SESSION 9A

EXPLOITATION, IN DISEASE MANAGEMENT, OF NEW MOLECULAR UNDERSTANDING OF RESISTANCE AND PATHOGENICITY

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The impact of the genomics era on our development of disease control strategies

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ABSTRACT

Genomics has made a hugely beneficial impact on our understanding of many aspects of biology, and plant pathology is no exception. The complete genome sequences of plant pathogens are providing blueprints of the entire repertoire of genes required for successful infection. Moreover, they are revealing insights into the lives of pathogens when they are not infecting their host plants. Similarly, genomic approaches are informing us about how plants defend themselves from pathogens. Armed with this knowledge, we can seek novel ways to combat disease, or better direct the efforts of conventional disease resistance breeding programmes to utilise the natural defence strategies of plants. We will illustrate the exciting potential of genomics in developing disease control strategies for two pathogens of potato: the bacterium *Erwinia carotovora* subsp. *atrosepticum*, which causes soft rot and blackleg and the oomycete *Phytophthora infestans*, which causes late blight.

WHAT IS GENOMICS?

In the last decade we have seen the first genomes sequenced to completion to reveal the entire complement of genes required for life. This has given rise to the era of genomics which, put very simply, is biology on a very large scale. Genomics has challenged the traditional reductionist approaches in biology of sequencing and studying the role of a single gene in a defined process. The question arises: if you can reveal the primary DNA structure of all genes, can you also study their expression and their translation into proteins on a global scale? Moreover, can you measure how those proteins act collectively to provide the myriad interacting biochemical processes needed for a life that is complicated by a changing environment? Genomics has thus posed a number of technological challenges that have led to new, high-throughput approaches to investigate the expression of many (or all) genes in the genome (the transcriptome), of the proteins that they synthesis (the proteome), and of the metabolites that they generate (the metabolome). A bewildering array of such high-throughput methods has been termed 'omics technologies. In recent years we have seen an increase in the application of these technologies to investigate plant-pathogen interactions.

UNDERSTANDING PLANT-PATHOGEN INTERACTIONS: THE CHALLENGE FOR GENOMICS

Plants face a constant daily barrage from millions of microorganisms, many of which are equipped with an arsenal of weapons, such as plant cell wall degrading enzymes, that can cause extensive damage to plant tissues. Nevertheless, in the vast majority of cases, plants are able to fend off the invaders. Most potential plant pathogens, including viruses, bacteria, oomycetes, fungi and nematodes, activate local and systemic defences in plants. These defences constitute a basal, innate immune system that is effective against the majority of microorganisms and which

must be evaded, manipulated or overcome to successfully establish infection. If we can find out more about how pathogens manipulate plant defences, then we may be able to prevent them from doing so. Similarly, a deeper understanding of natural plant disease resistance mechanisms, and how their expression is regulated, may allow us to better employ or activate them to the detriment of normally successful pathogens.

The interaction of a plant cell with a pathogen cell involves complex and dynamic exchanges of signals which influence regulatory networks and biochemical pathways in the interacting organisms (Figure 1).

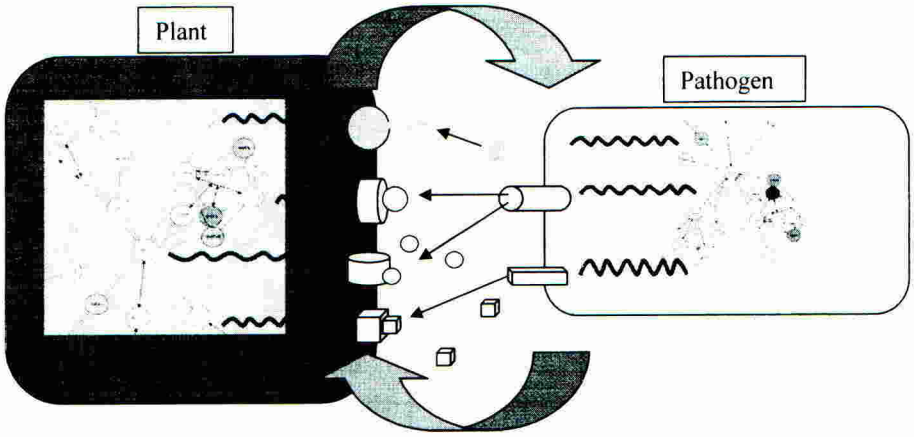

Figure 1. The interaction between a plant cell and a pathogen cell involves a complex and dynamic exchange of signals

Such signalling and regulatory systems provide an investigative challenge, since they comprise multiple inter-dependent networks whose combined behaviour is difficult to predict from an examination of the individual components alone. Genomics approaches can help us to unravel the complexities of plant-pathogen interactions. Complete genome sequencing of plant pathogens has led to the discovery of hitherto unknown virulence genes that reveal the strategies employed to manipulate host defences. Similarly, investigations of plant transcriptomes are revealing the genes that are activated in response to pathogen challenge. Nevertheless, the scale of the data generated in genomics projects, which often involve large international collaborations, presents a further challenge in analysis and interpretation to identify those genes with a key regulatory or mechanistic role. The next phase of the genomics era is thus attracting mathematical and computational biologists whose expertise is being harnessed to develop new approaches to integrate and analyse genomic data. This is an exciting period in biology as it is engendering the formation of truly multidisciplinary teams and a philosophy of collaboration and cooperation that is accelerating our understanding in areas such as plant-pathogen interactions. In the remainder of this paper, I will describe how genomics is providing important information about two potato pathogens, *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *Phytophthora infestans*, which may help to develop new strategies to combat the diseases that they cause.

THE IMPACT OF GENOMICS ON OUR UNDERSTANDING AND MANAGEMENT OF POTATO DISEASES

Resistance to the soft rot and blackleg pathogen, *Eca*

Erwinia carotovora subsp. *atroseptica* (*Eca*) is the number one bacterial plant pathogen in the UK, causing soft rot and blackleg disease of potato. There is no chemical control for *Eca* and little significant genetic resistance in the cultivated potato to utilise in breeding programmes. A year ago, the *Eca* genome was completely sequenced in collaboration with the Sanger Centre (Bell *et al.*, 2004). We developed new software to allow the *Eca* genome to be compared to all other bacterial genomes (Figure 2).

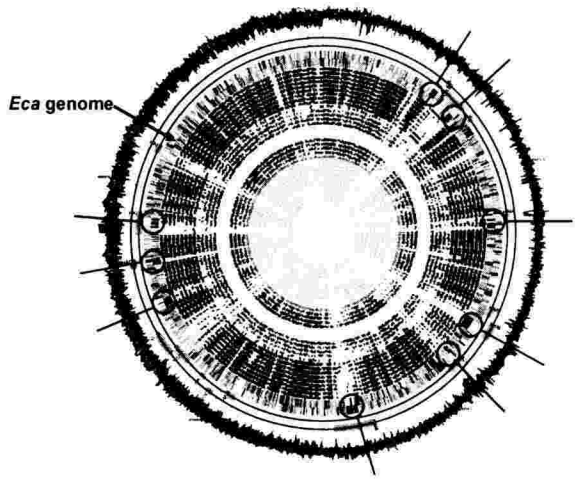

Figure 2. The *Eca* genome was compared to other bacterial genomes, and shaded inner circles indicate which *Eca* genes are conserved in other bacteria (each inner circle represents a different bacterial genome). Indicated are the locations of putative pathogenicity genes, often residing in *Eca*-specific genomic regions.

The comparisons revealed genomic regions specific to *Eca* that contained putative virulence genes, including those encoding phytotoxins that could damage plant tissues. In addition to the phytotoxins, genes encoding a type III secretion system (T3SS) were identified. The T3SS is involved in injecting pathogen effector proteins inside host cells where they act to manipulate plant defence proteins. To help us identify T3SS-delivered effector proteins, a key gene regulating the T3SS was mutated to knock out its function. Comparison of the transcriptomes in the mutant and the wild-type *Eca* revealed genes that were dependent on the T3SS regulator for their expression during infection. These genes included two effector proteins that are injected into potato cells. When these genes were mutated, the resulting *Eca* mutants were unable to fully infect potato. To investigate the roles of these effectors in infection, we investigated the transcriptome of potato after challenge with either wild-type or effector-mutant *Eca* strains. In this way, we discovered a potato regulatory gene that was being manipulated by the *Eca* effector proteins. In transgenic plants we over-expressed the potato regulator gene and found that this provided strong resistance to *Eca*. The potato regulator can be switched on and primed for action simply by spraying plants with plant cell wall breakdown products.

Triggering resistance to the late blight pathogen, *P. infestans*

Phytophthora infestans is an oomycete and the number one potato pathogen, causing the disease late blight. Oomycetes are the most devastating pathogens of dicotyledenous plants. Control of *P. infestans* involves the most extensive use of agrochemicals for any plant pathogen. Such chemical treatments represent an economic burden and are often overcome by resistant pathogen strains. Moreover, the desire by the EU and UK government for sustainable agriculture with decreased inputs presents significant challenges. Genetic disease resistance to *P. infestans* exists in the cultivated potato, although resistance (*R*) genes introgressed from wild potato species have been rapidly overcome by new pathogen strains.

The transcriptome of *P. infestans* was investigated and many candidate effector genes were identified that were expressed specifically during infection. One of these genes encoded a protein that triggered *R* gene-dependent resistance in potato, as a form of the gene was recognised by the R protein (Armstrong *et al.*, 2005). Studies of this gene in a range of *P. infestans* isolates gathered from around the world and, particularly, from Mexico, the potential origin of this pathogen, revealed that there were two forms, one which triggered resistance one which did not. The existence of only one (virulent) form of the gene that evades recognition by the potato *R* gene represents a potential Achilles heel for *P. infestans*: if a different form of the potato *R* gene that recognises the virulent form of the pathogen effector protein can be found from a wild potato species, then this can be introgressed into the cultivated potato to provide durable disease resistance.

What can genomics tell us about pathogen lifestyles?

The bacterial soft rot pathogen of potato, *Eca*, has long been regarded simply as a pathogen. However, a close look at its genome revealed a wide range of genes offering different metabolic capabilities and a potentially varied lifestyle. Among these genes are those required, in symbiotic microorganisms, for fixing nitrogen. This is important for plant growth and development as the nitrogen fixed by symbiotic bacteria helps to provide the building blocks for new proteins following photosynthesis, a process that provides food for all life on the planet. The presence of these genes in *Eca* raises speculation of an alternative lifestyle and a very different interaction with plant hosts.

A deeper look at the genome revealed a gene that, in other bacteria, is required for attachment to plant roots. This we mutated to investigate its role in potato pathogenesis. We were surprised to find that the absence of the gene did not affect the ability of *Eca* to attach to potato roots. Indeed, wild-type *Eca* attached only poorly to potato roots. However, the knock-out severely impaired the ability of *Eca* to attach to brassica roots. Indeed, in natural environments, *Eca* has been found associated with brassica roots. We are now investigating the association between brassica and *Eca* to see if the bacterium plays a symbiotic role. However, the implications of this finding on disease management are important; if the symbiotic association exists in nature, then crop rotation needs to be carefully considered (Figure 3).

Figure 3. The potato fields around Dundee are interspersed with fields containing the brassica oil seed rape. If *Eca* shows a non-pathogenic association with brassica, then a serious potato pathogen may be harbored in neighbouring fields.

CONCLUSIONS

In conclusion, genomics approaches are providing new insights into the lives of plants and their pathogens. Harnessing the knowledge gained from these studies may provide alternative disease control strategies and will inform our disease management practices. Scientifically, we are accelerating our fundamental understanding of mechanisms and processes involved in plant-pathogen interactions, many of which are generic across plant species. Such knowledge can only help us to better utilize the natural defences of plants to prevent such devastating diseases as potato late blight.

ACKNOWLEDGEMENTS

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New approaches for durable disease resistance in wheat

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ABSTRACT

To achieve durable control of wheat diseases, additional knowledge is required on the molecular genetic basis of plant-pathogen co-evolution. Little is known about the components regulating defence responses in bread wheat (*Triticum aestivum*) due to its huge and hexaploid genome. In an attempt to circumvent these difficulties, reverse and association genetics studies in the diploid Einkorn wheat species *Triticum monococcum (Tm)* with a less complex genome are in progress. There is a high level of micro-colinearity in different genome regions between *Tm*, wheat, rice and barley. Therefore, studies in *Tm* as a reference species should help to elucidate gene functions in *T. aestivum*. Initial phenotyping analyses indicate that *Tm* is a rich source of resistance to many of UK wheat fungal and viral pathogens. To explore gene function, the *Tm* homologues of known global regulators of defence responses *RAR1*, *SGT1* and *NPR1* were identified and gene variants screened for using a TILLING (Targeting Induced Local Lesions IN Genomes) platform in *Tm* and bread wheat. Through association genetics, the specific resistance/susceptibility phenotype–novel allele relationships will be explored in order to identify whether specific genes and gene variants may confer disease resistance in wheat to one or more pathogen species. In this article, we also discuss how this new combined approach could help to achieve durable resistance to multiple pathogens in polyploid species.

INTRODUCTION

In plants, resistance to a disease is considered durable when it is effective for a long period of time and over a large area in environmental conditions favouring the disease (Johnson, 1984). Wheat (*Triticum aestivum*) is the major crop in UK agriculture and is more valuable than any other arable crops. Many pathogens infect wheat lowering grain yield and quality. In Europe there is strong social, environmental and economic pressure to minimise chemical usage in crops. In addition, the emergence of pathogen variants which are resistant to specific chemicals can suddenly leave huge areas of crops vulnerable to these diseases. These concerns have heightened interests in the identification of novel germplasm held in the global seed banks which exhibits a high level of disease resistance, the establishment of the genetic basis of disease resistance to each pathogen and in providing plant breeders with linked

molecular markers to the disease resistance traits to help their introgression into the modern elite wheat cultivars. However, to complete this process it can typically take 7-10 years. Therefore, alternative approaches need to be developed.

Advances in molecular biology and genomics, particularly the acquisition of whole genome sequence and expressed sequence tags for *Arabidopsis,* rice and several fungal pathogens of plants, have greatly enhanced our understanding of plant-pathogen interactions at the molecular level. Many genes involved in pathogen perception, signal transduction, and the defence responses have been identified and well characterised (Hammond-Kosack & Parker, 2003; Thatcher et al., 2005). Several conserved regulators are now recognised to co-ordinate defence in a range of plant species (Muskett & Parker, 2003; Shirasu & Schulze-Lefert, 2003; Dong, 2004). These discoveries provide an opportunity to transfer knowledge from reference species to crops as well as among crop species and thereby accelerate genetic improvement.

In 2003, through a Department for Environment, Food and Rural Affairs (Defra) funded initiative, the Wheat Genetic Improvement Network (WGIN) was formed with the goal of uniting the UK public-sector research activity on genetic improvement of wheat and promoting a close working relationship between researchers and breeding companies (http://www.WGIN.org.uk/). WGIN aims are to transfer cutting edge knowledge obtained from reference species (i.e., *Arabidopsis,* tobacco, barley and rice) and to employ advanced molecular and genetic techniques and thereby enhance key traits including durable disease resistance, canopy architecture and resource use efficiency. We aim to develop domesticated Einkorn wheat, *Triticum monococcum* (*Tm*) into a reference species for functional genomics in hexaploid wheat. *Tm* was selected because it is a relatively easy threshing, diploid wheat, which possesses an A genome (A^m) closely related to the A genome progenitor of hexaploid wheat. *Tm* is known to be a good source of resistance to many pathogens (Anker et al., 2001). In this study, *Tm* is used to (1) detect novel traits through various field and laboratory-based phenotype screens, (2) identify genes and allele variants in candidate genes of interest, (3) establish by combining the data obtained from activities 1 and 2, specific resistance/susceptibility phenotype–variant allele associations, and (4) map the genetic location of traits of interest. A new reverse genetic technique first developed for the reference plant species *Arabidopsis* termed TILLING (Targeted Induced Local Lesions IN Genomes) (Henikoff et al., 2004) is employed to identify gene variants in a high throughput manner in chemically mutagenised or natural *Tm* populations.

In this paper, we describe our initial efforts to establish *Tm* as a reference species to explore the molecular genetic basis of wheat resistance to fungal and viral pathogens. A collection of *Tm* accessions was obtained and screened for resistance against major UK wheat pathogens. The homologues of three key defence signalling regulators, *RAR1*, *SGT1* and *NPR1* have been identified in *Tm*, and gene regions selected for TILLING to identify allele variants. These combined activities provide the foundation for high-throughput gene function studies in *Tm*.

MATERIALS AND METHODS

Pathogen screens completed under field and controlled environment conditions

Different *Tm* field experiments were conducted in 2003 - 2005 at Rothamsted Research to screen for resistance to *Mycosphaerella graminicola* which causes *Septoria* leaf blotch

disease (natural inoculum), and *Fusarium* ear blight (*FEB*) disease by mixed artificial inoculation with *Fusarium graminearum* and *F. culmorum*. Symptom scoring followed standard procedures (Hilton et al., 1999; Lovell et al., 1997). Screens for resistance to *Septoria* leaf blotch, *Soil-borne cereal mosaic virus* (*SBCMV*) transmitted by the vector *Polymyxa graminis* and cereal eyespots caused by *Oculimacula yallundae* and *O. acuformis*, were conducted in duplicate in controlled environment rooms as previously described (Scott, 1971; Shetty et al., 2003; Kanyuka et al., 2004).

Assessment of the genetic diversity of the *T. monococcum* accessions by NBS profiling

Four seedlings from each *Tm* accession were grown in a glasshouse and leaf tissue was harvested separately from each plant for DNA isolation using the QIAgene DNA mini-kit. The genomic DNA was subject to restriction with *Mse*I and used for Polymerase Chain Reaction (PCR) amplifications as described (van der Linden et al., 2004).

Targeted Induced Local Lesions IN Genomes (TILLING)

High-throughput variant screening was performed in natural *Tm* accessions using TILLING essentially as outlined by Slade et al. (2005). Two primer sets spanning the targeted sequences in *TmRAR1* were selected for PCR amplification.

RESULTS

Features and genetic diversity of a global *T. monococcum* collection

We obtained 26 *Tm* accessions from the Vavilov Research Institute of Plant Industry, 94 *Tm* accessions from the USDA Agricultural Research Service, and a further 4 accessions from elsewhere (Table 1). The earliest original collection date was 1904. Fifty-four countries and regions were included and most accessions are of European origin (n=124). Two thirds of the collection have a spring growth habit. Several accessions offer valuable molecular resources. For example, a BAC library is available for line DV92 (Lijavetzky et al., 1999), and accessions MDR001 and MDR002 are transformable by Biolistics (H. Jones, Rothamsted Research, unpublished).

Table 1. Rothamsted Research collection of *Tm* accessions

Item	Numbers
Total number	124
Origin of countries and regions	54
Original collection time	Since 1904
Spring habitat	93
Winter habitat	31
Accession with BAC library	1
Transformable accessions	2
Accession with EMS population	1

We have explored the variation of resistance (*R*) gene and *R* gene analogues (*RGA*) using a Nucleotide Binding Site (NBS) profiling technique (van der Linden et al., 2004). Figure 1 shows representative images obtained from the targeting of three distinct domains of NBS-LRR *R* genes, by using different PCR primer combinations. The NBS2 and NBS5 primers generated images with multiple polymorphic bands among the different *Tm* accessions.

However, no polymorphic bands were observed using the NBS3 primer set. Thus, the NBS3 class *R* genes or *RGA*s appears to be highly conserved in *Tm*. However, the NBS2 and NBS5 classes are clearly are more diverse and have the potential for further exploitation.

Figure 1. NBS profiling in *Tm* accessions. The genomic DNA from the *Tm* collection was fragmented with *Mse*I restriction enzyme, and then used for PCR amplification with a linker primer plus a ^{33}P labelled NBS domain specific primer. After electrophoresis, the polymorphic bands were visualised on radiographs. Gel images deciphering the NBS2, NBS3 and NBS5 profiles are shown. Each lane represents a *Tm* accession. Many common bands are detected, but some bands are polymorphic in NBS2 and NBS5 profiles.

Resistance and susceptibility of *T. monococcum* to major UK wheat pathogens

Field and/or glasshouse screens were performed to evaluate resistance and susceptibility to the major UK wheat pathogens. Table 2 shows that *Tm* exhibited a high degree of resistance to *Mycosphaerella graminicola (Mg)*. All the tested *Tm* accessions developed no disease symptoms under field conditions whilst the control hexaploid wheat (cv Avalon) plots showing 100% of plants with leaf blotch symptoms by early February each year. The resistance of *Tm* accessions to *M. graminicola* was unexpected because of results generated by others (Yechilevichauster et al., 1983; McKendry & Henke, 1994), but the resistance was subsequently confirmed in controlled glasshouse tests using a mixed inoculation of nine differential isolates (data not shown). Approximate one quarter of the collection had good resistance to *SBCMV*, even though all accessions but 2 were susceptible to the viral vector *Polymyxa*. A few accessions displayed a high level of resistance to either *Fusarium* ear blight or cereal eyespot. Thus, the *Tm* collection assembled is a valuable source of resistance to major UK wheat pathogens. Field-based screens to explore the responses of *Tm* accessions following inoculation with either yellow rust (*Puccinia striiformis* f.sp. *tritici*) or the Take-all fungus (*Gaeumannomyces graminis*) are currently in progress (Lesley Boyd, John Innes Centre and Richard Gutteridge, Rothamsted Research, UK).

Table 2. Overall summary of *Tm* resistance and susceptibility screens

Pathogen/Vector	Susceptible	Intermediate	Resistant	Total screened
*Polymyxa graminis**	122		2	124
SBCMV	93		31	124
Mycosphaerella graminicola			29**	29
Fusarium graminearum/ F. culmorum	26		2	28
Oculimacula yallundae	23	2	4	29
Oculimacula acuformis	23	3	3	29

*Vector for *SBCMV*. **Four *Tm* accessions displayed lesion formation with immature pycnidia briefly in February 2004 just prior to leaf senescence.

Identification and isolation of *Tm* homologues of *RAR1*, *SGT1* and *NPR1* genes

By interrogating the EST sequence databases for barley, rice and hexaploid wheat, primers were designed to amplify from both genomic and cDNA *Tm* templates by PCR and RT-PCR, respectively, sequences for the genes *TmRAR1*, *TmSGT1* and *TmNPR1*. The *TmRAR1* and *TmSGT1* sequences were confirmed to be single copy genes by DNA gel blot analysis (data not shown) and their predicted primary gene structures were similar to the homologous barley, rice and *Arabidopsis* genes (Figure 2). In particular, the three-nucleotide exon 2 of the *RAR1* gene found in barley and other species is also present in the *Tm* gene. Two *NPR1* homologues have previously been documented for hexaploid wheat (Patent WO0166755). Both genes were amplified from *Tm*. Using the deduced amino acid sequences for alignment (ClustalW), the TmRAR1 and TmSGT1 proteins are predicted to share 92% and 91% identity to barley RAR1 and SGT1, respectively, whilst the TmNPR1a and TmNPR1b proteins share 36% and 48% identity to *Arabidopsis* NPR1, respectively. These results imply that the identified *RAR1* and *SGT1* genes may represent the orthologues of these defence global regulators.

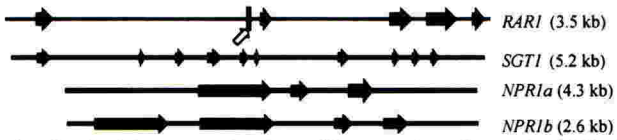

Figure 2. A diagram showing the intron-exon structure of *Tm* genes homologous to *RAR1*, *SGT1* and *NPR1*. The open arrow indicates the conserved three nucleotide exon in the *RAR1* gene. Dark arrow bars represent exons and grey bars introns. Scales of each gene diagram are different.

The cDNAs of *TmRAR1* and *TmSGT1* were isolated from fifteen independent *Tm* accessions and sequenced. Although some nucleotide substitutions were observed in *TmSGT1* coding regions, all were silent and did not change the encoded protein sequences. Amongst the fifteen sequenced *TmRAR1* cDNAs, one accession MDR037 possessed two nucleotide substitutions, which caused a lysine to arginine and a valine to isoleucine amino acid substitution and one silent mutation. These two amino acid substitutions are predicted to cause only small changes in the functionally important CHORD II domain of the RAR protein (Shiraru & Schulze-Lefert, 2003).

Establishing a TILLING protocol

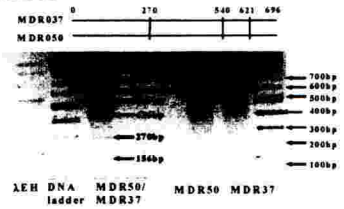

Figure 3. Proof of TILLING concept in *T. monococcum*. See text for details.

As a proof of concept, *TmRAR1* was selected as the candidate gene for establishing a TILLING protocol. Initial experiments were focused on detecting mismatches in *TmRAR1* using a CEL I double strand cutting assay and unlabelled templates (Sokurenko et al., 2001). The cDNAs from two *Tm* accessions, MDR037 and MDR050 that had three nucleotide mismatches, were used as templates. In addition to the original PCR products, at least five CEL I cleavage products, generated from the heteroduplex formed between the two PCR products, were evident solely in the MDR50/MDR37 lane (Figure 3). Thus, it is possible to detect simple mutations and polymorphisms in *Tm* utilising the unique property of CEL I. We

are currently establishing a high-throughput platform by combining the single strand cleavage activity of CEL I with denaturing electrophoresis on a Li-Cor 4300 DNA Analyser and will apply this platform to screen for novel variants of *TmRAR1* in our natural *Tm* collection and in an EMS-mutagenised population of the accession MDR050 generated by Kay Denyer (John Innes Centre, UK).

DISCUSSION

Wheat functional genomics combining the A^m subgenome and TILLING

In this article, we address the use of a diploid wheat species *Triticum monococcum* and TILLING as functional genomics means to unravel the molecular genetic basis of wheat resistance to the major UK fungal and viral pathogens. The fungal pathogens evaluated in this study are all classified as hemi-biotrophs since during the initial stages of infection the host tissue in direct contact with fungal hyphae remains alive for extended periods and only late on in the infection (10 days +) widespread host cell death occurs. Despite the identification of genetic loci conferring resistance to *M. graminicola (Septoria leaf blotch)*, *Fusarium* ear blight and cereal eyespots (reviewed in Jahoor et al., 2004), the molecular identity of an *R* gene conferring resistance to a wheat-attacking hemi-biotrophic fungal species is unknown. So far, only one locus conferring resistance to *SBCMV* has been reported in a UK commercial hexaploid variety (Kanyuka et al., 2004). Hence, additional new sources of resistance are needed. More importantly, the signalling components regulating wheat defence responses are poorly understood, although some can be inferred from reference species. Identification of global defence regulators such as *RAR1*, *SGT1* and *NPR1* may overcome taxonomic gene function restriction and offer new ways to potentiate disease resistance, increase the success rate of *R* gene introgression from wild-species, and hence provide durable disease controls to multiple pathogens.

A sub-genome approach utilising *Tm* as a model species is taken for several reasons. (1) Wheat presents a huge challenge to molecular genetic studies. Its allohexaploid genome consists of three distinct sub-genomes (A, B and D) with a combined haploid genome size of ~16,000 million bps. The potential for genetic redundancy is high and this makes pinpointing the function of a specific gene and its alleles difficult. (2) Several of the pathogens affecting UK wheat production have a restricted host range, for example *Mycosphaerella graminicola* and *SBCMV* solely infect wheat, which may complicate the situation when rice and barley are used as the reference species. (3) *Tm* has escaped intensive breeding selection and most likely retained a high level of genetic diversity. (4) There are successful examples of novel traits transferred from *Tm* to wheat (Cox et al., 1991). The results of phenotype screens described in this paper suggest that genes conferring high levels of resistance to the tested pathogens reside in *Tm*. Thus, *Tm* is a rich source of novel traits, genes and alleles accessible to breeders.

The TILLING technique in combination with mutagenesis is particularly suitable for functional genomic studies in crop plants. This is because many novel alleles of a gene of interest can be identified in a high-throughput manner and then commercially exploited through conventional breeding. By combining a mutagenised diploid species, TILLING and phenotype screening it is possible to establish a particular resistance phenotype:variant allele relationship in a relative short time. The recover of a range of weak alleles for the candidate genes of interest may help gene function studies. Mutagenised hexaploid wheat populations

are currently being developed within the WGIN project for the spring cultivars 'Paragon' and 'Cadenza' (http://www.WGIN.org.uk/).

New approaches to achieve durable resistance to multiple pathogens in polyploid species

In the 20th Century, considerable success in wheat breeding for disease resistance came from the introgression of chromosomal regions containing resistance loci from its wild relatives. This route continues to prosper and by using molecular markers only the smallest alien segments are introgressed thus reducing the risk of co-introducing disadvantageous genes. However, new approaches are required to evaluate gene / allele functions and thereby advance the effective exploitation of the existing *T. aestivum* as well as the wild species germplasm used for trait introgression. The advantage of using the diploid wheat species *Tm* is that it should be possible to identify novel gene variants possessed by individual plants in a mutagenised population, and then to test these plants to determine whether specific shifts from resistance to susceptibility to each pathogenic species have occurred. Once a functional association has been established, the hexaploid wheat genotypes can then be surveyed by TILLING, to establish the gene variation that exists on the three homoeologous wheat genomes (AA, BB and DD). This will determine whether one, two or all three gene copies may be functional. Thus, through TILLING it should be possible to develop for plant breeders, precise 'within-the-gene' molecular markers, which can be used to ensure that functional alleles of key genes are retained in the elite germplasm and that weak / non-function gene variants are eliminated because they have the potential to weaken the resistance trait. For example, genes like *RAR1* and *SGT1* code for downstream signalling components. If a plant only possesses a weak or non-functional allele variant of defence signalling genes, then when a disease resistance gene is introgressed from an exotic source or from another elite by marker assisted selection, the desired resistance trait may either not be inherited or only weakly expressed because the crucial signalling components is missing in the recipient plant genome. This new approach should in the next few years provide plant breeders with a suite of tools and novel information on gene function to enable the gradual enhancement of hexaploid wheat's natural resistance to a range of pathogenic microbes.

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Plant activators / induced resistance elicitors – their time has come?

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ABSTRACT

Enhanced resistance to pathogen infection can be induced in plants by treatment with a variety of abiotic and biotic inducers, including plant growth promoting rhizobacteria, cell wall fragments, chemicals and water stress. The resistance induced by these agents (resistance elicitors) is broad spectrum and long-lasting, but is rarely expressed as complete control of infection. In fact, disease control provided by resistance elicitors is variable, with elicitors providing greater than 90% control in some situations and no control in others. This variability in efficacy is likely to be due, in part, to the influence of plant genotype and environment on the expression of induced resistance, but this area is little understood. If induced resistance is to become a useful part of mainstream crop protection, such interactions need to be better understood and work needs to be directed towards determining how best to incorporate induced resistance into crop protection programmes.

INTRODUCTION

Plants need a range of defence mechanisms in order to protect themselves against microbial pathogens. The co-ordination and timing of these defence mechanisms is crucial to effective defence. Early in the interaction between plant and pathogen, elicitor molecules are released. These molecules can be derived from the plant or the pathogen and include carbohydrate polymers, lipids, glycopeptides and glycoproteins. Following perception of these elicitor molecules by plant cells, a signalling pathway is activated, leading in turn to the formation of defence mechanisms (Walters *et al.*, 2005a). These defences can include production of reactive oxygen species (ROS), phytoalexin biosynthesis, formation of physical barriers e.g. reinforcement of the plant cell wall, and accumulation of pathogenesis related (PR) proteins (Walters *et al.*, 2005a).

In a plant with genetic resistance to a particular pathogen, recognition occurs quickly, defences are activated rapidly and the progress of pathogen infection is stopped. In a plant that is genetically susceptible to a particular pathogen, the crucial recognition process occurs very slowly. This means that defence activation occurs much later in the interaction, allowing successful pathogen infection and colonisation (Walters *et al.*, 2005a). Susceptible plants therefore, possess the genetic machinery for defence, but in interactions with a virulent pathogen, the defences are not activated quickly enough (Walters *et al.*, 2005a).

But triggering of defences does not necessarily require specific recognition of the pathogen or its products. Defences can be activated non-specifically by environmental stimuli like heat shock, water stress and cold stress (Walters *et al.*, 2005b), suggesting a general resistance response to a range of biotic and abiotic stress conditions.

INDUCED RESISTANCE

In fact, it is well known that treatment of plants with a variety of agents [e.g. virulent or avirulent pathogens, non-pathogens, cell wall fragments, plant extracts, synthetic chemicals] can lead to the induction of resistance to subsequent pathogen attack, both locally and systemically (Walters *et al.*, 2005a). This induced resistance can be split broadly into systemic acquired resistance (SAR) and induced systemic resistance (ISR).

Systemic acquired resistance (SAR)

SAR is characterised by a restriction of pathogen growth and a suppression of disease symptom development compared to non-induced plants infected with the same pathogen (Hammerschmidt, 1999). The onset of SAR is associated with an accumulation of salicylic acid (SA) at sites of infection and systemically and with the coordinate activation of a specific set of genes encoding PR proteins, some of which possess antimicrobial activity (Hammerschmidt, 1999). Treatment of plants with SA or one of its functional analogues e.g. S-methylbenzo[1,2,3]thiadiazole-7-carbothiate (acibenzolar-S-methyl; ASM), induces SAR and activates the same set of PR genes (Hammerschmidt, 1999). A central role for SA became apparent with the use of NahG transformants. NahG plants constitutively express the bacterial *NahG* gene which encodes the enzyme salicylate hydroxylase. This enzyme converts SA into inactive catechol and so transgenic plants expressing the *NahG* gene cannot accumulate SA. Crucially, tobacco and *Arabidopsis thaliana* plants expressing *NahG* show enhanced susceptibility to a wide range of fungal, bacterial and viral pathogens (see Pieterse *et al,* 2001). In addition, a number of *Arabidopsis* mutants *[e.g. sid1, sid2* and *pad4]* are defective in SA accumulation in response to pathogen infection and as a result, display enhanced susceptibility to various pathogens (see Pieterse *et al.*, 2001). Interestingly, transgenic NahG plants and the *sid1, sid2* and *pad4* mutants are incapable of developing SAR and do not exhibit PR gene activation following pathogen infection. These results indicate that SA is a necessary intermediate in the SAR signalling pathway.

Induced systemic resistance (ISR)

ISR develops as a result of colonisation of plant roots by plant growth promoting rhizobacteria (PGPR) and is mediated by a SA-independent pathway (Pieterse *et al.*, 2001). Saprophytic rhizosphere bacteria are present in large numbers on plant root surfaces and strains isolated from naturally disease suppressive soils, mainly *Pseudomonas* spp., have been shown to promote plant growth by suppressing soil-borne pathogens (Pieterse *et al.*, 2001). This activity can be the result of competition for nutrients, siderophore-mediated competition for iron, antibiosis, or secretion of lytic enzymes. Some of these bacterial strains reduce disease through ISR. ISR has been shown to function independently of SA and activation of PR genes, requiring instead jasmonic acid (JA) and ethylene (ET), since *Arabidopsis* mutants impaired in their ability to respond to JA or ET cannot express ISR (Ton *et al.*, 2001). ISR is not associated with increases in expression of known defence-

related genes, but following challenge with a pathogen, plants expressing ISR exhibit an enhanced expression of certain JA-responsive genes (Pieterse *et al.*, 2001).

INDUCED RESISTANCE: HOW EFFECTIVE IS IT?

Induced resistance offers the prospect of broad spectrum disease control using the plant's own resistance mechanisms. This has given rise to increasing interest in the development of agents which can mimic natural inducers of resistance (Walters *et al.*, 2005a). Of interest to researchers are elicitor molecules released during the early stages of the plant-pathogen interaction, the signalling pathways used to trigger defences locally and systemically and the use of PGPR. It is impossible in a paper of this size to deal with all of the elicitors of induced resistance that have been studied and so a few selected examples are described briefly below.

Induced resistance using synthetic chemicals

ASM is considered to be a functional analogue of SA and has been shown to elicit SAR in a wide range of plant-pathogen interactions (Vallad & Goodman, 2004). For example, there are reports of successful control of foliar pathogens of wheat using ASM (e.g. Stadnik & Buchenauer, 1999a, b). The latter authors found that ASM reduced powdery mildew infection on wheat by up to 77 %, whereas infection by *Septoria tritici* was reduced by between 46-52 %. They also found that use of ASM did not result in yield improvements compared to non-treated controls (Stadnik & Buchenauer, 1999a, b). However, although ASM provided control against a range of important pathogens on a number of crops, reductions in infection intensity usually ranged between 4-70 % (Vallad & Goodman, 2004).

Probenazole has been used in Asia for more than 20 years to control blast caused by *Magnaporthe grisea* and other diseases of rice, including bacterial blight caused by *Xanthomonas oryzae* (Iwata, 2001). The probenazole containing product Oryzemate provides long lasting control of rice blast and despite its extensive use since the 1970s, there have been no reports of resistance to the chemical in the blast fungus (Iwata, 2001). Early research suggested that probenazole activated defence responses in rice and this was confirmed by later work showing that probenazole and its active metabolite 1,2-benzisothiazole-1,1-dioxide induce SAR by triggering signalling at a point upstream of salicylic acid accumulation (Nakashita *et al.*, 2002).

Induced resistance using PGPR

A number of PGPR strains have provided effective control of various diseases in field trials. In field trials on a range of crops, PGPR provided control of plant disease ranging from 6-89 %, with the majority of studies showing reductions in disease severity of less than 80 % (Vallad & Goodman, 2004). Nevertheless, some PGPR strains proved to be remarkably effective under field conditions, providing consistently high levels of disease control. Thus, *Bacillus pumilus* INR-7 and *Serratia marcescens* 90-166 provided 86 % and 89 % control respectively, of the bacterial pathogen *Erwinia tracheiphila* on cucumber (see Vallad & Goodman, 2004).

Induced resistance using naturally derived compounds and other agents

Oligosaccharides are well known to act as elicitors of plant defences. For example, chitosan, a common polymer in shells of crustaceans, exoskeletons of insects and cell walls of fungi, has been shown to control pathogen infection in a range of crops (Hadwiger, 1999). Elexa™, a commercial formulation containing 4 % chitosan as its active ingredient, has been shown to reduce downy mildew severity by 58 % when used as a seed treatment, by 75 % when used as a foliar spray and by 77 % when used as a combined treatment (Sharathchandra *et al.*, 2004). Elicitors derived from the yeast *Saccharomyces cerevisiae* have also been shown to control plant disease. For example, yeast-derived elicitors reduced powdery mildew infection on barley by 85 % in field trials, although interestingly, elicitor applied with a reduced rate of fungicide gave better disease control than the elicitor used on its own (Reglinski *et al.*, 1994).

β-aminobutyric acid (BABA), a simple, non-protein amino acid, has been shown to induce broad spectrum disease resistance in many crops (Cohen, 2002). BABA activates a variety of resistance mechanisms, including callose deposition, lignification and hypersensitivity and has been reported to induce the formation of PR proteins in some plants, but not others (Cohen, 2002). Levels of disease control in the field using BABA range between 92 % against grape downy mildew to 78 % against late blight on potato (Cohen, 2002). It interacts synergistically with fungicides and plant activators like ASM, providing high level disease control, as well as control of pathogens against which BABA used singly is ineffective (Cohen, 2002). As a result, BABA may usefully be incorporated into disease management programmes.

VARIABILITY IN THE EFFICACY OF INDUCED RESISTANCE

The preceeding paragraphs indicate that the efficacy of induced resistance in the field is variable, with levels of disease control ranging from 4 % to greater than 90 %. This variability in efficacy is a serious impediment to the practical use of induced resistance and demands some explanation of the underlying mechanisms. Induced resistance is a complex plant response to pathogen attack and as such, will be modified by many factors including genotype and environment.

Influence of genotype on induced resistance

Perhaps surprisingly, little is known about the influence of genotype on induced resistance. Work using the synthetic chemical 2,6-dichloroisonicotinic acid (INA) showed that greatest protection against powdery mildew on cucumber was obtained in partially resistant cultivars (Hijwegen & Verhaar, 1994). Cultivar-dependent differences in the expression of induced resistance have also been reported in other systems e.g. soybean (Dann *et al.*, 1998), wheat (Stadnik & Buchenauer, 1999b) and barley (Newton *et al.*, 2003).

Influence of the environment on induced resistance

Abiotic stress is known to influence plant resistance to pathogens (Ayres, 1984). When Ayres (1984) reviewed the interaction between stress and plant disease he proposed two

possible outcomes of the interaction (1) the possibility that the negative effects of pathogens and abiotic stress might be additive and (2) that abiotic stress might alter plant resistance to pathogen infection. Indeed, water stress has been reported to enhance resistance to powdery mildew in barley (Ayres, 1984), while Wiese *et al.* (2004) found that osmotic stress and proton stress led to the induction of active defences against powdery mildew in barley.

It has been suggested that induced resistance is associated with costs to the plant, for example, a diversion of resources away from plant growth towards defence (Walters *et al.*, 2005b). If so, it seems reasonable to suggest that any constraints on the availability of such resources should affect the expression of induced resistance. Indeed, the magnitude of costs associated with induced resistance was found to be dependent on environmental conditions, including nitrogen, water stress and inter-plant competition (Dietrich *et al.*, 2005). An interesting observation from the latter work is that plants treated with ASM showed reduced growth rates during the first week following induction, but appeared to compensate thereafter by increasing growth rates (Dietrich *et al.*, 2005).

But not all studies have found an effect of nitrogen on the efficacy of induced resistance. Wiese *et al.* (2003) could find no effect of nitrogen on resistance induced by ASM against powdery mildew on barley in field trials. Instead, they found that resistance induced by ASM was most profoundly affected by the organic matter content of the soil, with soils containing low organic matter content exhibiting the greatest induction of resistance. The authors suggested that high microbial activity in soils with high organic matter content may have led to a high degree of resistance, possibly induced by rhizobacteria and which could not be further enhanced by ASM (Wiese *et al.*, 2003). In soils with low organic matter content and hence low microbial activity, any rhizobacteria-induced resistance would be much less, allowing ASM induced resistance to be expressed.

INDUCED RESISTANCE: HAS ITS TIME REALLY COME?

Induced resistance has the potential to revolutionise disease control in crops. It utilises the plant's own resistance mechanisms, it has broad spectrum activity and the resistance induced can be long lasting. But after decades of research and many thousands of papers, induced resistance remains on the sidelines of mainstream crop protection. Why? The answer lies in much of what has been presented above. It is inconsistent, providing high levels of disease control in some situations, but not others and it rarely provides levels of control that can be achieved with modern fungicides. But perhaps we are asking too much of induced resistance. In the western world, we have come to expect very high levels of disease control provided by fungicides. But in many parts of the world, agriculture is practised differently, with different pressures, different diseases and different levels of resources. Even in the west, there are many crop-pathogen interactions for which there are no effective control measures. There is increasing concern for the environment and as a result a desire to reduce pesticide use. There is also the ever-present problem of fungicide resistance. Viewed from this perspective, induced resistance could be useful. So what needs to be done in order to move induced resistance from the sidelines and into mainstream crop protection?

There is a real need for information on and understanding of the effects of genotype and environment on the expression of induced resistance and its efficacy in the field. Although it is possible that induced resistance could be used on its own to control certain diseases, for which no other effective control exists, it is more likely that induced resistance will be incorporated into crop protection programmes. However, this will require information on how best to fit it into existing programmes for particular crops and diseases. There is much work demonstrating the effectiveness of combining fungicides and agents that elicit induced resistance, either alternating their use in the same programme or applying them together (see Vallad & Goodman, 2004). Combined use of induced resistance and fungicides should extend the effectiveness and lifespan of fungicides. However, work by Bousset & Pons-Kuhnemann (2003) on the effects of ASM on the population structure of *Blumeria graminis* f.sp. *hordei* showed that although ASM alone did not exert a direct effect on the genetic composition of the pathogen population, its combined use with fungicide (ethirimol in this case) had a greater effect on composition of the pathogen population than use of fungicide alone. Clearly, the effect of induced resistance on pathogen population biology is an important area for future investigation.

Although a great deal is known about the mechanisms underlying resistance induced by prior inoculation with necrotizing pathogens and use of plant activators like ASM, much less is known (in some cases nothing is known) about the mechanisms underlying resistance induced by other agents. This is an important area for future work, which will be greatly aided by developments in gene array technologies. In some recent work, von Rad *et al.* (2005) studied the induction of defence genes in *A. thaliana* treated with a number of agents that induce resistance, including ASM, natural plant extracts and microbial extracts. Using a microarray based on 750 genes involved in several aspects of plant defence and/or stress, they found that rather than mimicking one of the known defence pathways induced by SA or JA, the response to the inducers showed aspects of both major defence systems. A common feature was transient activation of JA biosynthesis genes, combined with a more sustained SA-associated induction of defence genes. These results highlight the both the complexity of plant responses to different inducers and how little we know about these responses. This lack of understanding is further highlighted by research on wheat using a microarray of 600 genes (Pasquer *et al.*, 2005). These workers found that under glasshouse conditions, ASM induced expression of defence-related genes very strongly, while two fungicides, azoxystrobin and fenpropimorph, also induced defence gene expression, albeit weakly. Moreover, when plants were grown under field conditions, defence-related genes were already expressed at a high level before ASM or fungicides were applied (Pasquer *et al.*, 2005). These data demonstrate the importance of plant growth conditions for studying the effects of agrochemicals and lends support to the suggestion that in the field, plants may always be induced, although they may not be fully induced (Hatcher & Paul, 2000). These results highlight the need for research on mechanisms to be undertaken in the field. Carrying out meaningful mechanistic research on plant defence under field conditions is not easy, but the availability of molecular tools makes it less difficult than previously. By understanding the pathways activated and resistance mechanisms triggered by different agents, it should be possible to use cocktails of elicitors to provide effective and reliable protection.

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Metabolic diversity in plants

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ABSTRACT

Natural products protect plants against pests and pathogens. They can also have positive or negative effects on crop quality. In addition to their functions in plants they are exploited for commercial use as drugs, dyes, flavourings, perfumes and for a variety of other purposes. Many of these compounds have complex chemical structures and their synthesis involves multi-step pathways. The genetic and biochemical dissection of natural product pathways in plants, many of which are completely uncharacterised, represents a substantial challenge. We have recently shown that genes for at least five unrelated enzymes required for the synthesis of defence-related natural products are physically clustered and co-expressed in oat. The genetic determinants of this natural product pathway therefore have features in common with bacterial operons. Although the genes for other characterised natural product pathways are generally unlinked, there is increasing evidence to suggest that functional gene clusters of this kind may be more common in plants than has previously been anticipated. These findings will facilitate the dissection and manipulation of natural product pathways.

INTRODUCTION

Plants produce a huge array of natural products (Dixon, 2001), many of which are specialised metabolites associated with particular species. These secondary metabolites often have important ecological roles, facilitating pollination and seed dispersal and/or providing protection against attack by pests and pathogens (Wink, 2003). Although the ability of plants to perform *in vivo* combinatorial chemistry by mixing, matching and evolving the genes required for different secondary metabolite biosynthetic pathways is likely to have been critical for survival and diversification of the Plant Kingdom we know very little about the mechanisms underpinning this process. The current explosion of platform technology-derived information, in combination with classical genetics and other complementary "traditional" strategies, now offers an unprecedented opportunity for intensification of investigations of secondary metabolism in reference, crop and other species. This will allow us to gain a better understanding of the function and synthesis of plant natural products and of the origins of metabolic diversity. In addition to their ecological functions, natural products often influence crop quality (in desirable or undesirable ways) by affecting flavour, benefit to human or animal health and/or amenability to processing. They also have a wealth of other applications, for example as pharmaceuticals. These compounds are therefore of considerable strategic importance for crop protection and improvement and for their commercial value.

THE TERPENES – A CASE STUDY

The terpenes are one of the largest and most diverse groups of plant natural products (Chappell, 2002). The terpene group includes sterols and triterpenes, compounds that are synthesized from mevalonate and that often accumulate in plants as glycosides. These glycosides (saponins) have a range of diverse biological activities that include antimicrobial, anti-feedant and allelopathic activity and have been shown to contribute to plant defence (Morrissey & Osbourn 1999; Papadopoulou *et al.*, 1999; Haralampidis *et al.*, 2001; Bouarab *et al.*, 2002). Saponins also have a variety of important pharmacological applications, for example as anti-cancer agents (Hanausek *et al.*, 2001; Haridas *et al.*, 2001a,b). The biological properties, structural complexity and metabolic diversity of these molecules across the Plant Kingdom make them an exciting and important challenge for natural product biology. The study of natural product pathways also has much broader implications for fundamental processes. For example, by understanding the mechanisms underpinning metabolic diversity we can expect to learn more about genome plasticity, diversification of function of enzymes and multi-component pathways, and about adaptive evolution. This article will focus on the triterpene glycosides and will consider recent progress in this field.

Triterpene glycosides appear to be absent from the model plant species *Arabidopsis* and rice although exhaustive analysis of large collections of accessions is required in order to be confident of this. Progress in characterizing the biosynthetic pathways for these compounds has consequently been slow until recently. Now, however, new developments are allowing major inroads to be made in this area. These include advanced chromatographic and spectroscopic techniques that allow more effective separation and analysis, so paving the way for profiling and identification of these structurally complex amphiphilic compounds in plant extracts (Marston *et al.*, 2000; Huhman & Sumner, 2002; Joshi *et al.*, 2002; Dixon & Sumner, 2003). EST analysis represents a relatively inexpensive and highly effective means of gene discovery and is ideal for investigating secondary metabolism in diverse plants ranging from models through to crop plants and exotic species (Ohlrogge & Benning, 2000). This approach is particularly effective when coupled with knowledge of the site and/or conditions under which specific metabolites are synthesised. For example, we have used an oat root tip EST collection to isolate sequences encoding enzymes involved in the synthesis of defence-related triterpene glycosides (avenacins) in oat (Haralampidis *et al.*, 2001). Avenacins are produced constitutively and are localised in the epidermal cells of the root tip (Haralampidis *et al.*, 2001). Sophisticated single-cell sampling technologies hold considerable potential for analysis of plant natural products that are synthesized in specific organs or cell types at the DNA, RNA, protein and metabolite levels (Tomos & Sharrock, 2001; Kehr, 2003). Synthesis of triterpene glycosides in *Medicago truncatula* can be induced by methyl jasmonate in cell suspension cultures, providing a powerful route for isolation of saponin biosynthesis genes using microarray-based approaches (Suzuki *et al.*, 2002).

Fully sequenced plant genomes allow global surveys of gene content and gene expression and also provide valuable information about genomic context. Opportunities to survey complete plant genome sequences are currently restricted to *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000) and rice (The Rice Genome Sequencing Project, 2005) although the amount of genomic sequence data available for *M. truncatula* (Bell *et al.*, 2001) and other plant species is increasing rapidly. *Arabidopsis* enzymes capable of the synthesis of simple triterpenes when expressed in yeast have been identified based initially on homology searches of the genome, although the functional significance of these enzymes *in planta* remains to be established

(Husselstein-Muller *et al.*, 2001; Herrera *et al.*, 1998; Kushiro *et al.*, 2000a; Ebizuka *et al.*, 2003; Fazio *et al.*, 2004).

The generation of mutant collections represents an extremely powerful way of dissecting natural product pathways and their regulation and does not require any *a priori* information about the pathway components. However detection methods for triterpene glycosides are problematic, generally relying on relatively non-specific chromogenic stains or on HPLC-based methods (Hostettmann & Marston, 1995; Marston *et al.*, 2000). An exception is the oat root saponin avenacin A-1, which is esterified with *N*-methyl anthranilate and so fluoresces bright blue under ultra-violet illumination. This property, which is highly unusual amongst triterpenes, has enabled simple screens based on reduced root fluorescence to be used to generate a valuable resource of biosynthetic mutants following chemical mutagenesis of diploid oat (Papadopoulou *et al.*, 1999). Remarkably, genetic analysis of these mutants has revealed that the genes for triterpene glycoside biosynthesis are clustered and map to a region of the oat genome that is not conserved in other cereals (Qi *et al.*, 2004). Subsequent investigation of bacterial artificial chromosome (BAC) contigs spanning this region has revealed that genes for at least five unrelated enzymes in the pathway are physically clustered (Osbourn *et al.*, unpublished data). Current evidence indicates that the cluster has not been introduced into oat by horizontal gene transfer, and that the biosynthetic components that have been characterised to date have arisen relatively recently by shuffling and accelerated evolution of existing components of the genome (Qi *et al.*, 2004; Osbourn *et al.*, unpublished data). One of the major challenges now is to understand how this gene cluster was formed.

Mechanisms that act to disperse genes are well known in eukaryotes and genes associated with common metabolic pathways are generally unlinked. This raises the question of how and why gene clusters of the kind that we have observed are maintained in the genome. One obvious explanation is that clustering will facilitate inheritance of the genes for the pathway components as a functional unit and so may be favoured by selection. Disruption of the gene cluster may also have additional detrimental effects by leading to accumulation of deleterious intermediates. Oat mutants that are defective in triterpene glycosylation have aberrant root morphology, an observation that is consistent with the latter hypothesis (Papadopoulou *et al.*, 1999; Mylona & Osbourn, unpublished data). A further benefit of clustering is that it is likely to facilitate co-regulation of gene expression at the higher order levels of chromatin and nuclear organisation (Finnegan *et al.*, 2004; Hurst *et al.*, 2004; Qi *et al.*, 2004; Williams & Bowles, 2004). This may be particularly important for secondary metabolism since synthesis of natural products in plants is usually under strict control. This is certainly the case in oat, where we have shown that the gene for the first committed enzyme in the triterpene glycoside pathway is expressed specifically in the epidermal cell layer of young roots coincident with the tissue-specific localisation of the avenacins (Haralampidis *et al.*, 2001). The other genes in the pathway that we have characterised are also expressed preferentially in the root tips.

Within the cereals and grasses the ability to synthesise avenacins (and apparently triterpene glycosides in general) is restricted to oat (*Avena* species) (Hostettmann & Marston, 1995). Introduction of this pathway into other major cereal crops such as wheat, barley, maize and rice has clear potential for improved broad-based disease resistance in crops. The fact that the genes are physically clustered in the oat genome has greatly facilitated identification of new pathway components. It may also prove possible to exploit this feature to transfer the complete pathway into other species using interspecies hybridisation strategies or, with improved technology, by transformation.

"OPERONS" IN PLANTS – A COMMON THEME?

In bacterial genomes functionally related genes are often clustered and controlled as units. These co-regulated functional units are referred to as operons. Until recently the genomes of eukaryotes have generally been regarded as "disorganised" with randomly distributed genes. The exceptions include repeats of similar sequences that have arisen by tandem gene duplication (e.g. genes encoding leucine-rich repeat proteins required for race-specific disease resistance in plants). However there is increasing evidence to suggest that the order of genes along chromosomes in many eukaryotes is not random. As outlined above, we have demonstrated that genes for the synthesis of defence-related natural products in oat show complete genetic co-segregation and are physically clustered in the genome (Qi *et al.*, 2004; Osbourn *et al.*, unpublished data). These genes are co-expressed in the root tips, the site of synthesis of the compounds. The genetic determinants of this natural product pathway therefore have some "operon-like" features.

To date, the genes that have been characterised for natural product pathways in plants have generally been found to be unlinked (such as those for anthocyanin biosynthesis), although genetic approaches have only been applied to a limited number of pathways. However there is at least one other well-characterised example of a gene cluster for a natural product pathway in plants – that of the benzoxazinoids, compounds that are produced by maize and some other cereals, that are structurally distinct from triterpenes, and that, like the oat compounds, are involved in plant defence (Frey *et al.*, 1997; Gierl & Frey, 2001; Frey *et al.*, 2003). Hints are also emerging from *Arabidopsis* and other plants that this phenomenon may be more common than first anticipated (Aubourg *et al.*, 2002; Lange & Ghassemian, 2003; Lee & Sonnhammer, 2003; Hurst *et al.*, 2004). It seems increasingly likely that functional gene clusters that exist as co-adapted gene complexes will emerge as a common theme in plant genomes. These may include not only gene clusters for complete natural product pathways but also clusters that determine other adaptive traits.

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