REVIEW

A unified approach to the estimation and interpretation of resistance costs in plants

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Plants exhibit a number of adaptive defence traits that endow resistance to past and current abiotic and biotic stresses. It is generally accepted that these adaptations will incur a cost when plants are not challenged by the stress to which they have become adapted—the so-called 'cost of adaptation'. The need to minimise or account for allelic variation at other fitness-related loci (genetic background control) is frequently overlooked when assessing resistance costs associated with plant defence traits. We provide a synthesis of the various experimental protocols that accomplish this essential requirement. We also differentiate those methods that enable the identification of the trait-specific or mechanistic basis of costs (direct methods) from those that provide an estimate of the impact of costs by examining the evolutionary trajectories of resistance allele frequencies at the population level (indirect methods). The advantages and disadvantages for each proposed experimental design are discussed. We conclude that plant resistance systems provide an ideal model to address fundamental questions about the cost of adaptation to stress. We also propose some ways to expand the scope of future studies for further fundamental and applied insight into the significance of adaptation costs. *Heredity* advance online publication, 4 May 2011; doi:10.1038/hdy.2011.29

Introduction

Plants are continuously exposed to a suite of novel and ever-changing abiotic and biotic stresses. Local plant adaptive responses to these stresses may occur through the evolution of defence strategies. Plant defence mechanisms elicit constitutive and inducible traits that prevent or limit damage or mortality (resistance) and/or improve the ability to recover from damage (tolerance) (Restif and Koella, 2004; Núñez-Farfán *et al.*, 2007) (no distinction between resistance and tolerance to stresses will be made in this study unless otherwise stated).

Central to evolutionary biology, population genetics and plant physiology is the principle that novel adaptive defence traits are not cost free (Herms and Mattson, 1992; Bergelson and Purrington, 1996). Evolved resistance alleles, by their nature, endow a fitness advantage over the wild allele in the presence of the stress. However, it is argued that resistance alleles will have pleiotropic fitness costs when the stress factor is removed from the environment—the so-called 'cost of resistance'. Pleiotropic costs of resistance may pose limits on evolution by natural selection and their likely existence is based on observed polymorphism at alleles for pathogen, herbivore and herbicide resistance in natural and agricultural

systems (Fritz and Simms, 1992; Preston and Powles, 2002; Neve and Powles, 2005).

A wealth of studies have sought to test the resistance cost hypothesis for adaptation to herbicide, herbivore and pathogen pressure (see reviews by Bergelson and Purrington, 1996; Coustau *et al.*, 2000; Purrington, 2000; Strauss *et al.*, 2002; Burdon and Thrall, 2003; Vila-Aiub *et al.*, 2009a, b). These studies have acknowledged the importance of resistance costs in predicting the spread of plant resistance alleles and determining their population equilibrium frequencies under a range of environmental conditions. A better understanding of the cost/benefit trade-offs of resistant and susceptible alleles has practical implications for the management of crop resistance to diseases, insects and herbicides in modern agriculture. There are also benefits for transgenic crop technology in understanding the plant fitness consequences of defence-related resistance alleles (Brown, 2002; Neve, 2007; Green, 2009; Vila-Aiub *et al.*, 2005b, 2009a, b) and for minimising risks of transgene dissemination into the wild flora (Ellstrand *et al.*, 1999).

There are inherent difficulties in measuring the expression and magnitude of costs of resistance/tolerance defence genes, meaning that the choice of experimental design and plant material is critical. Control of genetic background is essential to unequivocally ascribe fitness costs to those genes that endow resistance (Bergelson and Purrington, 1996). By controlling the genetic background, the genetic variability between resistant and susceptible genotypes is restricted, so that only the effect of the allelic substitution at the resistance locus on plant fitness traits is assessed. This applies for

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the effect of traits derived of single allele substitutions at one locus, such as target-site herbicide resistance genes (Yu *et al.*, 2007) or allele replacements in multilocus system-based host–parasite resistance interactions (Thrall and Burdon, 2003). Where control of genetic background is not achieved, it is likely that differential fitness will result from polymorphisms at non-resistance alleles and from other effects caused by inbreeding depression, non-random mating and linkage disequilibrium processes (Marshall and Ellstrand, 1986; Falconer and Mackay, 1996; Keller and Waller, 2002).

Despite its importance, the lack of genetic background control has been a major obstacle to interpretation of studies attempting to define the cost of plant resistance to environmental stresses (Bergelson and Purrington, 1996; Coustau *et al.*, 2000; Strauss *et al.*, 2002; Vila-Aiub *et al.*, 2009a, b). Analyses have revealed that 50 and 25% of studies evaluating resistance costs associated with herbivory (Strauss *et al.*, 2002) and herbicides (Vila-Aiub *et al.*, 2009a, b), respectively, have not complied with this essential experimental condition.

In order to estimate the expression and magnitude of resistance costs, comparison of fitness traits has been repeatedly carried out by comparing *R* and *S* phenotypes from distinct plant populations ('between 2 populations' approach). Although the 'between 2 populations' approach has been the most widely used method found in the last four decades of the literature, it presents the greatest limitations when assessing resistance costs (Bergelson and Purrington, 1996; Strauss *et al.*, 2002; Vila-Aiub *et al.*, 2009a, b). For studies assessing costs associated with herbicide resistance alleles, the 'between 2 populations' approach comprises *c.* 41% of the examined literature (Supplementary Table S1). The major and fundamental limitation of this methodology is that putative costs of resistance may be artefacts, as phenotypes originating from different geographical locations or environments will likely have been selected for different fitness traits and this would impede any valid fitness comparison between them in a common environment (Bergelson and Purrington, 1996; Strauss *et al.*, 2002; Vila-Aiub *et al.*, 2009a, b). Furthermore, inbreeding depression, non-random mating and linkage disequilibrium may also be present in field-collected *R* and *S* populations resulting in other confounding effects (Marshall and Ellstrand, 1986; Keller and Waller, 2002).

The motivation for the present contribution is to provide a synthesis of the various experimental designs and techniques available to account for allelic differences at loci other than those conferring resistance and tolerance. In particular, we will focus on those methods that (i) examine resistance costs within a random homogenous genetic background, (ii) quantify the effects of different genetic backgrounds on costs and (iii) eliminate or reduce the effects of genetic background on costs. We will also show that experimental methods can be distinguished on the basis of whether they are able to determine the trait-specific or mechanistic basis of costs (that is direct methods) or whether they provide an estimate of the impact of costs by examining the evolutionary trajectories of resistance allele frequencies at the population level in the absence of selection (that is indirect methods). The advantages and disadvantages for each proposed experimental design are discussed (Table 1).

For the particular case of herbicide resistance alleles in weed species, the extent to which each of the above methods have been used in the published literature is summarised in Table 1. Ninety-three experimental protocols contained in 90 published studies were identified in citation databases (Scopus (Elsevier, Amsterdam, the Netherlands) and Web of Science (Thomson Reuters, Philadelphia, PA, USA)) (Supplementary Table S1).

Resistance to pathogens and herbicides represents good models to study costs of resistance, as the molecular and the genetic basis of resistance is often known and, therefore, most recent methodological advances have been made in studying these traits. Our review will mainly be illustrated by studies in these research areas. Finally, we propose a number of research areas that require further understanding and evaluation in the context of costs associated with plant adaptive defence traits.

Methodological approaches to control for genetic background

Direct methods: comparison of traits

Direct methods enable the identification of the modified plant traits that result in the expression of resistance costs. For instance, the expression of costs associated with the chloroplastic *psbA* gene mutation (Ser-264–Gly) leading to triazine herbicide resistance in many weed species has been shown to originate from limited electron transfer within the PSII complex. This leads to reduced photosynthesis, lower growth rates, less competitive ability and reduced seed production (reviewed by Holt and Thill, 1994).

The most prevalent experimental design in the literature to assess resistance costs involves the comparison of plant survival and growth traits between resistant (*R*) and susceptible (*S*) genotypes (Bergelson and Purrington, 1996; Strauss *et al.*, 2002; Vila-Aiub *et al.*, 2009a, b). Biomass allocation to vegetative and reproductive organs has often been regarded as the 'common currency' to compare relative fitness between *R* and *S* genotypes. These comparisons enable the identification of life history stages in which resistance costs are expressed (direct methods), and this knowledge can be useful for the management of resistance in human-related activities, such as agriculture (herbicide-resistant weeds and disease resistance in crops).

Assessing costs within a random homogenous genetic background: *Discrete* R *and* S *phenotypes from within single populations:* Recently evolved resistance traits will rarely be fixed in plant populations. Therefore, providing that some random mating between *R* and *S* phenotypes has taken place under relaxed selection, *R* and *S* phenotypes will exist in a homogenous genetic background in a single population. If suitable experimental protocols are available to phenotype *R* and *S* individuals within these populations (Vila-Aiub *et al.*, 2005b), then these individuals can be grown to maturity, so that *R* and *S* seed can be collected for subsequent experiments.

A number of recent studies evaluating resistance costs associated with target and non-target-site herbicide

Table 1 Summary of the experimental protocols that enable to account for allelic differences at loci other than those conferring resistance among compared resistant and susceptible plant genotypes

Method	Direct methods							Indirect methods	
	Single population	Pedigreed lines	Segregating population	Multiple populations	Segregating cross	NILs	Transgenic lines	Multi-generational	Cline
(a) Prevalence[a]	4.5	1.0	18.2	18.2	2.0	4.3	2.0	7.5	1.0
(b) Advantages/disadvantages									
Mechanistic bases[b]	+	+	+	+	+	+	+	−	−
Fitness cost[c]	−	−	−	−	−	−	−	+	+
Time consumption (generation)	2	2	2–3	0	2–3	6–7	3–4[d]	5–6	1
Genetic background control[e]	+	+	+	++	++	+++	+++	[f]	[f]
Assumptions	No sub-population structure	Quantitative traits only		No population structure					
Genotyping and phenotyping cost[h]	+	+[g]	+	+	+	+	+	−	−
(c) Recommendations									
Model organism							x		x
Selfing species		x	x		x				x
Outcrossing species	x	?	x		x				x

Abbreviation: NILs, near-isogenic lines.
A number of advantages, disadvantages and recommendations are described for each protocol (for more details see text).
[a]Use (%) of experimental designs in published studies ($n = 93$) assessing pleiotropic effects associated with herbicide resistance alleles.
[b] '+' and '−' stand for methods that provide or not an understanding of the particular trait involved in the expression of cost, respectively.
[c]'+' and '−' stand for methods that evaluate or not the impact of costs on the evolutionary trajectories of resistance allele frequencies, respectively.
[d]It will depend on the reproductive system of the species.
[e]Methods that examine costs within a random homogenous genetic background (+), provide an understanding of the effects of different genetic backgrounds on costs (++) and eliminate the effects of genetic background (+++).
[f]The genetic background control for the indirect methods depends on the plant material used.
[g]Difficult to determine resistance genotype.
[h]'+' and '−' stand for low and high genotyping and phenotyping cost, respectively.

resistance alleles have employed this technique (Vila-Aiub et al., 2005a, b; Pedersen et al., 2007; Vila-Aiub et al., 2009a, b). In these studies, vegetative clones of individual plants were propagated and herbicide was applied to a single clone to enable individuals to be phenotyped as herbicide R or S. R and S plants were then individually bulk crossed to produce R and S seed populations. We have termed this the 'single population' approach (Vila-Aiub et al., 2005b).

A potential pitfall of the 'single population' protocol is that it is unable to discriminate between heterozygous (RS)- and homozygous (RR)-resistant individuals. In the case where RS plants are not associated with resistance costs (that is the cost of resistance is recessive; Roux et al., 2004), an underestimation of resistance costs associated with RR plants may occur. This drawback can be overcome by plant genotyping using allele-specific PCR techniques and thus enabling the identification of RS and RR plants (Délye et al., 2002).

Pedigreed lines: A range of possibilities exist for estimating costs of resistance based on the production of pedigreed seed populations. This method does not require any prior knowledge of the mechanism of resistance and is best suited to estimating costs of resistance traits that are inherited in a quantitative or semi-quantitative fashion. In selfing species, seeds collected from a single plant represent an inbred line. Then, once appropriate seed families (maternal lines)

have been produced, this method enables the quantification of costs through the statistical correlation between the resistance level of the plant families or phenotypes and any measured fitness trait.

Baucom and Mauricio (2004) used this approach to determine costs associated with increased glyphosate tolerance in maternal families of *Ipomoea purpurea*. They collected seed from 32 individual plants (grandmaternal lines), grew five seeds from each of these lines in a common garden and selfed these plants for one generation. Seeds were collected from each selfed plant and seeds from the five plants from each grandmaternal line were bulked to create 32 maternal lines. For each of these lines, mean glyphosate tolerance and mean fitness in the absence of glyphosate was determined. A clear trade-off between fitness costs and benefits was apparent: those individuals with higher glyphosate tolerance expressed the higher resistance costs. Similarly, the 'pedigree lines' approach has also enabled the identification of a trade-off between reproduction and herbivory defence traits in *Arabidopsis thaliana* inbred lines: those lines with higher trichome density and glucosinolate concentration have shown an impaired production of fruits (Mauricio, 1998).

In the above studies, allelic differences among different inbred or family lines were not completely accounted for. To reduce the impact of different genetic backgrounds on the expression of resistance costs, intermating among field-collected plants should have been required before production of maternal lines.

Controlled segregating populations: This protocol requires the formation of a segregating F_2 (or F_3) population resulting from either a single-parent mating (that is crossing of multiple paired homozygous *RR* plants with homozygous *SS* plants) (Jordan, 1996; Roux *et al*, 2004; Roux and Reboud, 2005) or from crosses among multiple parents (that is crossing several *RS* plants) (Menchari *et al*., 2008). As expected for crosses involving a greater control of genetic background, fitness costs have been more frequently reported in progeny originated from single-parent mating than when using crosses among multiple parents (Bergelson and Purrington, 1996). Despite of this, the use of single-parent mating has limitations, as it will depend on the extent to which costs are expressed in the selected genetic backgrounds.

The various 'segregating population' protocols have been employed in *c.* 20% of studies evaluating costs of herbicide resistance alleles (Table 1). For instance, an F_2 segregating *A. thaliana* population (single-parent mating approach) has been used to demonstrate a 37% recessive fitness cost (*RR* vs *RS = SS*) associated with the chlorsulfuron resistance endowing Pro-197–Ser acetolactate synthase mutation (Roux *et al*., 2004). Production of F_2 and F_3 lines has also enabled the assessment of the first documented ecological cost associated with a herbicide resistance allele: increased susceptibility to herbivorous insects associated with a chloroplastic *psbA* mutation in D1 protein of photosystem II (Gassmann, 2005).

An important attribute of the 'segregating population' approach is that it may enable the estimation of the dominance of the resistance cost (Carrière *et al*., 2001; Vacher *et al*., 2003; Roux *et al*., 2004), which, despite its importance in determining the initial spread and establishment of resistance alleles in populations, has seldom been estimated in plants (Roux *et al*., 2004; Menchari *et al*., 2008). However, the dominance of the cost can only be determined when it is possible to distinguish between *SS*, *RS* and *RR* genotypes in a segregating population by allele-specific PCR.

The control of genetic background effects achieved through the use of the 'segregating population' approach does not, however, completely overcome the linkage disequilibrium around the resistance gene. It is, therefore, possible that fitness costs may result from genetic variation at loci linked to the resistance gene in the two parents and not from the resistance allele itself.

Quantifying the effects of different genetic backgrounds on costs: *Field-collected* R *and* S *populations:* Comparison of multiple field-collected *R* and *S* populations provides some statistical power to account for the effect of differences in genetic background (Cousens *et al*., 1997; Strauss *et al*., 2002). This experimental design assumes that a statistically significant difference in mean trait values between compared *R* and *S* populations indicates that those differences are likely caused by pleiotropic effects of resistance gene(s) and not as a result of a type I error. This experimental approach was followed by Tardif *et al*. (2006), who identified a significant resistance cost correlated with the Trp-574-Leu acetolactate synthase mutation in six *Amaranthus powellii* populations. The resistance allele conferred pleiotropic effects on plant morphology and anatomy resulting in impaired resource acquisition in a comparison of six field-collected *R* and six *S* populations. For a proper comparison to be made, this method requires all resistant genotypes to have identical resistance alleles (that is Trp-574-Leu) as different alleles may be associated with different resistance costs (Roux *et al*., 2004; Vila-Aiub *et al*., 2009a, b).

While the multiple population approach does not control for differences in genetic background between *R* and *S* lines, one of its advantages is that helps determine the importance of different genetic backgrounds in influencing the fitness of resistance genes. This experimental protocol has been the second most widely used to estimate costs associated with herbicide resistance alleles (Table 1).

Multiple segregation crosses: Another method that enables the evaluation of the influence of the genetic background on variations in the magnitude and expression of resistance costs consists of pairing either the same resistant line (*RR* or resistant) with different *SS* (or susceptible) plants collected from different environments (locations, regions and so on) (Paris *et al*., 2008) or multiple *RS* plants for a particular resistance allele, but originated from different populations (Menchari *et al*., 2008). By analysing the same 2,4-D herbicide resistance mutation in eight different segregating genetic backgrounds, Paris *et al*. (2008) report that the fitness cost of a resistance gene is variable depending on the local genetic composition. The study provides evidence for the existence of compensatory genes that are segregating in different genetic backgrounds (see below Compensatory evolution of resistance costs).

The multiple segregation crosses method has seldom been used in the literature, although its significant power to detect the variable effect of genetic background on resistance costs (Table 1).

Eliminating the effects of genetic background on costs: *Introgression of resistance allele into a susceptible background: backcross (near-isogenic lines):* Isogenic lines, in which resistance alleles are expressed in a susceptible background, can be produced by crossing a single *R* and *S* plant followed by repeated backcrossing of *R* progeny into the *S* background. Introgression of the *R* allele is completed by selfing or crossing the resulting *RS* plants, in order to produce homozygote *RR* plants (the so-called 'near-isogenic lines (NILs)'), whose fitness is compared with the maternal *SS* line. It is rarely possible to produce lines that are entirely isogenic at all loci other than those conferring resistance. The production of 'NILs' aims to produce *RR* plants having a maximum percentage of genes of the susceptible genetic background. The degree to which this can be achieved is correlated with the number of generations of backcross.

Despite its ability to unambiguously identify resistance costs (Bergelson and Purrington, 1996), the NILs protocol has rarely been used (pathogen resistance: Heidel *et al*., 2004; Korves and Bergelson, 2004; Laughlin *et al*., 2009; herbicide resistance: McCloskey and Holt, 1990, 1991; Salzmann *et al*., 2008; Wang *et al*., 2010) (Table 1; Supplementary Table S1). Several plant generations are required to produce NILs in order to disrupt linkage disequilibrium between resistance and other genes. If, however, polymorphisms physically related to the resistance gene are still present after several $RS \times SS$ backcrosses, then these gene linkages may be

thought as genuine causes of resistance costs (Strauss *et al.*, 2002).

Introgression of resistance allele into a susceptible background: transgenic plants: The classical transgenic approach involves the introduction of a resistance gene *via* a vector into a susceptible genetic background in order to obtain plants whose genomes differ solely due to the presence of the studied resistance gene. Transgenic lines are usually backcrossed with the *S* wild type to obtain homozygous lines with and without the transgene. Any fitness differences between *S* and transgenic (*R*) lines may not, however, be entirely due to the effect of the resistance gene. The introduction of foreign DNA may modify the expression of the genome near the site of insertion and this may have fitness implications unrelated to expression of the resistance allele. Thus, several independent transgenic lines have to be generated to account for these positional effects (Bergelson *et al.*, 1996). Inclusion of marker genes, such as antibiotic resistance, as part of the transgene makes it possible to identify plants that have been successfully transformed. However, these linked marker genes may also impose their own pleiotropic fitness costs.

A small number of published studies have generated resistant transgenic plants and these studies have demonstrated the enormous potential of this technique for assessing resistance costs in a completely controlled genetic background (Bergelson *et al.*, 1996; Purrington and Bergelson, 1997, 1999; Burke and Rieseberg, 2003; Jackson *et al.*, 2004; Al-Ahmad *et al.*, 2005, 2006; Al-Ahmad and Gressel, 2006). After successfully controlling the effect of the vector plasmid and insertion position, Bergelson *et al.* (1996) demonstrated the expression of environmentally dependent negative pleiotropic effects on fecundity associated with the Pro-197–Ser acetolactate synthase herbicide resistance mutation in *A. thaliana*. Similarly, transgenic *A. thaliana* lines overexpressing cold resistance genes (CBF_{2-3}) have been shown to exhibit costs in cold-free environments (Jackson *et al.*, 2004).

The *Cre-lox* method makes it possible to eliminate the positional effect of the transgene on resistance cost expression (Tian *et al.*, 2003). The creation of *Cre-lox* lines has been described in detail elsewhere (Bayley *et al.*, 1992; Osborne *et al.*, 1995). Briefly, *Cre-lox* lines enable the insertion of resistance alleles into *S* backgrounds in constructs, where they are flanked by DNA sequences that enable subsequent recombinational excision of the *R* allele to produce *SS* and *RR Cre-lox* isolines. The *Cre-lox* protocol allows measurement of the fitness costs of the resistance gene only by comparison of individuals with the vector and transgene (*RR*) with individuals with the vector (inserted in the identical position), but without the transgene (*SS*). To date, the *Cre-lox* method has been used only once and has enabled the identification of a resistance cost associated with the pathogen resistance gene *RPM1* in *A. thaliana* in the absence of pathogens (Tian *et al.*, 2003).

Indirect methods: dynamics of resistance allele frequencies

Resistance costs may express in some but not all plant life history stages (Boege *et al.*, 2007; Vila-Aiub *et al.*, 2009a, b). As a result, pleiotropic effects of resistance genes should ideally be examined across all plant life history stages. A general approach that achieves this goal is the study of herbicide resistance allele frequencies in isolated or interconnected populations over a number of generations. This method relies on the expectation that a costly resistance allele will decrease in frequency over time, so that significant deviations of observed from expected resistance frequencies (allelic/genotypic/phenotypic) under no herbicide selection provides clear evidence for the expression and magnitude of resistance costs.

A limitation of indirect methods is that they require the genotyping (if the molecular genetic basis of resistance is known) or phenotyping of several hundreds or thousands of individuals in order to ascertain changes in resistance frequency. Where the allelic basis of resistance is known, the availability of high throughput genotyping methods makes these approaches possible (Giancola *et al.*, 2006).

As for direct methods, the validity of the results obtained from these approaches depends on proper control of genetic background. Unlike 'direct methods', these approaches do not allow the identification of the particular plant traits involved in the expression of costs of resistance. Despite its enormous power to detect resistance costs in insects and pathogens (Cochran, 1993; Bahri *et al.*, 2009; Li *et al.*, 2009), this experimental protocol has not proved to be popular in published literature to date in plants (Table 1; Supplementary Table S1).

Multigenerational study: This protocol assesses resistance frequencies over multiple discrete, isolated (no migration) and non-overlapping generations. For detecting relatively small resistance costs, the protocol requires large population sizes, and several replicates and/or plant generations to overcome the stochastic effects of genetic drift. Depending on the plant material used (segregating populations, NILs and so on), it may also be necessary to employ molecular techniques for genotyping analysis.

Using F_2 segregating populations, Roux *et al.* (2005) studied allele frequencies of acetolactate synthase, cellulose synthase and auxin-induced target-site resistance genes over seven *A. thaliana* generations under controlled environmental conditions. Clear deviations of observed from expected resistance allele frequencies (with the assumption of no fitness cost) for some but not all resistance alleles enabled the assessment of corresponding resistance costs, which varied from 0 to 94% (Roux *et al.*, 2005). Other studies that have used this approach under field conditions (using F_2 segregating populations ('segregating population') or discrete *S* and *R* phenotypes ('single population')) have successfully identified significant resistance costs associated with glyphosate (Preston *et al.*, 2009) and P450-based enhanced herbicide metabolism endowing resistance alleles (Vila-Aiub, Neve and Powles, unpublished).

Cline and migration-selection balance: In natural populations located along an ecological gradient, a gradient of allele frequency (that is, cline) is expected for the genes selected by the corresponding ecological factor (Nagylaki, 1975). In the case of resistance, the shape of the cline step depends on the fitness difference

among the *SS*, *RS* and *RR* genotypes in the regions with the selective pressure and the regions without the selective pressure, as well as the amount of gene flow between the two types of regions (Lenormand *et al.*, 1999; Lenormand and Raymond, 2000; Labbé *et al.*, 2009).

By experimentally fixing the migration rate among experimental populations, Roux *et al.* (2006) built up an artificial cline for estimating the resistance costs associated with *csr1-1* (Pro-197–Ser) acetolactate synthase and *axr2-1* (Pro-87–Ser) AXR2 herbicide resistance alleles in *A. thaliana*. The evolution of the resistance allele frequency was estimated along a transect across treated and untreated areas over discrete generations. Only three generations were sufficient to precisely estimate the resistance cost of those two herbicides resistance alleles: 10 and 92% for the *csr1-1* and *axr2-1* alleles, respectively. In contrast with isolated populations in multigenerational studies, control of gene flow among populations also allows the effect of genetic drift on resistance cost estimates to be reduced. This point has been illustrated for the *csr1-1* resistance allele. While the use of 12 isolated populations (each of 120 plants) precluded the detection of costs lower than 15% (Roux *et al.*, 2005), adding gene flow among experimental populations (each of 100 plants) allowed estimation of a fitness cost of 10% for the *csr1-1* allele (Roux *et al.*, 2006).

Future research

To this point we have reviewed and evaluated the various methods available for control of genetic background in order to perform studies to estimate costs of evolved defence mechanisms in plants. Most previous studies, many which have used flawed methodologies, have been primarily concerned with estimating the presence and extent of these costs either to explain the maintenance of polymorphism for resistance traits within plant populations or to assess the potential for costs of resistance to moderate the rate of resistance evolution in agricultural settings. Plant resistance systems provide an ideal model to address fundamental questions about the cost of adaptation, particularly as, in many cases, the molecular genetic basis of resistance is known. In the remaining sections, we propose some ways in which the scope of these studies can be expanded to provide further fundamental and applied insight into the significance of these costs of adaptation. Four relevant research areas are proposed below.

Genome-wide control of false positives when using natural populations

In the area of genome-wide association scans, it is well known that spurious genetic marker-trait associations may arise from confounding population structure, leading to a high false-positive rate (Lander and Schork, 1994). When using natural plant material for estimating resistance fitness costs, similar effects may confound results when *R* and *S* plants originate from different plant populations (that is 'between 2 populations' and 'multiple populations' methods). Moreover, population structure can also lead to a biased estimation of the effect of the gene under study. For example, a mutation in the *Vgt1* flowering time gene was initially found to explain

17% of the phenotypic variation in flowering time in maize (Ducrocq *et al.*, 2008). When population structure was taken into account, this percentage decreased to 4%. So, 13% of the flowering time variation was accounted for by population structure. We believe that there is an urgent need for estimating genetic relatedness among individuals phenotyped for resistance using genetic markers distributed across the genome. Those genetic relatedness estimates may then be integrated in appropriate statistical methods for reducing the false-positive rate and adequately estimating the additive effect of the resistance gene (Kang *et al.*, 2008).

Compensatory evolution of resistance costs

A number of experimental protocols have been proposed to enable more meaningful evaluations of resistance costs and many of these focus on removing the confounding effects of genetic background; however, it should be recognised that resistance alleles are expressed in a range of genetic backgrounds in natural populations. Significant effects of dissimilar genetic backgrounds on the expression and magnitude of several resistance mutations have been documented (Bergelson, 1994; Bergelson and Purrington, 1996; Menchari *et al.*, 2008; Paris *et al.*, 2008). Given this, we may easily speculate that pleiotropic resistance costs may be compensated by natural selection at other polymorphic loci within the genome (Maisnier-Patin and Andersson, 2004). This process creates the opportunity for co-adaptation or integration of the new resistance allele into the genome (Cohan *et al.*, 1994; Guillemaud *et al.*, 1998). By crossing an herbicide-resistant line (*axr1-3* resistance allele) to nine natural accessions, Paris *et al.* (2008) showed a potential compensation of both the resistance cost and its associated dominance by the genetic diversity present within *A. thaliana*. To our knowledge, no study has been specifically designed to detect the genetic basis of resistance cost modifiers in plants. Given the common and quick development of molecular markers in plant species, quantitative trait loci mapping or genome-wide association mapping might be used to find the genetic basis of compensation (Nordborg and Weigel, 2008).

Dominance and frequency dependence of resistance costs

Dominance and frequency dependence both influence the expression of resistance costs and thus the evolutionary dynamics of the corresponding resistance genes. Little attention has been given to understanding these effects and further research is required. The adaptive value of newly arisen herbicide resistance alleles depends on the dominance of the fitness cost. For resistance endowed by major genes, the first evolved resistant mutants in populations will be heterozygous (*RS*). Thus, an understanding of the effect of the number of allele copies on the expression of costs is pivotal. Given the very few studies designed to address this question, it is not possible to discern a general pattern. The very few relevant studies have shown that most resistance costs associated with herbicide resistance alleles are recessive, an indication that few constraints to the initial selection of resistance alleles exist (Roux *et al.*, 2004; Menchari *et al.*, 2008).

The magnitude of resistance costs may also be dependent on the frequency of resistance alleles in populations. If costs are less evident when the resistance frequency is relatively small (negative frequency dependence), then the resistance allele frequency prior to selection will be higher than if costs are positively frequency dependent. While the *axr2-1* herbicide resistance mutation has been shown to be associated with higher fitness costs when its frequency increased (Roux et al., 2006), the cost of *ixr1-2* herbicide resistance mutation increased with decreased resistance frequency (Roux et al. 2005). Frequency-dependent changes in resistance costs may result from density-dependent changes in the intensity and importance of competitive interactions among individuals carrying resistance alleles (Vila-Aiub et al., 2009a, b).

Expression of resistance costs in the presence of the selective agent

Usually, it is assumed that costs of resistance will only be detected in the absence of the selective agent. Korves and Bergelson (2004) found a net cost of resistance under pathogen pressure in the *A. thaliana–Pseudomonas syringae* pathosystem. Pathogen-resistant plants demonstrated a resistance cost when exposed to low pathogen pressure. This result might be explained by infection cost-free developmental response to infection in susceptible plants, or by an inappropriate triggering of defence mechanisms in resistant plants, so that the benefits of resistance are outweighed by the cost at low infection levels. This phenomenon should be investigated in other resistance systems. Since the intensity of the selective pressure is heterogeneous within natural populations (Goss and Bergelson, 2007), it will be interesting to observe if there is a benefit–cost trade-off of a resistance allele under a gradient of selective pressure (Barrett et al., 2009).

Final remarks

A number of methodological protocols exist to control for the confounding effects of genetic background when assessing plant resistance costs. Advantages and disadvantages exist for all protocols, and the final decision on which to use will depend on available plant material, study objectives, knowledge of the molecular basis of resistance and laboratory facilities. We encourage careful consideration of the most appropriate and meaningful protocol in all studies attempting to measure plant resistance costs.

Estimation of resistance costs is the initial step for the identification of constraints to evolution and fixation of novel adaptive alleles in plants. However, and as reviewed above, quantitative changes in plant costs as a result of genetic compensatory effects, dominance, plant frequency and intensity of the selective factor are possible and need to be addressed by further research efforts to comprehend the dynamics of resistance alleles in natural populations and agroecosystems.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)