POSITION PAPER

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New Breeding Techniques: Necessary tools to address forthcoming challenges in plant breeding

The "Groupement d'Intérêt Scientifique Biotechnologies Vertes" (GIS BV), the "Animation Scientifique Biotechnologies Végétales" of the INRA Division "Plant Biology and Breeding" and the "Plant Biology and Biotechnologies" working group (GT4) of the AllEnvi Alliance organized a scientific workshop on April 1st 2014 on the topic of «New Breeding Techniques» in Paris (France). Fifty-seven scientists from the public and private members of the GIS BV gathered at this event. Scientists presented research work carried out on the technologies listed in the JRC scientific and technical report, which are zinc finger nuclease (ZFN), oligonucleotide directed mutagenesis (ODM), cisgenesis, intragenesis, RNA-dependent DNA methylation (RdDM), grafting on GM rootstock, reverse breeding, agroinfiltration (agro-infiltration "sensu stricto", agro-inoculation, floral dip) and synthetic biology. Due to their significant interest for plant breeding, saturation mutagenesis, recombination and fast breeding were also addressed during this workshop. The presentations were followed by a global discussion. This paper summarizes the strategies identified and addresses the research opportunities for the French public-private community, in the light of the scientific and socio-economic issues regarding the implementation of the three most promising new breeding techniques.

Socio-economic context

Since its first day, mankind has been facing challenges such as food security. Massal selection was a first answer to provide sufficient food and feed. In the last century, the development of modern plant breeding allowed countries like France to implement the green revolution, increasing yields at an unprecedented scale notably through the introduction of hybrid seeds and semi-dwarf cereals. More recently, the emergence of GM-crops opened the way to a reduction of pesticide use in countries adopting the technology. For example, *Bt* crops reduced insecticide applications in the USA by 56 Mt between 1996 and 2011.

Nowadays, the challenge of food security coupled with energy supply and sustainable chemistry is even more difficult with agricultural systems that have to be economically viable, socially acceptable and respective of the environment. There is urgent need to produce in an agro-ecological framework, to mitigate climate change, to set aside arable land for non-food uses and to overcome the stagnation of genetic progress. The community gathered in the GIS BV strongly believes that plant breeding can help to address these challenges with the help of technological breakthroughs.

Plant breeders are strongly investigating a variety of entry points to meet the challenges they are facing today. Their main objectives are:

(i) to reduce the time to market (diminishing the number of generations, shortening the generation time)

(ii) to minimise genetic drag when introducing novel traits in already optimised germplasm

(iii) to capture the largest possible gene pool (screening of germplasm, enlarging the gene pool to non-related species)

(iv) to compensate the slow-down of genetic progress with innovative approaches

(v) to include demands for durability in breeding schemes

To meet these challenges new breeding techniques (NBT) offer themselves to plant breeders. They include genomic selection, which is taking marker assisted selection to a new level and allows predictions of agronomic performance, Site Directed Nucleases SDN), which allows to carry out genome modifications of unprecedented precision, saturation mutagenesis, which is the tool of choice for the optimisation of alleles and the modulation of recombination to combine loci in novel ways.

New breeding techniques for a necessary breakthrough

SDN, saturation mutagenesis and enhanced recombination are "knowledge based" NBTs that allow exploiting rapidly and efficiently genetic, physiological, biochemical, agronomical or phenotypic knowledge linked to a particular gene or allele, including from other species. They are complementary to "black box" techniques such as genomic selection, which rely on high throughput genotyping and phenotyping without any knowledge of the identity or the biological function

of the loci involved. Knowledge based NBTs allow to enlarge the gene pool and optimise the alleles available to black box strategies, thereby enhancing trait performance.
Hereafter we will focus on contributions of the 3 techniques appearing as the most innovative and most pertinent to meet future challenges of agriculture.

(1) Site directed nucleases (SDN) overcome a major drawback a classical transgenesis, i.e. the random insertion of transgenes in the genome. SDN act at a predetermined site of the genome and allow creating small deletions (SDN1), to replace a gene by an optimised allele of the same species (SDN2) or to insert foreign DNA (SDN3).

(2) Saturation mutagenesis multiplies natural genetic variability by the use of chemical mutagens such as the alkylating agent ethylmethane sulfonate (EMS). Theoretically up to 144 alleles can be created for a protein of 100 aa. To reach this limit with realistic population sizes of <20000 plants, a density of approximately one mutation every 500 kb needs to be attained. Mutagenised populations are used either in forward genetics by screens for desired agronomic traits or in reverse genetics by providing allelelic series for a gene underlying a valuable trait. Allele performance can either be predicted or tested experimentally by appropriate phenotyping.

(3) The rate of **meiotic recombination** can be increased by the knockout of genes such as *FANCM* or *FidgetinL1*, overcoming regulatory cellular mechanisms such as the interference between cross-overs, thereby enhancing genetic diversity. The knockouts increase naturally rare class II cross-overs without detrimental effects on meiosis or fertility. In the model plant *Arabidopsis*, double mutants show synergy and a 6 times increased cross-over rate. The influence of the *fancm* and *figL1* mutations on the distribution of cross-overs between "hot" (often close to telomers) and "cold" regions (mainly close to centromers) remains to be determined, just like efforts to change both cross-over distribution by implementing in plants GAL4-SPO11 system working in yeast.

These three techniques can significantly contribute to breeding towards agro-ecological demands and renewed genetic progress, which are the main challenges of plant breeding for the mid-long term. They provide the necessary means to achieve goals such as the shortened breeding schemes, enlarged breeding gene pools or limited yield drag.

Technologies that meet the breeders' challenges

1. Shortening breeding schemes.
SDN mediated gene replacement (no need for backcross) or the use of specific and characterized landing pads (one-off deregulation, gene stacking) considerably shorten the time necessary to achieve a desired phenotype in a variety. In addition, improved recombination levels allow breeders to reduce the number of generations necessary to create a highly diverse pre-breeding gene pool.

2. Enlarging the gene pool.
All three technologies allow building new phenotypes beyond natural variability. SDN technologies and saturation mutagenesis will create completely new alleles not present in natural germplasm, whereas highly recombinant plants allow to decrease linkage desequilibrium and to create novel genetic frameworks for the breeder.

3. Limiting the genetic drag.
The genetic drag is commonly observed in case of wild QTL or transgene introgression into elite germplasm. By using SDN technologies in a context of gene replacement or gene stacking at a specific and characterized landing pad, breeders could drastically decrease the risk of genetic drag. In parallel any system improving the recombination level at the genome scale will increase the probability of recombination events close to the introgressed region of interest and thus shorten the time necessary to minimize the genetic drag.

4. Increasing genetic gain.
Increased genetic gain has been achieved by hybrid systems and heterosis. SDN technologies or saturation mutagenesis provide tools to manipulate sex determination and fertility restoration, which are the main bottlenecks in crop species lacking hybrid system of breeding.

Scientific and Technological Bottlenecks

Whereas EMS based mutant collections exist for many species, true **saturation mutagenesis** getting close to the theoretical limits for allele number and population size remains a challenge just like the detection of mutations in a gene of interest by next generation sequencing or the "cleanup" of the resulting genetic background with a mutation every 500 kb. However, decent technical solutions exist for all these points and the main problem today resides in the important upfront investment and the logistics of creating, screening, handling and maintaining this type of collection. Nevertheless, alternatives are demanded for the reduction of the very elevated level of background mutations by other means than backcrosses, especially for species with long life cycles or clonal propagation.

Concerning **SDN technologies**, it appears clearly that several aspects need to be improved in order to fully master the entire process and enlarge their use to all crops of agricultural interest. The major bottlenecks are the low rates of SDN2 and SDN3 (in the percent range of the transformants obtained) as well as the absence, low efficiency or genotype dependence of plant transformation systems for numerous crop species. Minor bottlenecks concern (i) the type and rate of SDN delivery into the cell (direct or *via Agrobacterium*, as DNA, RNA or protein), (ii) the predictability of gene expression at the predetermined site, in particular in the context of gene stacking (iii) the characterisation and possible reduction of "off-target" activity in other regions of the genome and (iv) the establishment of efficient molecular and/or cellular screening methods to detect the rare SDN events among other outcomes of the transformation process.

Finally concerning **enhanced recombination techniques**, notwithstanding the current scientific progress, the technique is still in its infancy. In a first instance, proof of concept needs to be provided in agricultural crops. The adoption in agricultural crops will then probably depend on an increased efficiency in a heterozygous context, the availability of a dominant system (for example by RNAi knockdowns) and the establishment of an inducible system allowing to switch the system on or off on demand. A major challenge is the further improvement of recombination frequency by the identification and exploitation of additional molecular players. Another challenge of great interest to the breeding community is the manipulation of the recombination landscape (distribution of cross-over frequency in the genome) leading to new gene rearrangements.

Identification of research targets for future investments.

The evaluation of the above scientific and technological bottlenecks allowed the identification of the following research targets and necessary future investments:

- **Decipher the machinery of homologous recombination by comparative functional analysis across species** in order to understand and overcome its extremely low rate in higher plants.
- **Establish in a variety of crop plants efficient, genotype-independent transformation systems optimised for the delivery of SDN** and suitable for high throughput screen of gene deletion, replacement or insertion.
- **Achieve saturation mutagenesis at the gene rather than genome scale**, for example by attaching suitable enzymes to Transcription Activator Like (TAL) domains recognising the targeted gene.
- **Develop saturation mutagenesis resources for a number of crops of interest for agriculture** and assure the long-term access, screening, handling and maintaining of collections by public-private partnerships.
- **Decipher the meiotic recombination machinery by the identification of more recombination limiting factors** in order to optimize the efficiency or achieve dominance for inclusion in a breeding process.
- **Understand the meiotic recombination landscape** and modify the interplay between chromatin structure and molecular players (FANCM, FIG, SPO11) in ways useful for efficient breeding.

Genomic selection and apomixis were not addressed during that workshop due to the considerable size of the scientific communities involved and the diversity of the scientific aspects but will be treated in dedicated future analyses as they are also new techniques of significant interest for plant breeding.

This paper is endorsed by the strategic committee of the GIS BV, the "Plant Biology and Biotechnologies" working group (GT4) of the AllEnvi Alliance.

ANNEX 1. Presentation of NBTs and their characteristics

NBT	Short description	Strengths	Weaknesses
Cisgenesis	introduction of native DNA from the same or a crossable species; *sensu strictu* implies elimination of all vector DNA	no genetic drag faster than introgression from wild species, especially in the case of perennials can easily be assimilated to natural process of introgression	insertion does not necessarily occur at the locus where introgression would occur complete elimination of vector (for example T-DNA borders) remains challenging
1. Intragenesis	introduction of re-arranged DNA (other promoter, RNAi configuration) from the same or a crossable species; *sensu strictu* implies elimination of all vector DNA	genetic modification is limited to the modulation of expression of a native trait	more limited than classical transgenesis without offering scientific adavantages complete elimination of vector (for example T-DNA borders) remains challenging assimilation to naturally occurring variability is far fetched
2. Fast breeding	cross to a rapid cycling transgenic variant for backcrosses, then return to non-transgenic line	faster than backcrosses in standard line, especially in the case of perennials	rapid cycling transgenic variant not always available trait of interest may be influenced by transgene
3. SDN1	creation of short deletions at a predetermined site of the genome via the action of site-directed nucleases	robust way to obtain true loss-of-function alleles, especially in species without mutant resources resulting plants can be assimilated to naturally occurring mutants	possibility of "off-target" modifications at other loci in the genome mutations are recessive compared to dominant RNAi
4. SDN2	insertion of a native or modified DNA fragment from the same or a crossable species at its native locus via the action of site-directed nucleases	replacement of a gene by superior variant no interference with resident gene resulting plants can be assimilated to naturally occurring mutants	very low frequency of the targeted insertion relative to other outcomes requires efficient screening systems possibility of "off-target" modifications at other loci in the genome
5. SDN3	insertion of a foreign DNA fragement at a predetermined site of the genome via the action of site-directed nucleases	targeted insertion of transgenes one-off characterisation of an insertion site (land pad) enhanced predictability of transgene expression prime tool for transgene stacking	very low frequency of the targeted insertion relative to other outcomes requires efficient screening systems possibility of "off-target" modifications at other loci in the

			genome
NBT	**Short description**	**Strengths**	**Weaknesses**
6. Oligonucleotide directed mutagenesis (ODM)	mutation of one bp via the introduction of an oligonucleotide that serves as template during repair	gene modification without DNA integration in the genome	extremely low frequency more limited in possible modifications than SDN1 or SDN2 requires efficient screening systems
7. RND dependent DNA methylation (RdDM)	gene silencing by the expression of a hairpin construct with similarity to the promoter	heritable gene silencing after segregation of the triggering construct	severe doubts on the stability of the phenotype (obtained by gene silencing) over generations
8. Enhanced Recombination	increased overall recombination (cross over) rate by loss-of-function mutants in genes such as FANCM or FIDGETIN	faster exchange of genetic material, for example during introgressions of QTLs	awaiting proof of concept beyond *Arabidopsis* less efficient in hybrid backgrounds ideally should be inducible
9. Saturation mutagenesis	collection of chemically induced mutants that affects up to 80% of the amino acids encoded in a genome	allelic series for a gene of interest source of loss-of-function mutants source of interesting traits (forward screen)	necessitates production and characterisaiton of the resource is less suited for polyploids
10. Agroinfiltration	transient expression (agro-infiltration *sensu strictu*) of a gene of interest to produce large quantities of the gene product or to screen many gene constructs	valuable screening tool for transgene collections high yield confined production of value added products high flexibility for changes in the construct	
11. Grafting	protection against pathogens or increased yield of aerial parts by grafting of non-GMO scion on GMO rootstock	absence of transgene in the harvested parts absence of transgenic pollen or seed possible action of small RNAs produced in rootstock on targets in scion	interest limited to species where grafting is used in routine modified phenotype is not always detected in the non-GM scion
12. Reverse breeding	obtention of inbred lines that allow to reconstitute a plant of interest by simple cross; necessitates blockage of recombination, induction of haploids, duplication of haploids.	eternalisation of a genetic material of interest	limited to sexually propagated crops labour, time and cost intensive compared to standard doubled haploid lines

Annex 2. List of Participants

ATANASSOVA Ana (Bayer CropScience), BAULAND Cyril (INRA), BECKERT Michel (MESR), BENDAHMANE Abdel (INRA), BERTHE Grégoire (Céréales Vallée), BLARY Aurélien (INRA), BOUSSOUF Lise (SYNGENTA), BRANCOURT Maryse (INRA), CARANTA Carole (INRA), CASTELLETTI Sara (INRA UMR GV), CHARDIN Camille (INRA), CHAUVIN Laura (INRA), CHEVREAU Elisabeth (INRA), COURSOL Sylvie (INRA), DEVAUX Pierre (Florimond Desprez), DI BERARDINO Julien (INRA), DUCHENE Christiane (Limagrain), FALQUE Matthieu (INRA), FIERLEJ Yannick (INRA), FITAMANT Lydie (SYNGENTA), SEGUIN Marie-Paule (MAISADOUR SEMENCES), GALLAIS André (AgroParisTech), GEFFROY Valérie (INRA), GIGER-JANSKI Natacha (GIS BV), GIRIN Thomas (INRA), GUIHARD Gabriel (INRA), GUIDERDONI Emmanuel (CIRAD), HAMON Céline (Vegenov), HERNOULD Michel (INRA/Univ Bordeaux), HUOT Priscilla (SYNGENTA), JOUANIN Lise (CNRS / INRA), LASARACINA Olivia (GIS BV), LEMONTEY Claire (INRA Transfert), LUCAS Olivier (RAGT), LUCAS Hélène (GIS BV), MALPICA Anna (SYNGENTA), MATHIS Fabienne (Vegepolys), MERCIER Raphaël (INRA), MOQUET Frédéric (GAUTIER SEMENCES), MUNOS Stephane (INRA), NOGUE Fabien (INRA), OCHATT Sergio (INRA), PAUL Wyatt (Biogemma), PIGNARD Annie (SYNGENTA), QUILLERE Isabelle (INRA), REYMOND Matthieu (INRA), RICHARD Manon (IBP), RIVIERE Pierre (INRA), ROBERT Olivier (Florimond Desprez), ROGOWSKY Peter (INRA), SZAMBIEN Maxime (GIS BV), TEPFER Mark (INRA), THOMAS Gérard (SYNGENTA), THOMPSON Richard (INRA), VAUCHERET Hervé (INRA), WEILL Ariane (UPS).