POSITION PAPER

April 2013

Cereals benefiting from nitrogen fixation: strategies for the French public-private research community

The "Groupement d'Intérêt Scientifique Biotechnologies Vertes" (GIS BV) and the "Plant Biology and Biotechnologies" working group (GT4) of the AllEnvi Alliance organized a scientific workshop on March 14th 2013 on the topic of «Nitrogen fixation: applications to cereals» in Clermont-Ferrand (France). Forty-five scientists from the public and private members of the GIS BV gathered at this event. Invited as keynote speaker, Jean-Michel Ané (University of Wisconsin, USA) gave an overview of research strategies and current international initiatives to test the feasibility of engineering nitrogen-fixing cereals. Twelve scientists from France's public institutes presented the research work carried out at the national level on various aspects of nitrogen-fixing symbiotic interactions. 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 nitrogen fixation in cereals.

Socio-economic context

Synthetic nitrogen fertilizers are the main source of nitrogen for cereals. Because the production of these fertilizers is based on the very energy consuming Haber-Bosch process, their financial cost is high and varies according to fossil fuel prices. Depending on the crop and region, synthetic nitrogen fertilizers can represent up to 30% of the cereal production costs. Besides financial consideration, nitrogenous fertilizers also have a high ecological cost due to greenhouse gases, particularly CO_2 and N_2O released during their production and utilization and also to leaching following their application, leading to land and water pollution.

Reducing the use of nitrogenous fertilizers is therefore a major target to improve agricultural sustainability, to reduce pollution and to reduce costs to farmers and consumers. Although, in recent years increasing Nitrogen Use Efficiency through adopting "Best Nutrient Management Practices" has led to an overall stabilization in nitrogenous fertilizer use in the European Union at about 13 million tonnes, on a global scale this use is still rising[1].

In contrast to cereals, legume crops and actinorhizal plants can obtain up to 100% of their nitrogen requirements through establishing symbioses in root nodules with specific bacteria. These bacteria containing the nitrogen-fixing enzyme nitrogenase (encoded by *nif* genes) are able to convert the dinitrogen in the air to ammonia, directly usable by the plant. This attribute has led to renewed interest in increasing the use of legumes in agriculture, through improved breeding and management strategies[2]. However as cereals represent 55% of the crops grown globally for food and feed, international interest has also turned to whether cereals could benefit more from nitrogen fixation[3]. Recent scientific and technological advances suggest several biotechnology strategies through which this may be achieved (Table 1).

Strategies and international interest

1. Improving endophytic/epiphytic interactions with nitrogen-fixing bacteria

Most plants, including cereals, form epiphytic or endophytic associations with free-living diazotrophs which can lead to some nitrogen from fixation being transferred to the plant. However the benefit for the

[1] Good & Beatty 2011 PLoS Biol. 9 e1001124; FAOStat, 2011 (http://faostat.fao.org/)

[2] ANR PeaMUST (2012-2020); EU FP7 KBBE 2013.1.2-02

[3] Charpentier & Oldroyd (2010) Curr Opin Plant Biol 13:556-64; Beatty & Good (2011) Science 333:416-7

plant is highly variable depending on the type of association, the plant per se and the growing conditions. Whether the benefit comes from enhanced nitrogen fixation or from other microbial activities remains a debate[3]. Recently a new approach has shown that the plant genotype strongly influences rhizosphere interactions and potential benefits to the plant. In parallel, synthetic biology is being used to genetically improve or introduce nitrogen-fixation ability into competitive cereal rhizospheric bacteria.

Thus this strategy can be tackled from both the plant and the microbe's genetic variability to identify the best associations. It is a very explorative approach that may deliver new plant varieties with a better adaptation to microbial associations on the one hand, and on the other, it could deliver new microbial strains that could be associated with given crop varieties to enhance nitrogen fixation and transfer for plant growth.

2. Developing an endosymbiosis with nitrogen-fixing rhizobia

Legumes and actinorhizal plants have evolved well developed root nodule bacterial endosymbioses with Rhizobia and Frankia, which can fix and supply all the plants' nitrogen needs. Recent advances strongly involving French groups[4] have shown that the establishment of these endosymbioses requires bacterial activation of a common symbiotic signaling pathway (CSSP), which is also used for establishing the more widespread (non-nitrogen-fixing) arbuscular mycorrhizal (AM) symbiosis which affects most plants, including cereals. For most Rhizobia this activation is achieved through secretion of specific lipochitooligosaccharide (LCO) signals, which mimic AM signals. French groups are also active in studies to unravel the steps leading from bacterial recognition to infection, nodulation and metabolic exchange. Strategies to exploit the cereal CSSP to progressively form a cereal rhizobial endosymbiosis are currently funded for maize by the Bill & Melinda Gates Foundation (BMGF - in which French groups participate). Parallel work on wheat is funded by the Biotechnology and Biological Science Research Council (BBSRC, UK) (Table 1).

The goal of this approach is to have the target crop develop nodule-like structures able to host nitrogen-fixing bacteria. There are three requirements for this approach: (1) development of the symbiotic tissue, (2) colonization by desired bacteria and (3) transfer of nitrogen fixed by the bacteria to the plant.

[4] *e.g.* Gherbi et al (2008) PNAS 105:4928-32; Maillet et al. (2011) Nature 469:58-64

3. Developing a nitrogen-fixing AM fungal tripartite symbiosis

AM fungi benefit plants, including cereals, by improving uptake of minerals including nitrogen solutes and phosphate, and transferring them to the plant through well-developed fungal and plant transport systems. A related fungus has been shown to form a symbiotic relationship with a nitrogen-fixing cyanobacterium and some AM fungi have been shown to contain bacterial endosymbionts[5]. Cereals could potentially benefit from nitrogen fixation (in addition to other nutrients) through the identification or development of AM fungi which contain nitrogen-fixing endosymbiotic bacteria. Promoted by French groups, there is currently no major funding of this strategy.

This highly speculative strategy aims at (1) identifying endosymbiotic bacteria in AM fungi and/or (2) engineering an AM fungal/bacteria symbiosis, in order to favor the establishment of a tripartite association that could provide nitrogen from fixation to the targeted crop.

4. Engineering nif genes in plants

Recent work on the assembly of active nitrogenase protein has determined the minimum number and role of bacterial *nif* genes required for the production of this complex, oxygen-sensitive enzyme[6]. These genes could be expressed in the plant with the aim of assembling an active nitrogenase protein either as an oxygen-insensitive form or in an oxygen-shielded tissue or compartment. Amenable to synthetic biology, a large project with this aim is funded by the BMGF.

This approach focuses on the plant only as a target for expressing synthetic or bacterial *nif* genes to enable the plant to fix the dinitrogen by itself.

Future investment and research targets

The French scientific community working on symbiotic interactions has made major contributions to recent advances in the knowledge of the mechanisms of symbiotic development and functioning, using both legume and actinorhizal models and analyses on various interacting microbes[4]. This work is at the cutting edge of research in this field and combines integrated approaches on both the microbes and the plant, including genetics, omics, physiology, cell and structural biology and biochemistry. Links already exist

[5] Bonfante & Anca (2009) Annu Rev Microbiol 63:363-83
[6] Rubio & Ludden (2008) Annu Rev Microbiol 62:93-111

with the highly productive groups working on the physiology and modeling of nitrogen assimilation. Initiatives have already been taken, in some of the groups, to apply the knowledge on diverse symbiotic systems towards developing cereals which benefit from nitrogen fixation. In view of the importance and complexity of the studies much more could be done in France to exploit the recent advances in our knowledge through mobilizing the research community towards common goals. Various public and private laboratories with complementary expertise on nitrogen-fixing bacteria, plant symbioses, cereal breeding and genetic engineering and cereal nitrogen assimilation, are ready to take up the challenge and to unite their efforts towards the long term aim of developing “nitrogen-fixing cereals” (Table 1).

French public and private research have developed expertise valuable for all four strategies. The most researched areas are related to strategies 1 to 3 but the scientific and technical knowledge for the fourth strategy is also present. Further assessment is required of the choice of strategies which should be pursued, but studies with short and medium term goals are already evident (Table 2). Any of the potential future efforts should involve researchers specialized in these processes in collaboration with cereal geneticists and breeders. Internationally, the main funding efforts have been centered on maize. France is the 5th largest wheat producer in the world with about 40 million tonnes produced compared to 15 million tonnes of maize[1]. The French public and private research community is a world leader in the development of genetic and genomic resources and tools for wheat. Thus French research efforts should probably be centered on wheat, although for some analyses *e.g.* forward and reverse genetics, adoption of a model cereal plant may be judicious.

Cereal genetic variability is currently underexploited in terms of its potential to lead to benefit from nitrogen fixation and should be analyzed. Exploration through strategies 1 and 2, of the genetic variability of this trait in wheat could lead to the development of new genotypes. These genotypes could also be used in further studies in strategy 2 to use a step-by-step, knowledge-based approach to improve the plant-microbe interaction and the benefit obtained from it. For this strategy, studies in cereals on the first steps, should be undertaken in parallel to more fundamental studies on model symbiotic systems, which would provide the knowledge and materials for medium term goals (Table 2). The third strategy requires an initial, feasibility study. The fourth strategy, using a synthetic biology approach, could almost be addressed independently from the other strategies.

It must be emphasized that the development of “nitrogen-fixing cereals” requires long term research, with no guarantees of even partial success. However, the international research community, particularly in the USA and the UK, has realized that the potential socio-economic benefits outweigh the risks and are investing in large scale projects. The French public-private research community, with its broad and complementary range of expertise, could make a major impact at the International level, through better coordination and funding and by exploiting the genetic diversity of both wheat and nitrogen-fixing bacteria on projects with clearly-defined goals and strategies. Concordant with the BMGF philosophy to stimulate additional public and private investment through strategic funding of novel ideas, National funding of research on “nitrogen-fixing cereals” would be internationally welcomed and would be integrated and complementary to the current International projects.

This paper is endorsed by the strategic committee of the GIS BV, the “Plant Biology and Biotechnologies” working group (GT4) of the AllEnvi Alliance, and the participants of the GIS BV- AllEnvi workshop (Appendix 3)

GIS Biotechnologies Vertes
c/o Genoplante Valor - 28 rue du Docteur Finlay - 75015 Paris - France
Tél : +33 (0)1 42 75 95 83 - Fax : + 33(0)1 45 75 63 45

Table 1. Biotechnology strategies by which cereals could benefit from nitrogen fixation

Strategy	Characteristics	Challenges	Funded international projects (see Appendix 1)	Expertise and interest of French laboratories (see Appendix 2)
Improving endophytic/epiphytic interactions with nitrogen-fixing bacteria	Exploits natural interaction between plants and rhizosphere bacteria Requires plant selection of efficient N_2-fixing partners Potential to exploit genetic variability	Competitivity and stability of plant-bacterium interaction Maintaining stability of yield		EcoMic Biogemma GDEC Limagrain LIPM Agroécologie
Developing an endosymbiosis with nitrogen-fixing rhizobia	Potentially high rates of N_2 fixation Based on well-studied endosymbioses Control of plant-bacterium specificity by symbiotic signaling Potential to optimize both plant and bacterium	Some steps to endosymbiosis require further information Complex genetic information needs to be introduced in cereals Bacterium-plant couple needs to be optimized for each cereal species	BMGF BBSRC BBSRC/NSF	LIPM Biogemma LRSV Limagrain Rhizogènese LSTM ISV EcoMic GDEC Agroécologie
Developing a nitrogen-fixing AM fungal tripartite symbiosis	Requires AM fungal inoculum Exploits well-known AM symbiosis Applicable to all cereal crops	Unknown feasibility of identifying/developing “N_2-fixing AM fungi” Unknown potential for N_2 fixation rates Unknown costs to the plant		LRSV LSTM
Engineering nif genes in plants	Trait transmission by seed (independent of interacting microorganism) Applicable to all transformable crops	Complex genetic information needs to be introduced in cereals Unknown N_2 fixation rates Overcoming oxygen sensitivity of nitrogenase	BMGF	Biogemma

Table 2. Strategies and goals for future research in France

Strategy	Approach	Short term goals	Medium term goals
1.Exploiting cereal genetic diversity to benefit from nitrogen fixation	Cereal studies	Analysis of cereal genetic variability to benefit from N_2 fixation (by ^{15}N isotope enrichment)	Identification of trait loci
		Identification of interacting N_2-fixing bacteria	Introduction of trait into elite genotypes
		Analysis of plant-bacterium interaction	
2.Developing a symbiosis with nitrogen-fixing rhizobia	Cereal studies	Analysis of cereal genetic variability for interaction with rhizobia	Genetic improvement of plant genotypes through selection and/or genetic engineering
		Identification of best rhizobia from rhizosphere or known symbionts	Genetic improvement of bacterium by directed and experimental evolution approaches
		Studies on activation of the common symbiotic signaling pathway	Analysis of colonization and associated genetic and metabolic responses, including N_2 fixation and assimilation
		Studies on mechanisms for producing symbiotic tissue	Analysis of competitivity of bacterial inoculum
		Modeling of feasibility and cost of the interactions required to associate bacterial N_2 fixation with cereal assimilation capacities	Analysis of defense responses to pathogens
	Fundamental studies on model systems	Studies on mechanisms of endosymbiotic infection	
		Studies on symbiont's defense avoidance without defense compromise	
		Studies on coordination of the production and invasion of symbiotic tissue	
		Studies on the control of development of N_2 fixing capacities	
3.Developing a nitrogen-fixing AM fungal tripartite symbiosis	Cereal and fundamental studies	Survey of AM fungi and their bacterial N_2-fixing endosymbionts by high throughput PCR	Analysis of N_2 fixation and nitrogen transfer to the plant
		Studies on uptake and persistence of N_2-fixing bacteria in AM fungi	Analysis of persistence of interactions
4.Engineering *nif* genes in plants	Cereal studies	Introduction of bacterial or synthetic *nif* genes	Improve *nif* gene expression, activity of nitrogenase and benefit to plant
	Fundamental studies	Site-directed or directed evolution mutagenesis to generate improved variants of nitrogenase protein complex	

Appendix 1. Major International Projects

Funding Agency	Name of project	Coordinator/partners	Amount	Dates
BMGF	Engineering Nitrogen Fixation for Africa	G. Oldroyd (JIC, UK) with J. Stougaard (Aarhus, Denmark), T. Brutnell (USA), J. Dénarié (Toulouse), J.M. Ané (Wisconsin, USA)	$9.8m	2012-2017
BBSRC	First step in engineering cereal that is less reliant on fertiliser	G. Oldroyd (JIC, UK)	£2.5m	2012-
BMGF	NFIX: introduction of biological nitrogen fixation in cereals	L. Rubio (Madrid, Spain)	$2.9m	2011-2016
BBSRC/NSF Ideas Lab	Nitrogen: improving on nature	Under evaluation	$12m	2013-

Appendix 2. French Laboratories

Abbreviation	Full-name	Location	Public/Private
Agroécologie	UMR 1347 Agroécologie INRA/Université de Bourgogne/AgroSup	Dijon	Public
Biogemma	Biogemma	Clermont-Ferrand	Private
EcoMic	Ecologie Microbienne, UMR 5557 CNRS/Université de Lyon	Lyon	Public
GDEC	UMR INRA-UBP 1095 Genetics, Diversity and Ecophysiology of Cereals	Clermont-Ferrand	Public
IJPB	Institut Jean-Pierre Bourgin	Versailles	Public
ISV	CNRS - Institut des Sciences du Végétal	Gif-sur-Yvette	Public
LIPM	Laboratoire des Interactions Plantes Micro-organismes UMR CNRS-INRA 2594/441	Toulouse	Public
Limagrain	Limagrain Europe	Clermont-Ferrand	Private
LRSV	Laboratoire de Recherche en Sciences Végétales UMR 5546 CNRS/Université Paul Sabatier	Castanet-Tolosan	Public
LSTM	Laboratoire des Symbioses Tropicales et Méditerranéennes UMR 113 CIRAD/INRA/IRD/SupAgro/UMII	Montpellier	Public
Rhizogènese	Equipe Rhizogenèse - Institut de Recherche pour le Développement (IRD)	Montpellier	Public

Appendix 3. List of Participants

Jean-Michel Ané (University of Wisconsin); Patrick Armengaud (IJPB, AgroParisTech-INRA); Guillaume Bécard (LRSV, CNRS/Université Paul Sabatier) ; Gregoire Berthe (Céréales Vallée) ; Didier Bogusz (Rhizogenèse, IRD) ; Aurelia Boulaflous (GDEC, Université Clermont) ; Sébastien Caiveau (SYNGENTA) ; Julie Cullimore (LIPM, INRA) ; Laure David (IJPB, INRA) ; Frans de Bruijn (LIPM, INRA) ; Jean Dénarié (LIPM, INRA) ; Delphine Denoizé (Céréales Vallée) ; Pierre Devaux (Florimond Desprez) ; Laure Duchalais (RAGT 2n) ; Frédéric Fantin (AGRI-OBTENTIONS) ; Sébastien Faure (Biogemma) ; Claudine Franche (Rhizogenèse, IRD) ; Pierre Frendo (Université Nice Sophia Antipolis) ; Florian Frugier (ISV, CNRS) ; Olivier Gardet (Agri Obtentions) ; Hassen Gherbi (Rhizogenèse, IRD); Thomas Girin (IJPB, INRA); Clare Gough (LIPM, INRA) ; Marie-Hélène Jeuffroy (UMR Agronomie, INRA) ; Anne Krapp (IJPB, INRA) ; Fabien Le Couviour (Limagrain Europe) ; Jacques Le Gouis (GDEC, INRA) ; Pierre-Marie Le Roux (Secobra Recherches) ; Benoit Lefebvre (LIPM, INRA) ; Hélène Lucas (GIS BV) ; Pierre Martre (GDEC, INRA) ; Catherine Masson (LIPM, INRA) ; Yvan Moenne-Loccoz (Ecologie Microbienne, CNRS/Université de Lyon) ; Lionel Moulin (LSTM, IRD); Nico Nouwen (LSTM, IRD); Pascual Perez (GIS BV) ; Pierre Pin (Syngenta Seeds SAS) ; Francis Quetier (Génopole-Evry) ; Pascal Ratet (ISV, CNRS) ; Catherine Ravel (GDEC, INRA) ; Jacques Rouster (Biogemma) ; Baptiste Soenen (ARVALIS Institut du Végétal) ; François Torney (Biogemma) ; Florence Wisniewski-Dyé (Ecologie Microbienne, CNRS/Université de Lyon) ; Céline Zimmerli (GIS BV)