Chapter 4
Gene Identification: Reverse Genetics

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Introduction

The number of sequenced genes whose function remains unknown continues to climb with the continuing decrease in the cost of genome sequencing. Comparative genetics and bioinformatics have been invaluable in investigating the function of the genes that have been sequenced, but the elucidation of gene function *in planta* remains a huge challenge. Many gene functions have been defined through the use of forward genetics, where a phenotype is identified and used to clone the gene responsible. However, in most instances, genes of known sequence are not associated with a phenotype. This is particularly true in non-model species where forward genetics can be more challenging due to genetic redundancy. Reverse genetics is a powerful tool that can be used to identify the phenotype that results from disruption of a specific sequenced gene, even with no prior knowledge of its function. Several approaches have been developed in plants that have led to the production of resources including collections of T-DNA insertion mutants, RNAi-generated mutants, and populations carrying point mutations that can be detected by TILLING, direct sequencing or high resolution melting analysis (Table 4.1). These reverse genetics resources allow for the identification of mutations in candidate genes and subsequent phenotypic analysis of these mutants. In addition, new advances in technology and reduction in technical costs may soon make it practical to use whole genome sequencing or gene targeting on a routine basis to identify or generate mutations in specific genes in a variety of different plant species. This chapter will present the current status and promising prospects for the future of reverse genetics in plants.

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Table 4.1 Advantages and disadvantages of different reverse genetics techniques in plants

Technique	Advantages	Disadvantages	Species	References
Chemical mutagenesis and TILLING	Loss-of-function, reduction-of-function, and gain-of-function phenotypes	Requires the construction of a mutagenised population	Arabidopsis	Till et al. (2003), Martin et al. (2009)
			Barley	Caldwell et al. (2004), Talame et al. (2008)
			Brassica species	Wang et al. (2008), Himelblau et al. (2009), and Stephenson et al. (2010)
	Not labelled as genetic engineering	Few cost-effective ways of screening for individuals from the mutagenised population	Legumes	Perry et al. (2003), Dalmais et al. (2008), and Calderini et al. (2011)
			Melon	Dahmani-Mardas et al. (2010)
			Oats	Chawade et al. (2010)
			Potato	Elias et al. (2009)
		Position of mutation is random	Rice	Wu et al. (2005), Till et al. (2007)
			Sorghum	Xin et al. (2008)
			Soybean	Cooper et al. (2008)
			Tomato	Minoia et al. (2010), Piron et al. (2010)
			Wheat	Slade et al. (2005), Uauy et al. (2009)
T-DNA mutagenesis	Individuals carrying an insertion can be identified using PCR	Very large populations must be screened to achieve genome saturation	Apple	Smolka et al. (2010)
			Arabidopsis	Clough and Bent (1998)
			Banana	Sun et al. (2011)

Can be used for eliminating (knock-out) or enhancing (activation) of gene function	Position of mutation is random	Birch tree	Zeng et al. (2010)
		Blueberry	Song and Sink (2004)
	Requires transformation	Brachypodium	Thole et al. (2010)
		Brassica rapa	Lee et al. (2004)
	Labelled as genetically engineered	Carrot	Chen and Punja (2002)
		Cassava	Taylor et al. (2004)
		Chickpea	Indurker et al. (2010)
		Cucumber	Unni and Soniya (2010)
		Eucalyptus	Chen et al. (2007)
		Grape	Bouquet et al. (2007)
		Jute	Chattopadhyay et al. (2011)
		Lettuce	Michelmore et al. (1987)
		Lotus	Imaizumi et al. (2005)
		Medicago	Tadege et al. (2005)
		Peanut	Anuradha et al. (2006)
		Pear	Sun et al. (2011)
		Perilla	Ghimire et al. (2011)
		Pigeonpea	Krishna et al. (2010)
		Pine	Grant et al. (2004)
		Poplar	Busov et al. (2005)
		Potato	Barrell and Conner (2011)
		Rice	Jeon et al. (2000), Wan et al. (2009)
		Safflower	Belide et al. (2011)

(continued)

Table 4.1 (continued)

Technique	Advantages	Disadvantages	Species	References
			Sorghum	Kumar et al. (2011)
			Soybean	Widholm et al. (2010)
			Strawberry	Oosumi et al. (2010)
			Sugarcane	Arencibia and Carmona (2007)
			Switchgrass	Li and Qu (2011)
			Tomato	Mathews et al. (2003)
Transposon mutagenesis	Individuals carrying an insertion can be identified using PCR	Very large populations must be screened to achieve genome saturation	Arabidopsis	D'Erfurth et al. (2003), Marsch-Martínez (2011), and Nishal et al. (2005)
			Aspen	Kumar and Fladung (2003)
			Barley	Ayliffe and Pryor (2011)
	Can be used for eliminating (knock-out) or enhancing (activation) of gene function	Position of mutation is random	Beet	Kishchenko et al. (2010)
			Legumes	D'Erfurth et al. (2003)
		Can be epigenetic effects	Maize	May and Martienssen (2003)
			Rice	Upadhyaya et al. (2011), Zhu et al. (2007)
	Produce many unique insertion lines from a few initial plant lines		Tobacco	D'Erfurth et al. (2003)
Radiation or fast-neutron mutagenesis	Completely eliminates gene function	Very large number of plants must be screened	Arabidopsis	Li and Zhang (2002)
			Clementine	Rios et al. (2008)
			Legumes	Rios et al. (2008), Rogers et al. (2009)

		Size limitation of deletions detected	*Noccaeacaerulescens*	Lochlainn et al. (2011)
			Rice	Bruce et al. (2009)
			Soybean	Bolon et al. (2011)
			Tomato	Dor et al. (2010)
Virus-induced gene silencing (VIGS)	Relatively inexpensive	Not heritable	Apple	Sasaki et al. (2011)
			Arabidopsis	Burch-Smith et al. (2006)
	Homologous genes may be affected with a single construct	Homologous genes may be affected with a single construct	Barley	Scofield and Nelson (2009)
			Brachypodium	Demircan and Akkaya (2010)
			Brassica nigra	Zheng et al. (2010)
	Phenotype is transient	Phenotype is transient	California poppy	Wege et al. (2007)
			Cassava	Fofana et al. (2004)
	Does not require transformation	Side-effects of the infection may interfere with phenotype	Chilli pepper	Chung et al. (2004)
			Columbine	Gould and Kramer (2007)
	Delivers rapid results		Cotton	Tuttle et al. (2008)
		Level of silencing of target gene is variable	Cucurbit species	Igarashi et al. (2009)
			Ginger	Renner et al. (2009)
			Haynaldia	Wang et al. (2010)
			Jatropha	Ye et al. (2009)
			Legumes	Igarashi et al. (2009)
			Maize	Ding et al. (2006)
			Ornamental plants	Jiang et al. (2011)

(continued)

Table 4.1 (continued)

Technique	Advantages	Disadvantages	Species	References
			Pea	Constantin et al. (2004)
			Pear	Sasaki et al. (2011)
			Potato	Brigneti et al. (2004)
			Rice	Ding et al. (2006)
			Soybean	Zhang and Ghabrial (2006), Yamagishi and Yoshikawa (2009)
			Tomato	Fu et al. (2005)
			Wheat	Scofield and Nelson (2009)
RNA interference (RNAi)	Heritable	Some genes are resistant to silencing	Arabidopsis	Ossowski et al. (2008)
			Artemesinin	Zhang et al. (2009)
	Partial loss of function can be achieved	Expression is rarely completely silenced	Banana	Angaji et al. (2010)
			Barley	Angaji et al. (2010)
			Brassica species	Wood et al. (2011)
	Silencing is directed against a specific gene(s)	Long-term expression levels are variable	Coffee	Angaji et al. (2010)
			Cotton	Angaji et al. (2010)
			Tomato	Fernandez et al. (2009)
	Transcripts of multiple genes can be silenced by a single construct	Silencing level may vary	Wheat	Fu et al. (2007)
		‘Off-target’ silencing		
	Induced phenotypes are dominant			

New generation sequencing (NGS)	Vast amount of information obtained directly	High cost of data analysis and storage	Oat	Oliver et al. (2011)
			Tomato	Rigola et al. (2009), Tsai et al. (2011)
High resolution melting curve analysis (HRM)	Simple technology	Only small PCR fragments can be screened in one reaction	Almond	Wu et al. (2008)
			Barley	Hofinger et al. (2009)
	Inexpensive		Chilli pepper	Park et al. (2009)
			Maize	Li et al. (2010)
			Oat	Oliver et al. (2011)
			Olive	Muleo et al. (2009)
			Peach	Chen and Wilde (2011)
			Potato	De Koeyer et al. (2010)
			Ryegrass	Studer et al. (2009)
			Tomato	Gady et al. (2009)
			Wheat	Dong et al. (2009)
Gene targeting	Mutations in single, targeted genes	Difficulty and high cost of designing target zinc finger motifs	Arabidopsis	Zhang and Voytas (2011)
			Maize	Shukla et al. (2009)
			Soybean	Curtin et al. (2011)
			Tobacco	Townsend et al. (2009)

Established Techniques

Chemical Mutagenesis

Chemical mutagenesis was used to generate populations of mutants for forward genetics long before the advent of DNA sequencing and reverse genetics. Point mutations are, generally, less deleterious than large rearrangements and so a high degree of saturation can be achieved in a mutant population using chemicals that generate single base pair changes or small insertions and deletions. This approach is, therefore, useful for the examination of gene function using genome-wide approaches. Two chemicals, in particular, are known to cause primarily single base pair mutations in DNA in all organisms in which they have been tested: ethylmethane sulphonate (EMS) and ethyl nitrosourea (ENU).

While many reverse genetics techniques provide only loss-of-function alleles, chemical mutagenesis can result in either loss-of-function, reduction-of-function, or gain-of-function phenotypes. In fact, the frequency of induced missense alleles is, on average, three times higher than that of nonsense alleles. Many missense alleles will not have an effect on gene function since they may not alter the gene product(s) significantly, but examples of dominant point mutations caused by missense alleles have been well documented, including ones that affect plant hormone responses (Wang et al. 2006; Biswas et al. 2009), leaf polarity (Juarez et al. 2004; Byrne 2006), and host-pathogen defence (Eckardt 2007). The difficulty with using point mutations for reverse genetics screens is that there are few cost-effective ways of screening the mutagenised population for individuals that carry mutations in specific genes. The advent of TILLING, New Generation Sequencing (NGS), and High Resolution Melting (HRM) analysis, however, have made possible the screening of large populations, at a reasonable cost, within an acceptable time frame (Fig. 4.1). TILLING operations use a variety of techniques for creating mutant populations and screening them, including that described by (Colbert et al. 2001) which employs a mismatch-specific endonuclease for identifying point mutations in the target gene of interest. Generally, in this procedure the mutagenised generation (M_1) is grown up and then the progeny of these plants (the M_2 generation) are used for screening. This ensures that the mutations that are identified in this process are heritable and eliminates the background somatic mutations that may be present in the M_1 generation. After collecting seeds and DNA from the M_2 plants, the DNA from several mutagenised individuals is pooled, and then the polymerase chain reaction (PCR) is used to amplify a target gene of interest. In conventional TILLING, the PCR products (amplicons) are denatured and allowed to randomly re-anneal before being digested with a celery juice extract (CJE) (Till et al. 2003). Mismatches in the amplicons occur when mutant and wild-type strands of DNA are re-annealed together to form a heteroduplex. This heteroduplex then becomes a target for the mismatch-specific enzyme. Only the samples carrying a mismatch are cleaved, and these novel fragments can be detected using DNA separation technology such as the LI-COR DNA Analyser (LI-COR Biosciences, Lincoln, NE, USA), or AdvanCE

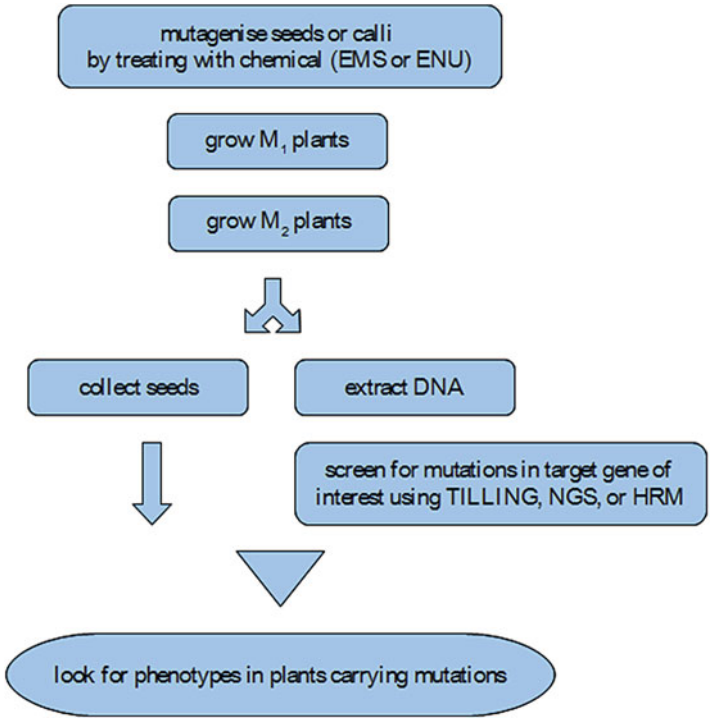

Fig. 4.1 Chemical mutagenesis for reverse genetics. Flow chart describing the procedure for TILLING, high-throughput new generation sequencing (NGS) or high resolution melting (HRM) analysis of a mutagenised population. M_1 refers to the mutagenized generation; M_2 refers to the progeny of the mutagenized generation

F96 (Advanced Analytical Technologies, Inc., Ames, IA, USA). The drawbacks of TILLING are that it requires the construction of a mutagenised population, and for many species the development of such a population is challenging. Further, the technique itself is labour-intensive, relatively expensive, and requires a high rate of mutagenesis to make the effort cost-effective. Nonetheless, TILLING has worked well in a wide variety of model and non-model plants as listed in Table 4.1.

Insertional Mutagenesis

One of the most established methods for reverse genetics is the production of populations of individuals that have insertions that disrupt gene function at unique sites in their genomes (Fig. 4.2). The advantage of insertional mutagenesis is that individuals carrying an insertion of known sequence in a specific gene can be identified in a population using PCR, a simple and relatively inexpensive technique.

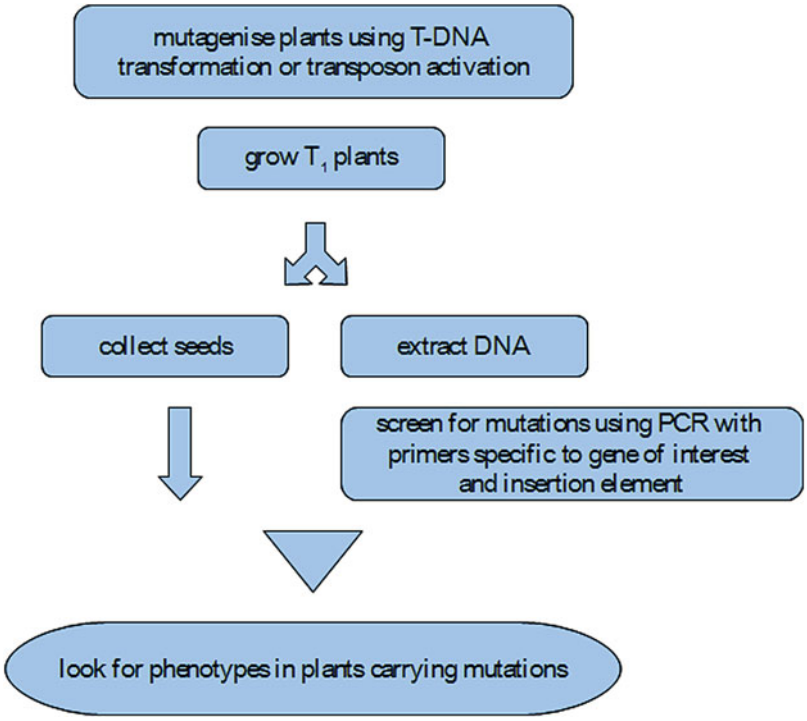

Fig. 4.2 Insertional mutagenesis for reverse genetics. Flow chart describing the procedure for insertional mutagenesis using either T-DNA transformation or transposon activation. *T_1* refers to the first generation after transformation of the T-DNA or transposon

PCR amplification is performed with one host gene-specific primer and one vector-based primer from the insertion element. Thus, an amplification product will only be observed, when the insertion is present in close proximity to the target gene (from which the host primer was designed). Gene disruption using this technique typically results in a total loss of gene function.

Insertional mutagenesis can also be used for activation tagging. Activation tagging is a method of causing over-expression or ectopic expression of a gene of interest. A construct is engineered such that it carries a strong promoter or enhancer element which, when introduced into the genome, can insert at random positions. Some of these insertion sites will be upstream of the target gene of interest where they can enhance transcription of that gene. The position of the insertion is determined using PCR as described above. Such enhanced expression can create a phenotype even in cases where loss-of-function mutations are not able to do so because of redundancy or lethality.

For either activation or disruption of gene function, the insertion can be detected in the first generation following transformation (T_1), and can be easily followed in a population of plants where the element will segregate in Mendelian fashion.

Insertional mutagenesis is generally generated either by transformation using an *Agrobacterium*-derived T-DNA construct or by transposon activation. Each of these options is discussed below.

Transfer-DNA (T-DNA) Mutagenesis

There are a number of different transformation techniques that can be used in plants, but by far the most established is *Agrobacterium*-mediated gene transfer using some form of T-DNA construct. In this process, the T-DNA segment of the tumour-inducing (T_i) plasmid from an *Agrobacterium* species integrates randomly into the plant genome and causes disruption or activation of the gene of interest depending on the construct used (Hellens et al. 2000). It is technically difficult to clone a gene directly into the T-DNA region of the Ti plasmid because the plasmid is large, making it challenging to isolate directly from *Agrobacterium*. Therefore, a binary vector system is typically employed (Lee and Gelvin 2008). This technique involves the use of two separate plasmids, one carrying the insert DNA flanked by the left and right border sequences of the T-DNA, and the other carrying the virulence genes from the Ti plasmid needed for infection and transfer of the of the T-DNA into the host. Using this system, the first vector can be constructed and grown in *E. coli* before transformation into an *Agrobacterium* strain that has been engineered to transfer the cloned DNA fragment into the plant without causing the typical symptoms of *Agrobacterium* infection.

There are several transformation techniques that can be used depending on the host plant (for review see Meyers et al. (2010)). The simplest of these is the floral dip method that involves simply dipping developing flowers into media containing the transgenic *Agrobacterium* and then planting the seeds from these plants on selective media so that only transgenic plants can germinate. This is the technique most commonly used in the model *Arabidopsis* (Clough and Bent 1998). For most plants, leaf-disc inoculation is used instead. This technique involves soaking the leaf discs in the *Agrobacterium* solution and then placing them on callus-inductive media containing the herbicide against which one of the transgenes on the T-DNA confers resistance (Barampuram and Zhang 2011). For plants that are resistant to *Agrobacterium*, electroporation or biolistic transformation of plant protoplasts is sometimes used, where the transforming DNA is introduced using an electrical pulse or bombardment with particles to which the transforming DNA constructs are attached respectively (Meyers et al. 2010).

One of the disadvantages of using T-DNA vectors to create insertional libraries is that very large populations must be screened to achieve genome saturation (a mutation in every gene). In addition, insertion is generally random so that activation of the introduced DNA may or may not be successful depending on the site of integration. For some species, large insertion libraries have been generated allowing researchers to access mutations in almost any gene of interest through comprehensive databases that have been set up for this purpose (for examples see Alonso et al. 2003; Krishnan et al. 2009). T-DNA transformation strategies have

been used successfully in many plants for both applied and basic research purposes. Aside from the model *Arabidopsis*, some plant species where this technology has been successful are listed in Table 4.1.

Transposon Mutagenesis

Transposon mutagenesis has been used for over half a century to create mutations that were originally detected using forward genetic screens. For the past three decades it has been used in reverse genetic screens that identify disruptions in target genes of interest (May and Martienssen 2003). Transposon-based reverse genetics usually involves two components: an autonomous element that includes the transposase gene, and one or more non-autonomous elements that are only active when the transposase produced by the autonomous element is active.

The first gene to be cloned using transposon tagging employed the *Activator*/*Dissociation* (*Ac*/*Ds*) transposon system from maize (reviewed in May and Martienssen (2003)). *Ac* is a member of the *hAT* cut-and-paste family of transposable elements, some of which have been shown to be controlled by environmental factors. Another *hAT* element, *Tam3*, has been extensively used in *Antirrhinum* because of its unique temperature-controlled characteristic activation at 15°C but not at temperatures above 25°C (Schwarz-Sommer et al. 2003). While, originally, transposon mutagenesis was only possible in plants like maize and *Antirrhinum* which had active and well-understood transposon systems, technological and intellectual advances in the understanding of transposition have made it possible to use some elements heterologously. The *Ac*/*Ds* system, along with the maize *Suppressor-mutator*(*Spm*) has been shown to work in many species other than maize, (for review see Candela and Hake (2008)). Systems in which *Ac*/*Ds* or *Spm* transposon-tagging has been effective include aspen trees (Kumar and Fladung 2003), barley and other cereals (Ayliffe and Pryor 2011), beet (Kishchenko et al. 2010), rice (Upadhyaya et al. 2011), and *Arabidopsis* (Marsch-Martínez 2011) among others.

Another transposon family, *Mu* (and *Mu-like* elements), includes the most widely spread and most mutagenic transposons found in plants. This transposon system is commonly used for reverse genetics in maize (Lisch and Jiang 2009), but the high activity level of the transposon can lead to deleterious somatic mutations and so *Mu* has been difficult to use in some heterologous systems, including rice, because epigenetic silencing occurs within a few generations (Diao and Lisch 2006).

The *Tos* retrotransposons were the first endogenous transposons demonstrated to be active in rice and remain the most commonly used in this species for a number of reasons, not the least of which is that because *Tos17* is derive from rice, affected lines can be grown and used without the regulatory problems associated with genetically modified organisms (GMOs) (Miyao et al. 2007). Several other transposons have also been used to create tagged populations in rice (Zhu et al. 2007).

Other transposons such as the Tobacco *Tnt1* element have also been used for transposon-tagging in systems such as in tobacco itself (Grandbastien et al. 1989),

and in *Arabidopsis* (Courtial et al. 2001) as well as in the legume *Medicago trunculata* (D'Erfurth et al. 2003) and lettuce (Mazier et al. 2007).

While most approaches to transposon mutagenesis result in random insertion of elements throughout the genome, transposition from a T-DNA construct carrying both the transposase and the non-autonomous element is effective for generating multiple insertion events within one region of the genome. In this system if transposition is inducible, for example through the use of a heat shock promoter, then induction of the transposase can result in transposition of the non-autonomous element from the T-DNA into flanking genomic regions, generating new mutant lines that have insertions in close proximity to the site of insertion of the T-DNA construct. Subsequent heat shock treatments can generate novel mutations by causing reactivation of the transposase, and the cycle can be repeated as many times as necessary to achieve saturation of mutations in this region (Nishal et al. 2005).

While T-DNA insertion systems are more popular than transposons, efficient transformation systems are still lacking in many monocot crop species so that transposon-tagging continues to hold a useful position in the arsenal of reverse genetics techniques (Ayliffe and Pryor 2011). In addition, transposon-generated populations have the advantage of being able to produce many unique insertion lines from a few initial plant lines and lack epigenetic changes associated with T-DNA-based insertions (Upadhyaya et al. 2011).

Fast-Neutron Mutagenesis

Another form of mutagenesis that causes physical disruption in genes is radiation or fast neutron bombardment (Li and Zhang 2002). In this technique, seeds are irradiated using fast neutrons and deletions are identified using PCR primers that flank the gene of interest (Fig. 4.3). Amplification time is restricted so as to preferentially allow amplification of the mutant (deleted) DNA where a smaller PCR product is synthesised. One advantage of using this technique is that the deletions produced via physical mutagenesis will almost certainly completely eliminate any gene function. The most useful benefit of this technology, however, may be the fact that tandemly linked gene duplications may be deleted in the same line. Mutation of tandemly-linked genes in the same line is difficult to achieve with other reverse genetics technologies commonly used in plants. The limitations of this technique include the fact that a very large number of plants must be laboriously screened, and that there are constraints on the size of deletions that can be recovered. Nonetheless, this technology has been effective in creating mutant populations in *Arabidopsis* (Li and Zhang 2002), legumes (Rios et al. 2008; Rogers et al. 2009), rice (Bruce et al. 2009), soybean (Bolon et al. 2011), tomato (Dor et al. 2010), the citrus clementine (Rios et al. 2008), and in the metal-tolerant plant species *Noccaeacae rulescens* (Ó Lochlainn et al. 2011).

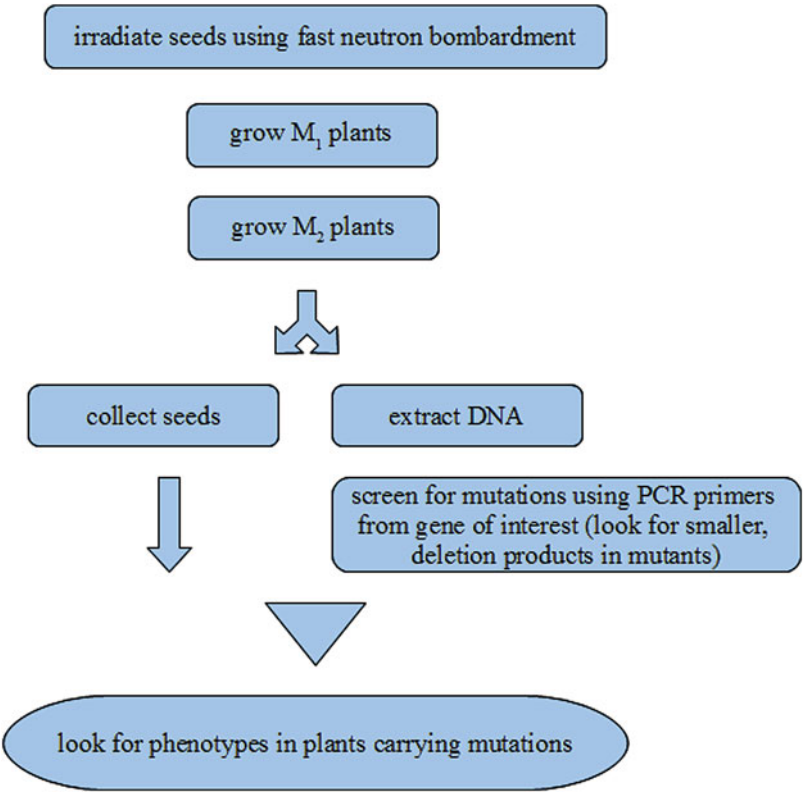

Fig. 4.3 Fast neutron mutagenesis. Flow chart describing the procedure for fast neutron mutagenesis. M_1 refers to the mutagenized generation; M_2 refers to the progeny of the mutagenized generation

Virus-Induced Gene Silencing (VIGS)

This technology began to be used extensively in the 1990s and is based on post-transcriptional gene silencing (PTGS) (Burch-Smith et al. 2004). The term VIGS was created by van Kammen (1997) to describe the development of a plant's resistance to virus infection after introduction of a viral transgene. VIGS is a very adaptable technique and has been used in many species. It has some advantages over other reverse techniques, such as the fact that it is relatively inexpensive, delivers rapid results and does not require transformation. In addition, because the phenotype is transient, deleterious effects of loss of gene function may be observed without causing lethality or infertility. The drawbacks include the fact that transient effects cannot be following using classical genetic studies, and that the vectors exhibit some host and/or tissue specificity. There can also be side-effects of the infection that may interfere with the silencing phenotype. In addition, the function

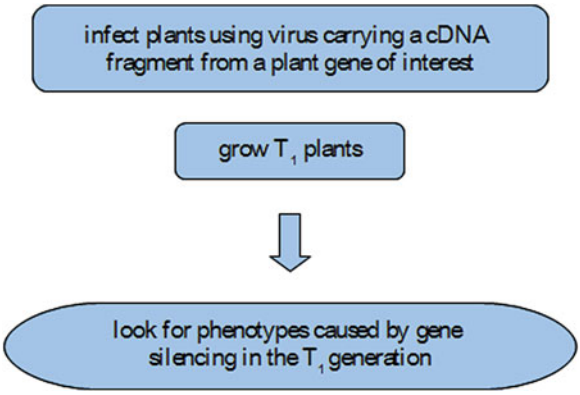

Fig. 4.4 Virus-induced gene silencing (*VIGS*). Flow chart describing the procedure for using virus-induced gene silencing to create transient loss-of-function mutations in specific genes. The cDNA fragment is part of the coding region of the gene. T_1 refers to the first generation after transformation of the cloned fragment into the plant

of several homologous genes may be affected with a single construct, complicating the interpretation of observed phenotypes. Finally, the level of silencing of the target gene is variable depending on the construct and the growth conditions used, and it is rare that genes will be completely silenced.

The protocol for VIGS involves cloning a 200–1,300 bp cDNA fragment from a plant gene of interest into a DNA copy of the genome of a plant virus (usually a RNA virus) and transfecting the plant with this construct (Hayward et al. 2011) (Fig. 4.4). Double-stranded RNA from the viral genome, including the sequence from the gene of interest, is formed during viral replication. The double-stranded RNA molecules are degraded into small interfering RNA (siRNA) molecules by the plant Dicer-like enzymes, thus activating the siRNA silencing pathway (for review see Chen (2009)) resulting in the degradation of the target gene transcript leading to a knockout or knockdown phenotype for the gene of interest.

The earliest vectors used for VIGS in plants included the *Tobacco mosaic virus* , *Potato virus X*, and *Tomato golden mosaic virus* but these had disadvantages such as infection symptoms that interfered with or complicated a mutant phenotype, or lack of infection in certain tissues (Ratcliff et al. 2001). Currently the most widely-used VIGS vector for dicotyledonous species is the *Tobacco rattle virus* (TRV) which has a broad plant host range, infects many different tissue types, and produces relatively mild disease symptoms in most plants (Hayward et al. 2011). The TRV vector has been used successfully for VIGS in the model species *Arabidopsis* (Burch-Smith et al. 2006), as well as a number of crop species including tomato (Fu et al. 2005), potato (Brigneti et al. 2004), *Jatropha* (Ye et al. 2009), chilli pepper (Chung et al. 2004), and *Brassica nigra* (Zheng et al. 2010). This vector has also been used for VIGS in a number of ornamental plants such as petunia, *Impatiens* and chrysanthemum (Jiang et al. 2011), California poppy (Wege et al. 2007), and columbine (Gould and Kramer 2007) to name a few.

In spite of it's broad host-range, however, some dicotyledonous and all monocotyledonous plants are resistant to infection by TRV. The main virus vector used for VIGS in monocots has been the *Barley stripe mosaic virus* (Holzberg et al. 2002).

This is currently the vector most commonly used in barley and wheat (reviewed in Scofield and Nelson 2009) and *Brachypodium* (Demircan and Akkaya 2010), and it has also been shown to be effective in less well-studied monocots such as the wheat-relative *Haynaldia* (Wang et al. 2010) and culinary ginger (Renner et al. 2009). More recently, the *Brome mosaic virus* has been used efficiently for VIGS in both rice and maize (Ding et al. 2006) and continued improvements to both of these vectors show promise for future studies using VIGS in monocot species.

There are also several other new virus vectors that are being used or studied for VIGS in plants. The *Apple latent spherical virus* is reported to be even broader in its host range than TRV, and to have minimal side effects. It has been used in both model and non-model dicot plants including legumes and cucurbit species (Igarashi et al. 2009), soybean (Yamagishi and Yoshikawa 2009) and fruit trees (Sasaki et al. 2011) as well as many species that are also susceptible to TRV.

In addition to the established VIGS vectors that can be used in many different species, new species-specific vectors are being developed for a diverse range of plants that will allow this technique to be used to study gene function in an even wider range of species. These include the *Pea early browning virus* for *Pisum sativum* (Constantin et al. 2004), the *African cassava mosaic virus* for cassava (Fofana et al. 2004), *Bean pod mottle virus* for soybean and other P*haseolus* species (Zhang and Ghabrial 2006) and the *Cotton leaf crumple virus* for cotton (Tuttle et al. 2008).

RNA Interference or Artificial MicroRNA Gene Silencing

RNA interference (RNAi), or RNA-induced gene silencing is similar to VIGS mechanistically, but the former is heritable in nature and so offers a different scope for investigation (McGinnis 2010). For the RNAi technique, a construct that produces double-stranded RNA complementary to the gene of interest is introduced into a cell where it activates the RNA silencing pathway and degrades some or all of the transcripts from the gene(s) of interest (for review see Chen 2009; Huntzinger and Izaurralde 2011) (Fig. 4.5). There are several techniques commonly used to activate the RNAi pathway in plants but the most popular strategies involve transformation with a construct encoding a hairpin RNA structure (hpRNA) or the use of an artificial microRNA (amiRNA) targeting the gene of interest (Eamens and Waterhouse 2011). For the hpRNA technique, reverse transcriptase PCR (RT-PCR) is used to amplify a region in the gene of interest, which is then cloned into a vector that creates inverted repeats of this region. The vector will also, typically, carry a promoter that will allow expression of the transgene at the time and in the tissue desired, along with a selectable marker for detection (Doran and Helliwell 2009). When this region is transcribed, the products act as dsRNA targets for the small RNA silencing pathway genes that normally target endogenous transcript(s) for degradation.

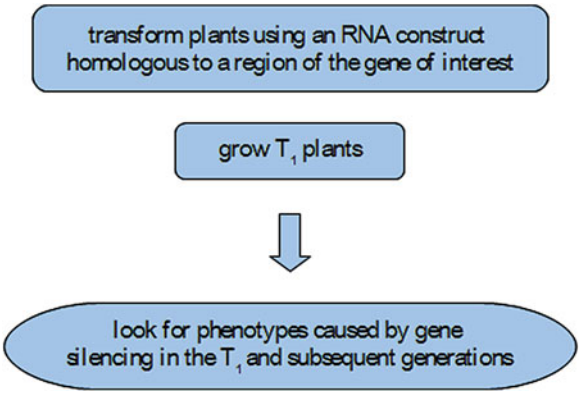

Fig. 4.5 RNA interference (*RNAi*). Flow chart describing the procedure for using RNA interference to create (usually) heritable loss-of-function mutations in specific plant genes. T_1 refers to the first generation after transformation of the RNAi construct into the plant

For amiRNA gene silencing, either ectopic or constitutive expression of an endogenous miRNAs is used to silence a target gene of interest (Alvarez et al. 2006), or an artificially constructed microRNA gene carrying a 21 bp insert complementary to the target gene is transformed into the plant where it acts in the endogenous miRNA silencing pathway (Ossowski et al. 2008). In addition, modern RNAi techniques may involve the use of promoters that are temporally or spatially specific, or that are inducible by some chemical or abiotic factor (Masclaux and Galaud 2011).

The advantages of using RNAi and amiRNA-based technology for reverse genetics in plants include the fact that a partial loss of function can be achieved when complete loss of function might be lethal, and that silencing is directed against a specific gene(s) so the screening of large populations is not required. In addition, transcripts of multiple genes from the same family can be silenced by a single construct (Alvarez et al. 2006; Schwab et al. 2006). This latter advantage is especially useful in plants since many plants have undergone partial or complete polyploidisation at some stage during their evolution, and a public website has been created to assist in the design of amiRNAs in more than 90 different plant species: http://wmd3.weigelworld.org (Ossowski Stephan, Fitz Joffrey, Schwab Rebecca, Riester Markus and Weigel Detlef, pers. comm.). Other advantages of this technique are that the induced phenotypes are dominant, they can be observed in the T_1 generation, and that stable inheritance of the transgenic RNAi gene makes the technique suitable for genetic engineering of traits into crop species in a manner that can be propagated from generation to generation.

Some disadvantages of the RNAi technology include the fact that some genes are resistant to silencing, possibly because of sequence or structural features of these genes. In addition, transcripts of genes that are similar in sequence to the target locus may be concomitantly down-regulated as well as the transcripts from the actual target gene. Although this is less of a problem in plants than in animals, 'off-target' silencing must be considered when planning experiments (Senthil-Kumar and Mysore 2011). The silencing level also may vary depending on the construct and the species, and gene expression is rarely completely silenced. The long-term

effects of RNAi are also variable and expression of the transgenic RNAi constructs is often less effective in succeeding generations of transgenic lines.

In spite of these disadvantages, however, RNAi has been used in many plant species for both experimental and applied purposes such as nutritional improvement, pest resistance, reduction of toxins and improved response to abiotic stresses (Jagtap et al. 2011). In addition, RNAi constructs that target pathogenesis genes in insects, nematodes, or fungal parasites has been very successful at creating crops and other plants that are resistant to infection by these pathogens (Niu et al. 2010). This technique has been used successfully to improve a number of crops including several Brassica species (reviewed in Wood et al. (2011)), banana, cotton, barley, and coffee (Angaji et al. 2010), wheat (Fu et al. 2007), tomato (Fernandez et al. 2009), and the anti-malarial *Artemesinin* (Zhang et al. 2009).

Emerging Techniques

Promising Technologies for Screening Mutagenised Populations

New Generation Sequencing (NGS)

Direct sequencing would be the simplest method for screening mutagenised populations, and this possibility may become a reality in the near future as sequencing costs continue to decline spurred by the astounding advances in NGS techniques (Niedringhaus et al. 2011). Two types of sequencing technologies have now been tested on mutant TILLING populations and both groups report success using this strategy on tomato (Rigola et al. 2009; Tsai et al. 2011). Most recently, sequencing of whole genomes using this new technology has also proven that single-nucleotide polymorphism analysis is possible using sequencing, even in complex genomes such as oat where there is no previous reference sequence available (Oliver et al. 2011).

High Resolution Melting Curve Analysis (HRM)

Melting curve analysis has been used to identify DNA variants since the late 1990s (Wittwer et al. 1997), but was of limited use because of the technical limitations imposed by instrumentation and dye technologies. With the development of more sensitive double-stranded DNA (dsDNA) dyes and improvements in instrumentation that allow more accurate measurements of amplicon melting behaviour (Vossen et al. 2009) it is now possible to use HRM analysis for genomic-scale screening such as is required for TILLING or other SNP-detection or genotyping projects. The process is based on the fact that a dsDNA binding dye is intercalated between each base pair of a double-stranded PCR amplicon. When the DNA is heated it starts to denature, thus releasing the encaptured dye which then no longer fluoresces. This

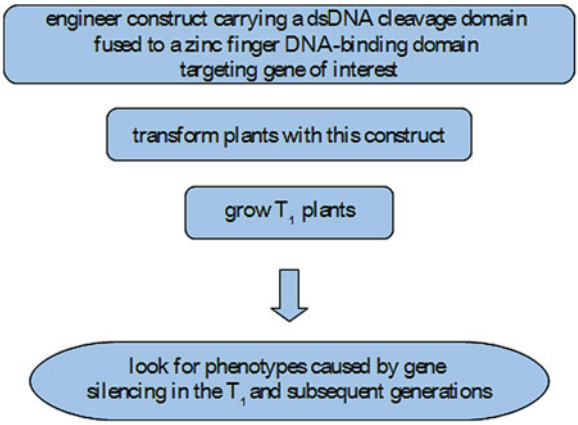

Fig. 4.6 Gene targeting using zinc-finger nucleases (*ZFNs*). Flow chart describing the procedure for using zinc-finger nuclease technology to create heritable loss-of-function mutations in specific genes. T_1 refers to the first generation after transformation of the ZFN construct into the plant

decrease in fluorescence is recorded by a camera and visualized on the screen. The rate of fluorescence decay is dependent on the sequence of the DNA, but also on the fidelity of the match of the two strands. Thus, any amplicon containing a mutation will produce a mismatch when paired with a wild-type amplicon, and this can be detected by a more rapid decrease in fluorescence than with homozygous wild-type amplicons. Only PCR fragments of a few hundred base pairs can be screened in one reaction, but this technology has been used for many medical applications and has now been tested in several plant species for detecting SNPs in both mutagenized and natural populations. This technology appears to be very versatile, inexpensive and has been successful in most systems in which it has been tested including almond (Wu et al. 2008), tomato (Gady et al. 2009), wheat (Dong et al. 2009), barley (Hofinger et al. 2009), ryegrass (Studer et al. 2009), olive (Muleo et al. 2009), chilli pepper (Park et al. 2009), maize (Li et al. 2010), potato (De Koeyer et al. 2010), peach (Chen and Wilde 2011), and oat (Oliver et al. 2011).

Gene Targeting

Random mutagenesis often results in mutations in single loci that do not have an effect on phenotype, and knock-down strategies rarely silence a gene or gene family entirely. Thus, in plants where gene redundancy is high and where polyploidy is often the rule rather than the exception, gene targeting allows for the isolation of plants carrying mutations in single, defined genes or multiple genes of a gene family within the same plant. Gene targeting involves the integration or removal of a piece of DNA from a specific target sequence in the host plant (Fig. 4.6). In theory, this enables the generation of specific alleles of any gene in the plant. It has been successfully used in fungi for many years, but remained elusive in plants until the recent improvements in synthetic Zinc finger nucleases (ZFNs) that were

first created in the late 1990s (Chandrasegaran and Smith 1999). Modern ZFN's are engineered by combining two zinc finger proteins that recognise a specific DNA sequence, with an endonuclease that causes non-specific double-stranded breaks in DNA. They were first used in plants in 2005 where they were shown to cause mutations at site-specific locations at a rate of approximately 20% (Lloyd et al. 2005). Most mutations created by this approach are small deletions or insertions of a few base pairs that can be attributed to the nonhomologous end joining (NHEJ) DNA repair mechanism found in all species (reviewed in Mladenov and Iliakis (2011)). Recently, this system has been improved so that targeting of specific ZFNs to plant genes can cause mutation rates of from 30 to 70% at these sites when the toxicity of the construct is controlled by making them heat or hormone-inducible (Zhang et al. 2010). One of the drawbacks of this technology has been the difficulty and high cost of designing appropriate zinc finger motifs to target the selected region in the genome, but this has been simplified by the creation of the publicly available OPEN (Oligomerized Pool Engineering) platform for engineering zinc-finger constructs (Maeder et al. 2008). A new development in ZFN technology is the context-dependent assembly (CoDA) platform recently published by the Zinc Finger Consortium (Sander et al. 2011). This strategy uses archived information from several hundred existing zinc finger arrays to automatically design new ZFN constructs that have different sequence specificity without requiring technical expertise beyond standard cloning techniques. CoDA appears to be as specific and less labour-intensive than OPEN and holds much promise for gene targeting in non-model, polyploid species (Curtin et al. 2011). ZFN technology has, to date, been successfully employed in a number of animal and plant species with equal success. Plants in which it has demonstrated utility include *Arabidopsis* (Zhang and Voytas 2011), soybean (Curtin et al. 2011), maize (Shukla et al. 2009) and tobacco (Townsend et al. 2009). Transcription activator-like (TAL) DNA-binding proteins have been developed for targeted gene modulation in plants as an alternative to ZFN technology. TAL proteins have been shown to be useful as a tool to study for gene activation (for review see Bogdanove et al. 2010). More recently, constructs known as TALENs have been successfully used for sequence-specific gene disruption by combining the catalytic domain of the FokI nuclease with specific TAL effector constructs (Cermak et al. 2011).

Conclusion

There are more and more resources available for reverse-genetic studies in plants. Each has its advantages and disadvantages depending on the species being targeted and the questions addressed (Table 4.1). Both VIGS and RNAi remain attractive, in part, because of their low cost. They are very useful for studying genes of unknown function in species for which these techniques have been developed. The availability of T-DNA and transposon insertion lines that are accessible to the public makes those resources attractive as well. It is more expensive to perform physical

mutagenesis using, for example, radiation, followed by reverse genetic analysis of the mutagenised population, but this approach is, nonetheless, useful in cases where other techniques have not been successful. Chemical mutagenesis and TILLING provide more varied types of mutations than other techniques, but can be more time-consuming and costly. One of the newest and most promising techniques is the fine-tuning of the zinc-finger nuclease and TALEN techniques that, for the first time, allow targeted mutagenesis in plants at an acceptable cost and in a reasonable amount of time using technology that is available in most laboratories. Finally, with the continuous development of new technologies, the most efficient technique for examining gene function in plants in the future may involve direct sequencing of part or complete genomes of individual plants, or some completely novel technology that has yet to be developed.

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