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REVIEW PAPER

Mutation discovery for crop improvement

Martin A. J. Parry^{1,*}, Pippa J. Madgwick¹, Carlos Bayon¹, Katie Tearall¹, Antonio Hernandez-Lopez¹, Marcela Baudo¹, Mariann Rakszegi², Walid Hamada³, Adnan Al-Yassin⁴, Hassan Ouabbou⁵, Mustapha Labhilili⁶ and Andrew L. Phillips¹

¹ Centre for Crop Genetic Improvement, Department of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

² Agricultural Research Institute of the Hungarian Academy of Sciences, PO Box 19, 2462 Martonvásár, Hungary

³ INAT, 43 Avenue Charles Nicolle, Cite Mahrajene Tunis, Tunisie

⁴ NCARE, PO Box 639 Baqa 19381, Amman, Jordan

⁵ INRA, BP 589, CRRA, Settat, Morocco

⁶ INRA, UR de Biotechnologie, Boulevard de la Victoire, Rabat, Morocco

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Abstract

Increasing crop yields to ensure food security is a major challenge. Mutagenesis is an important tool in crop improvement and is free of the regulatory restrictions imposed on genetically modified organisms. The forward genetic approach enables the identification of improved or novel phenotypes that can be exploited in conventional breeding programmes. Powerful reverse genetic strategies that allow the detection of induced point mutations in individuals of the mutagenized populations can address the major challenge of linking sequence information to the biological function of genes and can also identify novel variation for plant breeding. This review briefly discusses recent advances in the detection of mutants and the potential of mutagenesis for crop improvement.

Key words: Breeding, crop, mutagenesis, TILLING, wheat.

Introduction

Increasing crop yields to ensure food security is a major challenge. Amongst the obstacles against this are the changing climate (increasing temperatures and more erratic rainfall) which most often compromise crop productivity (Parry *et al.*, 2005) and the need to produce additional food and crops for bioenergy whilst minimizing the carbon costs of production (Powlson *et al.*, 2005). There is therefore an urgent requirement for new higher yielding varieties (Parry *et al.*, 2007; Reynolds *et al.*, 2009) with improved nutrient (Lea and Azevedo, 2006) and water use efficiency (Richards, 2000).

In this century, there has been a dramatic increase in the amount of genome sequence data available for world major food crops, their pests and pathogens. Complete genome sequences have been reported for rice (Matsumoto *et al.*, 2005) and sorghum (Paterson *et al.*, 2009) and also for

several crop pathogens (e.g. *Agrobacterium tumefaciens*, Wood *et al.*, 2001; *Phytoplasma*, Oshima *et al.*, 2004; *Fusarium graminearum*, Cuomo *et al.*, 2007; *Magnaporthe grisea*, Dean *et al.*, 2005). For the other major global crop, wheat, and other crop pests and pathogens the sequences of expressed sequence tags have become available. The exploitation of these sequence data for crop improvement is limited by the complexity of many of the traits that determine agronomic performance (Parry *et al.*, 2005; Parry and Reynolds, 2007). However, reverse genetics approaches allow progress to be made on the major challenge of linking sequence information to the biological function of genes and on determining their contribution to important characters and traits. Typically, these approaches rely on the disruption of candidate genes by mutagenesis, transposons, and T-DNA tagging or RNA interference (RNAi).

* To whom correspondence should be addressed: E-mail martin.parry@bbsrc.ac.uk

Abbreviations: RNAi, RNA interference; EMS, ethylmethane sulphonate; MNU, *N*-nitroso-*N*-methylurea; TILLING, Targeting Induced Local Lesions In Genomes; HRM, High resolution Melt analysis.

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Exploiting natural or induced genetic diversity is a proven strategy in the improvement of all major food crops, and the use of mutagenesis to create novel variation is particularly valuable in those crops with restricted genetic variability. Historically the use of mutagenesis in breeding has involved forward genetic screens and the selection of individual mutants with improved traits and their incorporation into breeding programmes. Over the past 70 years, more than 2500 varieties derived from mutagenesis programmes have been released, as listed in the IAEA/FAO mutant variety database, including 534 rice lines, 205 wheat lines, and 71 maize lines (<http://www-infocris.iaea.org/MVD/>). Although this approach has clearly proved very successful, there are limitations imposed by, for example, the difficulty of identifying a small number of individuals with novel phenotypes within a large population, or by the genetic redundancy present in many plant species as a result of gene duplication and polyploidy, such that many mutations have no detectable effect on the plant. Recently, reverse genetic approaches have permitted the silencing or interruption of individual candidate genes, providing the opportunity to investigate gene function and to relate sequence information to traits. However, these approaches have disadvantages: methods based on post-transcriptional gene silencing, such as RNAi, have variable success rates and rely on time-consuming vector construction and plant transformation. T-DNA insertional mutagenesis is also dependent on efficient plant transformation, while insertional mutagenesis by endogenous transposons is only available in a small number of crops, notably maize, although there has been some success in transferring these into other species such as rice (Kolesnik *et al.*, 2004). In any case, these insertional methods are likely to result in the complete disruption of gene function rather than in generating allelic series of mutants with partial loss-of-function, and thus will not produce the range of mutation strengths necessary for crop improvement. Furthermore, the insertion sites within the genome may not be distributed randomly (Zhang *et al.*, 2007), increasing the number of insertion lines required for full genome coverage to unrealistic levels. Chemical or physical mutagenesis have a number of advantages over such approaches, as mutagens introduce random changes throughout the genome, generating a wide variety of mutations in all target genes, and a single plant can contain a large number of different mutations, resulting in manageable population sizes.

Whilst mutations occur spontaneously in nature, the frequency of such mutations is too low to rely on alone for accelerated plant breeding. However, mutations can also be induced by physical and chemical mutagens and are applicable to all plant and animal species. Mutations may be gross, resulting in large-scale deletions of DNA, or only involve point mutations. Mutation can be induced by irradiation with non-ionizing (e.g. UV) or ionizing radiation (e.g. X and gamma rays, alpha and beta rays, fast and slow neutrons); such physical mutagens often result in the larger scale deletion of DNA and changes in chromosome structure. By contrast, chemical mutagens most often only affect single

nucleotide pairs. For plants, some of the more widely used mutagens include ethylmethane sulphonate (EMS), methylmethane sulphonate (MMS), hydrogen fluoride (HF), sodium azide, *N*-methyl-*N*-nitrosourea (MNU), and hydroxylamine. The degree of mutation is dependent on the tissue and degree of exposure (dosage \times time). Mutations at single nucleotide pairs are generally of the most interest to breeders because large-scale changes to chromosome structures usually have severely negative results. However, the use of mutagens that alter chromosome structure to increase the number of recombination events and break undesirable linkages is also extremely valuable.

Critically, mutations in important traits or genes (e.g. in nutritional quality, resource use efficiency, architecture or phenology) can be readily exploited by plant breeders without the legislative restrictions, licensing costs, and societal opposition applied to GM approaches. This is despite the fact that transcriptomic analyses have shown that large-scale plant mutagenesis may induce greater changes in gene expression patterns than transgene insertion (Batista *et al.*, 2008). This review briefly discusses recent advances in the detection of mutations and the potential of this approach for crop improvement.

Mutagenized populations

The creation of mutagenized populations forms the foundations of the reverse genetic approaches, although collections of accessions containing natural polymorphisms can also be used. To ensure that any gene of interest carries sufficient significant mutations, the populations of induced mutations may need to be very large; the size required is dependent on the dosage of mutagen and the level of gene duplication created by recent or ancient polyploidization events. Mutant populations have now been created for many cereal crops, including rice (Suzuki *et al.*, 2008), maize (Till *et al.*, 2004b), sorghum (Xin *et al.*, 2008), barley (Caldwell *et al.*, 2004; Talamè *et al.*, 2008), and both hexaploid bread wheat (Slade *et al.*, 2005; A Phillips *et al.*, unpublished data) and durum wheat (MAJ Parry *et al.*, unpublished data), mostly created by treating seeds or pollen with chemical mutagens (Weil, 2009). To increase the efficiency of mutation detection it is advisable to optimize mutagen dose to achieve a high mutation rate while avoiding serious effects on germination and plant development. In diploid species this can be difficult as even relatively low levels of mutation can result in the almost complete loss of male or female fertility, resulting in a need for very large populations if saturated coverage is required (Table 1). By contrast, polyploid species such as wheat have a very high tolerance of mutations due to the complementation of essential genes by homeologous copies and thus populations saturated with mutations can be much smaller, resulting in more cost-effective screening for mutations. However, a consequence of this genetic buffering is that recessive mutations in single homeologues of genes in polyploid species are less likely to show a phenotype, and it becomes necessary to identify

mutations in each homeologous copy of the target gene and bring these together by crossing. The choice of chemical mutagen will also influence the maximum permissible mutation rate achievable: EMS creates a larger proportion of non-sense mutations, involving the introduction of novel stop codons, than a mutagen such as MNU, due to the specificity of EMS in creating mainly G–A and C–T transitions, and any individual mutations is therefore more likely to have a phenotypic effect. This may partly explain the high mutation frequency achieved in rice by Suzuki *et al.* (1997) using MNU (Table 1), although the mutation rate in this population was also enhanced by the selection of M_1 plants with reduced fertility, which had previously been shown to be associated with higher rates of visible phenotypes in the M_2 .

The general strategy used to create a mutant population of self-fertilizing crops (e.g. barley, durum, wheat, and rice) as a resource for both forward and reverse genetic approaches is indicated in Fig. 1. Most mutagenized populations are generated by exposing seeds (M_0) to the

mutagen and allowing the resultant M_1 plants produced to self-fertilize and give rise to M_2 seed. The seeds must be exposed to sufficient mutagen to ensure a high level of mutations but without affecting viability and fertility. To ensure the greatest number of unique novel mutations it is recommended that only one seed is taken from each M_1 plant. Leaf material is taken from the resultant M_2 plants for the isolation of genomic DNA that is used as the resource for mutation detection. The M_2 plants are allowed to mature and the M_3 seeds are archived so that gene function can be studied in any plants in which mutations are identified. However, at this stage, the population is still segregating and not all M_3 plants will carry the mutations identified in the M_2 parent. The mutagenized population may be taken through further generations by single seed descent to generate near-homozygous material ($\sim 3\%$ heterozygous mutations at M_6), although up to half of all mutations present in the M_1 are lost in the process (Fig. 1). The mutagenized populations also form a valuable resource for forward genetic screening approaches for traits such as

Table 1. Estimated numbers of mutagenized lines required to identify a truncation mutation in mutagenized populations of different plant species

The table shows the number of lines screened to be 95% confident of identifying a non-sense (truncation) mutation in a typical 1.0 kbp of coding region, assuming that such mutations represent 5% of the total.

Species	Ploidy	Mutagen	Mutation frequency (per 10^6 bp)	Number of lines required	Reference
<i>Arabidopsis</i>	Diploid	EMS	3.3	$\sim 18\,000$	Greene <i>et al.</i> 2003
Rice	Diploid	MNU	7.4	$\sim 8\,000$	Suzuki <i>et al.</i> , 2008
Barley	Diploid	EMS	1.0	$\sim 60\,000$	Caldwell <i>et al.</i> , 2004
		NaN_3	2.6	$\sim 23\,000$	Talamè <i>et al.</i> , 2008
Durum wheat	Tetraploid	EMS	25	$\sim 2\,400$	Slade <i>et al.</i> , 2005
Bread wheat	Hexaploid	EMS	42	$\sim 1\,400$	Slade <i>et al.</i> , 2005

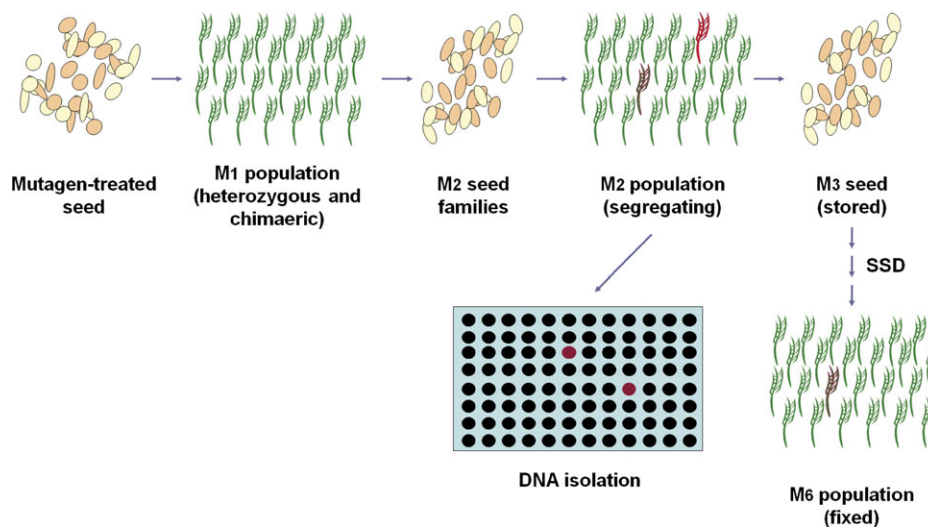


Fig. 1. The general strategy used to create a mutant population as a resource for forward and reverse genetic approaches. The seeds must be exposed to sufficient mutagen to ensure a high level of mutations without affecting viability and fertility; for diploid species much larger M_1 populations may be required to achieve saturated coverage of the genome. The M_2 and successive generations are produced by self-fertilization under single-seed descent, resulting in $\sim 97\%$ of the mutations being homozygous at M_6 , while losing about half of all mutations through segregation.

plant architecture (Fig. 2), yield, quality, resource use efficiency, stress tolerance, and pest and pathogen resistance. Detailed and comprehensive documentation of phenotypes is essential in order to increase the opportunity for the incorporation of novel traits into breeding programmes (Talamè *et al.*, 2008).

Mutant lines, whether identified by forward or reverse screening, will also be carrying numerous additional mutations in addition to that selected. For a diploid species such as rice with its relatively small genome the number of extraneous mutation may well be in the tens of thousands, and in the case of a mutation-tolerant species such as bread wheat, it is estimated that in any one M_2 individual within our population the total number of mutations may approach half a million. While the vast majority of these probably have no effect, it is obviously unwise to conclude that any observed phenotype observed at M_3 or later generations is due to the single point mutation under study. At the very least, it is essential to show genetic linkage of the mutation and phenotype, and some degree of back-crossing will almost certainly be necessary.

Mutation detection by TILLING

A number of techniques have been developed that can be used to exploit mutagenized populations and diversity collections through identification of point mutations and

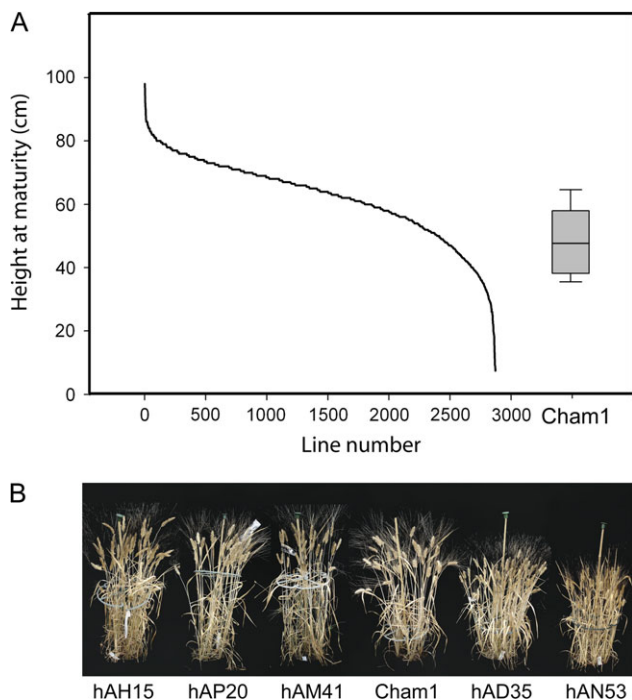


Fig. 2. (A) Stature profile of the M_4 generation of an EMS-mutagenized population of durum wheat cv. Cham1, ranked in order of height. A box plot of the range (bars), quartiles (box), and mean of plant height within a group of unmutagenized parental plants is also shown. (B) Examples of stature phenotypes within the Rothamsted durum wheat EMS population of Cham 1.

small indels in specific genes. Although a number of different methods of genotyping single nucleotide polymorphisms are available, having been developed largely for human studies, low allele frequencies at individual loci within most mutagenized populations is a major issue and thus sensitivity and throughput at reasonable cost are important factors in the selection of a suitable platform. One method that has been used is denaturing HPLC (DHPLC), a chromatographic technique that can discriminate between homoduplex and heteroduplex (mismatched) DNA, generated by re-annealing a mixture of wild-type and mutant DNA fragments. This was first used to detect single nucleotide polymorphisms in the human Y chromosome (Kuklin *et al.*, 1997) and was adopted by McCallum and colleagues, working at the University of Washington in Seattle, to identify point mutations in an EMS-mutagenized population of *Arabidopsis* (McCallum *et al.*, 2000a, b). These authors introduced the acronym TILLING (Targeting Induced Local Lesions IN Genomes) to describe their overall approach. To ensure the presence of heteroduplex DNA from lines homozygous for mutations, DNA samples from several lines were pooled up to 5-fold, thereby also increasing the throughput of the technique. However, a serious limitation of DHPLC was the number of samples that could be processed concurrently, as most instruments possess only a single HPLC column.

To increase sample throughput for population screening, the Seattle group established endonuclease cleavage for the detection of mismatches in heteroduplexes. The principle had been demonstrated by Oleykowski *et al.* (1998), who showed that many plant extracts contained endonucleases that specifically cleaved on the 3' side of mismatched bases in double-stranded DNA. Celery was selected as the source of the enzyme ('Cell') due to ready availability and mismatch detection was established using Cell cleavage of synthetic double-stranded oligonucleotides, the radiolabelled fragments being separated on denaturing polyacrylamide gels. It was also shown that polymorphisms in PCR-amplified exons of the human *BRCA1* gene could be detected, using Cell cleavage of fluorescent-labelled heteroduplex DNA in conjunction with a gel-based sequencer. The method was shown to be capable of detecting a range of different point mutations and also small deletions and insertions. The Seattle TILLING group developed this method further and showed that Cell digestion of heteroduplexes was sufficiently sensitive to allow large-scale screening of mutant populations of *Arabidopsis*: DNA samples from individual plants could be pooled up to 8-fold, representing 16-fold dilution of heterozygous mutations, without loss of detection sensitivity (Colbert *et al.*, 2001). Using fluorescent labelled primers on a Li-Cor 4300 DNA Analyzer, up to 96 pools, or 768 samples, could be analysed in a single run, although a subsequent TILLING analysis of each of the samples within a positive pool was necessary to deconvolute the pools and identify the mutated individuals. The use of fluorescent labelling, with different fluorophores on the forward and reverse primers, allowed the products of heteroduplex cleavage to be detected in

separate channels of the DNA analyser and thus provided confirmation of the mutation as the sum of the fragment sizes should equal that of the original amplicon, while the sizes of the respective fragments yielded the approximate position of the mutation within the amplicon, facilitating subsequent identification by sequencing. Pooling of DNA samples, as described above, allows efficient screening of even large mutagenized populations such as may be required for saturated coverage of diploid organisms. However, at the higher mutations frequencies that are achievable in polyploid species such as wheat, a pooling depth of 2-fold has been found to be most efficient, as this generates a realistic number of mutations per run and the lines within a positive pool can each be sequenced to identify the mutation without further TILLING. An example of a TILLING gel used to identify mutations in the *TaGA20ox1A* gene within an EMS-mutagenized population of bread wheat is shown in Fig. 3.

Under a grant from the NSF, the Seattle group offered training courses in TILLING which promoted the rapid spread of the technology into other model and crop plants

and in animal species. Thus, there now exist TILLING platforms and associated mutagenized populations in *Lotus* (Perry *et al.*, 2003), pea (Triques *et al.*, 2007), sorghum (Xin *et al.*, 2008), soybean (Cooper *et al.*, 2008b), oilseeds (Wang *et al.*, 2008), bread wheat (Slade *et al.*, 2005; A Phillips *et al.*, unpublished data), durum wheat (MAJ Parry *et al.*, unpublished data), barley (Caldwell *et al.*, 2004; Talamè *et al.*, 2008), rice (Suzuki *et al.*, 2008), maize (Till *et al.*, 2004b), *Drosophila* (Winkler *et al.*, 2005; Cooper *et al.*, 2008a), and zebrafish (Moens *et al.*, 2008).

Various groups have modified or improved upon the Cel1-based TILLING method to increase its fidelity or throughput. A range of different mismatch cleavage enzymes were tested by Till *et al.* (2004a), who showed that even very crude plant extracts could be used successfully. Triques *et al.* (2008) tested five different single-strand-specific nucleases from *Arabidopsis* by transient heterologous expression in leaves of *Nicotiana benthamiana* and showed that Endo1, an enzyme closely related to Cel1, was equally efficient at detecting all types of mutation. Within our wheat TILLING programme at RRes, Endo1 has been assessed against both commercial preparations and crude extracts of Cel1 and the former enzyme was found to give more consistent results (C Bayon, unpublished results). A number of different platforms for the separation and detection of the endonuclease cleavage products has also been developed. Capillary electrophoresis on platforms such as the ABI 3730 DNA sequencer has advantages of automation and, in principle, more sensitive detection of mutations, and has been established for TILLING by several groups (Cross *et al.*, 2008; Suzuki *et al.*, 2008). At the other extreme, agarose gel electrophoresis of unlabelled Cel1 cleavage products is also possible (Raghavan *et al.*, 2007), rendering the TILLING process feasible without major capital expenditure.

A number of bioinformatics tools has been created to aid in the selection of amplicons for TILLING and in the prioritization of the resulting mutations for subsequent analysis. CODDLE (www.proweb.org/coddle/) uses alignments of related sequences and takes into account both the mutagen used and the coding sequence to identify regions of the target gene most likely to generate deleterious mutations; CODDLE sends this information to Primer3 (Rozen and Skaletsky, 2000) to generate suitable primers. GelBuddy (www.proweb.org/gelbuddy/) can be used to automate band calling in the electrophoretic gel images, while PARSESNP (www.proweb.org/parsesnp/) analyses the resulting sequenced mutations and attempts to predict the likely consequence for gene function using a protein homology model.

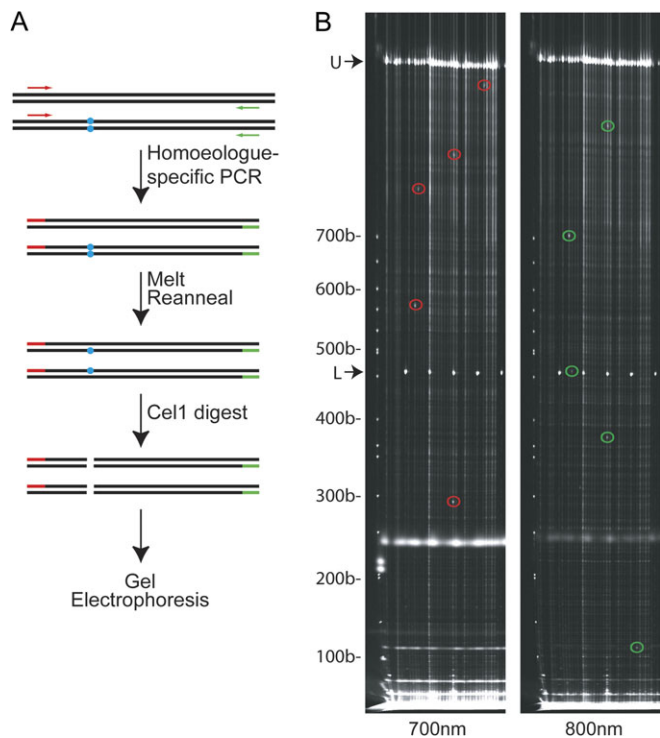


Fig. 3. Overview of mutation detection in wheat by Cel1-based TILLING. (A) Schematic of PCR and Cel1 digestion. (B) Typical TILLING run in wheat. The target was 1289 bp of the bread wheat *GA20ox1A* gene, amplified using homeologue-specific primers labelled with the Li-Cor dyes IRD-700 and IRD-800. The DNA samples were from 80 M_2 lines of an EMS-mutagenized population of bread wheat cv. Cadenza, pooled 2-fold. After PCR, melting/re-annealing, Cel1 digestion and clean-up, the 40 samples were run on a Li-Cor 4300 Genetic Analyser; fragments generated by Cel1 are ringed in the 700 nm (red) and 800 nm (green) channels. Lane (L) and size markers were also loaded onto the gel and the full-length (undigested) PCR product is indicated (U).

Alternative mutation detection platforms

Although Cel1-based TILLING is very efficient for detecting mutations in large (1–2 kbp) exon-rich amplicons from target genes, it is less productive when used to screen genes with multiple small exons separated by larger introns, as

mutations in introns, except those at splice junctions, rarely affect gene function. High-resolution melt analysis (HRM) has been established as an alternative screening platform for such targets. HRM depends on the loss of fluorescence from intercalating dyes bound to double-stranded DNA during thermal denaturation (Ririe *et al.*, 1997). Accurate control of temperature and continuous monitoring of fluorescence in instruments such as the Lightscanner (Idaho Technology Inc., Salt Lake City, USA) or the Rotor-Gene (Qiagen, Hilden, Germany) allows detection of single base mismatches in amplicons up to 500 bp. The method has been used both for genotyping and SNP discovery in medical genetics (Zhou *et al.*, 2004, 2005), and SNP genotyping in plants has been demonstrated. Mutation scanning by HRM in hexaploid wheat requires a two-step amplification process, first, using homeologue-specific primers to amplify a larger amplicon containing several coding regions, followed by HRM analysis using primers specific for each exon or part thereof; an example is shown in Fig. 4. As the melt analysis following PCR is extremely rapid, the throughput of this technique is equal to or greater than that of Cell-based TILLING and is, arguably, easier to establish.

As the costs of large-scale DNA sequencing continue to fall dramatically, this more direct approach to mutation discovery seems likely to become a serious competitor to the

methods based on mismatch detection. The current DNA sequencing technologies generate between 500 Mb and 20 Gb of data per run, suggesting that it should be feasible to sequence several targets across a large population at reasonable cost, even allowing for the oversampling required to eliminate sequencing errors and uneven pooling. In polyploid species such as wheat, the choice of sequencing platform is restricted to those generating longer reads (e.g. Roche 454), which are necessary to connect the identified mutations with homoeologue-specific polymorphisms. There are a number of different pooling strategies that could be used to sample large mutagenized collections. A simple three-dimensional pooling based on plates, rows, and columns of standard 96-well PCR plates condenses 960 samples to 30 pools (10 plate pools, 8 row pools, and 12 column pools), which can be amplified using bar-coded primers for sample tracking. An alternative strategy is to sequence pooled amplicons from all individuals without sample tracking and subsequently locate a small number of selected mutations in the 3D pools using allele-specific primers. We are currently assessing next generation sequencing for mutation discovery in our bread wheat population.

Prospects for crop improvement

The last few years have seen the release of the first genome sequences for model plant species and the more genetically simple crops (Arabidopsis Genome Initiative, 2000; International Rice Genome Sequencing Project, 2005). Exploitation of these sequence data and associated tools for functional genomics has led to rapid progress in understanding the roles of individual genes, particularly in plant development and defence against pathogens. However, utilization of this knowledge for genetic improvement of more complex crops has been variable due to a combination of factors including lack of complete genome sequences, lack of genomic resources such as insertional knockout libraries and, for some species, the absence of high throughput transformation systems. However, the mutation screening technologies described above should make progress much more rapid by facilitating both the deployment of candidate genes identified in model species and also increasing available variation in genes of known agronomic value, as illustrated in Table 2. This will be further enhanced by the explosion in sequence data for crop species that will undoubtedly follow the development of next generation sequencing technologies, enabling facile identification of genes for modification. One negative factor is that random mutagenesis is much more likely to generate loss of function mutations rather than conferring improved or new properties on the targeted gene, and thus will only be an appropriate strategy in a proportion of cases. Furthermore, in contrast to technologies such as RNAi which, in principle, can target multiple genes simultaneously, point mutagenesis can only target a single copy of a group of related genes, whether paralogues or

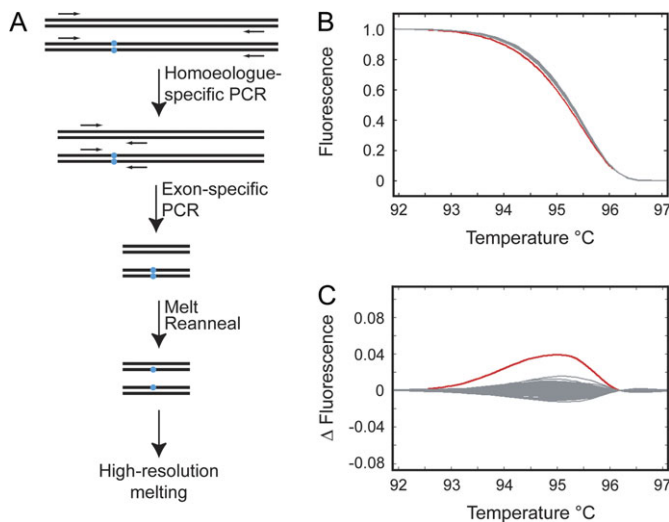


Fig. 4. Mutation detection in wheat using high-resolution melt analysis (HRM). (A) Schematic of PCR and heteroduplex production. The first-round homeologue-specific PCR targeted 971 bp of the bread wheat *GA20ox1D* gene; the second round amplicon was 201 bp of its first exon. LCGreen Plus dye was included in the second round PCR for melt detection and heteroduplex melt analysis was carried out on the Lightscanner (Idaho Technology). (B) Temperature-normalized fluorescence and (C) difference fluorescence plotted against temperature from melt analysis of the second-round PCR product from 192 samples (pooled 2-fold) from an EMS-mutagenized population of bread wheat cv. Cadenza. Wild-type samples are in grey, the single putative mutant pool identified through its different melt profile (and confirmed by sequencing of the two individual samples) is in red.

Table 2. Examples of reverse genetic screening of mutagenized populations of crop species for genes involved in agronomic and quality traits

Crop	Target trait	Target gene	Amplicon length	Lines screened	Mutants identified	Reference
Rice	Leaf emergence	<i>PLA1</i>	1327 bp	767	10	Suzuki <i>et al.</i> , 2008
Sorghum	Forage digestibility	<i>COMT</i>	1013 bp	768	2	Xin <i>et al.</i> , 2008
Barley	Grain hardness	<i>Hin-a</i>	420 bp	4608	4	Caldwell <i>et al.</i> , 2004
	Virus resistance	<i>eIF4E</i>	525 bp	3148	5	Talamè <i>et al.</i> , 2008
Bread wheat	Starch quality	<i>Wx-A1</i>	2114 bp	1152	106	Slade <i>et al.</i> , 2005
	Plant height	<i>GA20ox1A</i>	1289 bp	1152	17	A Phillips <i>et al.</i> , unpublished data
Durum wheat	Photosynthesis	<i>RCA-A1</i>	1435 bp	480	13	MAJ Parry <i>et al.</i> , unpublished data
Oil seed rape	Oil quality	<i>FAE1</i>	852 bp	930	15	Wang <i>et al.</i> , 2008

homeologues, and mutations in different gene copies may have to be brought together by crossing in order to achieve the desired effect.

Reverse genetic screening of mutagenized populations is thus unlikely completely to displace alternative crop improvement technologies such as genetic manipulation. However, varieties derived from mutagenized material will have a major economic advantage over those involving GM: while the technical development costs of the two approaches are probably broadly similar, bringing a GM variety to market is extremely costly due to the huge expense of licensing for commercial production and human consumption. Mutagenesis, on the other hand, is currently not regarded as genetic manipulation, as the random base changes involved are identical to those occurring naturally; indeed, as described above, many current crop varieties contain genetic material with origins in mutagenesis programmes. It seems likely, therefore, that reverse genetic selection of beneficial mutations through the screening approaches outlined above will have a major impact on crop breeding in the near future. The Seattle-based originators of the Cell-based TILLING technique established a commercial spin-out, Anawah, which was subsequently acquired by Arcadia Biosciences, an agricultural biotechnology company focusing on products with environmental and human benefits. Although no TILLING-derived crop varieties have yet been released, Arcadia is directing this technology into areas such as shelf life of tomatoes and wheat with reduced gluten content for sufferers of coeliac disease. TILLING and similar techniques have, therefore, reawakened interest in random mutagenesis for crop improvement, as illustrated by the rapid development of mutagenized populations and reverse genetic screening platforms in many crop species as shown in Table 1. An equally rapid deployment of novel alleles for crop improvement is certain to follow.

Conclusions

Recent developments in mutation scanning permit the identification of novel alleles of target genes within both

germplasm collections and mutagenized populations, allowing significant progress in functional genomics within model species and in the assessment of candidate genes for crop improvement. The Cell/Li-Cor-based TILLING platform is widely applicable, but a number of alternative technologies have also been developed, including agarose gel separation of cleavage products and high-resolution melting of heteroduplex DNA molecules. However, DNA sequencing costs continue to fall while throughput increases, and it seems likely that this more direct approach to mutation discovery will eventually replace other methods. A major obstacle in many crop species is the lack of complete genome sequence information, which slows the development of suitable TILLING targets. However, advances in sequencing technology are also likely to relieve this bottleneck within the relatively near future, as sequencing even very complex crop genomes such as wheat becomes practicable.

Mutagenized populations can be created at relatively low cost, although diploid species that are intolerant of high mutation frequencies require much larger populations for full coverage. Conversely, highly-mutagenized lines of polyploid species may require significant backcrossing to remove extraneous mutations before they can be assessed for phenotypes or used in plant breeding. Importantly, the use of such novel alleles in crops will not be impeded by the tough regulatory regimes that cover GM crops; this alone should assure the rapid deployment of this technology in plant breeding.

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