailoring enzymes, modularity, collinearity and moonlighting

The tailoring enzymes encoded within metabolic clusters may variously include CYPs, sugar transferases, methyltransferases, acyltransferases, dioxygenases, carboxylesterases, dehydrogenases/reductases and transaminases (King *et al.*, 2014; Nützmann & Osbourn, 2014; Shang *et al.*, 2014; Boutanaev *et al.*, 2015; Schneider *et al.*, 2016) (Fig. 2). It is likely that this list will continue to grow as more natural product clusters are discovered. The arbitrary definition of a metabolic gene cluster requires that it should contain genes for at least three different types of enzymes (Nützmann & Osbourn, 2014). In this regard, it is important to note that the CYP superfamily is divided into families based on sequence similarity (family members having > 40% amino acid sequence identity) (Nelson & Werck-Reichhart, 2011), and that for the purposes of defining clusters each CYP family should be regarded as a different type of biosynthetic enzyme.

The *Arabidopsis thaliana* thalianol and marneral clusters are the smallest plant metabolic clusters characterized so far. These are compact clusters that are 35–38 kb in size (Field & Osbourn, 2008; Field *et al.*, 2011). The largest clusters are several hundred kb (Nützmann & Osbourn, 2014). In some cases, the genes in the pathway are contiguous, with no other obvious intervening genes (e.g. the thalianol cluster in *A. thaliana* and the avenacin cluster in diploid oat) (Qi *et al.*, 2004, 2006; Field & Osbourn, 2008; Mugford *et al.*, 2009, 2013) (Fig. 2). In other cases, some of the pathway genes are less tightly linked and there may be intervening genes with no obvious function in specialized metabolism between these ‘peripheral’ genes and the core cluster. For example, the maize *Bx7* gene, which encodes an *O*-methyltransferase required for DIMBOA biosynthesis (Jonczyk *et al.*, 2008), lies within 15 Mb of the core cluster. Another gene, *Bx9*, which encodes a sugar transferase that is active towards DIBOA/DIMBOA, is located on a different chromosome (von Rad *et al.*, 2001). In diploid oat, the *Sad3* and *Sad4* loci have been shown by mutation to be required for avenacin glucosylation (Papadopoulou *et al.*, 1999; Qi *et al.*, 2004; Mylona *et al.*, 2008) but not yet cloned. *Sad3* is essential for avenacin glucosylation but is only loosely linked to the core cluster (genetic linkage distance 3.6 cM) (Qi *et al.*, 2004). Full characterization of the wider avenacin cluster region will reveal the nature of *Sad3* and the physical distance between this locus and the core cluster. *Sad4* is unlinked to the cluster, and unlike *Sad3* has only a partial effect on avenacin glucosylation when mutated and also glucosylates other oat metabolites (Papadopoulou *et al.*, 1999; Mylona *et al.*, 2008). *Sad4* is therefore not a dedicated pathway component and appears to be ‘moonlighting’.

While it is important to emphasize that plant metabolic gene clusters contain at least three nonhomologous genes encoding different types of enzymes in order to distinguish them from regions of the genome that contain only tandem arrays of highly homologous genes, some of the characterized clusters do contain duplicated genes that, in some cases, have evolved different functions. A noteworthy example is the maize DIMBOA cluster, which contains genes for four CYPs belonging to the CYP71C subfamily (Frey *et al.*, 1997) (Fig. 2). These closely related CYP genes (*Bx2-5*) have almost certainly arisen from a common ancestor by tandem gene duplication (Dutartre *et al.*, 2012). However, functional analysis following expression in yeast has revealed that each carries out a highly specific and dedicated step in the conversion of indole (the product of the signature enzyme, BX1) to

DIBOA (Frey *et al.*, 1997). This group of four genes therefore provides some intriguing insights into the evolution of sequential CYP-mediated steps in this pathway.

A further intriguing finding is the observation that the genes required for a particular modification can be grouped within a cluster as a module. This is exemplified by a set of three genes within the oat avenacin cluster that are required for triterpene acylation (Fig. 2). The major avenacin, A-1, is acylated with an *N*-methyl anthranilate group. This acyl group is introduced by the acyltransferase SAD7, which is a member of the SCPL family of acyltransferases (Mugford *et al.*, 2009). Unlike the BAHD acyltransferases, which use CoA-thioesters as the acyl donor, SCPL-acyltransferases use acyl sugar donors (Milkowski & Strack, 2004). The acyl glucose donor used by SAD7 is *N*-methyl anthranilate glucose, which is generated by methylation of the readily available precursor anthranilate (a product of primary metabolism) by the methyl transferase SAD9 followed by glucosylation by the glucosyl transferase SAD10 (Mugford *et al.*, 2013; Owatworakit *et al.*, 2013). The genes encoding SAD7, SAD9 and SAD10 are adjacent in the avenacin cluster, so forming an 'acylation module' (Mugford *et al.*, 2013).

Interestingly, the genes within the noscapine cluster in poppy appear to show collinearity in that they are organized in groups that roughly correspond to the early, middle and late steps of the pathway (Winzer *et al.*, 2012) (Fig. 2). Occasional examples of apparent collinearity of metabolic gene clusters have been noted in bacteria and filamentous fungi (Donadio *et al.*, 1991; Cary *et al.*, 2005; Erlich *et al.*, 2005; Osbourn, 2010). Collinearity appears to be the exception rather than the rule in plants. Nevertheless, the fact that it occurs at all is noteworthy and suggests that, at least for the noscapine pathway, gene order (and by extrapolation temporal control of gene expression) may be significant for pathway function and/or potentially also for pathway assembly.

3. Core clusters and satellite subgroups

Several examples of pathways that have core metabolic clusters along with one or two peripheral pathway genes have been mentioned earlier (e.g. the *O*-methyltransferase gene *Bx7* and the sugar transferase gene *Bx9* in maize, and the *Sad3* locus required for glucosylation of avenacins in oat). In some cases, the pathways are more fragmented. For example, as already mentioned, most of the genes for the synthesis of the steroidal glycoalkaloid α-tomatine in tomato are clustered on chromosome 7. These include two early pathway genes – the dioxygenase gene *GAME11*, and the CYP72 gene *GAME6* – along with the four sugar transferases, *GAME1, GAME17, GAME18* and *GAME2*. Two further pathway genes encoding the transaminase GAME12 and the CYP88D GAME4 are adjacent to each other on a different chromosome (Itkin *et al.*, 2013) (Fig. 2). Note that, of these regions, only the first contains genes for at least three different types of biosynthetic enzyme, i.e. fulfils the requirements to be classified as a metabolic cluster. The genes for the synthesis of the related steroidal glycoalkaloids α-solanine and α-chaconine are similarly organized in potato on chromosomes 7 and 12 in regions that are syntenic to those in tomato (Itkin *et al.*, 2013). Thus it appears that these pathways share a common evolutionary origin but have acquired different functions in tomatoes and potatoes.

By the same token, in cucumber (*Cucumis sativus*) five genes encoding enzymes required for the biosynthesis of cucurbitacins (triterpenoids that confer bitterness) are clustered on chromosome 6 – the gene for the oxidosqualene cyclase that synthesizes the triterpenoid scaffold cucurbitadienol, along with three different types of CYP genes (CYP81, CYP87, CYP89) and an acyltransferase gene. Four other CYP genes that are also required for cucurbitacin biosynthesis are located elsewhere (a CYP71 gene and two clustered CYP88 genes on chromosome 3; and a CYP87 gene on chromosome 1) (Shang *et al.*, 2014) (Fig. 2). By extending such analyses out across the wider Solanaceae (for the steroidal glycoalkaloids) and the Cucurbitaceae (for the cucurbitacins), it should be possible to unearth other related pathways and enzymes that represent further variations on these themes. Such investigations will also help to establish whether these pathways may represent evolutionary snapshots of clusters in the process of assembling/disassembling/diversifying.

In maize, the five genes that are necessary and sufficient for the synthesis of the cyclic hydroxamic acid DIBOA are located in a single core cluster, the *Bx* cluster (Frey *et al.*, 1997, 2003; von Rad *et al.*, 2001; Jonczyk *et al.*, 2008). The *Bx* cluster is believed to be of ancient origin. However, in wheat and rye, which also make cyclic hydroxamic acids, the genes for DIBOA biosynthesis are found as two subclusters located on different chromosomes. The organization of these genes in these two other cereals has been attributed to a reciprocal translocation in a common ancestor of wheat and rye (Nomura *et al.*, 2002; Sue *et al.*, 2011).

4. Summary – the architectures of plant metabolic clusters

The archetypal plant metabolic cluster (Fig. 4a) is compact and contains contiguous pathway genes, including the gene encoding the enzyme for the first committed step in the pathway. In other cases, the genes for most of the metabolic pathway steps (including the gene for the first committed step) are clustered and contiguous, but there may be one or more peripheral genes encoding other pathway steps. These peripheral genes may be loosely linked to the core cluster (*in cis*) or unlinked (*in trans*) (Fig. 4b). As more plant metabolic clusters are characterized, the relative importance of *in cis* and *in trans* peripheral genes should become clearer. It will also be interesting to establish whether these peripheral genes tend to encode particular types of enzymes (the earlier examples suggest that sugar transferase genes may have a tendency to be peripheral), although it is too early to make any definitive generalizations. In other cases, the pathways are more fragmented. In these situations, the bulk of the pathway genes, including the gene for the first committed step, are organized in a core cluster with other genes present as satellite subgroups elsewhere in the genome (Fig. 4c). For the examples reported so far, these satellite subgroups contain genes for one or two different types of tailoring enzyme. In a fourth scenario the gene for the first committed step may be peripheral, as is the case for the α-tomatine cluster (Fig. 4d). These peripheral genes would, however, be expected to be strongly coexpressed with the clustered genes encoding the other pathway steps.

III. Cluster regulation

Clustered pathways for natural product biosynthesis are common in bacteria and fungi, and often contain genes encoding pathway-specific transcription factors that regulate the biosynthetic genes within the cluster (Chater & Bibb, 1997; Brakhage, 2013). Loss-of-function mutations in these transcription factors result in loss of expression of pathway genes, while overexpression can lead to coordinate up-regulation and enhanced metabolite production (Uguru *et al.*, 2005; Bergmann *et al.*, 2007). Such transcription factors are therefore very useful tools, both for pathway delineation and for metabolic engineering. By contrast, the clusters that have been reported so far in plants do not appear to contain genes for pathway-specific transcription factors. However, recent advances are beginning to yield insights into how plant metabolic gene clusters are regulated.

In some cases, quite a bit is known about the temporal and spatial expression patterns of particular clusters. For example, the major product of the avenacin pathway for the synthesis of antimicrobial triterpene glycosides in oats, avenacin A-1, has strong autofluorescence under ultraviolet illumination and can be readily localized to the epidermal cells of the root tips and lateral roots, consistent with its role in defence against soilborne pathogens (Osbourn *et al.*, 1994). mRNA *in situ* hybridization indicates that the characterized avenacin pathway genes, including the first and last steps in the pathway, are all expressed specifically in these cell types (Qi *et al.*, 2004, 2006; Mugford *et al.*, 2009, 2013; Wegel *et al.*, 2009). Regulation of the avenacin pathway is therefore under strict developmental control and does not appear to be altered in response to pathogen attack or abiotic stress treatment. The avenacin pathway has evolved relatively recently in evolutionary time (since the divergence of oats from other cereals some 25 million yr ago) and the ability to synthesize avenacins is restricted to oat. Interestingly, however, the promoters of the avenacin pathway genes retain very similar expression patterns when introduced into *A. thaliana*, rice and other plant species as reporter fusion constructs, i.e. they are expressed in the epidermal cells of the tips of the primary and lateral roots, suggesting that this pathway has hooked into an ancient root development process that is conserved across monocots and eudicots (Kemen *et al.*, 2014). The class IV homeodomain leucine zipper transcription factor AsHDZ1 has been implicated in regulation of the avenacin gene cluster, but its function has not yet been validated in oat (Kemen *et al.*, 2014).

The momilactone and phytocassane/oryzalide clusters in rice make diterpenes that are implicated in plant defence. Momilactones, oryzalexins and phytocassanes are phytoalexins (i.e. are produced in the leaves in response to pathogen attack or elicitor treatment), while oryzalides are present in unchallenged plants and so are regarded as phytoanticipins (Schmelz *et al.*, 2014). Importantly, momilactones are also produced constitutively in the roots as part of normal growth and development, where they have been implicated in allelopathy (Xu *et al.*, 2011; Kato-Noguchi & Peters, 2013). These rice diterpene clusters are therefore subject to different types of regulation, both developmental and inducible. A chitin oligosaccharide elicitor-inducible basic leucine zipper (bZIP) transcription factor (OsTGAP1) coordinately regulates the gene clusters for the synthesis of momilactone and phytocassane/oryzalide diterpenes in rice (Okada *et al.*, 2009; Miyamoto *et al.*, 2014). However, this effect is not direct, as the promoters of the diterpene cluster genes are not

targets for OsTGAP1 binding (Miyamoto *et al.*, 2014). A conserved APETALA2/ethylene response factor (AP2/ERF) transcription factor (GAME9) is involved in regulation of synthesis of the cholesterol-derived steroidal glycoalkaloids α-tomatine in tomato, and α-solanine and α-chaconine in potato (Cárdenas *et al.*, 2016). GAME9 control of steroidal glycoalkaloid biosynthesis is not restricted to the clustered pathway genes and also encompasses upstream genes of the cholesterol pathway. Although concerted changes in cluster expression are observed in *GAME9* knockdown and overexpression lines, it is not yet known whether GAME9 directly regulates each cluster gene. Initial data suggest that GAME9 binds to the promoter sequences of some of the cluster genes (*GAME4* and *GAME7*) cooperatively with an MYC2 transcription factor (Cárdenas *et al.*, 2016).

In cucumber, two transcription factors that regulate cucurbitacin C synthesis in leaves and fruit, respectively, have been identified – *Bl* (*Bitter leaf*) and *Bt* (*Bitter fruit*) (Shang *et al.*, 2014). *Bl* encodes a basic helix–loop–helix (bHLH) transcription factor required for cluster regulation. Yeast one-hybrid analysis, tobacco transient reporter (luciferase) activation assays, chromatin immunoprecipitation analysis and electrophoretic mobility-shift assays (EMSAs) have collectively shown that Bl can selectively bind to *cis* elements within the promoter of the gene encoding the first step in the cucurbitacin C pathway *Bi*. *Bt* is also a bHLH transcription factor and lies within 8.5 kb of *Bl*. The *bt* allele confers the nonbitterness trait of cultivated cucumber and has been selected during domestication. *Bl* and *Bt* can specifically bind to the promoters of nine different cucurbitacin C pathway genes, including those of the core cluster and satellite subgroup. *Bl* and *Bt* are on a different chromosome to the cucurbitacin C core biosynthetic cluster and satellite pathway genes.

In some cases, the genes within reported clusters do not show consistent coexpression. For example, the cyanogenic glycoside cluster in *Lotus japonicus* and the terpene cluster in tomato deviate from the general coregulation pattern of metabolic gene clusters. The former is characterized by overlapping expression of all clustered genes in young leaf tissues. The transcript abundances of *CYP79D3* and *CYP736* are reduced in older leaf tissue but remain constant for the third pathway gene, *UGT85K3* (Takos *et al.*, 2011). The terpene cluster in tomato shows coregulation of subsets of genes. Expression of *Sl-CPT1*, *Sl-TPS19*, *Sl-TPS20* and *Sl-TPS41* is primarily restricted to the stem and leaf trichomes, while *Sl-CPT2*, *Sl*-CYP71BN1 and *Sl*-TPS21 show highest expression in the petiole and *Sl-CPT18* is expressed in the roots (Matsuba *et al.*, 2013, 2015). Other examples of inconsistencies in expression patterns of cluster genes have also been reported (von Rad *et al.*, 2001; Swaminathan *et al.*, 2009).

In filamentous fungi there is good evidence that gene clusters for natural product pathways are subject to regulation by chromatin-based mechanisms (Schwab *et al.*, 2007; Bok *et al.*, 2009; Brakhage, 2013). There is a growing body of evidence to indicate that this is also the case in plants. High-resolution DNA fluorescence *in situ* hybridization (FISH) analysis indicates that expression of the oat avenacin cluster is associated with chromatin decondensation (Wegel *et al.*, 2009). Metabolic gene clusters in *A. thaliana*, maize, oat and rice show pronounced trimethylation of histone H3 lysine 27 (H3K27me3) and the levels of this chromatin mark are inversely correlated with cluster expression (Yu *et al.*, 2016). H3K27me3 is a conserved histone modification in eukaryotes and is generally associated

with gene silencing. Consistent with this, the thalianol and marneral clusters in *A. thaliana* show increased expression in Polycomb mutants that are compromised in H3K27 trimethylation, and reduced expression in mutants for PICKLE, a positive regulator that counteracts H3K27me3 silencing (Yu *et al.*, 2016). These two *A. thaliana* clusters are also positively regulated (either directly or indirectly) by the SWR1 chromatin remodelling complex, which is required for the deposition of the histone 2 variant H2A.Z into nucleosomes, suggesting that H2A.Z is required for normal cluster expression. A model is emerging based on analysis of chromatin modifications and mutants in which H3K27me3 is implicated in cluster silencing and H2A.Z in cluster activation (Nützmann & Osbourn, 2015; Yu *et al.*, 2016). Importantly, these features can be used in genome-wide analyses to mine for new metabolic gene clusters (Yu *et al.*, 2016). Although mutations in chromatin regulators have been reported to affect the biosynthesis of phenylpropanoids and glucosinolates, compounds that are made by unclustered pathways (Rider *et al.*, 2004; Bennett *et al.*, 2005; Walley *et al.*, 2008), it is not clear whether these effects are direct or indirect. RNAseq analysis of H2A.Z mutant lines did not support a role for this chromatin mark in the regulation of nonclustered glucosinolate and flavonoid pathways in *A. thaliana* (Yu *et al.*, 2016).

IV. Why are the genes clustered?

The amount of metabolic diversity represented within the plant kingdom is huge. Very little is known by comparison about the locations of the genes encoding specialized metabolic pathways in plant genomes. Indeed, only a handful (< 50) of plant specialized metabolic pathways have been fully characterized in terms of both their biochemistry and the genomic locations of the pathway genes. As this inventory grows and more plant genome sequences become available, it will be possible to gain a wider overview of the organization of specialized metabolism in plants. The genes for well characterized pathways such as the flavonoid, carotenoid and glucosinolate pathways are patently unlinked (Sonderby *et al.*, 2010; Saito *et al.*, 2013; Nisar *et al.*, 2015). Why, then, should the genes for the natural product biosynthetic pathways shown in Table 1 be clustered? It is clear that for the clusters that have been reported so far there is no evidence of horizontal gene transfer from microbes (Nützmann & Osbourn, 2014). This raises intriguing questions about why some pathways are clustered and others are not.

1. Coinheritance, toxic/bioactive intermediates, coexpression and metabolons

Various theories have been put forward to explain clustering. These theories are not mutually exclusive – neither are they likely to be exhaustive. The overriding matter is the benefit of clustering for the plant, not for the pathway. This brings in the issue of population genetics and the environment, as the benefit to the plant will depend on its ability to compete and survive in nature. Another key point is that these clusters are likely to be dynamic and so in their current manifestation represent a moment in evolutionary time. Thus the clustering phenomenon should be considered in the wider context of the birth, life and death of clusters.

The coinheritance argument—Genetic linkage of genes encoding complex traits that confer a selective advantage will reduce the risk of disruption of these favourable gene sets by recombination. Metabolic diversification in plants is likely to be a reflection of adaptation to survival in particular ecological niches. Many of the compounds shown in Table 1 and Fig. 1 have been variously reported to provide protection against pathogens and herbivores (Arneson & Durbin, 1968; Da Costa & Jones, 1971; Niemeyer, 1988; Fewell & Roddick, 1997; Papadopoulou *et al.*, 1999; Tattersall *et al.*, 2001; Balkema-Boomstra *et al.*, 2003; Toyomasu *et al.*, 2014; Sohrabi *et al.*, 2015), to confer competitiveness against competing plant species (allelopathy) (Nicollier *et al.*, 1983; Xu *et al.*, 2011; Kato-Noguchi & Peters, 2013; Toyomasu *et al.*, 2014), to form protective leaf waxes (Schneider *et al.*, 2016) and to determine bitterness or antinutritional properties (Friedman, 2002, 2006; Shang *et al.*, 2014). Many of the plant metabolic gene clusters reported so far appear to have arisen relatively recently in evolutionary time and are restricted to narrow taxonomic subgroups (e.g. Qi *et al.*, 2004; Field *et al.*, 2011; Matsuba *et al.*, 2013). This ability to assemble new metabolic pathways is quite remarkable, and suggests that plant genomes are highly plastic and versatile. Once a favourable combination of alleles of different pathway genes is found, it will presumably be beneficial to inherit these genes together. Clustering may therefore be a feature of extreme selection for coinheritance and optimization of the best combinations of genes for new metabolic pathways that confer selective advantages. The arguments for cluster formation and maintenance are complex and sit at the interface of molecular biology, population and evolutionary genetics and genomics (Yeaman & Whitlock, 2011; Takos & Rook, 2012).

Toxic/bioactive intermediates—Clusters that are not optimal owing to the presence of maladapted alleles of pathway genes, or because of deletion, insertion or mutation, will be compromised in their ability to make protective chemicals. Importantly, there is growing evidence to indicate that interference with clustered pathways in plants through mutation, RNAi-mediated silencing or overexpression of pathway genes can result in accumulation of toxic intermediates (von Rad *et al.*, 2001; Kristensen *et al.*, 2005; Field & Osbourn, 2008; Mylona *et al.*, 2008; Field *et al.*, 2011; Itkin *et al.*, 2011; Xu *et al.*, 2011). Interestingly, in some cases, accumulation of pathway intermediates can have more subtle effects, such as increased root length in *A. thaliana* (Field & Osbourn, 2008) and altered cell specification, leading to super-hairy roots in oat (Kemen *et al.*, 2014). Clustering may therefore not only enable coinheritance of genes for complete metabolic pathways, but may also protect against ectopic production of toxic/bioactive intermediates. The cause of various human disorders has been attributed to the accumulation of toxic intermediates, which occurs when the product of an enzyme is not used as a substrate by a downstream neighbour in the metabolic network. It has been proposed by analogy that clustering of metabolic pathway genes in filamentous fungi may be an adaptation against the accumulation of toxic intermediates (McGary *et al.*, 2013). Further research is required to establish whether or not this may be the case in plants. Nevertheless, the various phenotypes observed in plants when metabolic pathways are disrupted provide some tantalizing glimpses into possible scenarios in pathway evolution and the likely consequences for plant growth, development and survival.

Coexpression—Physical clustering may provide opportunities for pathway regulation at the levels of localized chromatin modifications and nuclear organization, as discussed earlier. This could be important in order to avoid expression of incomplete pathways with consequent reduced product formation and accumulation of bioactive intermediates. Colocalization of pairs or groups of genes is known to increase the likelihood of coregulation of genes. Bidirectional promoters, locally spreading gene silencing mechanisms and formation of local chromatin domains have all been shown to be important for concerted transcription of colocalized genes in eukaryotes (Hurst *et al.*, 2004). Large-scale expression analysis also indicates a tendency towards coregulation of neighbouring genes in different eukaryotic species (Cohen *et al.*, 2000; Kruglyak & Tang, 2000; Boutanaev *et al.*, 2002; Lercher *et al.*, 2002; Spellman & Rubin, 2002; Kosak & Groudine, 2004). Underlining this pattern, a recent study reports a significant correlation of the expression profiles of neighbouring genes during evolution (Ghanbarian & Hurst, 2015). Furthermore, a number of examples throughout the eukaryotes provide evidence for local regulation patterns associated with clusters of functionally related genes.

Localized chromatin modification has been implicated in the stringent regulation of other types of eukaryotic gene clusters, including the major histocompatibility (*MHC*) locus, the β-globin locus and the homeodomain (Hox) transcription factor cluster in animals and a catabolic gene cluster in yeast (the *DAL* cluster) (Meneghini *et al.*, 2003; Gialitakis *et al.*, 2006; Garrick *et al.*, 2008; Osbourn & Field, 2009; Montavon & Duboule, 2013). Like the *A. thaliana* thalianol and marneral clusters, the *DAL* cluster has pronounced H2A.Z marking that readily distinguishes it at the genome-wide level (Meneghini *et al.*, 2003; Guillemette *et al.*, 2005; Wong & Wolfe, 2005). Metabolic clusters in filamentous fungi are also demarcated by chromatin marks that are associated with activation and silencing (Bok *et al.*, 2009; Reyes-Dominguez *et al.*, 2010; Nützmann *et al.*, 2011; Connolly *et al.*, 2013). DNA-FISH and chromosome conformation capture analysis have shown that local three-dimensional chromatin domains are formed at animal gene clusters (Tolhuis *et al.*, 2002; Simonis *et al.*, 2006; Noordermeer *et al.*, 2011; Tena *et al.*, 2011; Vieux-Rochas *et al.*, 2015). It has been suggested that these domains may separate cluster regions from the surrounding chromosomal areas and facilitate efficient and concerted silencing and activation of the clustered genes.

Work on a second catabolic cluster from yeast, the *GAL* gene cluster for galactose assimilation, indicates that changing the conformation of this three-gene cluster by placing one of the genes *in trans* results in reduced coregulation but does not incur a fitness cost, leading to the conclusion that genetic linkage is probably the selective force driving the origin and maintenance of *GAL* gene clusters (Lang & Botstein, 2011). By contrast, disruption of gene order in the *DAL* gene cluster, which is required for allantoin degradation under nitrogen limitation conditions, leads to both changes in expression of cluster genes and reduced fitness in starvation conditions (Naseeb & Delneri, 2012).

Investigation of the regulation of plant metabolic gene clusters at all levels – from analysis of promoters and transcription factors through to chromatin modifications, DNA looping and higher-level nuclear organization – is likely to reveal important new insights into the

regulation of specialized metabolism and should also shed light on how new coregulated clustered pathways become established in plants (Fig. 5).

Metabolic channelling/metabolons—The formation of multienzyme complexes (metabolons) in which pathway components are held together by noncovalent interactions has been proposed as a mechanism for enhancing local accumulation of substrates and avoiding the release of toxic intermediates through metabolic channelling (Jorgensen *et al.*, 2005). There is evidence to suggest that the pathway for the biosynthesis of the cyanogenic glycoside dhurrin in sorghum is highly channelled and that the pathway enzymes are organized as a metabolon (Møller & Conn, 1980; Kristensen *et al.*, 2005; Nielsen *et al.*, 2008; Møller, 2010). It has also been suggested that the STORR fusion protein that has recently been shown to catalyse the first committed step in morphinan biosynthesis may facilitate channelling of unstable or reactive pathway intermediates (Winzer *et al.*, 2015). By contrast, for several other clustered pathways including the maize DIBOA/DIMBOA and oat avenacin pathways, the pathway enzymes are not colocalized and are distributed across several subcellular compartments (Frey *et al.*, 1997, 2009; Jonczyk *et al.*, 2008; Mugford *et al.*, 2009, 2013). Thus metabolic channelling does not appear to be a general feature of clustered metabolic pathways. Furthermore, it is not clear why physical clustering of pathway genes may be expected to be beneficial for the formation of multienzyme complexes, although conceivably coordinate transcription of genes in distinct nuclear areas may facilitate cotranslation and subsequent colocalization of the cognate protein products.

In summary, the above arguments cannot readily be untangled and are unlikely to represent the full story. Further work is needed to shed light on the selective forces and mechanisms that drive the assembly and maintenance of plant metabolic gene clusters and on the significance of clustering for pathway function. It will also be important to explore the exceptions to the rule – where clusters are split (e.g. the DIBOA/DIMBOA pathway in wheat and rye) or have additional peripheral or satellite genes – as these examples are likely to be helpful when investigating the above possibilities.

2. Assembly of clustered pathways

Based on knowledge of the plant metabolic gene clusters reported so far, several scenarios for the assembly of clustered pathways can be envisaged. Although these are illustrated separately here (Fig. 6), based on existing evidence, these different scenarios are in reality likely to represent snapshots in an evolutionary continuum that, once fully understood, will reveal the events underlying the full life cycle – the birth, life and death – of metabolic clusters. In some cases the gene for the first committed step in plant metabolic clusters is likely to have been recruited either directly or indirectly from primary metabolism and to have ‘seeded’ the formation of a multigene cluster (Fig. 6a). An example of this is the avenacin cluster in oat. The first step in the avenacin pathway is the cyclization of 2,3-oxidosqualene to the triterpene scaffold β-amyrin, catalysed by the oxidosqualene cyclase enzyme β-amyrin synthase (SAD1) (Haralampidis *et al.*, 2001). The oat β-amyrin synthase gene has been recruited from the sterol pathway and is a divergent relative of cycloartenol synthase (Haralampidis *et al.*, 2001; Qi *et al.*, 2004). The avenacin pathway is restricted to oats (*Avena* species) and has arisen some 25 million yr ago, after the divergence of oats from

other cereals and grasses (Qi *et al.*, 2004). This duplicated gene then appears to have served as the founder gene for the *de novo* formation of the avenacin cluster. The second step in the avenacin pathway (the divergent CYP51 enzyme SAD2) has also been recruited from sterol biosynthesis, from obtusifoliol 14α-demethylase. However, the cycloartenol synthase and obtusifoliol 14α-demethylase genes are not linked to the avenacin cluster or to each other in oat (Qi *et al.*, 2004, 2006) and there is no evidence for linkage of these highly conserved sterol biosynthetic genes in *A. thaliana* or rice.

In other cases, there is evidence that cluster founding events may involve pairs of genes rather than single ones (Fig. 6b). For example, the *A. thaliana* thalianol and marneral clusters, both of which make triterpenes, may have been founded by the duplication of an ancestral gene pair consisting of an oxidosqualene cyclase gene and a CYP705 gene. Independent events involving gene rearrangement and recruitment of additional genes would then have led to the formation of the present-day clusters (Field *et al.*, 2011). These clusters formed relatively recently in evolutionary time after the α-duplication event within the Brassicales 4–20 million yr ago. Phylogenetic analysis of the DIBOA/DIMBOA gene cluster in maize suggests a similar cluster assembly path to that proposed by Field *et al.* (2011) for the thalianol and marneral clusters. A cluster founding event involving colocalization of the *Bx1* and *Bx2* genes, which together are required for the first two steps in the pathway, is believed to have been followed by recruitment of additional biosynthesis genes and cluster extension (Frey *et al.*, 2009; Dutartre *et al.*, 2012). In contrast to the thalianol and marneral clusters, the DIBOA/DIMBOA cluster is thought to be of ancient origin. A complex terpene cluster in tomato only partially follows this pattern. Here, after the colocalization of an original pair of biosynthesis genes (TPS41 and diTPS1), local duplications, rearrangements, gene recruitment and neofunctionalization have led to the entanglement of two functionally active clusters with different products and expression patterns (Matsuba *et al.*, 2013).

A recent study of terpene diversification and the evolution of terpene biosynthetic genes across multiple plant species has provided interesting insights into mechanisms of metabolic cluster formation (Boutanaev *et al.*, 2015). Systematic analysis of 17 sequenced plant genomes was carried out to determine the organization and evolution of terpene pathway genes. This study revealed that terpene synthase (TS) and CYP genes are clustered far more often than would be expected by chance, indicating nonrandom association of TS and CYP gene pairs within the surveyed genomes. All previously characterized terpene metabolic clusters were identified in this analysis along with other new candidate clusters, several of which were then experimentally validated (for *Ricinus communis*, *A. thaliana* and *C. sativus*). These experiments further demonstrated that certain TS/CYP pairings predominate, so providing signals for key events that are likely to have shaped terpene diversity. Importantly, comparison of the TS/CYP pairs from this analysis has provided evidence for different mechanisms of terpene cluster assembly in eudicots and monocots. In the eudicots, microsyntenic blocks of TS/CYP gene pairs duplicate and provide templates for the evolution of new clustered pathways. By contrast, in the monocots, new terpene clusters arise by mixing and matching of TS and CYP genes through dynamic genome rearrangements. These findings are consistent with the proposed model for the origin of the thalianol and marneral clusters from a common ancestral gene pair in *A. thaliana* (Field *et*

al., 2011) and also with the single founder gene scenario proposed for the oat avenacin cluster (Qi *et al.*, 2004, 2006; Mugford *et al.*, 2009, 2013).

The thalianol and marneral clusters are located in regions of the *A. thaliana* genome that are significantly enriched in transposable elements and are located in dynamic chromosome regions (Field *et al.*, 2011; Field & Osbourn, 2012). Other plant metabolic clusters also contain transposable elements that may have some function in gene rearrangement for cluster formation (e.g. Qi *et al.*, 2004; Wilderman *et al.*, 2004; Shimura *et al.*, 2007; Takos *et al.*, 2011; Winzer *et al.*, 2012; Krokida *et al.*, 2013). The flanking regions of the monocot TS/CYP gene pairs identified by Boutanaev *et al.* (2015) were significantly enriched in transposable elements relative to the norm for all TS and CYP genes across the monocot genomes analysed. A small but significant enrichment in transposable elements was also observed for eudicots when clustered and dispersed genes were compared (Boutanaev *et al.*, 2015). Thus transposable element-mediated recombination may contribute to cluster formation in both monocots and eudicots.

A third scenario for cluster formation is the apparent diversification of a founder cluster in the syntenic regions of closely related taxonomic lineages (Fig. 6c). Here there is clear synteny between the metabolic clusters within the related species but each species makes slightly different compounds (Table 1; Fig. 1). This is exemplified by the steroidal glycoalkaloid clusters in tomato and potato. Interestingly, these pathways are the most fragmented of the clustered pathways reported so far. It is not yet clear whether the satellite/peripheral genes may have formed part of an ancestral progenitor cluster or whether they represent more recent acquisitions.

V. Conclusions and outlook

Technological advances in sequencing and bioinformatics coupled with the growing body of knowledge about the organization of metabolic pathways in plant genomes now hold great promise for accelerating the discovery and elucidation of new natural product pathways. Genetics-driven, trait-based approaches have proved to be very powerful and have led to the discovery of clustered pathways that determine crop traits that are important for food security, food quality and medicine (Fig. 7). In the cases of the oat avenacin cluster and the poppy noscapine cluster, this was achieved using BAC contigs in the absence of publicly available genome sequence information (Qi *et al.*, 2006; Osbourn *et al.*, 2012; Winzer *et al.*, 2012; Mugford *et al.*, 2013). Strategies for pathway discovery are now beginning to shift from characterization of individual pathway steps to genomics-based prediction and validation of entire pathways (Fig. 8). Increased knowledge of plant metabolic gene clusters and the systematic analysis, curation and standardization of these clusters and their parts (Medema *et al.*, 2015; Patron *et al.*, 2015) will feed back into future genome mining efforts, so enhancing the genome to natural product discovery pipeline. It will also establish how common this clustering phenomenon is in plants and shed further light on the raison d'être for metabolic clusters. Last but by no means least, it is intriguing to consider whether plant genomes might harbour other types of nonhomologous gene clusters that have functions other than in secondary metabolism.

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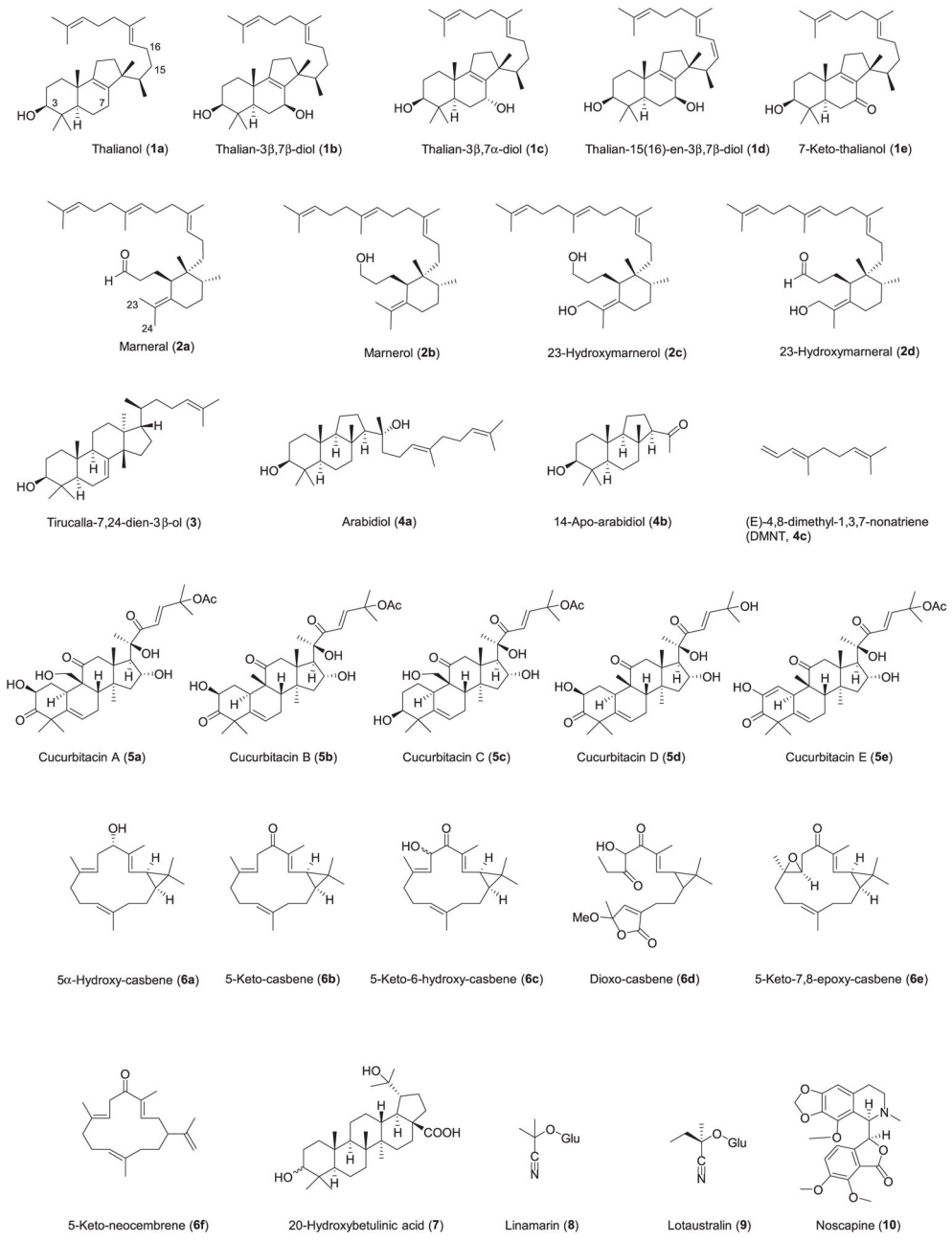

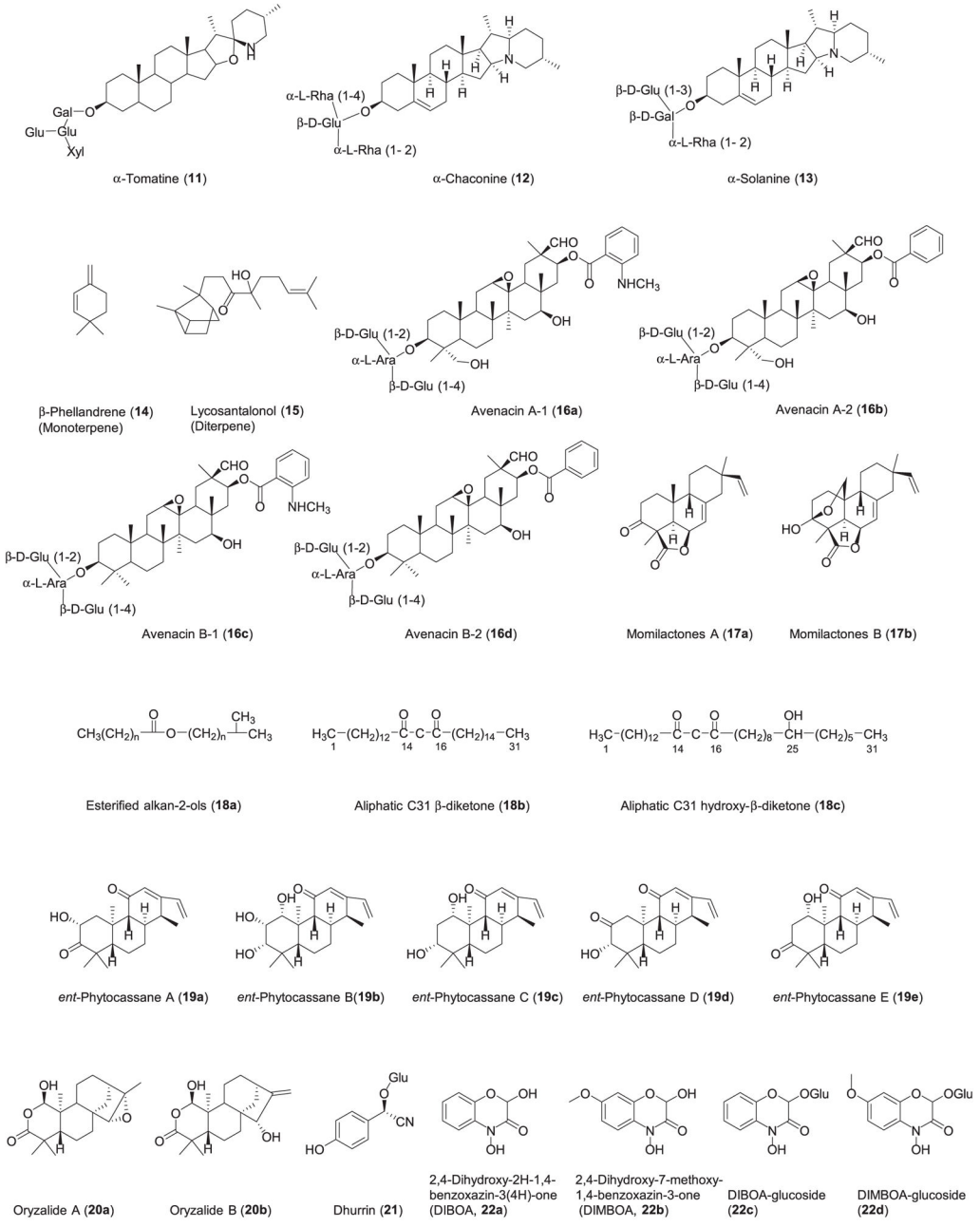

Fig. 1.

Structures of the products of characterized clustered plant metabolic pathways. The structures are numbered according to Table 1.

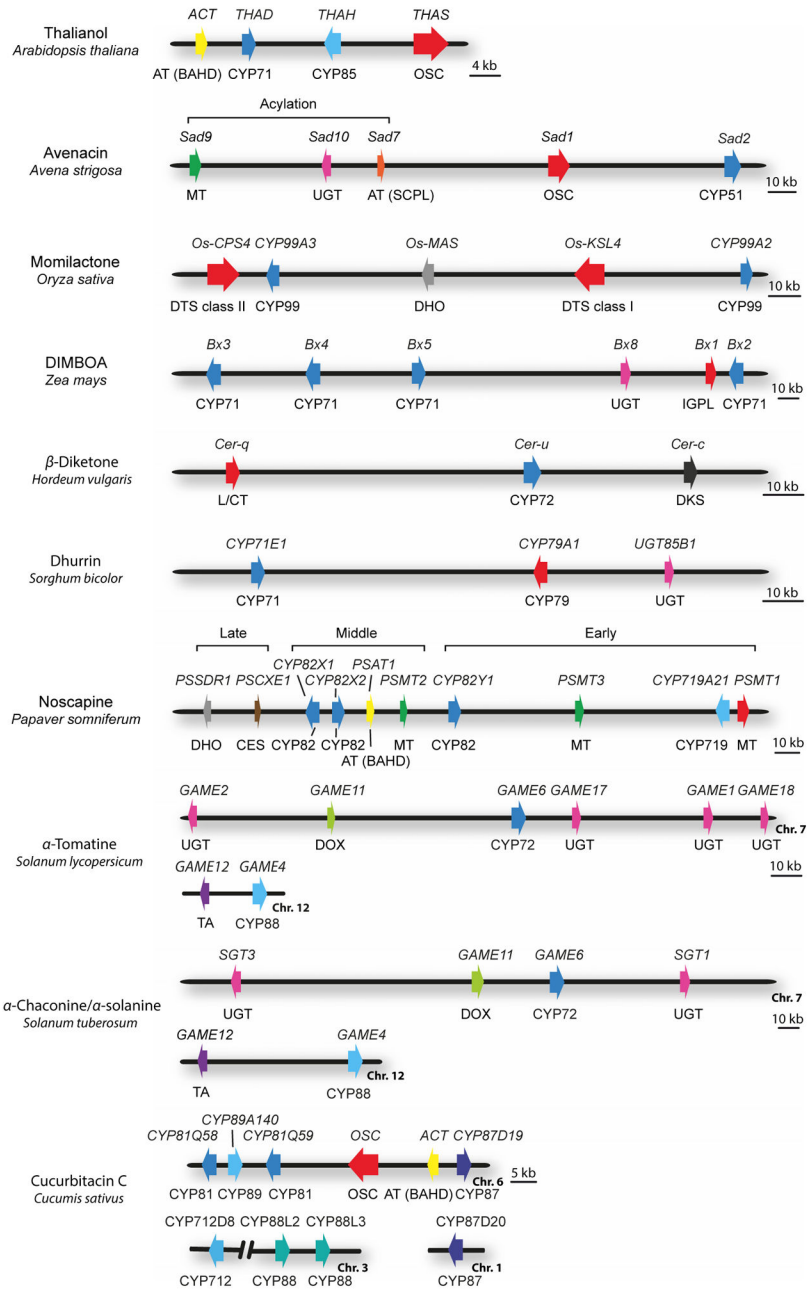

Fig. 2.

Genomic organization of plant metabolic gene clusters. Examples that exemplify different cluster types and biosynthetic pathways are shown. The genes are indicated by arrows. The gene(s) for the first committed pathway step are indicated in red. Gene names are indicated above the clusters, and class of biosynthetic enzyme below. Abbreviations: OSC, oxidosqualene cyclase; DTS, diterpene synthase (class I and class II shown); IGPL, indole 3-glycerol phosphate lyase; DKS, diketone synthase; AT (BAHD), BAHD-acyltransferase; AT (SCPL), SCPL-acyltransferase; MT, methyltransferase; UGT, UDP-dependent sugar transferase, DHO, dehydrogenase/reductase; L/CT, lipase/carboxyltransferase; CES,

carboxylesterase; DOX, dioxygenase; TA, transaminase; CYP, cytochrome P450. The maize DIMBOA pathway includes three genes that are not shown in the figure: *Bx7*, which is separated from the core cluster by an intervening region of 15 Mb; the sugar transferase gene *Bx9*, which is located on a different chromosome; and finally *Bx6*, which is not shown because its genomic location has not yet been established. [Correction added after online publication 26 April 2016; β-Diketone *Hordeum vulgaris* cluster has been corrected.]

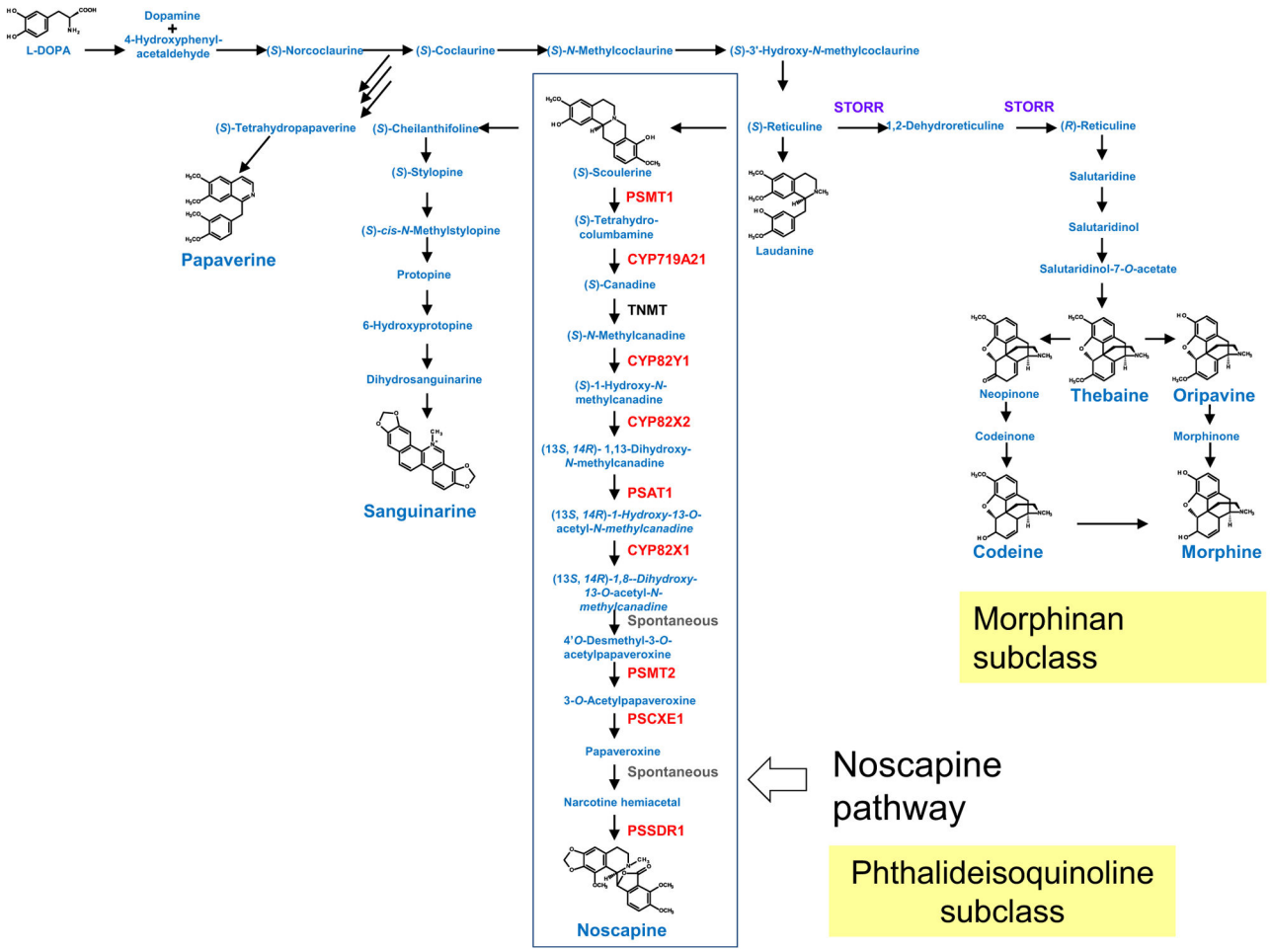

Fig. 3.

Benzylisoquinoline alkaloid biosynthesis in poppy. Poppy synthesizes a variety of different benzylisoquinoline alkaloids, including noscapine, codeine and morphine. The first committed step of the noscapine pathway (boxed) is the conversion of *(S)*-scoulerine to *(S)*-tetrahydrocolumbamine by PSMT1. The enzymes encoded by the genes within the noscapine cluster are indicated in red. The gene for one of the noscapine pathway steps (tetrahydroprotoberberine cis-*N*-methyltransferase; TNMT) is not represented within the cluster. Unlike the rest of the pathway genes, *TNMT* is present and expressed in HN1, HT1 and HM1 poppy varieties and so is likely to have other functions in addition to its role in noscapine biosynthesis (Winzer *et al.*, 2012). The P450 oxidoreductase fusion protein STORR, which catalyses the first step in morphinan biosynthesis in poppy – the conversion of (*S*)-reticuline to (*R*)-reticuline – is indicated in purple.

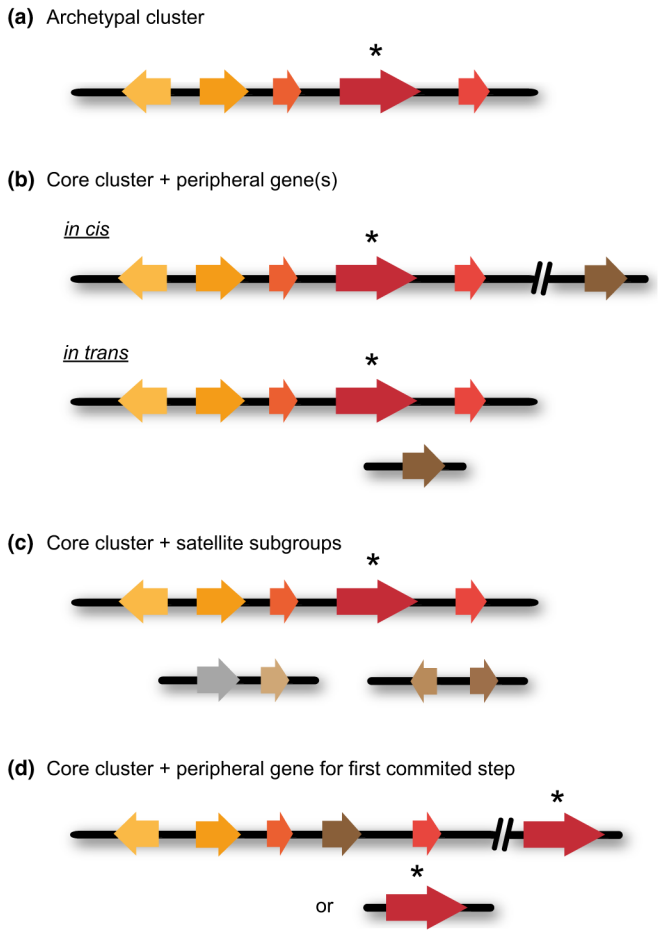

Fig. 4.

Types of cluster organization. (a) Archetypal cluster. The archetypal plant metabolic cluster is compact and contains contiguous pathway genes, including the gene encoding the enzyme for the first committed step in the pathway. (b) Core cluster plus peripheral gene(s). The genes for most of the metabolic pathway steps (including the gene for the first committed step) are clustered and contiguous, but there may be one or more peripheral genes encoding other pathway steps. These peripheral genes may be loosely linked to the core cluster (*in cis*) or may be unlinked (*in trans*). (c) Core cluster plus satellite subgroups. The bulk of the pathway genes, including the gene for the first committed step, are organized in a core cluster with small groups of two to three other genes present as satellite subgroups elsewhere in the genome. For the examples reported so far, these satellite subgroups contain genes for one or two different types of tailoring enzymes. (d) Core cluster plus peripheral gene for first step. In this scenario the core cluster encodes three or more different types of tailoring enzyme and the gene encoding the first committed step is elsewhere in the genome (either *in cis* or *in trans).* In each case the gene encoding the first committed pathway step is indicated in dark red and marked with an asterisk.

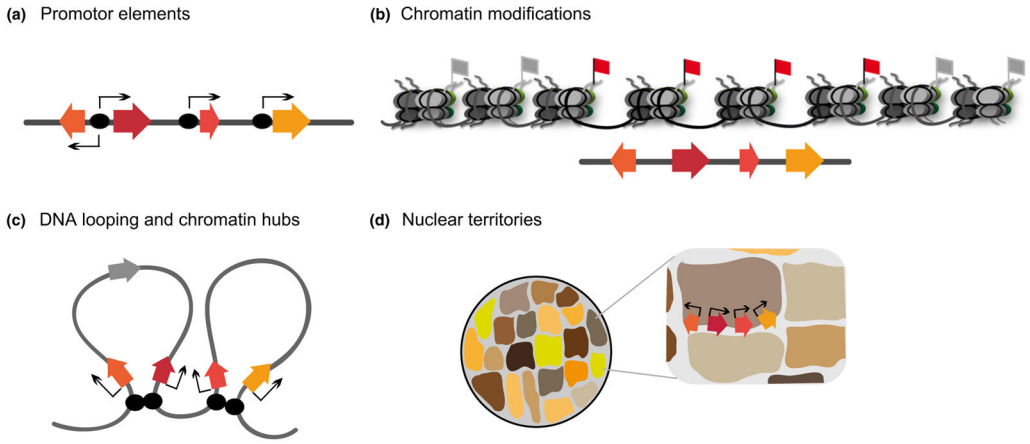

Fig. 5.

Schematic representation of potential levels of gene cluster regulation. (a) Coregulated promotor motifs (black ovals) are located upstream of each cluster gene. (b) Distinct chromatin modifications facilitate the formation of readily accessible open chromatin structures (red flag) and closed condensed chromatin regions (grey flag). An open chromatin structure will allow transcription, whereas a closed structure will suppress it. (c) At the tertiary level, *cis*-acting regulatory elements (black ovals) associate to form chromatin hubs, looping out the intervening DNA and bringing the cluster genes together in close proximity. The genes close to the regulatory elements are expressed (coloured), whereas genes located further away are silent (grey). (d) Gene clusters may be located in discrete nuclear territories that are characterized by distinct chromosomal conformations.

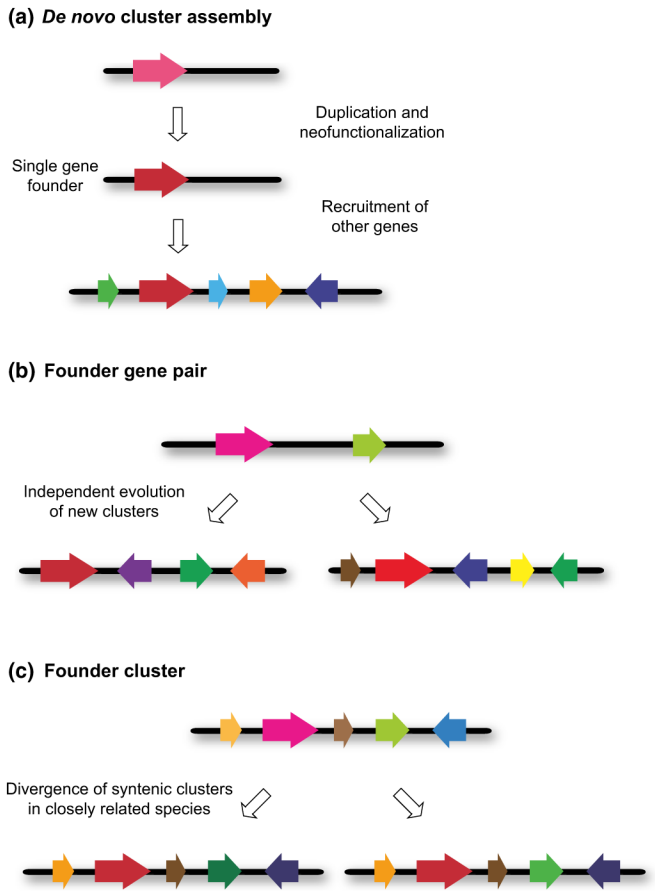

Fig. 6.

Origins of plant metabolic clusters. The scenarios shown are based on current knowledge of characterized plant metabolic clusters (see text). (a) *De novo* cluster assembly. The first step in this process is the recruitment of the gene for the first committed step in the pathway by gene duplication and neofunctionalization. This gene then 'seeds' the formation of a multigene cluster for a new metabolic pathway by an as yet unknown mechanism. (b) Formation of new metabolic clusters from a founder gene pair template. In this scenario, an ancestral gene pair (e.g. encoding a terpene synthase and a cytochrome P450 (CYP) enzyme) may give rise to different metabolic clusters for new natural product pathways as a result of independent events involving gene rearrangement and recruitment of new genes. This is exemplified by the *Arabidopsis thaliana* thalianol and marneral clusters (Field *et al.*, 2011). These are both triterpene pathways, but the scaffolds are not the same and the subsequent modifications are different. For both (a) and (b) the process of cluster formation will be accompanied by refinement and optimization of individual pathway components and establishment of a functional gene neighbourhood that allows for coordinate pathway control. (c) Diversification of an ancestral founder cluster in syntenic regions of closely related taxonomic lineages. In these cases, these pathways may have a common scaffold but make slightly different compounds.

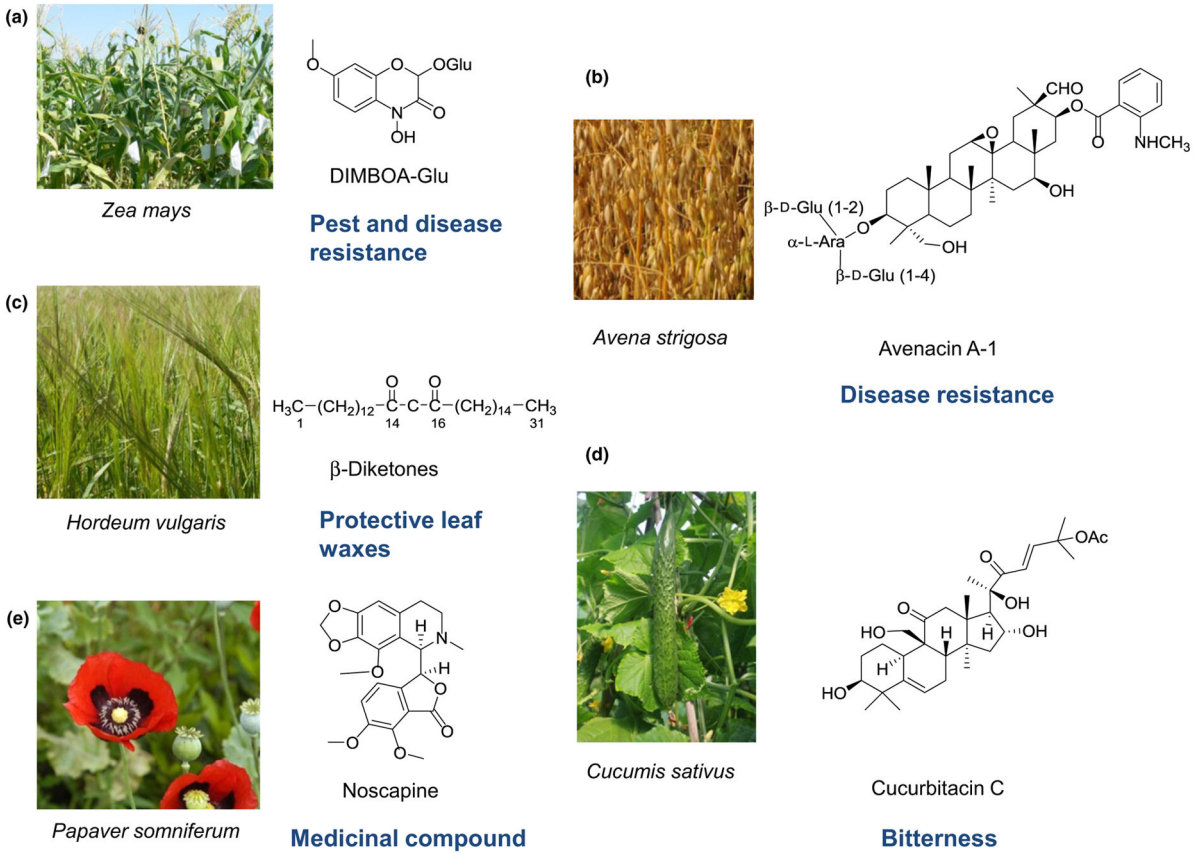

Fig. 7.

Trait-based cluster discovery. Examples of compounds of agronomic/medicinal importance for which the underlying metabolic pathways were characterized bytrait-based cluster discovery are shown. The images of plants are reproduced with the kind permission of: (a) Paul Cristou, Institució Catalanade Recerca I Estudis, Lleida, Spain; (b) Anthony Pugh, Institute for Biological, Environmental and Rural Sciences, Aberystwyth, UK; (c) John Innes Centre Photography; (d) Sanwen Huang, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences; (e) Tanja Niggendijker/Creative Commons.

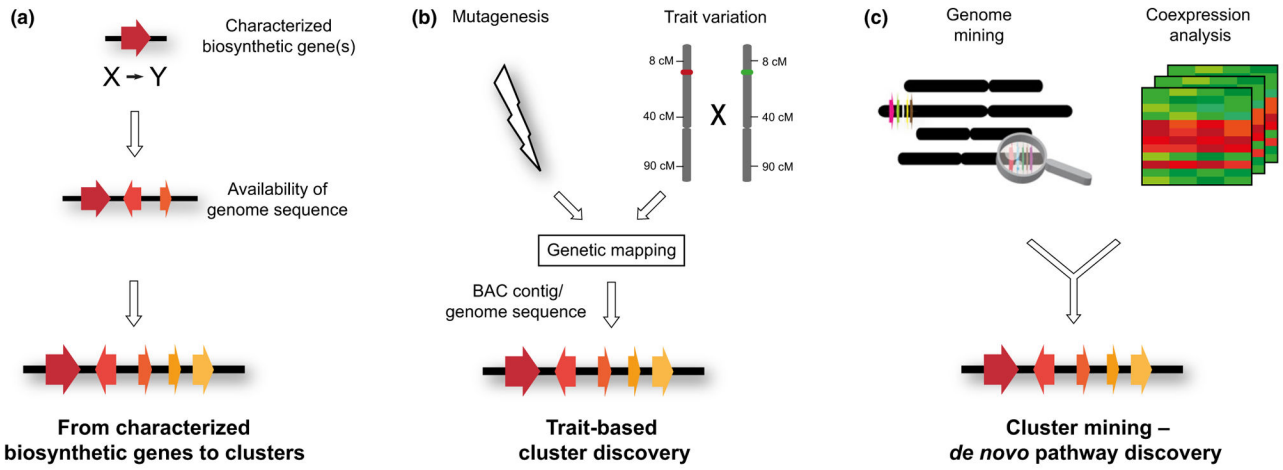

Fig. 8.

Strategies used for identification of plant metabolic clusters. (a) Biochemical characterization of one or more pathway steps in the pregenome era. The existence of clustering may then become apparent once genome sequence data for the species of interest is available. (b) Investigation of induced or natural variation in a trait provides genetic evidence for a multigene locus. The relevant region is then further defined by mapping. The underlying cluster is identified by assembly and sequencing of bacterial artificial chromosome (BAC) contigs spanning the region (in the absence of genome sequence information) or by exploiting available genome sequence data in the region of the locus. (c) Discovery of metabolic clusters for new pathways by genome mining. Physical clustering of genes implicated in a specialized metabolic pathway in combination with transcriptomics data enables the delineation of new candidate metabolic gene clusters.

Table 1

Clustered pathways for the biosynthesis of plant natural products

Pathway	Class of compound	Plant species	Annual/perennial	Method of cluster discovery	Reference
Eudicots[a]					
Thalianol (**1a–e**)	Triterpene	*Arabidopsis thaliana*	Annual	Cluster mining	Field & Osbourn (2008)
Marneral (**2a–d**)	Triterpene	*Arabidopsis thaliana*		Cluster mining	Field *et al.* (2011)
Tirucalla-7,24-dien-3β-ol (**3**)	Triterpene	*Arabidopsis thaliana*		Cluster mining	Boutanaev *et al.* (2015)
Arabidiol (**4a–c**)	Triterpene	*Arabidopsis thaliana*		Cluster mining	Castillo *et al.* (2013); Sohrabi *et al.* (2015)
Cucurbitacins (**5a–e**)	Triterpene	*Cucumis sativus*	Annual	Cluster mining; trait-based discovery (characterization of the *Bi* locus for bitterness)	Boutanaev *et al.* (2015); Shang *et al.* (2014)
Casbene diterpenoids (**6a–b**)	Diterpene	*Euphorbia peplus*	Annual	Characterized biosynthetic genes to clustering[c]	King *et al.* (2014)
Casbene diterpenoids	Diterpene	*Jatropha curcas*	Perennial	Genome mining; genetics	King *et al.* (2014)
20-Hydroxybetulinic acid (**7**)	Triterpene	*Lotus japonicus*	Annual	Cluster mining	Krokida *et al.* (2013)
Linamarin (**8**)/lotaustralin (**9**)	Cyanogenic glycoside	*Lotus japonicus*	Annual	Isolation of *cyanogenesis deficient* mutants; genomics	Takos *et al.* (2011)
Linamarin (**8**)/lotaustralin (**9**)	Cyanogenic glycoside	*Manihot esculenta*	Perennial	Characterized biosynthetic genes to cluster	Takos *et al.* (2011)
Noscapine (**10**)	Benzylisoquinoline alkaloid	*Papaver somniferum*[b]	Annual	Trait-based discovery (characterization of *High Noscapine 1* locus)	Winzer *et al.* (2012)
Casbene diterpenoids (**6a–f**)	Diterpene	*Ricinus communis*	Perennial with annual forms	Cluster mining; characterized biosynthetic genes to cluster	Boutanaev *et al.* (2015); King *et al.* (2014)
α-Tomatine (**11**)	Steroidal alkaloid	*Solanum lycopersicum*	Perennial with annual forms	Characterized biosynthetic genes to cluster	Itkin *et al.* (2013)
α-Chaconine (**12**)/α-solanine (**13**)	Steroidal alkaloid	*Solanum tuberosum*	Perennial	Characterized biosynthetic genes to cluster; synteny with *Solanum lycopersicon*	Itkin *et al.* (2013)
Monoterpenes (**14**)/diterpenes (**15**)	Terpenes	*Solanum lycopersicum*	Perennial with annual forms	Characterized biosynthetic genes to cluster	Falara *et al.* (2011); Matsuba *et al.* (2013, 2015)
Monocots					
Avenacins (**16a–d**)	Triterpene	*Avena strigosa*[b]	Annual	Trait-based discovery (forward screen for avenacin-deficient mutants)	Qi *et al.* (2004, 2006); Mugford *et al.* (2009, 2013)
β-Diketones (**18a–c**)	Polyketide	*Hordeum vulgare*	Annual	Trait-based discovery (characterization of the *Cer-cqu* leaf wax locus)	Schneider *et al.* (2016)

Pathway	Class of compound	Plant species	Annual/perennial	Method of cluster discovery	Reference
Momilactones (**17a, b**)	Diterpene	*Oryza sativa*	Annual	Characterized biosynthetic genes to cluster	Wilderman *et al.* (2004); Shimura *et al.* (2007)
Phytocassanes (**19a**–**e**), oryzalides (**20a, b**)	Diterpene	*Oryza sativa*	Annual	Characterized biosynthetic genes to cluster	Swaminathan *et al.* (2009)
Dhurrin (**21**)	Cyanogenic glycoside	*Sorghum bicolor*	Annual	Characterized biosynthetic genes to cluster	Takos *et al.* (2011)
2,4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA, **22a**–**d**)	Hydroxamic acid	*Zea mays*	Annual	Trait-based discovery (screen for *bx1* mutants)	Frey *et al.* (1997); von Rad *et al.* (2001); Frey *et al.* (2003); Jonczyk *et al.* (2008)

[a]The products of the pathways are shown in Fig. 1.

[b]Species for which genome sequences are not publicly available. Clusters were therefore defined by assembling and sequencing bacterial artificial chromosome (BAC) contigs (Mugford *et al.*, 2009; Mugford *et al.*, 2013; Qi *et al.*, 2006; Winzer *et al.*, 2012).

[c]For casbene diterpene biosynthesis in *E. peplus*, only two adjacent genes have been discovered so far (a casbene synthase and CYP72A19). Although this region does not contain genes encoding a minimum of three different types of enzymes, it has been included in the table since it emerged from a wider investigation of production of casbene diterpenoids in the wider Euphorbiaceae (see entries for castor (*Ricinus communis*) and jatropha (*Jatropha curcas*)) (King *et al.*, 2014).