Chapter 13
Allopolyploid Speciation in Action: The Origins and Evolution of *Senecio cambrensis*

Matthew J. Hegarty, Richard J. Abbott and Simon J. Hiscock

Abstract *Senecio cambrensis* is one of a few allopolyploid plant species known to have originated in the recent past and, therefore, provides excellent material for analysing allopolyploid speciation. This allohexaploid species originated in the UK within the last 100 years following hybridization between diploid *S. squalidus* and tetraploid *S. vulgaris*. In this chapter, we first describe the events leading up to hybridization between these two species, focusing mainly on the origin and spread of *S. squalidus* in the UK. We then consider alternative pathways by which *S. cambrensis* might have originated and conclude that current evidence suggests an origin via formation of the triploid hybrid (*S. x baxteri*) followed by chromosome doubling. We next review our investigations into levels of genetic diversity and also changes to gene expression and the possible causes of this (epigenetic effects) during the origin of *S. cambrensis*. High levels of genetic diversity, assessed by surveys of allozyme and AFLP variation, have been recorded in *S. cambrensis*, and it is likely that intergenomic recombination was an important generator of this diversity. Our studies of 'resynthesized' *S. cambrensis* have shown that the initial genome merger (hybridization) producing *S. x baxteri* generates genome-wide, non-additive alterations to parental patterns of gene expression and DNA

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methylation, with genome duplication resulting in a secondary burst of both transcriptional and epigenetic modification. In synthetic allohexaploid lines of *S. cambrensis* phenotypic changes become apparent from the second to fifth generations, possibly as a consequence of recombination or epigenetic effects; these include changes in ray flower form and emergence of self-incompatible individuals. We conclude by considering the future of *S. cambrensis* from the standpoint of it being a model species for further study of allopolyploid speciation, and second its long-term success in the wild. Ongoing work to produce a draft reference genome for *S. squalidus* will underpin future research in *S. cambrensis*, enabling a more thorough survey of changes to DNA methylation, small RNA activity and promoter binding in the hybrids, as well as comparison with the related allotetraploid *S. eboracensis* to determine the effects of genome dosage. The future of the species in the wild is currently uncertain. The population in Edinburgh that represented a separate origin of the species in the wild during the 1970s is now extinct, and there has been a marked decline in the number of populations and individuals of the species in its heartland, North Wales, since the 1980s. An analysis of how its ecology compares with those of its parents is lacking. However, it appears to share the same habitats in the wild with its parents, which might have contributed to its decline. Although *S. cambrensis* may become extinct in the wild in the near future, the potential will remain for it to originate again in the UK providing that conditions prevail for its parents to hybridize.

13.1 Introduction

13.1.1 General Introduction

Polyploidization (genome duplication) is an important evolutionary process in plants (Grant 1981) that appears to have accompanied major transitions in land plant evolution, including the evolution of the seed habit and the evolution of angiosperms (Jiao et al. 2011). All angiosperms are thought to have a polyploid ancestry (Jiao et al. 2011) but most of these are paleopolyploids that now function essentially as diploids. Nevertheless, there are also numerous examples of recently formed polyploids (Adams and Wendel 2005; Wood et al. 2009). Most recent polyploids have formed in association with interspecific hybridization (Grant 1981; Leitch and Bennett 1997; Soltis and Soltis 1999; Otto and Whitton 2000; Leitch and Leitch 2008; Hegarty and Hiscock 2008)—allopolyploidy, which is now recognised as perhaps the most important mechanism of abrupt speciation in plants (Grant 1981; Leitch and Leitch 2008; Hegarty and Hiscock 2008). Allopolyploidy confers rapid fertility to hybrids because duplicated parental chromosomes pair 'normally' during meiosis; it also confers reproductive isolation of the hybrids from their parental species because of aberrant or failed chromosome pairing (Soltis and Soltis 1999; Rieseberg et al. 2003;

Rieseberg and Willis 2007). Numerous examples of allopolyploid speciation have been described in the literature, including at least six new plant species that have arisen in the last century: *Tragopogon mirus* and *T. miscellus* (Novak et al. 1991, Chap. 14, this volume); *Spartina anglica* (Gray et al. 1991, Chap. 12, this volume), *Senecio cambrensis* and *S. eboracensis* (Ashton and Abbott 1992, Abbott and Lowe 1996, 2004) and *Cardamine schulzii* (Urbanska et al. 1997). In this chapter, we review the origins of the *Senecio* allopolyploid species *S. cambrensis* (Welsh groundsel). We show how resynthesised forms of this allopolyploid can reveal important insights into the genetic and genomic consequences of allopolyploidization and how parental traits, both morphological and physiological, recombine in neopolyploids.

13.1.2 *The Genus* Senecio *and the Origins of UK Allopolyploids*

The genus *Senecio* (Asteraceae), which includes ragworts and groundsels, is one of the largest and most morphologically diverse genera of flowering plants. With between 1000 and 3000 species, the genus has a worldwide distribution with species described from almost every land mass on Earth (Vincent 1996). Recent revisions, following molecular phylogenetic analysis, of the genus have assigned many former *Senecio* species to other genera leaving a conservative 1200 species of *Senecio sensu stricto* (Pelser et al. 2007). Within the genus there are numerous examples of hybridization and polyploidy, and many species have been proposed to have an allopolyploid origin (Abbott and Lowe 1996; Coleman et al. 2001; Kadereit et al. 2006; Pelser et al. 2012). In the UK, three new polyploid taxa have arisen within the last 100 years as a consequence of hybridization between native tetraploid *Senecio vulgaris* (common groundsel) and the introduced invasive diploid species *S. squalidus*. The origins of these new polyploid taxa (see below) provide one of the best examples of 'evolution in action' (Abbott and Lowe 1996, Hegarty and Hiscock 2008). The introduction and rapid spread of alien *S. squalidus* across the UK was the main catalyst for this burst of hybrid speciation, which was further facilitated by the obligate outcrossing mating system of *S. squalidus* increasing the frequency of interspecies pollinations (Brennan and Hiscock 2010).

13.1.3 *Oxford Ragwort in the British Isles: Introduction and Spread*

Senecio squalidus ($2n = 2x = 20$), commonly known as Oxford ragwort, is itself a recently evolved (homoploid) hybrid species (James and Abbott 2005; Abbott et al. 2010; Brennan et al. 2012) that originated on Mount Etna, Sicily, as a result of hybridization between *S. aethnensis* ($2n = 2x = 20$, a Mount Etna endemic of higher altitudes) and *S. chrysanthemifolius* ($2n = 2x = 20$, a native Sicilian

species of lower altitudes). At mid-altitudes on the volcano the distribution of these species overlaps, leading to the formation of a stable hybrid zone (Brennan et al. 2009). Material from this hybrid zone was introduced to the Oxford Botanic Garden in the early 1700s, but records of the collection locality or localities, methods of cultivation, and numbers of plants cultivated in the garden have been lost. Plants subsequently escaped from the Botanic Garden and colonised the masonry of the old college walls from the end of the eighteenth century (Harris 2002). During the industrial revolution of the nineteenth and early twentieth centuries *S. squalidus* moved rapidly out of Oxford by colonising the clinker beds of the expanding UK railway, of which Oxford was a key hub. The chronology of this famous plant invasion is meticulously documented in a set of papers by Kent (reviewed in Abbott et al. 2009), which record that it began to spread northwards in the late nineteenth century, reaching different parts of northern England during the middle of the twentieth century, before becoming established in the Central Belt of Scotland by the mid-1950s. Today, *S. squalidus* continues to spread north in Scotland and across Northern Ireland.

The rapidity of this invasion is intriguing because *S. squalidus* is self-incompatible (Abbott and Forbes 1993; Hiscock 2000a, b, and obligate outcrossers are generally thought not to make good colonizers or invasives (Baker 1967). Most invasive species tend to have uniparental reproduction, either sexual (selfing) or asexual (apomixis or vegetative reproduction), although there are exceptions, most notably other species of Asteraceae, such as yellow star thistle, *Centaurea solstitialis* (Sun and Ritland 1998). According to Baker's 'rule' (Baker 1967), successful colonising and invasive plants are usually self-fertile (self-compatible [SC]) (Stebbins 1957; Baker 1967). Studies of the mating system of *S. squalidus,* however, have shown that individuals exhibit strong self-incompatibility (SI) across its entire British range, and, as in other species of Asteraceae, this SI is regulated sporophytically by a single polymorphic *S* locus (Hiscock 2000a, b; Brennan et al. 2002, 2005, 2006). The finding of strong SI in *S. squalidus* is intriguing, particularly in the light of the extreme population bottleneck that its ancestors must have experienced during its introduction and early colonisation. Following a population bottleneck, allelic diversity at the *S* locus will be lowered and opportunities for mating (between individuals carrying different *S* alleles) correspondingly reduced (Hiscock 2000b; Brennan et al. 2002). An extensive survey of SI in *S. squalidus* across the UK showed that a combination of substantial between-population sharing of the seven *S* alleles contained in the entire UK population and low levels of selfing ('pseudo-self-compatibility') were the most likely cause of *S. squalidus'* reproductive success as a colonizer (Brennan et al. 2005, 2006).

13.1.4 The Origins of Senecio cambrensis

During its spread across the UK, *S. squalidus* ($2n = 2x = 20$) hybridized with the self-compatible native groundsel, *S. vulgaris* ($2n = 4x = 40$), resulting in the recent

Fig. 13.1 The neoallohexaploid *Senecio cambrensis* (*centre*) flanked by its parents, tetraploid *S. vulgaris* (*left*) and diploid *S. squalidus* (*right*). Here, *S. cambrensis* has flower heads (capitula) of intermediate type to its parents; however, its ray florets can vary in length, and occasionally non-radiate forms (lacking ray florets) are also found in the wild

origin of three hybrid taxa. These are the allohexaploid *S. cambrensis* ($2n = 6x = 60$), the recombinant tetraploid *S. eboracensis* ($2n = 4x = 40$), and the stabilized introgressant radiate form of *S. vulgaris, S. vulgaris* var. *hibernicus* ($2n = 4x = 40$). Interestingly, all three new hybrid taxa are self-compatible, suggesting that this SC mating system, inherited from *S. vulgaris*, is 'dominant' over the SI mating system present in *S. squalidus*. Detailed descriptions of these new taxa and what is known about their origins are presented elsewhere (see Abbott et al. 1992; Lowe and Abbott 2003; Abbott and Lowe, 2004; Kim et al. 2008).

Here, we briefly summarise the information available on the origin of *S. cambrensis* (Fig. 13.1) focussing particularly on the possible pathways of its origin. Knowing the pathway of origin of a polyploid taxon is helpful in regard to understanding the species' potential to generate genetic diversity during its initial stages of development, and also for accurate production of synthetic forms of the polyploid used to study possible genetic and epigenetic changes that occurred in the taxon immediately following its origin in the wild (Hegarty et al. 2006, 2008, 2011; Lukens et al. 2006; Buggs et al. 2009).

Senecio cambrensis was described by Rosser (1955) from material provided by H.E. Green, who first observed the plant growing at Ffrith and Ceffn-y-bedd, North Wales, UK, in 1948. The plant was described as an annual or short-lived perennial herb that was hexaploid with flower heads (capitula) containing ray florets having short ligules ($\sim$4.8 mm in length). Rosser (in Crisp 1972) later determined a herbarium specimen, collected in Denbigh, North Wales in 1925 and originally named as *S. squalidus* x *S. vulgaris*, to be *S. cambrensis*. However, in the absence of a chromosome count there remains some doubt as to whether this specimen is *S. cambrensis* or, alternatively, a fertile hybrid of *S. squalidus* and *S. vulgaris* (see below). Fertile, hexaploid plants with similar morphology to the wild form of

S. cambrensis can be produced by treating synthetic triploid hybrids between *S. squalidus* and *S. vulgaris* with colchicine (Harland 1955, Weir and Ingram 1980, Hegarty et al. 2005). On this basis, Rosser (1955) concluded that *S. cambrensis* was a new species that originated by hybridization between native *S. vulgaris* and introduced *S. squalidus* followed by chromosome doubling. The species is likely to have originated shortly before it was first recorded in North Wales and after *S. squalidus* had spread to the region in the early part of the twentieth century (Kent 1963).

In 1982, *S. cambrensis* was found growing in Edinburgh, UK (Abbott et al. 1983), and subsequent molecular analysis involving surveys of allozyme and chloroplast DNA variation showed that it had originated independently in Edinburgh rather than being dispersed there from North Wales (Ashton and Abbott 1992; Harris and Ingram 1992). Herbarium records indicate that the Edinburgh lineage may have existed since at least 1974; however, it is now thought to be extinct as the species has not been recorded in the Edinburgh area or nearby since 1993 (Abbott and Forbes 2002).

Because *S. cambrensis* is readily synthesised by treating the triploid hybrid between *S. squalidus* and *S. vulgaris* with colchicine, it has been assumed that chromosome doubling of the triploid hybrid was the likely pathway of origin of the allopolyploid species in the wild (Rosser 1955). In theory, however, the species could have originated along several possible pathways (Table 13.1) with the first step involving formation of a triploid, tetraploid, pentaploid or hexaploid hybrid. Of these alternatives, the formation of a triploid hybrid is more likely in that it results from fusion of normal haploid gametes produced by each parent. In contrast, formation of higher ploidy hybrids relies on the production of unreduced gametes, which will be generated at a much lower frequency in each parent species.

There are many records of the triploid hybrid (*Senecio x baxteri* Druce) occurring in the wild (Crisp 1972; Benoit et al. 1975; Marshall and Abbott 1980). It is easily recognised because of its intermediate morphology and its almost complete seed sterility. Progeny tests of *S. vulgaris* plants have shown that this hybrid is generated regularly but at very low frequencies in the wild where *S. vulgaris* and *S. squalidus* co-occur (Marshall and Abbott 1980). In contrast, the tetraploid hybrid has never been reported unequivocally in the wild, although Crisp (1972) described a plant likely to have been such a hybrid based on morphology and an analysis of its offspring. Although no chromosome count was made of the plant, all of its offspring were approximately tetraploid and segregated for a range of morphological, reproductive and disease resistance traits. Crisp (1972) suggested that such offspring could become stabilized in the wild to form distinct taxa, and it is feasible that the tetraploid *S. eboracensis* originated in this way (Lowe and Abbott 2000). Whether such a hybrid might have contributed to the origin of *S. cambrensis* as detailed in Table 13.1 remains unknown, but seems less likely than an origin involving the triploid hybrid, given the apparent rarity of the tetraploid hybrid in the wild. Similarly, origins involving the formation of either a pentaploid or hexaploid hybrid are less parsimonious than one involving the triploid hybrid, although cannot be ruled out entirely. Further support for the hypothesis that formation of a triploid hybrid was the first step in the origin of

Table 13.1 Some possible pathways of origin for *Senecio cambrensis* in the wild

(1) Via Triploid hybrid (2*n*=30)
• Step 1 - Formation of triploid hybrid (2*n*=30) through fusion of haploid gametes produced by each parent species.
• Step 2 - Chromosome doubling of triploid hybrid by: (i) fusion of 'unreduced' triploid gametes (*n*=30); or (ii) doubling of chromosome number of a somatic cell ancestral to a floret or flower head producing 'reduced' triploid gametes (*n*=30).
(2) Via Tetraploid hybrid (2*n*=40)
• Step 1 - Formation of tetraploid hybrid (2*n*=40) by: (i) fusion of a haploid gamete (n=20) of *S. vulgaris* and a diploid (unreduced) gamete (*n*=20) of *S. squalidus*; or (ii) fusion of an 'unreduced' triploid gamete of triploid hybrid (n=30) and haploid gamete of *S. squalidus* (*n*=10).
• Step 2 - Production of hexaploid hybrid by: (i) fusion of an 'unreduced' tetraploid gamete (*n*=40) generated by tetraploid hybrid and a 'reduced' diploid gamete (*n*=20) of same hybrid or of *S. vulgaris* or an unreduced gamete of *S. squalidus*; or (ii) fusion of 'balanced' triploid gametes (*n*=30) produced by same hybrid.
(3) Via Pentaploid hybrid (2*n*=50)
• Step 1 - Formation of pentaploid hybrid by: (i) fusion of a haploid gamete of *S. squalidus* (*n*=10) and an unreduced gamete of *S. vulgaris* (*n*=40); or (ii) fusion of unreduced gamete of triploid hybrid (*n*=30) with haploid gamete of *S. vulgaris* (*n*=20) or unreduced gamete of *S. squalidus* (*n*=20).
• Step 2 - Production of hexaploid hybrid by: (i) fusion of gametes with same or different 'balanced' chromosome numbers (i.e. *n*=10, *n*=20, *n*=30, *n*=40, *n*=50) generated by pentaploid hybrid such that the zygote produced is hexaploid (2*n*=60); (ii) fusion of tetraploid 'balanced' gamete (2*n*=40) produced by pentaploid hybrid with reduced gamete (*n*=20) of *S. vulgaris* or unreduced gamete of *S. squalidus*. (iii) fusion of diploid 'balanced' gamete (*n*=20) produced by pentaploid hybrid with unreduced gamete (*n*=40) of *S. vulgaris*.
4) Direct formation of Hexaploid hybrid (2*n*=60)
• Step 1 - Formation of hexaploid hybrid by fusion of unreduced gametes from both *S. squalidus* (*n*=20) and *S. vulgaris* (*n*=40).

S. cambrensis comes from reports by Vosa (in Crisp 1972) and Ingram (1978) that rare allohexaploid offspring were produced spontaneously by natural selfing of the synthetic triploid hybrids they made. However, Weir and Ingram (1980) also reported the production of an allohexaploid plant directly from a cross between *S. vulgaris* and *S. squalidus*. This could have been formed by fusion of unreduced gametes or alternatively by chromosome doubling of a triploid hybrid early in its development. We shall never know exactly how the different lineages of *S. cambrensis* originated in the UK, but given that the triploid hybrid is regularly encountered in the wild and is capable of producing allohexaploid offspring spontaneously, an origin involving doubling of the chromosome number of a triploid hybrid seems the most likely route of origin.

Because *S. cambrensis* is self-fertile, one newly formed individual of the species would have been able to reproduce sexually and successfully following the species' origin, i.e. without need of a mate. Moreover, if the species had originated through chromosome doubling of a triploid hybrid, it would be expected initially to be homozygous at all loci within its parental genomes, but to exhibit frequent fixed heterozygosity at duplicated loci among parental genomes. Somewhat

surprisingly, however, the species has been shown through surveys of allozyme variation (Ashton and Abbott 1992) and particularly AFLP variation (Abbott et al. 2007) to contain high levels of genetic diversity, indicating that it rapidly generated this diversity following its origin. Abbott et al. (2007) considered the ways in which such genetic diversity was produced and concluded that intergenomic recombination would most likely have been an important mechanism, although other mechanisms such as aneuploidy, gene conversion, activation of transposons and retroelements, other forms of mutation and gene flow from parental species could not be ruled out. The occurrence of radiate and non-radiate forms of *S. cambrensis* as well as variation in the ligule length of ray florets have been attributed to intergenomic recombination (Ingram and Noltie 1984), while the observation of multivalent formation occurring at low frequency in meiotic cells of the species (Ingram and Noltie, 1989) provides a mechanism for such recombination to occur. Although not reported by Ingram and Noltie (1989), Crisp (1972) observed up to eight chromosomes with subterminal centromeres in the somatic complement of *S. cambrensis* plants (based on root tip squashes). He pointed out that as neither parent species possessed such chromosomes they were probably the products of chromosome rearrangements following meiotic abnormalities. In addition to generating genetic diversity, intergenomic recombination resulting in chromosome rearrangements could lead to the formation of reproductive barriers between divergent lineages of *S. cambrensis*. Clearly, further work on the frequency of intergenomic recombination and its possible effects in *S. cambrensis* would be worthwhile.

13.2 Consequences of Hybridization and Polyploidy in Natural and Resynthesised *Senecio cambrensis*

13.2.1 Transcriptome Shock

In common with many other allopolyploid species, the merger of two divergent genomes during the formation of *Senecio cambrensis* has had a dramatic impact at the level of gene expression. As part of our investigation of the allopolyploid origins of *S. cambrensis*, we conducted gene expression analysis using a custom cDNA microarray platform (Hegarty et al. 2005) to survey the transcript levels of floral genes in both the intermediate triploid hybrid *S. x baxteri* and wild *S. cambrensis*, relative to their progenitor species. The experimental design of this comparison is shown in Fig. 13.2. This experiment revealed an initial large change in floral gene expression in *S. x baxteri*, with approximately 475 cDNA clones showing up- or down-regulation relative to its parental taxa or, also importantly, relative to natural *S. cambrensis*, from which it differs primarily by a change in ploidal level (Hegarty et al. 2005). Thus, the greatest changes in gene expression relative to the parents appeared to be associated with the hybridization step to form

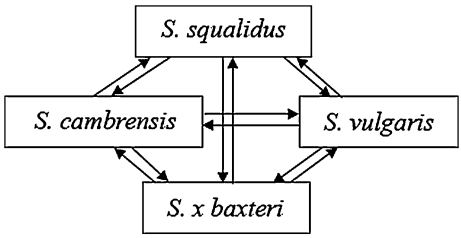

Fig. 13.2 Experimental design employed in microarray comparisons of gene expression between the allopolyploid *S. cambrensis* and its progenitor taxa, *S. vulgaris* and *S. squalidus*, and their sterile triploid F1 hybrid, *S. x baxteri*. Experimental details can be found in Hegarty et al. (2005), but, briefly, mature flower bud tissue was harvested from a mixed population of approximately 30 plants and pooled prior to RNA extraction to create an 'average' for each taxon. Labelled cDNA for each taxon was hybridized to a custom floral cDNA microarray. Two taxa were differentially labelled and compared per array hybridization (with 10 replicate hybridizations performed per comparison) using dye swaps to account for any bias in labelling efficiency. Each taxon was compared with the other three, for a total of 30 array hybridizations per taxon. Raw expression data for each taxon were extracted from these 30 replicates and imported separately into the GENESPRING microarray analysis software (Silicon Genetics) to enable comparison between all four taxa. Figure reproduced from Hegarty et al. (2008)

S. x baxteri. This initial burst of altered gene expression we termed "transcriptome shock", after the phenomenon of "genome shock" described by McClintock in her seminal work on transposable elements in plant hybrids (McClintock 1984). The 'transcriptome shock' effect in *S. x baxteri* was confirmed in our later analysis of resynthesised *S. cambrensis*, which further showed that the polyploidization event (here induced by colchicine) had an immediate calming (ameliorating) effect on altered patterns of gene expression detected in *S. x baxteri* (Hegarty et al. 2006). Importantly, this altered pattern of gene expression, apparent in first-generation allopolyploids, was preserved in four successive generations of the synthetic allopolyploids and in wild *S. cambrensis* (Hegarty et al. 2006). Previous research in resynthesised wheat (Feldman and Levy 2005) identified separate effects of hybridization and polyploidization on the genome and transcriptome, but our findings in *S. cambrensis* represented one of the first indications that these changes in gene expression were genome-wide. Interestingly, the putative functional classes of genes affected by hybridization and allopolyploidization were remarkably similar, with no functional class of genes being overly affected by hybridization or allopolyploidization (Fig. 13.3). However, perhaps not surprisingly, when compared with functional classes of genes not affected by either process, there was a greater representation of genes potentially involved in flower/inflorescence and pollen developments, which may reflect the transitions in floral phenotypes observed after hybridization and allopolyploidization.

We later reassessed the data (Hegarty et al. 2008) in light of a new approach used by Wang et al. (2006a) in their studies of allotetraploid *Arabidopsis suecica*. In this study, they focused on the identification of genes whose expression in hybrids differed from the additive expression midpoint of the two different parental

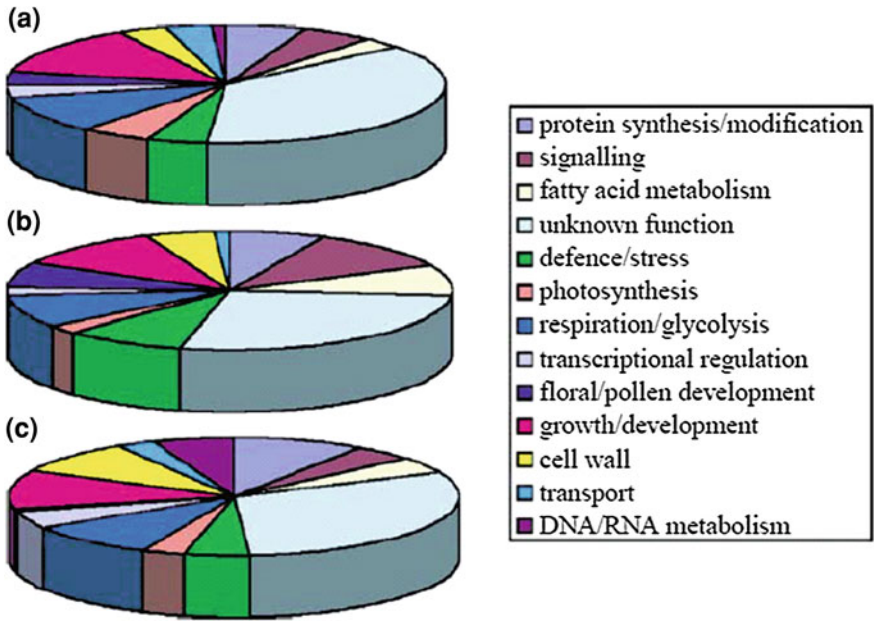

Fig. 13.3 Functional classes of genes affected by allopolyploidization and hybridization. Basic gene ontologies for cDNA clones displaying **a** conserved expression changes in both wild and synthetic *Senecio cambrensis* relative to *S. x baxteri* (genes affected by allopolyploidy, 540 clones), **b** expression changes relative to the parental taxa *S. squalidus* and *S. vulgaris* in both hybrid taxa (genes affected by hybridization, 99 clones) and **c** genes showing no expression difference between the parental and hybrid taxa (unaffected by hybridization or polyploidy, 289 clones). With the exception of a higher proportion of floral/pollen-related genes in (**a, b**) compared with (**c**), there are no substantial differences between the classes of affected genes (adapted with permission from Hegarty et al. 2006)

gene copies. A similar approach had also been used to analyse gene expression change in maize hybrids (Stupar et al. 2007). Such an approach provides a consistent and unified methodology for identifying genes affected by hybridization and/or polyploidization in different model study systems. We therefore reanalysed the microarray data from our original study to identify specific genes and classes of genes affected by hybridization and polyploidization. Using methods similar to Stupar et al. (2007), we tested whether changes in gene expression observed in synthetic *S. x baxteri* and wild allohexaploid *S. cambrensis* were additive or non-additive (Hegarty et al. 2008). By averaging the parental expression values for each feature on the array showing differential expression in the hybrids, a parental midpoint expression value (MPV) was obtained. The derived midpoint values were then used to calculate a ratio of hybrid and parental expression values compared to the MPV for each array feature. A ratio of −0.33 indicates additive gene expression, whilst ratios below −1 or above 1 represent expression in the hybrid outside the range of either parent. Statistical analysis of differentially expressed genes from our previous microarray experiment (Hegarty et al. 2005) showed that,

for both hybrids, the median ratio was significantly different from −0.33, allowing us to reject the null hypothesis of largely additive gene expression changes. Instead, for both hybrids, the majority of the data were skewed towards one of the parents; in the case of *S. x baxteri*, expression was skewed towards that of the lower expressing parent, whereas in *S. cambrensis* it was skewed towards that of the higher expressing parent. Further analysis of the data showed that for both hybrids *S. vulgaris* was the lower expressing parent in 70 % of cases. Expression outside the parental range was observed in a substantial proportion of cases in both hybrids: 7.42 % in *S. x baxteri* and 3.03 % *S. cambrensis* (Hegarty et al. 2008).

Having identified a pool of cDNA clones displaying non-additive changes to gene expression in both hybrid taxa, we then tested these clones for evidence of expression beyond the parental ranges, i.e. transgressive gene expression. In *S. x baxteri*, 80.4 % of non-additively expressed clones differed from the MPV by >1.5-fold, with 42.2 % of clones in *S. cambrensis* showing the same effect. Within both of these groups, the majority of cases involved upregulation compared with the MPV (66.9 and 70.4 % in *S. x baxteri* and *S. cambrensis*, respectively). Aside from the genes for which no functional class could be ascribed (49.2 %), the major functional groups affected in *S. x baxteri* were genes involved in development (6.6 %), nucleotide binding (6.1 %), mitochondrial activity (4.76 %) and cell wall function (3.97 %). Within the development category, a high proportion of clones (32 %) were found to encode tubulins, profilins or senescence-associated proteins. Of the clones involved in nucleotide binding, 34 % were transcription factors. In *S. cambrensis*, the majority of clones (58 %) could not be assigned to a functional category. Of the remainder, the largest categories were defense (11.1 %) and cell wall-related genes (6.17 %).

Our reanalysis of the microarray data therefore revealed a relatively high proportion of non-additive gene expression change in the hybrids relative to their parental expression levels (Hegarty et al. 2008). In addition, the degree of non-additive gene expression was lower in allohexaploid *S. cambrensis* compared with its triploid intermediate *S. x baxteri*. This finding was consistent with our previous observation that the "transcriptome shock" resulting from allopolyploidization is largely due to hybridization, with polyploidization resulting in a distinct secondary shift (amelioration) in gene expression (Hegarty et al. 2006). The fairly diverse nature of the genes affected was consistent with other findings in *Arabidopsis* (Wang et al. 2006b), cotton (Adams et al. 2004) and maize (Stupar et al. 2007) that non-additive changes to gene expression are genome-wide.

Interestingly, similar functional classes of genes were affected by hybridization in *Senecio*, *Arabidopsis* and maize (Fig. 13.4), suggesting that certain gene networks may be particularly susceptible to perturbation by hybridization; the functional categories of nucleotide binding, defense and mitochondria being good examples. In terms of the classes of genes affected in *Senecio*, it is also noteworthy that one of the major affected groups in *S. x baxteri* was nucleotide binding. In addition to a number of (primarily down-regulated) transcription factors that have shown similar alterations in expression pattern in the polyploid *Arabidopsis suecica* (Wang et al. 2006b), this category also contained clones encoding cytidine

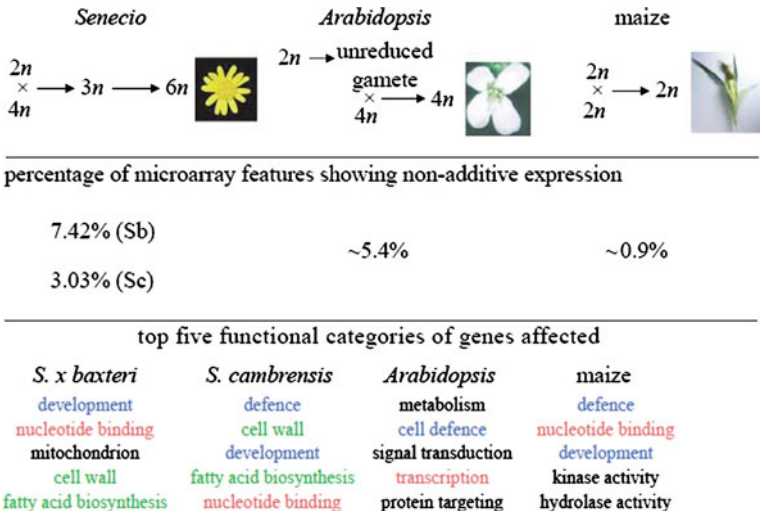

Fig. 13.4 Comparison of non-additive expression changes resulting from hybridization in maize and hybridization/polyploidy in *Senecio* and *Arabidopsis*. The formation of the hybrids is shown in each case, together with the level of non-additive gene expression in the hybrids expressed as a percentage of the features on the microarray platform used. Finally, the top five functional gene classes affected (ignoring unknowns) for each hybrid are displayed for comparison. *Red* indicates a functional gene class affected in all four hybrid systems, *blue* indicates a functional class affected in at least one of the *Senecio* hybrids and one of the other two hybrid taxa, and *green* indicates a functional class affected in both *Senecio* hybrids but not in either *Arabidopsis* or maize. For *Arabidopsis suecica*, gene function data were taken from Wang et al. (2006b), and for maize from an extrapolation of the supplementary data given in Stupar et al. (2007). Sb, *S. x baxteri*; Sc, *S. cambrensis*. Reproduced from Hegarty et al. (2008)

deaminase (CDA) and 8-oxoG-DNA glycosylase (OGG1). OGG1 has been implicated in DNA base excision repair (García-Ortiz et al. 2001), while CDA has been suspected to be involved in RNA editing, although it now appears that pentatricopeptide repeat proteins that contain CDA-like domains are the more likely candidates (Salone et al. 2007). These genes were of interest, given that we also observed a relatively high number of clones encoding proteins involved in either DNA modification or cell division. In addition to cytidine deaminase and OGG1 (both of which were upregulated compared with the parental midpoint in *S. x baxteri*), there was also up-regulation of adenosylhomocysteinase and adenosyl kinase, the genes involved in S-adenosylmethionine (SAM) dependent methylation (Moffatt et al. 2002; Mull et al. 2006). The expression of SAM synthetase was also increased relative to both parents. SAM-dependent methylation is used in gene silencing and also in pectin methylation (Pereira et al. 2006), and, indeed, we observed an increase in the expression of pectin methylesterase as well as another SAM-dependent enzyme, caffeic acid 3-O-methyltransferase (Hegarty et al. 2008).

It is clear from these analyses that hybridization and polyploidy have separate, distinct effects on gene expression in *Senecio*. These changes in gene expression

affect a wide spectrum of transcripts from a number of functional groups and are non-additive in nature. A good proportion of these changes are also transgressive—outside the range of either parent species—representing a possible source of heterotic success in interspecific hybrids. To investigate possible mechanisms for these changes in gene expression, we focused our attention on epigenetic modification in *Senecio* hybrids.

13.2.2 Epigenetic Modification

While we were unable to demonstrate complete silencing of any clones in our microarray analyses, it is important to note that the cDNA-based microarrays used could not distinguish between different parental homeologues. Therefore, in the cases where a hybrid showed down-regulation of a gene relative to its parental taxa, it may have been due to either homeologue loss or silencing as a consequence of DNA methylation. Both phenomena were observed using cleaved amplified polymorphic sequence (CAPS) analysis in natural and resynthesised allotetraploid *Tragopogon miscellus* (Buggs et al. 2009) and suggested that homeologue loss was not an immediate consequence of hybridization or polyploidy but occurs slightly later due to recombinational events. Silencing was also not observed to occur immediately in *Tragopogon* synthetics, but observations in natural populations suggest that silencing of one homoeologue is more prevalent than sequence loss. Studies in other allopolyploid systems such as wheat, *Arabidopsis* and *Spartina* have demonstrated that methylation status in F1 hybrids and first-generation allopolyploids displays similar changes to those observed for gene expression. To determine whether methylation in *Senecio x baxteri* and *S. cambrensis* was affected in a similar manner, we undertook a methylation-sensitive AFLP (MSAP) analysis of the parental taxa, three triploid lines and their S_0–S_1 allohexaploid derivatives (Hegarty et al. 2011). The MSAP technique (Xiong et al. 1999) involves digestion of genomic DNA with a standard, rare-cutter enzyme (i.e., *Eco*RI) and one of a pair of isoschizomeric enzymes that share a restriction site but are either sensitive or insensitive to cytosine methylation. In this case, we employed *Hpa*II, which is sensitive to methylation of either cytosine in its recognition site (CCGG) and *Msp*I, which is sensitive only to methylation of the external cytosine. Comparing the two AFLP profiles produced from each isoschizomer enables identification of the methylation status of a given locus, as described in Table 13.2. Five selective primer combinations were used to screen 33 plants as detailed in Table 13.3.

We successfully amplified MSAP products from all 33 individuals and obtained a total of 408 reliable MSAP loci, which were then surveyed to determine levels of non-additivity in the hybrids. Of the 408 loci, 264 (64.7 %) showed polymorphisms between the parental taxa *S. squalidus* and *S. vulgaris*. In the remaining 144 loci that were monomorphic in the parents, 75.7 % were monomorphic across all hybrid samples tested. Surveying the polymorphic loci, it was found that the

Table 13.2 HpaII% MspI banding patterns and locus methylation state (reproduced from Hegarty et al, 2011)

HpaII	MspI	Methylation status
+	-	CCGG (unmethylated)
-	+	C^{m}**C**GG (methylation of internal cytosine)
-	-	^{m}**C**CGG or ^{m}**C**^{m}**C**GG (methylation of external cytosine)
+	-	^{hm}**C**CGG (hemimethylation of external cytosine)

Note: In the case of methylation of the external cytosine, it is not possible from MSAP alone to determine whether the internal cytosine is also methylated

Table 13.3 Oligonucleotides for methylation-sensitive AFLP and number of loci (reproduced from Hegarty et al, 2011)

Oligo	Sequence (5'-3'	Primer Combination	# Loci
EcoRI adaptor 1	CTCGTAGACTGCGTACC	Eco+AGC/Hpa+CTG	118
EcoRI adaptor 2	AATTGGTACGCAGTC	Eco+AAC/Hpa+CTT	88
HpaII adaptor 1	GATCATGAGTCCTGCT	Eco+AGC/Hpa+CTT	80
HpaII adaptor 2	CGAGCAGGACTCATGA	Eco+AAC/Hpa+AAG	Failed
Eco+A	GACTGCGTACCAATTCA	Eco+ACG/Hpa+AAG	69
Hpa	ATCATGAGTCCTGCTCGG	Eco+AAC/Hpa+CTT	53
Eco+AAC (NED)	GACTGCGTACCAATTCAAC		
Eco+ACG (FAM)	GACTGCGTACCAATTCACG		
Hpa+CTG	ATCATGAGTCCTGCTCGGCTG		
Hpa+CTT	ATCATGAGTCCTGCTCGGCTT		
Hpa+AAG	ATCATGAGTCCTGCTCGGAAG		

triploid hybrids each displayed similar overall methylation patterns (Table 13.4), with a strong bias in favour of the *S. vulgaris* (maternal) methylation state for each locus (an average of 57.4 % of loci between the three triploid lines). In addition, the triploids also displayed approximately equivalent levels of non-additive methylation (13.4 % on average). To determine whether methylation patterns were maintained following genome duplication, the methylation status of the S_0 allohexaploids was compared to the triploid lines from which they were derived. In the vast majority of cases, the synthetic allohexaploids retained the methylation state observed in the triploid. On average, 78.2 % of these cases involved additive methylation, whereas 9.7 % of loci involved retention of a non-additive methylation profile. An average of 10.1 % of loci displayed a shift relative to the triploid in the specific parental methylation state favoured (4.5 % shift to *S. squalidus*, 3.8 % shift to *S. vulgaris*, 1.8 % shift to both). Finally, an average of 2 % of loci showed novel non-additive methylation not observed in the triploids.

The analysis was then extended to the S_1 allohexaploids, comparing them to both their triploid ancestors and the preceding S_0 generation. As with the S_0 allohexaploids, overall methylation state was highly similar among the three lines. Again, the most common result was retention of methylation status compared to both the triploid and the S_0 allohexaploid, with an average of 70.3 % of loci showing retention of additive methylation patterns and 7.3 % of non-additive

Table 13.4 Summarised methylation status of hybrid lines (reproduced from Hegarty et al. 2011)

Methylation	Percentage of Loci (triploids)		
State	Line 1	Line 2	Line 3
Additive (SS)	24.58%	24.58%	22.26%
Additive (SV)	56.15%	57.14%	58.80%
Additive (monomorphic)	4.65%	6.98%	4.65%
Nonadditive	14.62%	11.30%	14.29%
	Percentage of Loci (S_0 allohexaploids)		
Same as triploid (additive)	76.17%	78.86%	79.53%
Same as triploid (nonadditive)	9.73%	9.73%	9.73%
Differs from triploid (additive SS)	5.03%	4.36%	4.03%
Differs from triploid (additive SV)	5.37%	3.69%	2.35%
Differs from triploid (monomorphic)	2.01%	0.67%	2.68%
Novel nonadditive methylation	1.68%	2.68%	1.68%
	Percentage of Loci (S_1 allohexaploids)		
Same as triploid+S_0 (additive)	72.06%	72.38%	66.35%
Same as triploid+S_0 (nonadditive)	7.62%	7.94%	6.35%
Same as S_0 not triploid (additive)	7.62%	5.71%	3.49%
Same as S_0 not triploid (nonadditive)	0.32%	1.27%	0.63%
Same as triploid not S_0 (additive)	3.81%	3.17%	1.90%
Same as triploid not S_0 (nonadditive)	0.95%	0.00%	2.54%
Differs from triploid+S_0 (additive SS)	2.54%	4.76%	7.62%
Differs from triploid+S_0 (additive SV)	3.49%	2.22%	7.62%
Novel nonadditive methylation	1.59%	2.54%	3.49%

Note: SS = *S. squalidus*, SV = *S. vulgaris*. "Monomorphic" refers to loci with a common methlyation state in the parents, rather than across hybrid lines

methylation. A further 5.6 % of loci showed retention of additive methylation in the two allohexaploid generations, whereas the triploid had displayed non-additivity. Methylation status was not always consistent between the two allohexaploid generations; however, in 4.1 % of cases, loci displayed a shift in methylation state to that seen in the triploid but not in the S_0 allohexaploid. Furthermore, the S_1 generation showed a return to additivity in 9.4 % of cases, where both the triploid and the S_0 allohexaploids had been non-additive. As with the S_0 generation, though, there was a small (2.5 % average) degree of novel non-additivity.

The results were in accordance with our previous studies of gene expression (Hegarty et al. 2006, 2008): we found that, while cytosine methylation in both hybrid taxa was largely additive between the two parental patterns, a significant degree of non-additivity also exists. Overall methylation status was well conserved between different hybrid lines; while individual loci displayed differences, the global percentages of different methylation states were highly similar between lines (Hegarty et al. 2011). In all three triploid lines, approximately 13.4 % of loci showed non-additive methylation, although the precise type of methylation was not identical between lines in all cases. Levels of non-additive methylation observed in other allopolyploid systems are variable: 8.3 % in *Arabidopsis*

(Madlung et al. 2002), 9 % in *Brassica* (Gaeta et al. 2007), 13–20 % in wheat (Dong et al. 2005; Pumphrey et al. 2009) and as high as 30 % in *Spartina* (Salmon et al. 2005). It has been speculated that the higher genome copy number in wheat and *Spartina* might explain their greater levels of methylation, although Doyle et al. (2008) point out that both species are monocots, which tend to possess a higher GC content (and thus greater potential for methylation) than eudicots. The fact that conserved methylation changes between the *Senecio* hybrids are more on a par with the levels seen in *Arabidopsis* and *Brassica* suggests that this latter hypothesis may be correct, as *S. cambrensis* exhibits the same ploidy as wheat. We should note, however, that the wheat genome is significantly larger than that of *Senecio* (2C genome sizes of 33.96 Gbp wheat; 5.05 Gbp *S. cambrensis*) and is known to contain a significant amount of repetitive DNA including large numbers of retroelements. It is therefore probable that alterations to methylation are more necessary to prevent widespread activation of these genetic regions in wheat and similar polyploid monocots. Indeed, studies of methylation in *Spartina* (Parisod et al. 2010) showed that such changes frequently occur in the vicinity of transposable elements and, perhaps as a result, no transposition burst was detected in the *Spartina* hybrids analysed. Methylation change therefore appears to play a frequent role in genome mergers, but there are exceptions: despite significant differential gene expression in the allotetraploid *Gossypium hirsutum*, almost no differences in methylation could be observed between the hybrid and its parental taxa (Liu et al. 2001) nor do the parental genomes undergo any significant rearrangement. In this situation, it appears that subfunctionalization of the two genomes is the primary cause of phenotypic variation (Adams et al. 2004; Liu and Adams 2007), although a recent study by Chaudhary et al. (2009) demonstrated that neofunctionalization and divergence in parental *cis*-regulatory sequences also play a significant role. Exactly what factors determine the response of the parental genomes to hybridization are largely unknown, although the degree of parental divergence is speculated to play a large role (Chapman and Burke 2007; Buggs et al. 2008; Paun et al. 2009).

In further accordance with our expression analyses, we observed that non-additive methylation in *S. x baxteri* triploids was maintained, on average, in only 73.6 and 55.6 % of cases in the S_0 and S_1 allohexaploids, respectively (Hegarty et al. 2011). In approximately 73 % of cases observed in our microarray studies, the resynthesised allohexaploid lines (S_0–S_4) displayed either an immediate or gradual shift towards an expression pattern similar to that of wild *S. cambrensis* (Hegarty et al. 2006). It seems likely, therefore, that our previous observation that non-additivity results from hybridization but can be partially reduced by genome duplication, also holds true when applied to DNA methylation. This again matches observations from MSAP analysis of *Spartina* allopolyploids (Salmon et al. 2005), which showed that the allopolyploid *S. anglica* retained 71.4 % of the non-additive methylation patterns observed in the F_1 hybrid. These findings were again confirmed when assessing methylation changes associated with transposable elements (Parisod et al. 2010), with the additional observation that many of the changes seen in the F_1 hybrid involved loss of parental markers (usually in the maternal

genome), indicating that such changes involved structural rearrangements to the parental genomes. A similar process may be at play in *S. x baxteri*, because MSAP markers also detect structural changes: indeed, wild populations of *S. cambrensis* show evidence of intergenomic recombination (Abbott et al. 2007, and above), again favouring the *S. vulgaris* genome as with our triploid lines here. By contrast, most of the differences observed in allopolyploid *Spartina* involved alterations to methylation status, rather than structural changes. Similar findings have also been identified in *Brassica*, where most of the methylation changes identified in the S_0 allotetraploid were maintained in S_5 lines, but with a number of revertants and novel changes present as well (Gaeta et al. 2007).

A key finding from our analysis was that the global patterns of DNA methylation change observed in our experiments strongly mirror the global changes in gene expression observed in our earlier microarray analyses, indicating a possible underlying causation. Whilst further investigation of specific loci showing methylation differences is required to make a definitive case, the similarities between changes in gene expression and DNA methylation are nevertheless striking. For example, we noted that a number of loci displayed novel non-additivity in both the S_0 and S_1 allohexaploids (2.01 % on average in the S_0 lines, 2.54 % in the S_1), again a point of consistency between the methylation study and our earlier microarray expression analysis (Hegarty et al. 2008). The overall level of non-additive methylation may therefore not actually decrease as a consequence of genome duplication, but instead the level of conserved methylation may be lessened. A proportion of loci also displayed unstable methylation patterns across generations in the hexaploids, with an average of 16.08 % of loci showing differences between the S_0 and S_1 lines (including the aforementioned novel non-additive methylation). Of these, the majority consisted of cases where the S_1 allohexaploids revert to additivity or favoured a different parental methylation state to the S_0 line. This reversion to an additive profile was also observed between the triploids and the S_0 plants and agreed with observations from the microarray data that wild *S. cambrensis* often showed an opposing expression pattern to *S. x baxteri*.

However, approximately one-quarter of loci also displayed a shift relative to the S_0 allohexaploids to favour the methylation state seen in the triploid. These findings suggest that the methylation state of some loci may vary as a consequence of segregation. This may explain the novel changes observed by Gaeta et al. (2007) in their S_5 allopolyploids of *Brassica napus*. Similarly, an analysis of natural populations of the allopolyploid *Tragopogon miscellus* (Buggs et al. 2009), where hybridization occurred at least 40 generations ago, identified a random loss of one parental homoeologue at a rate of 3.2 % across 10 loci in 57 natural hybrids from five populations. In addition, a further 6.8 % of loci showed evidence of gene silencing in one parental copy. The loci lost/silenced were not consistent across populations or individuals, although within populations, there was some conservation in the loci affected. Whilst Buggs et al. (2009) did not note any homoeologue loss/silencing in resynthesised S_0 hybrids, the variability in silencing after such an extended period of time since hybrid formation suggests that

independently formed hybrids can still display significant levels of epigenetic variation. This study, as well as that of Gaeta et al. (2007), was based on a survey of a limited number of loci using cDNA-AFLP or CAPS assay. A later study by Buggs et al. (2011) used the Sequenom MassARRAY allelotyping technology to survey a much larger set of loci in *Tragopogon miscellus* and confirmed that natural hybrids displayed altered patterns of tissue-specific gene expression, whilst resynthesised hybrids demonstrated relaxed control of tissue-specificity. This latter finding suggests a possible mechanism for the 'transcriptome shock' effect we observed in *Senecio x baxteri* and *S. cambrensis*, resulting from a loss of tissue-specific expression patterns seen in the parent taxa. Further work will be needed to confirm if this is the case in *Senecio*. The Sequenom assay used by Buggs et al. (2011) shows the benefits of new molecular tools for studies of allopolyploid systems: with the advent of new technologies for global analysis of DNA methylation such as MSAP and next-generation sequencing (Salmon and Ainouche 2010), it would also be interesting to analyse our later-generation allohexaploid derivatives at a global scale to investigate the longer term changes in methylation as hybrid genomes undergo recombination and adaptation. Such studies may therefore provide further insights into which epigenetic changes are mandated by hybridity, and which may vary between populations and serve as a source of novelty upon which selection may act.

13.2.3 Phenotypic Change

Senecio x baxteri F_1 hybrids generated by crossing *S. vulgaris*, as female, with *S. squalidus* were all self-sterile in contrast to previous studies which reported some self-fertility (Crisp 1972; Ingram 1978). Treating shoots of *S. x baxteri* plants with colchicine produced 'chimeric' plants with allohexaploid branches that produced flower heads that were fully self-fertile. Seed from these flower heads was then used to found the synthetic *S. cambrensis* lines (S_0–S_5) used in the transcriptomic and epigenetic analyses described above. The first wholly allohexaploid plants generated from this seed (S_0 lines), and their progeny (S_1 lines), showed similar ray flower structure and self-compatibility (Hiscock and Hegarty, unpublished). However, in subsequent lines, from the S_2 onwards, variation in ray flower form was detected between individuals within and between the nine independent lineages of synthetic *S. cambrensis*. Some individuals were observed with no ray flowers, some had short partially tubular ray flowers, while in others ray flowers were observed of different lengths and number (Fig. 13.5.) Observations on the progeny of these different individuals showed that these various forms of ray flower are heritable (Hiscock, unpublished). Comparable variation in *S. cambrensis* ray flower form was previously attributed to intergenomic recombination (Ingram and Noltie 1984), but here we suggest another possibility for such abrupt changes to ray flower phenotype—epigenetic effects associated with the epigenetic instability observed in early-generation synthetic *S. cambrensis*

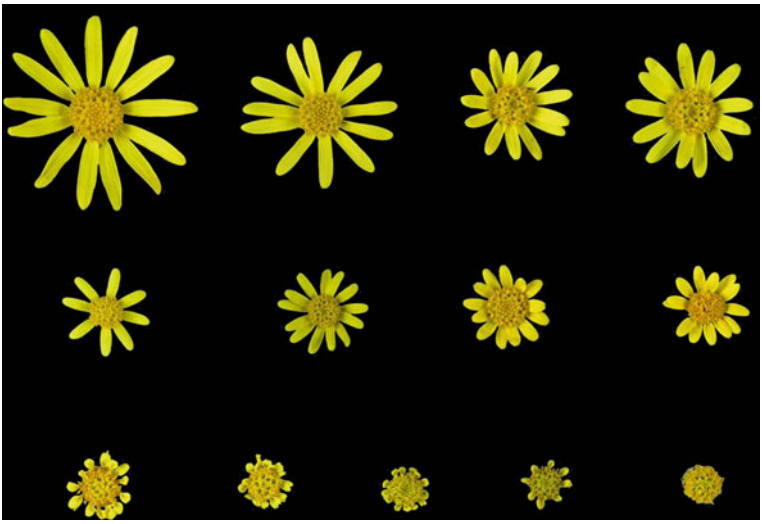

Fig. 13.5 Variation in ray flower morphology observed in individuals of synthetic lines of allohexaploid *S. cambrensis* from the S2 to S5 generation

(Hegarty et al. 2011). Genes shown to be involved in ray flower development in *Senecio* (*RAY1* and *RAY2*) are orthologues of *CYCLOIDEA* (*CYC*) (Kim et al. 2008), which has been shown to occur as a stable mutant (hypermethylated) epiallele that in *Linaria* (toadflax, Plantaginaceae) manifests itself in a change in flower symmetry from bilateral to radial symmetry (Cubas et al. 1999). We therefore hypothesise that the observed changes in ray flower form may in part be associated with epigenetic modifications to *RAY1* and/or *RAY2*. Testing this hypothesis will be a focus for future work.

Another unexpected observation in the synthetic *S. cambrensis* lines was the appearance of self-sterile individuals in otherwise SC lines, again from the S_2 generation onwards. Subsequent analyses using controlled self- and cross-pollinations confirmed that these self-sterile individuals possessed functional sporophytic self-incompatibility (Brennan and Hiscock 2010). This is the first time that sporophytic SI has been shown to be inherited and expressed in an allopolyploid and raises intriguing questions about the mechanism regulating this important trait. All S_0 and S_1 lines of synthetic *S. cambrensis* were highly self-fertile (SC) indicating that the SI system, present in parental *S. squalidus*, was repressed in these allopolyploids, only to be reactivated/derepressed later. The emergence of SI individuals may be a consequence of recombination or might also be associated with epigenetic changes observed in the early-generation synthetic allopolyploids. Most observations of wild *S. cambrensis* have reported it to be SC (Abbott and Lowe 2004), but the finding of SI in synthetic *S. cambrensis* prompted a re-examination of the mating system of wild *S. cambrensis.* An analysis of selfing rates in 41 wild *S. cambrensis* individuals from Edinburgh and North Wales

identified five SI individuals (Brennan and Hiscock 2010) implying, albeit from a relatively small sample size, that SI may be present in wild *S. cambrensis* at a frequency of ~12 %. This important finding means that *S. cambrensis* should now be considered as possessing a mixed mating system that has the potential to evolve towards either outcrossing or selfing.

13.3 Future Prospects

13.3.1 Next-Generation Approaches to Studying Evolution in S. cambrensis

We are currently engaged in the generation of a draft reference sequence of the *Senecio squalidus* "gene-space" (low-copy, non-methylated regions of the genome). Next-generation sequencing (NGS) platforms enable a variety of potential experiments to examine the consequences of polyploidy and hybridization in *Senecio* further. For example, we intend to identify promoter regions using chromatin-immunoprecipitation sequencing (ChIPseq) to enrich for DNA fragments bound by enzymes involved in transcription. We can then determine whether hybrids and polyploids display alterations in promoter sequence/binding that may explain the altered patterns of expression observed in our microarray experiments. Further, once a reference sequence is available, we can consider bisulphite sequencing to identify genomic regions which show differential methylation in hybrids relative to their progenitors. One key target for bisulphite sequencing will be the *RAY1* and *RAY2* genes (Kim et al. 2009) which we suspect may show differential methylation associated with the observed variation in ray flower morphology that appears in synthetic *S. cambrensis* lines. Identification of small interfering RNAs (siRNAs) and their targets will enable analysis of changes to epigenetic regulation of gene expression in hybrids. At the structural level, resequencing of hybrid "gene-space" and comparative sequence analysis may allow us to detect genomic rearrangements and sequence loss, plus the activity of transposable elements. The increasing capability of genotyping-by-sequencing (GBS) approaches such as restriction-associated DNA (RAD) sequencing (Baird et al. 2008) may also prove useful in detecting structural rearrangements in hybrid genomes. Finally, comparative sequencing of *Senecio cambrensis* and the two other hybrid derivatives of *S. vulgaris* and *S. squalidus*, i.e. *S. vulgaris* var. *hibernicus* and *S. eboracensis,* may enable identification of dosage effects, since the hybrids share parental genomes but differ in the specific combinations thereof.

13.3.2 S. cambrensis in the Wild

Senecio cambrensis now exists in the wild only in North Wales, UK, following extinction of the Edinburgh population in 1993 (Abbott and Forbes 2002).

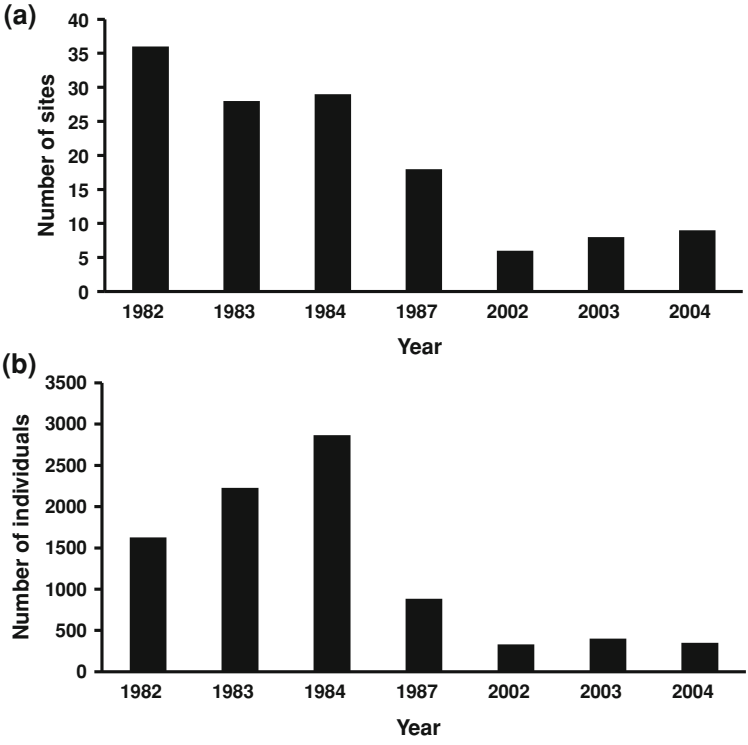

Fig. 13.6 Decline in the number of **a** sites and **b** flowering individuals across sites, recorded for *S. cambrensis* in North Wales in different years from 1982 to 2004. Records are from Abbott et al. (2007) and were made in June each year except in 1987 when they were collected in May

Although the species was recorded at numerous sites and in relatively high numbers in North Wales in the early 1980s (Ingram and Noltie 1995; Fig. 13.6), it has undergone a significant decline in this region since then. In the most recent census of the species undertaken in 2004, it was found at only nine sites in North Wales with a total of 349 individuals recorded across all sites (Abbott et al. 2007; Fig. 13.6). It has been speculated that this decline in numbers has been caused by a reduction of available sites for colonisation by the species (e.g., waste ground) and also to increased use of herbicide to control weed populations (Abbott et al. 2007). Its disappearance from the thin layer of soil that collects along roads between the road edge and verge, where it was found often in the past, is most likely due to increased use of herbicides on plants growing along road sides. However, it has also been noted that plants of the species are frequently heavily infected with the rust *Puccinia lagenophorae*. Although this rust also infects both parent species, it is possible that its effects on *S. cambrensis* are more dramatic in terms of production of offspring for colonising new sites, given that numbers of *S. squalidus* and *S. vulgaris* that reproduce each year in North Wales and elsewhere in the UK are vast relative to those of *S. cambrensis*. Although no detailed analysis has been

undertaken on whether *S. cambrensis* is ecologically divergent from its two parents, it tends to grow in sympatry with one or both parent species in the wild. Thus, there is likely competition between the three species for occupation of available open sites, and this might place *S. cambrensis* at a disadvantage if the seed it produces is less numerous relative to that of *S. vulgaris* and/or *S. squalidus* occurring in the same area. Whatever the cause of its marked decline in numbers in North Wales over the last 25 years or so, it is clear that *S. cambrensis* has reached the stage where its presence in the wild is endangered and that a conservation plan is required to prevent it from possibly becoming extinct in the near future.

Given the decline in numbers of *S. cambrensis* in recent years it is important to gain a better understanding of the nature of its mating system and mating dynamics, especially in the light of our findings of SI in wild populations (Brennan and Hiscock 2010). If there has been a recent shift in mating system from predominantly SC towards SI, it is possible that mating potential has become compromised due to obligate outcrossing. Given the possibility of a single hybrid origin for *S. cambrensis* in Wales, it is likely that wild populations possess very few *S* alleles. Whilst a small number of shared *S* alleles are not necessarily a problem when populations are large, it becomes a problem when populations are in decline and when stochastic effects can lead to *S* allele loss (Brennan et al. 2003; Pickup and Young 2008). This can then exacerbate decline, leading to an uncontrollable spiral of extinction. A reappraisal of the mating system of *S. cambrensis* is thus urgently needed.

Acknowledgments Our research on *Senecio cambrensis* and its parents has been supported by grants from the NERC, BBSRC and Leverhulme Trust to whom we are grateful. We would also like to acknowledge our long-term collaboration with Keith Edwards and the considerable efforts of former research students, technicians and postdocs who contributed to the research over the past 25 years. These include Alexandra Allen, Paul Ashton, Garry Barker, Tom Batstone, Adrian Brennan, Mark Chapman, David Forbes, Amanda Gillies, Helen Ireland, Juliet James, Joanna Jones, Andrew Lowe, David Tabah, and Ian Wilson.

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