Deployment of new biotechnologies in plant breeding

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The first crops obtained through new plant breeding techniques are close to commercialization. Regulatory issues will determine the adoption of the techniques by breeders.

The global food crisis of 2008 reminded us of the importance of innovation in agriculture to address global challenges such as population growth and climate change. The projections presented in a report of the Food and Agricultural Organization of the United Nations (proceedings of a high-level expert forum) show that feeding a world population of 9.1 billion people in 2050 would require raising overall food production by some 70% between 2005/07 and 2050 (ref. 1). Additionally, farmers will have to hit targets for reducing greenhouse gas emissions, improving water use efficiency and meeting the demands of consumers for healthful food and high-value ingredients. In this context, new plant breeding techniques are needed to contribute improvements in crop productivity and sustainability.

Clearly, an important aspect of technology adoption and dissemination is how such approaches relate to regulatory oversight and whether such breeding techniques fall under present rules for genetically modified organism (GMO) legislation. In the case of EU the issue is currently being analyzed[2–4]. Although studies analyzing new plant breeding techniques from the point of view of risk assessors and regulators are available[4–8], data are lacking on the refinement and/or maturation of technology and the extent of adoption in commercial breeding programs (and thus likely contribution to new crop varieties in the short or medium term).

To close this gap, we have conducted a study on new plant breeding techniques (beyond traditional genetic modification), under the aegis of the European Union's Joint Research Centre (JRC), that encompasses state-of-the-art technology and their prospects for commercial development, including zinc-finger nuclease (ZFN) technology[9,10], oligonucleotide-directed mutagenesis (ODM)[11,12], cisgenesis and intragenesis[13], RNA-dependent DNA methylation (RdDM)[14], grafting (on genetically modified (GM) rootstock)[15], reverse breeding[16] and agro-infiltration (encompassing agro-infiltration 'sensu stricto', agro-infection and floral dip)[17]. Our primary focus is on the current development status of these approaches, the main actors exploiting them in R&D (both public and private), the patenting landscape and the current use of these techniques by the commercial breeding sector. We also address the main drivers and constraints for the further adoption of these techniques. Finally, we analyze the possibilities for detecting and identifying crops produced using them (to fulfill possible regulatory requirements).

Historical backdrop

Since the beginning of the twentieth century various tools have been introduced to broaden the possibilities for breeding new plant varieties. Chemical- and radiation-induced mutagenesis increases the frequency of genetic variations, and hybrid seed technology generates heterozygous plants with improved yield and disease resistance[18]. Applying the principles of cell biology and tissue culture—micropropagation, embryo rescue and double-haploid techniques—allows the rapid production of many uniform plants and the crossing of incompatible plants[18].

The latest wave of innovation in plant breeding, dating from the 1980s, came from 'modern biotech'. Molecular marker–assisted selection is now widely used to map and select commercially important agricultural traits[19]. Genetic modification, also known as genetic engineering, exploits recombinant DNA technology to expand the gene pool available to plant breeders. The earliest crops produced by genetic modification technologies (pest-resistant and herbicide-tolerant varieties) reached commercial cultivation in the mid-1990s and currently the global area sown with GM varieties measures over 148 million hectares[20].

In the past two decades, additional applications of biotech and molecular biology in plants have emerged, with the potential to further enlarge the plant breeder's toolbox. Several recently described techniques allow for site-directed mutagenesis of plant genes (to knock out or modify gene functions) and the targeted deletion or insertion of genes into plant genomes[5,9–12]. Another innovative trend is the use of transgenes solely as a tool to facilitate the breeding process.

In this application, transgenes are used in intermediate breeding steps and then selected for removal during later crosses, eliminating them from the final commercial variety. Among these tools are accelerated breeding techniques, where genes that promote early flowering are used to speed up breeding[21], and reverse breeding, a technique that produces homozygous parental lines from heterozygous elite plants[16].

The potential of these and other new techniques to produce innovative crop varieties will likely be affected by the regulatory framework of the regions where they are to be introduced. The application of modern biotech in the 1980s resulted in new forms of regulation and governance of certain plant breeding techniques (in particular genetic modification technologies) and of the release of GM crops into the environment. Various legal and regulatory approaches have been adopted worldwide, which include differing definitions of GM crops[22].

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Box 1 Definitions of new techniques and applications

The seven techniques we focused on are described below.

• **ZFN technology.** ZFNs are synthetic restriction endonucleases, custom designed to cut DNA at specific sequences. They consist of a zinc-finger domain that recognizes specific DNA sequences and a nuclease domain. Genes encoding the ZFNs are delivered to plant cells in an expression plasmid. Depending on the method, the expression plasmid may additionally contain a short template sequence or a stretch of DNA to be inserted. The ZFNs create a double-strand break (DSB) at a specific site in the DNA. The double-strand break stimulates the cell's repair mechanism, the process of homologous recombination and the insertion of DNA. Essentially three methods are in development:

ZFN-1, ZFN genes are delivered to plant cells without a repair template. The ZFN binds to a specific DNA sequence and generates a site-specific DSB. Gene repair mechanisms of the plant cell intervene to repair the break and generate site-specific mutations, which consist of changes of single or few base pairs, short deletions or insertions.

ZFN-2, ZFN genes are delivered to plant cells along with a short repair template, consisting of a DNA sequence homologous to the targeted area with the exception of a point mutation. The ZFN binds to a specific DNA sequence and generates a site-specific DSB. Gene repair mechanisms of the plant cell intervene to repair the break and generate site-specific point mutations by copying the repair template.

ZFN-3, ZFN genes are delivered to plant cells along with a large stretch of DNA (e.g., a gene of interest). The ZFN binds to a specific DNA sequence and generates a site-specific DSB. The ends of the DNA stretch are homologous to the sites flanking the DSB; therefore, the DNA stretch is site-specifically inserted into the plant genome.

The rationale of ZFN technology is to create site-specific mutations or gene inactivation leading to the desired phenotype, like herbicide resistance. The ZFN-3 approach can be used for targeted addition of genes of interest, gene replacement and trait stacking. Specific gene targeting can prevent so-called 'position effects' caused by random insertion of genes in the genome.

• **Oligonucleotide directed mutagenesis (ODM)**. Also known as targeted gene repair, oligonucleotide-directed gene targeting, genoplasty and chimeraplasty. Oligonucleotides are chemically synthesized to share homology with a target sequence, with the exception of a few nucleotides. Oligonucleotides induce site-specific mutation at the target sequence. The genetic changes include the introduction of a new mutation (replacement of one or a few base pairs), the reversal of an existing mutation or the induction of short deletions.

• **Cisgenesis and intragenesis**. Cisgenic and intragenic plants are produced by the same transformation techniques as transgenic plants, but the DNA transferred belongs to the same species of the transformed plant, or to a cross-compatible species. In cisgenesis, the DNA sequence includes the gene of interest flanked by its own promoter and terminator. In intragenesis, the gene of interest can be combined with regulatory elements from the species itself or from a cross-compatible species. Both approaches aim to confer a new property to the modified plant. By definition only cisgenics could achieve results also possible by traditional breeding methods, whereas intragenesis offers more options for modifying gene expression and trait development. Intragenesis can also include the use of silencing approaches, for example, RNA interference, by introducing inverted DNA repeats.

• **RNA-dependent DNA methylation (RdDM).** RdDM induces transcriptional gene silencing by methylation of promoter sequences. Genes encoding RNAs homologous to promoter regions are delivered to the plant cells. These genes give rise to the formation of small double-stranded RNAs that induce methylation and silencing of the homologous sequences. RdDM allows breeders to produce plants that do not contain foreign DNA sequences and in which no changes or mutations are made in the nucleotide sequence but in which gene expression is modified epigenetically.

• **Grafting.** A chimeric plant is produced by grafting a nongenetically modified scion on a genetically modified rootstock. Consequently, the fruits of the plant do not contain the inserted DNA sequence.

The rootstock can be modified to improve its rooting capacity or resistance to soil-borne diseases, resulting in a substantial increase in the yield of harvestable components. The rootstock can also be modified for obtaining gene silencing through the technique of RNA interference. In grafted plants, the small RNAs can also move through the graft so that the silencing signal can affect gene expression in the scion.

• **Reverse breeding.** Homozygous parental lines of a selected heterozygous plant are reproduced. The genes involved in the meiotic recombination process are silenced through transgenesis. Consequently, nonrecombined haploid lines are obtained from the heterozygous plant and their chromosomes are doubled through the double-haploid technique. The doubled haploids obtained are screened to find a pair that, would reconstitute the original heterozygous plants. Only nontransgenic plants are selected, thus the offspring of the selected parental lines would not carry any additional genomic change.

• **Agro-infiltration.** Three types of agro-infiltration can be distinguished:

'Sensu stricto,' nongermline tissues, mostly leaves, are infiltrated with a liquid suspension of *Agrobacterium* carrying a gene of interest. The gene is locally expressed at a high level, without being integrated into the plant genome;

Agro-infection, nongermline tissues, typically leaves, are infiltrated with a full-length virus vector containing a gene of interest. Through the virus vector, the expression of the gene of interest is spread in the entire plant;

The floral dip technique involves immersion of germline tissues, typically flowers, into a suspension of *Agrobacterium* carrying a gene of interest so as to obtain stable transformation. Transformed embryos are then selected at the germination state.

Agro-infiltration can be used to screen for plants with valuable phenotypes that can then be used in breeding programs, for instance, with specific genes from pathogens to evaluate plant resistance. The technique has also been developed as a production platform for high-value recombinant proteins. However, the technique is mostly used in a research context, for example, to study plant-pathogen interaction in living tissues (leaves) or to test the functionality of regulatory elements in gene constructs.

Regulators, advisory bodies and scholars have recently turned their attention to the legal classification and governance of some of the new plant breeding techniques[4–8]. The main question addressed is whether they differ from existing techniques and how the resulting products should be classified for regulatory purposes, according to current definitions of genetic modification.

With this in mind, the European Commission (Brussels) has assembled a group of experts from national regulatory agencies to evaluate whether certain new techniques constitute genetic modification and, if so, whether the resulting organisms fall within the scope of the EU GMO legislation[2,3]. (For a detailed description of the legal definition of GMO according to EU legislation, see **Supplementary Note 1**.) This group is evaluating the seven techniques studied in our paper (zinc finger nuclease technology (ZFNs), oligonucleotide directed mutagenesis (ODMs), cisgenesis and intragenesis, RNA-dependent DNA methylation, cisgenesis and intragenesis, RdDM, grafting, reverse breeding and agro-infiltration), which are regarded as technically advanced enough to merit legal evaluation as well as synthetic genetics (**Box 1**). In our study, we elected not to cover synthetic genomics because we deem it insufficiently advanced in plant research.

Research in new plant breeding techniques

We analyzed the research landscape through a keyword search in the bibliographic database ISI (now Thomson Reuters) Web of Science (**Supplementary Methods 2**). Research papers and reviews were screened individually for their relevance to plant breeding. The search was finalized in April 2010; therefore, results include all scientific publications on new plant breeding techniques until the end of 2009.

We identified a total of 187 relevant scientific publications. The picture emerging is that of a young sector with growing interest on the part of researchers. Most papers on new plant breeding techniques were produced in the past decade (with the exception of grafting on GM rootstocks) and the total number of papers is on the rise (**Fig. 1**). Considering individual techniques, the highest number of publications was identified for cisgenesis and intragenesis (36 papers), followed by RdDM and grafting on GM rootstock (31 papers each), agro-infiltration (26 papers), ODM (25 papers) and ZFN technology (20 papers). Only four papers were identified for reverse breeding, which is also the most recent technique according to publication dates.

EU public institutions have the largest share of publications, followed by North America (with publications mainly from the United States) (**Fig. 2**). The EU leads in research publications on cisgenesis/intragenesis, reverse breeding, RdDM and grafting on GM rootstock. The United States has the highest number of research papers on ZFN technology, ODM and agro-infiltration. The ten leading institutions publishing research on new plant breeding techniques (**Table 1**) are all public institutes with the exception of one.

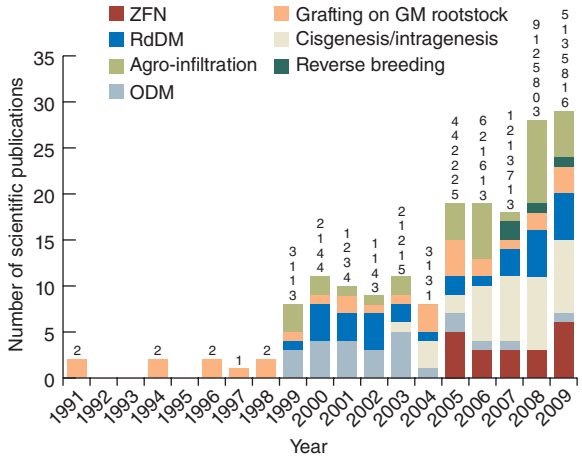

Figure 1 Number of scientific publications on new plant breeding technologies 1991–2009.

For each publication, we analyzed the plant species used and the traits introduced with the seven techniques. This permits a preliminary comparison of the stage of development of each technique and their potential application to crop plants. The majority of papers report proof-of-concept demonstration of the new techniques, mainly by introducing marker traits or traits for herbicide tolerance and pest resistance. **Table 2** presents publications with most relevance to actual deployment of a crop species (that is, model plants and marker genes are not included in the table. (More detailed information, including the inserted/modified genes and the complete references, are available in **Supplementary Note 2**.)

We find substantial differences among techniques in terms of their current applicability to crop species. For example, only one mutagenesis technique, ODM, has been proven to work on a variety of crop plants (that is, maize, wheat, canola and even banana), whereas other mutagenesis approaches, such as ZFN technology, have only been reported in model plants (maize and, very recently, soybean)[23,24]. Grafting on GM rootstock, cisgenesis and intragenesis, on the other hand, have already been used on several crop plants because they rely on existing tools for genetic modification (transformation by *Agrobacterium* or biolistics whereby the genetic information is delivered into the cell through particles coated with genetic material). RdDM has been applied in a few crop plants (maize, potato and carrot) for the silencing of several marker genes. Agro-infiltration, as a tool to screen for phenotypes in the breeding process (usually resistance to pathogens), has been described in important crops, such as rice, potato, tomato and beans. Finally, reverse breeding, has not yet been the subject of any scientific research papers, only a handful of reviews.

Patents in new plant breeding techniques

Whereas R&D in plant breeding is carried out both by the private sector and by public institutions, a search of the scientific literature, although useful for assessing the current knowledge about new techniques, will not provide insight into industry activities because most published data come from academic institutions. Therefore, in addition to searching the literature, we carried out a patent search to provide an overview of the applications for inventions related to the seven new plant breeding techniques. A patent landscape analysis based on the number of patents per technique can identify the main actors interested in the commercial exploitation of a technique and its potential applications.

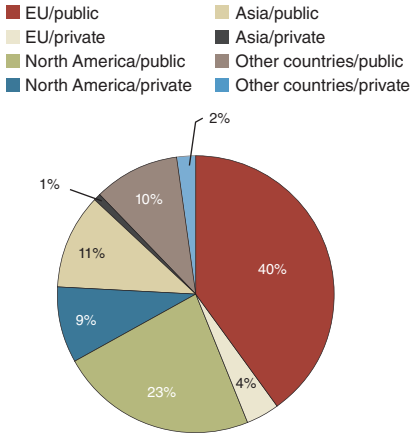

Figure 2 Country of origin and sector (public or private) of institutions authoring scientific publications on new plant breeding techniques.

Table 1 Ten leading institutions developing new plant breeding technologies ranked according to absolute number of publications and number of covered techniques

Institution	Location	Number of publications	Techniques[a]
Wageningen University	Wageningen, The Netherlands	21	C,R,G,B,A
University of California, Riverside	Riverside, CA, USA	11	O,R,G,A
John Innes Centre	Norwich, UK	9	C,R,G,A
J.R. Simplot[b]	Boise, Idaho, USA	9	C
Austrian Academy of Sciences	Salzburg, Austria	9	R
University of Amsterdam	Amsterdam	6	Z,O,C,R
Iowa State University	Ames, Iowa, USA	6	Z
Max-Planck Institute	Koln, Germany	4	O,R,G
University of Michigan	Ann Arbor, Michigan, USA	4	C,Z
Institute of Plant Genetics and Crop Plant Research (IPK)	Gatersleben, Germany	4	O,G

[a]Each technique is represented by a letter. Z, ZFN; O, ODM; C, cisgenesis/intragenesis; R, RdDM; G, grafting; B, reverse breeding; A, agro-infiltration. [b]Private institution.

We conducted a keyword analysis of three public databases: the World Intellectual Property Organization (WIPO; Geneva), the European Patent Office (EPO; Munich) and the United States Patent and Trademark Office (USPTO; Alexandria, VA, USA). We screened the patents for the relevance of their contents to plant breeding (**Supplementary Note 3**).

The patent search was finalized in November 2010. Because patent applications are published 18 months after filing, only patents filed by the end of 2008 were included in the findings. Both patent applications and granted patents were analyzed; therefore, when we refer to a patent, we could be talking about either. Each patent listed represents all members of its patent family (a patent family is defined as a set of patents—taken in various countries—that protect the same invention[25]).

We identified 84 patents, most of them filed after the year 2000 (**Fig. 3**). The most patents were filed for ODM (26 patents), followed by cisgenesis/intragenesis and ZFN technology (16 patents each). Grafting on GM rootstock (13 patents) and agro-infiltration (11 patents) followed closely, whereas only two patents were identified for reverse breeding and just one for RdDM (**Fig. 3**). The analysis of the patent claims shows some patents with rather general claims (in which the new technique is described without indicating a specific crop plant or trait) and other more specific patents claiming a final product (crop/trait combination). The crops and traits identified in patent claims on new plant breeding techniques are similar to the findings of the scientific literature search.

Figure 4 shows the distribution of patent applications to the USPTO and the EPO, and additionally the patent applications that went through the Patent Cooperation Treaty (PCT) route and are administered by WIPO. PCT is a route followed to obtain protection in any or all contracting states. Within 18 months of the PCT application, the inventor can select the patent offices of the countries in which to protect the invention, including the EPO and the USPTO. Therefore, the same application can be submitted to the three offices. Our search shows that most applications (94%) are found in the WIPO database, meaning that applicants followed the PCT route. The percentage of patents submitted to the USPTO (68% of the total) and to the EPO (65% of the total) is similar, suggesting that applicants see commercial interest in both the European and North American markets.

When looking at the country of origin of patent assignees, we found that the majority (65%) are US institutions (mainly private companies). Assignees based in the EU comprise 26% of the patent applications (**Fig. 5**). The search identified 50 assignees that are active in patenting new plant breeding techniques. **Table 3** shows the ten leading organizations ranked according to the number of patents assigned. Seven are US assignees (six private and one public institution) and the remainder are based in the EU.

These results differ from the findings of the scientific literature search in that US companies and universities are more active in patenting, despite the considerable research activity in the EU in the field of new plant breeding techniques. This result might be due to the generally stronger tradition of patenting innovation (public or private) related to plants in the United States compared to the EU. US-based inventors have been able to patent certain plants since 1930 (the Townsend-Purnell Act), and biotech-generated plants and processes since the mid-1980s. In the EU, the possibility of patenting plants dates only from 1998 (Directive 98/44/EC).

Another finding is the high degree of specialization of the companies active in this field. Most of the companies identified have patents covering just one of the seven techniques analyzed in our study (**Table 3**). In terms of specific techniques, US-based assignees are dominant in ZFN technology, ODM and grafting on GM rootstock. The results for cisgenesis/intragenesis and agro-infiltration show a similar activity in patenting for the United States and the EU. In contrast, all patents for RdDM and reverse breeding belong to assignees from the EU.

Adoption of the new techniques by plant breeders

We have shown that there has been activity in both research and patenting in new plant breeding techniques over the past ten years, suggesting that these techniques may be used by commercial breeders. To ascertain to what extent the new plant breeding techniques have already been adopted by the private breeding sector and to estimate the status of the development of commercial products, we conducted a written survey of plant breeding companies (**Supplementary Note 4**). The data obtained from this survey were confirmed during a workshop in which companies, stakeholders and regulators participated (**Supplementary Note 5**).

The written survey was carried out in March 2010 and was directed at companies already familiar with the use of biotech for plant breeding and at companies that provide

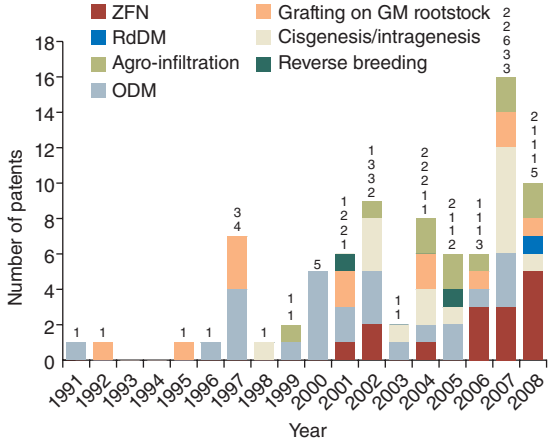

Figure 3 Patents on new plant breeding technologies 1991–2008. Priority date (date of first application) of each patent is given on the *x* axis. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

technology (that is, dedicated biotech companies providing techniques for plant breeding companies). We identified suitable companies and contacted them with the support of European and national seed breeders' associations. Twenty-seven companies agreed to participate and were sent the written survey; 17 completed and returned the questionnaires. Both large and small companies returned the questionnaires; employee numbers at each company ranged from 10 to 100,000. Most were individual companies, but some were branches of international groups or parts of complex business structures. In the case of these multinational operations, questionnaires were sent only to the EU-based branch to avoid duplications. Two companies were dedicated technology providers and 15 were active in plant breeding. Most companies

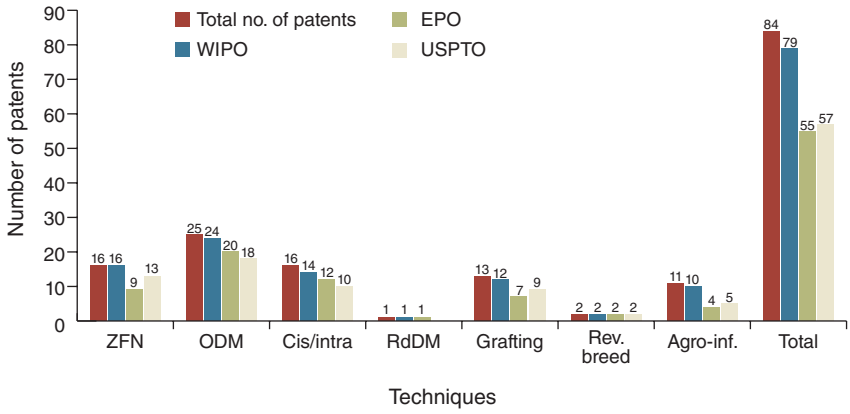

Figure 4 Patents on new plant breeding technologies at EPO and USPTO, and PCT applications (administered by WIPO), distributed per technique. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

Table 2 Most relevant crops and traits resulting from the use of new plant breeding techniques, according to literature findings[a]

Technique	Crop	Traits	Number of research papers
ZFN	Maize	Herbicide tolerance	1
	Tobacco	Herbicide tolerance	3
ODM	Maize	Herbicide tolerance	2
	Rice	Herbicide tolerance	1
	Tobacco	Herbicide tolerance	3
	Oilseed rape	Herbicide tolerance	1
Cisgenesis Intragenesis	Potato	Fungal resistance; black spot bruise tolerance; lower acrylamide levels	5
	Apple	Fungal resistance	3
	Melon	Fungal resistance	1
RdDM	Maize	Male sterility	1
	Potato	Modified starch content	1
	Petunia	Reduced flower pigmentation	1
Grafting on GM rootstock	Grapevine	Resistance against bacteria, fungi and virus; rooting ability	6
	Potato	Resistance against fungi and virus; changed composition	5
	Apple	Rooting ability	4
	Watermelon	Robust growth; virus resistance	4
	Orange	Fungal resistance; osmotic control	2
	Cucumber	Virus resistance	1
	Tomato	Insect resistance	1
	Plum	Resistance against fungi and nematodes	1
	Walnut	Rooting ability	1
	Pea	Virus resistance	1
	Rose	Rooting ability	1
	Tobacco	Resistance against bacteria	1
Agro-infiltration	Tomato	Production of vaccines (hepatitis B); screen for virus resistance	2
	Tobacco	Production of vaccines (hepatitis B, HIV, diabetes, influenza, toxoplasma, tetanus, tuberculosis, SARS, New Castle disease, Norwalk virus), antibodies (HIV, hepatitis, cancer, blood typing, crinivirus), therapeutic proteins and enzymes; screen for resistance against fungi and virus	23
	White clover	Production of vaccines (bovine pasteurellosis)	1
	Lettuce	Production of vaccines (SARS)	1
	Rice	Screen for virus resistance	2
	Bean	Screen for virus resistance	1
	Potato	Screen for resistance against fungi and virus	3

[a]Reverse breeding is not included because no research papers on specific plants have been identified.

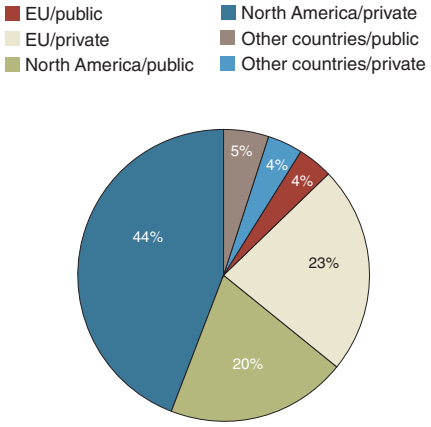

Figure 5 Country of origin and sector (public or private) of patent assignees on new plant breeding techniques. 'Patents' refer to both granted patents and patent applications and each patent represents all members of its family.

focused on the production of major arable crops (cereals, oilseeds and potatoes) with only a few companies active in minor crops such as vegetables.

Breeding companies were asked if they used any of the seven techniques. Additionally, they had to specify for what crops and traits the techniques were used, as well as the phase of development of the eventual commercial products based on these techniques.

The survey shows that each of the seven new plant breeding techniques is being used in breeding programs by two to four of the 17 surveyed plant breeding companies. Crops developed with some of these techniques have reached commercial development up to phase 3 (**Box 2**).

In the case of targeted mutagenesis techniques, ODM-derived products are in phases 2 and 3, namely oilseed rape and maize with tolerance to herbicides. ZFN technology is being used in breeding maize, oilseed rape and tomato in projects ranging from research phase to phase 3. Traits were not disclosed. ZFN-2 seems to be the least developed of the three ZFN variants (**Box 1**).

Cisgenesis/intragenesis-based products in phases 1–3 included maize, oilseed rape (traits not disclosed) and potatoes (fungal resistance). With this technique, we were able to complement the results of the survey with an analysis of field trials done in the EU[26] because cisgenesis/intragenesis are subject to mandatory notification in the EU and information is stored in a searchable public database. The EU database revealed field trials of potatoes for starch production and late-blight resistance using cisgenesis and intragenesis.

We did not identify any product in an advanced development phase with regard to grafting in the survey. Even so, this technique is also subject to mandatory notification and a search of the EU field trials database revealed trials with apple and pear GM rootstocks with improved rooting ability, grapevine GM rootstocks resistant to viruses, and orange tree GM rootstocks with dwarf phenotype and resistant to fungal diseases.

According to the survey, agro-infiltration is being used in breeding potatoes, oilseed rape and lettuce. In the case of lettuce, the aim was to test breeding lines for resistance to downy mildew. Finally, RdDM was reported to be used in maize and oilseed rape (up to phase 3) and reverse breeding was adopted in several crops but only at a research stage.

The questionnaire included an open-ended question concerning the use of other new plant breeding techniques not contemplated in the study. The meganuclease technique[27] was mentioned, which can be used in a similar way to ZFN technology for site-specific mutagenesis or for targeted gene insertion in plant breeding. This technique was reportedly used in maize breeding (trait not disclosed, phase 1). Another technique mentioned concerned the delivery of DNA-modifying enzymes (e.g., ZFNs or other nucleases like transcription activator–like effectors) directly into the plant cells without introducing nucleic acids. Breeding companies also appear to be very interested in a set of techniques that fall into the category of 'transgenic construct-driven breeding techniques'. This heterogeneous group of techniques has as its common feature the use of a transgenesis step during the breeding process that is subsequently eliminated by crossing and selection in the final commercial line.

Drivers and constraints for further adoption

We used several sources to obtain information on drivers and constraints for the adoption of new plant breeding techniques. These include feedback during the workshop, available literature and discussions with experts from Plant Research International, which is part of Wageningen University in The Netherlands.

Most of the new plant breeding techniques discussed in this report can be used for producing genetic variation, the first step in plant breeding. Those aiming at targeted mutagenesis (ZFN-1 and ZFN-2 technology (**Box 1**) and ODM), or targeted introduction of new genes (ZFN-3 technique), provide technical advantages compared with older techniques. Unlike mutagenesis induced by chemicals or irradiation and transgenesis, which result in random changes in the genome, the application of ODM or ZFN techniques leads to ostensibly site-specific mutations or insertions.

Cisgenesis uses the same transformation method as transgenesis and therefore benefits from whatever technical advantages particular transformation systems provide. With cisgenesis, only DNA fragments from the species itself or cross-compatible species are involved, resulting in plants which, in principle, could be created by conventional breeding. But cisgenesis has the advantage of introducing only the desired gene, thereby avoiding any linkage drag that can result from conventional crossbreeding and eliminating tedious and time-consuming backcrossing to recover the initial quality traits of the parent.

For some of the techniques (e.g., ZFNs, RdDM and reverse breeding) the genetic information coding for the desired trait is only transiently present in the plants or stably integrated only in intermediate plants. Segregating progeny carrying the gene creates new lines without the transgene.

Economic advantages are also driving the adoption of new plant breeding techniques. The time saved, when compared with conventional breeding, is rated highly by experts. Some new plant breeding techniques speed up the breeding process and consequently returns from the market can be generated earlier, increasing the value of the investment in R&D. Cisgenesis uses the same gene pool as conventional breeding, but is much faster when the appropriate gene is inserted directly into elite gene pool progenitor(s), saving backcrossing time. For example, development of cisgenic apples by Plant Research International for resistance against apple scab required 12 years. In contrast, the conventional methods of crossing the elite variety with wild ones (carrying the gene of resistance) took around 50 years (http://www.cisgenesis.com/content/view/7/35/lang,english/). Because apples are not self-compatible, the initial variety needs to be crossed many times with different varieties, but not all the initial qualities can be maintained. In contrast, the cisgenic counterpart maintains those qualities because the elite variety is the one that is transformed.

On the basis of the information obtained from workshop participants, an important technical constraint for the use of new plant breeding techniques is their generally low efficiency[9,28]. However, estimates for the efficiency of the techniques are difficult to make for various reasons—it depends on the crop, the method, the genes involved and marker genes in case they are used. Information in the literature is usually very specific in terms of the plant and genes involved and results are highly variable. For ZFN-induced mutations, frequencies

Box 2 Definitions of commercial development phases

In the present study, we have analyzed new plant breeding technologies in the context of their progress in commercial development. We have categorized the various stages of development into the following four phases:

- Phase 1. Construct optimization, use in target crop
- Phase 2. Trait development, preregulatory data, large-scale transformation
- Phase 3. Trait integration, field testing, regulatory data generation (if applicable)
- Phase 4. Regulatory submission (if applicable), seed bulk-up, pre-marketing

of 2% were reported in *Arabidopsis*[29], whereas in tobacco, a value of 40% efficiency was reported[10]. Additionally, given the current state-of-art of the technology, nontarget mutations resulting from nonspecific binding of the ZFNs are likely to occur[30,31].

Other techniques for which efficiency improvements are needed according to experts are RdDM—here the instability of methylation status is seen as the biggest hurdle for commercial applications—and cisgenesis and intragenesis—for which the efficiency of the technique ranges from low to high depending on species and cultivar. The concept of cisgenesis and intragenesis also considerably restricts the choice of promoters and the use of selectable marker genes, which—in the case in which they are used—have to be removed in the final breeding steps[32].

Therefore, further R&D of the techniques is required. An area of particular interest is developing efficient methods of delivering desired constructs into a plant cell. Suitable techniques of delivery have to be developed or adapted specifically for each technique as well as for each crop modified by these techniques.

Uncertainties regarding the regulatory status worldwide and possible high regulatory costs are other constraining factors for the adoption of the new techniques. If a technique or its products are classified as GMO, it will generate additional time and financial costs compared with nonregulated classic breeding techniques. Several studies have evaluated the time and cost of the regulatory research associated with transgenesis (GM crops)[33–35]. A recent study by Crop Life International (Brussels) found that it takes 5.5 years to generate the data for a regulatory dossier at the cost of $35 million per new GM event[36]. Thus, as with GM techniques, the high entrance costs may be a disincentive to the use of these new techniques by smaller companies, and their application may be limited to traits and high-value crops[34,37]. The additional time delay associated with the launch of a new event under the GM legislation also has major implications on the expected time to profits. Launching a variety one year earlier results in a present estimated added net value of $1–100 million, depending on the commercial value of the crop[38].

Regulatory uncertainty may be particularly cumbersome for establishing new techniques, as they generally are used in the early stages of the breeding process, which can take up to 15 years. Therefore, it may be difficult for plant breeders to invest in projects using these new techniques when regulatory costs would affect the economic potential, particularly for orphan crops, or where concerns associated with the use of GM approaches might compromise local consumer acceptance.

Challenges for detecting plants derived by new techniques

Another consequence of plants being classified as GMOs is the need to develop methods for quantitative detection or identification of the final product put on the market. This is a mandatory requirement in some regions, such as the EU[5] but comes into play elsewhere given the global trade of agricultural commodities and the differences in requirements for approval between trading partners[39].

Current standard methods for GMO detection are largely based on DNA and rely on PCR. An expert group evaluated the changes in the genomes of plants produced by these new techniques as an important element for risk assessment[40]. In addition, an expert group from the European Network of GMO Laboratories—a network of enforcement laboratories from EU countries—looked at the possibilities for detecting and identifying crops produced with new plant breeding techniques[41]. This group made the distinction between possibilities for detecting a change (the possibility of determining the existence of a change in DNA by reference to an appropriate comparator) and identifying the change (the possibility of determining that a particular change in the DNA has been intentionally introduced by the breeding technique). A key factor affecting whether detection and identification are feasible is the availability of prior knowledge of the DNA sequence of the particular product.

For plants produced with ZFN-1 and ZFN-2 techniques (targeted modification of a single or few nucleotides; **Box 1**), detection with DNA-based methods, like PCR, would be possible only with prior information on the nucleotide sequences flanking the introduced modification. Even so, identification is not possible because the same changes could be generated by other mutagenesis techniques or by natural genetic variation. The same conclusions can be applied to ODM technique.

For plants produced with ZFN-3 technology (targeted insertion of larger sequences, even whole genes; **Box 1**) detection and identification would be possible if prior information on

Table 3 Ten leading organizations in patents on new plant breeding techniques ranked according to absolute number of patents and number of covered techniques

Institution	Country	Entity	Number of patents	Techniques
Sangamo Biosciences	US	Private	11	Z
Dow Agrosciences	US	Private	5	Z
University of Delaware	US	Public	5	O
J.R. Simplot	US	Private	5	C
Cornell Research Foundation	US	Private	5	G
Keygene	The Netherlands	Private	4	O
Pioneer Hi Bred	US	Private	3	Z, O
Cibus Genetics	US	Private	3	O
Wageningen University	The Netherlands	Public	3	C
Plant Bioscience	UK	Private	2	C, A

Z, ZFN; O, ODM; C, cisgenesis/intragenesis; G, grafting; A, agro-infiltration.

flanking sequences were available. Similarly, for cisgenic/intragenic plants, detection and identification is feasible as long as appropriate prior information on the event is available to design specific PCR primers. For plants produced by grafting non-GM scions onto GM rootstocks, detection and identification of the scion-derived products is currently not possible, whereas detection and/or identification of GM rootstocks is possible with standard methods used for GMO detection.

In the case of plants derived using the RdDM technique, gene silencing is obtained through DNA and/or histone methylation but the DNA sequence itself is not modified. A typical enforcement laboratory will not be able to differentiate between naturally induced methylation patterns and those induced by the deliberate use of RdDM, so products from this technique cannot be routinely detected or identified.

The same can be said for reverse breeding (and, by extension, new techniques using 'negative segregation' transgenes in the final product). The end product of reverse breeding will not contain a genetic modification for which a routine DNA-based detection method can be developed.

Finally, in the case of agro-infiltration, if the constructs introduced into plants are not integrated or replicated, their presence can be detected only in the agro-infiltrated tissue and not in the progeny plant. Detection and identification of agro-infiltrated plants that contain inserted fragments would be possible with standard methods used for GMO detection.

Conclusions

Interest in the new breeding techniques by those who make regulatory and oversight decisions is based on the assumption that these techniques are being used by the breeding sector and that commercialization is imminent. Yet evidence is lacking on whether this assumption is supported by facts. We show here that products derived from several new techniques are in late stages of development, which indicates that the commercial sector has indeed incorporated them into breeding programs of important crops.

We conclude that biotech use in plant breeding has evolved quickly over the past decade, incorporating new techniques for targeted mutagenesis, using epigenetics, reverse breeding and other applications in which transgenesis is only used in an intermediate step of the breeding process. Transgenesis and marker-assisted selection, techniques behind many commercial varieties of agricultural crops produced in the past 20 years, are now joined by new tools derived from modern biotech.

Public research institutes based in the EU play a prominent role in the R&D of new plant breeding techniques. However, US-based companies and public institutions are more active in patenting these techniques. Overall, the activity in R&D and patenting reveals a strong interest in the plant breeding sector in modern biotech. Our industry consultation and survey, the first on this topic, reveals that these techniques are already incorporated into commercial breeding programs. ODM, cisgenesis/intragenesis and agro-infiltration appear to be the most often used techniques and the first crops developed with these techniques have reached an advanced commercial development phase. ZFN technology, RdDM, grafting on GM rootstocks and reverse breeding are less often used and are still mainly applied in research. The most advanced crops could reach the commercialization stage in the short to medium term (2–3 years). The first products will likely correspond to simple agronomic traits (e.g., herbicide tolerance) that have been used widely in the process of developing the technology. However, the examination of the stage of adoption by plant breeders reveals more complex traits at earlier development stages.

The fact that the field is evolving quickly is supported by the identification in our survey of additional techniques used by breeders and not included in our study. These include new approaches to targeted mutagenesis (e.g., engineered meganucleases). Application of engineered nucleases for targeted mutagenesis is a particularly active research field where systems based on different nucleases are constantly being developed[42].

The new breeding techniques are adopted by the industry because of the potential technical and economic advantages they offer compared with alternative techniques. The extent of the adoption, and the application of the techniques to a wider range of crops, will depend on many factors, including the need to increase the technical efficiency of some processes and the decisions on their regulatory status worldwide.

In the next few years, many regulatory jurisdictions around the world will make decisions on the governance of new plant breeding techniques, which will have implications for technology adoption, but also for the global agricultural supply chain. Decisions on regulatory oversight governed by both scientific principles and political expediency are also complicated by the fact that the products of many of these techniques are not detectable or identifiable with standard methods used for GMO detection.

The differences worldwide in the regulatory regimes for GM crops have resulted in asynchronicity in approvals of new crops. Because agriculture is an open process, the presence of unauthorized GM material cannot be excluded in traded commodities. If the importing country operates a 'zero tolerance' policy, imports may be rejected if they contain traces of unauthorized GMOs[43]. A global discussion on the governance of these new techniques seems necessary in the light of previous experiences with current biotech-derived crops and trade disruptions.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING FINANCIAL INTERESTS

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