

# Deployment of disease resistance genes by plant transformation – a ‘mix and match’ approach

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**Breeding for disease resistance has often resulted in the evolution of a matching virulence within the pathogen population, leading to an apparent ‘breakdown’ of resistance. In general, plant breeders have responded by introducing new genes for resistance, with similar consequences. This has led to ‘boom–bust’ cycles, where varieties possessing effective resistance are grown on an expanding acreage (boom) until matching virulence evolves and spreads within the pathogen population (bust). A variety of resistance genes have recently been identified and characterized in model systems. Together with the development of efficient plant transformation systems these genes offer an alternative means to introduce specific resistance into a crop improvement programme. However, unless the resistance genes are deployed with care, the boom–bust cycle is likely to be perpetuated.**

Resistance to a plant disease, yellow rust (*Puccinia striiformis*) of wheat, was first shown to be a mendelian character controlled by a single locus at the beginning of the 20th century<sup>1</sup>, and many disease resistance genes have since been described in numerous plant species. It was found that genes at different loci determined resistance to different ‘pathotypes’ of a pathogen, and this specificity was shown by Flor<sup>2</sup> to be determined by a ‘gene-for-gene’ relationship between resistance genes in the host and virulence genes in the pathogen.

The deployment of single resistance genes in a crop monoculture exerts strong selection pressure for matching virulence within the pathogen population. In the majority of cases this has resulted in the evolution and spread of the matching virulence allele within the pathogen population, so rendering the resistance ineffective. The breeder’s response has been to introduce another resistance gene, which has often resulted in a repetition of these events – the ‘boom–bust’ cycle. The time taken for evolution of virulence is unpredictable, but has occurred in some cases even before a resistant variety has been released<sup>3</sup>. Breeding a new variety requires considerable time and effort, particularly when the source of resistance is poorly adapted (e.g. a related wild species) and requires a lengthy backcross breeding programme to eliminate deleterious genes.

Plant transformation has become a tool for crop improvement, and lines expressing genes conferring resistance to viruses, insects and herbicides have all found commercial application<sup>4</sup>. Plant transformation therefore offers an efficient means to introduce specific genes,

determining disease resistance, into crop species without the introduction of deleterious ‘background’ genes, that in a few cases might be harmful. However, unless consideration is given to how these transgenes are to be deployed, the boom–bust cycle is likely to be perpetuated and valuable genetic resources (i.e. the isolated genes) will be wasted.

## Lessons to be learnt from the deployment of insect resistance genes

Although there is a major difference between pest and pathogen resistance in that a pest has mobility and can exhibit a choice of host, there are sufficient similarities to enable lessons to be learned from the deployment of transgenes for pest resistance.

Even before the advent of insect resistant crops expressing *Bacillus thuringiensis* (*Bt*) insecticidal crystal protein genes, insect populations insensitive to the effects of *Bt* had evolved in Hawaii following repeated foliar applications of commercial formulations. (We have used the term insensitivity when referring to pest resistance to a toxin to avoid confusion with host resistance to the pest.) This highlighted the fact that the development of insensitivity in pest populations was an important consideration for the deployment of *Bt* genes<sup>5</sup>. The usefulness of crops expressing *Bt* genes would clearly be short-lived if insects evolved insensitivity to the toxin. One particular concern was that the frequency of insensitivity could build up in a small number of generations. The selection pressure on the pest population resulting from a crop composed entirely of plants expressing the same *Bt* gene would be constant.

Research scientists have considered strategies that could delay the build-up of insensitivity within insect pest populations, such as the relative merits of refugia (stands of toxin-free plants grown beside the transgenic crop) and crop mixtures of ‘toxin’ and ‘toxin-free’ plants<sup>6</sup>. It has been shown that provision of toxin-free plants slows the development of insensitivity<sup>7</sup>. Under the conditions of the model tested, refugia were generally more effective than seed mixtures but the relative merits of either strategy depend upon the frequency of alleles for insensitivity in the pest population, inheritance of insensitivity (recessive versus dominant), pest movement and mating<sup>7</sup>. Where a pest avoids the toxin (i.e. has a choice of host) this would increase the durability of the resistance due to a *Bt* gene deployed in a crop mixture<sup>6</sup>. Thus, the breeder can control deployment via crop mixtures but if refugia are the better strategy it might be more difficult to persuade growers to adopt this approach<sup>6</sup>.

Transgenic broccoli expressing a *Bt* gene [*CryIA(c)*] have been produced<sup>8</sup>. Laboratory experiments revealed that *Bt*-insensitive Diamond back moth (*Plutella xylostella*) larvae could effectively colonize these transgenic plants. The nature of insensitivity in the larvae is due to recessive alleles and it has been demonstrated that transgenic plants expressing the *CryIA(c)* gene killed those larvae that were heterozygous at the loci determining insensitivity<sup>8</sup>. Under these conditions, insensitivity would take several hundred generations to evolve, provided that toxin-free plants, which susceptible insects could colonize, were mixed with transgenic plants<sup>6,7</sup>. Practical considerations specific to the crop also need to be taken into account. Thus for many horticultural crops where harvesting costs are relatively high and tolerance of insect pests is low, refugia might be preferred, because the sorting of insect resistant (i.e. clean) and susceptible plants in a mixture in the field would significantly add to production costs.

Deployment strategies require further consideration where a pest can be controlled by two different genes. Provided that build-up of insensitivity in the pest population to one of the toxins does not confer resistance to the other (i.e. cross-resistance) then the sequential use of insensitivity genes can be expected to provide more durable control than the deployment of both genes simultaneously. Repeating the sequence of deployment of resistance genes favours the maintenance of heterozygotes at loci for insensitivity, thereby keeping the frequency of alleles for insensitivity low. Mixtures of two *Bt* genes have not slowed or retarded development of resistance in pest populations compared with the sequential use of the same two genes<sup>9</sup>, because pests display choice of their host and temporal separation reduces selection pressure on any one gene.

Thus it is clear that the practical use of transformation technology for the deployment of resistance genes needs to be carefully managed if the genes are to remain useful. Management strategies need to take into account specific features of the targeted pathogen or pest (frequency of resistance/virulence alleles in the population, inheritance, host preferences, pest movement and mating) and the nature of the crop (perennial versus annual, hybrid versus inbred line). It is also important to utilize diverse genes for resistance and to recognize that transformation technology should augment rather than replace conventional breeding.

#### Alternative deployment strategies for disease resistance genes

Alternatives to the deployment of single disease resistance genes have been proposed<sup>10</sup>. However, in general these have had limited success because of the constraints on plant breeders. For example, gene pyramiding, the simultaneous deployment of several genes within a single variety has been of limited value when the matching virulence genes were already present in the pathogen population. The evolution of a pathogen to a race possessing the matching combination of virulence genes has generally been much quicker than the time taken to produce a variety possessing several resistance genes by conventional breeding techniques. Attempts to incorporate more than one 'new' resistance gene for which no matching virulence gene existed in the pathogen population, have foundered. Also, in the past it was difficult to distinguish between plants possessing either one or several resistance genes, because both are resistant to all existing races of the pathogen<sup>11</sup>. This can now be addressed by using marker assisted selection techniques, but this approach is constrained by linkage between loci for resistance, which limits the possible combinations of resistance genes<sup>12</sup>. Although in general, gene pyramiding has not resulted in greater durability, there are reported exceptions, and the subject has been keenly debated<sup>13-15</sup>.

Strategies to reduce the selection pressure for matching virulence genes have included the use of multilines<sup>16</sup> and cultivar mixtures<sup>17</sup>. In both cases the crop comprises components that possess different resistance genes. In a multiline, the components are near isogenic lines with identical agronomic and morphological characteristics. A major restriction on their use has been the length of time needed to breed the component lines, which can make it difficult to respond to changes in market requirements. Mixtures were proposed as a way to reduce the time needed to deploy a crop that is heterogeneous for resistance genes. The components are

varieties differing in their resistance gene complement. However, they also differ for other characters, such as time to harvest. Variety mixtures have been successful for some crops<sup>18,19</sup> but they have been less useful where crop uniformity and quality is important.

The theoretical and practical benefits of growing a heterogeneous crop in comparison to a pure stand are well documented<sup>19</sup>. Disease development in mixtures and multilines is reduced as a consequence of several possible mechanisms including both physical (such as barrier effects) and physiological (such as systemic acquired resistance) effects. The strategy of deploying a crop that is heterogeneous for resistance genes relies on the hypothesis that multiple virulences in the pathotype would carry a fitness deficit (i.e. the *Avr* loci are functionally beneficial to the pathogen and so mutations within these genes would confer insensitivity, but at a price)<sup>20</sup>.

#### The future – deployment of disease resistance genes via plant transformation

Isolation of genes for resistance to several plant pathogens<sup>21</sup> (with the prospect of more to come) make gene pyramiding and multilines feasible strategies for the practical deployment of resistance genes. The relative merits of the two strategies can be assessed for the deployment of genes for resistance to pathogens of vegetable crops, such as *Brassica oleracea* (Fig. 1). A particular concern is the efficient deployment of genes for resistance present within *B. oleracea* itself. In the past, breeders were limited in their use of the *B. oleracea* gene pool because a lengthy backcross breeding programme was required to introgress a single resistance gene from one crop type to another. Transformation technology eliminates this requirement and opens up the complete gene pool for exploitation. The *Arabidopsis* genome can also be exploited in a similar manner as diploid *Brassica* spp. such as *B. oleracea*, display a high level of synteny between their genomes and that of *Arabidopsis*.

The task of introducing a gene into a plant species is no longer a major obstacle. Techniques exist for the delivery of isolated or modified single genes into almost all cultivated species<sup>22,23</sup>. However, among individually transformed plants the expression patterns of an introduced gene can vary. A further complication is that the transmission of introduced genes to progeny might not always follow mendelian patterns of inheritance. Typically, individuals with the desired phenotype and stability have to be selected from several independently produced transformed lines<sup>24</sup>. The stability of transgene expression is critical to commercial success.

#### Gene pyramiding

Currently available gene transfer technologies have limitations that could effect the success of multiple gene insertions. Particle bombardment technology can deliver large numbers of genes into plant cells in a single step. However, the fragmentation of plasmids, the integration of multiple copies and the presence of ancillary DNA sequences complicate the practical use of this technology. In contrast, the use of *Agrobacterium* leads to the integration of only a small number of copies per cell. Also, the method is only effective for delivery of DNA sequences of up to 25 kb. A binary bacterial artificial chromosome vector in combination with enhanced virulence functions of *Agrobacterium*, has been used to deliver DNA sequences in excess of 150 kb in tobacco<sup>25</sup>. This method could prove to be suitable for delivering gene clusters into crop plants.

An alternative approach is to add genes singly. However, there are two practical problems with this approach. Firstly, most transformation protocols rely on the use of a selectable marker gene (typically *nptII*, conferring resistance to kanamycin) whose expression confers a selective advantage to the transformed cells. The presence of a particular selectable marker gene in a transgenic cultivar precludes the future use of that marker in subsequent rounds of transformation<sup>26</sup>. There are alternative selectable marker genes<sup>26</sup>, but for many plants their use would compromise the efficiency of transformation and in species that are recalcitrant to transformation, such as *Brassica*<sup>27</sup>, they are not viable alternatives.

Single genes added independently into the same homozygous genetic background (e.g. a doubled haploid or inbred line) can be combined together through hybridization, so avoiding the need for multiple rounds of transformation. However, the pyramiding of transgenes that share homologous sequences, by either repeated rounds of transformation or crossing, can lead to altered expression levels of the inserted genes<sup>28-30</sup>. Consequently, methods for the elimination of ancillary DNA sequences (such as selectable markers), which would otherwise contribute to the build up of sequence homology and instabilities in transgene expression<sup>31</sup>, are an important feature of gene pyramiding. Transformation systems for generating marker-free transgenic plants have been developed<sup>26</sup>. One of the simplest strategies is co-transformation, where marker-genes and genes of interest are inserted on separate plasmids. When they integrate independently, segregation in the subsequent generation allows recovery of marker-free plants. The strategy requires a high frequency of co-transformation with integration of genes at unlinked sites. A co-transformation strategy for *Agrobacterium rhizogenes* is currently being optimized for the production of marker-free plants.

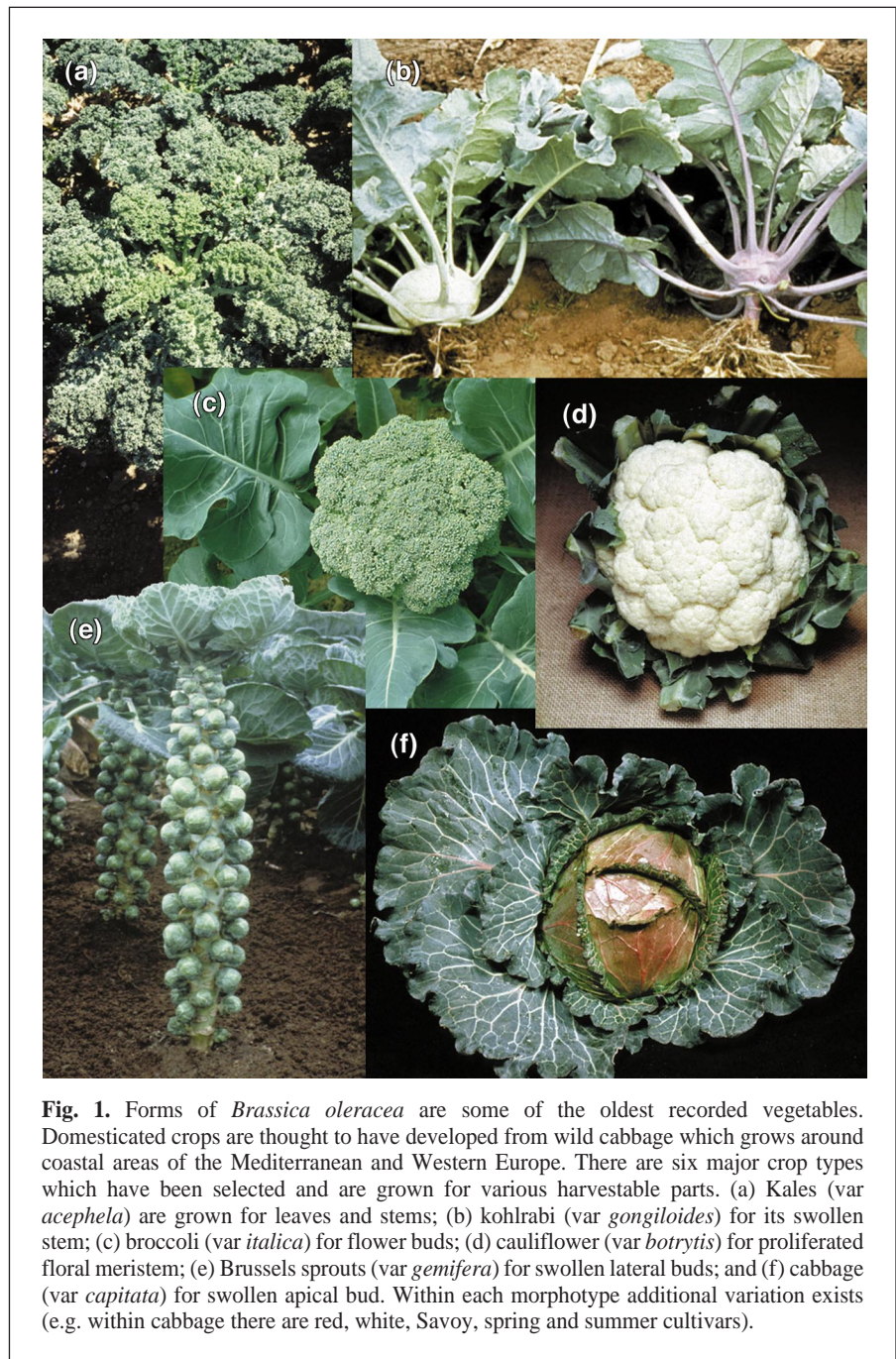
Pyramiding of resistance genes for which matching virulence already exists might be effective in some cases. Transformation provides an opportunity to create new combinations of resistance genes free from the constraints of linkage. Some of these new combinations could eventually prove to give durable resistance. However, in the absence of a detailed knowledge of the mode of action of resistance genes and how their gene products interact with *Avr* genes, it is not possible to predict durability.

The pyramiding of new resistance genes is superficially attractive because it gives rise to completely clean crops whilst resistance is effective. The likelihood of the matching combination of virulence genes evolving is reduced in proportion to the number of genes in the pyramid. However, if the same combination of resistance genes is widely deployed (i.e. a 'boom') then there will be a strong selection pressure for this to happen. In addition, gene pyramids are vulnerable if the component genes are deployed singly in other varieties such that selection for matching virulence occurs within the pathogen population<sup>11</sup>. If the resistance 'busts' then the genes will be of limited value for future use in protecting the crop. Monitoring the pathogen population for evolution of virulence genes that match single gene components of the pyramid will give some indication of the risk of this happening. A tendency to build bigger pyramids ('Pharaoh phenomenon') by adding more and more genes would hopefully prevent loss of resistance, but it is a similar treadmill to the boom-bust cycle.

#### 'Mix and match' multilines

Plant transformation can be combined with conventional breeding techniques and studies of the pathogen population to formulate a deployment strategy that reduces the selection pressure on the pathogen and combines the advantages of multilines (uniformity) and mixtures (speed of deployment). This can be done by producing a multiline that is heterogeneous for resistance genes but homogeneous for quality and other characters. The resistance genes in the crop are therefore 'mixed' to 'match' the frequency of virulence alleles in the pathogen population. Theoretical studies show that the likelihood of a 'super race' evolving with matching virulence to all resistance genes in a multiline, reduces as the number of components increases<sup>32</sup>. However, practical studies have demonstrated a significant reduction in disease levels with as few as three components<sup>17</sup> and there is a diminishing return in terms of disease reduction on the effort required to produce additional component lines.

Different strategies can be used to deploy resistance genes in pure line and clonally

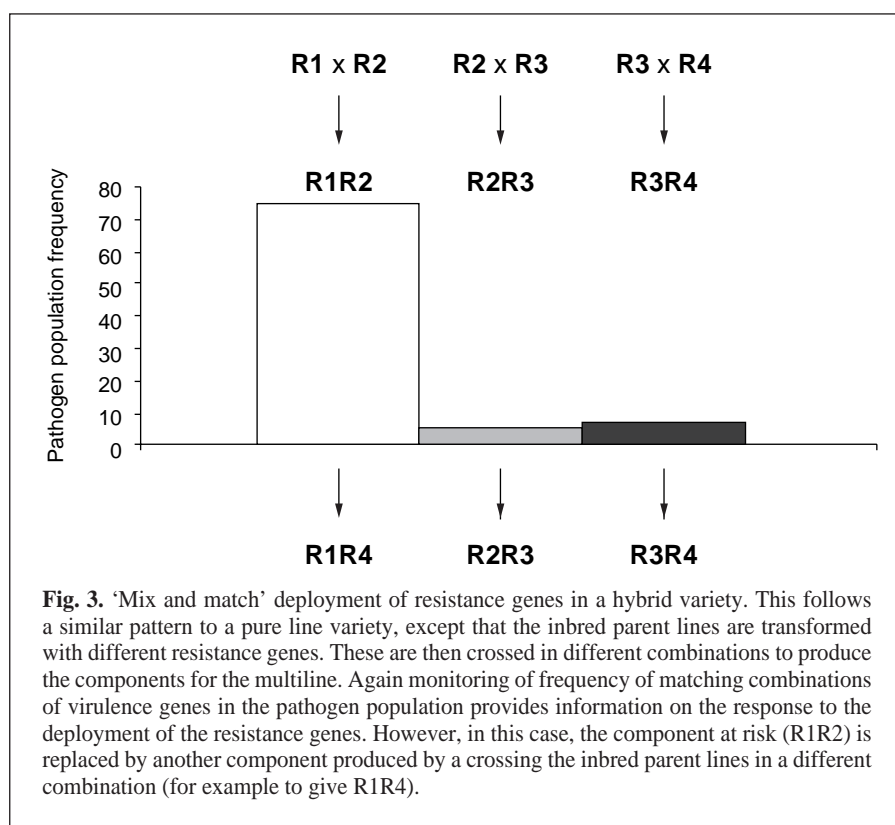
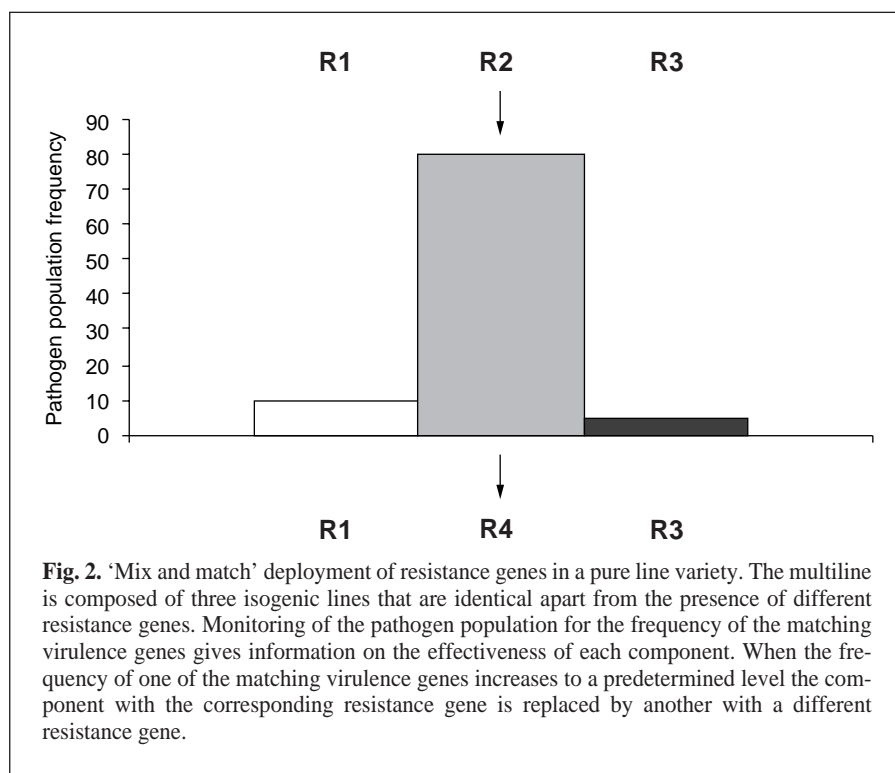


**Fig. 1.** Forms of *Brassica oleracea* are some of the oldest recorded vegetables. Domesticated crops are thought to have developed from wild cabbage which grows around coastal areas of the Mediterranean and Western Europe. There are six major crop types which have been selected and are grown for various harvestable parts. (a) Kales (var *acephala*) are grown for leaves and stems; (b) kohlrabi (var *gongiloides*) for its swollen stem; (c) broccoli (var *italica*) for flower buds; (d) cauliflower (var *botrytis*) for proliferated floral meristem; (e) Brussels sprouts (var *gemifera*) for swollen lateral buds; and (f) cabbage (var *capitata*) for swollen apical bud. Within each morphotype additional variation exists (e.g. within cabbage there are red, white, Savoy, spring and summer cultivars).

propagated crops (Fig. 2) compared to hybrid crops (Fig. 3). In both cases there is no requirement for multiple rounds of transformation. In pure line crops (such as lettuce), resistance genes are deployed singly in the components of the multiline that are produced by transformation of the elite variety. Thus at any one time, three different resistance genes are deployed within the crop, so reducing the selection pressure for the matching virulence for any one gene. In addition, seed of additional components possessing other genes for resistance are held in reserve. There is concern that the continuous cultivation of a multiline will select for complex races of the pathogen<sup>32</sup>. However, monitoring the frequency of matching

virulence genes in the pathogen population provides information on the response of the pathogen population to the selection pressure being exerted. If the frequency of the virulence matching one of the resistance genes in the multiline increases, the component carrying that gene is replaced with one of the reserve components. Deployment in response to the dynamics of the pathogen population can be relatively rapid compared to conventional breeding (i.e. the following year or sooner depending upon the time taken to grow the crop).

For clonally propagated crops, response time is dependent upon the nature of the crop. For an annual crop such as potato, a response to changes in the pathogen population can be



made the following year. However, for plantation/orchard crops such as apple, where the plant goes through a juvenal phase before producing any marketable product, the ability to respond rapidly is limited. Nevertheless deployment of resistance genes in a multiline is still of value in such crops because the selection

pressure on the pathogen to evolve matching virulence is substantially reduced compared to a homogeneous crop.

For hybrid varieties (such as the vegetable brassicas), resistance genes are introduced singly via transformation of the inbred parents (Fig. 3). These are then crossed in different

combinations to give three hybrid components of the multiline that are identical to one another, except that each possesses a different pairwise combination of resistance genes. The potential durability of the multiline might be increased if the components do not share any resistance genes<sup>33</sup>, but the availability of isolated resistance genes could be a limiting factor in this respect. Again monitoring of the pathogen population gives information on the response to deployment. A component for which matching virulence is increasing in frequency is replaced with one carrying a different pairwise combination. This is produced by crossing the appropriate combination of the inbred parents and no additional gene needs to be utilized (i.e. no additional transformation events are required).

A potential disadvantage of a multiline strategy compared to gene pyramiding is that it reduces the rate of increase in disease in the crop but does not necessarily give total disease control. Studies with barley variety mixtures<sup>17</sup> have shown that disease is restricted to an acceptable level. However, if the level of disease control is insufficient, the mix and match approach is a stable and sustainable approach that can be combined with other control measures (such as limited use of chemicals and biocontrol measures) in an integrated disease management programme. A great advantage of the strategy is that it does not depend upon a supply of new resistance genes, and is a dynamic strategy for the management of resistance genes, which can preserve their usefulness. Existing resistance genes can be recycled. For example, if a gene, or gene combination, is withdrawn because the frequency of the matching virulence increases in the pathogen population it can be re-introduced when the frequency of the matching virulence allele(s) reduces.

**Perspective**

In the past, strategies such as gene pyramiding and multilines were impractical, and even strategies such as variety mixtures, which were feasible in some crops, were in general not as effective as they might have been. This was partly due to a lack of control over the deployment of resistance genes. Under plant variety rights, once a variety possessing a new resistance gene was released it was freely available to other breeders to use in their programmes (i.e. the original breeder lost control of the destiny of the gene). Greater control can be achieved using patents to protect isolated gene sequences. The owner of the patent on the gene(s) has control over how the gene(s) are deployed, either directly or through licensing agreements. Thus, in spite of the controversy surrounding the patenting of genes, it provides a mechanism for the rational exploitation and deployment of resistance genes

via transformation. We propose that this should be through the 'mix and match' approach described above, which preserves the usefulness of resistance genes for the future, rather than via gene pyramiding, which runs the risk of perpetuating the boom–bust cycle.

#### Acknowledgement

David Pink and Ian Puddephat are funded by the UK Biotechnology and Biological Sciences Research Council and the Ministry of Agriculture, Fisheries and Food.

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